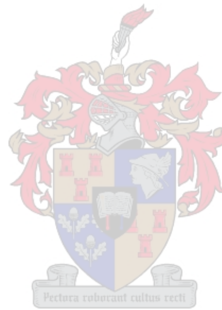
Mutagenesis studies of a Glycoside Hydrolase Family 2 enzyme

Izak Jacques de Villiers



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Supervisor: Prof. J. Kossmann

Co-Supervisors: Dr J.R. Lloyd and Dr S.W. Peters

Laboratory Supervisor: Mr. M.L. Smith

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Abstract

Galactooligosaccharides are produced by the transglycosylation activity of β-galactosidases (β-gal, EC 3.2.1.23) when utilising lactose as a substrate. They have emerged as important constituents used in the food and pharmaceutical industries owing to their prebiotic properties. Although transglycosylation was discovered in 1951 (Wallenfels 1951), and a number of β-gals have had their transglycosylation activity characterised, the activities of these enzymes are not optimal for industrial use. Their tendency to favour the hydrolytic reaction over the transglycosylation reaction, coupled with the production of shorter chain oligosaccharides has driven scientists to investigate altering protein structure both to increase chain lengths and the amount of oligosaccharide produced at lower substrate concentrations.

In an attempt to alter the amount of oligosaccharide produced by a metagenomically derived β-gal belonging to the glycosyl hydrolase 2 family, random and site-directed mutagenesis were used. A randomly mutagenised library was screened on SOB agar plates containing 5% (w/v) lactose which should select for clones that synthesise oligosaccharides at relatively low concentrations. No such activity was detected. Site-directed mutagenesis was also utilised to alter protein structure. It was confirmed that the β-gal utilised in this study belonged to the glycosyl hydrolase 2 family through mutation of the predicted catalytic acid/base glutamic acid to a non-catalytic residue, thus removing activity. Another mutation was utilised to investigate if it was possible to increase the degree of polymerisation of oligosaccharides produced by the β-gal. This mutation was successful in increasing the degree of polymerisation.

Biochemical characterisation of the β-gal revealed that it exhibited optimal activity at pH 8.0, with a temperature optimum of 30°C. The β-gal exhibited a K_m and V_{max} of 54.23 mM and 2.26 µmol/minute^{-1}/mg protein^{-1} respectively, similar to kinetic parameters that have been determined for a number of previously characterised enzymes.

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Opsomming

Galaktooligosakkariede word geproduseer deur die transglikosileering aktiwiteit van β-galaktosidase (β-gal, EG 3.2.1.23) wanneer hulle laktose as 'n substraat gebruik. Hierdie oligosakkariede het na vore gekom as 'n belangrike bestandeel vir gebruik in die voedsel en farmaseutiese bedryf as gevolg van hulle prebiotiese eienskappe. Alhoewel transglycosylation al in 1951 ontdek is (Wallenfels 1951) en 'n aantal β-gals se transglycosylation aktiwiteit gekenmerk is, is hierdie ensieme nie ideaal vir industriële toepassings nie. Die geneigdheid om die hidrolitiese reaksie oor die transglycosylation reaksie bevoordeel, tesame met die produksie van korter oligosakkariede het wetenskaplikes ondersoek genoop om die proteïenstruktuur te verander om ketting-lengte en die kwantiteit van oligosakkaried geproduseer teen laer substraat konsentrasies te verhoog.

In 'n poging om die opbrengs van die oligosakkaried wat deur 'n metagenomiese β-gal wat aan die glycosyl hidrolase 2 familie behoort te verander, is lukraak en terrein gerigte-mutagenese gebruik. Die mutagenese biblioteek is op SOB agarplate met 5% (w/v) lactose gekeur, om klone wat die fenotipe wat verband hou met die produksie oligosakkaried teen relatiewe lae konsentrasies te selekteer. Geen aktiwiteit is opgemerk nie. Terrein gerigte-mutagenese is ook gebruik om die proteïenstruktuur te verander. Deur ‘n bioinformatiese voorspelling, is dit bevestig dat die β-gal wat in hiedie studie gebruik word tot die glycosyl hidrolase 2 familie behoort. Dit is gedoen deur mutasie van die voorspelde katalitiese suur/basis glutamiensuur na 'n nie-katalitiese oorskot, dus die verwydering van aktiwiteit. Nog ‘n mutasie is gebruik om te ondersoek of dit moontlik was om die ketting-lengte van die oligosakkaried wat deur die β-gal geproduseer is te verhoog. Die mutasie was suksesvol in die verhoging van die oligosakkaried wat geproduseer was.

Biochemiese karakterisering van die β-gal het getoon dat hierdie β-gal optimale aktiwiteit het by pH 8.0, met 'n optimum temperatuur van 30°C. Die β-gal het 'n K_m en V_{max} van 54.23 mM en 2.26 µmol/minute^{-1}/mg proteïen^{-1} onderskeidelik, soortgelyk aan kinetiese parameters wat bepaal word vir ensieme wat voorheen gekenmerk is.

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List of Abbreviations

ATP: Adenosine triphosphate

β-gal: β-galactosidase

Bis-Tris: 2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol

BLAST: Basic local alignment tool

BSA: Bovine serum albumin

°C: degrees Celsius

CAZy database: Carbohydrate active enzymes database

ddH_2O: Distilled deionized water

DNA: Deoxyribose nucleic acid

DP: Degree of polymerisation

DTT: Dithiothreitol

E. coli: *Escherichia coli*

EDTA: Ethylenediaminetetraacetic acid

g: Gram

G6PDH: Glucose-6-phosphate dehydrogenase

Gal: Galactose

GalDH: Galactose dehydrogenase

GalM: Galactose mutarotase

GGL: Glucose/galactose/lactose

GH: Glycosyl Hydrolase

GIT: Gastrointestinal tract

Glc: Glucose

GOS: Galactooligosaccharides

GRAS: Generally regarded as safe

HEPES: 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid

HK: Hexokinase

HPLC: High Performance Liquid Chromatography

IPTG: Isopropyl β-D-1-thiogalactopyranoside

Kb: Kilobase

kDa: Kilo Dalton

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K_m: Michaelis Menten constant

kPa: Kilo Pascal

Lac: Lactose

LacZ: *Escherichia coli* β-galactosidase

LB: Lysogeny broth

LC-MS: Liquid chromatography-mass spectrometry

M: Molar

MES: 2-(N-morpholino)-ethanesulfonic acid

N: Normal

NAD^+: Nicotinamide adenine dinucleotide (oxidised)

NADH: Nicotinamide adenine dinucleotide (reduced)

$NADP^+$: Nicotinamide adenine dinucleotide phosphate (oxidised)

NADPH: Nicotinamide adenine dinucleotide (reduced)

NDO: Non-digestible oligosaccharides

NMR: Nuclear magnetic resonance

-OH: Hydroxyl

oNPG: ortho-nitrophenyl-β-galactoside

PAGE: Polyacrylamide gel electrophoresis

QTOF: Quadrupole time of flight

RPM: Revolutions per minute

SDM: Site-directed mutagenesis

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOB: Super optimal broth

Sp: Species

TIM barrel: Triose Phosphate Isomerase Barrel

TLC: Thin Layer Chromatography

U: Units

UPLC: Ultra performance liquid chromatography

UV: Ultraviolet

v/v: volume/volume

V_{max}: Maximum rate

WT: wild type

w/v: weight/volume

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X-gal: 5-bromo-4-chloro-3-indoyl-β-galactopyranoside

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1. General Introduction

Oligosaccharides are food ingredients that can be classed as prebiotics as they selectively stimulate the proliferation of *Bifidiobacteria* and other probiotic microorganisms in the gastrointestinal tract, that infer numerous health benefits to the host (Roberfroid and Slavin 2000; Macfarlane *et al*. 2008; Roberfroid *et al*. 2010). Their commercial importance has been steadily increasing over the last decade. Market analysts Frost & Sullivan estimate that the demand for pre- and probiotic products in the European market will reach €766.9 million in 2015, up from €295.5 million in 2008 – a compound annual growth of 14% (Feick 2009).

One class of oligosaccharides, galactooligosaccharides (GOS) can be produced from the transglycosylation activity of β-galactosidase (β-gal) utilising the disaccharide lactose as a substrate, and are considered to be prebiotic oligosaccharides (Gosling *et al*. 2010). Novel biological treatments to convert lactose into GOS have been receiving increased attention, due both to the health benefits they can confer when ingested (Yang and Silva 1995; González Siso 1996; Prazeres *et al*. 2012) and an increase in consumer health awareness (Mattila-Sandholm *et al*. 2002; Grunert and Wills 2007). These benefits include increased mineral uptake (Chonan *et al*. 1995; Scholz-Ahrens *et al*. 1998, 2001; Mussatto and Mancilha 2007), modifying the intestinal microflora (Gibson *et al*. 2004; Roberfroid *et al*. 2010), and reducing the risk of intestinal cancer (Guarner and Malagelada 2003; Bruno-Barcena and Azcarate-Peril 2015). The enzymatic treatment of lactose with β-gal is the main method utilised by industry to synthesise GOS, but the use of this enzyme has drawbacks as the oligosaccharide products can also be hydrolysed by it. This has led scientists to isolate and characterise new β-gals that favour transglycosylation over hydrolysis, as well as to utilise protein engineering to modify existing enzymes so that the oligosaccharides they produce have a longer degree of polymerisation.

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1.1. Oligosaccharides

Carbohydrates can be classified according to their molecular weight or degree of polymerisation (DP), into monosaccharides, oligosaccharides or polysaccharides (Crittenden and Playne 1996; Mussatto and Mancilha 2007). Oligosaccharides are defined as carbohydrates consisting of between three and ten monosaccharide units linked together by a glycosidic bond (Crittenden and Playne 1996). They can be further subdivided into hetero- and homo-oligosaccharides, which can be made up of anionic (hexoses) or neutral (pentoses and hexoses) sugars (Badel *et al.* 2011). Oligosaccharides can be branched or linear, with each monosaccharide being connected by either an α or β glycosidic linkage (Sears and Wong 2001). This means that there are a very large number of potential oligosaccharide structures that might exist due to the diversity of sugars that can be incorporated, bonds that can exist between them and branch points that can be introduced.

Oligosaccharides are water soluble and can taste sweet, although less so than sucrose. The sweetness depends on the DP of the oligosaccharide as well as its structure (Crittenden and Playne 1996; Voragen 1998). Owing to these characteristics, oligosaccharides are utilised as bulking agents and thickeners in the food industry (Crittenden and Playne 1996; Rivero-Urgell and Santamaria-Orleans 2001). Due to their selective degradation and fermentation, they are considered low cariogenic sugar substitutes, and can be used to alter the freezing temperature of foods (Lamsal 2012).

Oligosaccharides are synthesised either through chemical or enzymatic reactions (Perugino *et al.* 2005). Because of the immense structural complexity that they can possess chemical synthesis is highly complex. The presence of a number of -OH groups with similar reactivity can result in highly branched structures being formed (Perugino *et al.* 2004). Selective synthesis of oligosaccharides can be achieved through the use of protection groups that bind to -OH radical, but the difficulty of this process and relatively low yields of end product have resulted in enzymatic synthesis being favoured (Sears and Wong 2001). This has received the most attention for applications in industry as enzymes possess very tight regio- and stereoselectivity. This means that the final products can be manufactured in a reproducible manner on a large scale.

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1.1.1. Galactooligosaccharides

GOS structures can differ in a number of ways; namely saccharide composition, DP and type of glycosidic linkage (Gosling *et al.* 2010). These structural differences can alter the properties of GOS and, thus, how they are utilised in the food industry (Mahoney 1998; Gosling *et al.* 2010; Sangwan *et al.* 2011). There is evidence that suggests that microorganisms propagate differently when fermenting different oligosaccharide structures; this needs to be taken into account when formulating prebiotic products for the market to ensure propagation of probiotic microflora in the gastrointestinal tract (GIT) of hosts (Gosling *et al.* 2010; Marín-Manzano *et al.* 2013).

The general composition of GOS consist of a terminal glucose unit linked to one or more galactose units (Gosling *et al.* 2010; Gänzle 2012; Yu and O'Sullivan 2014), however branched structures as well as GOS lacking a terminal glucose moiety (Gal-Gal) also exist (Table 1) (Mahoney 1998). GOS are highly stable compounds, capable both of being stored for long periods of time and being resistant to high temperatures. These properties allow them to be utilised in foods that undergo pasteurization (Klewicki 2007).

Table 1: Oligosaccharide structures commonly found in GOS mixtures (Mahoney 1998).

Degree of Polymerisation	**Linkage**
Disaccharides	β-Gal (1→6)-Glc
	β-Gal (1→6)-Gal
	β-Gal (1→2)-Glc
	β-Gal (1→3)-Glc
	β-Gal (1→3)-Gal
Trisaccharides	β-Gal (1→6)-β-Gal (1→6)-Glc
	β-Gal (1→6)-β-Gal (1→6)-Gal
	β-Gal (1→6)-β-Gal (1→4)-Glc
	β-Gal (1→3)-β-Gal (1→4)-Glc
	β-Gal (1→4)-β-Gal (1→4)-Glc
Tetrasaccharides	β-Gal (1→6)-β-Gal (1→6)-β-Gal (1→4)-Glc
	β-Gal (1→6)-β-Gal (1→3)-β-Gal (1→4)-Glc
	β-Gal (1→3)-β-Gal- (1→6)-β-Gal (1→4)-Glc
Pentasaccharides	β-Gal (1→6)-β-Gal (1→6)-β-Gal (1→6)-β-Gal(1→4)-Glc

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1.1.2. Industrial production of galactooligosaccharides

The commercial production of GOS utilises high concentrations of lactose as a substrate, in the form of either whey permeate or refined lactose (Lamsal 2012). Whey permeate is an attractive substrate for use, as it is readily available as a waste product from the cheese manufacturing process. The GOS manufacturing process usually utilises batch mode fermentation in conjunction with β-gal enzyme immobilised on a matrix; although hollow fibre membrane reactors, fixed bed reactors and continuous stirred tank reactors with “free enzyme” have all been used as methods for production (Boon *et al.* 2000b; Gänzle *et al.* 2008; Gänzle 2012). Although the free enzyme approach is the simplest to implement, enzyme isolation and purification in conjunction with the enzyme preparations not being reusable result in high input costs (Gosling *et al.* 2010). The batch mode is the manufacturing method of choice due to its ease of implementation, its minimisation of contamination, and relatively low cost as the enzyme can be reused (Boon *et al.* 2000b; Gänzle 2012; Lamsal 2012). GOS manufacturers also have to address problems with the β-gals that they use for GOS synthesis – the enzymes do not produce oligosaccharides at high enough rates. This has led to research being conducted to identify and characterise novel β-gals with high transglycosylation activity. At the end of a production run, all GOS mixtures go through the same decolourisation and demineralisation treatments before being filtered and concentrated in an evaporator (Sako *et al.* 1999; Otieno 2010). Commercial GOS products are available in either syrup or powder form, and contain a mixture of DP as well as residual lactose, glucose and galactose (Torres *et al.* 2010). Table 2 shows some producers of GOS, as well as the origin of the enzymes that they utilise in their GOS production.

Table 2: Manufacturers of commercial GOS (Tzortzis and Vulevic 2009; Torres *et al*. 2010; Sangwan *et al*. 2011; Bruno-Barcena and Azcarate-Peril 2015).

