Production of Novel Enzymes and Peptides Using a Synthetic Biology Approach in *Lactococcus lactis*

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Dedication

I dedicate my Ph.D. thesis to my family. I would like to express my gratitude to my advisor Dr. O'Sullivan for his advises and help. I am thankful to Dr. Schoenfuss, Dr. Gallaher, Dr. Smith and Dr. Barney for their advises and comments. I thank all the lab colleagues, Xiulan Li, Wilfredo Dominguez, Xindi Liu, Omer Celik and Yang Yang for their help and supports.

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History of *Lactococcus lactis*

Lactococcus lactis strains are non-pathogenic, low GC, Gram-positive bacteria in the *Firmicutes* phylum. They are important members of the lactic acid bacteria (LAB) and are used worldwide for food fermentations, particularly dairy. They were first described in 1857 by Louis Pasteur who observed them as the primary organisms in soured milk (1). The first pure culture was isolated by Joseph Lister in 1873 and was designated as *Bacterium lactis*. Subsequently, it was included in the genus *Streptococcus*, with two proposed species, *S. lactis* and *S. cremoris.* An early classification system for *Streptococcus* divided the genus into the four different groups, Pyogenic, Viridans, Lactic and Enterococcus*,* based on serological, physiological and biochemical characteristics (2). Subsequent nucleic acid hybridization studies and sequencing studies demonstrated that some species were not as closely related to each other leading to the generation of the three genera, *Streptococcus, Lactococcus* and *Enterococcus* based primarily on molecular characteristics (3–5). The subspecies *diacetylactis* is a strain of *L. lactis* that synthesizes diacetyl from citrate metabolism and was first described by Matuszewski et al. in 1936 (6, 7). It was initially given the species name, *S. diacetylactis*, but later as a subspecies of *L. lactis* due to the high degree of genetic similarity and the only phenotypic difference being production of diacetyl. Later it was found that the phenotype difference is solely the result of a plasmid and therefore reclassified as a biovar of *L. lactis* ssp. *lactis* (8).

Currently, the *L. lactis* species consists of three subspecies, *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *hordniae* (9). The first two subspecies are very important for dairy fermentations, especially cheese production, and they were isolated from milk sources. Some studies have proposed that they originally came from plant sources (10, 11). Both of them are associated with dairy fermentations as starter cultures that play important roles in acidification, production of aroma and flavor, preservation and development of texture. Early research with *L. lactis* noted that it would lose its ability to grow on lactose if grown for extended periods in glucose (12–15). Following the discovery of plasmids, this instability was found to be the encoding of lactose metabolism on a plasmid as well as the other metabolic characteristics, citrate utilization and proteinase activity (16–20). Therefore, plasmid free strains were developed to enable characterization of these important metabolic traits, and three of them are widely used as laboratory strains, *L. lactis ssp. cremoris* MG1363*, L. lactis ssp. lactis* IL1403 and *L. lactis. ssp lactis* LM0230 (21–23). The complete chromosome sequence of *L. lactis* was first obtained from *L. lactis ssp. lactis* IL1403 and consisted of 2,365,589 bp and a G+C content of 35.4% (24). The third subspecies, *L. lactis* ssp. *hordniae*, is unable to use lactose as a carbon source and it was isolated from a leafhopper (25).

Cloning vectors developed for *L. lactis*

Replication origins (*ori*) that function in *L. lactis* were obtained from naturally occurring lactococcal plasmids such as pWV01 and pSH71. These two plasmids exhibited a rolling circle replication mechanism, in which a single strand DNA is generated during the replication and then used to make a double strand DNA (26) (Figure 1. 1a). Both plasmids are lactococcal cryptic plasmids without specific phenotypes. Another source of ori's came from plasmids from other Gram-positive bacteria, such as pAMβ1 from *Enterococcus faecalis* and pIP501 from *Streptococcus agalactiae*, both of which exhibit a unidirectional theta-replication mechanism, in which a theta structure occurs during the replication of circular DNA (27, 28) (Fig 1. 1b). Parental plasmids used for vector construction for *L. lactis* are listed in Table 1. 1. A number of vectors have been constructed based on these plasmids and used for cloning of genes in *L. lactis* (Table 1. 2). Some plasmids only replicate in a number of Gram-positive bacteria, including members of the LAB, a number of bacilli and some species of *Streptococcus*. However, a number of vectors could either replicate in Gram-positive or Gram-negative bacteria due to the broad host range of the *ori* or the introduction of *E. coli ori's.* More details are provided in Table 1. 2.

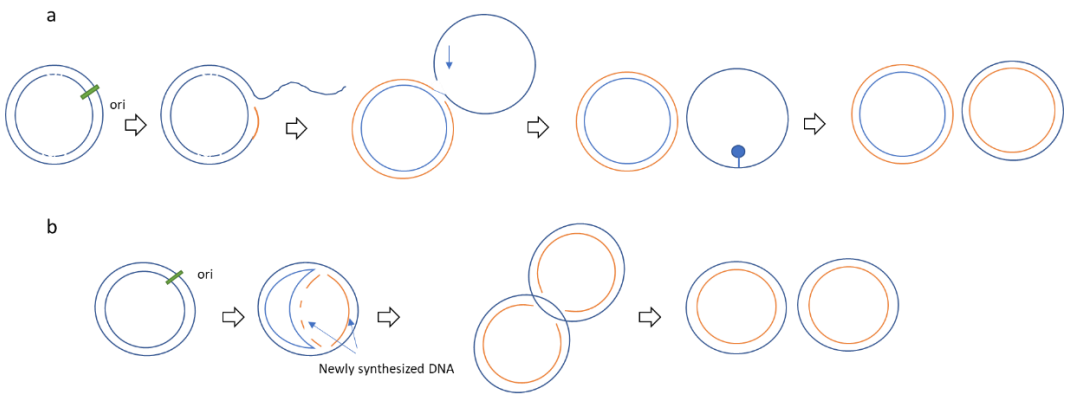

Figure 1. 1. Diagrammatic representation of plasmid replication models. a) Rolling circle replication, where one DNA strand is nicked at the *ori* site by the rep protein. This enables DNA polymerase to elongate the strand using the intact strand as template. The displaced nicked strand remains bound to the Rep enzyme and its single strand DNA ligase activity functions to seal the nick, thus releasing Rep from the single stranded circular DNA molecule. A double strand DNA secondary structure is formed from the inverted repeats on the single strand ori sequence, providing RNA polymerase a platform to synthesize RNA primer. The single strand acts as template and new double strand DNA is synthesized by DNA polymerase. b) Theta-model; The replication initiates at *ori* site, in which double strand DNA is denatured, resulting in a replication bubble. DNA polymerase initiates the replication of leading strand DNA continuously. While the synthesis of lagging strand DNA is discontinuous. Eventually the replication finish and two plasmids are generated.

Antibiotic Resistance Marker Genes

Antibiotic resistance genes from Gram-positive bacteria were introduced into *L. lactis* vectors to enable selection and maintenance of plasmid vectors. *L. lactis* strains are naturally very susceptible to erythromycin and chloramphenicol, making them the most widely used selection markers (29). More details about antibiotic resistance genes used for *L. lactis* vectors are provided in Table 1. 3.

Food grade vectors

To make plasmid vectors more acceptable for food applications, food grade vector plasmids were further developed by replacing the antibiotic markers with other selectable markers. Genes encoding resistance to nisin, heavy metals (copper and cadmium) or some other genes involved in lactococcal metabolism have been used as food grade markers. The *thyA* gene has been used as a positive selection marker in food grade vectors for *thyA* mutant strains, which were obtained by selecting for resistance to the antibiotic, trimethoprim (30). More examples of food grade vectors are provided in Table 1. 4.

