University of Natural Resources and Life Sciences

Department of Food Science and Technology

PhD Thesis

Characterization and engineering of β-glycosidases for biotechnological applications

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ABSTRACT

β-Galactosidases catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides, such as lactose. These features make these enzymes interesting for biotechnological applications, such as the production of galacto-oligosaccharides (GOS) from lactose. As so-called prebiotics, GOS are non-digestible and positively impact the intestinal microflora. GOS are essential components of human breast milk and are of great interest for artificial infant milk formulas, which are among the top industrially produced prebiotics.

In the first part of this work, we investigated the transgalactosylation potential of *Streptococcus thermophilus* β-galactosidase for valorization of lactose in whey. Whey, a by-product of the cheese industry, rich in lactose, is of low economic value and poses a huge environmental burden, when released to waste water. *S. thermophilus* β-galactosidase was shown to be suitable for the enzymatic valorization of lactose in whey, due to its ability to completely hydrolyze lactose in whey, as well as its high transgalactosylation activity. A GOS yield of up to 50 % of total sugars was obtained.

The second part of this work addressed the characterization of *Halothermotrix orenii* β-glucosidase variants with respect to their transgalactosylation products. *H. orenii* β-glucosidase shows an optimal activity over a wide temperature range and at low pH values. The enzyme is active with cellobiose and lactose and has a naturally high transgalactosylation activity. These characteristics, along with its high thermostability, make it an ideal candidate for applications in the food industry. Therefore, *H. orenii* β-glucosidase is of interest for mutagenesis, in order to enhance its transferase activity while reducing its hydrolytic activity. Aim is the generation of a transglycosidase utilizing cheap and easily available substrates, such as lactose. The enzyme was modified by rational design in order to improve the transgalactosylation yield. Five variants (N222F, N294T, F417S, F417Y and Y296F) were generated. In transgalactosylation using lactose, F417S and F417Y showed the highest total GOS yield out of all variants at 70 °C, which was increased by about one third compared to the wild type (WT).

The third part of this work was the generation of a site-saturation library, in order to investigate the influence of position 417 on the catalytic activity in more detail. Most importantly, one variant, F417T, preferred lactose 3-fold over cellobiose, whereas the WT favored cellobiose 15-fold over lactose as substrate – a β-glucosidase was turned into a β-galactosidase.

ZUSAMMENFASSUNG

β-Galaktosidasen katalysieren sowohl die Hydrolyse, als auch die Transgalaktosylierung von β-D-Galaktopyranosiden wie Laktose. Diese Eigenschaften machen diese Enzyme interessant für biotechnologische Anwendungen, wie die Produktion von Galaktooligosacchariden (GOS) aus Laktose. Als sogenannte Präbiotika, unverdauliche Lebensmittelbestandteile, haben GOS einen positiven Einfluss auf die intestinale Mikroflora. GOS sind wesentliche Bestandteile der menschlichen Muttermilch, als solche sind sie von großem Interesse für künstliche Säuglingsmilchprodukte, welche zu den am häufigsten industriell produzierten Präbiotika gehören.

Im ersten Teil dieser Arbeit untersuchten wir das Transgalaktosylierungspotential der *Streptococcus thermophilus* β-Galaktosidase für die Valorisierung von Laktose in Molke. Molke ist ein laktosereiches Nebenprodukt der Käseindustrie, es ist von geringem ökonomischem Wert und stellt eine enorme Umweltbelastung dar, wenn es ins Abwasser gelangt. Es wurde gezeigt, dass die *S. thermophilus* β-Galaktosidase aufgrund der Fähigkeit, Laktose in Molke vollständig hydrolysieren, sowie der hohen Transgalaktosylierungsaktivität für die enzymatische Valorisierung von Laktose in Molke geeignet ist. Es wurde eine GOS-Ausbeute von bis zu 50 % der Gesamtzucker erzielt.

Der zweite Teil dieser Arbeit befasste sich mit der Charakterisierung von Varianten der *Halothermotrix orenii* β-Glukosidase in Bezug auf ihre Transgalaktosylierungsprodukte. Die *H. orenii* β-Glukosidase weist eine optimale Aktivität über einen weiten Temperaturbereich und bei niedrigen pH-Werten auf. Das Enzym ist mit Zellobiose und Laktose aktiv und hat eine natürlich hohe Transgalaktosylierungsaktivität. Diese Eigenschaften, sowie die hohe Thermostabilität, machen es zu einem idealen Kandidaten für Anwendungen in der Lebensmittelindustrie. Daher ist die *H. orenii*-β-Glukosidase interessant für die Mutagenese, um die Transferaseaktivität zu verbessern und gleichzeitig die hydrolytische Aktivität zu verringern. Ziel ist die Generierung einer Transglykosidase, welche billige und leicht verfügbare Substrate wie Laktose umsetzt. In dieser Arbeit wurde das Enzym durch rationales Design modifiziert, um die Transgalaktosylierungsaktivität zu erhöhen.

Fünf Varianten (N222F, N294T, F417S, F417Y und Y296F) wurden generiert. Von allen Varianten zeigten F417S und F417Y die besten GOS-Ausbeuten bei 70 °C, welche verglichen mit dem Wildtyp (WT) um ein Drittel erhöht waren.

Im dritten Teil dieser Arbeit wurde eine Site-saturation Mutagenese durchgeführt, um den Einfluss der Position 417 im Detail zu untersuchen. Zusammenfassend lässt sich sagen, dass eine Variante der *H. orenii* β-Glukosidase, F417T eine 3-fach höhere Preferenz für Laktose gegenüber Zellobiose als Substrat aufwies, wohingegen der WT eine 15-fach höhere Selektivität für Zellobiose gegenüber Laktose zeigte – aus einer β-Glukosidase wurde eine β-Galaktosidase generiert.

DECLARATION OF CONTRIBUTION

Geiger, B., Nguyen, H.M., Wenig, S., Nguyen, H. A., Lorenz, C., Kittl, R., Nguyen, T.H. (2016). From by-product to valuable components: Efficient enzymatic conversion of lactose in whey using β-galactosidase from *Streptococcus thermophilus*. Biochem Eng J. 116: 45-53.

Barbara Geiger performed the following experiments that are part of the publication: Expression of recombinant β-galactosidase in *L. plantarum*, fermentation of recombinant *L. plantarum,* β-galactosidase assays, temperature dependence of stability in phosphate buffer and in whey, determination of protein concentrations, lactose hydrolysis and GOS synthesis. Barbara Geiger drafted a part of the manuscript.

Hassan, N., Geiger, B., Gandini, R., Patel, B.K C., Kittl, R., Haltrich, D., Nguyen, T.H., Divne, C., Tan, T.C. (2015). Engineering a thermostable *Halothermothrix orenii* β-glucosidase for improved galacto-oligosaccharide synthesis. Appl Microbiol Biotechnol. 100: 3533–3543.

Barbara Geiger performed the following experiments that are part of the publication: TLC screening for GOS production by *H. orenii* β-glucosidase variants using cell lysates, analysis of transgalactosylation and GOS production by *H. orenii* β-glucosidase variants. Barbara Geiger drafted a part of the manuscript.

Geiger B., Jandova Z., Nguyen T. H., Oostenbrink C., Haltrich D, Kittl R. Elucidating structure-function relationships of *Halothermotrix orenii* β-glucosidase by probing the active site. Manuscript in preparation for Appl Microbiol Biotechnol.

Barbara Geiger performed the following experiments that are part of the manuscript: Generation of the *H. orenii* β-glucosidase site saturation library on position 417, fast screening of variants, expression and purification of the proteins, β-galactosidase assays, kinetic measurements, stability measurements, analysis of transgalactosylation and transglucosylation activity. Barbara Geiger drafted the manuscript.

Nguyen, T. T., Nguyen, H. M., Geiger, B., Mathiesen, G., Eijsink, V. G., Peterbauer, C. K., Nguyen, T. H. (2015). Heterologous expression of a recombinant lactobacillal β-galactosidase in *Lactobacillus plantarum*: effect of different parameters on the sakacin P-based expression system. Microb Cell Fact. 14, 30.

Barbara Geiger performed enzyme measurements that are part of the publication.

SCOPE AND AIMS OF RESEARCH

Overall, the aim of this research was the biochemical characterization of *S. thermophilus* β-galactosidase and *H. orenii* WT β-glucosidase and variant β-glucosidases with a particular focus on transglycosylation. Variants *of H. orenii* β-glucosidase were generated by rational design.

CHAPTER 1

Whey is a by-product in the food industry and poses a huge environmental burden. In this work crude cell-free extracts of *S. thermophilus* overexpressed in the food-grade organism *Lactobacillus plantarum* were analyzed for efficient lactose hydrolysis and GOS formation. Use of the crude cell-free extract eliminates the need for extensive and cost intensive enzyme purification procedures.

Research question

Can *S. thermophilus* β-galactosidase efficiently valorize lactose in whey?

CHAPTER 2

Thermostable *H. orenii* β-glucosidase shows transgalactosylation activity with lactose as substrate. Here, we aimed to enhance the already high transgalactosylation activity of *H. orenii* β-glucosidase with lactose as substrate, using a rational design approach to generate variants N222F, N294T, F417S, F417Y and Y296F.

Research question

Can the transgalactosylation activity of *H. orenii* β-glucosidase be improved by rational design?

CHAPTER 3

Among all variants of *H. orenii* β-glucosidase generated by rational design, variants F417S and F417Y showed the highest GOS yield at 70 °C. Therefore, this position located in the active site was further investigated by generation of a site saturation library.

Research question

How do amino acid exchanges on particular position 417 impact enzymatic activity?

CHAPTER 4

Food-grade expression systems such as the sakacin P-based expression system (pSIP) are regarded as safe and yield high amounts of the recombinant target protein. Safe organisms, such as *L. plantarum* or *L. reuteri* are used as expression host. Various parameters that affect recombinant protein production were investigated.

Research question

What is the effect of different parameters on the pSIP expression system?

INTRODUCTION

Glycoside hydrolases

Glycoside hydrolases (EC 3.2.1.x) mainly hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. As a side reaction these enzymes also catalyze glycosidic bond formation (Cantarel et al. 2009). Currently there are about 144 members of the glycosidase families classified (http://www.cazy.org). Depending on their interaction with the substrate, they can be classified into chain end-cleaving exo-glycosidases or internally cleaving endo-glycosidases (Naumoff et al. 2011). The catalytic mechanism of endo- and exoglycosidases is illustrated in Figure 1.
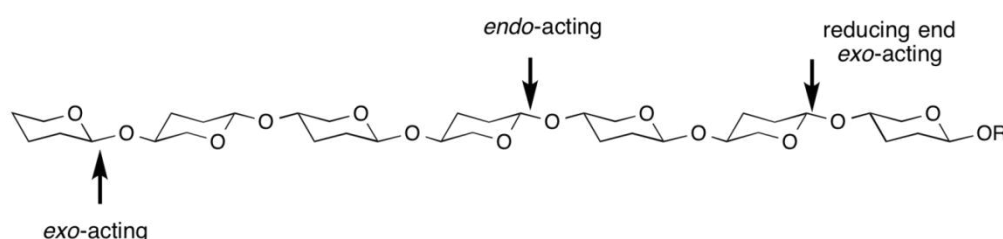

Figure 1 Catalytic Mechanism of endo- and exoglycosidases
(https://www.cazypedia.org/index.php/Glycoside_hydrolases)

Glycoside hydrolases can be further grouped based on the reaction mechanism. There are four groups of glycoside hydrolases distinguished, depending on whether they hydrolyze equatorial or axial glycosidic bonds and whether they invert or retain the anomeric configuration (Sinnott et al. 1990). Retaining as well as converting glycosidases possess two carboxylate amino acid residues in the catalytic center acting as nucleophile and acid/base catalyst. In retaining glycosidases, the two carboxylate residues are located in a distance of about 5.5 Å, whereas in inverting glycosidases the residues are positioned about 10 Å apart. The enzymatic reaction of a retaining β-glycosidase is a double displacement mechanism. The nucleophilic residue attacks the anomeric center in order to form an enzyme-substrate complex. Subsequently the general acid/base residue protonates the glycosidic oxygen and then activates the water molecule. The enzyme converts the substrate under retention of the anomeric configuration of the glycosidic bond of the substrate. The enzymatic

mechanism of an inverting β-glycosidase is a single displacement mechanism. The glycosidic bond oxygen is protonated by the nucleophile and subsequently attacked by a water molecule activated by the base residue. The enzyme converts the substrate under retention of the anomeric configuration of the glycosidic bond of the substrate (Figure 2).
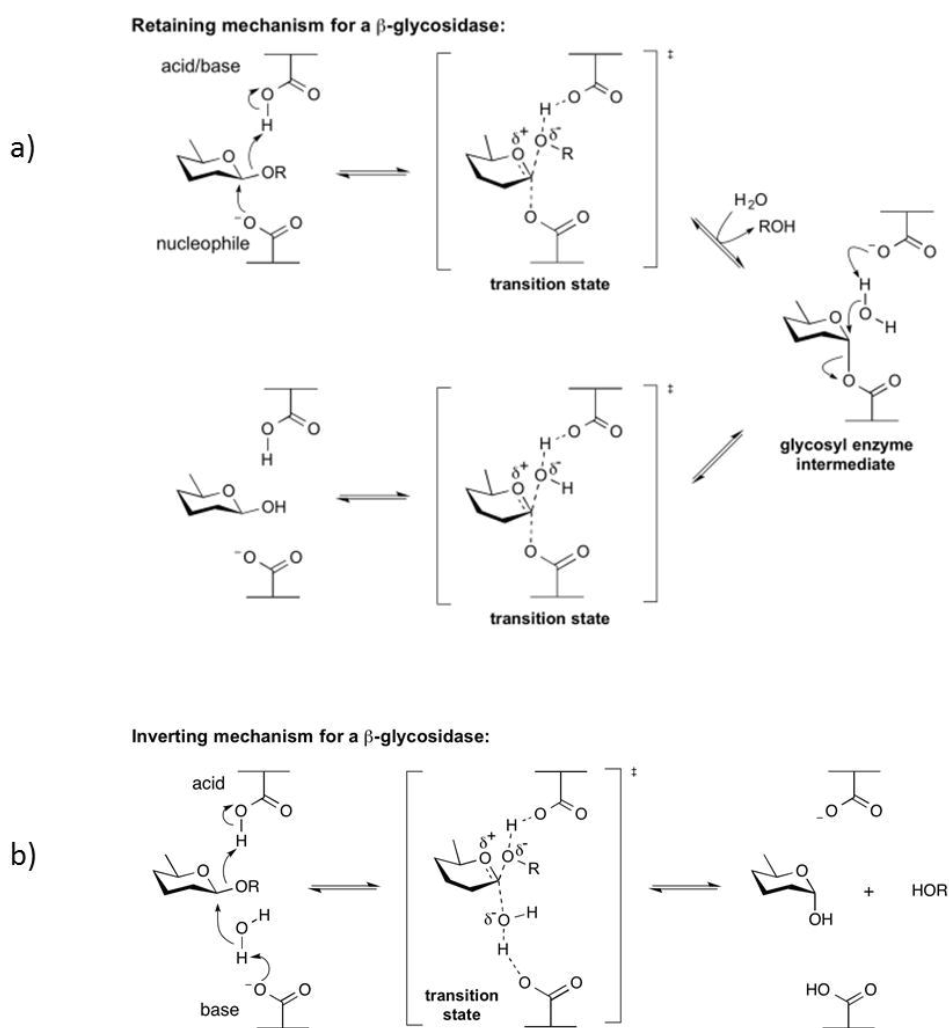

Figure 2

a) Mechanism of a retaining β-glycosidase, b) Mechanism of an inverting β-glycosidase

(https://www.cazypedia.org/index.php/Glycoside_hydrolases)

Henrissat and Davies introduced a classification system of glycoside hydrolases dependent only on the amino acid sequence. Information about structural and mechanistic characteristics can be gained from amino acid sequence only. The catalytic mechanism of an enzyme is dependent on its molecular structure which is conserved within each family. Similar folds of sequence-related families enable homology modelling of related sequences, and also direct the choice of suitable search models for molecular replacement. In the Carbohydrate-Active Enzyme (CAZy) database, enzymes that catalyze formation and degradation of complex carbohydrates are compiled and classified based on amino acid sequence similarity. The enzymes are grouped in families based on experimentally characterized proteins and are enriched by sequences from public databases with significant similarity. This classification allows a better characterization of the structural features of the enzymes, than their substrate specificities only. Moreover, the classification according to amino acid similarities contributes to the understanding of evolutionary relationships and mechanistic properties (Henrissat and Davies 1997).

Transglycosylation and hydrolysis

Hydrolysis is the naturally occurring reaction mechanism for glycosidases. However, under kinetically controlled conditions these enzymes can be used for glycosidic bond formation due to their transglycosylation activity. If lactose is the substrate, GOS are formed. GOS are β-linked oligosaccharides with a chain length of 2-10 monosaccharides, consisting of 1-7 linked galactose units and a terminal glucose. Galactose dimers are also considered as GOS (Tzortzis et al. 2009). In the enzymatic synthesis of GOS, lactose acts both as donor and acceptor of the galactosyl residue to yield trisaccharides which then can act as acceptors to yield oligosaccharides of longer chain length. Development of efficient scale processes for oligosaccharide synthesis is of great interest for the food and pharmaceutical industry. For GOS production, retaining glycosidases (such as β-galactosidase), in particular exo-glycosidases have been applied. Typically β-galactosidases catalyze the formation of GOS, but also β-glucosidases are capable of transgalactosylation (Gosling et al. 2010). GOS formation can be controlled by thermodynamic and kinetic factors. In order to shift the reaction towards transglycosylation, high substrate concentrations and high temperatures are required. A benefit of reactions at high temperatures is the improved solubility of the substrates which enables the use of higher substrate concentrations (Boon et al. 2000). An

increase in the initial lactose concentration results in a small increase in total yield of GOS by β-galactosidase, as long as the lactose remains dissolved (Vera et al. 2012). Most of these transglycosylation approaches are limited by enzyme stability. The ratio of transferase to hydrolase activity is dependent on the concentration of the acceptor, as well as on the ability of the enzyme to bind the sugar acceptor and to exclude water. Hydrolysis of lactose and transgalactosylation occur concomitantly, thereby galactose and glucose are produced, as well as GOS are formed (Prenosil et al. 1987). The GOS synthesis can take place by intramolecular or by intermolecular reactions. The intramolecular reaction mode is also called direct transgalactosylation. In this transglycosylation mode the glucose does not leave the active site after hydrolysis, and the galactose is transferred onto the C6 position of glucose yielding allolactose [(β-D-Gal-(1→6)-D-Glc)], a regioisomer of lactose [(β-D-Gal-(1→4)-D-Glc)]. By intermolecular or indirect transgalactosylation disaccharides, trisaccharides, tetrasaccharides and longer GOS are formed. Here a galactose is transferred onto a sugar acceptor outside the catalytic site of the enzyme (Huber et al. 1976). The potential of β-galactosidase for the hydrolysis of the lactose is determined by the isomeric orientation of the lactose. A greater diversity in formed GOS can be achieved by increasing the initial lactose concentration (Otieno et al. 2010). The general mode of lactose hydrolysis and GOS synthesis is illustrated in Figure 3.
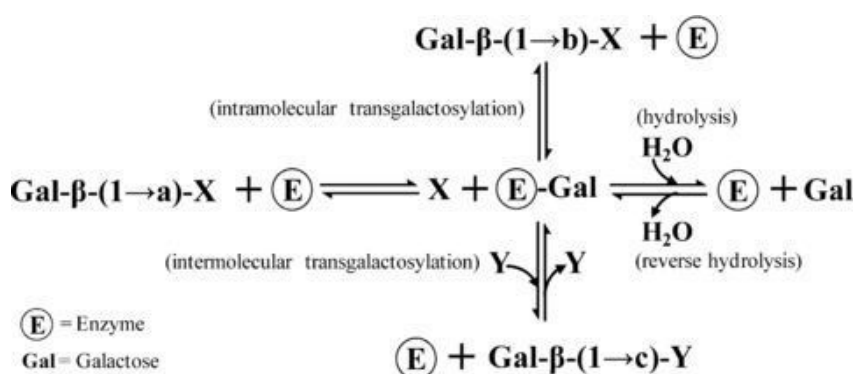

Figure 3 General mode of lactose hydrolysis and GOS synthesis (Torres et al. 2010)

β-Galactosidases

β-Galactosidases belong to the enzyme family of glycoside hydrolases. These enzymes predominantly degrade the disaccharide lactose [β-D-Gal-(1→4)-D-Glc], but also catalyze GOS formation. Moreover, β-galactosidases hydrolyze many different aglycones, such as oNPG, which is routinely used to assay enzyme activity (Juers et al. 2012). β-Galactosidase is produced by prokaryotic microorganisms, as well as by eukaryotes. Enzymatic activity of β-galactosidase is crucial for plenty of physiological processes, such as the release of stored energy for rapid growth by lactose hydrolysis in mammals and bacteria, and the release of free galactose during metabolic recycling of galactolipids and glycoproteins. In mammals, β-lactase is produced in the milk glands and in the pancreas, as well as in the epithelial cells of the jejunum. In food industry β-galactosidase is of great importance in the production of lactose-free dairy products, as well as for the production of GOS which are important components of infant milk formulas. Enzymes of commercial interest are isolated mainly from the yeasts *Kluyveromyces lactis*, *K. fragilis, K. marxianus*, *Candida kefyr* and the fungi *Aspergillus niger* or *A. oryzae* (Grosová et al. 2008), but also bacteria are a source of β-galactosidases for industrial use (Table 1). Enzymes with β-galactosidase activity are classified into GH families 1, 2, 35 and 42 (Lombard, Golaconda Ramulu et al. 2014). Most β-galactosidases belong to the GH2 family, which comprises retaining β-galactosidases. GH2 family members have a narrow substrate specificity and are only active with lactose and β-1,3 and β-1,6 galactosides (Cantarel et al. 2009). Among all galactosidases, the *E. coli* β-galactosidase is the most prominent member. The enzyme is best known for its activity with X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside), which is composed of a galactose linked to a substituted indole. When X-gal is hydrolyzed, the substituted indole is released, resulting in an intensively blue product. On a growth medium supplemented with X-gal, colonies of *E.coli* containing an active β-galactosidase become blue. *E.coli* β-galactosidase cleaves lactose [(β-D-Gal-(1→4)-D-Glc)], releasing glucose and galactose. By transgalactosylation allolactose [(β-D-Gal-(1→6)-D-Glc)] can then be formed further on. Allolactose binds to the lacZ repressor and creates the positive feedback loop regulating the amount of β-galactosidase in the cell. The allolactose in turn can be degraded to the monosaccharides again (Figure 4). *E coli* β-galactosidase needs Na^+ and Mg^{2+} for full activity (Juers et al. 2012).
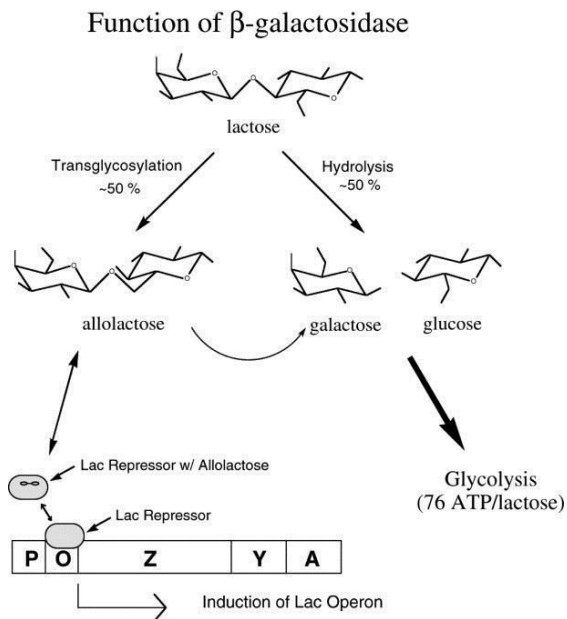
Figure 4 Function of *E.coli* β-galactosidase (Juers et al. 2012)

E.coli β-galactosidase is configured as a tetramer of four identical polypeptide chains, each of 1023 amino acids. In *E.coli* β-galactosidase E461 acts as nucleophile, and E537 is the acid/base catalyst (Figure 5).

Figure 5 *E. coli* β-galactosidase (http://www.rcsb.org/pdb/explore.do?structureId=1JYN)

a) *E.coli* β-galactosidase homotetramer

b) Zoom into the active site
E461 is the nucleophile, E537 the acid/base catalyst

In our work, we focused on *S. thermophilus* β-galactosidase, which is routinely used for yoghurt production together with *Lactobacillus bulgaricus* (Guarner et al. 2005). *S. thermophilus* β-galactosidase belongs to GH family 2. It configured as homotetramer

consisting of four 118 kDa monomers. As described in CHAPTER 1, at 50 °C the enzyme has a half-life of more than one day, the GOS yield of 50 % achieved by this enzyme is high compared to the amount produced by commercial enzymes (Geiger, Nguyen et al. 2016). GOS yields of different β-galactosidases derived from lactobacilli are shown in the Table 1 below.
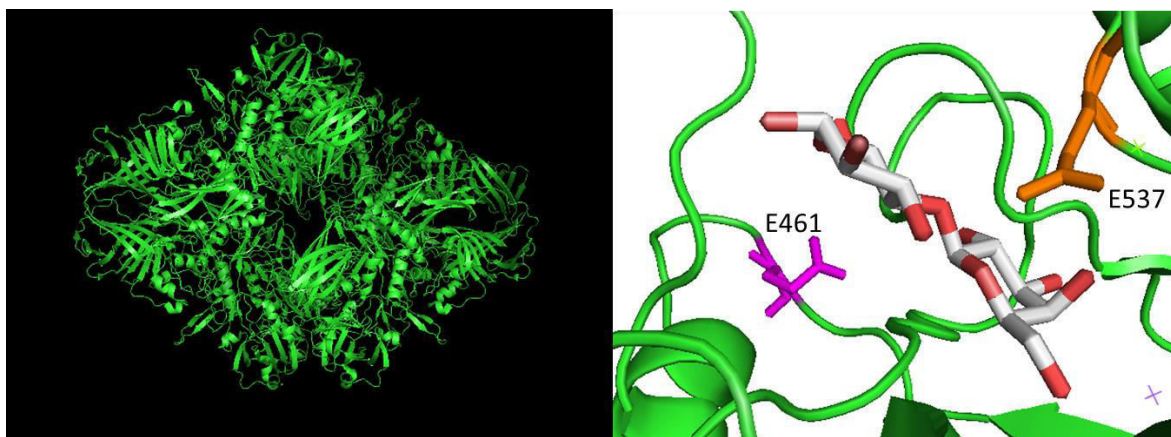
β-galactosidases					
Organism	°C	Initial lactose	GOS yield in %	β-linkages	References
L. reuteri	30	205 g/l	38	1,3 1,6	(Splechtna et al. 2006)
L. sakei	37	215 g/l	41	1,3 1,6	(Iqbal et al. 2011)
L. plantarum	37	215 g/l	41	1,3 1,6	(Iqbal et al. 2010)
L. pentosus	30	205 g/l	31	1,3 1,6	(Maischberger et al 2010)
L. bulgaricus	30	215 g/l	49.5	1,3 1,6	(Nguyen et al. 2012)

Table 1: GOS yield of different commercially used β-galactosidases from Lactobacilli

β-Glucosidases

β-Glucosidases are the major group among glycoside hydrolases (Bhatia et al. 2002) and are ubiquitous in bacteria, archaea and eukaryotes, playing key roles in biological processes such as metabolizing cellulose and other carbohydrates, developmental regulation as well as in defense mechanisms against pathogen invasion. Practical applications of these enzymes are diverse. β-Glucosidases are used in various industrial applications, such as the improvement of flavor, the production of bio-degradable non-ionic surfactants, synthesis of diverse oligosaccharides, glycoconjugates, and alkyl-aminoglycosides, as well as in lignocellulose hydrolysis and biofuel production. Based on the amino acid sequence similarity which reflects structural and catalytic mechanisms, β-glucosidases are grouped by CaZy into glycoside hydrolase (GH) families 1 or 3. GH1 and GH3 β-glucosidases both belong to the family of retaining β-glucosidases. GH3 family members have aspartic acids as nucleophile/base in the active center, whereas GH1 β-glucosidases have glutamic acids as nucleophile/base in the active center (McCarter et al. 1994, White et al. 1994). β-Glucosidases (E.C. 3.2.1.21) show activity with natural substrates, as well as with synthetic aryl-glycosides and some aglycons. According to their substrate specificity, β-glucosidases are classified in three groups: (1) aryl-β-glucosidases with a high specificity for substrates such as pNPG, (2) true cellobiases with a high specificity towards cellobiose and (3) broad substrate specificity enzymes. Most β-glucosidases are members of the broad substrate specificity family with diverse catalytic mechanisms including cleavage of β-1,4, β-1,6, β-1,2, α-1,3, α-1,4, and α-1,6 glycosidic bonds (Bhatia et al.2002, Cairns et al. 2015, Tokpohozin et al. 2016)

An ideal β-glucosidase should be active over a wide pH range and stable at elevated temperatures. *H. orenii* is a thermophilic and halophilic gram negative bacterium. It grows at temperatures of up to 70 °C and at NaCl concentrations of 5-10 % (Kori et al. 2011). *H. orenii* β-glucosidase is an unspecific β-glycosidase with mixed activities for different substrates and prominent activity with various galactosides such as lactose, the enzyme is highly active and stable within a broad pH range. The temperature optimum is in the range of 65-70 °C, and *H. orenii* β-glucosidase also shows excellent thermostability at this temperature range. The enzyme retains 90 % of its activity after 3 h of incubation at 65 °C. Half-life times of activity (τ1/2) are 18 hours and 6 hours at 65 and 70 °C (Hassan et al. 2015).