Manufacturer	Product	Enzyme Origin
Corn Products Intl. USA	Purimune - powder	*Bacillus circulans*
Clasado Ltd. United Kingdom	Bimuno – syrup and powder	*Bifidiobacterium bifidum*
Fayrefield Food. United Kingdom	Promotiva – syrup	*Bacillus circulans*
Friesland Foods Domo. Netherlands	Vivinal GOS – syrup and powder	*Bacillus circulans*
Nissin Sugar Manufacturing Co. Japan	CUP-oligo – syrup and powder	*Cryptococcus laurentii*
Yakult Honsha. Japan	Oligomat 55NP – syrup and powder	*Aspergillus oryzae* *Streptococcus thermophilus* *Sporobolomyces singularis*

1.2. Functional foods

The term 'functional foods' was first utilised in Japan in the 1980's. They can be defined as food or food ingredients that, when consumed, impart physiological or health benefits to the organism ingesting them over and above the nutritional value of the food (Gibson *et al*. 2004; Sangwan *et al*. 2011). As the definition of a functional food is broad, a large number of different compounds fall into this category; prebiotics, probiotics, vitamins, minerals and antioxidants have all been classified as components of functional foods (Sangwan *et al*. 2011). Currently prebiotics and probiotics have received the greatest attention and are the most widely utilised and studied functional foods in the world (Figueroa-González *et al*. 2011; Sangwan *et al*. 2011).

Probiotics are defined as "non-pathogenic microorganisms which confer a health benefit on the host and are able to prevent or improve some diseases when administered in adequate amounts" (Gibson and Roberfroid 1995; Gibson *et al*. 2004; Fric 2007). It is accepted that they benefit human health through competing with pathogenic microbes that can cause disease, induce immune system responses and produce substances that influence the wellbeing of their hosts (Saier and Mansour 2005). Prebiotics on the other hand are defined as being non-digestible oligosaccharides (NDO's) that are selectively fermented by the beneficial bacteria that are already present in the GIT and which confer a health benefit to the host (Fric 2007; Otieno 2010; Figueroa-González *et al*. 2011). Among the microorganisms populating the GIT, *Bifidiobacterium* and *Lactobacilli* predominantly utilise NDO's to

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the benefit of the hosts health (Scholz-Ahrens *et al.* 1998). Common NDO's utilised as prebiotics include inulin, fructooligosaccharides and galactooligosaccharides. Due to the potential interaction between prebiotics and probiotics, food products that contain a mixture of both have been referred to as synbiotics, and are becoming more prevalent (Mattila-Sandholm *et al.* 2002; Gibson *et al.* 2004).

1.2.1. Health benefits of non-digestible oligosaccharides

Oligosaccharides have been shown to exhibit a number of health benefits, and studies have shown that they:

- Modify the microflora of the GIT. NDO's are selectively fermented by *Bifidiobacterium* which inhibit the growth of pathogenic bacteria (Gibson *et al.* 2004; Roberfroid *et al.* 2010).
- Increase the rate of synthesis of vitamin B in the GIT by *Bifidiobacterium* (Perugino *et al.* 2005).
- Can reduce adhesion of toxins produced by pathogenic bacteria in the GIT as well as the adhesion of these bacteria, protecting the host against illnesses (Sinclair *et al.* 2009; Searle *et al.* 2010; Quintero *et al.* 2011).
- Relieve symptoms of constipation in a similar manner to dietary fibre (Delzenne and Roberfroid 1994; Roberfroid and Slavin 2000; Mussatto and Mancilha 2007).
- Reduce cholesterol, phospholipid and triglyceride levels in blood of hosts, resulting in a decrease in the risk of diabetes and obesity (Alliet *et al.* 2007; Hanning *et al.* 2010; Roberfroid *et al.* 2010; Sanz and Santacruz 2010).
- Inhibit diarrhoea associated with GIT infections possibly due to the inhibiting effect of *Bifidiobacterium* on pathogenic bacterial species (Roberfroid and Slavin 2000; de Vrese and Offick 2010).
- Increase the rate of calcium absorption in the GIT as well as the rate of bone mineralisation, resulting in decreased risk of osteoporosis (Chonan *et al.* 1995; Scholz-Ahrens *et al.* 1998, 2001; Mussatto and Mancilha 2007).
- Reduce the risk of developing intestinal cancer through the reduction of carcinogenic precursors (Guarner and Malagelada 2003; Bruno-Barcena and Azcarate-Peril 2015).

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1.3. β-Galactosidase: a multifunctional enzyme

β-Galactosidases hydrolyse the non-reducing β-D-galactose ends of di- and oligosaccharides and are found in both eukaryotic and prokaryotic organisms (Table 3), performing a variety of different functions (Richmond *et al*. 1981; Bhatia *et al*. 2002; Husain 2010). They are secreted by microorganisms such as fungi, bacteria and yeast to hydrolyse oligosaccharides to fermentable monosaccharides (Richmond *et al*. 1981; Husain 2010). In plants they are found in a variety of tissues, playing roles in plant growth and fruit ripening (Li *et al*. 2001) and are also located in the membrane of the small intestines of mammals where they hydrolyse lactose into glucose and galactose (Mahoney 1997).

Table 3: Selection of the numerous sources of β-galactosidases in nature (Adapted from Mahoney, 1997; Richmond *et al*, 1981)

Plants	Mammal Organs	Bacterial Species	Fungal Species
Apricots Coffee Beans Peaches Alfalfa seeds Almonds Apples	Liver Brain Intestines	*Arthrobacter* sp. *Streptococcus thermophilus* *Streptococcus lactis* *Escherichia coli* *Thermus aquaticus* *Bacillus coagulans* *Bacillus megaterium* *Bacillus circulans* *Lactobacillus bulgaricus* *Lactobacillus helareticus*	*Alternaria alternata* *Aspergillus oryzae* *Aspergillus niger* *Aspergillus foetidus* *Kluyveromyces lactis* *Kluyveromyces fragilis* *Kluyveromyces bulgaricus* *Mucor meuhei* *Mucor pucillus* *Neurospora crassa* *Rhizobium meliloti*

The *Escherichia coli* (*E. coli*) β-gal (EC 3.2.1.23) is arguably one of the best studied proteins in the world (Mahoney 1997; Juers *et al*. 2012). It was used by the scientists Jacob and Monod (1961) to develop their operon model, was fully sequenced in 1970 (Fowler and Zabin 1978) and its three dimensional and crystal structures were determined in 1994 and 2001 respectively (Jacobson *et al*. 1994; Juers *et al*. 2001). Since the first crystal structure elucidation, the structures for β-gals originating from a variety of organisms have been determined and deposited in the Protein Data Bank (PDB, http://www.rcsb.org) (Table 4). It is utilised by molecular biologists throughout

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the world as it forms a blue dye when grown with X-gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside). This artificial substrate is a colourless substance which consists of a substituted indole linked to a galactose moiety. The β-gal enzyme is highly specific for the sugar within X-gal and, therefore, hydrolyses it causing the substituted indole to be released. This then dimerises, resulting in an insoluble blue product (Horwitz *et al*. 1964).

Table 4: Selection of organisms whose β-galactosidases have been isolated and used to produce crystal structures (Berman *et al*. 2000)

Organism	GH Family	PDB Code	Reference
Sulfolobus solfaticus	GH1	1UWQ	(Gloster *et al*. 2004)
Escherichia coli	GH2	1BGM	(Juers *et al*. 2001)
Arthrobacter sp. C2-2	GH2	1YQ2	(Skálová *et al*. 2005)
Kluyveromyces lactis	GH2	3OBA	(Pereira-Rodríguez *et al*. 2012)
Penicillium sp.	GH35	3D3A	(Rojas *et al*. 2004)
Trichoderma reesei	GH35	3OG2	(Maksimainen and Rouvinen 2011)
Homo sapiens	GH35	3THC	(Ohto *et al*. 2012)
Thermus thermophilus A4	GH42	1KWG	(Hidaka *et al*. 2002)
Bacillus circulans sp. *alkalophilus*	GH42	3TTS	(Maksimainen *et al*. 2012)

E. coli β-gal is a 464 kDa homotetrameric enzyme, each subunit of which consists of 1023 amino acids (Appel *et al*. 1965; Fowler and Zabin 1978; Kalnins *et al*. 1983). Individual subunits contain five different structural domains (Jacobson *et al*. 1994; Juers *et al*. 2000). The third of these domains contains the active site which is formed by a $\alpha_8\beta_8$ barrel (Juers *et al*. 2012), although there are a number of residues in the other domains that play critical roles in the activity of the enzyme. The catalytic reaction of β-gal is dependent on two amino acid residues; one acting as an acid or proton donor and the other as the nucleophile or base (Juers *et al*. 2012). In *E. coli* these two residues have both been shown to be glutamic acid (Glu461 and Glu537) which act as the proton donor and nucleophile, respectively (Bader *et al*. 1988; Gebler *et al*. 1992).

The Carbohydrate Active Enzymes database (CAZy, http://www.cazy.org) is an online resource where enzymes that are active on carbohydrates have been divided into five classes – glycoside hydrolases (GH), carbohydrate-binding molecules, glycosyltransferases, carbohydrate esterases, and polysaccharide lyases (Cantarel *et al*. 2009). The amino acid sequences of β-gals originating from a large number of different sources have been determined. Multiple sequence alignments indicate that

there are a number of highly conserved regions throughout these enzymes that have been utilised to divide them into groups with similar enzymatic activity (Henrissat 1991; Henrissat and Bairoch 1993, 1996; Davies and Henrissat 1995; Henrissat and Davies 1997). Owing to the correlation between sequence similarity and protein folding, classifying enzymes in this manner illustrates their structural characteristics more accurately than utilising only substrate specificity. It also provides scientists with a means of predicting the enzymatic mechanism of a protein and allows them to determine evolutionary relationships between different polypeptides. Glycoside hydrolases, which include β-gals, are currently classified into 133 different families (Lombard *et al*. 2014). β-Gals are classified within four of these, namely GH1, GH2, GH35 and GH42, based on sequence similarity. Those belonging to the GH1, GH2 and GH42 families have mainly been found in microorganisms (Kalnins *et al*. 1983; Cubellis *et al*. 1990; Ohtsu *et al*. 1998), whereas β-gals in GH35 have been identified in plants, animals and microorganisms (Oshima *et al*. 1988; Smith and Gross 2000; Zinin *et al*. 2002). Over and above β-gal activity, enzymes that fall into the GH1 family also possess β-glucosidase, β-mannosidase and β-glucuronidase activities. GH2 enzymes possess all of those activities as well as an additional α-arabinofuranosidase activity. Proteins classified in GH32 possess exo-β-glucosaminidase and exo-β-1, 4-galactanase activities while GH42 family enzymes possess α-arabinopyranosidase activity (Lombard *et al*. 2014).

β-Galactosidases are capable of hydrolysing a large number of different substrates as the enzyme is specific only for the β-D-galactose moiety (Brockhaus *et al*. 1979; Nam Shin *et al*. 1980). This leads to a degree of promiscuity by these enzymes when another molecule is present in the non-galactose position, resulting in a large number of aglycones being utilised by them as substrates. β-Gals can also form oligosaccharides under specific conditions through a process known as transglycosylation (Wallenfels 1951; Aronson 1952; Pazur 1953). They catalyse the reaction between a donor that contains a galactose moiety and an acceptor that contains an -OH group (Figure 1) through a double displacement mechanism where a covalent glycosyl-enzyme intermediate forms. When using lactose, this reaction proceeds in three steps:

- **Step 1**: the enzyme binds to lactose forming an enzyme-lactose complex.
- **Step 2**: glucose is released, while galactose remains covalently bound to the enzyme (intermediate form).
- **Step 3**: the bound galactose is released when an acceptor nucleophile containing a –OH group accepts the galactose molecule. When the nucleophilic acceptor is water, galactose is released as the final product (Acceptor 1; Figure 1). When the acceptor is a saccharide, the galactose and saccharide are bound together through the formation of a glycosidic bond, and an oligosaccharide is released (Acceptor 2, 3 or 4; Figure 1). As carbohydrates of different degrees of polymerisation present in the environment around the enzyme can fill the role of nucleophilic acceptor, the results of this transglycosylation reaction can vary between short chain oligosaccharides and long chain polymers.

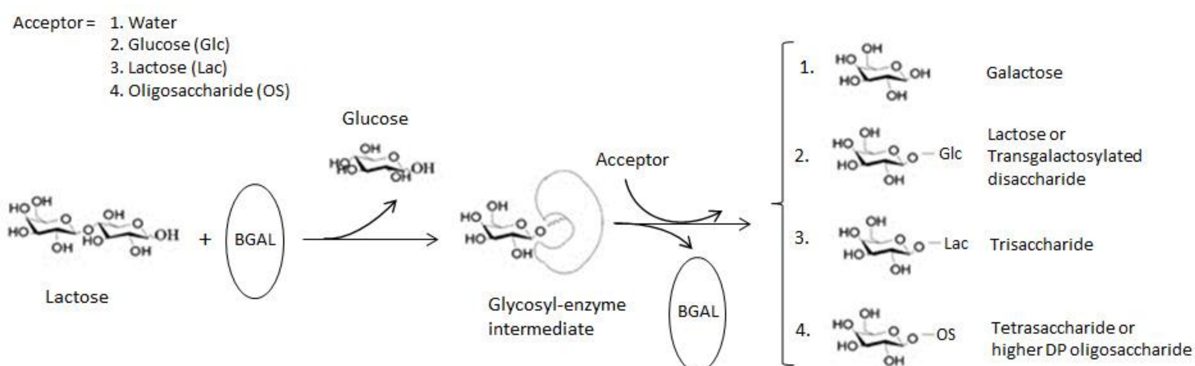

Figure 1: Mechanism of hydrolysis and transglycosylation of lactose. Different acceptors result in the formation of various end-products (Gosling *et al*. 2011; Wang *et al*. 2012).

A number of studies have been conducted to show that GOS yield increases with increasing initial lactose concentrations (Roberts and Pettinati 1957; Huber *et al.* 1976). This property has been observed both in enzymes that tend to favour hydrolysis over transglycosylation (such as the *E. coli* β-gal) (Aronson 1952), as well as those that produce large quantities of GOS (Boon *et al*. 2000a; Hansson and Adlercreutz 2001b). Two hypotheses exist that can be used to explain this observation – a reduction in competitive hydrolysis of oligosaccharides already formed or an increase in the rate of the transferase reaction. This could be explained by the reduction of water activity as a result of the high substrate concentrations, leading to reduced hydrolysis (Maugard *et al*. 2003; Gaur *et al*. 2006; Martínez-Villaluenga *et al*. 2008b). The second hypothesis predicts that increased initial

substrate concentrations make carbohydrate acceptors more available, resulting in an increase in oligosaccharide synthesis (Huber *et al.* 1976; Burvall *et al.* 1979; Akiyama *et al.* 2001; Hsu *et al.* 2007). It has also been shown that the linkage and degree of polymerisation produced during transglycosylation is dependent on a number of variables such as the source of the enzyme, pH, presence of enzymatic co-factors and reaction time (Mahoney 1997; Boon *et al.* 2000a; Tzortzis and Vulevic 2009; Gosling *et al.* 2010, 2011; Park and Oh 2010b; Wang *et al.* 2012). The various activities that β-gals possess, as well as their presence in many different species have led to their use in industry as biocatalysts.

1.4. Biocatalysts

1.4.1. Definition and characteristics of biocatalysts

Enzymes are becoming used more extensively as catalysts to generate products of interest such as biopolymers, biofuels and pharmaceuticals (Cobb *et al.* 2013). Characteristics of biocatalysts that can be utilised on an industrial scale include the need to have a high rate of catalytic turnover, stability under industrial conditions and stringent selectivity towards the formation of the product of interest (Panke *et al.* 2004; Pollard and Woodley 2007; Fox and Clay 2009; Turner 2009). Although these enzymes can perform complicated chemical reactions efficiently, industrial conditions differ from those found in nature with regard to temperature, substrate concentrations, presence of organic solvents and sheering forces (Otten and Quax 2005). The majority of native enzymes do not function optimally under these conditions and, therefore, cannot be utilised. Industrial biocatalysts with a desired activity can be acquired through optimising the conditions under which the reaction takes place, as well as through protein engineering to obtain a better biocatalyst (Otten and Quax 2005).