Table 1. 1. Plasmid *ori*s that function in *L. lactis*

Plasmid	Size (kb)	Relevant characteristics	Reference
pAMβ1	27	Originally from *E. faecalis*; Em^r, replicates in some Gram-positive bacteria; theta replication	(27)
pIP501	30	Originally from *S. agalactiae*; Cm^r; Em^r; replicates in some Gram-positive bacteria; theta replication	(31)
pWV01	2.3	Cryptic plasmid from *L. lactis* subsp. *cremoris* Wg2; replicates in both Gram-positive and Gram-negative bacteria; rolling circle replication	(32)
pSH71	2.1	Cryptic plasmid from *L. lactis* subsp. *lactis* 712; replicates in both Gram-positive and Gram-negative bacteria; rolling circle replication	(33)

Cm and Em: chloramphenicol and erythromycin, respectively

Table 1. 2. Cloning vectors and shuttle vectors developed for *L. lactis*

Plasmids	Size (kb)	Replication in *E. coli*	Relevant characteristics	Reference
pHV1301	13.0	-	Developed based on pAMβ1; $^{L}Em^{r}$ low copy number	(34, 35)
pIL252	4.6	-	Constructed based on pHV1301 (pAMβ1-derived); $^{L}Em^{r}$; low copy number	(36)
pIL253	4.8	-	Constructed based on pMT9 (pAMβ1-derived); $^{L}Em^{r}$; high copy number	(36)
pGB301	9.8	-	Derived from a spontaneous deletion mutant of pIP501; $^{L}Cm^{r}$, $^{L}Em^{r}$; low copy number	(37)
pSA3	10.2	+	Constructed based on pGB305 (pIP501-derived) by introducing *E. coli* p15A *ori* from pACYC184; $^{LE}Em^{r}$, $^{E}Cm^{r}$ and $^{E}Tc^{r}$;	(38) (39)
pGK12	4.4	+	Constructed based on pWV01; $^{E}Cm^{r}$; $^{LE}Em^{r}$; low copy number	(40, 41)
pCK1	5.7	+	Constructed based on pSH71; $^{L}Cm^{r}$; $^{E}Km^{r}$; high copy number	(42)
pNZ12	4.3	+	Constructed base on pSH71; $^{L}Cm^{r}$; $^{E}Km^{r}$; high copy number	(33)
pTRKL1	8.3	+	Constructed based on pIL252 (pAMβ1-derived) by introducing *E. coli* p15A *ori* from pACYC184; $^{LE}Em^{r}$; $^{E}Cm^{r}$; low copy number	(43)
pTRKH1	11.0	+	Constructed based on pIL253 (pAMβ1-derived) by introducing *E. coli* p15A *ori* from pSA34;$^{LE}Cm^{r}$; high copy number	(43)

Cm, Em, Tc and Km: chloramphenicol, erythromycin, tetracycline and kanamycin, respectively; ^{L}: Antibiotic resistant gene functions in *L. lactis*; ^{E}: Antibiotic resistant gene functions in *E. coli*

Table 1. 3. Selective antibiotic resistant marker genes used in *L. lactis*

Selective marker	Marker genes and source	Reference
Chloramphenicol	*cat* gene from pC194 in *Staphylococcus aureus*	(44, 45)
Erythromycin	*erm* gene from pE194 in *S. aureus*	(46)
Tetracycline	*tet* gene from chromosome of *E. faecium*	(47)
Kanamycin	*kan* gene from chromosome of *S. pyogenes*	(48)

Table 1. 4. Food grade vectors developed for *L. lactis*

Plasmids	Size (kb)	Food grade marker gene	Host used for selection	Reference
pFM011	7.8	Nisin resistant gene	Nisin sensitive *L. lactis* strains	(49)
pDN648	7.0	Cadmium and nisin resistant gene	Cadmium and nisin sensitive *L. lactis* strains	(50)
pDN969	14.7	Copper and nisin resistant gene	Copper and nisn sensitive *L. lactis* strains	(50)
pFG1	2.0	Ochre nonsense suppressor gene *supB*	nonsense mutant *L. lactis* strains	(51)
pNZ8045	6.0	*lacF* gene	*L. lactis* NZ3000, Δ*lacF*	(52)
pSUW611	3.9	Aspartate aminotransferase gene *aspC*	*aspC* mutant *L. lactis* strains	(53, 54)
pPR602	N/A	thymidylate synthase gene *thyA*	*thyA* mutant *L. lactis* strains	(30)

Vector transfer into *L. lactis*

To facilitate the transfer of plasmids, Kondo and McKay proposed protoplast transformation for *L. lactis*, in which cells were pre-treated by mutanolysin to degrade the cell wall. Plasmid DNA was mixed with protoplasts followed by polyethylene glycol (PEG) treatment. Protoplasts were recovered and the transformants were selected in M17-lactose media (55). it was found that proper formation of protoplasts was crucial for successful transformation. Subsequently, lysozyme was used for protoplast generation, resulting in an increased transformation efficiency of $4x10^4$ to $5x10^6$ transformants per μg of DNA (56, 57).

Although plasmid transformation can be completed via protoplasts, it is not feasible for all the Gram-positive bacteria. Therefore, another method, electroporation transformation, was proposed as an alternative method to protoplast transformation. Electroporation transformation was developed based on the phenomenon that cell membrane can be reversible and nondestructive electro-permeabilized following the short period of electronic field pulse (58). It was first used for gene transfer in 1982, in which a plasmid containing a thymidine kinase gene was transferred into mammalian cells with the help of electric pulsing, followed by successful expression (59). This method was subsequently used in a number of Gram-positive bacteria, including members of LAB, *Listeria*, *Bacillus*, *Staphylococcus*, and *Propionibacterium* (60). Powell proposed a simple electroporation method specifically for *L. lactis*, in which the

cells were treated by lysosome (61). Subsequently, a high transformation frequency (5.7 x10^7 transformants per μg of DNA) electroporation transformation protocol has been described, in which *L. lactis* was grown in media with a high concentration of glycine and 0.5M sucrose (62). In this method, glycine was added to inhibit cell wall formation and sucrose functioned as an osmotic stabilizer.

Development of gene expression vectors for *L. lactis*

The first expression system developed for *L. lactis* was used to express a proteinase gene from *L. lactis* subsp. *cremoris* (63). In 1989, another expression system was constructed to express an egg white lysozyme gene in *L .lactis* using a characterized promoter (64). Later, several expression systems have been explored and developed for *L. lactis*. Both constitutive and regulated promoters have been utilized.

Constitutive promoter expression vectors

Constitutive promoters are required when continuous expression of the target gene is needed. Generally, constitutive promoters can be isolated from *L. lactis* strains via screening vectors containing reporter genes, such as antibiotic resistant genes or β-galactosidase genes (65, 66). Efficient promoters isolated include, P45, p23 and p32 and they were used in *L. lactis* IL1403 and *L. lactis* MG1363 for expression of various proteins (67, 68). More recently, another 8

constitutive promoters were identified from *L. lactis* subsp *lactis* N8, and 4 of them (p8, p5, p3 and p2) showed a higher transcriptional activity than p45, when *nisZ* was used for as the reporter gene for investigating the efficiency of the promoter (69). The efficiency of these constitutive promoters that have been used is provided in Table 1. 5.

Table 1. 5. Efficiency of constitutive promoters and their transcriptional activities.

Promoters	Reporter gene used	Promoter efficiency	References
p45	*nisZ* from chromosomal DNA from *L. lactis* N8	*12	(69)
p32		*10.64	
p8		*16.24	
p5		*15.12	
p3		*14.22	
p2		*13.28	
p32	*luc* from plasmid pWL1	#70	(70)
p6C		#135	
p13C		#75	

*: Inhibition zone (mm) obtained on a bioassay; #: Relative luciferase units measured using luciferase assay

Regulated promoters

Regulated promoters have been characterized from *L. lactis* including those involved in sugar and protein metabolism. Regulated promoters are suitable for expression vectors when controlled production is needed.

The first regulated gene expression system for *L. lactis* was developed based on an inducible *lac* promoter from *L. lactis* (Fig.1. 2) (71). It was shown that transcription of the *lac* operon was repressed by the interaction of LacR , in a similar fashion to *E. coli* (71). However, the inducer was tagatose-6-phosphate that accumulated during its growth in lactose, thus removing LacR from the operator allowing transcription to occur (72).

The weaknesses of this expression system are the low induction level (less than 10 -fold) and difficulty of control since the inducer is an intracellular intermediate, tagatose-6-phosphate.