The *H. orenii* genome contains genes for two β-glucosidases Hore-19810 (GCCC-2224) and Hore-04820 with a length of 739 amino acids and 451 amino acids, respectively. β-Glucosidase A gene (Hore-04820) was previously crystallized and biochemically characterized. The purified recombinant protein overexpressed in *E.coli* appears as 53 KDa when analyzed by SDS-PAGE (Bhattacharya et al. 2014). In *H. orenii* β-glucosidase A E354 acts as nucleophile, E166 is the acid/base catalyst and E408 is the substrate binding site (Figure 6). The main transgalactosylation products from *H. orenii* β-glucosidase are 6'-galactosyllactose [β-D-Gal-(1→6)-D-Lac] and 3'-galactosyllactose [β-D-Gal-(1→3)-D-Lac] (Kori et al. 2011, Hassan et al. 2015).
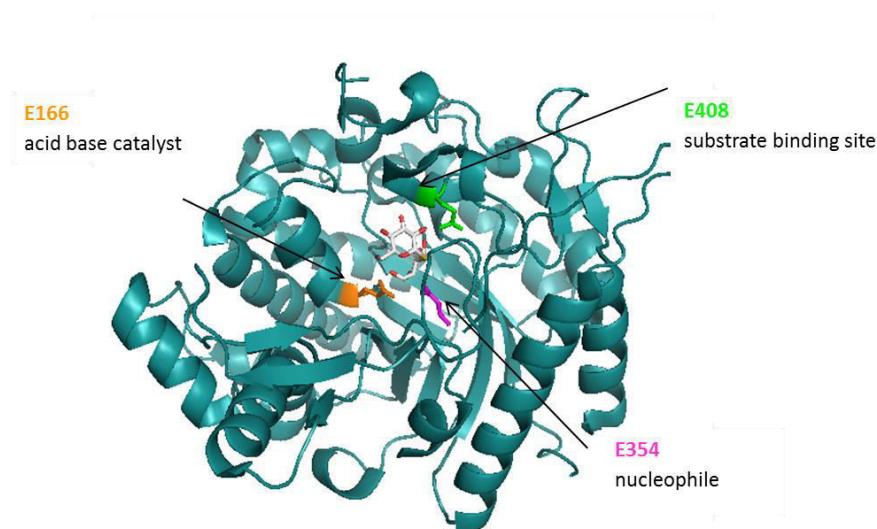

Figure 6: Crystal structure of monomeric *Halothermotrix orenii* β-glucosidase A (http://www.rcsb.org/pdb/explore.do?structureId=4PTV)

Fields of applications

In food industry β-galactosidases are commonly used to reduce the amount of lactose in dairy products. About 75 % of adults worldwide are lactose intolerant and therefore unable to digest lactose present in dairy products. Lactose intolerance is caused by the lack of the β-lactase in the mucosa of the small intestine. Consumption of milk products causes abdominal pain, diarrhea, cramps, or flatulence in lactose intolerant individuals. Lactose is degraded to the digestible sugars glucose and galactose by β-galactosidase. High lactose concentrations in milk products cause excessive lactose crystallization. Application of β-galactosidase in dairy industry reduces the amount of lactose. The texture, solubility and digestibility of dairy foods are improved, increasing their quality. In the cheese industry lactose-rich whey is a by-product, which causes several economic and environmental problems. Hydrolysis of lactose in whey converts it into sweet syrup, which can be used in food industry (Husain et al. 2010, Dutra Rosolen et al. 2015). Transgalactosylation activity of β-galactosidases is exploited for the synthesis of GOS, which are of particular value as they are an example of functional foods, so-called prebiotics. Functional foods are generally defined as foods or food ingredients which have a beneficial influence on health apart from the nutritional value. A prebiotic is defined as *"…being a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health"* (Roberfroid 2007). A further criterion is, that "prebiotics are not digested by humans or other animals" (Sangwan 2011). Since the early 1990s the use of GOS as food component has become increasingly popular. GOS are predominantly used as ingredients of beverages, confectionery bakery products, yoghurt and dairy desserts and infant milk powders (Crittenden et al. 1996). In human breast milk there are high amounts of GOS, which show a high structural diversity. About 7 % lactose and 1 % human milk oligosaccharides (HMOs) consisting of lactose with linked fucose, N-acetylglucosamine and sialic acid are contained, whereas in bovine colostrum, there are only traces of oligosaccharides (Bode 2009). In infant milk formulas, GOS are essential components. They are supplemented, in order to replace HMOs and exert a positive health effect on the infant by mimicking human mother milk (Schwab et al. 2011). Small amounts of GOS enhance the growth of intestinal bifidobacteria and lactobacilli in formula-fed infants (Ben et al. 2008). Harmfulness of most pathogenic microorganism such

as *E. coli* and *Campylobacter jejuni* is dependent on their ability to adhere to the epithelium of the host. HMOs can serve as decoy receptors by mimicking attachment sites for certain pathogens. Thereby colonization and invasion of pathogens is prevented and the breastfed child is protected against infection (Bode 2012). There are further fields of application of GOS, such as their use as low caloric sweeteners, their sweetness decreases with increasing chain length (Patel et al. 2011). Beside their use in food applications, GOS are becoming of increasing interest in the medical field (Belorkar et al. 2016). Upon ingestion GOS stimulate the growth of beneficial bacteria, which are so-called probiotics. GOS enhance in particular the proliferation of bifidobacteria, acting as so-called "Bifidus-growth factor". GOS mixtures containing β-1,3, β-1,4 and β-1,6 linkages have a better bifidogenic effect than GOS mixtures comprising β-1,4 and β-1,6 linkages only (Sangwan et al. 2011). Bifidobacteria are known for the secretion of soluble vitamins such as folate, nicotinic acid, thiamine, pyridoxine (Macfarlane et al. 2008) Lactic acid bacteria comprise a heterologous group of gram positive bacteria, lactococci, streptococci and lactobacilli and are of great interest for food applications due to their advantageous health effects and are commonly used in the production of human and animal nutrition (Caplice et al. 1999). Moreover these organisms are generally regarded as safe and might have probiotic activity, such as the improved digestion of lactose in the intestine (Husain et al. 2010). Probiotics and prebiotics have cholesterol lowering effects (Ooi et al. 2010).

Improvement of transglycosylation by rational design

Most enzymes industrially used today are derived from mesophilic organisms, their use is limited at harsh conditions. Therefore, there is a need for improved enzymes which can stand extremes of pH, temperatures and salinity. Enzymes derived from extremophile organisms, that are well adapted to extreme conditions might be a better alternative than mesophilic enzymes. The genome of *H. orenii* has a GC content of 38 %. Thermostability, adaption to a wide range of salt concentrations, and broad pH stability suggest *H. orenii* proteins to be suitable candidates for application in new enzymatic processes under extreme conditions, such as in the production of biofuels, food, textile chemicals and pharmaceuticals. The amino acid composition of *H. orenii* is comparable to thermophilic organisms, but distinct to other halophilic organisms. These characteristics enable this organism to survive in an environment with fluctuating salt concentrations. Restricted

availability of water affects the structure and function of most proteins. Halophilic proteins show unique characteristics that allow them to have multilayered hydration shells and maintain their functional conformation in presence of high salt concentrations. These are an increase in acidic amino acids (glutamic acid, aspartic acid and threonine), a higher amount of smaller hydrophobic amino acids (glycine, alanine and valine), and a decreased amount of lysine residues. These features allow a higher degree of cooperation between electrostatic interactions, as well as the formation of a higher number of salt bridges to enable halophilic enzymes to perform optimally at high salt concentrations. Among *H. orenii* proteins in contrast, there is an increased number of both positively and negatively charged amino acids. Moreover, there is a reduced number of thermolabile amino acids (glutamine, histidine and threonine), as well as an increase in the number of hydrophobic lysine residues. This amino acid composition is typical for thermostable proteins, and contradictive to the established concept of stable halophilic proteins. These changes are unique to *H. orenii* and indicate that the organism is well adapted to fluctuations of salt concentrations (Bhattacharya et al. 2014).

Random mutagenesis and directed evolution are tools to generate enzymes with altered properties for industrial applications. Glycosidases are essential for the synthesis of oligosaccharides by transglycosylation. Plenty of physiological events involve glycoconjugates, consequently oligosaccharides have a great therapeutic potential. However, the use of these enzymes is limited due to the enzymatic degradation of the formed oligosaccharides. Establishment of a screening assay is important in any directed evolution experiment in order to facilitate the screening of large libraries. Assaying for transfer activity remains a huge challenge, as no obvious change in fluorescence or absorbance is associated with glycosidic bond formation. High-throughput screening methods are of particular use for the enrichment and isolation of rare mutants with desired enzyme activity from large mutant libraries (Aharoni et al. 2006). A novel class of mutant glycosidases, the so-called glycosynthases were introduced in the late 1990s (Mackenzie et al. 1998). These enzymes are capable of the formation of new glycosidic linkages but they do not hydrolyze newly formed oligosaccharides. Glycosynthases have been developed in order to increase oligosaccharide synthesis, this was achieved by the mutation of a single catalytic carboxylate nucleophile to a neutral amino acid residue such as alanine. The resulting enzymes have no hydrolytic activity, but show enhanced transglycosylation activity with

glycosylfluorides as activated donors (Hancock et al. 2006). Application of glycosynthases in industrial processes for large-scale production of GOS is limited, due to their inactivity on natural substrates. Directed evolution of glycosidases is done in order to obtain variants with very little hydrolytic activity, but high transglycosylation activity with lactose, a cheap and simple substrate. There are few reports on β-glycosidases with improved transgalactosylation activity generated by directed evolution (Hansson et al. 2001, Placier G. et al. 2009). Some former publications report improved activity with artificial substrates only (Lundemo et al. 2013, Teze et al. 2014). Glucosidase variants with improved transglycosylation were shown to be created by single amino acid substitutions in the active site previously. Replacement of single amino acids in the active site of *Thermus thermophilus* GH3 β-glucosidase results in variants with an overall transglycosylation yield much higher than the WT. Variants Y284F, F401S and N282T are among the variants with the most improved transglycosylation activity with the artificial substrate o-nitrophenol-β-D-galactopyranoside-6-phosphate. (Teze et al. 2014). A further publication reports a variant of *Thermotoga neapolitana* β-glucosidase N220F with improved transglycosylation activity with alkyl-glycosides (Lundemo et al. 2013). Transglycosylation activity of the variant *T. neapolitana* β-glucosidase F412S is increased, due to a reduced hydrolytic activity when pNPG was used as substrate and arbutin as an acceptor (Choi et al. 2008). Variant F426Y of GH1-β-glucosidase from *Pyrococcus furiosus* shows a much higher transgalactosylation activity with lactose (Hansson et al. 2001). Homologous positions for all target sites for amino acid substitutions previously shown to increase transglycosylation in *T. thermophilus* β-glucosidase, *T. neapolitana* β-glucosidase and *P. furiosus* β-glucosidase are found in *H. orenii* β-glucosidase (Table 2). As described in CHAPTER 2, corresponding *H. orenii* β-glucosidase variants were analyzed for hydrolytic and transgalactosylation activity with lactose as substrate (Hassan, Geiger et al. 2015).

Target sides for improvement of transglycosylation

T. thermophilus β-glucosidase	*T. neapolitana* β-glucosidase	*P. furiosus* β-glucosidase	*H. orenii* β-glucosidase
N282T	-	-	N294T
Y284F			Y296F
F401S	F412S	F426Y	F417S, F417Y
(Teze et al. 2014)	(Choi et al. 2008)	(Hansson et al. 2001)	
-	N220F	-	N222F
	(Lundemo et al. 2013)		(Hassan, Geiger et al. 2015)

Table 2 Variants of β-glucosidases investigated for improved transgalactosylation

Structure and function

Bioinformatic tools such as molecular dynamics simulation might be a suitable approach in order to study the structure-function relationship if a high-resolution crystal structure of the enzyme of interest is available. GH family members are grouped according their amino acid sequence similarities, there is a strong correlation between sequence and mechanistic characteristics (Henrissat and Davies 1997). Consequently, assigned functions of amino acids in one GH family enzyme might be similar in another family member as well. Previously, the amino acid interactions with the substrate in *Zea maize* β-glucosidase were described. The aromatic amino acid W452 in the glycone binding site acts as the stacking site for glucose. Several amino acids are involved in hydrogen-bond interactions with the substrate among them Q33, H137, N185 and E459. Furthermore, the experimental replacement of a single amino acid E401D impairs protein assembly of *Zea maize* β-glucosidase (Zouhar et al. 2001). A more recent publication states, that mutations in the (-1) subsite of *T. thermophilus* β-glucosidase might lead to an enhanced transfer of the glycoside onto a sugar acceptor rather than water, due to repositioning of the glycoside in the (-1) subsite, along with an improved fit of the acceptor in the (+1) subsite. By molecular dynamics simulation, it was shown that Van der Waals forces are most important for substrate binding. F401S mutation affects its surrounding amino acids. Neighboring amino acid residues such as the acid/base catalyst E166 and N219 are rearranged, resulting in a displacement of both sugar monomers involved in transglycosylation. (Feng et al. 2005). Amino acid residues involved in substrate-

interactions that were previously reported, are shown in Table 3. As described in CHAPTER 3, interactions of amino acids in the active site of *H. orenii* β-glucosidase with lactose and cellobiose as substrate were investigated by molecular dynamics simulation.

Zea maize β-glucosidase	*T. thermophilus* β-glucosidase	Function
H137	-	Substrate interaction site
W373	W312	Substrate interaction site
E186	E164	Acid/base catalyst
E401	E338	Nucleophile
W452	W385	Substrate interaction site
Q33	-	Substrate interaction site
E459	-	Substrate interaction site
-	N219	Substrate interaction site
N185	-	Substrate interaction site
(Zouhar et al. 2001)	(Feng et al. 2005)	

Table 3 Assigned functions of amino acids in the catalytic site of
Zea maize β-glucosidase and *T. thermophilus* β-glucosidase

CHAPTER 1

From by-product to valuable components: Efficient enzymatic conversion of lactose in whey using β-galactosidase from *Streptococcus thermophilus*

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From by-product to valuable components: Efficient enzymatic conversion of lactose in whey using β-galactosidase from *Streptococcus thermophilus*

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Abstract

β-Galactosidase from *Streptococcus thermophilus* was overexpressed in a food-grade organism, *Lactobacillus plantarum* WCFS1. Laboratory cultivations yielded 11,000 U of β-galactosidase activity per liter of culture corresponding to approximately 170 mg of enzyme. Crude cell-free enzyme extracts obtained by cell disruption and subsequent removal of cell debris showed high stability and were used for conversion of lactose in whey permeate. The enzyme showed high transgalactosylation activity. When using an initial concentration of whey permeate corresponding to 205 g L^{-1} lactose, the maximum yield of galacto-oligosaccharides (GOS) obtained at 50°C reached approximately 50% of total sugar at 90% lactose conversion, meaning that efficient valorization of the whey lactose was obtained. GOS are of great interest for both human and animal nutrition; thus, efficient conversion of lactose in whey into GOS using an enzymatic approach will not only decrease the environmental impact of whey disposal, but also create additional value.

Keywords

Whey permeate; Galacto-oligosaccharides (GOS); β-galactosidase; Prebiotics; Transgalactosylation

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1 Introduction

Cheese whey is the most significant waste from the dairy industry and can cause significant environmental pollution problems [1]. This by-product is generated upon coagulation of caseins in cheese making, and corresponds to 85–95% of the milk volume. The liquid whey retains about 55% of milk nutrients [2], of which lactose (4.5 5% w/v) is the most abundant. Removal of valuable whey proteins leaves whey permeate, which can contain up to 85% lactose based on dry matter. Several studies have shown that lactose is largely responsible for the high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of whey [1]. Annual world-wide cheese whey production amounts to over 160 million tons per year, corresponding to approximately 6 million tons of lactose [3,4]. Whey poses significant challenges to the dairy industry's environmental protection strategies. High production of cheese whey and whey permeate as well as their high environmental impact and nutritional content make them an important subject for careful valorization studies. Different ways of whey valorization have been investigated, both decreasing environmental impact and exploring the possibilities of reusing nutrients [5].

Due to the abundant amount of lactose in whey, one approach for whey valorization that has attracted increasing attention is the bioconversion of lactose (β-D-Galactose-(1→4)-D-Glucose) to valuable products using β-galactosidases. β-Galactosidases (β-Gal; EC 3.2.1.23) catalyze both the hydrolysis and transgalactosylation of β-D-galactopyranosides, including lactose [6–8] and are found widespread in nature. They catalyze the hydrolysis of lactose and related compounds, and are thus used in dairy industry to remove lactose from various products. These enzymes often also show transgalactosylation activity [6,9], which is of interest because the resulting galacto-oligosaccharides (GOS) are non-digestible carbohydrates with known prebiotic activity. GOS generally comprise one or more galactose units that are typically linked to a terminal glucose. The degree of polymerization of GOS can vary quite markedly, ranging from 2 to 8 monomeric units. GOS are thus complex mixtures of different oligosaccharides, and the spectrum of the oligosaccharides making up these mixtures strongly depends on the source of the β-galactosidase used for the biocatalytic reaction as well as on the conversion conditions used in their production. GOS can serve as fermentable substrates for certain members of the gut microbiota, and have been found to modulate the colonic flora by stimulation of beneficial bacteria, such as bifidobacteria and lactobacilli, and inhibition of less desirable bacteria [10–12]. Potential health benefits of GOS include reduction of intestinal disturbances (constipation and diarrhea), cardiovascular disease and intestinal cancer, increased absorption and retention of several minerals, particularly magnesium, calcium, and iron, modulation of immune responses, as well as reduction of serum cholesterol levels [13–18]. Because of these benefits, GOS are of great interest for both human and animal nutrition. Furthermore, GOS are of special interest because of the presence of structurally related oligosaccharides in human breast milk [14,19,20].

In this paper, we describe the use of β-galactosidase from *Streptococcus thermophilus*, which is recombinantly produced in food-grade *Lactobacillus plantarum*, for the efficient conversion of lactose in whey to obtain GOS. Conversion of an important food waste into

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more valuable products is advantageous not only for the environment but also for sustainable economics.

2 Materials and methods

2.1 Chemicals and enzymes

All chemicals and enzymes were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise and were of the highest quality available. All restriction enzymes, T4 DNA ligase, and corresponding buffers were from Fermentas (Vilnius, Lithunia).

2.2 Bacterial strains and culture conditions

Streptococcus salivarius subsp. *thermophilus* DSM 20259 (synonym *S. thermophilus*) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). *L. plantarum* WCFS1, isolated from human saliva as described by Kleerebezem et al. [21], was originally obtained from NIZO Food Research (Ede, The Netherlands) and maintained in the culture collection of the Norwegian University of Life Sciences, Ås, Norway. *Escherichia coli* DH5α (New England Biolabs, Frankfurt am Main, Germany) was used in the transformation experiments involving the subcloning of DNA fragments. *S. thermophilus* and *L. plantarum* were cultivated in M-17 broth and in MRS media, respectively, at 37°C without agitation. *E. coli* NEB5α (New England Biolabs, Frankfurt am Main, Germany) was grown at 37°C in Luria–Bertani (LB) medium with shaking at 120 rpm. When needed, erythromycin was supplemented to media in concentrations of 5 μg/mL for *Lactobacillus* or 200 μg/mL for *E. coli*, whereas ampicillin was used at 100 μg/mL for *E. coli*.

2.3 Construction of β-galactosidase expression vectors

The *lacZ* gene, which encodes for a β-galactosidase from *S. thermophilus* DSM2 0259 (NCBI Reference No. CP000419), was amplified using proof-reading Phusion polymerase with the primer pair FwdStNcoI (5′-GCGGCCATGGACATGACTGAAAAAATTCAAAC-3′) and RevStXhoI (5′-GGCGCTCGAGCTAATTTAGTGGTTCAATCATG-3′). The forward primer, FwdStNcoI, contains an *Nco*I restriction site and the reverse primer, RevStXhoI, includes an *Xho*I recognition site (underlined). Genomic DNA of *S. thermophilus* isolated according to a previously described procedure [22] was used as template for the PCR reaction. The initial denaturation step at 98°C for 3 min was follow by 30 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s and extension at 72°C for 40 s, followed by a final extension step at 72°C for 5 min. The amplified gene was digested with *Nco*I and *Xho*I after which the PCR product was purified using the Wizard SV Gel and PCR Clean-up system kit (Promega, Madison, WI). The PCR fragment was subcloned into the pJET1.2 plasmid (CloneJET PCR cloning kit, Fermentas), and *E. coli* NEB5α was used as a host for obtaining the plasmids in sufficient amounts. The sequence of the insert was confirmed by DNA sequencing performed by a commercial provider (Microsynth, Vienna, Austria). The gene fragment of *lacZ* was then cloned into the expression vector pSIP409, which employs regulatory elements of the sakacin P operon of *Lactobacillus sakei* [23,24], using *Nco*I and *Xho*I cloning sites, resulting in the plasmid p409lacZSt. The constructed plasmid was transformed

into electrocompetent cells of *L. plantarum* WCFS1 according to the protocol of Aukrust and Blom [25].

2.4 Expression of recombinant β-galactosidase

For the heterologous overexpression of the *lacZ* gene from *S. thermophilus*, an overnight culture (~16 h) of *L. plantarum* WCFS1 harboring the expression plasmid p409lacZSt was used to inoculate 50 mL of fresh MRS medium containing erythromycin, with a starting OD_{600} of ~0.1. The culture was incubated at 30°C without agitation and the cells were induced at an OD_{600} of 0.3 by adding 25 ng/mL of the inducing peptide pheromone IP-673 [26]. Cells were harvested at an OD_{600} of 1.8–2.0, washed twice using 50 mM sodium phosphate buffer, pH 6.5 and resuspended in 1 mL (2% of the original culture volume) of the same buffer. Cells were disrupted in a bead beating homogenizer using 1 g of glass bead (Precellys 24; PEQLAB, Germany). Cell-free extracts were obtained after a centrifugation step at 9000*g* for 15 min at 4°C.

2.5 Fermentation of recombinant *L. plantarum*

L. plantarum WCFS1 harboring p409lacZSt was cultivated in 1-L fermentations to obtain sufficient amounts of LacZ. The cultivation conditions and the induction protocol were identical to those described above for small-scale cultivations. Expression of *lacZ* was induced at OD_{600} of 0.3, the cells were harvested at OD_{600} ~6 and washed twice with 50 mM sodium phosphate buffer, pH 6.5 and the cells were then disrupted by passing the 20 mL suspension 3 times through a French press (AMINCO, Silver Spring, MD) with an applied pressure of 1000 psi. Cell debris was removed by centrifugation (25,000*g*, 20 min, 4°C). The lysate (crude extract) was then stored at −20°C.

2.6 Gel electrophoresis analysis

The cell-free extracts were analyzed by SDS-PAGE using the Phast System with precast gels (Pharmacia Biotech, Uppsala, Sweden). The enzyme preparation was diluted to 1 mg protein mL^{-1} and mixed with an equal volume of 2×Laemmli buffer, followed by incubation at 90°C for 5 min. Protein bands were visualized by staining with Bio-safe Coomassie (Bio-Rad). Unstained Precision plus Protein Standard (Bio-Rad) was used as mass marker.

2.7 β-Galactosidase assays

The measurement of β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) or lactose as the substrates was carried out as previously described [27]. When chromogenic *o*NPG was used as the substrate, the reaction was initiated by adding 20 μL of enzyme solution to 480 μL of 22 mM *o*NPG in 50 mM sodium phosphate buffer (pH 6.5) and stopped after 10 min of incubation at 30°C by adding 750 μL of 0.4 M Na_2CO_3. The release of *o*-nitrophenol (*o*NP) was measured by determining the absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 μmol of *o*NP per minute under the described conditions.

When lactose was used as the substrate, 20 μL of enzyme solution was added to 480 μL of a 600 mM lactose solution in 50 mM sodium phosphate buffer, pH 6.5. After 10 min of incubation at 30°C, the reaction was stopped by heating the reaction mixture at 99°C for 5

min. The reaction mixture was cooled to room temperature, and the release of D-glucose was determined using the test kit from Megazyme. One unit of lactase activity was defined as the amount of enzyme releasing 1 μmol of D-glucose per minute under the given conditions.

2.8 Temperature dependence of stability

The catalytic stability of crude recombinant β-galactosidase from *S. thermophilus* overexpressed in *L. plantarum* was determined by incubating the enzyme in 50 mM phosphate buffer or in whey permeate solution, pH 6.5 at 37°C and 50°C and by subsequent measurements of the remaining enzyme activity (A) at various time points (t) using the standard oNPG assay. Residual activities (A_t/A_0, where A_t is the activity measured at time t and A_0 is the initial activity) were plotted versus the incubation time. The inactivation constants k_{in} were obtained by linear regression of ln(activity) versus time. The half-life values of thermal inactivation $t_{1/2}$ were calculated using $t_{1/2} = \ln 2/k_{in}$ [28].

2.9 Steady-state kinetic measurements

A small portion of the crude extract was used to purify the β-galactosidase to homogeneity by affinity chromatography using p-aminobenzyl 1-thio-β-D-galactopyranoside immobilized onto cross-linked 4% beaded agarose (Sigma), as previously described [27]. Steady-state kinetic data for the substrates lactose or oNPG were obtained at 30°C in 50 mM sodium phosphate buffer, pH 6.5, with concentrations ranging from 0 to 600 mM for lactose and from 0 to 22 mM for oNPG. The kinetic parameters were calculated using nonlinear regression, fitting the observed data to the Michaelis-Menten equation using SigmaPlot (SPSS, Chicago, IL).

2.10 Determination of protein concentration

Protein concentrations were determined by the method of Bradford [29] using bovine serum albumin as the standard.

2.11 Lactose hydrolysis and galacto-oligosaccharides synthesis

Batch conversion reactions were carried out with crude recombinant β-galactosidase from *S. thermophilus* using whey permeate powder (with approximately 65% of the dry matter being lactose) as the source of lactose. The influence of process parameters such as temperature (37°C, 50°C) and substrate concentration (50 and 200 g L^{-1} lactose) on the reaction was also studied. The substrate solution was prepared in 50 mM sodium phosphate buffer pH 6.5, containing 10 mM MgCl$_2$. Agitation during these conversions was applied at 300 rpm using a thermomixer (Eppendorf, Hamburg, Germany).

2.12 Analysis of mono- and oligosaccharides

The carbohydrate composition in reaction mixtures was analysed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), which was carried out on a Dionex DX-500 system consisting of a GP50 gradient pump, an ED 40 electrochemical detector with a gold working electrode and an Ag/AgCl reference electrode, and Chromeleon version 6.5 (Dionex Corp., Sunnyvale, CA). All eluents were degassed by

flushing with helium for 30 min. Separations were performed at room temperature on a CarboPac PA-1 column (4 mm × 250 mm) connected to a CarboPac PA-1 guard column (Dionex) [30]. Separation of D-glucose, D-galactose, lactose and allolactose (β-D-Galp-(1→6)-D-Glc) was carried out with an isocratic run (45 min) with 15 mM NaOH at 1.0 mL min^{-1}, followed by 25 min elution with 100 mM NaOH. For separation of GOS, eluents A (100 mM NaOH) and B (100 mM NaOH and 150 mM NaOAc) were mixed to form the following gradient: 98% A from 0 to 10 min, 98% A to 52% A from 10 to 40 min, and then 52% A for another 5 min. The column was washed with 20% B for 10 min and re-equilibrated for 15 min with the starting conditions of the employed gradient. Galacto-oligosaccharide standards of β-D-Galp-(1 → 3)-D-Glc, β-D-Galp-(1 → 6)-D-Glc, β-D-Galp-(1 → 3)-D-Gal, β-D-Galp-(1 → 4)-D-Gal, β-D-Galp-(1 → 6)-D-Gal, β-D-Galp-(1 → 3)-D-Lac, β-D-Galp-(1 → 4)-D-Lac, β-D-Galp-(1 → 6)-D-Lac were purchased from Carbosynth (Berkshire, UK).

3 Results

3.1 Expression of recombinant β-galactosidase from *S. thermophilus*

The yields of β-galactosidase activity when using the wild-type strain of *S. thermophilus* as a producer were rather low. For example, after cultivation of *S. salivarius* subsp. *thermophilus* DSM 20259 at 37°C for 24 h, the β-galactosidase yield, as measured in a cell-free extract, was only ~90 U$_{o\mathrm{NPG}}$ per L of culture (M17 containing 2% lactose) with a specific activity of 2.1 U/mg (data not shown), which is activity of enzyme per mg protein. Hence, we set out to establish heterologous overexpression in a food-grade organism. To do so, we cloned the *S. thermophilus lacZ* gene into the pSIP409 vector for subsequent overexpression in *L. plantarum*. Induced and non-induced cells carrying the expression plasmid were harvested at OD$_{600}$ of 1.8–2.0. SDS-PAGE analysis of cell-free protein extracts showed a unique band at ~100 kDa in induced *L. plantarum* cells (Fig. 1), which is in agreement with the calculated molecular mass of β-galactosidase from *S. thermophilus*.

L. plantarum harboring p409lacZSt was then cultivated on a larger scale (1-L cultivation volume). Analysis of cell-free extracts of such 1-L laboratory cultivations showed enzyme yield of approximately 11 ± 0.5 kU$_{o\mathrm{NPG}}$ of β-galactosidase activity. The specific activity of the extracts was ~12 U/mg. A small portion of the crude extract was used to purify the β-galactosidase to homogeneity by affinity chromatography using *p*-aminobenzyl 1-thio-β-D-galactopyranoside immobilized onto cross-linked 4% beaded agarose (Sigma) and the specific activity of the purified enzyme was determined to be 65 U/mg (data not shown), therefore the enzyme yield of 11 ± 0.5 kU$_{o\mathrm{NPG}}$ of β-galactosidase activity per liter of culture corresponds to approximately 170 mg of enzyme. The β-galactosidase activity in *L. plantarum* cells without plasmid was negligible (0.07 U/mg), and hence the enzyme activities obtained can be attributed solely to plasmid-encoded LacZ from *S. thermophilus*.