1.4.2. β-Galactosidase as a biocatalyst

β-Galactosidases are widely utilised by the food industry as biocatalysts to remove or reduce lactose from milk products for consumption by lactose intolerant individuals (Haider and Husain 2008). High concentrations of this sugar in refrigerated products are undesirable due to its tendency to form crystals that confer a sandy texture. By lowering the lactose content with β-gal, unwanted crystal formation can be reduced (Panesar *et al.* 2006). The addition of β-gal to whey

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hydrolyses the lactose that is present into glucose and galactose, converting an environmental pollutant into a sweet syrup that can be utilised by the soft drink, baking and dairy industries as a replacement to starch or sucrose based sweeteners (Shukla 1975; González Siso 1996; Gänzle *et al*. 2008).

Although there is an abundance of sources for β-gals available for scientists to study and characterise, those from only a small number of species have been utilised in the food and dairy industry. This is due to strict regulations requiring that any enzyme utilised in the food industry must be isolated from generally regarded as safe (GRAS) microorganisms (Mahoney 1997). Currently scientists and food technologists are trying to make use of recombinant DNA technology as a means of manufacturing β-gals with higher transglycosylation activity than current commercially available β-gals. This is being done using enzymes originating from microorganisms that are not GRAS in hosts such as *Saccharomyces cerevisiae*, although these products will still have to undergo comprehensive testing to ensure they are safe for consumption before being released (Mahoney 1997; Husain 2010; Oliveira *et al*. 2011). Due to these strict regulations, β-gals that are currently utilised in a commercial setting come from the yeasts *Kluyveromyces lactis*, *Kluyveromyces fragilis* and *Candida pseudotropicalis*, bacteria such as *Streptococcus thermophilus* and *Bacillus stearothermophilus* as well as fungal species such as *Aspergillus niger*, *Aspergillus oryzae*, *Cryptococcus* and *Bullera* (Table 5) (Mahoney 1997; Labrou 2005; Tzortzis and Vulevic 2009; Otieno 2010; Park and Oh 2010b). Clearly the β-gals that are currently used as industrial biocatalysts can be improved which has led scientists to undertake protein engineering as a way to enhance the transglycosylation activity of commercially available β-gals.

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Table 5: Some sources of commercial β-galactosidases and their suppliers (Panesar *et al.* 2006).

Source Organism	Supplier	GRAS
Bacteria		
Bacillus sp.	Novozyme, Denmark	Yes
Escherichia coli	Sigma-Aldrich, UK	No
Yeast		
Kluyveromyces sp.	SNAM Progetti, Italy	Yes
	Sigma-Aldrich, UK	
Candida pseudotropicalis	Novozyme, Denmark	Yes
	Pfizer, USA	
Saccharomyces fragilis	Sigma-Aldrich, UK	Yes
Fungi		
Aspergillus niger	Sumitomo Chemical, Japan	Yes
	Valio Labs, Finland	
	Megazyme, Ireland	
Aspergillus oryzae	Biocon, USA	Yes
	Sigma-Aldrich, UK	

1.5. Protein engineering of β-galactosidases

Recently biocatalysts have been altered through the process of protein engineering to optimise and increase the rate of production of the product of interest. This is the process whereby the DNA sequence of a gene is altered, resulting in an alteration in structure and activity of the protein it encodes (Farinas *et al.* 2001; Dougherty and Arnold 2009; Socha and Tokuriki 2013). There are a number of different methods that can be utilised to achieve this, which can be divided into random or targeted approaches (Lutz and Patrick 2004; Neylon 2004; Hibbert *et al.* 2005; Jäckel *et al.* 2008). Methods to induce random changes in the DNA sequence include the use of bacterial mutator strains that are deficient in DNA repair pathways, use of mutagens (such as UV radiation) to alter the DNA sequence, or error-prone PCR. Targeted approaches can be accomplished using commercially available kits that alter the nucleotide sequence resulting in the deletion, insertion or alteration of specific amino acids (Jäckel *et al.* 2008).

Initially, scientists utilised protein engineering to determine the location of residues, in *E. coli* LacZ protein, that were essential for catalysis. This was done by introducing point mutations and observing the change in hydrolytic activity with chromogenic substrates such as ortho-nitrophenyl-β-galactoside (oNPG). As more became known about the structure, function and characteristics of GH enzymes, focus has shifted to

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mutating these enzymes to increase specific activities, alter substrate specificities, confer increased tolerance to heat and pH changes, reduce substrate and product inhibition, or change the final product of an enzyme (Kittl and Withers 2010; Teze *et al.* 2014). Although our understanding of the mechanisms behind enzymatic reactions continues to grow, rational design of enzymes is a highly complex process as it is limited by comparing potential changes to protein structure models that have been determined by X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy (Kittl and Withers 2010). Changes in conformation of enzymes continuously occur during catalysis, resulting in deviation from modelled or predicted protein structures making rational design with the aim of altering activity a hit and miss affair (Jäckel *et al.* 2008; Li *et al.* 2012; Kries *et al.* 2013). This has led to researchers combining rational design and directed evolution to achieve the alterations that they are selecting for (Chica *et al.* 2005).

As the importance of oligosaccharides increases throughout industry, there has been a concerted effort by scientists to increase the rate of their synthesis through a targeted approach (Perugino *et al.* 2004). The main problem with using GH enzymes to produce them is that they have a tendency to favour the hydrolysis over the transglycosylation reaction. This leads them to both manufacture and degrade oligosaccharides, resulting in low yield. In an attempt to stop the hydrolysis reaction a novel class of enzyme was developed, known as a glycosynthase (Mackenzie *et al.* 1998). Through site-directed mutagenesis (SDM), the hydrolytic activity of a β-glycosidase from *Agrobacterium tumefaciens* was completely inactivated. This was achieved by mutating the codon responsible for the amino acid that acts as the catalytic nucleophile into one that encoded a non-nucleophilic residue (Table 6). In the presence of an external nucleophile such as sodium azide, activity is restored. Through the use of activated glycosyl donors (e.g. α-glycosyl fluoride) as substrates and external nucleophiles, oligosaccharides accumulate that cannot be hydrolysed by the glycosynthase.

Although glycosynthases can produce relatively large quantities of oligosaccharides, the requirement of activated substrates has limited their widespread use in industry as there is a preference to utilise cheaper substrates such as lactose (Perugino *et al.* 2004). This has led scientists to engineer GH enzymes with an increased synthetic

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activity that can utilise these cost effective substrates. A protein engineering approach was used to increase the rate of oligosaccharide synthesis by a β-glucosidase originating from *Pyrococcus furiosus* (Hansson and Adlercreutz 2001a; Hansson *et al*. 2001). Through altering amino acids in the active site of the enzyme, an increase in the total amount of oligosaccharides synthesized at low substrate concentrations as well an increase in the transglycosylation/hydrolysis ratio was observed (Table 6).

Feng *et al.* (2005) conducted studies on converting a GH1 β-glycosidase from *Thermus thermophilus* into a β-transglycosidase through both random and site-directed mutagenesis. A multistep screening process consisting firstly of isolating mutants displaying a reduction in hydrolytic activity and then selection of colonies that retained transglycosylation activity was performed. Using this Feng *et al*. (2005) were able to isolate and characterise a number of clones that exhibited increased transglycosylation/hydrolysis ratio (Table 6).

The mutation sites that Feng *et al*. (2005) and Hansson *et al*. (2001a) identified in their studies were utilised to investigate whether transglycosylation could be increased in a GH family 1 β-gal belonging to another organism, *Sulfolobus solfataricus* (Wu *et al*. 2013). Multiple sequence alignments showed that the sites were conserved throughout the GH family 1, and they separately altered the phenylalanine at position 359 to glutamine, as well as altering the phenylalanine at position 441 to tyrosine. The Phe359Gln and Phe441Tyr mutations increased total GOS yield to 58.3% and 61.7% respectively, from 50% total GOS yield utilising the WT protein (Table 6). Further analysis of the oligosaccharides produced by the transglycosylation reactions of the mutants compared to the WT enzyme indicated that both mutants also had increased yields of tri- and tetrasaccharides.

The enhancement of the transglycosylation process in conjunction with the reduction of hydrolytic activity by directed evolution strategies has not been fully explored, despite evidence that GH enzymes transglycosylation activity can be maintained while hydrolytic activity can be almost completely removed (Feng *et al*. 2005; Placier *et al*. 2009). Placier *et al*. (2009) explored this theory by randomly mutating the β-gal originating from *Geobacillus stearothermophilus* in an attempt to increase

oligosaccharide yields. The approach utilised was similar to Feng *et al.* (2005), whereby a multistep screening strategy based on the reduction of the hydrolysis of a potential transglycosylation product (lacto-sucrose) was used to identify mutants with increased transglycosylation/hydrolysis ratios. The screen yielded a number of mutants, all containing an arginine to lysine missense mutation at residue 109. Site saturation mutagenesis was carried out at that site, with all possible amino acid residues except methionine, asparagine and proline being produced. This site falls within a highly conserved region near the active site, and analysis of all the mutants demonstrated a decrease in hydrolysis. The arginine to tryptophan mutation at residue 109 resulted in the greatest increase in GOS yield (30%), as well as an overall decrease in product hydrolysis (Table 6).

Table 6: Mutations in β-galactosidases that resulted in increased oligosaccharide yield.

Enzyme source	GH family	Mutagenesis approach	Sites mutated	Oligosaccharide yield vs WT enzyme	Reference
Agrobacterium tumefaciens	GH42	SDM	Glu358Ala	29-54% increase in yield Complete loss of hydrolytic activity	(Mackenzie *et al.* 1998)
Pyrococcus furiosus	GH1	SDM	Met424Lys Phe426Tyr Met424Lys/ Phe426Tyr	1% increase in yield compared to WT enzyme 40-45% increase in yield 40% increase in rate of synthesis at 10% (w/v) lactose concentration	(Hansson and Adlercreutz 2001a; Hansson *et al.* 2001)
Thermus thermophilus	GH1	Random Mutagenesis & SDM	Phe401Ser Asn282Thr Phe401Ser/ Asn282Thr	60-74% increase in yield Increase in rate of synthesis 73% increase in yield	(Feng *et al.* 2005)
Geobacillus stearothermophilus	GH42	Random Mutagenesis & SDM	Arg109Trp	Decrease in product hydrolysis, resulting in 30% increase in GOS yield	(Placier *et al.* 2009)
Sulfolobus solfataricus	GH1	SDM	Phe359Gln Phe441Tyr	Increased total GOS yield to 58.3% Increased total GOS yield to 61.7% Overall increase in tri- and tetrasaccharide yield in both mutants	(Wu *et al.* 2013)

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1.5.1. Functional screening of a mutation library

Screening for a specific type of activity plays a central role in the identification of clones of interest from a mutation library. The functional screening process entails the use of either end product identification or some form of complementation to allow identification of a sequence encoding a specific activity (Aharoni *et al*. 2005). A number of demands need to be met by a screening system for it to be considered effective. Firstly, it should selectively identify the property of interest (Schmidt-Dannert and Arnold 1999; Aharoni *et al*. 2005). Secondly, the screening assay needs to be highly sensitive over a range of enzymatic activities so as to allow for identification and recovery of all improved clones – even if the improvement only results in a minor increase in activity when compared to the starting protein (Schmidt-Dannert and Arnold 1999). Finally, the screening system needs to be able to function in a high throughput format for it to be considered truly effective (Aharoni *et al*. 2005; Dougherty and Arnold 2009; Kittl and Withers 2010).

Current commonly used screening systems rely on heterologously expressed biocatalysts in a host organism cultivated on agar plates utilising fluorophoric or chromophoric substrates to detect activity (Kittl and Withers 2010). An example of such a screening system is the detection of β-gal activity with the chromogenic substrate X-gal. Drawbacks to this approach include the fact that, currently very few substrates are available, meaning that only a small number of activities can be examined. In addition, the hosts genetic machinery can fail to identify transcription or translation signals which results in little or no protein expression, meaning that the presence of an activity of interest can be difficult to identify due to only faint colour changes (Uchiyama and Miyazaki 2009).

Screening for transglycosylation activity is more complex than screening for other activities as there is currently no method to detect the formation of a glycosidic linkage (Kittl and Withers 2010). Oligosaccharide production by microorganisms is usually identified visually through a mucoid phenotype when cultivated on agar plates containing a substrate, or by evaluating the thickness or “ropiness” of a liquid culture (Vedamuthu and Neville 1986; Wahler and Reymond 2001; Ruas-Madiedo and de los Reyes-Gavilán 2005). These methods cannot be carried over to high-

throughput applications either due to limited production of the oligosaccharide producing protein, or to the oligosaccharide not being secreted from the cell.

A simple solution to overcome these problems is to utilise chemical dyes that selectively stain oligosaccharides. The fluorochrome aniline blue has been shown to possess a high affinity towards polysaccharides such as β(1→3), (1→4) and (1→6) glucans (Wood and Fulcher 1984), as well as β(1→3) and (1→6) galactans (Evans *et al*. 1984). It has been used successfully to detect the presence and formation of curdlan in bacterial, yeast and fungal colonies on agar plates (Nakanishi *et al*. 1976), and was used to screen a soil sample for bacterial strains producing glucans (Jung *et al*. 2007). Methylene blue on the other hand has been used previously to identify the presence of anionic polysaccharides in the red seaweed *Gymnogongrus torulosus* (Estevez *et al*. 2008), while Yariv phenylglycosides have been utilised to selectively stain β(1→3), (1→4) and α(1→4) glucans (Triplett and Timpa 1997), as well as the β(1→3) galactan of arabinogalactan proteins (Kitazawa *et al*. 2013).

1.6. Problem definition

Although there are numerous β-gals that produce GOS through the enzymatic transglycosylation of lactose, the activities of these enzymes are not optimal. The DP of the oligosaccharides that they produce are not very long, and they do not produce GOS in high enough quantities under industrial conditions to satisfy the need of manufacturers. This poses a serious problem to food scientists and biotechnologists, as the demand for GOS is steadily increasing due to their potential health benefits and an increase in consumer health awareness (Feick 2009; Ohr 2010). Two potential solutions to this problem are the screening of metagenomic libraries focussing on isolating and characterising novel β-gals with high transglycosylation activity; or utilising protein engineering to alter protein structure and function to increase the rate of transglycosylation of a β-gal that already produces GOS. This project aims to utilise both of these approaches to isolate β-gals with novel activities.

A novel β-galactosidase (BGAL32) has been previously isolated within the Institute of Plant Biotechnology from a metagenomic library that was created with genomic DNA isolated from a milk contaminated soil sample obtained from a dairy farm (Prof. Jens Kossmann, Unpublished Results). This site was chosen as soil has been

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shown to harbour a diverse mix of microorganisms (Streit *et al*. 2004; Curtis and Sloan 2005; Gans *et al*. 2006; Steele *et al*. 2008) and the presence of milk (and therefore lactose) would likely naturally enrich for microorganisms containing β-gals. Based on the mucoid colony morphology observed in cells expressing BGAL32, the clone appeared to encode an enzyme that produces oligosaccharides. The amounts and size of these oligosaccharides are, however, unknown. This project is aimed at identifying what carbohydrates BGAL32 produces from lactose and whether it is possible to mutate it to make it more useful to industry.