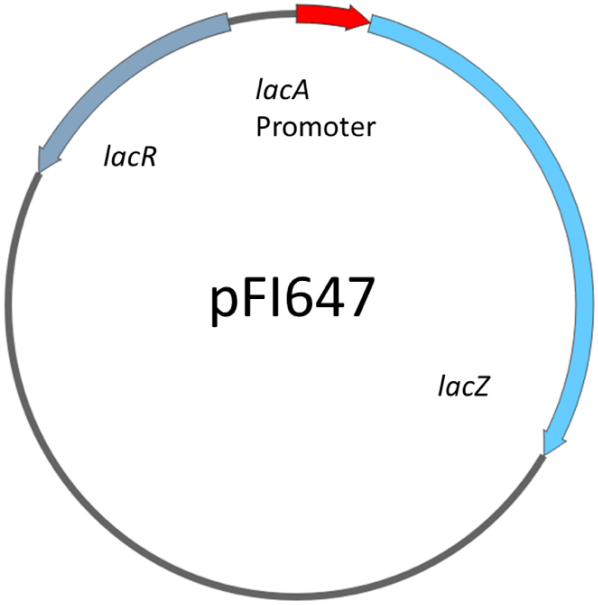

Figure 1. 2. Construction strategy of expression vector developed based on the *lac* operon from *L. lactis*. The reporter gene (*lacZ*) was fused downstream of the *lacA* promoter. The vector was subsequently introduced into an *L. lactis* strain without its original lactose plasmid.

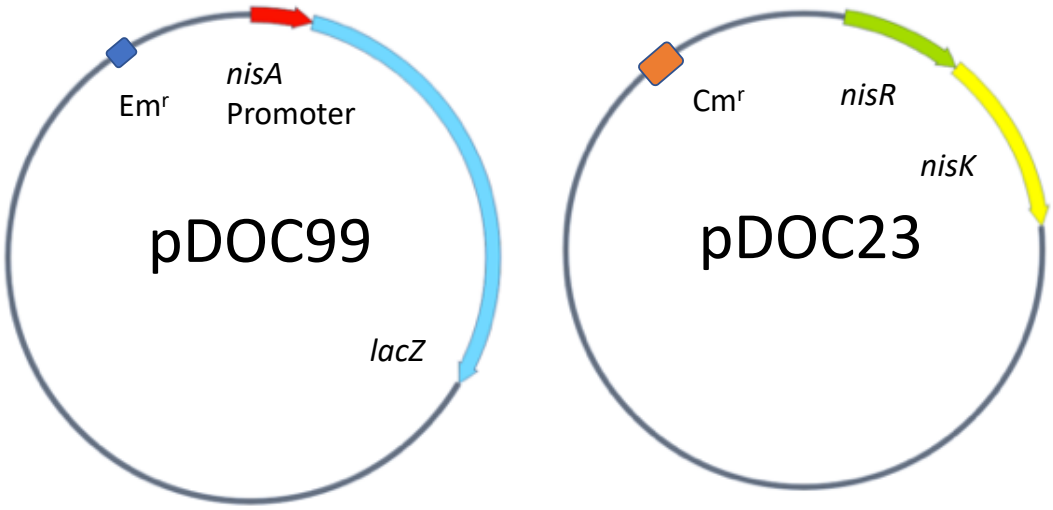

Figure 1. 3. Construction strategy of expression vector developed based on the nisin controlled system.

Another well-characterized expression system used in *L. lactis* is the nisin controlled gene expression system developed based on a two-component regulatory system, which is composed of *NisRK*. NisK is a transmembrane sensor protein with histidine kinase activity to activate the cytoplasmic NisR response regulator. When NisK senses the presence of the inducer (nisin) in the media, it will autophosphorylate. The phosphate group will be subsequently transferred into NisR for its activation. Activated NisR turns on the powerful *nisA* promoter (73).

Based on the understanding of the mechanism of nisin gene regulation, a nisin-controlled expression system was first developed by construction of a vector plasmid containing the *nisA* fused to an *E. coli gusA* gene. The resulting plasmids were then introduced into *L. lactis* strain NZ9700 and a β-glucuronidase activity was found during the growth with the maximum level at the stationary phase (52). The system was further improved by cloning the *nisA* promoter and *nisRK* into two plasmids respectively (Fig. 1. 3) (74). A β-galactosidase enzyme was expressed successfully upon the addition of nisin in the fully-grown culture. Due to its high efficiency and tight regulation, nisin-controlled expression systems have been widely used for overexpression of a variety of genes in *L. lactis*.

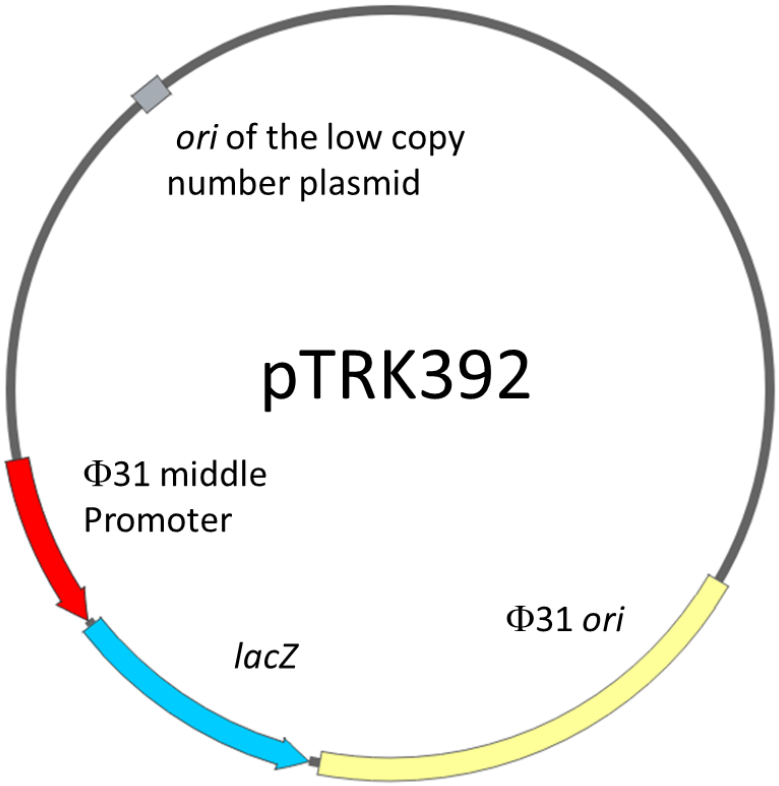

Figure 1. 4. Construction strategy of expression vector developed based on a phage inducible expression system. The reporter gene will be induced and expressed upon phage infection, resulting in explosive expression.

In addition to lac expression system and nisin-controlled expression system, some other inducible expression systems were also explored and developed for *L. lactis*. For example, a phage inducible system was developed based on the plasmid containing an origin of replicon of Φ31 and phage expression signals (Fig. 1. 4) (76, 77). A phosphate starvation-inducible expression system was developed, in which system, no inducing agents were required in the growth media since the cells of *L. lactis* could use up phosphate during the growth to induce promoter *pstF* (Fig. 1. 5) (78). Later, a zinc-inducible system has been developed with a promoter *pczcD* from *S. pneumoniae* D39 together with a repressor (Fig. 1. 6) (79).

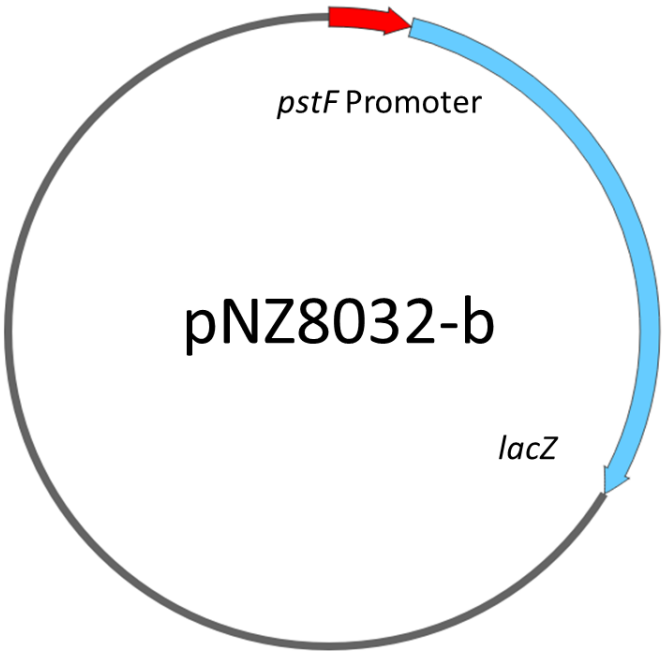

Figure 1. 5. Construction strategy of expression vector developed based on a phosphate starvation-inducible expression system.