The steady-state kinetic constants were determined for the hydrolysis of lactose and *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG). Kinetic analysis with lactose as the substrate showed Michaelis-Menten kinetics with the following parameters obtained by nonlinear regression: $v_{\max} = 17.67 \pm 0.36$ (μmol D-glucose released min^{-1} (mg protein)$^{-1}$) and $K_m = 5.12 \pm 0.53$ (mM). The experiments with *o*NPG showed substrate inhibition and the

following kinetic parameters were obtained: v_{max} = 151 ± 10 (µmol oNP released min^{-1} (mg protein)$^{-1}$), K_m = 0.55 ± 0.10 (mM), and $K_{i,s}$ = 14.1 ± 2.3 (mM). The k_{cat} values were 34.4 ± 0.70 (s^{-1}) and 294.7 ± 20.3 (s^{-1}) and the catalytic efficiencies (k_{cat}/K_m) were 6.7 and 539 (mM^{-1} s^{-1}) for lactose and oNPG, respectively.

3.2 Stability of the crude enzyme

Temperature stability of this crude enzyme preparation in sodium phosphate buffer (pH 6.5) and in whey permeate solution (dissolved in sodium phosphate buffer, pH 6.5) was measured at 37°C and 50°C (Table 1). The half-life time (t$_{1/2}$) of the crude enzyme in whey permeate solution was increased compared to sodium phosphate buffer. A possible explanation could be the presence of the substrate and various mono- and divalent ions, such as sodium or magnesium, in the whey, which can affect the stability of β-galactosidases. The effect of ions such as Na$^+$ on activity and Mg^{2+} on thermal stability seems common among GH2 β-galactosidases and is observed for *E. coli* β-galactosidase LacZ [31] as well as for some purified β-galactosidases from *Lactobacillus* spp. [27,32–34]. The effect of additional Mg^{2+} on stability of the crude enzyme in sodium phosphate buffer and in whey permeate was also tested and the results show that the addition of Mg^{2+} tends to have further stabilizing effect. Importantly, the data show that the recombinantly expressed enzyme is quite stable at 50°C.

3.3 Lactose hydrolysis and formation of GOS

Based on the observations of the stability of the crude enzyme preparation, the conversion of lactose in whey using the crude recombinant β-galactosidase from *S. thermophilus* was performed at both 37°C and 50°C to investigate the influence of process temperature. Whey permeate powder containing 65% (w/w) lactose was dissolved in 50 mM sodium phosphate buffer, pH 6.5 with 10 mM MgCl$_2$ to a concentration of 50 g L^{-1} lactose, which is about the concentration of lactose in liquid whey and in milk. Crude enzyme was added to a final concentration of 5 U$_{o\text{NPG}}$ (corresponding to 1.35 U$_{Lac}$) per mL of reaction mixture. Lactose hydrolysis was significantly faster at 50°C and was completed within 2 h of reaction, while at 37°C lactose was completely hydrolyzed only after 5 h (Table 2). A maximum GOS yield of 34.2% of total sugar mass was achieved at ~80% lactose conversion at 37°C. At ~94–95% lactose conversion, the GOS yield decreased to ~30%, and when lactose was completely hydrolyzed, the GOS yield was ~20%. The reduction in GOS content can be explained by the fact that GOS are also subject to hydrolysis. When continuing the reaction after complete lactose conversion had been achieved, GOS concentrations decreased even further to ~11% after 7 h at 37°C or after 3 h at 50°C.

3.4 GOS production

The formation of GOS described above happened even at low initial lactose concentration (50 g L^{-1}). To increase the GOS yields further, we also tested a higher initial lactose concentration for this reaction. Whey permeate powder containing 65% (w/w) lactose was dissolved in 50 mM sodium phosphate buffer, pH 6.5 with 10 mM MgCl$_2$ to an equivalent concentration of 205 g L^{-1} lactose. The reaction was again performed at 37°C and 50°C using 10 U$_{o\text{NPG}}$/mL (corresponding to 2.7 U$_{Lac}$/mL) of enzyme. At 50°C, it took 5 h and 6 h to achieve ~90% and ~95% lactose conversion, respectively. The conversion was slower at 37°C, with ~95% lactose conversion being obtained after 9 h of reaction (Fig. 2A). The

maximum GOS yield was ~50% of total sugar mass at ~90% lactose conversion, which was reached after 5 h of reaction at 50°C and 8.5 h of reaction at 37°C (Fig. 2B). Using authentic standards, we could identify the main GOS products of transgalactosylation, which are β-D-Gal*p*-(1 → 6)-D-Glc, β-D-Gal*p*-(1 → 3)-D-Lac, β-D-Gal*p*-(1 → 3)-D-Glc, β-D-Gal*p*-(1 → 3)-D-Gal, β-D-Gal*p*-(1→ 6)-D-Gal, β-D-Gal*p*-(1 → 6)-D-Lac (Fig. 3).

4   Discussion

Lactic acid bacteria (LAB) are important microorganisms in the food and beverage industry. Over the past few decades, LAB have been used not only as starter cultures but also as producers of flavoring enzymes, antimicrobial peptides or metabolites that contribute to the flavor, texture and safety of food products [35–37]. LAB have for a long time been used in the production of a wide range of foods without adverse effects on humans. Due to their food-grade status and probiotic characteristics, several LAB are considered as safe and effective cell-factories for food-application purposes [36,37]. Due to this potential, several constitutive or inducible gene expression and protein targeting systems have been developed for LAB [23,35,36,38]. One such expression system comprises the so-called pSIP vectors [24] and is based on promoters and regulatory genes involved in the production of the class-II bacteriocins sakacin A [39] and sakacin P [40,41] in *Lactobacillus* spp. One of the advantages of this system is that it is strictly regulated and leads to high production of the target protein.

Recently, we reported the overproduction of β-galactosidases from *Lactobacillus reuteri* and *Lactobacillus bulgaricus* in the food-grade expression host *L. plantarum* WCSF1 [34,42]. The heterodimeric β-galactosidase of *L. reuteri* is encoded by two overlapping genes, *lacL* and *lacM*, while the homodimeric β-galactosidase of *L. bulgaricus* is encoded by *lacZ.* These enzymes both belong to glycoside hydrolase family GH2. The predominant GH2 β-galactosidases found in lactobacilli are of the LacLM type, encoded by the overlapping *lacLM* genes [22,32,33,43], while GH2 β-galactosidases of the LacZ type, encoded by a single *lacZ* gene, are less frequently found in lactobacilli. These LacZ β-galactosidases are more frequent in other LAB including *S. salivarius* and *S. thermophilus* [44] or bifidobacteria including *Bifidobacterium bifidum* [45], *Bifidobacterium longum* subsp. *infantis* [46], or *Bifidobacterium breve* [47]. When overexpressing β-galactosidases from *L. reuteri* and *L. bulgaricus* in the host *L. plantarum* WCFS1, the highest yields obtained under optimized fermentation conditions were ~35–40 kU and ~53 kU of β-galactosidase activity per liter of culture, respectively [34,48]. The yield obtained for β-galactosidase from *S. thermophilus* overexpressed in *L. plantarum* WCFS1 was somewhat lower, namely ~11 kU per liter of fermentation medium. Notably, this yield was obtained using relatively simple fermentation conditions without optimization and is expected to improve significantly if the fermentation process is optimized, as previously shown for the overexpression of β-galactosidase from *L. reuteri* in *L. plantarum* WCFS1 [48].

The present study was performed with crude recombinant β-galactosidase obtained after cell disruption and separation of cell debris by centrifugation. Because of the GRAS status of *L. plantarum*, it is safe to use a crude extract in food and feed applications. The direct use of a crude extract can reduce the enzyme costs of the process by avoiding laborious and

expensive purification steps. An advantage of this recombinant β-galactosidase from *S. thermophilus* is that it is stable at high temperatures (up to 50°C) for an extended period of time. The enzyme is stable at 50°C with a $t_{1/2}$ of more than a day, whereas the process for GOS production reported in this study can be completed within 5 h to obtain the maximal GOS yield. One major drawback of using mesophilic biocatalysts in industrial processes is the threat of microbial contamination. Most GH2 β-galactosidases from LAB are mesophilic biocatalysts, with some exceptions, for example β-galactosidase from *L. bulgaricus* [34] and the enzyme reported here in this study. Performing these conversion experiments at increased temperature will obviously decrease the chance of microbial contamination.

We first looked at lactose hydrolysis at a low initial lactose concentration of 50 g L^{-1}, which is the concentration of lactose in liquid whey and in milk. This approach could reveal whether the enzyme has potential for removal of lactose in whey liquid waste or for applications in the dairy industry, such as production of low-lactose or lactose-free products or prevention of crystallization of lactose in dairy products. The recombinantly produced *S. thermophilus β*-galactosidase was found to be a promising candidate for these applications as complete lactose hydrolysis was obtained within less than 2 h at reasonable enzyme dosage. We did not detect lactose in our samples after 5 h at 37°C and 2 h at 50°C (Table 2) by using HPAEC-PAD for analysis, which confirms complete lactose hydrolysis. The detection limit of our HPAEC-PAD system for lactose is 0.1 g L^{-1}. The level of lactose was also analyzed with the enzyme-based lactose biosensor *Lactosens* (DirectSens GmbH, Vienna, Austria; http://www.directsens.com), which confirmed the concentration to be below 0.1 g L^{-1}, which is the limit recommended for dairy products to be labelled as 'lactose-free' [49].

A more attractive biocatalytic application of the β-galactosidase is based on its transgalactosylation potential. Indeed, recombinant β-galactosidase from *S. thermophilus* was found to be suitable for the production of GOS, with the highest total GOS yields reaching ~50% when the enzyme was used in batch conversion mode with an initial lactose concentration of 205 g L^{-1}. This yield can be considered as relatively high compared to the reported yields obtained with other β-galactosidases from LAB and some commercial β-galactosidases, for example *L. reuteri* (38%) [30], *L. sakei* (41%) [32], *L. plantarum* (41%) [33], *Lactobacillus pentosus* (31%) [43], *L. bulgaricus* (49.5%) [34], *Kluyveromyces lactis* (Lactozym 3000 L HP G from Novozymes, Bagsvaerd, Denmark) (30%) [50], *Bacillus circulans* (Biolacta FN5 from Daiwa Kasei K.K., Japan) (39%) [51]. GOS yields of over 50% are not often exceeded, and more typical optimized yields are between 30% and 40% (w/w) [52]. The increase in GOS yield is often observed with increased initial lactose concentration [52], therefore higher GOS yield than the yield reported here might be even achieved with higher initial lactose concentration. When looking at the individual components of the GOS mixture, it becomes evident that the recombinant enzyme from *S. thermophilus* has a propensity to synthesize β-(1 → 6) and β-(1 → 3)-linked GOS. Such a preference towards β-(1 → 3)- and β-(1 → 6)-bond formation has also been found for other β-galactosidases from LAB [30,32–34,43]. The predominant transgalactosylation products were identified as β-D-Gal*p*-(1 → 6)-D-Glc (allolactose) and β-D-Gal*p*-(1 → 3)-D-Lac (Fig. 3). It was reported that the administration of a GOS mixture containing β-(1 → 3) as well as

β-(1 → 4) and β-(1 → 6) linkages proved to have a better bifidogenic effect than a mixture containing GOS with β-(1 → 4) and β-(1 → 6) linkages [53].

The use of whey permeate powder as a cheap lactose source could lower the costs for production of GOS. We have previously reported a process for GOS production using crude enzyme extract from *Lactobacillus* sp. [54]. In this previously reported process, we used the native enzyme, whose production yield was in the order of 2.5 kU of β-galactosidase activity per liter of culture [27]. The reaction temperature was as low as 17°C to limit possible microbial contamination, and therefore the obtained GOS yield from whey permeate was only ~25% after 15 h [54]. The process described in the present study has significant improvements, which are: (1) higher production of recombinant β-galactosidase in a food-grade organism, which lowers the enzyme costs, (2) the use of a thermostable crude recombinant enzyme, which enables the process to be carried out at 50°C, reducing the risks of microbial contamination, and (3) high GOS yield amounting up to ~50% of total sugar, which can be obtained in a much shorter process time, that is within 5 h at 50°C.

5   Conclusion

We describe the valorization of whey using an enzymatic approach for the production of GOS. Overexpression of β-galactosidase from *S. thermophilus* in another food-grade organism as well as the direct use of a crude cell extract reduce the enzyme costs. The process is very efficient with 50% of GOS yield being obtained within 5 h at 50°C. Our results imply that 1 kg of GOS can be produced from 2 kg of lactose or 3 kg or whey permeate powder using ~100 kU of enzyme. The conversion of lactose in whey into valuable products such as GOS would substantially decrease the environmental impact of whey, while significant profits could be obtained.

Acknowledgement

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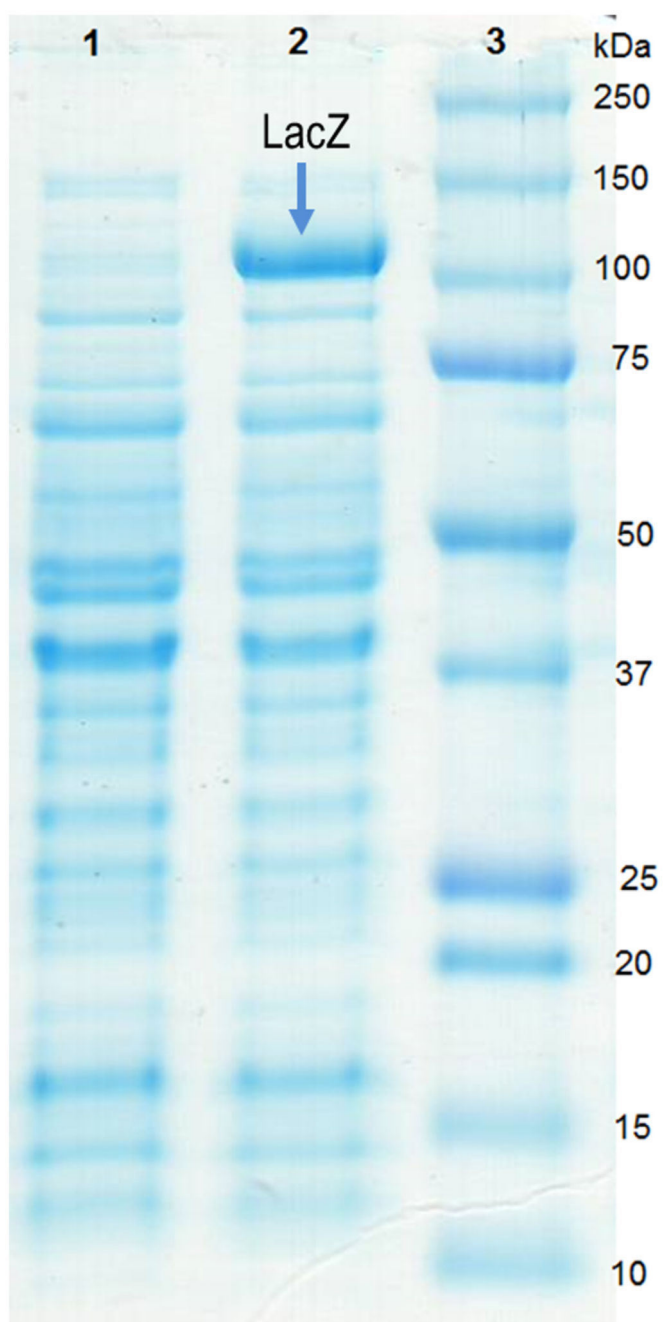
Fig. 1.
SDS-PAGE of crude protein extracts obtained from noninduced (lane 1) and induced (lane 2) *L. plantarum* cells carrying the plasmid pSIP409lacZSt. The precision plus protein™ dual color standard (lane 3) was from Bio-Rad.
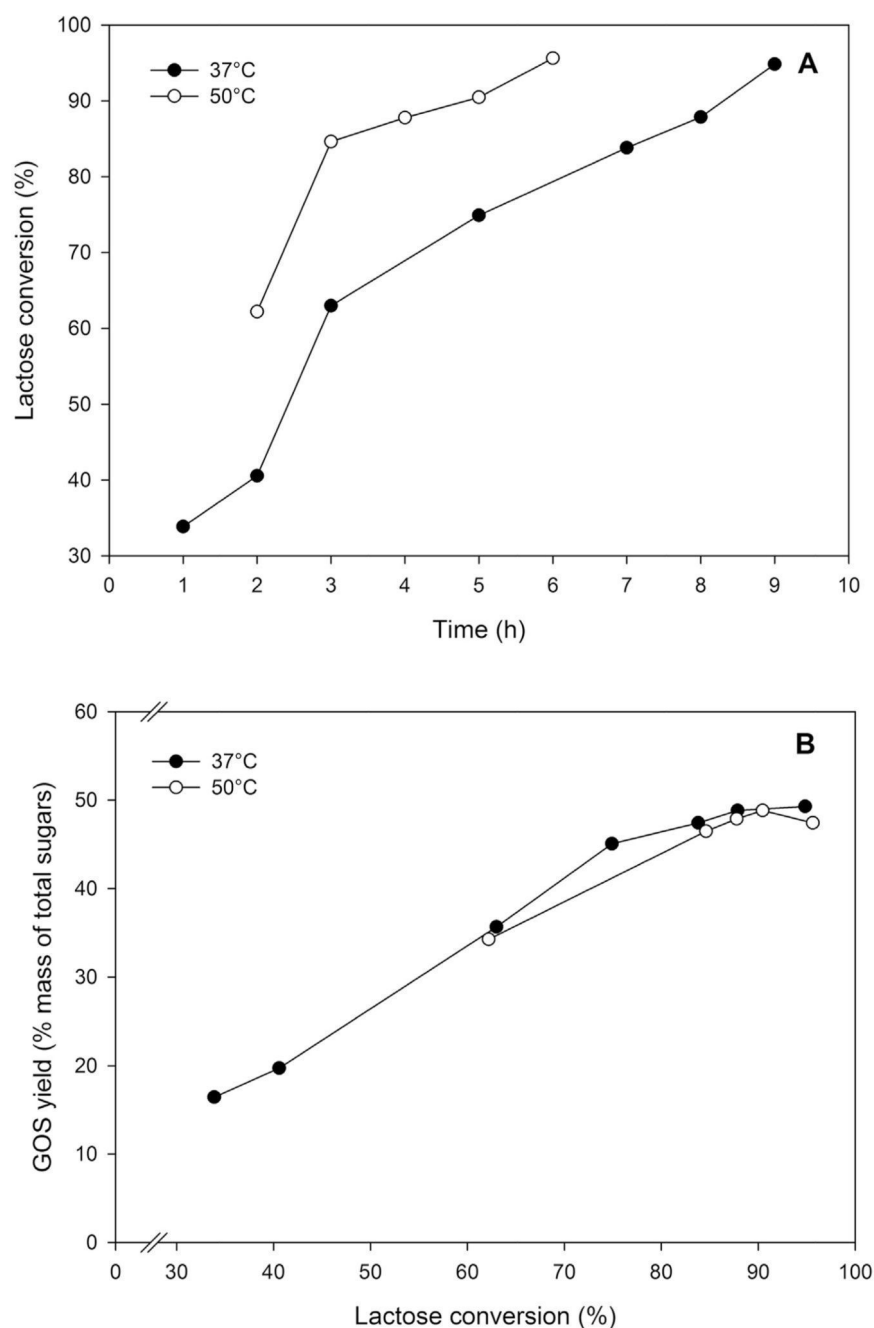
Fig. 2.
Time course of lactose conversion (A) and GOS yields (B) of reactions run at 37°C and 50°C using whey permeate dissolved in 50 mM sodium phosphate buffer, pH 6.5 with 10 mM $MgCl_2$ to a final concentration corresponding to 205 g L^{-1} lactose. Reactions were performed using 10 U_{oNPG}/mL of crude recombinant β-galactosidase from *S. thermophilus* overexpressed in *L. plantarum*.
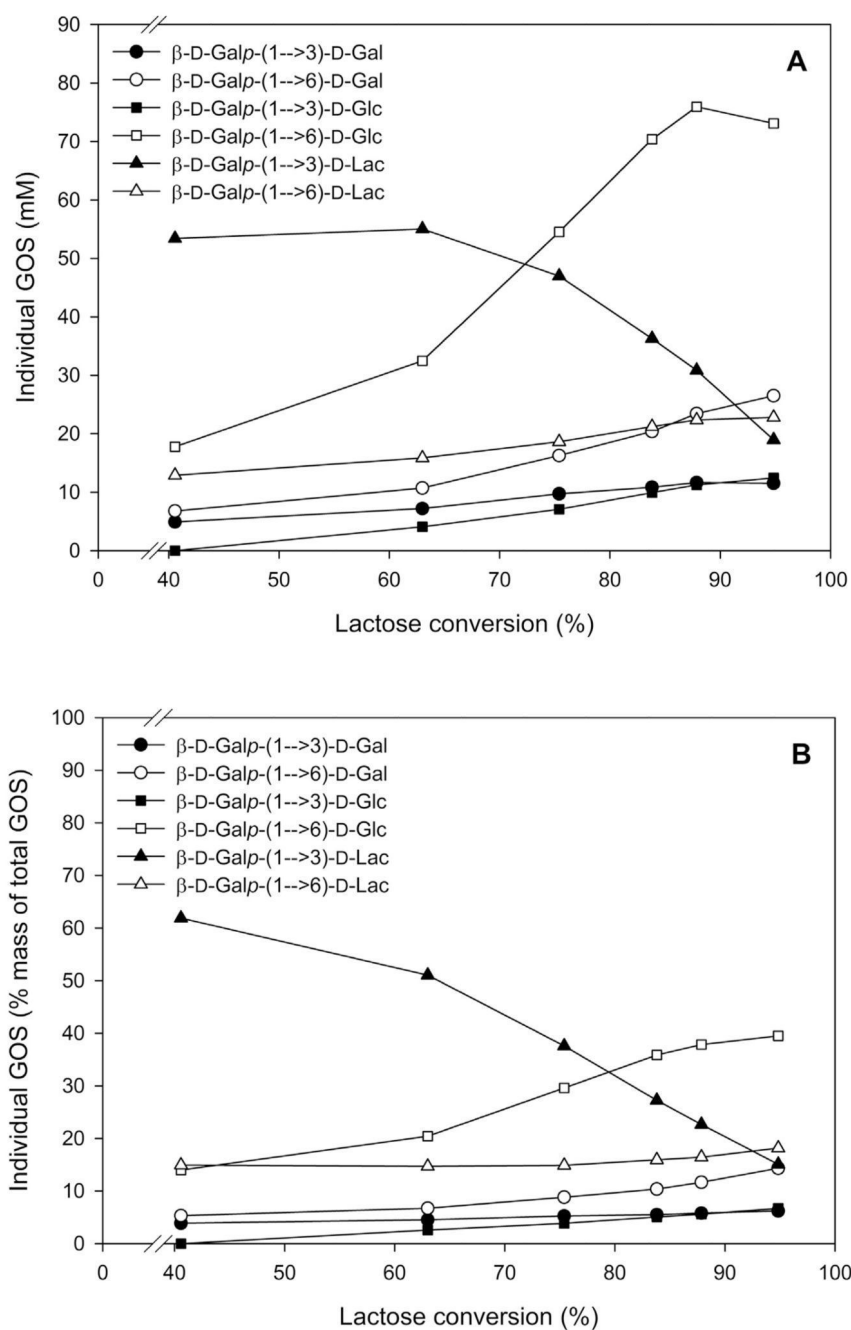
Fig. 3.
Formation and degradation of individual GOS, in mM (A) and as mass percentage of total GOS (B) during lactose conversion at 37°C. Reactions were performed as described for Fig. 2.

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Table 1

Half-life times ($t_{1/2}$) of activity (in) for crude recombinant β-galactosidase from *S. thermophilus* overexpressed in *L. plantarum*.

Temperature (°C)	Sodium phosphate buffer (pH 6.5)	Sodium phosphate buffer (pH 6.5) + 10 mM MgCl$_2$	Whey permeate (dissolved in sodium phosphate buffer, pH 6.5)	Whey permeate (dissolved in sodium phosphate buffer, pH 6.5 + 10 mM MgCl$_2$)
37	25.4	nd	33.6	39.6
50	21.0	27.5	25.1	28.1

nd: not determined.

Experiments were performed in duplicates, and the standard deviation was always <5%.

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Table 2

Lactose conversion and GOS formation during hydrolysis of lactose (50 g L^{-1}) in whey permeate solution dissolved in 50 mM sodium phosphate buffer (pH 6.5) and 10 mM MgCl$_2$, at 37 °C and 50 °C using 5 U$_{o\text{NPG/mL}}$ of crude recombinant β-galactosidase from *S. thermophilus* overexpressed in *L. plantarum.*

Time (h)	37 °C		50 °C	
	GOS (% mass of total sugars)	Lactose conversion (%)	GOS (% mass of total sugars)	Lactose conversion (%)
0	0.0	0.0	0.0	0.0
1	32.2	70.3	29.1	95.3
2	34.2	79.7	16.8	>99.8
3	31.2	93.7	11.1	—
4	25.0	96.6		
5	22.1	>99.8		
7	10.7	—		

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CHAPTER 2

Engineering a thermostable *Halothermothrix orenii* β-glucosidase for improved galacto-oligosaccharide synthesis

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† Joint first authorship

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Engineering a thermostable *Halothermothrix orenii* β-glucosidase for improved galacto-oligosaccharide synthesis

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Abstract Lactose is produced in large amounts as a by-product from the dairy industry. This inexpensive disaccharide can be converted to more useful value-added products such as galacto-oligosaccharides (GOSs) by transgalactosylation reactions with retaining β-galactosidases (BGALs) being normally used for this purpose. Hydrolysis is always competing with the transglycosylation reaction, and hence, the yields of GOSs can be too low for industrial use. We have reported that a β-glucosidase from *Halothermothrix orenii* (*Ho*BGLA) shows promising characteristics for lactose conversion and GOS synthesis. Here, we engineered *Ho*BGLA to investigate the possibility to further improve lactose conversion and GOS production. Five variants that targeted the glycone (−1) and aglycone (+1) subsites (N222F, N294T, F417S, F417Y, and Y296F) were designed and expressed. All variants show significantly impaired catalytic activity with cellobiose and lactose as substrates. Particularly, F417S is hydrolytically crippled with cellobiose as substrate with a 1000-fold decrease in apparent k_{cat}, but to a lesser extent affected when catalyzing hydrolysis of lactose (47-fold lower k_{cat}). This large selective effect on cellobiose hydrolysis is manifested as a change in substrate selectivity from cellobiose to lactose. The least affected variant is F417Y, which retains the capacity to hydrolyze both cellobiose and lactose with the same relative substrate selectivity as the wild type, but with ~10-fold lower turnover numbers. Thin-layer chromatography results show that this effect is accompanied by synthesis of a particular GOS product in higher yields by Y296F and F417S compared with the other variants, whereas the variant F417Y produces a higher yield of total GOSs.

Keywords β-Glucosidase · β-Galactosidase · Halothermophile · *Halothermothrix* · Lactose conversion · Galacto-oligosaccharides · Transglycosylation mutants

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Introduction

About 150–200 million tons of lactose are generated each year from liquid whey (Smithers, 2008). Lactose can be conveniently hydrolyzed into glucose and galactose by the use of β-galactosidases (BGALs). In cases where the enzyme displays significant transglycosylation activity, hydrolysis can be combined with the transfer of hydrolysis products onto suitable acceptor molecules to form new value-added compounds such as galacto-oligosaccharides (GOSs).

The main producers of GOSs are BGALs produced by various microorganisms such as *Aspergillus oryzae*, *Aspergillus niger*, *Kluyveromyces lactis*, and *Kluyveromyces fragilis* (Torres et al. 2010; Gosling et al. 2010). A number of commercial and non-commercial BGALs from different sources have been evaluated for their ability and efficiency

to produce GOSs (e.g., Petzelbauer et al. 2000; Jørgensen et al. 2001; Hung et al. 2002; Chockchaisawasdee et al. 2005; Nguyen et al. 2006; Nakkharat & Haltrich, 2006; Splechtna et al. 2007; Goulas et al. 2009; Neri et al. 2009; Maischberger et al. 2010; Iqbal et al. 2011; Liu et al. 2011; Rodriguez-Colinas et al. 2011; 2012; 2013; Nguyen et al. 2012; Osman et al. 2012; Urrutia et al. 2013; Wu et al. 2013; Yu & Sullivan, 2013; Arreola et al. 2014). *A. oryzae*, *Bacillus circulans*, *Cryptococcus laurentii*, *K. lactis*, and *Streptococcus thermophilus* are commercial sources of BGALs used for GOS production. When using these enzymes, yields of GOS formation ranging from 14 to 45 % are reported with 5 % initial lactose concentrations (Torres et al. 2010; Gosling et al. 2010). The use of rational design and enzyme engineering offers a means by which to improve the transglycosylation-to-hydrolysis (T/H) ratio to produce more useful enzyme variants giving higher GOS yields.

Alternatives to BGALs for these applications include many retaining *β*-glucosidases (BGL) that are capable of catalyzing transglycosylation as a side reaction to the functionally relevant hydrolytic cleavage of glycosidic bonds. Efforts to engineer BGLs for improved transglycosylation have been reported in the literature (Hansson et al. 2001; Feng et al. 2005; Wu et al. 2013), where the typical engineering strategy involves replacement of side chains in the aglycone (+1) and glycone (−1) subsites (Hansson et al. 2001; Feng et al. 2005). For engineering, it is advantageous to start with an enzyme that has evolved naturally to be functional under conditions that are relevant for the desired application, for instance high temperature. Thermophilic and hyperthermophilic bacteria are among the most useful microbial producers of highly stable and robust enzymes for bioprocesses. Among others, *Halothermothrix orenii* is a heterotrophic, halophilic, thermophilic, obligate anaerobic bacterium (Cayol et al. 1994) that produces a thermostable *β*-glucosidase, *Ho*BGLA (Mijts & Patel, 2001; Mavromatis et al. 2009; Kori et al. 2011; Hassan et al. 2015). We have reported previously the biochemical and structural characterization of *Ho*BGLA and shown that this GH1 glycosidase (www.cazy.org; Lombard et al. 2014) displays attractive properties relevant to GOS synthesis (Hassan et al. 2015). Specifically, the enzyme has *β*-galactosidase activity at high temperatures (65–70 °C) and

within a broad pH range (4.5–7.5), conditions under which the wild-type enzyme also displays significant transgalactosylation activity to efficiently convert lactose into mainly *β*-D-Gal*p*-(1 → 3)-D-Lac (3′-galactosyl lactose; 3GALA) and *β*-D-Gal*p*-(1 → 6)-D-Lac (6′-galactosyl lactose; 6GALA).