Objectives of this study

The objectives of this study are summarised below:

- Biochemical characterisation of β-galactosidase protein to determine optimum conditions for activity
- Random and site-directed mutagenesis of a oligosaccharide producing metagenomically derived β-galactosidase in order to:
    - increase the DP of the oligosaccharide that it produces
    - decrease the concentration of substrate required for oligosaccharide production
- Analysis of oligosaccharide produced by the wild type and mutated β-galactosidase

2. Materials and Methods

2.1. *Escherichia coli* genotypes, plasmids and constructs

DH5α (Invitrogen): F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoRnupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17($r_K^- m_K^+$), λ–

DH5α Turbo ΔlacZ: F´ proA+B+ $lacI^q$ ΔlacZ fhuA2 Δ(lac-proAB) glnV gal R(zgb-210::Tn10)Tet^S endA1 thi-1 Δ(hsdS-mcrB) (Kind gift of Mr Kyle Willard, Institute for Plant Biotechnology (IPB), Stellenbosch University, South Africa)

XL1 Red (Agilent Technologies): F- endA1 gyrA96(nal^R) thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT Tn10 (Tet^r)[a]

XL1 Blue (Agilent Technologies): endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ $lacI^q$ Δ(lacZ)M15] hsdR17($r_K^- m_K^+$)

Table 7: Plasmids used in this study.

Name	Promoter	Fusion Tag	Signal Peptide	Selection	Origin
pBluescript sk+	T7 promoter	None	None	Amp	Agilent Technologies, USA
pRSET A	T7 promoter	His-Tag N-terminal	None	Amp	ThermoFisher Scientific, USA
pRSET::BGAL32	T7 promoter	His-Tag N-terminal	Present	Amp	Jens Kossmann, IPB*
pRSET::E568Q	T7 promoter	His-Tag N-terminal	Present	Amp	This study
pRSET::W1092F	T7 promoter	His-Tag N-terminal	Present	Amp	This study
pRSET::lacZ	T7 promoter	His-Tag N-terminal	Present	Amp	ThermoFisher Scientific, USA

2.2. Plasmid DNA isolation

Minipreps of plasmid DNA were conducted with the GeneJET plasmid miniprep kit (Fermentas, USA) following the manufacturer's instructions. Maxipreps of plasmid DNA were conducted utilising the alkaline lysis maxiprep method of Sambrook and Russell (2000).

2.3. Preparation and transformation of competent cells

Chemically competent *E. coli* cells were prepared according to the method developed by Hanahan (1991). Electrocompetent *E. coli* cells were prepared according to the method of Sambrook and Russell (2000). Plasmid DNA was transformed into competent *E. coli* DH5α ΔlacZ mutant either by the heat-shock method (Sambrook and Russell 2000) or using a Gene Pulser Xcell (BioRad, USA) for electrocompetent cells. Transformants were selected on LB agar plates [1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) bacterial agar, 40 µg/ml X-gal, 0.1 mM IPTG] supplemented with appropriate antibiotics.

2.4. Random mutagenesis

E. coli XL1-Red was used to introduce random mutations following the manufacturer's protocol. Briefly, XL1-Red competent cells were thawed on ice and 1.7 µl β-mercaptoethanol was added to the competent cell mixture. Cells were incubated on ice for 10 minutes, with the contents gently mixed every 2 minutes. Plasmid DNA (30 ng) was added to the cells and incubated on ice for 30 minutes. Cells were then subjected to heat-shock at 42°C in a hot water bath for 45 seconds, and thereafter incubated on ice for 2 min. Pre-warmed (900 µl, 42°C) SOC medium [2% (w/v) tryptone, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, 0.5% (w/v) yeast extract] was added to the tube, and incubated at 37°C for 60 minutes with shaking at 200 RPM. The transformation mixture (150 µl) was plated out onto LB agar plates [1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) bacterial agar, supplemented with ampicillin (50 µg/ml)] and incubated for 36 hours at 37°C. Colonies were picked at random from the transformation plates using sterile toothpicks to inoculate 260 3 ml LB broth [1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, supplemented with ampicillin (50 µg/ml)], and were incubated overnight at 37°C with shaking at 200 RPM. Individual cultures were utilised to ensure that there was no over-representation of a particular mutation; these were then pooled and a plasmid maxiprep was performed to isolate the plasmids (Sambrook and Russell 2000).

2.5. Screening for oligosaccharide production

Approximately 200 000 colonies were screened using the DH5α ΔlacZ mutant on SOB agar plates [2% (w/v) tryptone, 10 mM NaCl, 0.5% (w/v) yeast extract, 1.5%

(w/v) bacterial agar, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 5% (w/v) lactose, 40 µg/ml X-gal, 0.1 mM IPTG, supplemented with chloramphenicol (34 µg/ml) and ampicillin (50 µg/ml)] containing varying amounts of lactose [between 5-20% (w/v)] and/or M9 minimal media plates [1x Micronutrients (Appendix 6.1), 1x M9 salts (Appendix 6.2), 5 mM $MgSO_4$, 10 mg/L Biotin, 10 mg/L Thiamine-HCl, 1.5% (w/v) bacterial agar, 40 µg/ml X-gal, 0.1 mM IPTG, 20% (w/v) lactose, supplemented with chloramphenicol (34 µg/ml) and ampicillin (50 µg/ml)] (Studier 2005). Chloramphenicol was added to the media as the *LacZ* gene has been replaced with a chloramphenicol resistance cassette in the *E. coli* DH5α ΔlacZ mutant. Plates were incubated at 28°C for a minimum of 7 days to allow for possible oligosaccharide production, which was visually identified by colony morphology associated with oligosaccharide production.

2.6. Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange II XL mutagenesis kit (Agilent Technologies, USA). Mutation primers were designed for specific sites in the BGAL32 gene (Table 8).

Table 8: Mutagenic oligonucleotide primers utilised for site-directed mutagenesis. Nucleotides that were altered to change the amino acids are highlighted in bold and underlined.

Desired mutation	**Forward Primer**	**Reverse Primer**
E568Q	CCTGTTGTACCTTCG**<u>C</u>**AATACTCGCACGCCA	TGGCGTGCGAGTATT**<u>G</u>**CGAAGGTACAACAGG
W1092F	CGGGCGTGCACC**<u>AA</u>**AGCTGTTGTATCCGGCTA	TAGCCGGATACAACAGCT**<u>TT</u>**GGTGCACGCCCG

Site-directed mutagenesis was performed following the manufacturer’s instructions. PCR reactions were carried out under cycling conditions summarised in Table 9. Amplicons were digested with *Dpn*I at 37°C for 1 hour to remove parental plasmid DNA. The digested PCR product was transformed into chemically competent *E. coli* (XL1 Blue), and plated out on LB agar plates [1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) bacterial agar, 40 µg/ml X-gal, 0.1 mM IPTG, supplemented with ampicillin (50 µg/ml)] and incubated overnight at 37°C. The next day, single colonies were inoculated into LB broth [1% (w/v) tryptone, 1% (w/v) NaCl,

0.5% (w/v) yeast extract, supplemented with ampicillin (50 μg/ml)] and grown overnight at 37°C with shaking at 200 RPM.

Table 9: Cycling parameters for mutation PCR.

Segment	Cycles	Temperature	Time
1	1	95°C	1 min
2	18	95°C	50 sec
		60°C	50 sec
		68°C	6 min 20 sec
3	1	68 °C	7 min

2.7. Sequencing

Plasmid DNA of the mutation library was sequenced using a commercial company (Macrogen, South Korea).

2.8. Heterologous protein expression in *E. coli*

E. coli DH5α ΔlacZ cells containing expression plasmids [pRSET::BGAL32 (WT), pRSET A, pRSET::LacZ, pRSET::E568Q, pRSET::W1092F] were inoculated into 5 ml LB [1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, supplemented with ampicillin (50 μg/ml)] and incubated overnight at 37°C with shaking at 200 RPM. The overnight culture was used to inoculate 200 ml ZYP-5052 auto-induction media [1% (w/v) N-Z amine, 0.5% (w/v) yeast extract, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.2x Micronutrients, 0.5% (v/v) glycerol, 0.05 % (w/v) glucose, 0.2 % (w/v) lactose, supplemented with ampicillin (50 μg/ml)] (Studier 2005). The culture was allowed to grow to saturation at 37°C with shaking at 250 RPM. Cells were then sedimented by centrifugation (6000 x *g*, 15 minutes, 4°C). Crude protein was extracted from the cells by resuspending the pellet in 2.5 ml protein extraction buffer [100 mM HEPES-KOH buffer, pH 7.5, 20 μg/ml lysozyme], and cells were lysed by sonication for 10 seconds 4 times. Cell debris and unlysed cells were removed by centrifugation (16 000 x *g*, 20 minutes, 4°C).

2.9. Determination of protein concentration

Protein concentration was determined using the Bradford method (Bradford 1976), with Bovine Serum Albumin (BSA) as a standard. Crude protein was diluted down to

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a working concentration of 10 µg/µl in protein extraction buffer [100 mM HEPES-KOH buffer, pH 7.5].

2.10. SDS-PAGE

Crude protein extracts were visualised on Bis-Tris SDS-PAGE gels under denaturing conditions. Protein samples were mixed with equal volumes of loading dye [50 mM Tris-HCl, 0.02% (w/v) bromophenol blue, 1% (w/v) DTT, 10% (v/v) glycerol, 12.5 mM EDTA, 2% (w/v) SDS] and denatured by incubating samples at 70°C for 10 minutes before being loaded onto a 10% SDS-PAGE gel. Gels were run at a constant 120V.

Gels were developed with colloidal Coomassie stain [0.02% (w/v) Coomassie Brilliant Blue G-250, 5% (w/v) $Al_2(SO_4)_3$, 2% (v/v) orthophosphoric acid, 10% (v/v) ethanol], and excess stain was removed with destaining solution [2% (v/v) orthophosphoric acid, 10% (v/v) ethanol] (Dyballa and Metzger 2009).

2.11. Enzyme activity determination

β-gal activity was determined in a stopped assay examining glucose or galactose release from lactose. 10 µl of enzyme was incubated with 100 mM lactose at 30°C for 10 minutes. The reaction was stopped by boiling at 100°C for 5 minutes and amounts of released glucose and galactose were determined. Glucose was measured by incubating 20 µl aliquots with 180 µl of reaction buffer [150 mM Tris-HCl (pH 8.1), 5 mM $MgCl_2$, 1.5 mM NADP, 1.0 mM ATP]. Boiled protein was utilised as a negative control. The reaction was started by addition of 1.0 U/ml HK and 1.0 U/ml G6PDH (Both from Megazyme, Ireland). Galactose was determined by incubating 20 µl aliquot with 180 µl buffer [150 mM Tris-HCl (pH 8.1), 1.5 mM NAD]. The reaction was started by addition of 1.0 U/ml GalDH and 1.0 U/ml GalM (Both enzymes from Megazyme, Ireland). Both reactions were followed by measuring the change in OD_{340} which was used to calculate the amount of sugar produced. Standard curves were prepared to determine the amount of sugar.

2.12. Determining optimal pH for enzyme activity

The pH optimum of the enzyme was determined by measuring glucose production (Section 2.11) in over a range of values from pH 5.0 to pH 10.0 [McIlvaine buffer, pH

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5.0 to pH 6.0; MES-KOH, pH 6.0 to pH 7.0; HEPES-KOH, pH 7.0 to pH 9.0; Sodium carbonate, pH 9.0 to pH 10.0, all 100mM].

2.13. Determining optimal temperature for enzyme activity

The temperature optimum of the enzyme was determined by measuring glucose production (Section 2.11) over a range, from 4°C to 60°C. Assays were performed in 100 mM lactose monohydrate for 10 minutes at the respective temperatures. Termination of the reactions was achieved by immersion in liquid N_2, and subsequent boiling at 100°C for 5 minutes.

2.14. Examination of enzymatic co-factors

Various divalent metal ions were tested as possible inhibitory or stimulating agents on β-gal activity. This was achieved by incubating the enzyme with 100 mM lactose monohydrate in the presence of various 5 mM metal ions (Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) and the chelating agent EDTA for 10 minutes at 30°C. The enzyme activity was compared to that of a blank control that had no co-factors added.

2.15. Michaelis-Menten kinetics

Michaelis-Menten kinetics for the enzyme was evaluated when the catalysed reaction followed a linear relationship, and determined by examining glucose production (Section 2.11) with increasing concentrations of lactose monohydrate from 5 mM to 200 mM. Kinetic constants K_m and V_{max} were determined utilising Michaelis-Menten non-linear regression plots.

2.16. Enzymatic synthesis of oligosaccharides in an aqueous system

To determine the optimal substrate concentration for GOS synthesis in an aqueous system, GOS synthesis was carried out with lactose monohydrate as a substrate in a total volume of 1 ml, over a range of lactose concentrations from 5 to 30% (w/v) at pH 8.0, crude protein (equivalent of 5 U β-gal activity), and 5 mM $MgSO_4$. Samples were incubated for 24 hours at 30°C with shaking at 250 RPM, and then stored at -20°C.

2.17. Oligosaccharide analysis

2.17.1. Thin layer chromatography

Supernatant from the enzymatic synthesis reaction was diluted to 7.5% (v/w) with ddH_2O, and 0.25 µl was spotted with a pipette onto aluminium backed silica gel 60 TLC plates (Merck, Germany), with butanol/ethanol/water (5:3:2, v/v/v) used as the mobile phase (Rabiu *et al*. 2001; Wang *et al*. 2012). Plates were developed by spraying a solution containing 0.5% (w/v) 3,5-dihydroxytoluene (Sigma-Aldrich, USA) and 20% (v/v) sulphuric acid and heating between two glass plates for 30 minutes at 100°C (Wang *et al*. 2012).

2.17.2. LC-MS

Oligosaccharide samples were desalted utilising AG 1-X8 Formate and AG 50W resin (Bio-Rad, USA) packed spin columns. Columns were packed, and washed twice with 2 ml ddH_2O at 1400 x *g* (2 minutes; 4°C). Carbohydrates were separated on a Waters Acquity UPLC system (Hewlett-Packard, USA), coupled to a Waters Synapt G2 QTOF (Hewlett-Packard, USA). Samples (2 µl) were injected and separated with a UPLC BEH Amide column (1.7 µm particle size; 2.1 x 150 mm; 35 °C) at a flow rate of 0.17 ml/minute. Elution was over a gradient utilising ddH_2O and acetonitrile as mobile phase, both containing 0.1% NH_4OH. The gradient changed from 20:80 (v/v) to 50:50 (v/v) over 22 minutes. The column was then returned to initial conditions for 8 minutes to equilibrate. Electrospray ionization was operated in negative mode, under the following MS conditions: nebulizing gas (N_2) pressure 260 kPa, cone voltage of 40V. Data was analysed in MassLynx version 4.0 (Hewlett-Packard, USA). Analytical grade standards were all purchased from Sigma-Aldrich (USA) unless stated otherwise: lactose (β-Gal-[1-4]-Gluc), 3' galactosyl-lactose (β-Gal-[1-3]-β-Gal-[1-4]-Gluc) (Carbosynth, UK), maltotetraose (α-Gluc-$[1\text{-}4]_3$-Gluc), maltopentose (α-Gluc-$[1\text{-}4]_4$-Gluc), maltohexaose (α-Gluc-$[1\text{-}4]_5$-Gluc), maltoheptahose (α-Gluc-$[1\text{-}4]_6$-Gluc).