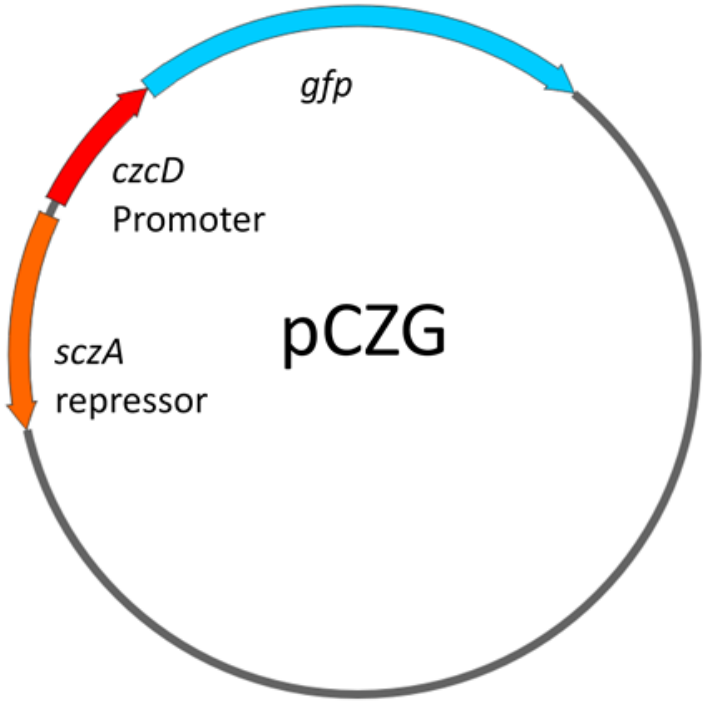

Figure 1. 6. Construction strategy of expression vector developed based on zinc inducible expression system.

Applications

The development of a variety of vectors, together with gene transfer systems and high efficiency gene expression systems, makes *L. lactis* a popular choice for gene expression in both the food and pharmaceutical industries. Numerous reports have demonstrated its ability to produce novel enzymes, including the hyper-thermophilic beta-galactosidase enzyme described in Chapter 2 and 3 of this thesis that can be used for efficient production of GOS. More details are illustrated in Table 1.6.

Table 1. 6. Proteins expressed using *L. lactis* and their applications.

Proteins expressed and function	Protein application	Gene/ subunits	Expression host strain used	Reference
Cyclodextrin glucanotransferase, enzyme to hydrolyze starch and cyclodextrins	Production of food ingredients, such as cyclodextrins and oligosaccharides	*CGTase* gene from *Bacillus* sp. G1	*L. lactis* NZ9000	(80)
β-galactosidase, enzyme to break down lactose	Galactooligosaccharide (GOS) production	*lacZ* gene from *Streptococcus thermophilus*	*L. lactis* LM0230	(74)
Acid urease, enzyme to degrade urea in rice wine	Elimination of urea in fermented products	*ureABCEFGD* from *Lactobacillus reuteri* CICC6124	*L. lactis* NZ9000	(81)
Glucansucrase, enzyme that catalyzes formation of glucans from sucrose	Oligosaccharide based ingredient production	*dsrI* from *Leuconostoc mesenteroides*	*L. lactis* NZ9000	(82)
Lipase, enzyme to hydrolyze milk fat	Synthesis of flavor components	*lpl* gene from *Burkholderia cepacia*	*L. lactis* NZ9000	(83)
Leucocin C, bacteriocin, class IIa	Promising use in pasteurized milk processing	*lecC* from *Leuconostoc carnosum* 4010	*L. lactis* NZ9000	(84)
Brazzein, sweet-tasting protein	Potential use as a low-calorie sweetener	*bra* fruit *Pentadiplandra brazzeana* Bailon	*L. lactis* IL 1403	(85)
Chitinases, enzyme to degrade chitin	Chitooligosaccharide production used in pharmaceutical filed	*chiA* from *Serratia marcescens*	*L. lactis* MG1363	(86)
Neutral protease, enzyme to hydrolyze proteins	Used for beverage clarification	*nprB* from *Bacillus subtilis*	*L. lactis* MG1363	(87)

Conclusions

The production of novel enzymes and other proteins from other organisms has received significant attention from researchers in Food Science, they can have a beneficial influence on the nutritional and shelf life characteristics of different foods. *L. lactis* strains are good candidates for heterologous gene expression, with a variety of expression vector systems developed for various purposes. A wide range of proteins with different of functions have already been successfully expressed in *L. lactis*. While the efficiency of heterologous protein expression is quite good, the future will likely see more efficient systems developed. It is inevitable that numerous other proteins with new functions will be produced using *L. lactis*.

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Chapter 2. Production of Galactooligosaccharides Using a Hyperthermophilic β-galactosidase in Permeabilized Whole Cells of *Lactococcus lactis*

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Galactooligosaccharides(GOS) are novel prebiotic food ingredients that can be produced from lactose using β-galactosidase, but the process is more efficient at higher temperatures. To efficiently express the *lacS* gene from the hyperthermophile *Sulfolobus solfataricus*, in *Lactococcus lactis* a synthetic gene (*lacSt*) with optimized codon usage for *Lc. lactis* was designed and synthesized. This hyperthermostable β-galactosidase enzyme was successfully overexpressed in *Lc. lactis* LM0230 using a nisin-controlled gene expression system. Enzyme-containing cells were then killed and permeabilized using 50% ethanol and were used to determine both hydrolysis and transgalactosylation activity. The optimum conditions for GOS synthesis was found to be at pH 6.0 and 85°C. A maximum production of 197 g/L of GOS tri- and tetrasaccharides was obtained from 40% initial lactose, after 55 h of incubation. The total GOS yield increased with the initial lactose concentration, whereas the highest lactose conversion rate (72%) was achieved from a low lactose solution (5%). Given that a significant proportion of the remaining lactose would be expected to be converted into disaccharide GOS, this should enable the future development of a cost-effective approach for the conversion of whey-based substrates into GOS-enriched food ingredients using this cell-based technology.

Introduction

Prebiotics are non-digestible or minimally digestible food ingredients, such as oligosaccharides and sugar alcohols, which are proposed to be fermented by certain intestinal microflora, such as bifidobacteria. This stimulation of beneficial colon bacteria is proposed to improve gut health by suppressing the levels of deleterious bacteria (Roberfroid, 2007). Galactooligosaccharides (GOS), which are composed of a terminal glucose unit and 1 or more galactose moieties, are novel prebiotics that are particularly effective growth factors for bifidobacteria (Ito et al., 1990; Barboza et al., 2009). Bifidobacteria are proposed to play an important role in modulating the gut microbiota, accompanied by other health benefits, such as diarrhea prevention, constipation relief, protection from colon cancer, and stimulation of antiinflammatory immune responses (Ito et al., 1993; O'Sullivan, 2001; Lee and O'Sullivan, 2010). Several in vivo experiments have suggested that the consumption of GOS promoted the growth of bifidobacteria and lactobacilli in the intestines of infants and adults (Macfarlane et al., 2008; Davis et al., 2011). In addition, infant formula supplemented with a low level of GOS (0.24 g/100 mL) can either stimulate the stool frequency or reduce fecal pH compared with those fed with formula without GOS (Ben et al., 2008). Thus, GOS have now been incorporated in infant formula in some countries and a lot of studies have looked at its incorporation into dairy products, such as yogurts and fermented milks, as well as fruit juices and bakery products (Torres et al., 2010; Sangwan et al., 2011).