Encouraged by the high natural transgalactosylation activity of *Ho*BGLA, and the general amenability of BGLs to be engineered toward improved T/H ratios, we set out in this study to investigate the possibility to further improve transgalactosylation activity and GOS yield for *Ho*BGLA by replacing amino acid side chains in, or near, the −1 subsite in positions previously shown to improve transglycosylation for related BGLs (Hansson et al. 2001; Feng et al. 2005; Lundemo et al. 2013; Teze et al. 2014). We present the production, biochemical characterization, and transgalactosylation analysis for six *Ho*BGLA active-site variants aimed at improving GOS production.

Materials and methods

Site-directed mutagenesis, expression, and purification of *Ho*BGLA variants

The cloning and expression of the *H. orenii* *bglA* gene (UniProtKB B8CYA8) have been reported previously (Hassan et al. 2015). The wild-type *bglA* gene cloned in the pNIC28-Bsa4 vector containing a cleavable N-terminal hexahistidine tag and the tobacco etch virus (TEV) protease cleavage site (sequence [23]MHHHHHHSSGVDLGTEN LYFQSM[−1]) (Savitsky et al. 2010) was used as template for site-directed mutagenesis to produce the single-replacement variants N222F, N294T, Y296F, N406I, F417Y, and F417S. Forward and reverse PCR primers were designed with the QuickChange® Primer Design Program from Agilent Technology. The forward primers are given in Table 1, and the reverse primers were the reverse complements of the forward primers. All PCR reactions, plasmid transformations, expression, cell harvest, and protein purification were performed as described for the *Ho*BGLA wild-type and active-site variants reported earlier (Hassan et al. 2015). Briefly, *Escherichia coli* BL21(DE3) cells were grown

Table 1 Forward PCR mutagenesis primers

N222F_fwd	5′-CGGGTAAGCAGGGGGTTAAGAAGAGAGTAATACCAATCTCC-3′
N294T_fwd	5′-CCATCCTGGAGTAGTAAGTAATGCCCAGGAAGTCAA-3′
Y296F_fwd	5′-ACCACCATCCTGGAGAAGTAATTAATGCCCAGGAA-3′
N406I_fwd	5′-GCCATAGGCCCATTCAAAAATATCCATCAATGACCACAC-3′
F417Y_fwd	5′-CCTATGGCTATAGCAAGCGCTATGGTCTCATTTATG-3′
F417S_fwd	5′-TAATCAACATAAATGAGACCACTGCGCTTGCTATAGCCATAGGC-3′

in Terrific Broth (TB) medium supplemented with 50 μg/mL kanamycin and glycerol as carbon source (60 mL per 600 mL), induced with 0.2 mM IPTG and cultivated at 18 °C for 16–18 h. Purification was performed as described previously (Hassan et al. 2015), using an initial step of Ni^{2+}-charged immobilized metal affinity chromatography (IMAC), after which the His_6 tag was removed using tobacco etch virus (TEV) protease. After tag removal, a second step of IMAC was performed (i.e., reverse IMAC) where the TEV-treated target protein lacking the His_6 tag was collected in the flow through and further purified using size exclusion chromatography on a HiLoad™ 16/60 Superdex™ 200 prep grade column (GE Healthcare Life Sciences) equilibrated with 20 mM HEPES (pH 7.0) and 150 mM NaCl.

Hydrolytic activity assays of the *Ho*BGLA variants on cellobiose and lactose

The hydrolytic activity of *Ho*BGLA variants on cellobiose and lactose was assayed using the coupled glucose oxidase/peroxidase assay (Kunst et al. 1988) as described earlier for wild-type and mutant *Ho*BGLA (Hassan et al. 2015), the only differences being the buffer and temperature at which the assay was performed. The reactions were carried out at 70 °C in 20 mM HEPES buffer (pH 7.0) and 0.15 M NaCl with substrate concentrations in the range 2 to 120 mM for cellobiose, and 5 to 400 mM for lactose. Enzyme concentrations used were as follows: wild type, 0.085 mg/mL; N222F, 1.7 mg/mL; N294T, 4 mg/mL; Y296F, 5 mg/mL; F417S, 8.5 mg/mL; and F417Y, 4 mg/mL. One unit of lactose-hydrolyzing activity was defined as the amount of enzyme releasing 1 μmol of D-glucose per minute under the given conditions. One unit of cellobiose-hydrolyzing activity was defined as the amount of enzyme releasing 2 μmol of D-glucose per minute under similar conditions as described for determination of *β*-galactosidase activity using lactose as the substrate. Non-linear regression was used to derive the kinetic parameters, and the data were fitted to the Michaelis–Menten model using GraphPad Prism 6.0 for Mac (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The apparent turnover values ($k_{cat,app}$) were calculated using the experimentally determined v_{max} values and a molecular mass of 53 kDa for the enzyme.

TLC screening for GOS production by *Ho*BGLA variants using cell lysates

To assess the transglycosylation activity of the *Ho*BGLA variants, synthesized GOS products were screened by thin-layer chromatography (TLC). To this end, the crude-cell extracts were incubated in the presence of 30 % lactose (w/v) at 70 °C and different durations, allowing denaturation of most endogenous *E. coli* proteins. Specifically, 2-mL overnight cultures of *E. coli* BL21(DE3) cells carrying the *p*NIC28-Bsa4-*HoBGLA* vectors were harvested by centrifugation, and the cell pellet resuspended in either 200 μL sodium phosphate buffer, pH 6.0, containing 300 g/L lactose, and 1 mM Mg^{2+} to increase enzyme stability (Nguyen et al. 2006; Iqbal et al. 2011; Hassan et al. 2015), or 200 μL sodium phosphate buffer, pH 6.0, containing 200 g/L cellobiose and 1 mM Mg^{2+}. Cells were lysed by ultrasonication on ice. The resulting cell lysates were incubated at 70 °C with shaking (700 r.p.m.). In the case of lactose as substrate, the reaction was run for 3 and 4.5 h, after which samples were taken for analysis of GOS products. The samples were heated at 95 °C for 5 min and diluted 1:10, followed by loading of 1 μL sample on a TLC plate. In the case of cellobiose as substrate, the reaction was carried out for 2 h after which the enzymes were heat-inactivated for 5 min at 95 °C, and the carbohydrate content analyzed using TLC (2 μL of 1:10 dilution). HPTLC Li Chrosper®Silica gel 60 F_{254}s (Merck) was used as adsorbent. Samples were applied on the plates and placed in the eluent (*n*-butanol–*n*-propanol–ethanol–water = 2:3:3:2). Visualization of the separated carbohydrates was performed by immersing the TLC plate in a staining solution (0.5 g thymol, 95 mL 96 % ethanol, 5 mL concentrated sulfuric acid) for 3 s and subsequent heating at 90 °C for approximately 1 min. Standards for lactose conversion included a mixture of glucose, galactose, and lactose (LGG); a purified GOS mixture with monosaccharides and lactose removed produced using *Lactobacillus* sp. *β*-galactosidase (Maischberger et al. 2008); and Vivinal®GOS (Borculo Domo, NL). For cellobiose conversion, cellobiose and glucose were used as standards.

For HPLC analysis of transglycosylation products obtained with purified enzymes, a volume of 50 μL enzyme (7 mg/mL) was mixed with 450 μL lactose (300 g/L) in 50 mM sodium phosphate buffer (pH 6) containing 1 mM $MgCl_2$ and incubated with shaking at 70 °C at 600 r.p.m. for 12 h. Samples were withdrawn at different time points and heat-inactivated for 5 min at 95 °C, followed by HPLC analysis as described previously (Spechtna et al. 2007).

Transgalactosylation of lactose using purified enzymes and analysis of galacto-oligosaccharides

A volume of 50 μL purified enzyme (7 mg/mL) of *Ho*BGLA variants was mixed with 450 μL lactose (300 g/L) in 50 mM sodium phosphate buffer (pH 6) containing 1 mM $MgCl_2$ and incubated with shaking at 70 °C at 600 r.p.m. for 12 h. Samples were withdrawn at specific time intervals and immediately transferred to 99 °C for 5 min to inactivate the enzyme. Samples were stored at −18 °C for subsequent analysis.

The GOS mixtures were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). HPAEC-PAD analysis was carried

out on a Dionex DX-500 system consisting of a GP50 gradient pump, an ED 40 electrochemical detector with a gold working electrode and an Ag/AgCl reference electrode, and Chromeleon version 6.5 (Dionex Corp., Sunnyvale, CA). All eluents were degassed by flushing with helium for 30 min. Separations were performed at room temperature on a CarboPac PA-1 column (4 mm × 250 mm) connected to a CarboPac PA-1 guard column (Dionex). Separation of D-glucose-, D-galactose, lactose, and allolactose was carried out with an isocratic run (45 min) with 15 mM NaOH at 1.0 mL/min, followed by 25-min elution with 100 mM NaOH (gradient 1). For separation of other GOSs, eluents A (100 mM NaOH) and B (100 mM NaOH and 150 mM NaAc) were mixed to form the following gradient: 98 % A from 0 to 10 min, 98 % A to 52 % A from 10 to 40 min, and then 52 % A for another 5 min (gradient 2). The column was washed with 20 % B for 10 min and re-equilibrated for 15 min with the starting conditions of the employed gradient.

Individual GOS components were identified by comparison to authentic material, specifically β-D-Gal$p$-(1 → 3)-D-Glc, β-D-Gal$p$-(1 → 6)-D-Glc, β-D-Gal$p$-(1 → 3)-D-Gal, β-D-Gal$p$-(1 → 4)-D-Gal, β-D-Gal$p$-(1 → 6)-D-Gal, β-D-Gal$p$-(1 → 3)-D-Lac, β-D-Gal$p$-(1 → 4)-D-Lac, and β-D-Gal$p$-(1 → 6)-D-Lac purchased from Carbosynth (Berkshire, UK). The degree of lactose conversion was calculated as percentage of lactose converted of initial lactose employed. The GOS yields were calculated as percentage of GOSs formed of total sugars.

Results

Construction and expression of *Ho*BGLA variants

Based on engineering studies on related GH1 β-glycosidases (Hansson et al. 2001; Feng et al. 2005; Wu et al. 2013), amino acids in the glycone (−1) and aglycone (+1) subsites of *Ho*BGLA were selected for mutagenesis with the aim to enhance the T/H ratio. The β-glucosidase *Tn*Bgl1A from *Thermotoga neapolitana* was engineered toward improved transglycosylation by replacing N[220] in subsite +1 with phenylalanine. In *Tn*Bgl1A, this substitution caused significant improvement of the T/H ratio and higher yields of alkyl glycosides through transglycosylation (Lundemo et al. 2013). The corresponding residue in *Ho*BGLA is N[222], and thus, the *Ho*BGLA variant N222F was considered an interesting candidate. Introducing a phenylalanine side chain would increase hydrophobicity of the +1 subsite and possibly increase the affinity for acceptors of less polar character than water, such as sugars, which theoretically may favor a higher T/H ratio.

In the case of *Thermus thermophilus* β-glycosidase Ttβ-gly, directed-evolution experiments identified the

replacements of N282T, N390I, and F401S in the vicinity of the −1 subsite as variants with improved T/H ratios (Feng et al. 2005; Teze et al. 2014). These residues are conserved in *Ho*BGLA and correspond to N[294], N[406], and F[417]. Moreover, the transgalactosylation activity increased by 22 % for the *Pyrococcus furiosus* β-glucosidase CelB variant F426Y (F[417] in *Ho*BGLA) at low lactose concentrations compared with the wild type (Hansson et al. 2001). A comparison of the crystal structures of Ttβ-gly N282T (PDB code 4BCE; Teze et al. 2014) and F401S (PDB code 3ZJK; Teze et al. 2014) with that of *Ho*BGLA (PDB code 4PTX; Hassan et al. 2015) suggested that these replacements may cause similar effects in *Ho*BGLA. Based on the above information, the *Ho*BGLA variants N294T, N406I, F417S, and F417Y were selected. Additional Ttβ-gly variants with promising transglycosylation characteristics have been reported, such as R75A, W120C, N163A, and Y284F (Teze et al. 2014). These positions correspond to R[77], W[122], N[165], and Y[296] in *Ho*BGLA, respectively, and of these, the *Ho*BGLA Y296F replacement in subsite −1 was considered for further work.

In total, six *Ho*BGLA variants were designed rationally, including N222F, N294T, Y296F, N406I, F417Y, and F417S (Fig. 1), of which all but N406I could be expressed. The expression yields for the purified proteins (after reverse IMAC) were as follows: wild type, 7.5 mg/L culture (0.8 mg/g of wet cell mass); N222F, 1.0 mg/L culture (0.14 mg/g of wet cell mass); N294T, 2.9 mg/L culture (0.5 mg/g of wet cell mass); N406I, no expression; F417S, 3.8 mg/L culture (0.9 mg/g of wet cell mass); F417Y, 2.5 mg/ L culture (0.4 mg/g of wet cell mass); and Y296F, 3.2 mg/L culture (0.5 mg/g of wet cell mass).

Characterization of the hydrolytic activity of *Ho*BGLA mutants

The kinetic parameters (v_{max}, K_m, k_{cat}/K_m) for hydrolysis of cellobiose and lactose were determined for the *Ho*BGLA wild type and variants (Table 2). We previously reported the kinetics for the *Ho*BGLA-catalyzed hydrolysis of cellobiose and lactose at 50 °C (Hassan et al. 2015). The turnover number for cellobiose hydrolysis catalyzed by the wild type increases slightly (~6 %) when raising the temperature to 70 °C, while a larger increase in apparent k_{cat} is observed for lactose hydrolysis (~60 %). The changes in the K_m values in response to increasing temperature are more pronounced with $K_{m[cellobiose]}$ decreasing 2.5-fold and $K_{m[lactose]}$ dropping 6-fold. These changes are manifested as a 2.7-fold and 9-fold increase in specificity constant for cellobiose and lactose, respectively. This shows that wild-type *Ho*BGLA achieves improved specificity and catalytic efficiency for both cellobiose and lactose at 70 °C. Compared with the wild-type enzyme, all expressed variants show impaired hydrolytic activity for both substrates.

Fig. 1 Structural details of mutation sites. **a** The active site of wild-type *Ho*BGLA with relevant residues shown (PDB code 4PTX; Hassan et al. 2015). The amino acid replacements were modeled in the crystal structure: **b** N222F, **c** N294T, **d** Y296F, **e** N406I, **f** F417Y, and **g** F417S. Mutated residues are highlighted in *green*. A previously modeled 3GALA molecule (Hassan et al. 2015) was shown to delineate the subsites −1, +1, and +2 and has been superimposed on the theoretical structural models of the variants (Color figure online)
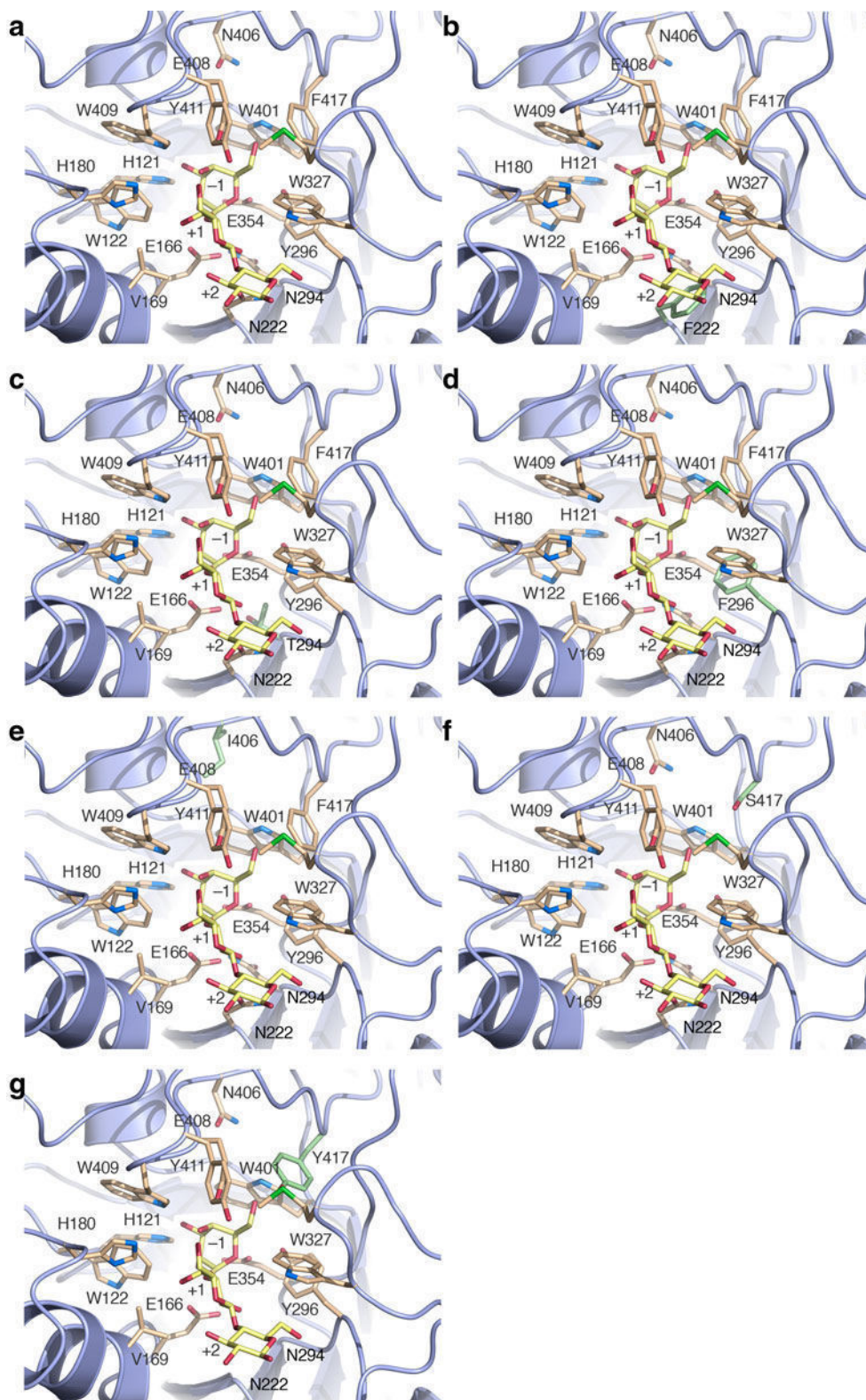
The variant N222F shows an ~23-fold decrease in k_{cat}, and 9-fold increase in K_m for cellobiose hydrolysis. The variant is also severely compromised catalytically when lactose is used as substrate (~250-fold lower k_{cat} and ~1.6-fold increase in K_m). For cellobiose hydrolysis, the variant N294T shows an ~23-fold decrease in k_{cat} (as for N222F), while K_m remains

Table 2 Kinetic parameters for β-glucosidase and β-galactosidase activities

	D-cellobiose			D-lactose			Selectivity, cellobiose over lactose[b]
	$k_{cat,app}$ (s^{-1})	K_m (mM)	$k_{cat,app}/K_m$ (mM^{-1} s^{-1})	$k_{cat,app}$ (s^{-1})	K_m (mM)	$k_{cat,app}/K_m$ (s^{-1} mM^{-1})	$(k_{cat,app}/K_{m[cel]})/$ $(k_{cat,app}/K_{m[lac]})$
WT[a]	366	25.4	14.4	231	154	1.54	9.4
WT	387 ± 15	10.0 ± 1.0	38.7	366 ± 17	26.3 ± 2.8	13.9	2.8
N222F	16.9 ± 1.3	93.2 ± 12.6	0.18	1.47 ± 0.05	41.3 ± 3.1	0.036	5.0
N294T	16.5 ± 0.4	9.0 ± 0.8	1.8	7.4 ± 0.2	30.8 ± 2.0	0.24	7.5
Y296F	8.5 ± 0.3	20.2 ± 1.6	0.42	6.9 ± 0.2	29.1 ± 2.3	0.24	1.8
F417Y	38.7 ± 0.6	3.5 ± 0.2	11.1	41.5 ± 1.2	10.3 ± 0.9	4.0	2.8
F417S	0.38 ± 0.01	11.9 ± 0.9	0.032	7.8 ± 0.4	23.2 ± 2.7	0.34	0.09

[a] Reaction performed at 50 °C; from Hassan et al. 2015; all other reactions performed at 70 °C

[b] A ratio >1 favors cellobiose hydrolysis, whereas a ratio <1 favors lactose hydrolysis

unchanged compared with the wild type. Using lactose as substrate, this mutant displays an almost 50-fold decrease in k_{cat} and only slightly higher K_m (17 %). The HoBGLA variant Y296F hydrolyzes cellobiose ~45 times slower than does the wild-type enzyme, and the affinity for cellobiose is reduced, as indicated by a twofold increase in K_m value. Turnover of lactose is affected slightly more (~53-fold lower k_{cat}), but with only minor impact on K_m (11 % increase).

The least affected HoBGLA variant is F417Y, which, compared to the wild type, retains 10 and 11 % of the cellobiose and lactose turnover numbers, respectively. A concomitant drop in K_m and 3.5-fold decrease in specificity constant for both substrates accompany the decrease in apparent k_{cat}. With cellobiose as substrate, the variant F417S shows the lowest turnover number (k_{cat} 0.38 s^{-1}), which corresponds to a 1000-fold decrease in k_{cat}, but an unperturbed K_m value. However, hydrolysis of lactose by this variant is less affected with a 47-fold decrease in turnover number (k_{cat} 7.8 s^{-1}) and similar K_m compared with the wild type. Thus, the F417S replacement in HoBGLA is detrimental for cellobiose hydrolysis whereas this variant remains relatively competent with respect to lactose hydrolysis.

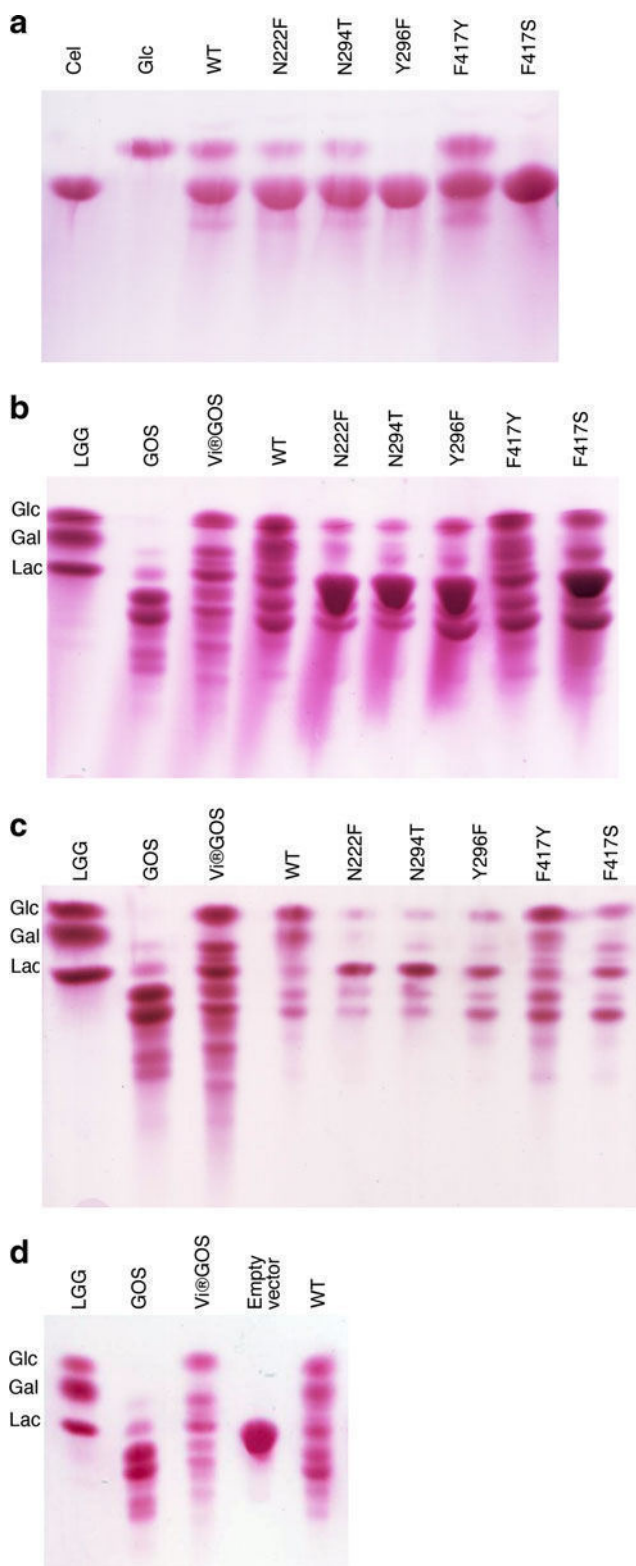## TLC screening for GOS production by HoBGLA variants using cell lysates

We have reported previously that wild-type HoBGLA is able to transform lactose into GOSs efficiently and in high yields (Hassan et al. 2015). As an initial evaluation of the capacity of the HoBGLA variants to synthesize GOSs, the crude lysates containing the respective variants were screened for their ability to hydrolyze cellobiose, and to convert lactose to products other than the hydrolysis products glucose and galactose using TLC (Fig. 2). As expected from the steady state kinetics (Table 2), all variants show hydrolytic activity on both cellobiose and lactose. The product patterns for cellobiose

hydrolysis are similar for wild type, N222F, N294T, and F417Y (Fig. 2a). The absence of hydrolysis products from cellobiose for Y296F and F417S (Fig. 2a) under the conditions considered and the low associated turnover numbers, especially for F417S (Table 2), are consistent with poor performance of these mutants with cellobiose as substrate.

The variant F417Y displays product patterns from lactose hydrolysis similar to those of the wild type, whereas N222F, N294F, Y296F, and F417S show different patterns of GOSs formed (Fig. 2b, c). The variants Y296F, F417Y, and F417S seem to generate especially one GOS product in higher yields (i.e., the lower GOS band in Fig. 2b, c). Of these, F417Y shows the weakest signal for residual lactose, which reflects the higher $k_{cat,lac}$ value of this variant compared with Y296F and F417S. As mentioned above, F417S shows a change in substrate selectivity from cellobiose to lactose, which could be useful for GOS production from substrate sources containing a mixture of cellobiose and lactose.

Analysis of transgalactosylation activity and GOS production by HoBGLA variants

The transgalactosylation activity of the HoBGLA variants was subsequently investigated in more detail using purified enzyme preparations. The reactions were performed at 70 °C with an initial lactose concentration of 300 g/L using the same amount of purified enzyme (~0.35 mg). Three variants, Y296F, F417S, and F417Y, show improved total GOS yields compared with the wild-type enzyme (Table 3). The highest GOS yield of ~57 % was obtained with F417Y, and F417Y also shows highest lactose conversion after 8 h among the variants tested. N294T is the least efficient in converting lactose and forming GOSs compared with the other variants and the wild-type enzyme under the conditions applied here.

Fig. 2 Cellobiose and lactose hydrolysis and transglycosylation using cell lysates. TLC analysis of *Ho*BGLA-catalyzed hydrolysis and transglycosylation in cell lysates of **a** cellobiose after 2 h, **b** lactose after 3 h, **c** lactose after 4.5 h; and **d** lactose after 4.5 h of reaction using cells carrying the expression vector without *Ho*BGLA insert as control. Standards used: LGG, lactose (Lac); galactose (Gal); glucose (Glc); GOS, purified GOS with monosaccharides and lactose removed; Vivinal®GOS; Cel, cellobiose

All variants yield β-D-Gal$p$-(1 → 6)-Lac as the predominant oligosaccharide product (Table 3), at levels ranging from 43 to 67 % mass of the total GOSs produced. Y296F and F417S show almost identical GOS yields as well as the highest relative amounts of β-D-Gal$p$-(1 → 6)-Lac. Differences between these two variants during lactose transformation are that F417S converts lactose faster and that F417S forms the disaccharide β-D-Gal$p$-(1 → 3)-Glc as its second most frequent GOS product, whereas this is β-D-Gal$p$-(1 → 3)-Lac in the case of Y296F. Only four main GOS components were found in detectable amounts in the GOS mixtures formed when using Y296F and F417S, which were β-D-Gal$p$-(1 → 6)-Lac, β-D-Gal$p$-(1 → 3)-Glc, β-D-Gal$p$-(1 → 3)-Lac, and β-D-Gal$p$-(1 → 4)-Lac. Interestingly, F417S shows the same relative composition of main GOS components (calculated as percentage mass of the total GOSs) throughout the conversion (Table 4). Separation and quantification by HPAEC-PAD of individual GOS produced during lactose conversion catalyzed by wild-type *Ho*BGLA and F417S are given in Fig. 3.

Discussion

When selecting the *Ho*BGLA variants to be engineered, the designs (Fig. 1) were guided by work performed on homologous GH1 enzymes. Compared with *Ho*BGLA N222F (Fig. 1b), the *Tn*Bgl1A variant N220F showed less dramatic effects on the kinetic parameters, i.e., k_{cat} and K_m increased by 4-fold and 2.8-fold, respectively, using *p*-nitrophenyl β-D-glucopyranoside (*p*NP-Glc) relative to the wild type (Lundemo et al. 2013). There is no crystal structure available for *Tn*Bgl1A, but molecular modeling suggests that the active site in *Tn*Bgl1A is sufficiently different compared with that of *Ho*BGLA to account for the discrepancy. In addition, different substrates were used, which makes a direct comparison difficult. That aside, the impact of the mutation appears to have a more drastic effect on *Ho*BGLA.