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3. Results

3.1. Bioinformatics analysis of BGAL32

The BGAL32 (WT) gene was provided by Prof. Jens Kossmann (Institute for Plant Biotechnology, University of Stellenbosch) and encodes a β-gal isolated from screening a metagenomic library. Blastx (http://www.ncbi.nlm.nih.gov/BLAST/) analysis of the DNA sequence shows that it has significant similarity to a number of known β-gals. Its highest identity is with sequences from *Proteiniphilum acetatigenes* (WP_026327044.1) (79%), *Porphyromonadaceae bacterium* (WP_045090556.1) (73%), *Elizabethkingia* sp. (WP_047034341.1) (65%), *Chryseobacterium piperi* (WP_034681323.1) (63%) and *Bacteroides acidifaciens* (WP_044654332.1) (63%). These β-gals all belong to the GH2 family as they all contained the consensus sequence of the putative active site [LIVMFS]-W-[GSV]-x(2,3)-N-E, as defined by the Carbohydrate Active Enzymes database (CAZy, http://www.cazy.org). The predicted amino acid sequence of BGAL32 was aligned with sequences of bacterial GH family 2 β-gals from *Streptococcus thermophilus* (WP_011226267.1), *Escherichia coli* (AAA24053.1), *Arthrobacter sp. C2-2* (AJ457162.1) and *Kluyveromyces lactis* (GI:343781100) utilising ClustalW (http://www.ebi.ac.uk/clustalw) (McWilliam *et al.* 2013) (Figure 2). Previous studies of the *E. coli* β-gal indicate that the general acid/base site is the glutamic acid at position 461 (Bader *et al.* 1988) while the glutamic acid at position 537 is the catalytic nucleophile (Gebler *et al.* 1992). Jacobson *et al.* (1994) reported that the glutamic acid at position 416 and the histidine at position 418 play roles in the binding of magnesium. Alignments of the bacterial GH2 enzymes indicate that these residues are fully conserved in BGAL32. The predicted molecular weight of the protein based on sequence analysis is 128.75 kDa (CLC Main Workbench, Qiagen). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) indicated the presence of a signal peptide which leads to secretion of the protein out of the cell after it is synthesised (Petersen *et al.* 2011). The signal peptide was not deleted from the pRSET::BGAL32 construct, nor any of the mutants.

```
                             ↓                                        ↓
BGAL32           491  PSVAIWSLGNEAGNGINFFHT 511   558  KGSDRPVVPSEYSHAMGNSSG 578
S.thermophilus   447  ASVIIWSCGNESYAGKDIADM 467   571  NKPQKPYISCEYMHTMGNSGG 591
E.coli           451  PSVIIWSLGNESGHGANHDAL 471   528  PGETRPLILCEYAHAMGNSLG 548
Arthrobacter     431  PSIVMWSLGNESGTGSNLAAM 451   511  RQRTKPFILCEYVHAMGNGPG 531
K.lactis         478  PSIIIWSLGNEACYGRNHKAM 498   548  GKFEKPLILCEYGHAMGNGPG 568
                      .*: :**:***:  * :                  :* : .** *:***. *

                                ↓ ↓
BGAL32           445  GLYVYDEANIESHGMYYGQESLA 467
S.thermophilus   394  GLYVIDEANLETHGTWQKLGLCE 416
E.coli           406  GLYVVDEANIETHGMVPMNRLTD 428
Arthrobacter     383  GFWVILECDLETHGFEAGGWVEN 405
K.lactis         410  GFWVIDEADLETHGVQEPFNRHT 432
                      *::*  *.::*:**
```

Figure 2: Alignments of putative active sites and Mg^{2+} binging sites from BGAL32 and β-galactosidases belonging to GH family 2. The predicted amino acid sequence of BGAL32 is compared to known GH2 β-gal sequences from *Streptococcus thermophilus* (WP_011226267.1), *Escherichia coli* (AAA24053.1), *Arthrobacter* sp. C2-2 (AJ457162.1) and *Kluyveromyces lactis* (GI:343781100). Grey arrows indicate the predicted catalytic acid/base site (E568) and the proton donor (E501); black arrows indicate predicted Mg^{2+} ligand binding sites (E455 and H457). Asterisks (*) indicate fully conserved residues, colons (:) indicate conservation between groups with highly similar properties, periods (.) indicate conservation between groups with dissimilar properties.

3.2. *Escherichia coli* DH5α ΔlacZ mutant

The functional β-gal enzyme can be split into two subunits, namely LacZα and LacZΩ. On their own these subunits are not active, but when both are present they recombine spontaneously to form an active enzyme (Juers *et al*. 2012). In many laboratory strains of *E. coli* there is a deletion of the LacZα subunit resulting in a non-functioning enzyme, even though the LacZΩ is still present and expressed. By transforming a plasmid containing the LacZα fragment into the cell, a functional β-gal will be expressed which will result in colonies turning blue on X-gal. This phenomenon is the basis of blue/white screening - as there is a multiple cloning site in the plasmid that disrupts the LacZα gene, an insertion will result in a loss of β-gal activity. As this project specifically examines novel β-gals an *E. coli* DH5α ΔlacZ mutant, where the complete *LacZ* gene has been replaced with a chloramphenicol resistance cassette, was utilised. This means that although it will not display a blue phenotype on X-gal when transformed with a plasmid that contains sequence encoding the α-fragment, it will when complemented with the full *LacZ* gene (Figure 3).

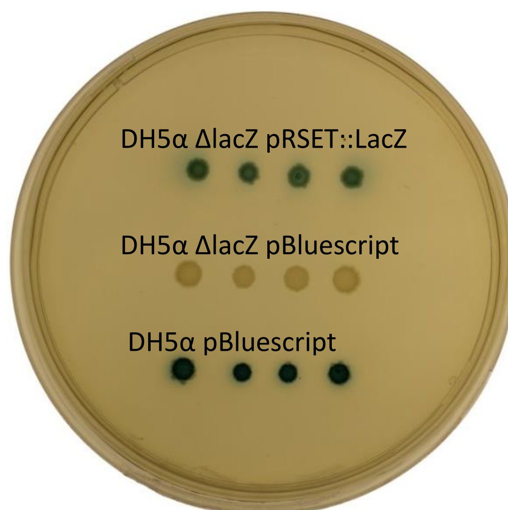

Figure 3: Demonstration of the lack of ability to complement *lacZ* mutations in *E. coli* strains DH5α and DH5α ΔlacZ. Cells were grown on LB agar containing X-gal. Deletion of the LacZ gene results in the loss of β-galactosidase activity, and as such the mutant does not display a blue phenotype when an α-complementation plasmid such as pBluescript is transformed into the cells. This activity can be reinstated by transforming the LacZ gene into the E. coli DH5α ΔlacZ mutant.

3.3. Minimum lactose concentration required for plate based oligosaccharide production

The minimal lactose concentration required for colony morphologies to visibly indicate oligosaccharides was investigated by spotting liquid cultures of *E. coli* DH5α ΔlacZ containing pRSET::BGAL32 onto plates supplemented with X-gal and varying concentrations of lactose. As can be seen in Figure 4, expression of BGAL32 results in a mucoid or slimy colony morphology that are associated with oligosaccharide production (Ruas-Madiedo and de los Reyes-Gavilán 2005) at 10%, 15% and 20% lactose (w/v), but not at 5% (w/v).

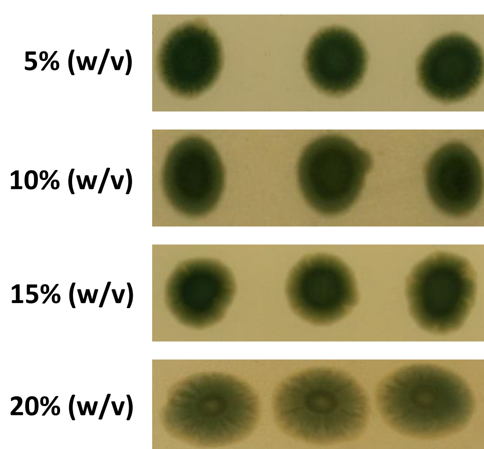

Figure 4: Oligosaccharide production of BGAL32 on agar plates over a range of lactose concentrations. Oligosaccharide production can be seen from 10% (w/v) lactose and higher. All plates contained X-gal, and were incubated at 28°C for 7 days.

3.4. Heterologous protein expression

Crude protein extracts from the *E. coli* DH5α ΔlacZ mutant expressing different genes were separated by SDS-PAGE. The LacZ protein has an estimated molecular weight of approximately 116 kDa, while BGAL32 has an estimated molecular weight of approximately 130 kDa. Proteins of those sizes were observed in gels stained with Coomassie brilliant blue (Figure 5), and their presence is evident comparing colony phenotypes in Figure 12. Low levels of BGAL32 expression are observed when compared to expression levels of LacZ in the *E. coli* DH5α ΔlacZ mutant.

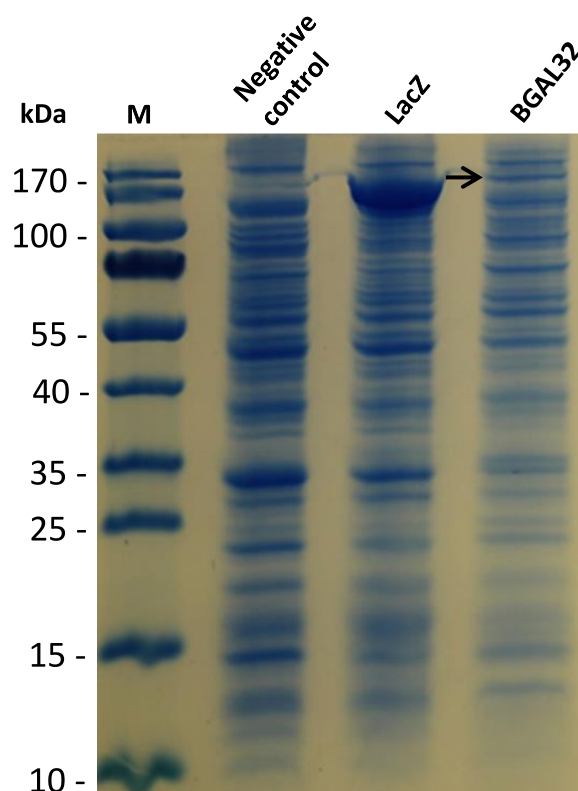

Figure 5: Separation of crude protein extracts by SDS-PAGE. DH5α ΔlacZ cells containing vectors driving expression of *lacZ* or BGAL32 were grown and crude proteins were extracted by sonication. These were denatured and were separated on a 10% (v/v) Bis-Tris denaturing SDS-PAGE gel and stained with colloidal Coomassie brilliant blue. M: PageRuler prestained protein ladder. Negative control: empty pRSET A. LacZ: pRSET::LacZ. BGAL32: pRSET::BGAL32. Arrow indicates BGAL32.

3.5. Enzymatic assays

3.5.1. β-galactosidase activity of *E. coli* DH5α ΔlacZ mutant crude protein extracts

Crude protein extracts were incubated with 100 mM analytical grade lactose to measure hydrolytic β-gal activity. The crude extracts of cells containing pRSET::LacZ and pRSET::BGAL32 exhibited β-gal activity, whereas those containing pRSET A did

not. This indicates that the hydrolytic activity observed is due to heterologously expressed proteins that possess β-gal activity (Figure 6).

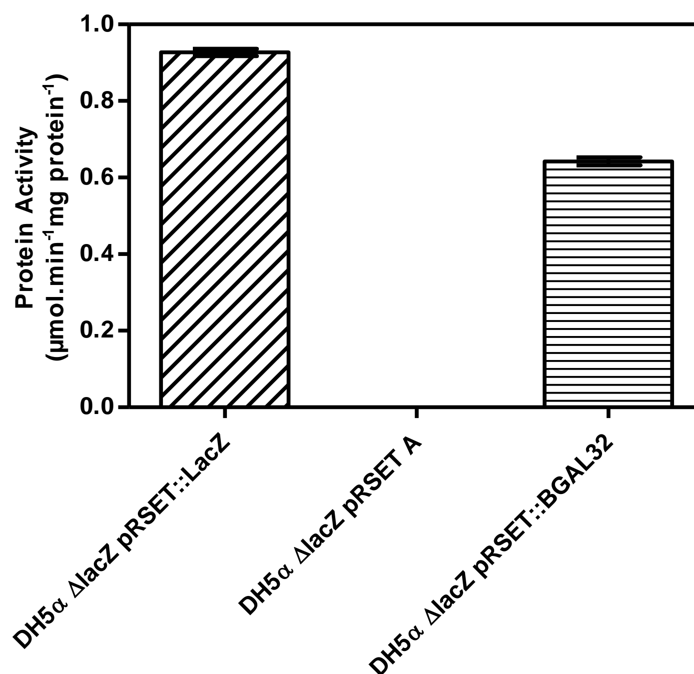

Figure 6: Determining background β-gal activity in the *E. coli* DH5α ΔlacZ mutant. Hexokinase/G6PDH assay to analyse background β-gal activity in the *E. coli* DH5α ΔlacZ mutant. pRSET::LacZ and pRSET::BGAL32 restored hydrolytic β-galactosidase activity; whereas pRSET A did not possess any activity indicating that there is zero background β-galactosidase activity in the *E. coli* DH5α ΔlacZ mutant.

3.5.2. Biochemical characterisation of lactose degradation by BGAL32

To examine the pH range under which the β-gal is active, assays were conducted in various buffers ranging from pH 5.0 to 10.0. Significant levels of β-gal activity were observed between pH 6.0 and 10.0, with maximal activity occurring between pH 7.5 and 9.0. The optimum pH for enzyme activity being approximately pH 8.0 (Figure 7).

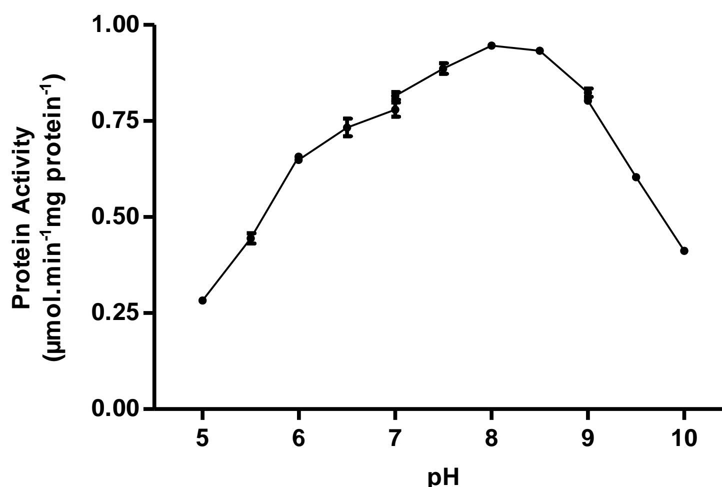

Figure 7: Effect of pH on enzyme activity. β-galactosidase activity was measured in different buffers from pH 5 to 10. Each data point represents 6 independent replicates ± S.E.M.

The optimal temperature for enzyme activity was also assessed over a range from 10°C to 60°C. Optimal enzyme activity was observed between 20°C and 40°C, with maximal activity being observed at 30°C (Figure 8).

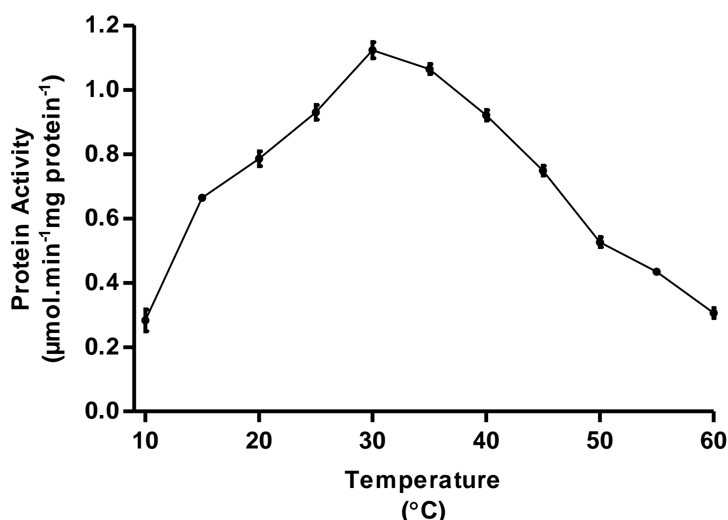

Figure 8: Effect of temperature on enzyme activity. β-galactosidase activity was measured over a range of temperatures (10°C – 60°C). Each data point represents 6 independent replicates ± S.E.M.

The effect of various enzymatic co-factors on β-gal activity was investigated by measuring activity in the presence of various divalent metal ions. The relative activity was determined by comparing activity to a control with no ions added. Ca^{2+} slightly inhibited enzyme activity, with Cu^{2+} and Zn^{2+} having much greater negative effects.