The production of GOS can be achieved by enzymatic transgalactosylation activity during the hydrolysis of lactose. It can be catalyzed by β-galactosidase enzymes from various sources, such as *Bifidobacterium longum* (Hsu et al., 2007), *Kluyveromyces lactis* (RodriguezColinas et al., 2011), *Aspergillus oryzae* (Albayrak and Yang, 2002), and *Lactobacillus pentosus* (Maischberger et al., 2010). The GOS yield, lactose conversion rate and composition of GOS vary depending on the β-galactosidase enzyme used. The highest yield of trisaccharides or longer chains of GOS that has been reported to date is produced by a hyperthermostable β-galactosidase from *Sulfolobus solfataricus*, which allowed GOS to be produced at a high temperature, resulting in a higher solubility of lactose and higher reaction velocity (Park et al., 2008). Most studies cannot determine the yield of disaccharide GOS by-products. However, recently, the synthesis of GOS catalyzed by a β-galactosidase from *A. oryzae* was studied, in which 70% of initial lactose was converted into GOS, with 40% of the GOS by-products consisting of disaccharides (Urrutia et al., 2013). Galactooligosaccharide production by *Lactococcus lactis* would be desirable, as it is a lactic acid bacterium with a long history of use in food fermentations for commercial production of dairy products, such as cheese and fermented milks. This bacterium has been well characterized and numerous efficient expression systems have been constructed. For instance, a phage inducible expression system containing a φ31 promoter and the phage origin of replication was developed for *Lc. lactis* for high-level, and tightly controlled, heterologous gene expression following phage infection (O'Sullivan et al., 1996). A sugar-inducible

system was constructed based on the xylose-inducible lactococcal promoter for controlled expression of a target protein (Miyoshi et al., 2004). The most widely utilized vector system for heterologous gene expression in *Lc. lactis* utilizes the nisin 2-component NisRK regulatory signals and the *nisA* promoter. This was first developed by fusing the *nisA* promoter to a target gene for expression in a plasmid vector and the *nisRK* genes were integrated into the chromosome, facilitating controlled and high-level gene expression upon induction with nisin (de Ruyter et al., 1996; Mierau and Kleerebezem, 2005). Subsequently, the *nisRK* genes were cloned in a multicopy plasmid (pDOC23), thus increasing the amount of the NisK sensor protein exposed to the outside of the cells for higher-level induction of the *nisA* promoter (Chandrapati and O'Sullivan, 1999).

The objective of this study was to use *Lc. lactis* as the host culture to overexpress a synthetic gene encoding the hyperthermostable β-galactosidase enzyme from *S. solfataricus* using the efficient nisin expression system. Following ethanol treatment, the nonviable and permeabilized cells could then be evaluated for GOS production from lactose containing substrates.

Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2. 1.

Table 2. 1. Bacterial strains and plasmids

Strain/plasmid	Features [1]	Source of Reference[2]
Baterial strains		
E. coli DH5 α	Plasmid free cloning host	Invitrogen
E. coli ER2925	Plasmid free cloning host, Dam^-	NEB
L. lactis LM0230	Plasmid cured derivative of *Lc. Lactis* C2	DCC
Plasmid		
pUC57	*E. coli* cloning vector; Amp^r	GenScript
pDOC99	Transcription fusion of *nisA* promoter to *lacZ* gene from *S. thermophiles*; Ery^r	Chandrapati and O'Sullivan (1999)
pDOC23	*nisR* and *nisK* genes cloned into pCI372; Cm^r	Chandrapati and O'Sullivan (1999)
pDOLY105	Transcription fusion of *nisA* promoter to synthetically engineered *lacSt* gene; Ery^r	This study

[1] Amp^r = ampicillin resistance; Ery^r = erythromycin resistance; Cm^r = chloramphenicol resistance. [2] Invitrogen = Invitrogen Corp. (Grand Island, NY); NEB = New England Biolabs Inc. (Ipswich, MA); DCC = Dairy Culture Collection (University of Minnesota, St. Paul); GenScript = GenScript USA Inc. (Piscataway, NJ).

Media and Culture Conditions *Lactococcus lactis* was inoculated into M17 (Becton, Dickinson and Co., Franklin Lakes, NJ) broth media with 0.5% glucose (M17G) and grown at 30°C for 12 h. *Escherichia coli* were grown at 37°C in lysogeny broth (LB) or brain-heart infusion (BHI) broth (Becton, Dickinson and Co.). When necessary, erythromycin was added at a level of 150 µg/mL for *E. coli* and 3 µg/mL for *Lc. lactis*. Chloramphenicol was supplemented at a level of 20 µg/mL for *E. coli* and 3 µg/mL *for Lc. lactis*, and ampicillin was used when needed at 150 µg/mL for *E. coli*. Nisin was induced at 1.0 IU/mL *for Lc. lactis*.

Molecular techniques

Two oligonucleotides, *LacSt* F 5c-GCGAATGCATCTAGATCCCC-3c and *LacSt* R 5c-GGTGGTGTCGACCAGCTATGACCATGATTACGCC-3c, were designed as primers to amplify the commercially synthesized *lacSt* gene (GenScript USA Inc., Piscataway, NJ), with underlines indicating restriction sites for *Xba*I and *Sal*I, respectively. Polymerase chain reaction was performed using a PTC-200 thermocycler (MJ Research, MN) with the following conditions: 1 cycle of 98°C for 30 s (initial denaturation), 35 cycles of 10 s at 98°C, 30 s at 62°C, and 30 s at 72°C, which was followed by 1 cycle of 72°C for 10 min for a final extension. The PCR reaction mixture consisted of 1× Phusion HF buffer, 200 µM deoxyribonucleotide triphosphates (dNTP), 0.5 µM concentration of each primer, 100 ng of template DNA, and 0.5 µL of Phusion polymerase (New England Biolabs Inc., Ipswich, MA) in a total volume of 20 µL. The amplified fragment was purified using a PCR purification kit (IBI Scientific, Peosta, IA)

according to the manufacturer's instructions; *Xba*I and *Sal*I (New England Biolabs Inc.) were used for digestion and T4 DNA Ligase (New England Biolabs Inc.) was used for ligation.

Plasmid transformation and electroporation

Commercial competent cells of *E. coli* DH5α (Invitrogen Corp., Grand Island, NY) were transformed according to the manufacturer's protocol. To generate electrocompetent cells of *Lc. lactis*, it was grown in 1 L of M17G broth with 20 mM threonine at 30°C. When the optical density at a wavelength of 600 nm (OD600) reached 0.4, the culture was centrifuged at 15,000 × g for 15 min at 4°C using an ultra-fixed angle type 19 rotor (Beckman Coulter Inc., Chaska, MN). The pellet was washed by resuspension in an equal volume of ice-cold 0.5 M sucrose and 10% glycerol solution 4 times and finally resuspended in 3 mL of the same solution and stored at −80°C. For electroporation, 50 to 100 ng of plasmid DNA was mixed with 50 µL of competent cells on ice. The mixture was transferred into a prechilled (−20°C) 0.2- cm electroporation cuvette (Molecular Bio-Products Inc., San Diego, CA) for an electronic pulse at 2.5 KV, 200 Ω, and 25 µF, using an electroporator 2510 (Eppendorf North America, Hauppauge, NY). Immediately afterward, 950 µL of recovery medium (M17G + 0.5 M sucrose) was added into the suspension and incubated for 1 h at 30°C before spread plating on selective M17G plates.

β-galactosidase measurements

β-Galactosidase activity was measured using orthonitrophenyl-β-galactoside (ONPG), essentially as described by Miller (1972). One drop of toluene was added to permeabilize the cell membrane in a mixture containing 100 µL of nisin-induced culture and 0.9 mL of Z buffer (60 mM Na2HPO4·7H2O, 40 mM NaH2PO4·H2O, 10 mM KCl, and 1 mM MgSO4·7H2O). The hydrolysis reaction was performed by adding 200 µL of ONPG (4 mg/mL). After development of sufficient yellow color, the reaction was stopped by adding 500 µL of a 1 M Na_2CO_3 solution at room temperature. The exact time for the reaction was recorded in minutes and used to calculate the Miller unit enzyme activity [Miller unit = 1,000 × (OD420 − 1.75 × OD550)/(volume of cells used × time of reaction × OD600 of cells used].

Enzyme preparation for GOS evaluation

Lactococcus lactis LM0230 (pDOLY105, pDOC23) cells were grown in 100 mL of M17G medium containing erythromycin and chloramphenicol at 30°C until an OD600 of 0.7 was reached. Nisin (Sigma-Aldrich, St. Louis, MO; 1.0 IU/mL) was added to the culture to initiate induction and the incubation was continued at 30°C for 2.5 h. The induced cells were centrifuged (10,000 × g for 15 min at 4°C) and washed with the same volume of ice-cold water. The cells were pelleted and resuspended with 50 mL of POM buffer (50 mM K2HPO4, 50 mM KH2PO4, and 1 mM MgCl2, pH 7.4; Somkuti and Steinberg, 1994). A 50-mL aliquot of 100% ethanol was then added to kill and permeabilize the cells. After

25 min of incubation at room temperature, the cells were washed with ice-cold POM buffer again and resuspended in POM buffer to the original culture volume. These permeabilized cells were stored at 4°C until used for GOS production.