For the Ttβ-gly N282T variant (corresponding to *Ho*BGLA N294T; Fig. 1c), *p*NP-Glc was hydrolyzed with 4-fold lower k_{cat} and 27-fold higher K_m (Feng et al. 2005), while hydrolysis of *p*NP-Gal was associated with a 8-fold lower k_{cat} and 6.5-fold higher K_m value compared with the wild-type enzyme. The crystal structure of Ttβ-gly N282T (PDB code 4BCE) did not reveal any significant structural changes that could explain the altered performance for this variant (Teze et al. 2014). The discrepancy in catalytic performance between the *Ho*BGLA and Ttβ-gly variants is difficult to

Table 3 Degree of lactose conversion, GOS yield, and individual GOS components

Variants	WT	N222F	Y296F	F417S	N294T	F417Y
Degree of lactose conversion (%)	99.2	49.9	70.4	79.5	39.3	97.4
GOS yield (% mass of total sugars)	39.3	33.9	52.3	52.5	29.4	57.4
GOS components (% mass of total GOS)						
D-Gal*p*-(1 → 3)-D-Gal	8.2	0.0	0.0	0.0	0.0	2.8
D-Gal*p*-(1 → 6)-D-Gal	7.0	2.6	0.0	0.0	0.0	4.7
D-Gal*p*-(1 → 3)-D-Glc	8.8	3.7	10.5	25.1	22.8	13.6
D-Gal*p*-(1 → 6)-D-Glc	16.7	24.8	0.0	0.0	0.0	8.8
D-Gal*p*-(1 → 3)-D-Lac	5.1	10.9	16.9	6.2	31.0	13.4
D-Gal*p*-(1 → 4)-D-Lac	2.9	0.0	1.4	2.0	3.3	3.3
D-Gal*p*-(1 → 6)-D-Lac	45.4	57.9	66.3	66.5	42.6	53.1

Degree of lactose conversion, GOS yield, and individual GOS components produced by the transgalactosylation reaction of wild-type *Ho*BGLA and the variants using lactose as substrate after 8 h of reaction. The reactions were performed at 70 °C with an initial lactose concentration of 300 g/L in sodium phosphate buffer (pH 6.0) and 1 mM MgCl₂ using 0.35 mg of purified enzyme

rationalize from a structural viewpoint since the active sites are nearly identical, and the differences may be mainly accounted for by the different substrates used. Hydrolysis was severely compromised in the Tt*β*-gly variant Y284F (Teze et al. 2014) and *Agrobacterium β*-glycosidase Abg Y298F (Gebler et al. 1995), which correspond to *Ho*BGLA Y296F (Fig. 1d), and the tyrosine residue was assigned an important role to fine-tune the position of the nucleophile and to stabilize its deprotonated state (Gebler et al. 1995).

F417S (Fig. 1f) is the most catalytically impaired *Ho*BGLA variant with respect to the hydrolytic reaction, but with lactose performing considerably better than cellobiose. Similarly, the corresponding Tt*β*-gly variant F401S is also severely crippled catalytically, with no detectable hydrolytic activity on *p*-nitrophenyl *β*-D-glucoside (*p*NP-Glc) and *p*-nitrophenyl *β*-D-galactoside (*p*NP-Gal) (Feng et al. 2005). As for N282T, the crystal structure of Tt*β*-gly F401S (PDB code 3ZJK) offered no

Table 4 Time course of lactose conversion and formation of GOS by F417S

Time (h)	1	2	3	6	8
Degree of lactose conversion (%)	30.2	46.2	55.8	73.3	79.5
GOS yield (% mass of total sugars)	23.7	36.7	43.3	55.6	52.5
GOS components (% mass of total GOS)					
D-Gal*p*-(1 → 3)-D-Glc	18.8	23.1	24.7	25.5	25.1
D-Gal*p*-(1 → 4)-D-Lac	2.7	2.6	2.8	3.0	2.0
D-Gal*p*-(1 → 3)-D-Lac	7.4	6.9	6.5	6.1	6.2
D-Gal*p*-(1 → 6)-D-Lac	70.9	67.3	65.8	65.3	66.5

Time course of lactose conversion and formation of GOS during lactose conversion by the variant F417S. The reactions were performed at 70 °C at an initial lactose concentration of 300 g/L in sodium phosphate buffer (pH 6.0) and 1 mM MgCl₂ using 0.35 mg of purified enzyme

explanation for the loss of hydrolytic activity or improved T/H ratio for the mutants (Teze et al. 2014), but the phenylalanine residue was suggested to be selectively important for transition state (TS*) stabilization during the hydrolysis reaction, but of less significance for the transglycosylation reaction (Teze et al. 2014).

The rate of catalysis for cellobiose and lactose hydrolysis is only marginally affected in *Ho*BGLA F417Y (Fig. 1g), and decreased K_m values are observed for both substrates. For the corresponding *P. furiosus* CelB variant F426Y, the turnover number decreased by approximately 30 % while K_m was essentially unchanged using *p*NP-Glc (Hansson et al. 2001). To improve the yield of transglycosylation products for *P. furiosus* CelB variant F426Y, the complementary replacement M424K was made, generating the variant M424K/F426Y (Hansson et al. 2001). As for F426Y, the double mutant displayed somewhat lower turnover number while K_m increased 1.5-fold for *p*NP-Glc compared with the wild type. The position in *Ho*BGLA equivalent to M[424] in CelB is K[415], and *Ho*BGLA F417Y should therefore be compared with CelB M424K/F426Y.

By dividing the specificity constant for cellobiose hydrolysis by that of lactose hydrolysis, a selectivity ratio [$(k_{cat,app}/K_{m[cellobiose]})/(k_{cat,app}/K_{m[lactose]})$] is obtained, where a smaller ratio means improved performance with lactose over cellobiose as substrate. The selectivity ratio for the wild-type reaction with cellobiose measured at 50 and 70 °C shows that the preference for lactose is improved at higher temperatures, i.e., at 50 °C, cellobiose is ~9 times favored over lactose, but at 70 °C, cellobiose is only 2.8 times more favored over lactose. Thus, in a reaction where both cellobiose and lactose are present, elevated temperature improves lactose hydrolysis relative to cellobiose hydrolysis. Interestingly, *Ho*BGLA F417S displays a change of substrate selectivity with lactose being
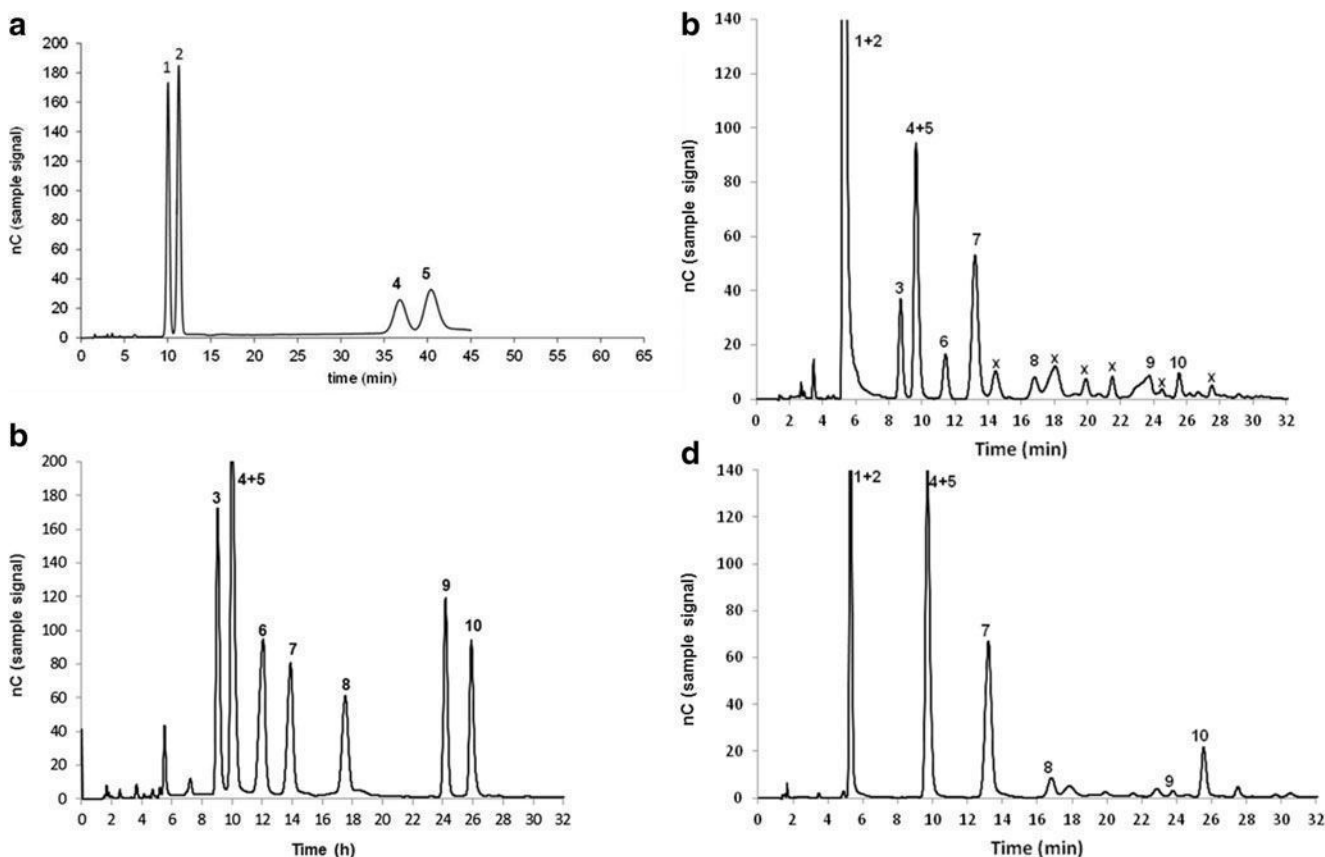
Fig. 3 Separation and quantification by HPAEC-PAD. Separation and quantification by HPAEC-PAD of authentic standards (**a**, **b**) and of the GOS mixtures produced during lactose conversion catalyzed by wild-type *Ho*BGLA (**c**) and *Ho*BGLA F417S (**d**). The identified compounds are (*1*) galactose, (*2*) glucose, (*3*) D-Gal*p*-(1 → 6)-D-Gal, (*4*) D-Gal*p*-(1 → 6)-D-Glc (allolactose), (*5*) D-Gal*p*-(1 → 4)-D-Glc (lactose), (*6*) D-Gal*p*-

(1 → 3)-D-Gal, (*7*) D-Gal*p*-(1 → 6)-Lac, (*8*) D-Gal*p*-(1 → 3)-D-Glc, (*9*) D-Gal*p*-(1 → 4)-Lac, and (*10*) D-Gal*p*-(1 → 3)-Lac. Products marked with an "*x*" were not identified. Different conditions were used for HPAEC-PAD analysis to separate D-glucose-, D-galactose, lactose, and allolactose (gradient 1, panel (**a**)) as well as the other oligosaccharides (gradient 2, panels (**b–d**)). Details are given in the "Materials and methods" section

favored 11-fold over cellobiose (selectivity ratio 0.09). Improved selectivity ratios for lactose are also observed for the other variants, although cellobiose remains the preferred substrate.

In this study, the objective was to evaluate whether *Ho*-BGLA can be engineered toward improved transgalactosylation. With respect to transglycosylation performance, the variants F417S and F417Y are of particular interest to address. The *Ho*BGLA variant F417S (Table 3) produces mainly the trisaccharides β-D-Gal*p*-(1 → 6)-Lac and β-D-Gal*p*-(1 → 3)-Lac, and the disaccharides β-D-Gal*p*-(1 → 3)-Gal and β-D-Gal*p*-(1 → 3)-Glc, whereas for wild-type *Ho*BGLA, several other disaccharides are also produced. The percentages of disaccharides versus trisaccharides were 33.2 versus 66.5 % for F417Y, and 27.1 versus 72.7 % for F417S. The degree of lactose conversion of *Ho*BGLA F417Y is similar to that of the wild type, while the total GOSs has increased. The yield of total GOSs for F417Y is also close to that observed for some of the most efficient transglycosylating BGALs, e.g.,

recombinant β-galactosidase from *Bifidobacterium infantis* for which 63 % GOSs of the total sugars in the reaction mixture has been reported (Hung et al. 2001).

Several of the *Ho*BGLA variants reported here show interesting properties for lactose conversion and GOS production. The variant F417S, as well as Y296F, produces β-D-Gal*p*-(1 → 6)-Lac in significantly higher amounts than the wild type and the other variants. Amino acid replacements in the −1 subsite, such as in F417S or Y296F, resulted in a significantly different spectrum of GOS components formed and could be of interest for tailoring GOS mixtures, e.g., for a higher content in trisaccharides. Variant F417Y is almost as efficient as wild-type *Ho*BGLA in converting lactose but more competent for overall GOS production. This work shows that the thermostable BGLA from *H. orenii* can be successfully engineered toward higher GOS production and different GOS distributions depending on the requirements. Although the present enzyme variants would not contribute to industrial GOS production, they offer molecular insights into improved GOS yields by *Ho*BGLA.

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CHAPTER 3

Elucidating structure-function relationships of *Halothermotrix orenii* β-glucosidase by probing the active site

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Abstract

β-Glucosidases (E.C. 3.2.1.21) are enzymes that catalyze the cleavage of glycosidic bonds and are ubiquitous in bacteria, archaea and eukaryotes, playing essential roles in biological processes such as metabolizing cellulose and other carbohydrates, developmental regulation and defence against pathogen invasion. Besides their functions in nature, β-glucosidases are of great interest for industrial applications. They are glycoside hydrolase (GH) family members and are evolutionary highly conserved among species. Several publications report the improved transglycosylation of β-glucosidases by single amino acid replacements in the -1 subsite. In this work, we investigated the impact of active site amino acid exchanges in the *Halothermotrix orenii* β-glucosidase (*Ho*BGLA) by probing position F417 to get further insight in the structure-function relationship of β-glucosidases, F417 is one of the amino acids interacting with the sugar in the -1 subsite. Seven variants with significant lactose activity were selected from a mutant library covering all possible amino acid exchanges. Kinetic parameters with lactose and cellobiose and temperature stability were determined. Moreover, individual galacto-oligosaccharide (GOS) and cello-oligosaccharide (COS) compositions produced by the purified enzyme variants were analyzed by HPLC. Most importantly, variant F417T preferred lactose over cellobiose as substrate. When lactose was used as substrate in transgalactosylation, variant F417T mainly produced allolactose, whereas the wildtype (WT) predominantly catalyzed the formation of 6'-galactosyllactose. When cellobiose was used in transglucosylation, variant F417T produced cellotetraose with cellobiose as substrate in addition to trisaccharides and disaccharides. In WT the only detected COS were disaccharides and trisaccharides. All variants were less thermostable than the WT. All in all these findings underline the importance of position F417 for enzymatic activity and thermostability of *Ho*BGLA, which belongs to the GH1 family.

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1 Introduction

β-Glucosidases have many applications in industry, such as the improvement of flavor, the production of bio-degradable non-ionic surfactants, synthesis of diverse oligosaccharides, glycoconjugates and alkyl-aminoglycosides, as well as in lignocellulose hydrolysis and biofuel production (Bhatia et al. 2002). An ideal β-glucosidase should be active over a wide pH range and stable at high temperatures. In industry, there is a need for improved and novel enzymes which are resistant to increased temperature, extremes of pH, and high salt concentrations. Its thermostability, adaption to a wide range of salt concentrations and broad pH stability make *H. orenii* proteins an interesting target for applications that require extreme conditions such as high temperatures and extremes of pH (Bhattacharya et al. 2014). *Ho*BGLA is a thermophilic and halophilic organism. It grows best at temperatures of 60-70 °C and at NaCl concentrations of 5-10 % (Kori et al. 2011). *Ho*BGLA is highly active and stable within a broad pH range (4.5–7.5), the temperature optimum is in the range of 65–70 °C, and the enzyme also shows good stability at this temperature range (Hassan et al. 2015). Its primary activity is the hydrolysis of cellobiose, but the enzyme shows significant activity with other substrates such as lactose and aryl-glycosides. With lactose as a substrate, the enzyme has a distinct transgalactosylation activity producing large amounts of GOS. Its high thermostability allows the use of elevated temperatures and high substrate concentrations, conditions which favor GOS formation. GOS are non-digestible carbohydrates, so-called prebiotics which positively impact human health upon ingestion (Roberfroid 2007, Sangwan et al. 2011, Bode 2012). We recently reported enzyme variants from *Ho*BGLA with altered transglycosylation activity, generated by exchanges of amino acids around the active center. The exchange of the phenylalanine at position 417 for tryptophan or serine leads to improved GOS yields (Hassan, Geiger et al. 2015). Previous publications report an improvement of transglycosylation activity of GH β-glucosidases derived from different organisms by single exchange of the amino acid homologous to F417 in *Ho*BGLA. This suggests this amino acid position to be crucial for transglycosylation activity in HoBGLA as well. Exchange of phenylalanine for serine on position 401 in *Thermus thermophilus* β-glucosidase results in a higher transglycosylation yield using artificial substrates, pNP-Fuc as donor and N-methyl-O-benzyl-N-(b-D-glucopyranosyl)-hydroxylamine as acceptor (Teze et al. 2014). In *P. furiosus* β-glucosidase, replacement of phenylalanine by tyrosine at the position 426 results in enhanced transgalactosylation activity with lactose (Hansson et al. 2001). Transglycosylation activity of variant

Thermotoga neapolitana β-glucosidase F412S is increased, due to reduced hydrolytic activity with pNPG as a substrate and arbutin as an acceptor (Choi et al. 2008). In this work, we aimed to further explore the structure-function relationship and particularly the transglycosylation activity of *Ho*BGLA by probing position F417. We present the production, purification and characterization of variants with different amino acid exchanges at this position.

2. Methods

2.1. Construction of the library

The *Ho*BGLA WT gene in vector pNIC28-Bsa4 (Savitsky et al. 2010) containing an N-terminal hexahistidine tag was used as a template for site-saturation mutagenesis to produce the single-replacement variant library on position 417. The following primers were used:
Forward primer
5´ CAAGCGCNNSGGTCTCATTTATGTTG 3´
Reverse primer
5´ TGAGACCSNNGCGCTTGCTATAGCC 3´

The initial denaturation step at 98 °C for 10 sec was followed by 34 cycles of denaturation at 98 °C for 20 sec, annealing at 60 °C for 20 sec and extension at 72 °C for 90 sec, followed by a terminal elongation at 72 °C for 5 min. Resulting PCR products were digested with *Dpn*I in order to eliminate template DNA and were subsequently transformed into *Escherichia coli* NiCo21 (DE3). Enzyme variants were identified by single colony sequencing (Microsynth AUSTRIA).

2.2 Fast Screening

Enzymatic activity of variants was analyzed by a fast screening procedure. Crude cell lysates of overnight cultures of transformed *E. coli* NiCo21 (DE3) single colonies were incubated in HEPES buffer 20 mM pH 7, containing 150 mM NaCl supplemented with cellobiose and lactose to a final concentration of 20 % (w/v). Reaction mixtures were incubated at 70 °C and shaking at 600 rpm for 3 hours. Subsequently, the reaction was stopped at 95 °C for 5 min. Samples were diluted 1:10, followed by loading of 1 µL sample on a TLC plate. HPTLC Li Chrosper®Silica gel 60 F_{254}S

(Merck) was used as an adsorbent. Samples were applied to the plates and placed in the eluent (*n*-butanol–*n*-propanol–ethanol–water = 2:3:3:2). Visualization of carbohydrates was performed by immersing the TLC plate in a staining solution (0.5 g thymol, 95 ml 96 % ethanol, 5 ml concentrated sulfuric acid) for 3 seconds and subsequent heating at 90 °C for approximately 1 min. Standards for lactose conversion included a mixture of glucose, galactose and lactose (LGG) (Maischberger et al. 2008).

2.3 Expression and Purification

For enzyme expression, *E.coli* NiCo21(DE3) cells were grown in Terrific Broth (TB) medium supplemented with 50 µg/ml kanamycin and glycerol as a carbon source (60 ml per 600 ml) at 37 °C to an optical cell density, OD_{600}, of 0.6. Subsequently, the culture was induced with 0.2 mM IPTG and cultivated at 18 °C for 16–18 h. The crude cell extracts were prepared by resuspending the cell pellets of a 250 ml induced cell culture of *E. coli* NiCo21(DE3) cells in 5 ml of HEPES buffer 20 mM pH 7, containing 150 mM NaCl. Cells were lysed by using a French press and centrifuged at 4 °C for 30 min at 20,000 g. The *Ho*BGLA was purified by Ni^{2+}-charged immobilized metal affinity chromatography (IMAC) (His Pur ™ Ni NTA chromatography cartridge 1 ml, Thermo Scientific). The clear lysate was loaded onto the column, followed by a washing step with 10 column volumes of HEPES buffer 20 mM pH 7, containing 10 mM imidazole, 150 mM NaCl and 20 % glycerol. *Ho*BGLA was then eluted with 2 column volumes HEPES buffer 20 mM

pH 7, containing 350 mM imidazole, 150 mM NaCl and 20 % glycerol. Protein concentrations were determined by the Bradford assay, bovine serum albumin was used as standard (Bradford 1976). Enzymatic activities of protein elutions were determined with lactose and cellobiose. For standard activity measurements, 480 µl of substrate (lactose and cellobiose respectively) 600 mM and 20 µl of enzyme solution were incubated at 50 °C shaking at 600 rpm for 10 min. The glucose release was measured by the GOD/POD Assay Kit (Megazyme). One unit of lactose-hydrolyzing activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions. One unit of cellobiose-hydrolyzing activity was defined as the amount of enzyme releasing 2 µmol of D-glucose per minute under the same conditions. The purity of *Ho*BGLA preparations was assessed by SDS-PAGE.

2.4 Analysis of kinetic parameters

Kinetic parameters of variants with lactose and cellobiose as a substrate were determined. For determination of the hydrolytic activity of *HoBGLA* with lactose as a substrate, 20 µl of enzyme solution and 480 µl lactose in HEPES buffer 20 mM pH 7, supplemented with 150 mM NaCl were incubated at 50 °C shaking at 600 rpm for 10 min. The concentration range of lactose was

0–800 mM. The glucose release was measured by the GOD/POD Assay Kit (Megazyme). One unit of lactose-hydrolyzing activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions. Measurement of the hydrolytic activity with cellobiose was performed by incubating 20 µl enzyme solution and 480 µl cellobiose in HEPES buffer 20 mM, pH 7

supplemented with 150 mM NaCl at 50 °C, shaking at 600 rpm for 10 min. The cellobiose concentration range was 0–600 mM. Glucose release was determined by the GOD/POD Assay Kit (Megazyme). One unit of lactose-hydrolyzing activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions. One unit of cellobiose-hydrolyzing activity was defined as the amount of enzyme

releasing 2 µmol of D-glucose per minute under the same conditions. Non-linear regression was used for determination of the kinetic parameters and the data were fitted to the Michaelis-Menten model using SigmaPlot Prism 11.0. The apparent turnover values (k_{cat}) were calculated using the experimentally determined v_{max} values and a molecular mass of 53 kDa for the enzyme.

2.5   Molecular dynamics simulation

As a starting configuration for molecular dynamics simulation we used *Ho*BGLA A in complex with thiocellobiose [PDB ID: 4PTV (Hassan et al. 2015)] at 1.85 Å resolution. However, the binding pose of thiocellobiose in this crystal structure has been described as an artefact of a PEG molecule bound in the active site and does not correspond to the actual position. For that reason and because of the high similarity of active sites, the binding pose of thiocellobiose was taken from a crystal structure of *Paenibacillus polymyxa* β-glucosidase in complex with thiocellobiose [PDB ID: 2O9R (Isorna et al. 2007)]. For the simulation of complexes with cellobiose, the sulphur atom in thiocellobiose was replaced by oxygen. The poses of cellobiose after 1 ns of equilibration run were taken as starting structures for lactose simulations. These cellobiose structures were modified into lactose by inverting the stereocenter at C4, which was followed by an energy-minimization. To study the interactions

between disaccharides and proteins, 10 simulations for 20 ns were performed in total. The WT and variants F417M, F417L, F417T, F417Y were simulated with both, cellobiose and lactose. All arginines, lysines and cysteines were protonated, while aspartic and glutamic acids were deprotonated. The protonation state of histidines was determined based on the hydrogen bonding possibilities in the surrounding. All MD simulations were carried out using the GROMOS11 software simulation package (Schmid et al. 2012), employing the 54a8glyc forcefield (Reif et al. 2013). Proteins were energy-minimized in vacuum using the steepest-descent algorithm and subsequently solvated in a rectangular, periodic and pre-equilibrated box of single point charge (SPC) water (Berendsen et al. 1981). This led to systems containing about 15,000 atoms. To achieve electroneutrality of the system that included the protein, 16 sodium ions were added to the system. Production runs of 20 ns were performed with constant numbers of

particles, constant temperature (300 K) and constant pressure (1 atm). To sustain a constant temperature, we used the weak-coupling thermostat (Hermans et al. 1984) with a coupling time of 0.1 ps. Solute and solvent were coupled to a separate temperature bath. The pressure was maintained using a weak coupling barostat with a coupling time of 0.5 ps and an isothermal compressibility of 4.575×10^{-4} $kJ^{-1} \cdot mol \cdot nm^{-3}$. Implementation of the SHAKE algorithm (Ryckaert et al. 1977) in constraining bond lengths of solute and solvent allowed for a 2-fs time-step. Coordinate trajectories were stored every 0.5 ps for subsequent analysis.

2.6   Transglycosylation

Initial enzymatic activities were determined by standard activity measurements. 20 µl of enzyme solution and 480 µl lactose 600 mM and cellobiose 600 mM respectively, were incubated in HEPES buffer 20 mM pH 7, supplemented with 150 mM NaCl at 50 °C, shaking at 600 rpm for 10 min. For transgalactosylation, 200 µl of enzyme solution (0.45 U_{Lac}/ml) and 200 µl 40 % lactose (w/v) in HEPES buffer 20 mM, pH 7 supplemented with 150 mM NaCl were incubated at 50 °C shaking at 600 rpm. For transglucosylation, 200 µl of enzyme solution (0.45 U_{Cell}/ml) and 200 µl 40 % cellobiose (w/v) in HEPES buffer 20 mM, pH 7 supplemented with 150 mM NaCl were incubated at 50 °C shaking at 600 rpm. Samples were drawn at different time points and heat-inactivated for 5 min at 95 °C, followed by HPLC analysis of transglycosylation products. The HPLC analysis of transgalactosylation products was performed as described previously, the same GOS standards were used (Splechtna et al. 2007). Transglucosylation products were analyzed by the HPLC as described formerly (Westereng et al. 2013). COS standards (Megazyme) included:

cellotriose

[D-β-Glc-(1→4)-D-Cel]

cellotetraose

[D-β-Glc-(1→4)-D-Glc-(1→4)-D-Cel]

cellopentaose

[D-β-Glc-(1→4)-D-Cel-(1→4)-D-Cel]

laminaribiose

[D-β-Glc-(1→3)-D-Glc]

Laminartriose

[D-β-Glc-(1→3)-D-Glc-(1→3)-D-Glc]

glucotriose

[D-β-Glc-(1→3)-D-Cel]

gentiobiose

[D-β-Glc-(1→6)-D-Glc]

3   Results

3.1   Fast screening of variants

Enzymatic activities of generated and identified *Ho*BGLA variants were analyzed by incubation of crude cell lysates of transformed *E. coli* cells for 3 hours at 70 °C in HEPES buffer 20 mM, pH 7 supplemented with 150 mM NaCl, containing 20 % (w/v) cellobiose or lactose respectively. Reaction products were then visualized by TLC. Replacement of F417 by A, D, E, G, K, N, P, Q, R, and V rendered the enzyme almost inactive for cellobiose and completely inactive for lactose. For variants F417D, F417E, F417K, F417N, F417P and F417Q, a faint band for glucose was observable, indicating a modest residual hydrolytic activity with cellobiose. Exchange of F417 for C, H, I, L, M, S, T, W and Y resulted in variants with retained activity with lactose and cellobiose. F417M shows a reaction product pattern with lactose comparable to the WT, whereas hydrolytic activity of the other variants was decreased and appeared to be lowest in variants F417I, F417S and 417T. Hydrolytic activity of variant 417M with cellobiose was comparable to the WT and reduced in the other variants. Hydrolytic activity of variants 417S and 417T with cellobiose appeared to be most impaired among all variants (Figure 1).

3.2   Temperature stability

Temperature stability of all variants was determined by incubation of the purified enzymes in HEPES buffer 20 mM, pH 7 supplemented with 150 mM NaCl at 50 °C and 60 °C respectively. All variants exhibited decreased temperature stabilities compared to the WT. F417M showed the best stability among all variants with a half-life of 10 h at 60 °C, whereas variants 417T and 417I had a half-life of shorter than 1 h. At 50 °C the half-life of all variants was at least 10 h (Table 1). Due to these limited temperature stabilities of variants, determination of kinetic parameters with lactose and cellobiose, as well as transglycosylation reactions were performed at 50 °C.

3.3   Hydrolytic activity of variants

The kinetic parameters (v_{max}, K_m, k_{cat}/K_m) for lactose and cellobiose were determined at 50 °C for the WT and the variants. All variants showed impaired catalytic efficiencies (k_{cat}/K_m) with lactose, in comparison to the WT. Catalytic efficiency of variant F417M with lactose was decreased by one third compared to the WT, but was the highest of all variants due to a turnover number (k_{cat}) similar to the WT. The turnover numbers of all other variants were decreased. Variant F417C showed the highest K_m value with lactose, with a 5-fold increase in comparison to the WT, while variants F417T and F417Y

exhibited the lowest K_m value with lactose in comparison to the WT. (Table 3).