Mg^{2+} and Mn^{2+} on the other hand increased the β-gal activity. The addition of EDTA to the reaction resulted in no change in β-gal activity (Table 10).

Table 10: Influence of various co-factors on β-galactosidase activity. Activity was measured in the presence of various 5 mM divalent metal ions to determine if they inhibited or stimulated activity. Relative activity was determined by comparing β-gal activity to a blank sample that did not have any ions added. Each data point represents 6 independent replicates.

Cation	Relative Activity (%)
Blank (none)	100
EDTA	100
Cu^{2+}	41
Ca^{2+}	89
Mg^{2+}	137
Mn^{2+}	123
Zn^{2+}	24

To determine kinetics for BGAL32, β-gal activity was measured in the presence of increasing concentrations of substrate (5 - 200 mM lactose) under optimum conditions (30°C, pH 8.0, 5 mM $MgSO_4$). The protein displayed classical Michaelis-Menten kinetics, with the K_m and V_{max} being 54.23 mM and 2.26 μmol/minute^{-1}/mg protein^{-1} respectively (Figure 9).

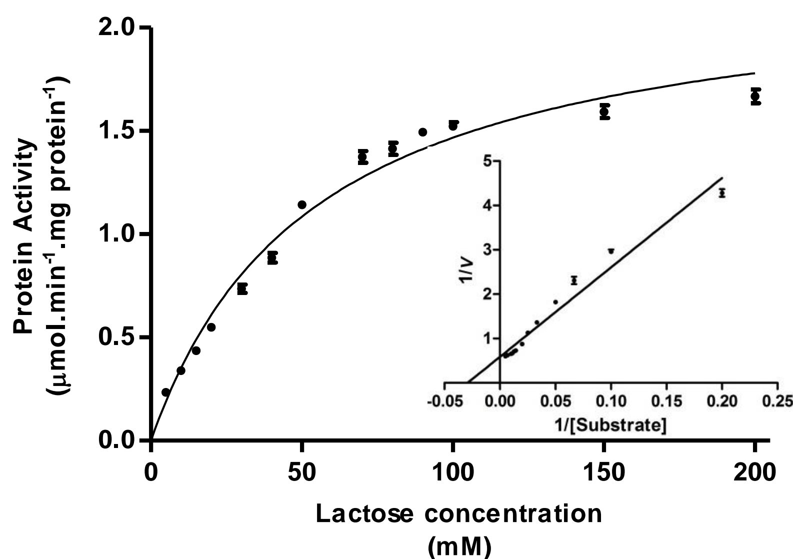

Figure 9: Velocity of BGAL32 at different lactose concentrations. Kinetic constants were determined by measuring β-galactosidase activity under optimal conditions over a range of lactose concentrations from 5 mM to 200 mM. Inset: Lineweaver-Burk plot.

3.5.3. Evaluating transglycosylation activity

Transglycosylation activity of BGAL32 and the W1092F site-directed mutant was determined by comparing the amount of glucose and galactose released when crude protein extracts were incubated with lactose (Figure 10). If only hydrolysis was occurring, then it would be expected that there would be an equimolar release of glucose and galactose. Any discrepancy in this 1:1 ratio is as a result of the transglycosylation of galactose into oligosaccharides by the β-gal, and has been used by a number of authors to mathematically define the affinity of a β-gal to favour transglycosylation over hydrolysis (Torres *et al*. 2010; Palai *et al*. 2012; Frenzel *et al*. 2015).

As can be seen in Figure 10, both BGAL32 and W1092F are polymerising lactose via transglycosylation activity. When comparing the glucose/galactose ratios, the BGAL32 ratio is 1.54, and the W1092F ratio is 1.63. There were no statistically significant ($P<0.05$) differences in glucose and galactose release between the two enzymes, indicating that both have the same rate of transglycosylation. The ratios determined for BGAL32 and W1029F are comparable to the ratios observed by Frenzel *et al*. (2015) for GOS synthesis under similar reaction conditions with the β-gal from *Kluyveromyces lactis*.

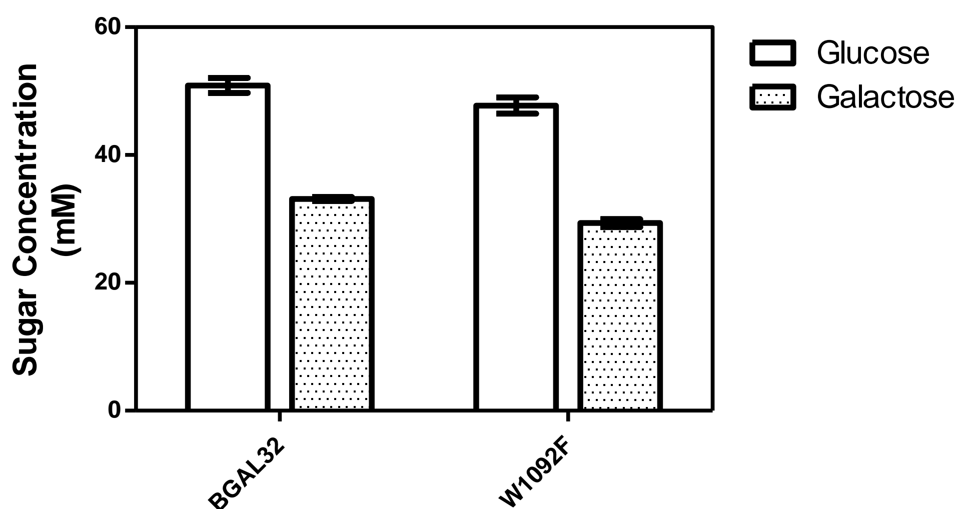

Figure 10: Glucose and galactose production of BGAL32 and W1092F mutant after incubation with lactose. 5U of β-gal was incubated in 100 mM lactose (pH 8.0), 5 mM $MgSO_4$ at 30°C for 24 hours, and then the glucose and galactose concentrations were determined.

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3.6. Random mutagenesis of BGAL32

A random mutagenesis library was created with the intention of screening for the production of oligosaccharides at low substrate concentrations using the *E. coli* XL1-Red mutator strain. To determine the rate of mutation, plasmid DNA was isolated from 96 individual colonies and sequenced. Alignments of the resulting data with BGAL32 sequence allowed quantification of the rate and type of mutations. A total of 28 kb of sequence was analysed, leading to the identification of 94 point mutations, 3 insertions, and 2 deletions. This equates to 95.92% of total mutations being point mutations, 3.06% being insertions and 1.02% being deletions, with the overall rate of mutation determined to be 3.52 per kb. This falls within the mutation rate observed in experiments conducted by Greener *et al.* (1994; 1997).

3.7. Functional screening of random mutagenesis library

As BGAL32 did not exhibit the mucoid phenotype associated with oligosaccharide production at 5% (w/v) lactose concentrations, it was decided to screen the mutagenesis library at this concentration to isolate a clone that exhibited oligosaccharide formation. As BGAL32 was isolated from a metagenomic library that was screened in the *E. coli* ΔlacZ mutant, and it is known to be active in this strain of *E. coli,* it was decided to continue utilising it. Approximately 200 000 *E. coli* colonies were screened, however, no colonies were identified that produced oligosaccharide at these reduced lactose concentrations. The library was screened to completion, as 170 900 colonies would be needed to theoretically screen for every possible mutation. Blue/white screening with X-gal was also utilised to assess mutations and loss of β-gal activity. Approximately 7% of the colonies exhibited a reduction in the intensity of the blue colour or complete loss of β-gal activity.

3.8. Site-directed mutagenesis of BGAL32

BGAL32 was mutated using the QuickChange II kit (Agilent Technologies, USA). As BGAL32 is metagenomically derived, to confirm that it belongs to the GH family 2 the predicted catalytic acid/base glutamic acid as determined by pairwise alignments with the *E. coli* LacZ gene at position 568 was mutated to a non-catalytic residue, glutamine, to try and inactivate activity. The other sequence that was mutated was chosen based on findings by Huber *et al.* (2003), where mutation of the tryptophan at position 999 in the *E. coli* LacZ gene resulted in alterations in the rate of

transgalactosylation due to the role it plays in stabilising the enzyme-glycosyl complex. Feng *et al.* (2005) showed that altering amino acids in these sites could lead to a reduction in the rate of hydrolysis, but increase the rate of transglycosylation. Mutants were confirmed by sequencing and alignments with BGAL32 (Figure 11).

```
BGAL32     KGSDRPVVPSEYSHAMGNSSG
LacZ       KGSDRPVVPSEYSHAMGNSSG
E568Q      KGSDRPVVPSQYSHAMGNSSG

BGAL32     KQQGVAGYNSWGARPLPEYSI
LacZ       KQQGVAGYNSWGARPLPEYSI
W1092F     KQQGVAGYNSFGARPLPEYSI
```

Figure 11: Multiple sequence alignments of mutated plasmids. Predicted amino acid sequence alignments of E568Q and W1092F with BGAL32 and *E. coli* LacZ. Mutation sites are highlighted in grey.

Mutated plasmids were transformed into *E. coli* DH5α ΔlacZ mutant, and grown on agar plates containing 20% (w/v) lactose (Figure 12) and M9 minimal media plates (Figure 13) containing different concentrations of lactose to observe alterations in phenotypes.

Figure 12: Growth of mutants on SOB agar containing 20% (w/v) lactose and 20 µg/ml X-gal. BGAL32, LacZ and the W1092F mutant expressed in DH5α ΔlacZ able to hydrolyse X-gal, but only BGAL32 and W1092F exhibited a mucoid phenotype associated with oligosaccharide production. The E568Q mutation results in loss of β-galactosidase activity. pRSET was included as a negative control.

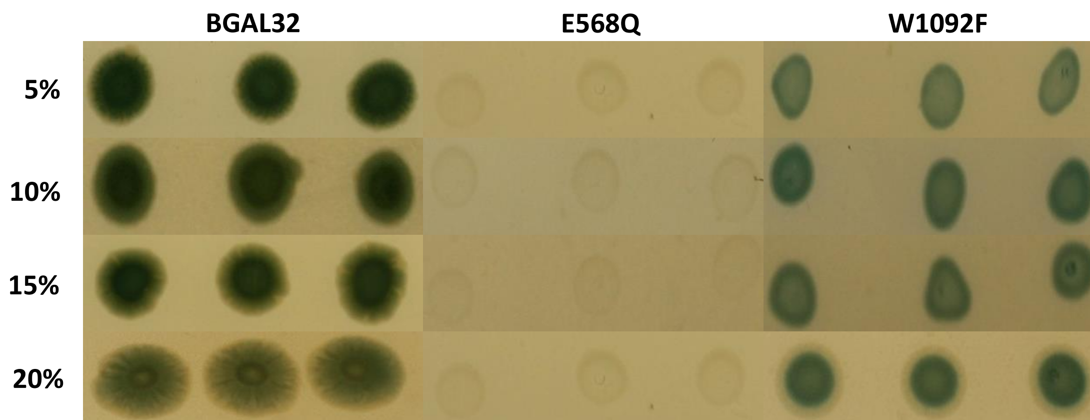

Figure 13: Phenotypes of mutants on M9 minimal media at different lactose concentrations. BGAL32 produces oligosaccharides at lactose concentrations of 10% (w/v). E568Q mutant exhibits minimal growth when lactose is the sole carbon source as all hydrolytic activity has been removed. W1092F mutant has retained β-galactosidase activity, and exhibits a mucoid phenotype at 20% (w/v) lactose.

As can be seen in Figure 12 and Figure 13, the E568Q mutation results in a loss of hydrolytic activity, leading to an inability to hydrolyze X-gal and minimal growth on M9 plates containing lactose as the sole carbon source. The W1092F mutant retained β-gal activity as it turns blue on agar plates containing X-gal and it has retained the ability to produce oligosaccharide based on colony morphology, however this is only observed at 20% (w/v) lactose.

3.9. Oligosaccharide analysis

3.9.1. Thin layer chromatography

To investigate the effect of lactose concentration on GOS synthesis by BGAL32, crude protein extract with the equivalent of 5U of β-gal activity was incubated overnight at 30°C with lactose ranging from 5 – 30% (w/v). The majority of lactose became hydrolysed in all samples (Figure 14), but there was an increase in GOS formation as lactose concentrations increase.

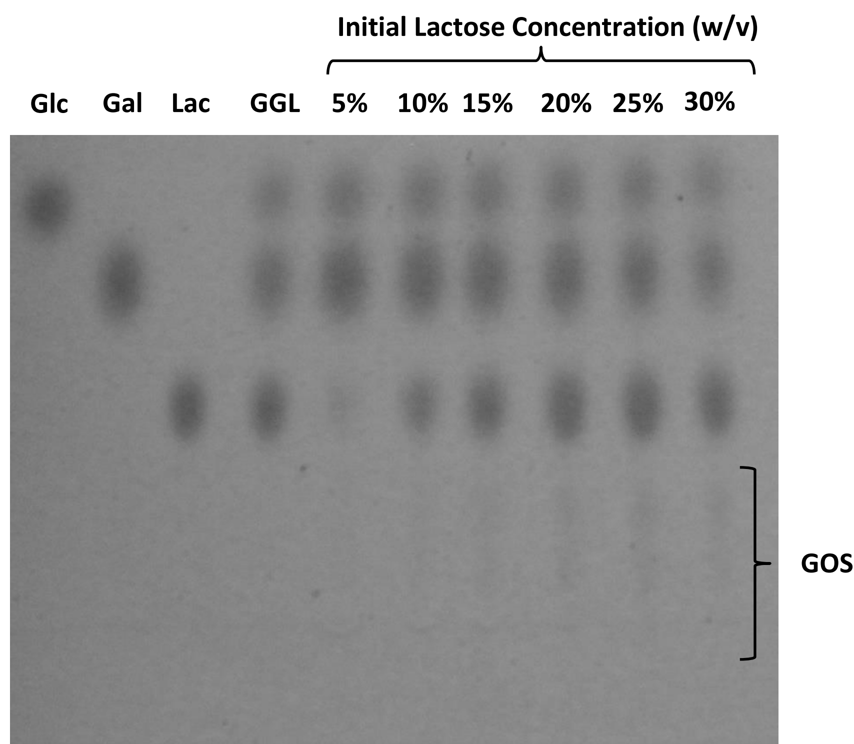

Figure 14: TLC comparing the ability of BGAL32 to form oligosaccharide at different concentrations of lactose. Although BGAL32 can produce low amounts of GOS at 10% (w/v) lactose, increased amounts form as lactose concentrations increase. All reactions were carried out at 30°C for 24 hours in increasing concentrations of lactose, pH 8.0, 5 mM $MgSO_4$, 5U of β-gal. Glc: glucose; Gal: galactose; Lac: lactose; GGL: mixture of equimolar amounts of glucose, galactose and lactose.

The ability of the two site-directed mutants to synthesize oligosaccharides was also investigated. An extract from cells expressing the null mutant E568Q neither hydrolyse lactose nor synthesises GOS while one from the W1092F mutant is still able to hydrolyse lactose although it appears to have reduced hydrolytic ability when compared to BGAL32 when comparing the intensity of the glucose and galactose spots on the TLC It also produces GOS at what appears to be greater concentrations than BGAL32 (Figure 15), although it is not clear if it produces GOS with a higher DP.