GOS Synthesis

Permeabilized cells containing thermostable β-galactosidase were incorporated in a reaction mixture (5 mL) containing 100 µL of permeabilized cells and 40% lactose dissolved in 0.05 M sodium phosphate buffer (pH 6) and incubated at 85°C for 25 h. The reaction was terminated by incubation at 4°C overnight. The samples were filtered through a 0.22-µm membrane and diluted 1:2,000 in molecular-grade water for further analysis.

Oligosaccharide analysis by liquid chromatograph-mass spectrometry

The carbohydrate composition of GOS samples was determined by liquid chromatography-mass spectrometry, using an Acquity ultra-performance liquid chromatography system coupled to an LCT premier XE time-of-flight mass spectrometer (Waters Corp., Milford, MA) with an Acquity ultra-performance liquid chromatography (UPLC) amide column (1.7-µm particle size and 50 × 2.1-mm dimensions). The mobile phase was composed of 0.1% formic acid in 80% acetonitrile (A) and 0.1% formic acid in 70% acetonitrile (B) and the chromatographic separation was performed in a gradient mode (A/B 40/60 to 100/0) at a flow rate of 200 µL/min for a total running time of 15 min per sample.

The MS data were analyzed using MassLynx software (Waters Corp.) for qualitative and quantitative analysis.

Lactose, globotriose, and 3α,4β,3α-galactotetraose (Sigma-Aldrich) were used as standards for disaccharide, trisaccharide, and tetrasaccharide analysis, respectively. The calibration curve of each standard was made and used to convert peak areas into concentrations.

Results

Codon optimization of the *lacS* Gene from *S. solfataricus* and construction of the *lacSt* gene for *Lc. lactis*

The complete genome information of *Lc. lactis* ssp. *lactis* IL1403 (accession number NC_002662.1) and *Lc. lactis* ssp. *cremoris* MG1363 (accession number NC_009004.1) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The Matlab (R2102b; The MathWorks Inc., Natick, MA) program was used to count the different codons and calculate the codon frequency in *Lc. lactis*, resulting in a codon usage table for *Lc. lactis* (see Supplemental Table S1, available online at http://dx.doi.org/10.3168/jds.2013- 7492). Based on the amino acid sequence of the hyperthermostable β-galactosidase enzyme from *Sulfolobus solfataricus* P2 (accession number NP_344331.1), a modified *lacSt* gene sequence containing an optimized ribosomal binding site for *Lc. lactis* (van de Guchte et al., 1991) was designed using the preferred codons for *Lc. lactis* (see Supplemental Figure S1,

available online at http://dx.doi.org/10.3168/jds.2013-7492). In addition, a 50-bp transcription terminator was designed and attached downstream of the *lacSt* gene (Figure 2. 1). The modified terminator was derived from the *ldh* gene terminator in *Lc. lactis* Il 1403 (Kim and Mills, 2007) by replacing the 13th and 30th nucleotides from T to C, thus decreasing the free energy from −11.34 to −19.19 kcal/mol, which were predicted and calculated by the UNAFold program (version 3.8; http://mfold. rna.albany.edu/?q=DINAMelt/software; Figure 2. 2).

Cloning of *lacSt* into a nisn-induced expression vector for *Lc. lactis*

The 1.5-kb fragment that contained the *lacSt* gene in pUC105 was amplified by PCR using *LacSt* F and *LacSt* R primers. It was digested with *Xba*I and *Sal*I, purified, and cloned into pDOC99 that was digested with the same restriction enzymes, replacing the existing *lacZ* gene from *Streptococcus thermophilus*. The ligation mixture was transformed into *E. coli* DH5α, selecting for erythromycin resistance (Ery^r). This resulted in the construction of a transcription fusion of the *nisA* promoter from *Lc. lactis* ATCC 11454 to the synthetic *lacSt* gene, termed pDOLY105 (Figure 2. 3).

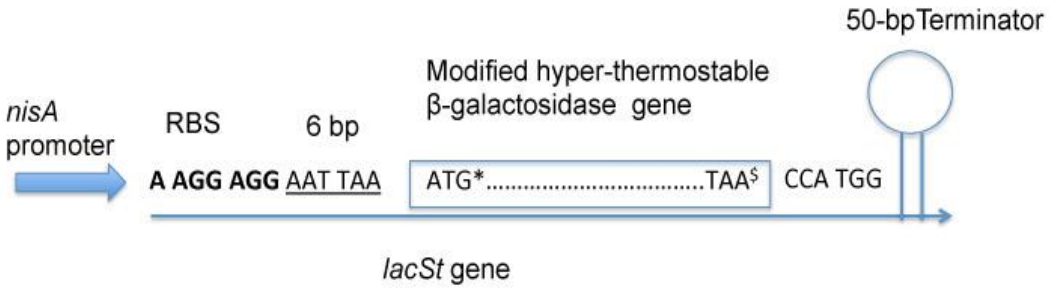


Figure 2. 1. Genetic organization of the designed *lacSt* gene. The arrow indicates the *nisA* promoter. * indicates start codon; $ indicates stop codon; RBS = ribosome binding site.

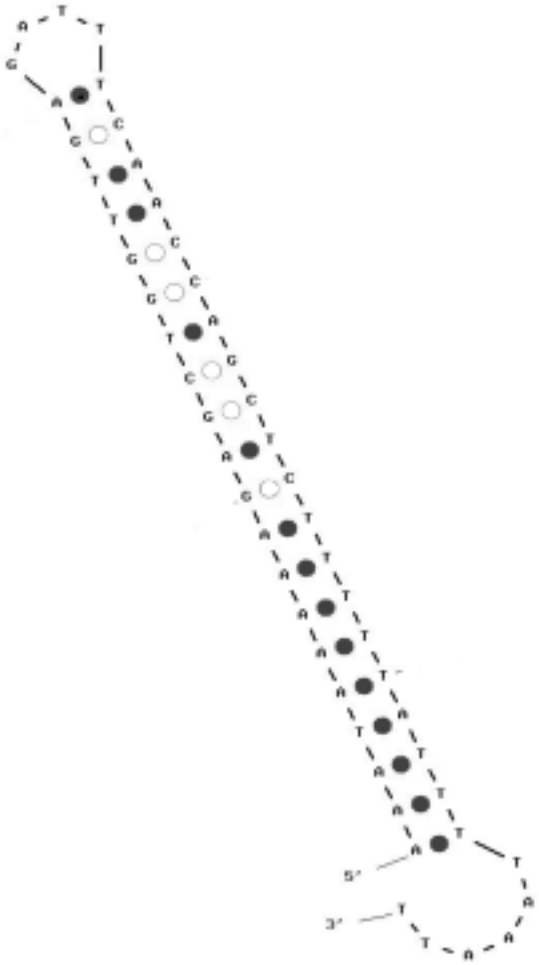


Figure 2. 2. Modified terminator structure derived from the *ldh* gene from *Lactococcus lactis* IL1403 with decreased free energy. Black and white circles indicate AT and GC bonds, respectively. The changed nucleotides at positions 13 and 30 bases are indicated by arrows.

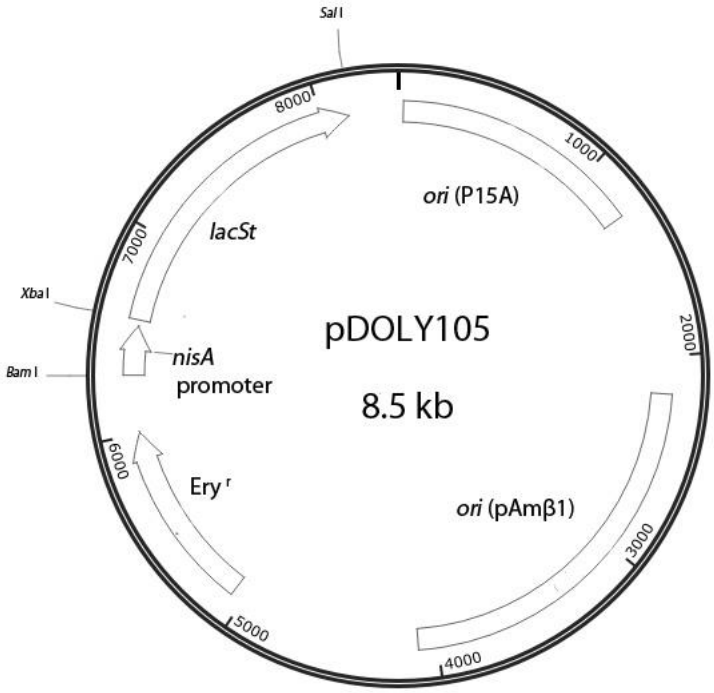


Figure 2. 3. Map of the *lacSt* expression plasmid pDOLY105. Ery^r = erythromicin resistance.