All variants showed a lower catalytic efficiency (k_{cat}/K_m) for cellobiose than the WT. Variant F417M, in contrast, was the least affected variant, retaining half of the catalytic efficiency of the WT. Variant F417C had a k_{cat} comparable to the WT, whereas all other variants showed decreased turnover numbers (Table 2). Variant F417T was most impaired for the substrate cellobiose, with a 200-fold decrease in catalytic efficiency, mainly due to an unfavorable K_m. Variant F417T showed the highest selectivity of lactose over cellobiose as substrate, with a 3-fold increase (Table 4). Most importantly, single amino acid replacement of F417 by T resulted in an enzyme which preferred lactose over cellobiose as substrate (Table 4).

3.4 Molecular dynamics simulation

To explore differences in binding of cellobiose and lactose in mutants of F417, we performed 10 independent plain MD simulations, each of 20 ns. Variants were selected, based on the K_m value. We have chosen mutants preferring either lactose (F417T and F417Y), cellobiose (WT and F417M) or binding both equally strongly (F417L). Figure 2 shows snapshots of the residues interacting with the substrates after 20 ns of simulation. It is, nevertheless, a single snapshot in 20 ns, so not all the hydrogen bonds are visible here. In general, the binding pose of the disaccharides depends more on the amino acid in position 417, than the disaccharide itself. However, in the case of bulky amino acids at 417, i.e. phenylalanine, tyrosine and threonine, cellobiose is more tilted towards E408 than lactose. The orientation of the second glucose ring differs most in F417M and F417L mutants from the WT. Furthermore, we monitored the occurrence of hydrogen bonds between the disaccharide substrates and the protein throughout the molecular dynamics trajectories (Table 5). The most persistent hydrogen bonding was seen between the O4 and O6 atoms and OE1, OE2 atoms of E408, in average 1.66 per residue. These bonds have been observed in both substrates in all above-mentioned variants, which explains their activity. On the outer side of the disaccharide binding, a strong interaction between E173 and O1 and O2 of the second glucose ring of both substrates has been observed in all variants, except for F417M. The biggest differences between variants occur in hydrogen binding of substrates O2, O3 and O4 to other catalytic residues, E166 and E354. While in simulations of F417T and F417Y lactose creates hydrogen bonds to these residues more often than cellobiose, in simulations of F417, F417L and F417M cellobiose is binding to these residues more frequently. This trend can be seen in the time series of the distances between the E166 and E354 side chain oxygen atoms and C1 and O4

atoms of disaccharide (Figure 3) as well. Preference of F417Y and F417T for lactose can be explained by the close distance of E354 oxygens to the disaccharide C1 atoms (~8 Å) and by the close distance of E166 oxygens to the lactose O4 atom (~4 Å). In the WT and F417M, however, lactose is located slightly further from the catalytic glutamates than cellobiose. Moreover, hydrogen bonding occurs between O3, O4 and O6 atoms of both substrates and Q20 and H121 in F417T and F417Y. Hydrogen bonding to W401 and W409

has been seen in all simulations and variants except for both substrates in F417M mutant, because of the shifted pose of the substrate. Interestingly, O3 and O4 oxygens of lactose in the WT and F417M create a stable hydrogen bond to Y296, which might be pulling the ring further towards E166 and E354. Overall, stronger interactions are observed for cellobiose than for lactose in mutants F417L and F417M, while in the WT and in F417T and F417Y lactose forms slightly more hydrogen bonds.

3.5 Transgalactosylation with lactose

Transgalactosylation reactions were performed at 50 °C using an initial lactose concentration of 200 g/l in HEPES buffer 20 mM pH 7 supplemented with 150 mM NaCl. For analysis, samples with a glucose release of (50 % ± 6 %) were chosen for better comparison. In variants F417Y, F417I, F417T and F417S allolactose [D-β-Gal-(1→6)-D-Glc] constituted the predominant GOS with a share of 47 % of total GOS, (F417I), 37 % (F417S), 56 % (F417T) and 51 % (F417Y). The main GOS component produced by the WT (36 %) as well

as by variants F417C (48 %), F417H (44 %) F417L (36 %), F417M (38 %) and F417W (51 %) was 6'-galactosyllactose [D-β-Gal-(1→6)-D-Lac]. All variants and the WT produced D-β-Gal-(1→6)-D-Gal, D-β-Gal-(1→3)-D-β-Gal, D-Gal-(1→3)-D-β-Glc, D-Gal-β- (1→4)-D-Lac and D-β-Gal-(1→3)-D-Lac in minor amounts (Table 6). Out of all variants F417C, F417W, F417I and F417H showed a slightly higher total GOS yield than the WT, exhibiting an increase of 16 %, 15 %, 10 % and 4 % respectively (Table 7).

3.6 Transglucosylation with cellobiose

Transglucosylation reactions were performed at 50 °C using an initial cellobiose concentration of 200g/l in HEPES buffer 20 mM pH 7, with 150 mM NaCl. Samples with a glucose release of (50 % ± 10 %) were chosen for analysis, due to a better comparison. All variants show a higher total COS formation

than the WT. Variants 417H, 417L, F417M and F417W showed a 2-fold increase in total COS yield with respect to the WT enzyme. In variant F417T total COS production was 2.5-fold increased, and in variants F417T, F417I and F417Y 3-fold higher than in the WT. In variants F417C and F417S total COS formation

is increased by about 4-fold in comparison to the WT enzyme. In all variants, cellotriose was formed in higher amounts than in the WT enzyme. Gentiobiose constituted the major share of total transglucosylation products in the WT (61 %), as well as in variants, F417H (72 %), F417L (54 %), F417M (70 %), F417T (51 %), F417W (50 %). The other COS components were produced in much lower amounts. In the variants F417C, F417I, F417S and F417Y, there was a greater variety and a more equal distribution of individual COS. The WT produced gentiobiose, cellotriose, glucotriose as well as laminaribiose, (Table 9). Variants F417M and F417H in contrast showed a limited diversity in COS formation, yielding only laminaribiose, gentiobiose and cellotriose. Laminaribiose accounted for one third of total COS formation in the WT and all variants, except in F417M where the laminaribiose contributed for 6 % of total COS. In contrast to the WT which produced only di- and trisaccharides, some variants produced COS longer than trisaccharides. Variants F417I, F417L, F417S, F417T F417W and F417Y produced cellotetraose. Cellopentaose was formed by variants F417C, F417I, F417S and 417Y (Table 8).

4   Discussion and Conclusion

Amino acid position F417 located in the -1 subsite of *Ho*BGLA was shown to be crucial for enzymatic activity. In a previous study transglycosylation activity of *T. thermophilus* β-glucosidase was reported to be improved upon exchange of F401 for N, K, G, P, and Q using oNPG-Gal as substrate (Feng et al. 2005). Replacement of homologous F417 in *Ho*BGLA by those reported amino acids, except S, eradicated enzymatic activity with lactose and cellobiose. Exchange of F417 for A, V, D, E and R also resulted in an inactive enzyme. Upon substitution of F417 with L, S, I, T, C, M, Y and W, the enzyme remained still active (Figure 1). In this work, we investigated the impact of active site amino acid exchanges in the β-glucosidase of *Ho*BGLA by probing position F417. In particular, the substrate interaction mechanisms with the natural substrates lactose and cellobiose were analyzed. Temperature stabilities of all variants were decreased, but all variants retained half of their initial enzymatic activity for at least 10 hours at 50 °C (Table 1). Therefore, kinetic measurements, as well as transglycosylation were carried out at 50 °C.

Variant F417T showed a 3-fold higher preference for lactose over cellobiose as substrate, whereas the WT favored cellobiose 15-fold over lactose as substrate (Table 4) – a β-glucosidase was turned into a β-galactosidase, however with a weaker efficiency for both. In variant F417M, single amino acid replacement resulted in an enzyme

which retained significant catalytic efficiency for cellobiose and lactose. Catalytic lactose. (Table 2, Table 3). Bioinformatic tools are a suitable approach in order to study the structure-function relationship in more detail. Molecular dynamics simulations were performed with the WT and variants F417M, F417T, F417Y and F417L. Results of molecular dynamics simulation of variant F417M and F417L and the WT showed a higher number of hydrogen-bond interactions of catalytic amino acid residues E166 and E354 with cellobiose than with lactose. For F417T there was a slightly higher number of hydrogen-bond formations and a closer binding to catalytic amino acid residues with lactose than with cellobiose observed. (Figure 2, Figure 3, Table 5). These bioinformatics results support the results of kinetic analysis, which showed a lower K_m for lactose than the WT and a preference for lactose over cellobiose as substrate for variant F417T. (Table 3, Table 4). In transgalactosylation experiments using lactose as substrate, allolactose was the predominant GOS component produced by variants F417I, F417S, F417T and F417Y. 6'-galactosyllactose constituted the main transgalactosylation product in the WT and the variants F417C, F417H, F417L, F417M and F417W (Table 6, Table 7). Enhanced formation of allolactose by variants F417S, F417T and F417I suggests that replacement of F417F by S, Y, T and I in *Ho*BGLA variants shifts the transglycosylation mechanism towards intramolecular transglycosylation. Thereby

efficiencies of all other variants were more severely impaired for cellobiose and lactose is immediately converted into allolactose after cleavage of the glycosidic linkage (Huber et al. 1976). Single amino acid replacements at position 417F in *Ho*BGLA were shown to alter the product specificity as well as the amounts of transglucosylation yields. COS are considered as so-called candidate prebiotics (Rastall 2013). With cellobiose as a substrate, the WT enzyme of *Ho*BGLA formed β-1,3-, β-1,4- and β-1,6-linked di-and trisaccharides. Single amino acid replacements at position F417 by L, T and W resulted in variants that produced tetrasaccharides as well. Exchange of F417 for C, I, Y and S created enzymes that formed cellopentaose (Table 8). We analyzed transglucosylation by *Ho*BGLA in detail. Previously, the generation of β-1,3, β-1,4 and β-1,6 linked di- tri-and tetrasaccharides from cellobiose by β-glucosidases from *Aspergillus niger* and *Sclerotinia sclerotiorum* has been reported (Smaali et al. 2004). The formation of cellopentaose has not yet been documented. *Ho*BGLA WT, as well as all variants, favored the formation of β-1,6 linked COS over β-1,3 and β-1,4 linkages. In COS synthesis, the *Ho*BGLA WT, as well as variants F417C, F417H, F417L, F417M, F417T and F417W preferred the production of β-1,6 linked gentiobiose over β-1,3 and β-1,4 linked COS. In variants F417I, F417Y and F417S, there was a shift towards synthesis of β-1,3 and β-1,4 linked

COS (Table 8 Table 9). Since GH family β-glucosidases are grouped according to their amino acid sequence similarities, there is a strong correlation between sequence and mechanistic characteristics (Henrissat and Davies 1997). Consequently, assigned functions of amino acids in one GH family enzyme might be similar for homologous amino acids in a member of this family. Former publications report the identification of amino acid residues involved in substrate-interactions in *Zea maize* β-glucosidase and *T. thermophilus* β-glucosidase by molecular dynamics simulation (Zouhar et al. 2001, Feng et al.2005). In *Ho*BGLA, the amino acid residues Q20, H121, E166, E173, Y296, E354, W401, E408, W409 were shown to participate in substrate interaction (Table 10). For detailed evaluation of altered stereoselectivity and regioselectivity of variants, further bioinformatic analysis is required.

5 Tables and figures

5.1 Temperature stability of variants

$T_{1/2}$ at 60°C	
wt	21 h
F417C	5 h
F417H	6 h
F417I	30 min
F417L	10 h
F417M	11 h
F417S	1 h
F417T	40 min
F417W	1 h
F417Y	1 h
$T_{1/2}$ at 50°C	
All variants	>10 h

Table 1 Temperature stability of WT and variant 417 *Ho*BGLAs

in HEPES buffer 20 mM and 150 mM NaCl at pH 7

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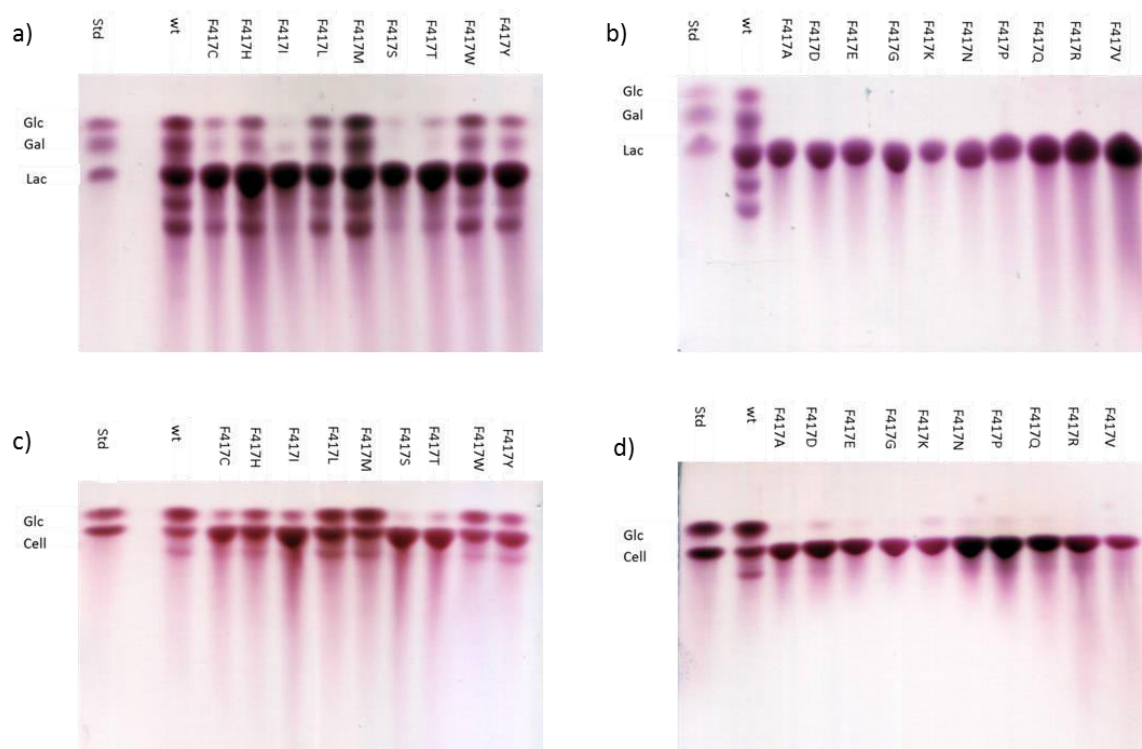
5.2   Fast Screening

Figure 1 Fast Screening for enzymatic activity of variant 417 *Ho*BGLAs –Visualization by TLC
a) hydrolytic activity with lactose, b) hydrolytic activity with cellobiose, c) inactivity with lactose,
d) impaired activity with cellobiose

5.3   Kinetic parameters

D-cellobiose

	k_{cat} (s^{-1})		K_m (mM)			$k_{cat.}/K_m$ (s^{-1} mM^{-1})
WT	291.0 ±	4.0	14.8	±	0.7	19.7
F417C	280.0 ±	0.0	508.5	±	5.8	0.6
F417H	157.0 ±	2.6	62.0	±	0.7	2.5
F417I	49.2 ±	0.9	127.3	±	4.5	0.4
F417L	174.0 ±	10.0	71.3	±	0.7	2.4
F417M	141.2 ±	2.4	14.8	±	1.3	9.5
F417S	116.8 ±	5.3	114.7	±	8.4	1.0
F417T	84.3 ±	2.5	1262.4	±	52.1	0.1
F417W	159.0 ±	2.0	74.0	±	7.0	2.1
F417Y	53.0 ±	0.7	137.8	±	2.6	0.4

Table 2 Kinetic parameters of WT and variant 417 *Ho*BGLAs with D-cellobiose at 50 °C

D-lactose

	k_{cat} (s^{-1})		K_m (mM)			$k_{cat.}/K_m$ (s^{-1} mM^{-1})
WT	185.4 ±	6.2	139.0	±	10.0	1.3
F417C	114.0 ±	3.0	764.3	±	31.2	0.1
F417H	64.9 ±	1.3	186.4	±	7.6	0.3
F417I	19.4 ±	0.2	98.6	±	3.9	0.2
F417L	59.2 ±	0.7	105.4	±	3.8	0.6
F417M	184.0 ±	1.0	207.7	±	0.3	0.9
F417S	66.7 ±	1.8	287.8	±	18.6	0.2
F417T	14.9 ±	0.2	52.3	±	2.7	0.3
F417W	84.3 ±	0.1	180.0	±	1.0	0.5
F417Y	19.9 ±	0.2	47.2	±	0.4	0.4

Table 3 Kinetic parameters of WT and variant 417 *Ho*BGLAs with D-lactose at 50°C

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	Substrate Selectivity, cellobiose over lactose $(k_{cat,}/K_{m[cel]})/ (k_{cat,}/K_{m[lac]})$
WT	15.1
F417C	3.7
F417H	7.3
F417I	2.0
F417L	4.3
F417M	10.7
F417S	4.4
F417T	0.3
F417W	4.6
F417Y	0.9

Table 4 Selectivity of cellobiose over lactose of WT and variant 417 *Ho*BGLAs
A ratio >1 favors cellobiose hydrolysis, whereas a ratio <1 favors lactose hydrolysis

5.4 Molecular dynamics simulations

F417L-cellobiose

F417L-lactose

F417T-cellobiose

F417T-lactose
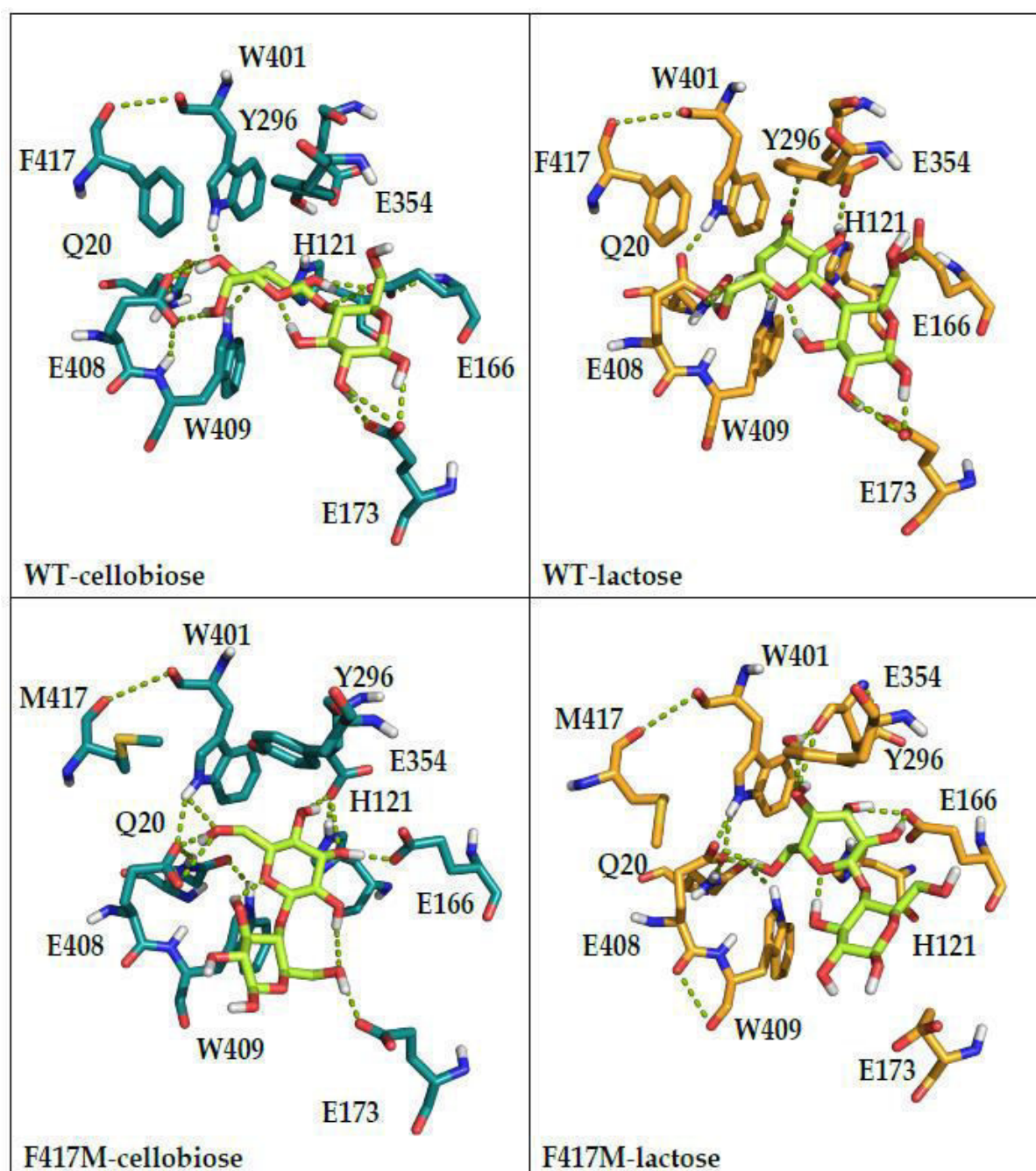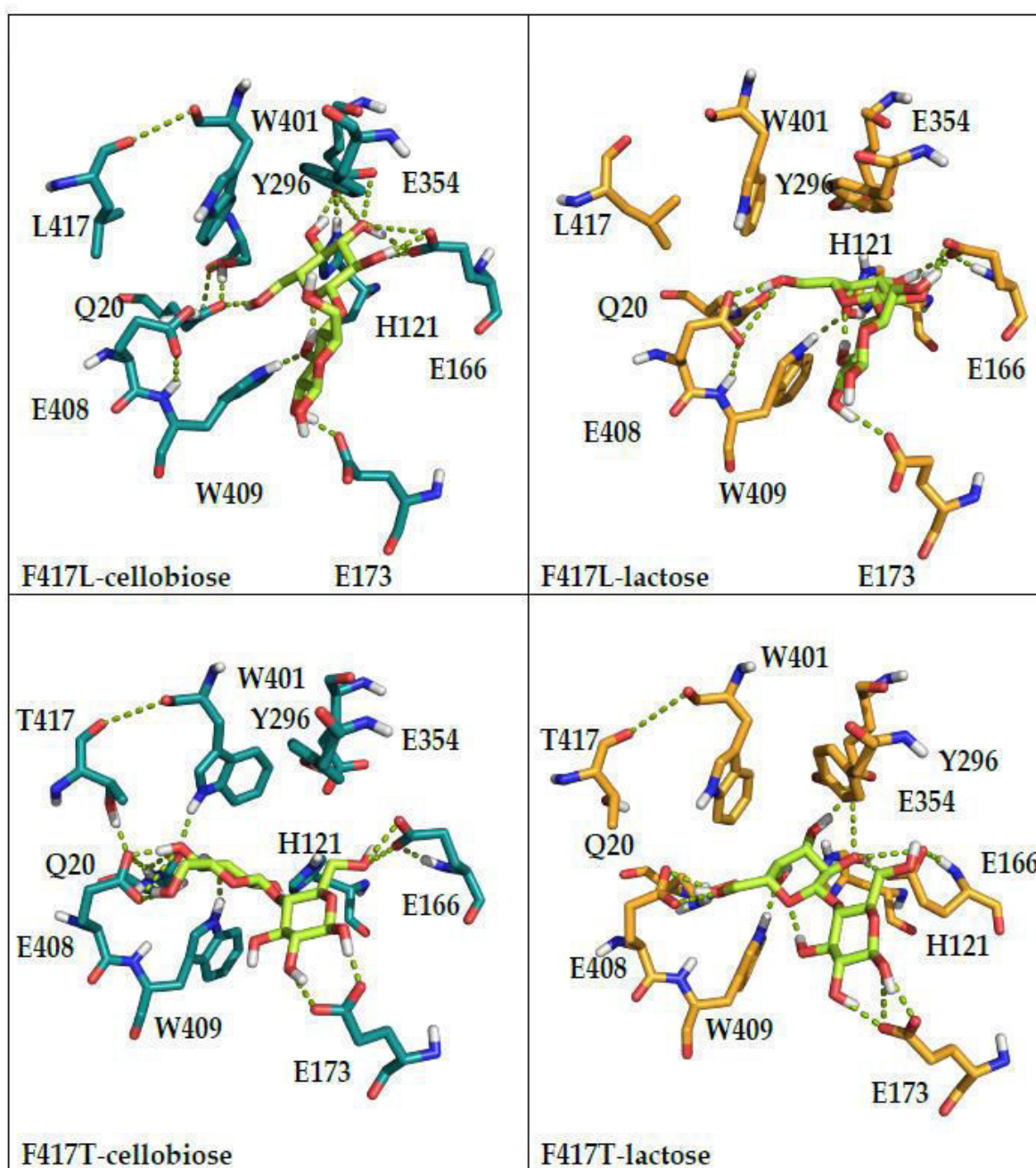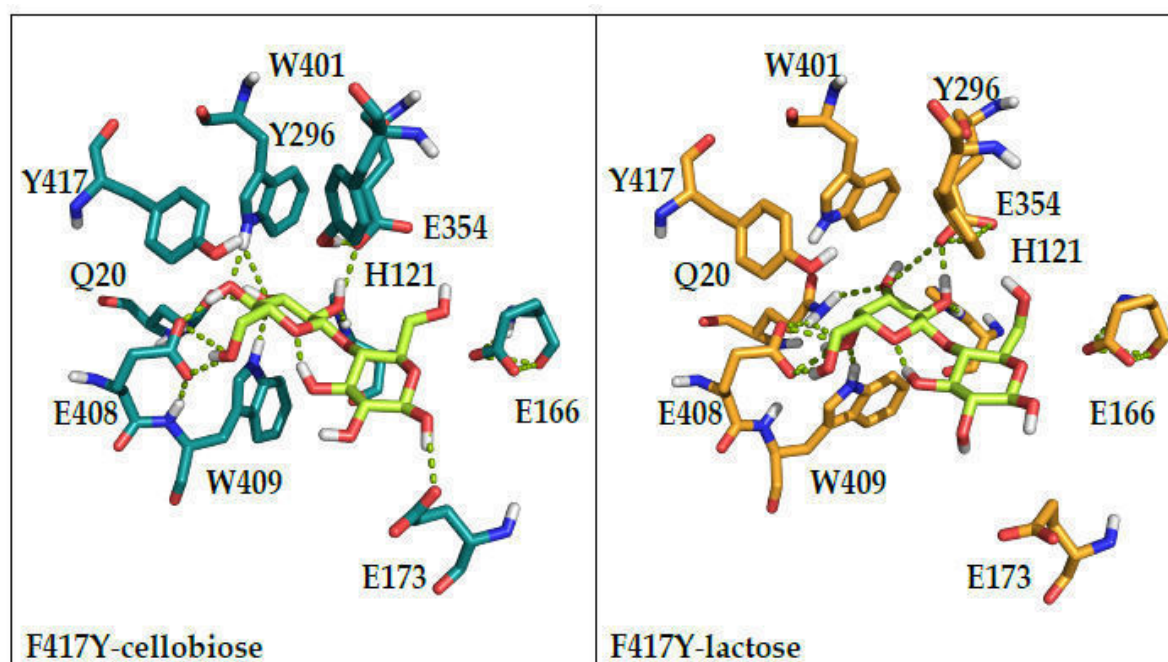
Figure 2 The active site of the WT and variant 417 *Ho*BGLAs with lactose and cellobiose. Polar contacts are shown in green dashes.

Figure 3 Time series of distances between the centre of geometry (cog) of OE1 and OE2 atoms of E166 and O4 atom of the first disaccharide ring of cellobiose (black line) and lactose (dashed black line) and between the centre of geometry (cog) of OE1 and OE2 atoms of E354 and C1 atom of the first disaccharide ring of cellobiose (red line) and lactose (dashed red line).