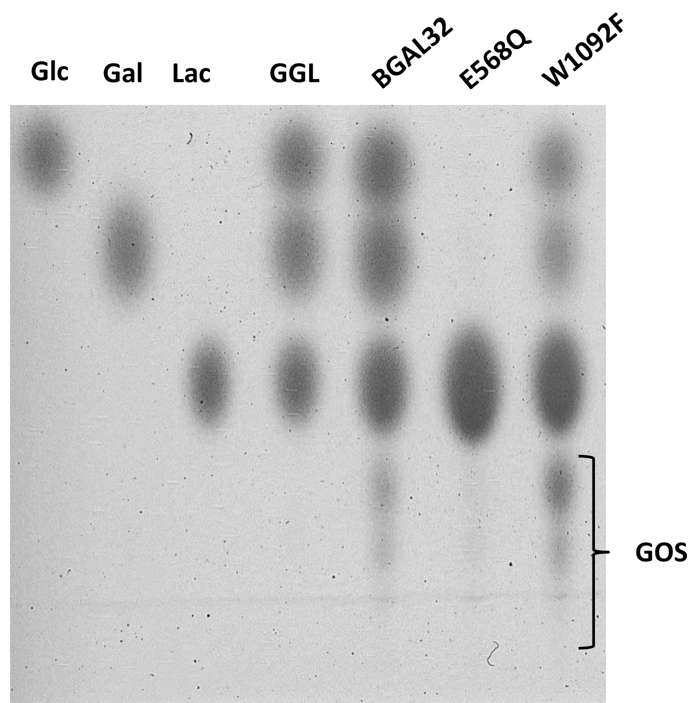

Figure 15: TLC comparing oligosaccharide formation between BGAL32 and E568Q and W1092F site-directed mutants. All reactions were carried out at 30°C for 24 hours in 30% (w/v) lactose, pH 8.0, 5 mM $MgSO_4$, 5U of β-gal. Glc: glucose; Gal: galactose; Lac: lactose; GGL: mixture of equimolar amounts of glucose, galactose and lactose.

3.9.2. LC-MS

To quantitatively assess the oligosaccharides being produced by the transglycosylation reaction of BGAL32 and the two site-directed mutants, LC-MS was utilised. Crude protein extracts were incubated overnight at 30°C in 30% (w/v) lactose with 5 mM $MgSO_4$, and then desalted with anion/cation exchange columns. Samples were diluted and analysed on a QTOF in ESI negative mode. The total ion chromatogram shows that the W1092F mutant produces longer DP oligosaccharides in an aqueous GOS synthesis system than BGAL32, with the null mutant E568Q not having any β-gal activity at all (Figure 16). To illustrate that W1092F produces longer chain oligosaccharides than BGAL32, the mass for DP4 oligosaccharides has been extracted. As can be seen in Figure 17, only the W1092F mutant exhibits peaks above background levels on the chromatogram at this mass, indicating that it is the only sample that possesses oligosaccharides at this molecular mass.

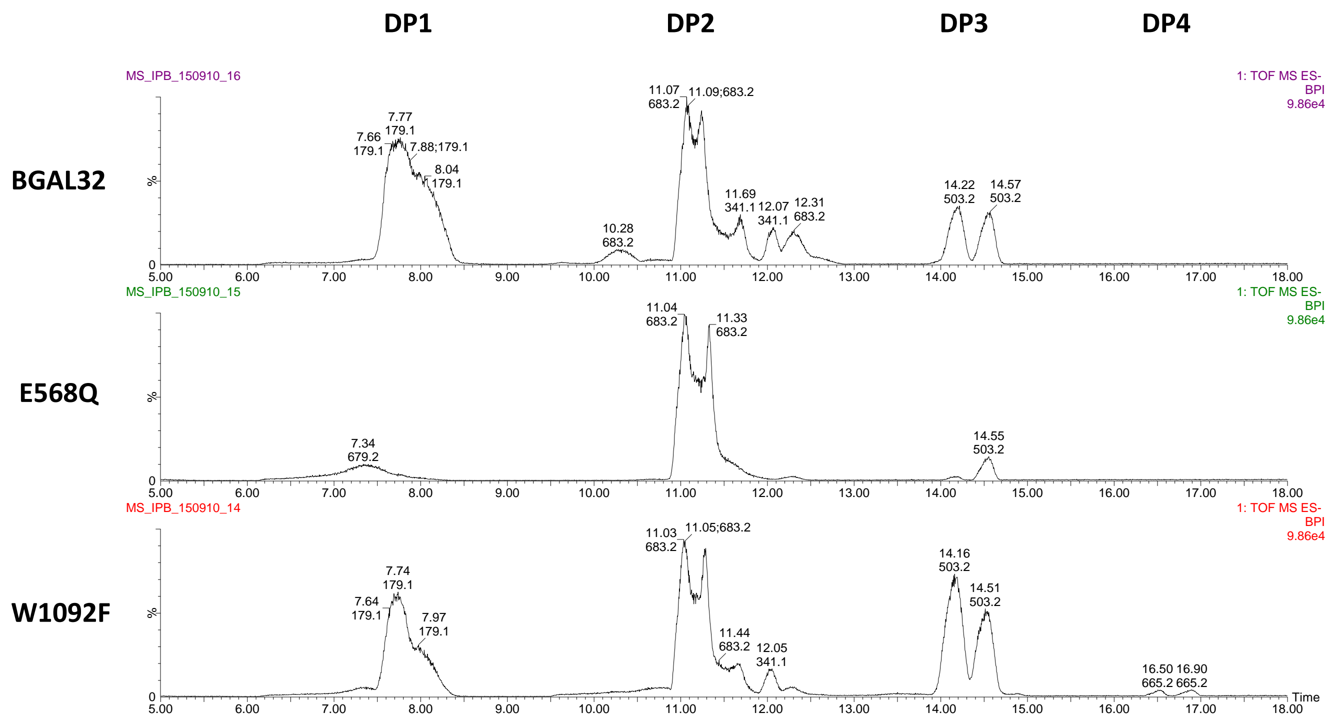

Figure 16: Total ion chromatogram illustrating seperation of oligosaccharides from aqueous incubation of crude protein extracts in lactose. All reactions were carried out at 30°C for 24 hours in 30% (w/v) lactose, pH 8.0, 5 mM $MgSO_4$, 5U of β-gal.

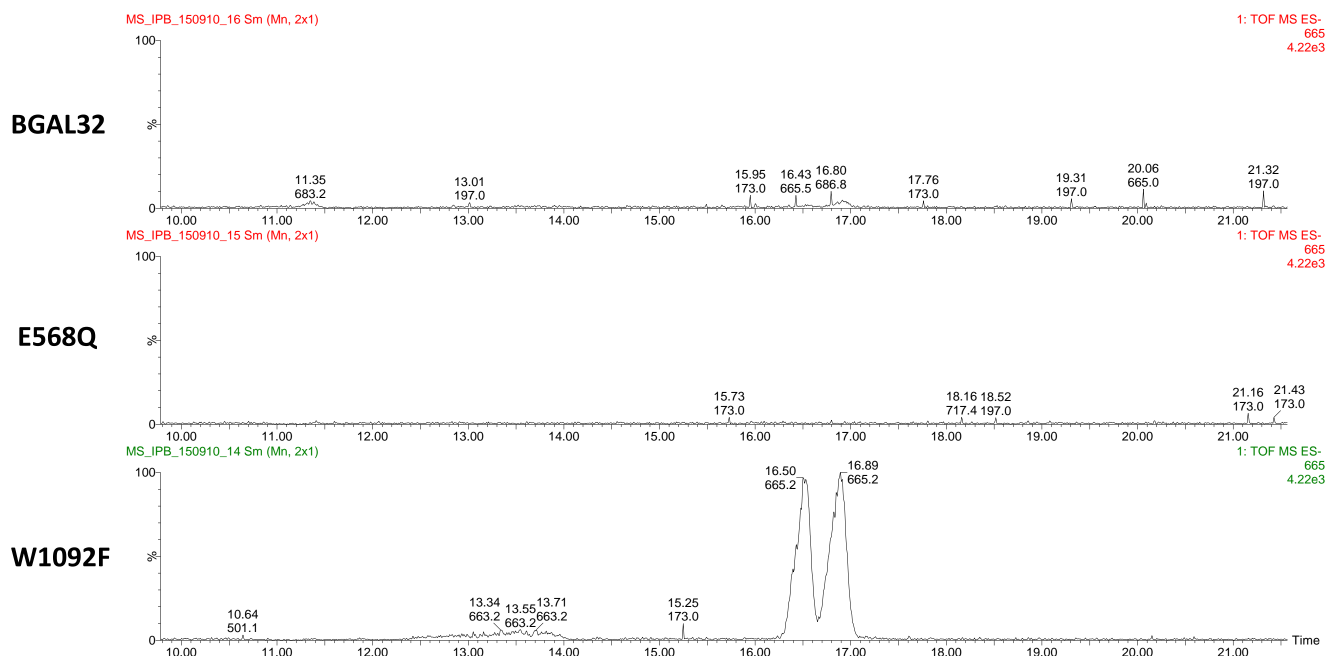

Figure 17: Extracted mass (665) indicating DP4 oligosaccharides. As can be seen above, BGAL32 and E568Q do not produce DP4 oligosaccharides but the W1092F mutant does. This indicates that the W1029F mutation was successful in increasing the DP of the oligosaccharide produced in the aqueous enzymatic incubation.

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Table 11: GOS identified in LC-MS samples by their molar mass and retention times.

Molar Mass (*m/z*) [M-H]	**Degree of polymerisation**	**Retention time (min)**
179	Monosaccharides	7.6 – 8.0
341	Disaccharides	11.60 – 12.05
503	Trisaccharides	14.22 – 14.55
665	Tetrasaccharides	16.50 – 16.89

These results indicate that transglycosylation with BGAL32 produces a mixed population of oligosaccharides over a range of DP, and confirms that the W1092F mutant produces longer chain oligosaccharides than BGAL32 under aqueous conditions (Figure 17).

4. Discussion

Galactooligosaccharides have been receiving increased attention due to their prebiotic activities, however, β-gals that are currently utilised to manufacture these oligosaccharides, tend to favour the hydrolytic reaction over the transglycosylation reaction. It was therefore decided to utilise both random and site-directed mutagenesis in an effort to increase the amount of oligosaccharide produced by a metagenomically derived β-gal belonging to the glycosyl hydrolase 2 family.

4.1. Background of BGAL32

Traditionally, β-gals that are used on an industrial scale for the hydrolysis of lactose and the formation of GOS are derived from *Kluyveromyces*, *Aspergillus* and *Bacillus* species (Husain 2010; Ansari and Satar 2012; Cruz-Guerrero *et al*. 2014). Furthermore, the majority of studies performed on β-gals are from cultured organisms. This means that a large potential source of enzymes has been ignored as it has been reported that up to 99% of microorganisms are unculturable under current laboratory conditions (Handelsman 2004; Xu 2006; Simon and Daniel 2009; Uchiyama and Miyazaki 2009; Iqbal *et al*. 2012), severely limiting the scope of scientists in terms of β-gals can be studied and utilised as biocatalysts. A metagenomic approach has been shown as a successful means to isolate biocatalysts with novel characteristics that can be utilised in a commercial setting (Steele *et al*. 2008), and has been utilised to isolate a diverse assortment including lipases, esterases, polysaccharide modifying enzymes, oxidoreductases and antibiotics (Henne *et al*. 2000; Rondon and Al 2000; Courtois *et al*. 2003; Schmeisser *et al*. 2003; Voget *et al*. 2003; Streit *et al*. 2004).

BGAL32 was previously isolated from screening a metagenomic library that was created from genomic DNA isolated from a soil sample obtained from a dairy farm in an area where large volumes of milk runoff occurred. To determine the functionality of the clone, the sequence was analysed with various bioinformatic programs. The analysis showed similarity between BGAL32 and a number of bacterial β-gals, indicating putative conserved domains at the active site and sugar binding domain. Multiple sequence alignments of the BGAL32 amino acid sequence with those of

various bacterial GH family 2 β-gals allowed the identification of amino acids that act as the acid/base and nucleophile as Glu568 and Glu501 respectively, while the magnesium binding site was predicted to be Glu416 and His418 (Figure 2). These residues are conserved in all GH family 2 β-gals that were used for the alignment.

4.2. Biochemical characterisation of BGAL32

β-Gals are found throughout nature and they play a variety of different roles. The main activity that they are known for is their ability to hydrolyse lactose into glucose and galactose, but they also possess promiscuous activity on other substrates and they have the ability to form oligosaccharides (Wallenfels 1951; Juers *et al.* 2001, 2012; Park and Oh 2010b). When observing the levels of heterologously expressed protein in the DH5α ΔlacZ mutant it is noted that BGAL32 exhibits low levels of expression. This is in all likelihood due to the presence of the signal peptide in the that exports the protein outside of the cell. However, there is still sufficient expression levels to synthesise oligosaccharide and restore β-gal activity to the DH5α ΔlacZ mutant as can be seen in Figure 4.The biochemical characterisation of BGAL32 was performed utilising 100 mM analytical grade lactose as a substrate. The majority of studies utilise artificial glycosides such as the chromogenic substance ONPG for characterisation (Table 12) and focus mainly on the hydrolytic action of the enzyme not its transglycosylation activity (Ly and Withers 1999; Gänzle *et al.* 2008; Gänzle 2012). As the investigation of transglycosylation is the main focus of this study it was decided to characterise BGAL32 utilising crude protein extract and lactose as a substrate; the *E. coli* DH5α ΔlacZ mutant lacks any background β-gal activity, any activity observed utilising lactose as a substrate comes from heterologously expressed protein.

The majority of β-gals exhibit bell-shaped pH activity curves (Ly and Withers 1999) over a broad range of pH values. β-Gals originating in fungi tend to exhibit maximal activity between pH 3.0 and 5.5, where β-gals isolated from bacterial and yeast sources are maximally active between pH 6.0 and 8.0 (Ly and Withers 1999; Husain 2010; Ansari and Satar 2012). The pH curve of BGAL32 exhibited optimal functionality in the range of pH 7.0 and 9.0, similar to a number of β-gals that have been previously characterised (Table 12). The genomic DNA utilised to construct the metagenomic library was isolated from a location where milk runoff occurred at a

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dairy farm and it is interesting to note that the optimal pH of BGAL32 is similar to those reported to be found in such runoff areas (Williamson *et al*. 1998; Ali *et al*. 2007; Carvalho *et al*. 2013). The temperature at which BGAL32 displayed highest activity is in the general range of mesophilic organisms, with optimal activity in the range of 25°C to 40°C; similar to the β-gals from *Arthrobacter* sp. 32cB (30°C) (Pawlak-Szukalska *et al*. 2014), and two metagenomically derived β-gals (38°C and 37°C, respectively) (Wang *et al*. 2010; Erich *et al*. 2015).

It is commonly known that the *E. coli* β-gal as well as numerous other β-gals require divalent cations as enzymatic co-factors which activate the enzyme (Huber *et al*. 1976; Harada *et al*. 1994; Sutendra *et al*. 2007). Investigations with BGAL32 revealed that the enzyme was able to hydrolyse lactose in the presence of 10 mM EDTA indicating that hydrolytic activity is not inhibited by the chelation of divalent ions, however, β-gal activity was increased by the inclusion of Mg^{2+} and Mn^{2+}. This is to be expected as the bioinformatic analysis of BGAL32 shows that there is an Mg^{2+} binding site in the enzyme (Figure 2). It is currently unclear exactly how the divalent cation activates enzymatic activity, but it is hypothesised its binding alters the conformation of the active site in such a way that the acid/base residue is located in the optimal position to allow nucleophilic attack of the bound glycosyl (Juers *et al*. 2001; Lo *et al*. 2010). It was previously observed that BGAL32 did not produce oligosaccharides on agar plates that were not supplemented with Mg^{2+}. This finding is supported by studies conducted on the *E. coli* β-gal transglycosylation reaction, which showed a significant decrease in transglycosylation in the absence of Mg^{2+} (Shifrin and Hunn 1969; Huber *et al*. 1976, 1979). Zn^{2+} and Cu^{2+} significantly inhibited β-gal activity to 42% and 21% respectively. Although it is not clear why that is the case, these cations could possibly interfere with substrate binding. Ca^{2+} inhibited the β-gal activity of BGAL32 by 10% and the overall concentration of calcium is relatively high in milk, however, the concentration of free Ca^{2+} ions in milk is low - approximately 2 mM – thus it may not result in high levels of inhibition on the enzymatic activity of BGAL32 if it was to be utilised to produce GOS from milk products (Gupta *et al*. 2012).