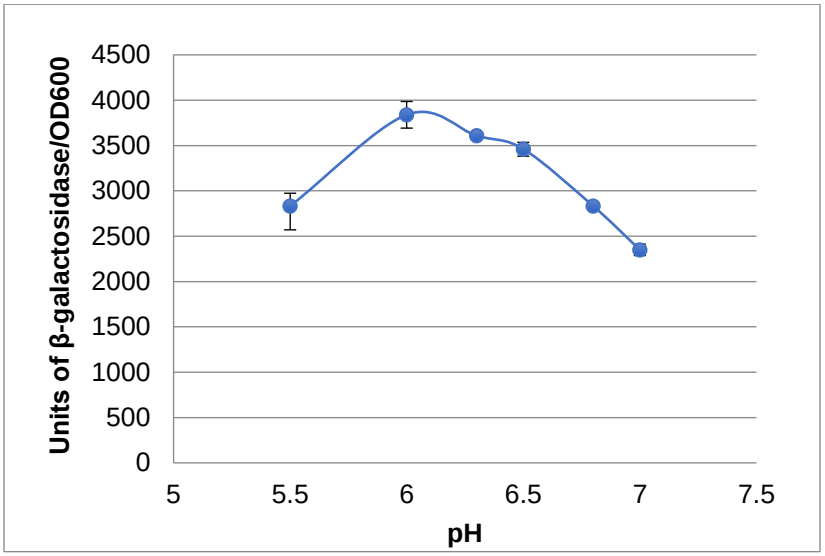
Effect of pH and temperature on β-galactosidase activity

The hyperthermophilic β-galactosidase expressed in *Lc. lactis* LM0230 was examined at different temperatures and pH (Figure 2. 4). The optimum pH for the β-galactosidase assay was found to be at 6 and the optimum temperature was in the range of 85 to 90°C. The hyperthermophilic β-galactosidase showed little activity at 60°C and activity was beginning to be impeded at 95°C.

Evaluation of the hyperthermophilic β-galactosidase enzyme in permeabilized whole cells for GOS formation

Galactooligosaccharide synthesis was initiated as described in the Methods section. The reaction mixtures containing various oligosaccharides were separated by ultra-performance liquid chromatography, giving different retention times (3.06 min for disaccharides, 3.80 min for trisaccharides, and 4.46 and 4.59 min for tetrasaccharides; Figure 2. 5) and their composition was further analyzed by MS (Figure 2. 6). The oligosaccharides contained disaccharides, trisaccharides, and tetrasaccharides with molecular weights of 341, 503, and 665, corresponding to the standard oligosaccharides.

(a)



(b)

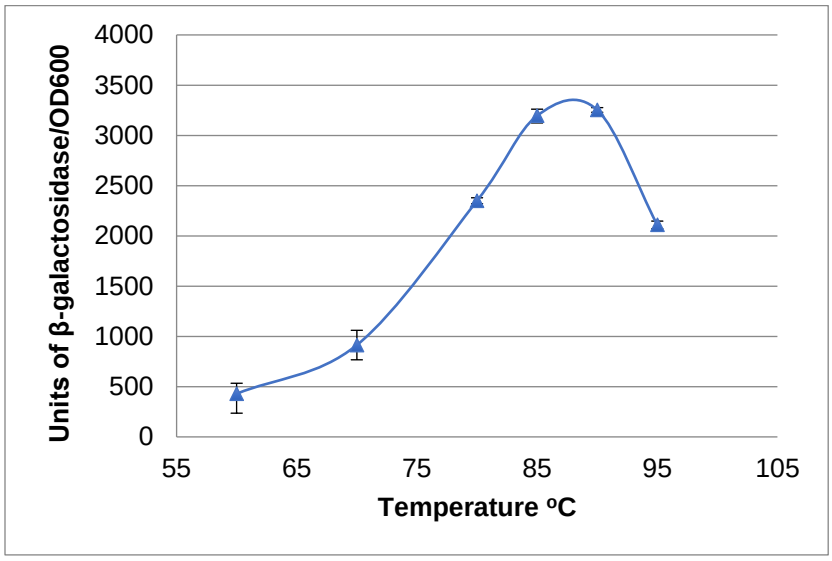


Figure 2. 4. Effect of temperature and pH on β-galactosidase activity when ortho-nitrophenyl-β-galactoside (ONPG) was used as substrate. The error bars indicate SD (n = 3). OD600 = optical density at a wavelength of 600 nm.

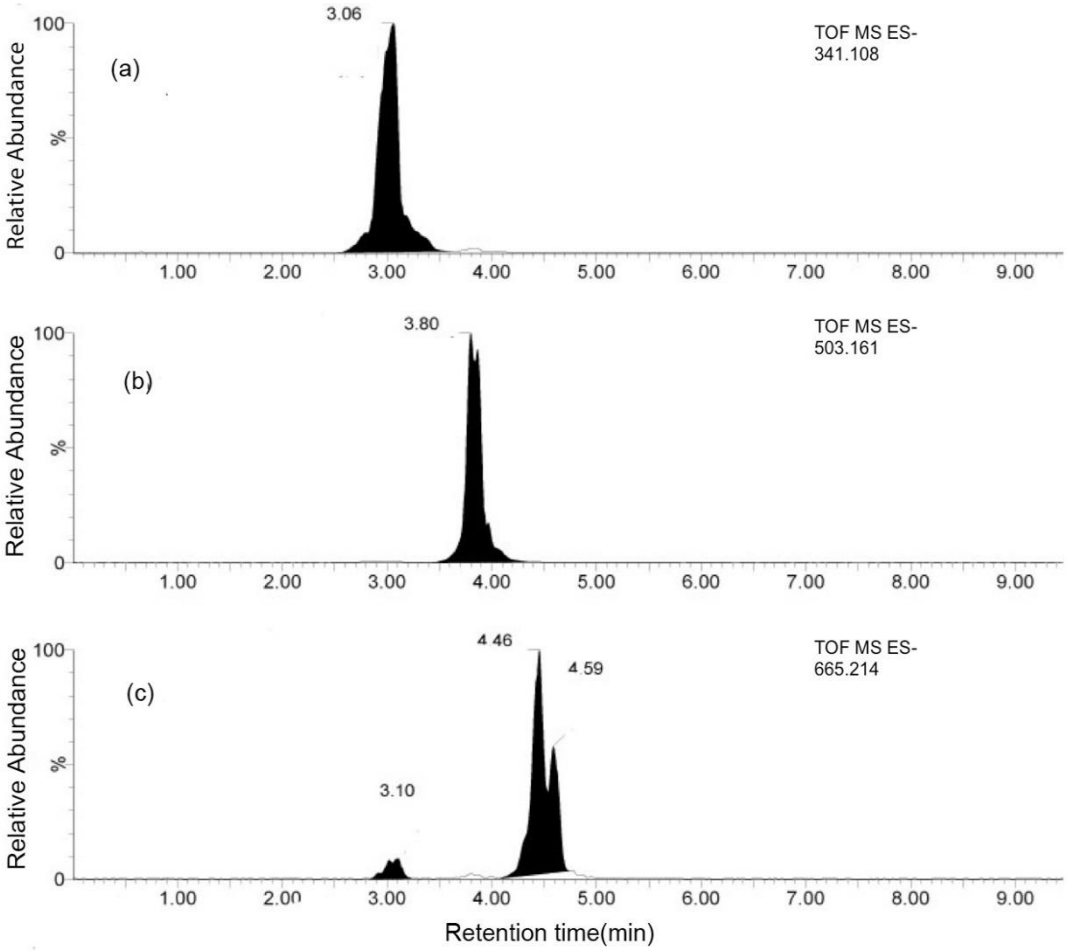


Figure 2. 5. Chromatogram of each galactooligosaccharide (GOS) composition. Different sugars were separated by ultra-performance liquid chromatography (UPLC) through a column over time: disaccharides (a), trisaccharides (b), and tetrasaccharides (c). TOF MS ES = time-offlight mass spectrometry electrospray.