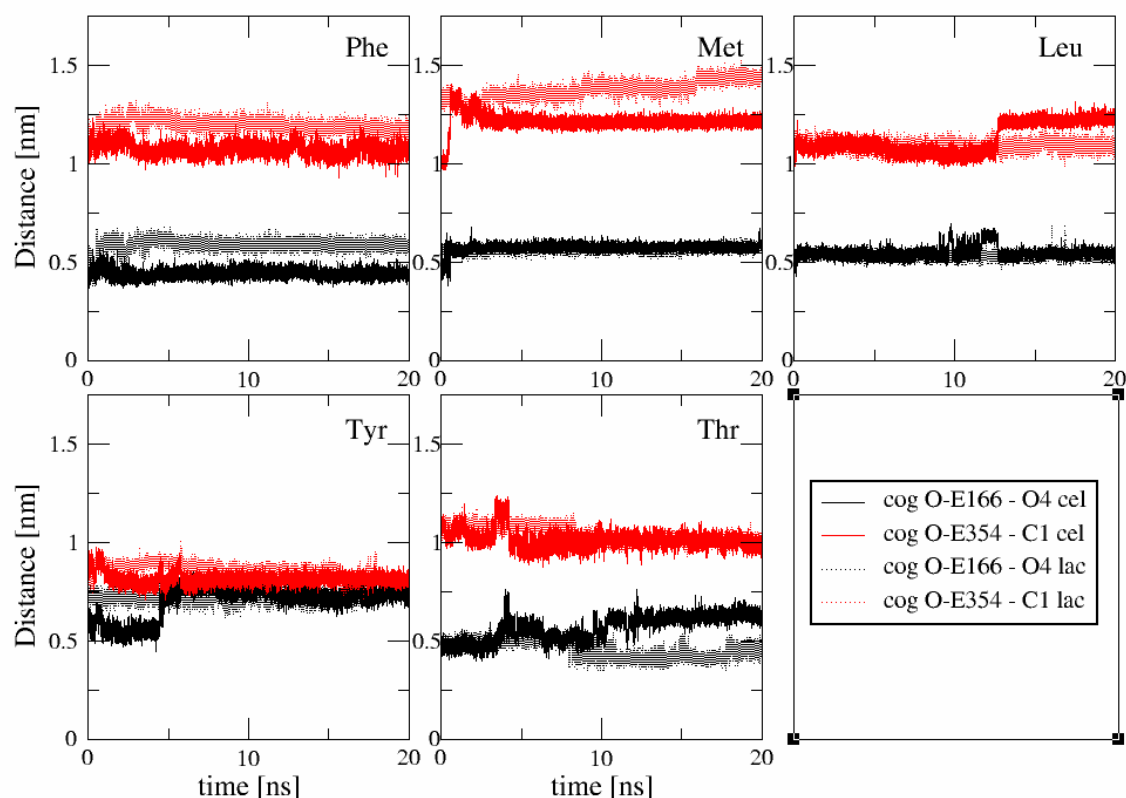| H-bond per residue	WT		F417M		F417L		F417T		F417Y		Average
	cel	lac	cel	lac	cel	lac	cel	lac	cel	lac	
Q20	0.00	0.00	0.00	0.00	0.69	0.00	0.56	1.11	0.56	0.20	0.31
H121	0.00	0.00	0.00	0.00	0.37	0.00	0.26	0.03	0.66	0.31	0.16
E166	0.99	0.07	2.45	2.21	2.17	2.20	0.98	1.42	0.00	0.00	1.25
E173	0.90	1.99	0.70	0.00	1.02	0.95	2.06	2.06	1.37	1.77	1.28
Y296	0.00	0.80	0.14	0.88	0.24	0.00	0.04	0.00	0.00	0.91	0.30
E354	0.00	0.00	0.93	0.00	1.02	0.00	0.34	0.99	1.01	1.68	0.60
W401	0.99	0.15	0.02	0.00	0.00	0.00	0.63	0.10	0.79	0.03	0.27
E408	2.05	2.38	1.25	1.31	0.29	1.07	2.21	1.53	2.13	2.35	1.66
W409	0.68	0.38	0.00	0.00	0.59	0.96	0.58	0.76	0.75	0.70	0.54
Sum	5.61	5.76	5.49	4.40	6.40	5.17	7.67	8.00	7.27	7.95	6.37

Table 5 Average number of hydrogen bonds per residue of the substrates with residues of the active site in different variant 417 *Ho*BGLAs over 20ns MD trajectory

5.5 GOS determination

Variants	WT	C	H	I	L	M	S	T	W	Y
GOS yield (% mass of total sugars)	32	37	37	33	31	27	30	27	35	30
GOS components (% of total GOS)										
D-β-Gal-(1→6)-D-Gal	3	2	3	5	2	4	2	2	2	3
D-β-Gal-(1→3)-D-Gal	2	0.5	1.5	0.8	1.0	3	0.3	0.4	0.9	0.5
D-β-Gal-(1→6)-D-Lac	36	48	44	16	36	38	22	14	51	13
D-β-Gal-(1→3)-D-Glc	19	21	21	14	27	23	23	16	19	15
D-β-Gal-(1→4)-D-Lac	6	11	9	10	10	4	8	5	7	8
D-β-Gal-(1→3)-D-Lac	16	5	9	8	8	13	8	7	9	10
D-β-Gal-(1→6)-D-Glc	19	13	13	47	16	16	37	56	12	51

Table 6 GOS yield and individual GOS components produced by the transgalactosylation reaction of the WT and variant 417 *Ho*BGLAs at a glucose release of 50 %. The reactions were performed at 50 °C with an initial lactose concentration of 200g/l lactose in HEPES buffer 20 mM and 150 mM NaCl at pH 7

Variants	C	H	I	L	M	S	T	W	Y
GOS yield (% mass of wt)	116	115	104	96	85	93	83	111	94
mass of total GOS (% of wt)									
D-β-Gal-(1→6)-D-Gal	0.3	0.9	0.4	0.5	1.3	0.2	0.2	0.5	0.3
D-β-Gal-(1→3)-D-Gal	33	92	42	50	125	17	17	50	25
D-β-Gal-(1→6)-D-Lac	154	139	46	94	89	55	32	155	33
D-β-Gal-(1→3)-D-Glc	124	126	77	135	102	109	68	109	73
D-β-Gal-(1→4)-D-Lac	237	186	180	180	57	137	74	134	131
D-β-Gal-(1→3)-D-Lac	34	68	52	47	68	50	37	63	60
D-β-Gal-(1→6)-D-Glc	79	80	264	82	74	184	250	68	257

Table 7 GOS yield and individual GOS components produced by the variant 417 *Ho*BGLAs relative to the yield of the WT at a glucose release of 50 %. The reactions were performed at 50 °C with an initial lactose concentration of 200g/l lactose in HEPES buffer 20 mM and 150 mM NaCl at pH 7

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5.6 COS determination

	WT	C	H	I	L	M	S	T	W	Y
COS yield (% mass of total sugars)	5	18	9	15	10	8	17	12	8	14
% of total GOS										
D-β-Glc-(1→4)-D-Cel	4	10	3	21	6	24	23	9	10	22
D-β-Glc-(1→4)-D-Glc-(1→4)-D-Cel	0	0	0	4	5	0	5	3	4	2
D-β-Glc-(1→4)-D-Cel-(1→4)-D-Cel	0	0	0	2	0	0	2	0	0	2
D-β-Glc-(1→3)-D-Glc	28	32	26	30	29	6	27	32	32	25
D-β-Glc-(1→3)-D-Glc-(1→3)-D-Glc	0	3	0	0	0	0	0	0	4	0
D-β-Glc-(1→3)-D-Cel	7	9	0	19	6	0	21	7	0	28
D-β-Glc-(1→6)-D-Glc	61	46	72	25	54	70	23	51	50	21

Table 8 COS yield and individual COS components produced by the WT and variant 417 *Ho*BGLAs at a glucose release of 50 %. The reactions were performed at 50 °C with an initial lactose concentration of 200g/l cellobiose in HEPES buffer 20 mM and 150 mM NaCl at pH 7

	C	H	I	L	M	S	T	W	Y
COS yield (% mass of wt)	391	191	327	224	168	369	250	171	294
D-β-Glc-(1→4)-D-Cel	890	115	1560	330	918	1970	507	374	1500
D-β-Glc (1→3)-D-Glc	447	172	349	229	37	351	278	195	257
D-β-Glc-(1→3)-D-Cel	517	0	905	206	0	1140	251	0	1220
D-β-Glc-(1→6)-D-Glc	294	226	134	199	195	138	208	140	103

Table 9 COS yield and individual COS components produced by variant 417 *Ho*BGLAs relative to the yield of the WT at a glucose release of 50 %. The reactions were performed at 50 °C with an initial lactose concentration of 200g/l cellobiose in HEPES buffer 20 mM and 150 mM NaCl at pH 7

5.7   Enzymatic activity

Zea maize BGLA	*T. thermophilus* BGLA	*Ho*BGLA	Function
H137		H121	Substrate interaction site
E186	E164	E166	Acid/base catalyst
E401	E338	E354	Nucleophile
W452	W385	W401	Substrate interaction site
Q33	-	Q20	Substrate interaction site
E459	-	E408	Substrate interaction site
-	-	E173	Substrate interaction site
-	-	Y296	Substrate interaction site
-	-	W409	Substrate interaction site
W373	W312	-	Substrate interaction site
-	N219	-	Substrate interaction site
N185	-	-	Substrate interaction site
(Zouhar et al.2001)	(Feng et al. 2005)		

Table 10 Assigned functions of amino acids in the catalytic site of different BGLAs (β-glucosidases)

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CHAPTER 4

Heterologous expression of a recombinant lactobacillal β-galactosidase in *Lactobacillus plantarum*: effect of different parameters on the sakacin P-based expression system

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[§] Both authors contributed equally in this work

RESEARCH

Heterologous expression of a recombinant lactobacillal β-galactosidase in *Lactobacillus plantarum*: effect of different parameters on the sakacin P-based expression system

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Abstract

Background: Two overlapping genes *lacL* and *lacM* (*lacLM*) encoding for heterodimeric β-galactosidase from *Lactobacillus reuteri* were previously cloned and over-expressed in the food-grade host strain *Lactobacillus plantarum* WCFS1, using the inducible lactobacillal pSIP expression system. In this study, we analyzed different factors that affect the production of recombinant *L. reuteri* β-galactosidase.

Results: Various factors related to the cultivation, i.e. culture pH, growth temperature, glucose concentration, as well as the induction conditions, including cell concentration at induction point and inducer concentration, were tested. Under optimal fermentation conditions, the maximum β-galactosidase levels obtained were 130 U/mg protein and 35–40 U/ml of fermentation broth corresponding to the formation of approximately 200 mg of recombinant protein per litre of fermentation medium. As calculated from the specific activity of the purified enzyme (190 U/mg), β-galactosidase yield amounted to roughly 70% of the total soluble intracellular protein of the host organism. It was observed that pH and substrate (glucose) concentration are the most prominent factors affecting the production of recombinant β-galactosidase.

Conclusions: The over-expression of recombinant *L. reuteri* β-galactosidase in a food-grade host strain was optimized, which is of interest for applications of this enzyme in the food industry. The results provide more detailed insight into these lactobacillal expression systems and confirm the potential of the pSIP system for efficient, tightly controlled expression of enzymes and proteins in lactobacilli.

Background

Lactic acid bacteria (LAB) have been known for a long time as important micro-organisms in the preparation and processing of a wide range of different foods, beverages and animal feed [1,2]. Being capable of rapidly converting glucose to lactic acid, LAB have been used as starter cultures in the production of a number of fermented foods in e.g. the meat and dairy industries, and have thus played an important role in human nutrition.

Some lactic acid bacteria are known as producers of processing enzymes, antimicrobial peptides, or metabolites that contribute to flavor, conservation or texture of various foods. Furthermore, some LAB, in particular *Lactobacillus* spp., have been used as commercial probiotic cultures with health-promoting properties [2-4]. Based on their long-time use in food, a number of LAB carry the 'generally recognized as safe (GRAS)' or 'qualified presumption of safety (QPS)' status for human consumption.

In addition, LAB are increasingly considered as safe and attractive expression hosts and cell factories, especially for food-application purposes [2,4]. They are also attractive vehicles for *in situ* delivery of antigens or other bioactive compounds in the GI-tract [5,6]. As a consequence, a variety of constitutive or inducible gene expression and

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protein targeting systems have been developed for LAB [2,5,7,8]. One of the most widely used gene expression systems derived from LAB is the NIsin-Controlled gene Expression system (NICE), which is based on the autoregulatory properties and the genes involved in the synthesis of nisin, an antimicrobial peptide produced by certain strains of *Lactococcus lactis* [9]. The NICE system has been adapted to lactobacilli, but this approach has not always been straightforward or successful [10,11]. An alternative expression system, the so-called pSIP system [12], was constructed for *Lactobacillus* spp. based on the promoter and regulatory genes involved in the production of the class-II bacteriocins sakacin A [13] and sakacin P [14,15]. The production of these two bacteriocins is regulated via quorum sensing mechanisms that are based on secreted peptide pheromones with little or no bacteriocin activity [5,16,17]. The peptide pheromone (also termed inducing peptide, IP) activates a two-component regulatory system consisting of a membrane-bound histidine kinase sensing the pheromone, and an intracellular response regulator that, upon activation by the histidine kinase, induces the promoters of the operons involved in bacteriocin synthesis. In the pSIP systems, expression of the gene of interest is under control of a strong, inducible bacteriocin promoter, and gene expression is induced by external addition of the peptide pheromone. An advantage of these systems is that they are strictly regulated and lead to high production of the target protein. The applicability of these sakacin-based expression systems was shown for the over-production of enzymes such as β-glucuronidase and aminopeptidase in several *Lactobacillus* hosts [7,12].

β-Galactosidases (lactases, EC 3.2.1.23) catalyse the hydrolysis of lactose into galactose and glucose, and are important enzymes for applications in the dairy industry [18-20]. They can among others be used to produce low-lactose or lactose-free products, or prevent crystallization of lactose especially at low temperatures [21]. Moreover, β-galactosidases can catalyse transgalactosylation reactions, transferring galactosyl moieties from e.g. lactose to a suitable acceptor molecule [18]. When lactose is the primary acceptor, galacto-oligosaccharides (GOS) are obtained, which are physiologically important and health-promoting prebiotic sugars [19,20,22,23]. Especially β-galactosidases obtained from known probiotic bacteria such as bifidobacteria or lactobacilli are of interest for the synthesis of these prebiotic GOS [24,25]. Nguyen *et al.* [19] screened a number of *Lactobacillus* isolates and found that one strain of *L. reuteri* exhibited high β-galactosidase activity with significant transferase activity [19]. This heterodimeric β-galactosidase of *L. reuteri* is encoded by two overlapping genes, *lacL* and *lacM.* The activity levels obtained with the wild-type strain (~2.3 kU per litre of cultivation medium, corresponding to 14 mg of β-galactosidase protein per litre) are too low to be attractive from an applied point of view.

To improve these low yields, the coding regions of the two overlapping genes *lacL* and *lacM* (*lacLM)* were cloned and over-expressed in a standard expression host, *Escherichia coli* [26]. Heterologous expression in *E. coli* resulted in efficient over-expression of β-galactosidase (~110 kU/l of fermentation broth, specific activity of 55 U/mg), yet *E. coli* might not be the preferred host for food-related enzymes. As calculated from the specific activity of the purified enzyme (~180 U/mg), β-galactosidase yield amounted to roughly 30% of the total soluble intracellular protein of the host organism, hence laborious chromatographic step is required for the purification of the enzyme for further applications.

We have reported the overproduction of this enzyme in the food-grade expression host *Lactobacillus plantarum* WCSF1 [27]. The *lacLM* genes from *L. reuteri* were cloned into the expression vectors pSIP403 and pSIP409, which are based on the sakacin P operon of *L. sakei* [7,12], differing only with respect to the bacteriocin promoter that drives *lacLM* expression (P_{sppA} and P_{sppQ}, respectively). This resulted in the two expression plasmids, pEH3R and pEH9R [27]. When over-expressed in the host *L. plantarum* WCFS1, cultivations of *L. plantarum* WCFS1 carrying these plasmids yielded up to ~23 kU of β-galactosidase activity, corresponding to the formation of approximately 100 mg of recombinant protein per liter of fermentation medium, and β-galactosidase levels amounted to 55% of the total intracellular protein of the host organism [27], without any optimisation of the fermentation process. The pSIP409-derived construct pEH9R was considered the better since this construct yielded lower pheromone-independent recombinant protein levels, indicative of a more strictly regulated promoter.

To further explore the (industrial) potential of the pSIP system in general and the use of lactobacilli for food-grade production of β-galactosidases in particular, we investigated the effects of various cultivation and induction conditions on gene expression. Among the factors studied were pheromone dose, timing of induction, culture pH and glucose concentration. Plasmid copy numbers during a cultivation were analyzed using reverse-transcriptase quantitative PCR. The results provide more detailed insight into these lactobacillal expression systems and show how high the expression of recombinant *L. reuteri* β-galactosidase may be achieved.

Results

Effect of inducer concentration, time of induction and glucose concentration

L. plantarum WCFS1 harbouring the plasmid pEH9R, which contains the *lacLM* genes under control of the pheromone-inducible P_{sppQ} promoter, was grown with and without pH control under various induction conditions. The concentration of the inducing pheromone (IP; a linear

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19-residue peptide sometimes referred to as IP-673) was varied and the inducer was added at different growth phases of the host organism.

Batch cultivations without pH control

Cultivations were performed without pH control at 37°C using MRS medium containing 20 g/l glucose. Despite the varying induction conditions, growth of the organism was in all cases very similar and reached an OD_{600} of ~4.5-5.0 after 12 h of cultivation (Figure 1). The volumetric activities of β-galactosidase (U per ml of fermentation broth) in induced cultures varied between 2 U/ml and 8 U/ml, and the specific activities ranged from about 20 U/mg to 50 U/mg, depending on the conditions employed. These production levels were generally reached at OD_{600} 2.0–3.0, regardless of the time of induction (immediately after inoculation, at OD_{600} of 0.4–0.5, or at OD_{600} of 1.5; Figure 1). The results show clear dose–response effects for the pheromone concentration, which level off at about 40 ng/ml. Maximum β-galactosidase levels were quite similar for cultures induced immediately after inoculation (Figure 1A) or at an OD_{600} of 0.4–0.5 (Figure 1B), but volumetric activities were clearly lower (2–4 U/ml rather than 4 – 8 U/ml) for cultures induced at OD_{600} of 1.5 (Figure 1C). These data also indicate that more pheromone is needed when induction takes place at a later growth phase. For example, induction with 20 ng/ml at OD_{600} of 0.4–0.5 maximally yielded 6 U/ml and 44 U/mg, whereas induction with 20 ng/ml at OD_{600} of 1.5 maximally yielded 2.6 U/ml and 34 U/mg. In the non-induced cultures very low enzyme activity was measured with approximately 0.2 U/ml of fermentation broth or 1.3 U/mg protein (Figure 1A). The average pH value of the fermentation media dropped from 6.5 to approximately 5.2 or 4.3 after 7 h (OD_{600} ~ 1.8-2.1) or 12 h (OD_{600} ~ 4.5-5.0) of growth, respectively.

Batch cultivations with pH control

In order to study the effect of the pH value on recombinant protein production when using the pSIP system, a series of cultivations was carried out where the pH was maintained at 6.5 by adding sodium hydroxide. Induction was performed using a non-saturating pheromone concentration of 20 ng/ml. The results, depicted in Figure 2A, B, show that culture pH had a strong positive effect on both growth and protein expression, and that the time of induction (immediately after inoculation, at OD_{600} of 0.3, or at OD_{600} of 3.0) hardly affected the outcome of the cultivations. OD_{600} values around 7 were reached after 10 hours of cultivation regardless of the induction time (Figure 2A) as compared to an OD_{600} of 4.5-5.0 obtained for growth without pH control (Figure 1). Accordingly, recombinant protein production was improved: β-galactosidase levels increased until the cells reached the early stationary phase to yield final

volumetric activities of 15–19 U/ml, which is a 2.5–3 fold increase compared to the cultivations without pH control. Interestingly, specific β-galactosidase activities also increased about two-fold, reaching values of around 90–100 U/mg. This indicates that the improved performance of pH-controlled cultivations is not just a matter of increased cell densities.

Subsequently, we studied the effect of varying glucose concentrations on β-galactosidase production under pH-controlled conditions (pH 6.5). Figure 3 shows that an increase of the glucose concentration from 20 g/l to 40 g/l approximately doubled the maximum OD_{600} values, which now reached 15–18. Concomitantly, the recombinant enzyme production also increased approximately two-fold; β-galactosidase levels continuously increased during the cultivation to reach a maximum of about 35 U/ml when the stationary growth phase was reached. Maximum specific activities were only slightly higher than those obtained with 20 g/l glucose, indicating that the increased volumetric yields are primarily caused by the increased cell densities. Dose–response effects for the pheromone were tested in a limited range only (20–80 ng/ml) and were generally small, as observed in other experiments for this concentration range. Comparison of the experiments displayed in Figure 3 further shows that under these conditions it may be favourable to induce somewhat later during growth since this yielded slightly higher specific activities. Higher concentrations of glucose (80, 120 g/l) were also tested, and this did not lead to a significant increase in enzyme yield even though higher cell densities were obtained (data not shown).

Effect of antibiotic concentrations

To examine the effect of different antibiotic concentrations on recombinant enzyme production, erythromycin concentrations of 1, 5 and 10 μg/ml (final concentration in the cultivation medium) were tested using cultivation conditions similar to those described in Figure 3B. Varying the erythromycin concentrations had no significant effect on growth or recombinant protein production (data not shown). When no antibiotic was added to the culture medium, the β-galactosidase yield was much lower (approximately 2 U/ml, data not shown) than with the antibiotic added, indicating the absolute necessity to keep the selection pressure for maintaining the expression plasmid.

Effect of temperature

Finally, we compared two different cultivation temperatures, 30°C and 37°C, with respect to growth as well as over-expression of β-galactosidase. When recombinant *L. plantarum* WCFS1 was grown in MRS medium with 40 g/l glucose and pH control at 6.5, growth and enzyme production were faster at 37°C than at 30°C. After 12 h
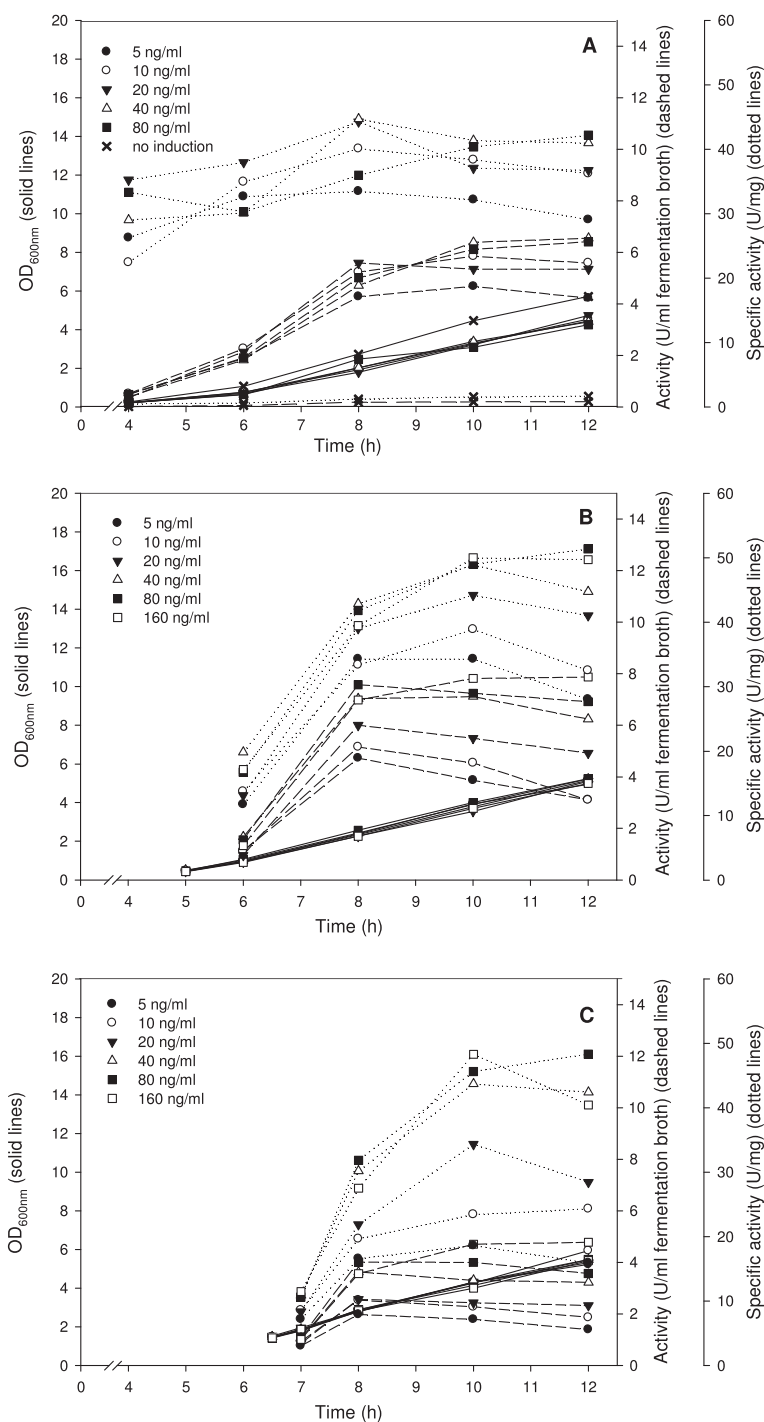
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Figure 1 Time course of the cultivations of *L. plantarum* overexpressing β-galactosidase from *L. reuteri* without pH control. *L. plantarum* WCFS1 harbouring the pEH9R plasmid was grown in 50-ml cultures using MRS medium with 20 g/l glucose, at 37°C. Recombinant protein expression was induced by the addition of varying amounts of the inducing pheromone IP (ng/ml fermentation broth; see inset) at different phases of the cultivation, i.e., different OD_{600} values: immediately after inoculation of the culture **(A)**, at OD_{600} of 0.4-0.5 **(B)**, or at OD_{600} of 1.5 **(C)**. All data points represent the average value from 2 independent experiments.

of growth, OD_{600} values were approximately 17 and 10 for cultivations at 37°C and 30°C, respectively (data not shown). The difference in cell densities also resulted in differences in volumetric β-galactosidase activity, which ranged from 35 U/ml for 37°C to 18 U/ml for 30°C, respectively, after 12 h.
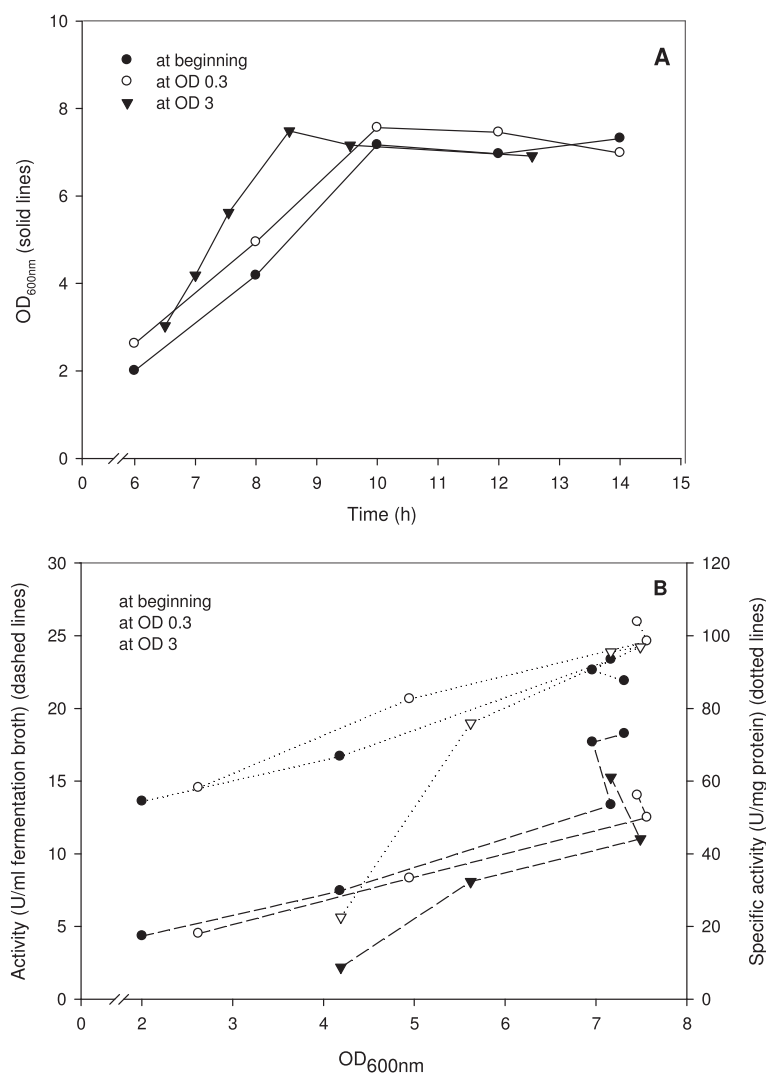
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Figure 2 Effect of pH control on the growth (A) and enzyme production (B) of *L. plantarum* overexpressing β-galactosidase from *L. reuteri*. *L. plantarum* WCFS1 harbouring pEH9R was cultivated in 400-ml laboratory fermentors at 37°C using MRS medium with 20 g/l glucose and pH control at pH 6.5. Expression of β-galactosidase was induced by adding 20 ng/ml pheromone at different OD_{600}: immediately after inoculation, at OD_{600} of 0.3 or at OD_{600} of 3.0. All data points represent the average value from 2 independent experiments.

Variation of the plasmid copy number during growth

Figure 4 shows more detailed cultivation data for an experiment run under optimal conditions. The data show that all glucose was consumed and that glucose depletion coincides with reaching maximum levels of β-galactosidase and lactic acid. To check whether the gene dose was constant during the cultivation, the plasmid copy number (PCN) was determined. The PCN was found to be at a constant level of ~4 throughout the whole exponential and stationary phase, with a slight dip in the late exponential phase.