Table 12: Biochemical characteristics of several β-galactosidase enzymes. 1 unit of enzyme activity is expressed as 1 μmol of product per minute, and is determined under the optimal conditions for the enzyme. *Note: total protein activity – crude extract utilised to determine activity.

Source organism	**Opt temp (°C)**	**Opt pH**	**Activity (ONPG) (U/mg)**	**Activity (Lactose) (U/mg)**	**Metal Ion Required**	**GOS Synthesis**	**Reference**
Metagenomic	30	8.0	-	1.5*	Mg^{2+}, Mn^{2+}	Yes	This study
Metagenomic	50	7.0	314	32	-	Yes	(Wang *et al.* 2014)
Metagenomic	65	8.0	148	2.76	Mn^{2+}, Zn^{2+}, Li^{2+}	Yes	(Gupta *et al.* 2012)
Metagenomic	78	6.8	185	47.6	Mg^{2+}, Ca^{2+}	No	(Zhang *et al.* 2013)
Metagenomic	38	7.0	243	86	Na+, K^{+}, Ca^{2+}	No	(Wang *et al.* 2010)
Metagenomic	37	7.0	-	6.7	Mg^{2+}	No	(Erich *et al.* 2015)
Alicyclobacillus acidocaldarius	65	5.5	13	-	-	No	(Di Lauro *et al.* 2008)
Arthrobacter sp. 32cB	30	8.0	213	42	Mg^{2+}	Yes	(Pawlak-Szukalska *et al.* 2014)
Bacillus megaterium	55	7.5	60	-	None	Yes	(Li *et al.* 2009)
Bacillus stearothermophilus	70	7.0	125	-	K^{+}, Mn^{2+}	No	(Chen *et al.* 2008)
Bacillus licheniformis DSM 13	50	6.5	270	13.7	Na+, K^{+}	Yes	(Juajun *et al.* 2011)
Caldicellulosiruptor saccharolyticus	80	6.0	211	9.8	None	Yes	(Park and Oh 2010a)
Deinococcus geothermalis	60	6.5	38	11	-	No	(Lee *et al.* 2011)
Geobacillus stearothermophilus	65	6.5	0.5	0.34	-	Yes	(Placier *et al.* 2009)
Halomonas sp. S62	45	7.0	118.5	-	Fe^{2+}, Mn^{2+}, Na^{+}, Co^{2+}, Ca^{2+}	No	(Wang *et al.* 2013)
Lactococcus lactis	15-55	6.0-7.5	36	-	Mg^{2+}, Fe^{2+}	No	(Vincent *et al.* 2013)
Lactobacillus crispatus	50	5.5-6.5	221	-	Mg^{2+}, Mn^{2+}, K^{+}	Yes	(Nie *et al.* 2013)
Pseudoaltermonas haloplanktis	10	8.5	-	20.4	Mg^{2+}, Mn^{2+}, Ca^{2+}, Li^{+}	No	(Hoyoux *et al.* 2001)
Kluyveromyces lactis	40	7.0	560	124	Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ca^{2+}	Yes	(Kim *et al.* 2003; Martínez-Villaluenga *et al.* 2008b)

The β-gal activity of BGAL32 exhibited classical Michaelis-Menten enzyme kinetics. The K_m of the enzyme was determined to be 54.3 mM and the V_{max} = 2.26 µmol/minute^{-1}/mg protein. This K_m value is relatively high when related to results obtained for some other β-gals that have been characterised which range between approximately 1 and 17 mM (Li *et al.* 2009; Placier *et al.* 2009; Vincent *et al.* 2013; Zhang *et al.* 2013; Wang *et al.* 2014; Erich *et al.* 2015). However, it is similar to that of a number of others (Di Lauro *et al.* 2008; Wang *et al.* 2010), as well as the commercial enzymes originating from *Aspergillus niger* and *Aspergillus oryzae* which range between 36 – 100 mM (De Roos 2004).

4.3. Mutagenesis of BGAL32

When a plasmid encoding BGAL32 was expressed in *E. coli*, the cells demonstrated a mucoid phenotype when grown on plates with lactose concentrations greater than 10% (w/v). This indicates that it produces GOS outside of the cell, albeit only when lactose concentrations are relatively high. In an attempt to increase the DP of the oligosaccharide produced by BGAL32, as well as decrease the concentration of lactose that is required to synthesise them, BGAL32 was randomly mutated using the *E. coli* XL1-Red strain. This strain lacks three of the primary DNA repair pathways – *mutT* (inability to hydrolyse 8-oxodGTP), *mutD* (deficient in 3´- to 5´-exonuclease of DNA polymerase III) and *mutS* (error-prone mismatch repair). The mutation rate within this strain is 5000 times higher than that of wild-type *E. coli* (Greener and Callahan 1994; Greener *et al.* 1997; Wong *et al.* 2006), with mutations being introduced with each cycle of DNA replication. A number of studies have utilised XL1-Red (Coia *et al.* 1997; Lu *et al.* 2001; Terao *et al.* 2006; Callanan *et al.* 2007; Rasila *et al.* 2009; Theriot *et al.* 2010) as it very fast and simple method to produce a gene library containing mainly point mutations. These are more desirable than insertions or deletions when the desire is to modify, rather than eliminate an activity, as they do not cause frameshifts and are less likely to result in the production of truncated proteins (Greener and Callahan 1994; Greener *et al.* 1997; Wong *et al.* 2006). Sequence analysis of the mutagenesis library indicated that the majority of the mutations were indeed point mutations. The library was screened on agar plates with 5% (w/v) lactose, which is also the approximate concentration of lactose present in bovine milk (Gänzle *et al.* 2008; Rodriguez-Colinas *et al.* 2014). Any clones encoding polypeptides that could synthesise oligosaccharides at this low

concentration would be highly favourable for use in the food industry and in the production of transglycosylated milk (Kunz and Rudloff 2006; Bauer *et al.* 2009; Rodriguez-Colinas *et al.* 2014). Despite screening 200 000 clones, none were found that appeared to produce oligosaccharides based on colony morphology. This could be due to the need for the alteration of more than one amino acid being required to alter the active site structure in a way that alters protein activity, the point mutations that occurred could be silent mutations, or a greater number of colonies need to be screened.

As random mutagenesis of BGAL32 yielded no clones with improved properties, a site-directed mutagenesis approach was utilised in an attempt to produce new variants of BGAL32. This has been shown to be a valuable tool that scientists can utilise to determine structure/function relationships and has been successfully utilised by a number of groups to rationally design biocatalysts to increase their rate of production or alter their activity (Hinz *et al.* 2005; Perugino *et al.* 2005; Di Lauro *et al.* 2008; Park and Oh 2010b; Tran *et al.* 2010; Dong *et al.* 2011; Shumway and Sheridan 2012; Wu *et al.* 2013; Teze *et al.* 2014). Recently, a number of studies have been conducted on β-gals belonging to GH1 (Hansson and Adlercreutz 2001a; Hansson *et al.* 2001; Feng *et al.* 2005; Wu *et al.* 2013) and GH42 (Mackenzie *et al.* 1998; Placier *et al.* 2009) families in an effort to increase the DP as well as the total amount of oligosaccharide that they produce by transglycosylation activity (see section 1.5 for more details, Table 6).

Specific sites selected for the mutation of BGAL32 were identified from literature, as a number of studies have focused on altering oligosaccharide synthesis of glycosyl hydrolases. To confirm that BGAL32 belongs to the GH family 2 the predicted catalytic acid/base glutamic acid, as determined by pairwise alignments with the *E. coli LacZ* gene, at position 568 was mutated to glutamine. Glutamine is similar in size to glutamic acid and thus will not alter the structure of the protein or active site, however, it is unable to carry a charge as glutamic acid can. As expected all β-gal activity was lost in the mutated enzyme, similar to what occurs with the *E. coli* enzyme (Bader *et al.* 1988). This confirms the bioinformatic analysis that the metagenomically derived BGAL32 belongs to the GH family 2 enzymes.

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In addition to the active-site residues, a number of other amino acids play roles in influencing enzymatic activity. These include those that orientate or stabilise the active site residues, as well as ones that are located close enough to the carbohydrate that they bind with it to form the intermediate enzyme-glycosyl complex (Ly and Withers 1999; Muraki 2002). The tryptophan at position 1092 is one of these amino acids and was selected based on findings by Huber *et al*. (2003), where mutation of the tryptophan at position 999 in the *E. coli LacZ* gene resulted in alterations in the rate of transgalactosylation. This residue is conserved in many GH family 2 β-gals and it has been determined that it helps to stabilise the enzyme-glycosyl complex (Jacobson *et al*. 1994; Juers *et al*. 2000; Muraki 2002). This site was selected due to its location and role it plays in stabilising the enzyme-glycosyl complex; Feng *et al*. (2005) showed that altering amino acids in these sites could lead to reducing the rate of hydrolysis, but increase the rate of transglycosylation.

4.4. Oligosaccharide analysis

The transglycosylation activity of β-gals can be utilised to synthesise novel compounds through attaching galactose moieties to other chemicals or oligosaccharides, opening the possibility of applying these enzymes to produce molecules that possess biological activities such as GOS (Panesar *et al*. 2006). Currently, the production of GOS is achieved by batch fermentation production runs, utilising aqueous lactose solutions as substrate (Boon *et al*. 2000b; Gänzle *et al*. 2008; Gänzle 2012; Lamsal 2012). The main drawback in utilising this method to synthesise GOS is that the reaction is shifted away from transglycosylation toward hydrolysis due to the high water content, ultimately resulting in low yield of GOS (Chen *et al*. 2001; Wang *et al*. 2012). This phenomenon was observed with BGAL32 – the TLC data clearly indicates that hydrolysis takes place at all substrate concentrations that the enzyme was incubated with (Figure 14, Section 3.9.1). There have been several studies that have attempted to circumvent this problem with the addition of solvents to the reaction mixture which have shown that the reaction can be shifted towards transglycosylation (Shin and Yang 1994; Chen *et al*. 2001; Maugard *et al*. 2003; Wang *et al*. 2012). This still needs to be explored with BGAL32 to determine whether a solvent-buffer system can be utilised to increase transglycosylation, and whether the protein will remain functional in the presence of

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solvents as most β-gals are both less active and stable under those conditions (Shin and Yang 1994; Chen *et al*. 2001; Maugard *et al*. 2003; Wang *et al*. 2012).

Transglycosylation of lactose can be easily identified by measuring the production of both glucose and galactose with enzymatically coupled assays. Hydrolysis of lactose alone results in equimolar amounts of glucose and galactose being released, however, when transglycosylation takes place relatively more glucose is liberated due to the galactose being used to produce oligosaccharides. Thin layer chromatography has also been widely utilised to qualitatively detect and visualise oligosaccharides (Jørgensen *et al*. 2001; Rabiu *et al*. 2001; Wang *et al*. 2012). It is, however, still possible to estimate the relative concentration of oligosaccharides produced by transglycosylation based on the intensity of the colour that develops from staining the TLC plate. As can be seen in Figure 14 there is an increase in GOS production with increasing initial lactose concentrations, as has also been observed with other β-gals (Chen *et al*. 2001; Maugard *et al*. 2003; Hsu *et al*. 2007; Gosling *et al*. 2011; Wang *et al*. 2012; Srivastava *et al*. 2015). This is most likely because increases in the initial concentration of lactose make acceptor and donor oligosaccharides more available than water to the enzyme, resulting in an increase in the hydrolysis/transglycosylation ratio (Gosling *et al*. 2011; Wang *et al*. 2012). The transglycosylation of BGAL32 was compared to the two mutant β-gals with TLC and it was observed that the W1092F mutant hydrolysed less lactose, but produced more oligosaccharides. The E568Q mutant has lost all hydrolytic activity, and thus only lactose is observed (Figure 15, Section 3.9.1).

LC-MS has become a powerful analytical tool in the study of glycobiology and can be used to accurately quantitate the different oligosaccharide populations that are synthesised by transglycosylation (Dwek 1996; Stick and Williams 2008; Blow 2009; Coulier *et al*. 2009). As there is evidence that suggests that microorganisms propagate differently when fermenting different oligosaccharide structures, it is important to study and evaluate the structure of oligosaccharides if they are to be utilised as prebiotics (Gosling *et al*. 2010; Marín-Manzano *et al*. 2013). A large number of studies have shown that lactic acid bacteria and *Bifidiobacteria* preferentially utilise oligosaccharides with β1-6 glycosidic linkages over β1-4 linkages (Gopal *et al*. 2001; Martínez-Villaluenga *et al*. 2008a, 2008c; Cardelle-

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Cobas *et al*. 2011). LC-MS has been utilised to determine the different linkages of oligosaccharides present in oligosaccharide samples. When comparing the total ion chromatograms of BGAL32 between the two site-directed mutants a difference can be seen. The E568Q mutant only has lactose present in the sample indicating a complete loss of hydrolytic activity, whereas the W1092F mutant produces longer DP oligosaccharides under aqueous reaction conditions than BGAL32, however, the structure of the DP4 oligosaccharide is yet to be elucidated. This result is significant as, in a commercial setting, GOS is typically synthesised by incubation of β-gal enzyme in a high concentration aqueous solution of lactose. Results from a previous study examining a β-gal belonging to the GH family 1 utilised the same sites for mutation between species of and achieved similar results (Wu *et al*. 2013). As this site is highly conserved amongst GH2 enzymes, further investigation is needed to determine whether or not this mutation will yield the same outcome with regard to oligosaccharide synthesis in other GH2 β-gals.

5. Conclusion

In conclusion, a metagenomically derived β-galactosidase that produced oligosaccharide on lactose was biochemically characterised to determine the optimal conditions under which it hydrolyses lactose. It was also subjected to random and site-directed mutagenesis in an effort to increase the amount of oligosaccharide it produced at low substrate concentrations. The random mutagenesis library was screened on agar plates containing 5% (w/v) lactose but no clones exhibiting oligosaccharide production were identified. Site-directed mutagenesis of BGAL32 allowed confirmation of the bioinformatic classification of BGAL32 into the GH2 family as the E568Q null mutant exhibited a loss in the ability to hydrolyse lactose. The W1092F oligosaccharide synthesis mutant exhibited an increase in the degree of polymerisation of the oligosaccharide that was produced in an aqueous GOS synthesis reaction, as well as an apparent increase in the amount of GOS produced. LC-MS experiments indicate that both BGAL32 and the W1092F mutant produce a mixed population of GOS through their transglycosylation activity on lactose, although these different structures need to be identified.

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The findings presented in this thesis are significant, as studies that have been conducted on GH1 and GH42 β-galactosidases with the sole purpose of increasing the DP of the oligosaccharide that they produce have not been carried out on GH2 β-galactosidases. Furthermore, research needs to be conducted to confirm whether or not the W1092F site is compatible with other β-galactosidases belonging to the GH2 family, and whether or not this mutation will cause a similar increase in the DP and amount of oligosaccharide produced in an aqueous system for those enzymes.

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6. Appendix

6.1. Trace Metals Mix

Chemical	Final Concentration
$FeSO_4$ - $7H_2O$	50 mM
$CaCl_2$	20 mM
$MnCl_2$ - $4H_2O$	10 mM
$ZnSO_4$ - $7H_2O$	10 mM
$CoCl_2$ - $6H_2O$	2 mM
$CuSO_4$ - $5H_2O$	2 mM
$NiCl_2$	2 mM
H_3BO_3	2 mM
Na_2SeO_3	2 mM
Na_2MoO_4	2 mM

6.2. M9 Salts

Chemical	Final Concentration
Na_2HPO_4 - $7H_2O$	47.7 mM
KH_2PO_4	22 mM
NaCl	8.5 mM
NH_4Cl	18.7 mM

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