Discussion

Progress in genetic engineering and better understanding of various regulatory mechanisms in lactobacilli have opened the perspective of engineering these bacteria to use them as microbial cell factories and delivery vehicles for proteins. The usefulness of the pSIP vector system for high protein production has previously been shown in several studies using *L. plantarum* and *L. sakei* as host strains [7,12,27]. Most of these studies were performed in acidifying cultures in flasks, and no detailed bioreactor studies have been performed to investigate these systems in more depth. In the present study we aimed at identifying parameters that influence heterologous protein production with the pSIP vectors by using controlled cultivation conditions, and by optimizing factors such as the time and dose of induction. We used heterodimeric β-galactosidase from *L. reuteri*, encoded by the overlapping *lacLM* genes, as reporter/target

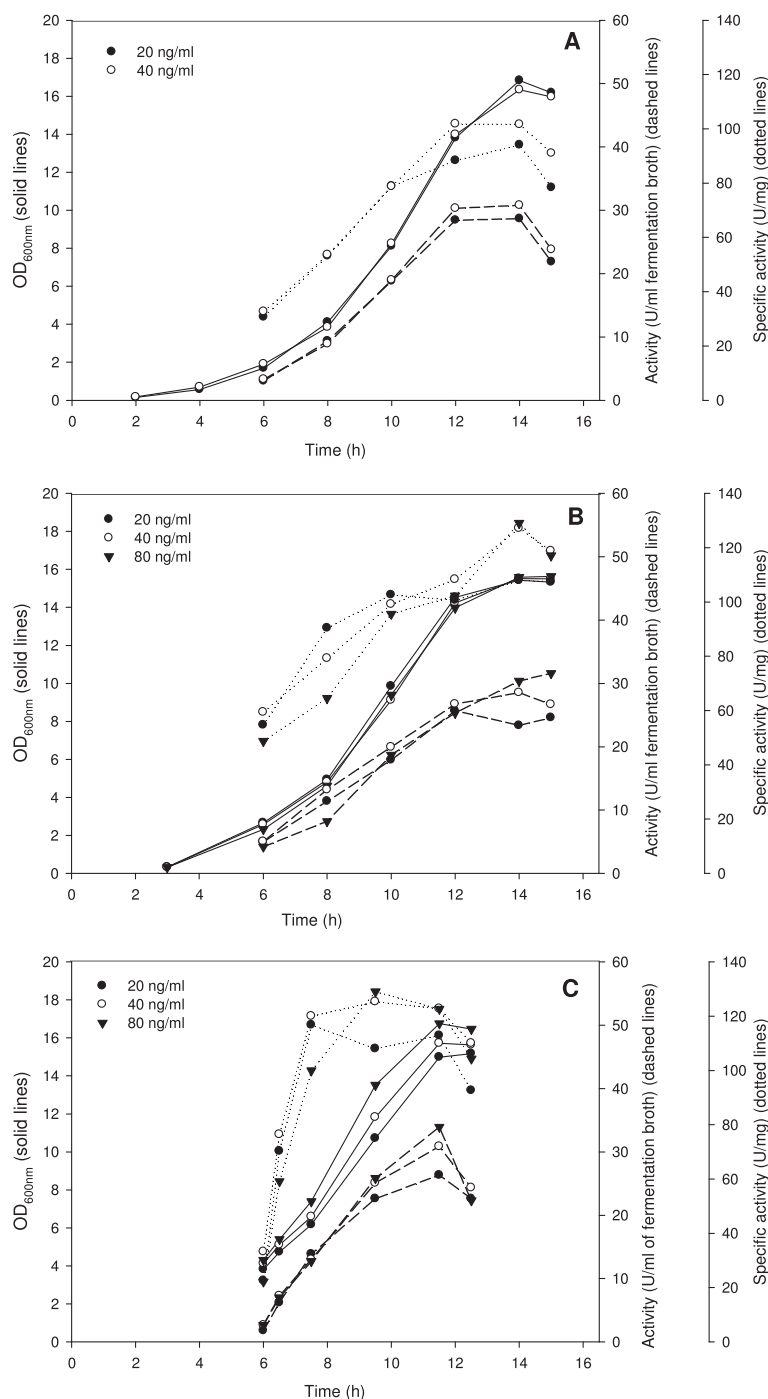Nguyen *et al. Microbial Cell Factories* (2015) 14:30

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Figure 3 Time course of the cultivations of *L. plantarum* overexpressing β-galactosidase from *L. reuteri* with pH control at increased glucose concentration. *L. plantarum* WCFS1 harbouring the pEH9R plasmid was cultivated in 400-ml laboratory fermentors at 37°C using MRS medium with 40 g/l glucose and pH control at pH 6.5. Expression of β-galactosidase was induced by the addition of varying amounts of pheromone (ng/ml fermentation broth; see insert) at different OD_{600} values: immediately after inoculation **(A)**, at OD_{600} of 0.3 **(B)**, or at OD_{600} of 3.0 **(C)**. All data points represent the average value from 2 independent experiments.

protein in the optimization studies as the highest expression levels were obtained in a laboratory cultivation of *L. plantarum* WCFS 1 harbouring the plasmids containing these genes [27].

As expected the β-galactosidase yield was very low in non-induced cultures, while specific activities of up to 130 U/mg were found under appropriate induction and growing conditions, giving typical induction factors (ratio of
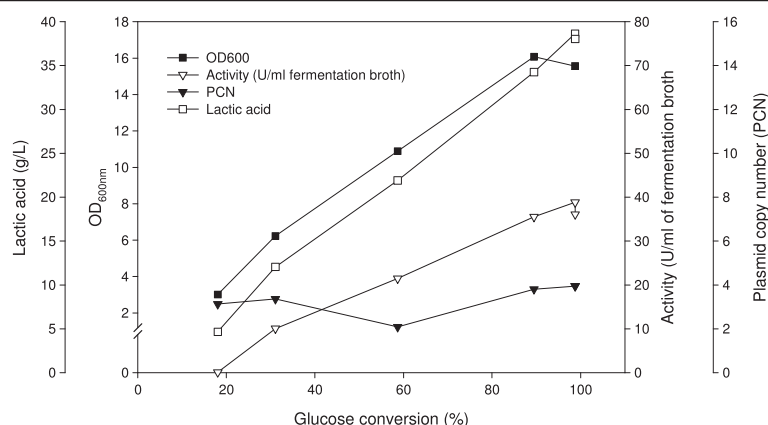
Figure 4 Variation of the plasmid copy number during the cultivations of *L. plantarum* overexpressing β-galactosidase from *L. reuteri*. *L. plantarum* WCFS1 harbouring the pEH9R plasmid was cultivated in 400-ml laboratory fermentors at 37°C using MRS medium with 40 g/l glucose, pH control at pH 6.5 and the cells were induced at OD ~ 3 with 80 ng/ml peptide pheromone. All data points represent the average value from 2 independent experiments.

specific activity under induced and non-induced conditions) of more than 100. This illustrates the tight control of the system, in agreement with previous studies of the pSIP expression system [7,12]. It should be noted that background β-galactosidase activity caused by expression of the chromosomal *lacLM* genes of *L. plantarum* are negligible (<0.1 U/mg) when the strain is grown on glucose. Hence, the activities reported in this paper can be considered as originating exclusively from heterologous expression of the vector-based *lacLM* genes. We observed a clear dose–response effect up to IP concentrations of ~40 ng/ml (Figures 1 and 3), and none of the tested IP concentrations had inhibitory effects on growth of *L. plantarum* as is evident from the almost identical growth curves depicted in Figures 1 and 3. Apart from showing that the IP itself is not inhibitory up to the highest tested concentration of 160 ng/ml, this also shows that the cells are capable of handling the high amounts of heterologous protein very well.

The yield of the recombinant protein was affected by the induction time point (growth phase), but only in the experiments without pH control. In these experiments, induction at high optical density (OD_{600} ~ 1.5) resulted in lower volumetric activities than induction at low OD_{600}, and higher pheromone concentrations were needed to reach maximum expression levels (Figure 1). The absence of this effect in cultures with pH control (Figures 2 and 3) indicates that the pH at the time of induction has influence on the induction efficiency, as has been suggested previously [14].

Maintaining the pH at a set value of 6.5 was clearly beneficial for β-galactosidase yields, both in terms of the volumetric and the specific β-galactosidase activities. This indicates that the decrease in pH during a non-controlled cultivation has a negative effect of the production of β-galactosidase. As expected the constant pH of 6.5 led to increased cell densities. However, this increase in biomass

cannot solely explain the higher yields of recombinant protein, as indicated by the considerably higher specific activities that were obtained. One possible beneficial effect of the constant pH could be higher effectiveness of the induction process, as mentioned above. The difference in specific activities between pH controlled and non-controlled fermentations was further confirmed by SDS-PAGE analysis of cell free crude extract obtained from these cultivations (Figure 5), with the bands for the recombinant β-galactosidase being more prominent for the samples obtained with pH control.

Even higher enzyme yields were obtained when the initial glucose concentration was increased, with maximum β-galactosidase levels being reached at 40 g/l glucose. This showed that glucose is the limiting factor in standard MRS medium. The maximum β-galactosidase levels obtained in these experiments (130 U/mg protein and 35–40 U/ml of fermentation broth) correspond to approximately 180 mg of recombinant protein produced per litre of fermentation medium as calculated from the specific activity of purified enzyme of 190 U/mg, which corresponds to roughly 70% of the total soluble intracellular protein being recombinant β-galactosidase. This is one of the highest expression levels obtained with gene expression systems in lactic acid bacteria to date [9].

It was previously reported that temperature can affect bacteriocin-related quorum sensing mechanisms in lactobacilli [28], and thus perhaps also expression levels for the pSIP system. We did, however, not observe significant differences in yield when comparing results at identical cell densities in pH-controlled cultivations performed at these two temperatures.

The expression system functioned well at antibiotic concentrations down to 1 µg/ml but the experiments also showed that the system does not work without antibiotics
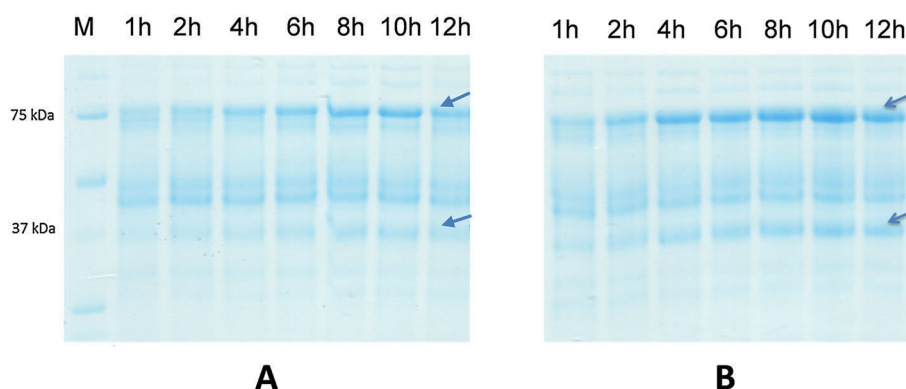
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Figure 5 SDS-PAGE analysis of cell free extract of crude *L. plantarum* overexpressing β-galactosidase from *L. reuteri* from the cultivations without pH control (A) and with pH control at pH 6.5 (B). *L. plantarum* WCFS1 harbouring pEH9R was cultivated in 400-ml laboratory fermentors at 37°C using MRS medium with 20 g/l glucose, and samples were taken at different time points. The arrows indicate the LacL and LacM subunits of the recombinant β-galactosidase. M denotes the Precision protein ladder (Biorad, CA, USA).

at all. Recent studies on segregational stability of pEH9R in *L. plantarum* WCFS1 showed that absence of erythromycin leads to a decrease in the number of cells harbouring plasmid pEH9R [29]. This indicates the absolute necessity to maintain strict selection pressure on the pSIP expression system during the cultivation. Because of the modular structure of the SIP system [5,12] it is easy to exchange selection markers e.g. with complementation markers such as the alanine racemase gene (*alr*) [30,31] or the lactose carrier *LacF* [32], which makes the addition of antibiotics redundant. We recently developed pSIP variants based on *alr* as selection marker, and tests done so far indicate that these vectors perform equally well as the original *ery*-based vectors in terms of protein expression and stability [29].

The pEH9R plasmid was found to be present in low copy numbers (approximately 2–4, depending on the growth phase), and this is in accordance with the findings that the 256_{rep} replicon is a low-copy-number replicon in *Lactobacillus* [33]. A decrease in the PCN was observed after approx. 10 h of cultivation, later during the exponential growth phase. A possible explanation for this could be that because of the fast duplication of the cells during this phase of rapid growth, the cellular machinery cannot provide the daughter cell with a sufficient number of the plasmids. When the growth rate subsequently decreased again, the PCN increased to the original value of approximately 4, and then stayed constant also during the stationary phase. It is interesting to note that the exceptionally high levels of recombinant protein, amounting to about 70% of total intracellular protein, were achieved with a gene dose not higher than approximately four.

Conclusion

We here described the optimization in terms of growth and induction conditions for the over-expression of a recombinant β-galactosidase using a pSIP409-based expression vector in *Lactobacillus plantarum* WCFS1. The highest β-galactosidase levels obtained were 130 U/mg protein and 35–40 U/ml of fermentation broth, which corresponds to roughly 70% of the total soluble intracellular protein being recombinant β-galactosidase.

Materials and methods
Bacterial strains and media; fermentations

L. plantarum WCFS1 [34] harbouring pEH9R [27], which contains the overlapping genes (*lacLM*) coding for β-galactosidase of *L. reuteri* L103 [26], was grown at 37°C in 5 ml of MRS containing 5 μg/ml erythromycin for 16–18 h. Such overnight cultures were used as inoculum for subsequent cultivations.

For batch fermentations without pH control, 1% (v/v) of inoculum was added to 50 ml of medium, and the cultures were grown in 50 ml tightly closed bottles at 37°C. Batch fermentations with pH control were carried out in 400 ml medium in HT-Multifors fermentors (Infors HT, Switzerland); also in this case cultures were inoculated with 1% (v/v) of an overnight preculture. The pH was controlled at pH 6.5 using sodium hydroxide when stated, and agitation was set at 200 rpm. Glucose concentrations in the MRS medium were varied as indicated. Gene expression was induced by adding varying levels of the synthetic pheromone IP-673 at different time points. IP-673 is a 19-amino acid peptide synthesised commercially according to the sequence of the original pheromone from *Lactobacillus sakei* LTH673 [35].

Samples were taken periodically to measure optical density at 600 nm, β-galactosidase activity and the PCN. For β-galactosidase measurements, cells from 1 ml of culture were harvested by centrifugation at 16000 *g* for 3 min, cell pellets were re-suspended in sodium phosphate buffer (buffer P) [22], and then disrupted by sonication (Bandelin

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Sonopuls HD60, Germany). Subsequently, debris was removed by centrifugation at 16000 *g* for 10 min. The crude cell extract was used to determine β-galactosidase activity and protein concentration. For PCN estimation, an appropriate volume of sample was taken depending on the densities of the cultures (OD_{600}) to ensure sufficient biomass for DNA isolation. Cells were pelleted by centrifugation and stored at –80°C until further use.

β-Galactosidase assay

β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) as the substrate as described previously [19]. In brief, the assay was performed at an *o*NPG concentration of 22 mM *o*NPG, pH 6.5, and 30°C. One unit of *o*NPG activity is defined as the amount of enzyme releasing 1 μmol of *o*NP per minute under these conditions. Protein concentration was determined by the method of Bradford using bovine serum albumin as the standard.

SDS-PAGE analysis

The cell-free crude extracts were analysed by SDS-PAGE following the previous protocol [29]. The protein bands were stained with Coomassie Brilliant Blue G250 (Sigma, Switzerland).

DNA isolation and purification for measurement of plasmid copy number (PCN)

DNA from bacterial cells was isolated and purified using the phenol-chloroform extraction method as described previously [36]. Purified bacterial DNA was stored at –2°C until further use.

Quantitative reverse transcriptase PCR (qPCR)
Oligonucleotide primers

The erythromycin resistance gene *ermB* and the *16S rRNA* gene were chosen as representatives for plasmid DNA and genomic DNA, respectively. The oligonucleotides Ery[R]-f, Ery[R]-r, 16 s-f and 16 s-r (Table 1) were used for qPCR. All primers were obtained from VBC-Biotech (Vienna, Austria).

qPCR using SYBR Green I

The thermal cycling system iCycler together with the myIQ single Color Real-Time PCR Detection system (Biorad, CA,

USA) were used for qPCR amplification and detection. The qPCR reactions were carried out in duplicates of 25-μl reaction mixtures in 96-well plates (iCycler, Biorad) sealed with optical adhesive covers (Microseal 'B' film, Biorad). Each reaction contained 250 nM of each primer, 12.5 μl of Perfecta SYBR Green Super mix of IQ (Quanta Biosciences, MD, USA) and 2.5 μl of template DNA (about 50 ng). Negative controls prepared by replacing template DNA with diethylpyrocarbonate (DEPC)-treated water, were included in each run to ensure the absence of DNA contaminants in the reagents. The concentration of primers, annealing temperature and template DNA concentrations had been optimized before the actual experiments as previously described [37]. The qPCR reactions were conducted as follows: initial denaturation at 95°C for 3 min followed by 50 cycles of 20 s at 95°C, 20 s at 60°C, and 72°C for 10 s. Fluorescence was measured at the end of each extension step at 72°C. The temperature was increased from 55°C to 95°C at a rate of 0.2°C per s to establish the melting curve. The threshold cycle values (C_t) were automatically determined by MyIQ Optical System software (version 2.0) (Biorad).

Calculation of the PCN value

Based on PCN definition, which is the number of copies of a plasmid present per chromosome in bacteria [38,39], the PCN can be calculated using equation (1) as previously reported [40]:

$$PCN = \frac{E_c{}^{C_{tc}}}{E_p{}^{C_{tp}}} \qquad (1)$$

where E_c, C_{tc} and E_p, C_{tp} are the amplification efficiencies and the threshold cycle values of the amplicon representing chromosome and plasmid, respectively. The equivalence between the amplification efficiency (*E*) of plasmid and chromosomal amplicons was confirmed in validation experiments as described previously [41].

Validation of the reverse transcriptase PCR reaction

A series of 10-fold dilutions of template DNA was used to run reverse transcriptase PCR reactions in order to estimate C_t values and to subsequently calculate the ΔC_t values for the two primer pairs. The amplification efficiencies for

Table 1 equences of the primers used for qPCR

Target gene	Primers	Sequence (5′ → 3′)	Reference
Plasmid DNA (Erythromycin resistant gene)	Ery[R]_f	CCGTGCGTCTGACATCTATC	This study
	Ery[R]_r	TGCTGAATCGAGACTTGAGTG	
Genomic DNA (16S-rRNA)	16s_f	TGATCCTGGCTCAGGACGAA	[42]
	16s_r	TGCAAGCACCAATCAATACCA	

f denotes forward primers.
r denote reverse primers.

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the 16S and Ery^R primer sets calculated based on the slope of the regression lines of the plots of C_t versus the logarithm of DNA dilution were found to be equivalent, i.e., 0.96 and 0.97, respectively. This is also corroborated by the plot of ΔC_t versus $\log_{10}$ (DNA dilution), where a regression line with a slope of 0.04 was obtained. This indicates that the $\Delta\Delta C_t$ method can be used in this study for the two primers sets, 16S and Ery^R [41].

High-performance liquid chromatography

Glucose and lactic acid in fermentation samples were analyzed by high performance liquid chromatography (HPLC) using a Dionex system (Sunnyvale, CA, USA) equipped with an Aminex HPX87-H column (300×7.8 mm) from Biorad and 0.005 M sulphuric acid as eluent at a flow rate of 0.6 ml/min, and separation temperature was at 60°C. Interested components were detected by RID detector.

Statistical analysis

All experiments and measurements were performed at least in duplicate, and the data are given as the mean ± standard deviation when appropriate. The standard deviation was always less than 5%.

Abbreviations
IP: Inducing pheromone; PCN: Plasmid copy number; MRS medium: de Man, Rogosa and Sharpe medium; *o*NP: *o*-nitrophenol; *o*NPG: *o*-nitrophenyl-β-D-galactopyranoside.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
TTN, THN and GM designed the experiments, TTN, HMN and BG performed the experiments, VGHE and DH conceived of the study, TTN and THN drafted the manuscript, GM, VGHE, CKP and DH contributed to the discussion, THN supervised the research and wrote the final version of the paper. All authors read and approved the final manuscript.

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DISCUSSION

Glycosidases such as β-glucosidases and β-galactosidases are important in many industrial processes. For instance, β-Glucosidases are applied for the conversion of cello-oligosaccharides in biofuel production (Bhatia et al. 2002). In food industry, β-galactosidases are commercially used for the hydrolysis of lactose in dairy products. A further field of application is the synthesis of GOS, which function as prebiotic food ingredients (Crittenden et al. 1996). In industry GOS are usually produced with β-galactosidase at high temperatures and at high substrate concentrations. A benefit of reactions at high temperatures is the improved solubility of the substrates which enables the use of higher substrate concentrations.

S. thermophilus β-galactosidase is already commercially used for yoghurt production (Guarner et al. 2005). Former studies suggest the practical applicability of *S. thermophilus* β-galactosidase for GOS production, due to its tolerance to temperatures of up to 50 °C. *S. thermophilus* β-galactosidase effectively hydrolyzes lactose in milk (Rao et al. 1977) and its stability was reported to be improved by addition of Mg^{2+} (Rhimi M. et al. 2010). As described in CHAPTER 1, our characterization data confirmed, that *S. thermophilus* β-galactosidase is an ideal enzyme for this approach, owing to its high hydrolytic activity towards lactose, as well as its high transgalactosylation activity and its activity and stability in a broad temperature range. The enzyme was recombinantly expressed in the food grade organism *L. plantarum*, for experiments crude cell-free extracts were used. The use of the food-grade organism *L. plantarum* for lactose conversions does not require laborious purification procedures. We determined enzymatic stability in phosphate buffer with and without $MgCl_2$ and in whey. Whey itself had already a stabilizing effect. At 50 °C the enzyme was shown to be stable for more than a day. Furthermore, the capability of the enzyme to efficiently hydrolyze lactose in whey was analyzed. For that purpose, whey containing 50 g/l lactose which corresponds to the concentration of lactose in bovine milk was used. Lactose hydrolysis took place much faster at 50 °C and was completed within 2 hours, whereas at 37 °C lactose was fully hydrolyzed after 5 hours. A product free from lactose and allolactose could be obtained. Complete lactose hydrolysis was confirmed by HPAEC-PAD, as well as by the enzyme-based lactose biosensor Lactosens (DirectSens GmbH, Vienna, Austria;

http://www.directsens.com). The enzyme was shown to be suited for transgalactosylation as well. For transgalactosylation, dissolved whey permeate containing 205 g/l lactose was used. A GOS yield peaking at 50 % was reached at a conversion temperature of 50 °C. The transgalactosylation yield obtained by *S. thermophilus* β-galactosidase is high compared to other commercially used β-galactosidases. *S. thermophilus* β-galactosidase was shown to be suitable for efficient degradation of lactose in whey into glucose and galactose for the production of lactose-free and allolactose-free dairy products, as well as for the production of milk products richer in GOS. (Geiger, Nguyen et al. 2016). Increased temperature allows the use of higher substrate concentrations which in turn result in a higher GOS yield (Boon et al. 2000). Most β-glycosidases used for commercial purposes are mesophilic enzymes. For a large number of processes, the use of extremely thermostable enzymes is favorable (Bhattacharya et al. 2014). For nutritional processing, enzymes derived from food-grade organisms such as lactic acid bacteria are commonly used (Macfarlane et al. 2008).

H. orenii β-glucosidase poses the ideal candidate enzyme for mutagenesis, in order to alter its transglycosylation activity. The main activity of the β-glucosidase from *H. orenii* is the hydrolysis of cellobiose. With lactose as substrate, the enzyme shows a distinctive transgalactosylation activity, producing large amounts of GOS. Its high thermostability of up to 70 °C enables the use of elevated temperatures and high substrate concentrations; conditions which favor GOS formation (Kori et al. 2011, Hassan et al. 2015). Generally, β-glucosidases are suitable to be engineered towards improved transferase/hydrolase ratios. Former publications report improved transglycoslation in related β-glycosidases (Hansson et al. 2001, Feng et al. 2005, Lundemo et al. 2013, Teze et al. 2014). Due to the good thermostability of *H. orenii* β-glucosidase, the reaction can be carried out at high temperatures, which reduces the risk for microbial contamination. High temperatures allow the use of higher substrate concentrations, resulting in a higher GOS yield (Hassan, Geiger et al. 2015). Overall all these characteristics allow a cheap production of valuable GOS from lactose.

As described in CHAPTER 2, we investigated the possibility to further enhance transgalactosylation activity and GOS yield by replacing amino acid side chains in the active center of *H. orenii* β-glucosidase. Five variants (N222F, N294T, F417S, F417Y and Y296F) were generated. At 70 °C two variants, F417S and F417Y produced the trisaccharide 6'-galactosyllactose [β-D-Gal-(1→6)-D-Lac] in much higher amounts and at lower lactose conversion rates than the WT. Variant F417Y performed best, achieving a total GOS yield of 57 % of total sugars, whereas total GOS yield of the WT accounted for 39 % of total sugars (Hassan, Geiger et al. 2015).

Due to the fact that the exchange of the phenylalanine at position 417 for tryptophane or serine in *H. orenii* β-glucosidase led to improved GOS yields, we aimed to further explore the effect of this amino acid residue on the activity of *H. orenii* β-glucosidase by probing position F417 as described in CHAPTER 3. Out of the variant library covering all possible amino acid exchanges, seven showing activity with lactose were selected. The variants were recombinantly expressed in *E. coli* for detailed characterization. Replacement of phenylalanine at position 417 was shown to affect temperature stability. All variants showed decreased temperature stability with respect to the WT, variant F417M was most stable of all variants. All variants retained half of their initial enzymatic activity for at least 10 hours at 50 °C, therefore, kinetic measurements, as well as transglycosylation were carried out at this temperature.

Replacement of F417 by T generated an enzyme with a 3-fold higher preference for lactose than for cellobiose. By molecular dynamics simulation, the amino acid residues participating in substrate interaction in *H. orenii* β-glucosidase were identified. Q20, H121, E166, E173, Y296, E354, W401, E408, W409 were shown to be involved in substrate binding. Most importantly, molecular dynamics simulation showed a higher number of hydrogen-bond interactions of the catalytic amino acid residues E166 and E354 with cellobiose than with lactose for the WT, whereas in F417T there were slightly higher numbers of hydrogen-bond formations and closer bonds to the catalytic amino acid residues with lactose than with cellobiose. These results confirm the results of kinetic analysis, which showed a lower K_m for lactose of variant F417T, as well as a higher preference for lactose over cellobiose as substrate.

In transgalactosylation experiments with lactose as substrate at 50 °C, GOS patterns of variants differ from the WT, as well as between each other. Allolactose is the main GOS product formed by variants F417I, F417S, F417T and F417Y, whereas 6'-galactosyllactose is the predominant transgalactosylation product in the WT and the variants 417C, 417H, 417L, 417M and 417W. All variants and the WT produce β-1,3 and β-1,4 linked GOS to a smaller extend. β-Galactosidases from lactobacilli, as well as *S. thermophilus* β-galactosidase produce β-1-3, and β-1-6 linked GOS (Geiger, Nguyen et al. 2016). *H. orenii* β-glucosidase in contrast formed β-1,3, β-1,6 as well as β-1,4 linkages (Hassan, Geiger et al. 2015). For the growth of bifidobacteria, GOS mixtures containing β-1,3, β-1,4 and β-1,6 linkages are more beneficial than GOS mixtures containing only β-1,3 and β-1,6 linkages (Sangwan et al. 2011).

A beneficial feature of β-glucosidases is that they show a broader substrate specificitiy for β-D-glycosides than β-galactosidases. *H. orenii* β-glucosidase was shown not only to form transgalactosylation products with lactose, but also transglucosylation products with cellobiose. In transglucosylation experiments with cellobiose as substrate at 50 °C, the WT produces β-1,3, β-1,6 and β-1,4 linked disaccharides and trisaccharides, whereas in variants F417C, F17Y, F417S, and F417I additionally the formation of cellotetraose and cellopentaose was detected. Gentiobiose is the main COS component produced by the WT enzyme as well as by variants F417H, F417L, F417M, F417T, and F417W. Other COS components are formed in smaller amounts. In the variants F417C, F417I, F417S and F417Y there is a greater variety and a more equal distribution of individual COS components synthesized.

Currently, COS are considered as candidate prebiotics, there is not enough evidence to classify them as prebiotics (Rastall et al. 2013). Further investigation, whether the transglucosylation components formed by *H. orenii* β-glucosidase have a growth stimulating effect on lactobacilli and bifidobacteria might be promising. There is a previous publication on the formation of β-1,3, β-1,6 and β-1,4 linked disaccharides, trisaccharides and tetrasaccharides from cellobiose by β-glucosidases of fungal origin (Smaali et al. 2004). *H. orenii* β-glucosidases produced β-1,3, β-1,6 and β-1,4 linked disaccharides and trisaccharides as well. In order to further elucidate relationships between structure and function also in terms of temperature stability, generation of corresponding variants in other GH family members might be a suitable approach, such as single amino acid exchanges at

the homologous position F426 in *P. furiosus* β-glucosidase. Like *H. orenii* β-glucosidase, *P. furiosus* β-glucosidase acts as β-galactosidase to a minor extent. The natural β-glucosidase activity of *P. furiosus* β-glucosidase accounts for about one third of its β-glucosidase activity. Previously, the shift of enzymatic activity of *P. furiosus* β-glucosidase towards transglycosylation by single amino acid exchange on position F426 for Y has been reported (Hansson et al. 2001). Temperature optimum for *P. furiosus* β-glucosidase is 100 °C, with a half-life of 85 h (Kengen et al. 1993). Therefore, this enzyme is by far more thermostable than *H. orenii* β-glucosidase with a half-life time of activity of 18 hours at 65 °C, its temperature optimum (Hassan et al. 2015). *P. furiosus* β-glucosidase produces a lactose-free product from milk at pasteurization conditions of 100 °C (Li et al. 2013). Molecular dynamics simulation is the method of choice for identification of amino acids in the active site, participating in the substrate interaction (Zouhar et al. 2001, Feng et al. 2005). Crystal structures for both, *H.orenii* β-glucosidase as well as for *P. furiosus* β-glucosidase are available in a good resolution of 1.85 Å and 1.7 Å, which makes this bioinformatic approach feasible.

Cloning of *H. orenii* β-glucosidase into a food grade expression vector such as the pSIP, and recombinant expression in a safe organism such as *L. plantarum* or *L. reuteri* could be a future approach. This might be advantageous, in many aspects. Food-grade expression systems are regarded as safe and are able to produce high amounts of the recombinant target protein (Sorvig et al. 2005). Different parameters impact the the pSIP expression system.

In CHAPTER 4, it was shown, that the yield of the recombinant protein can be further improved. Maintenance of a pH of 6.5 resulted in a higher β-galactosidase yield. The yield of the recombinant protein was affected by the induction time point, but only in experiments with no pH control. Induction at high OD resulted in lower volumetric activities. If no antibiotic was added, the β-galactosidase yield was much lower as well. Volumetric activity at 37 °C after 12 hours was 2-fold higher than at 30 °C. Until a concentration of 40 ng/ml there was a dose-dependent effect of IP protein. Higher initial glucose concentrations were shown to increase the maximum β-galactosidase yield, β-galactosidase yields were highest at 40 g/l glucose (Nguyen et al. 2015).

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