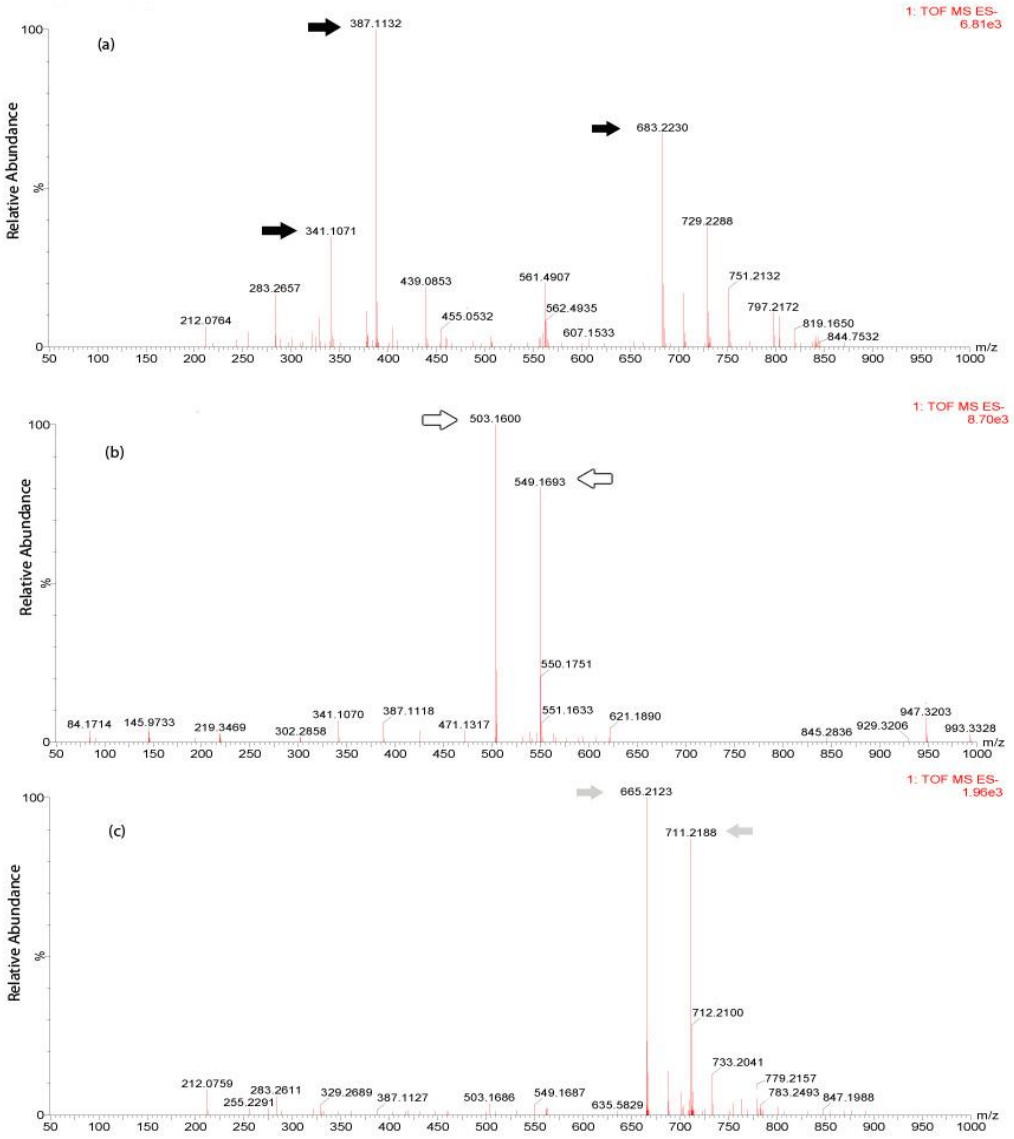
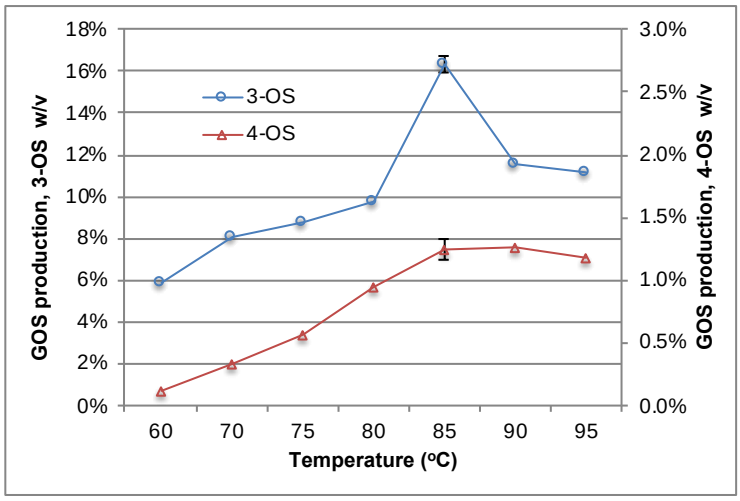


Figure 2. 6. Mass spectrometry profile of galactooligosaccharide (GOS) samples. Black arrows indicate disaccharides, disaccharide formate, and disaccharide dimer (a), white arrows indicate trisaccharides and trisaccharide formate (b), and gray arrows indicate tetrasaccharides and tetrasaccharide formate (c). TOF MS ES = time-of-flight mass spectrometry electrospray.

Effect of temperature and pH on GOS formation

The effect of temperature on GOS production was measured under a range from 60 to 95°C with 400 g of lactose/L at pH 6 for 25 h of incubation. The transgalactosylation activity was low at 60°C. Increased temperatures contributed to a higher transgalactosylation activity, which resulted in higher GOS production (Figure 2. 7a). The optimum temperature for GOS production was 85°C. Once the temperature exceeded 85°C, the yield of GOS decreased for both trisaccharides and tetrasaccharides. The effect of pH on GOS production was investigated in a range from 5 to 7.5 with 400 g of lactose/L as substrate at 85°C for 25 h (Figure 2. 7b). The optimum pH value for GOS production was 6, resulting in 174.1 g/L total tri- and tetrasaccharide GOS formation, which was composed of 94% trisaccharides and 6% tetrasaccharides.

(a)



(b)

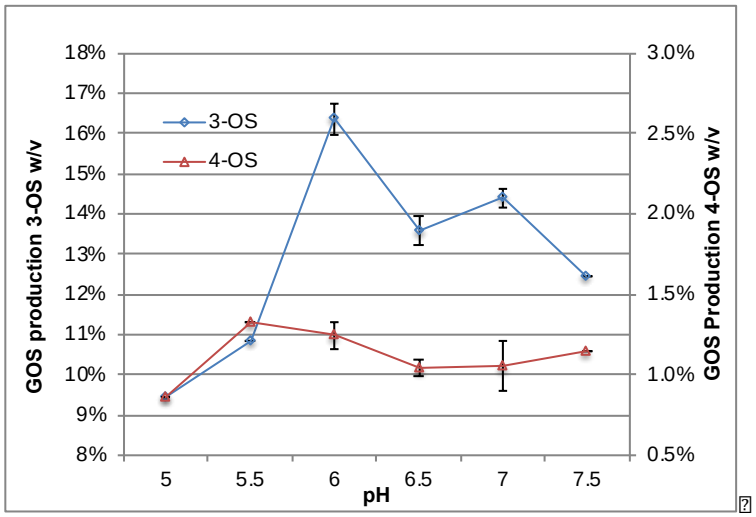


Figure 2. 7. Effect of temperature and pH on galactooligosaccharide. Activity was measured at different temperatures and pH 6 for 25 h with 400 g of lactose/L (a); activity was determined at 85°C with various pH for 25 h with 400 g of lactose/L (b). 3-OS = trioligosaccharides; 4-OS = tetraoligosaccharides. The error bars represent SD of duplicated samples.

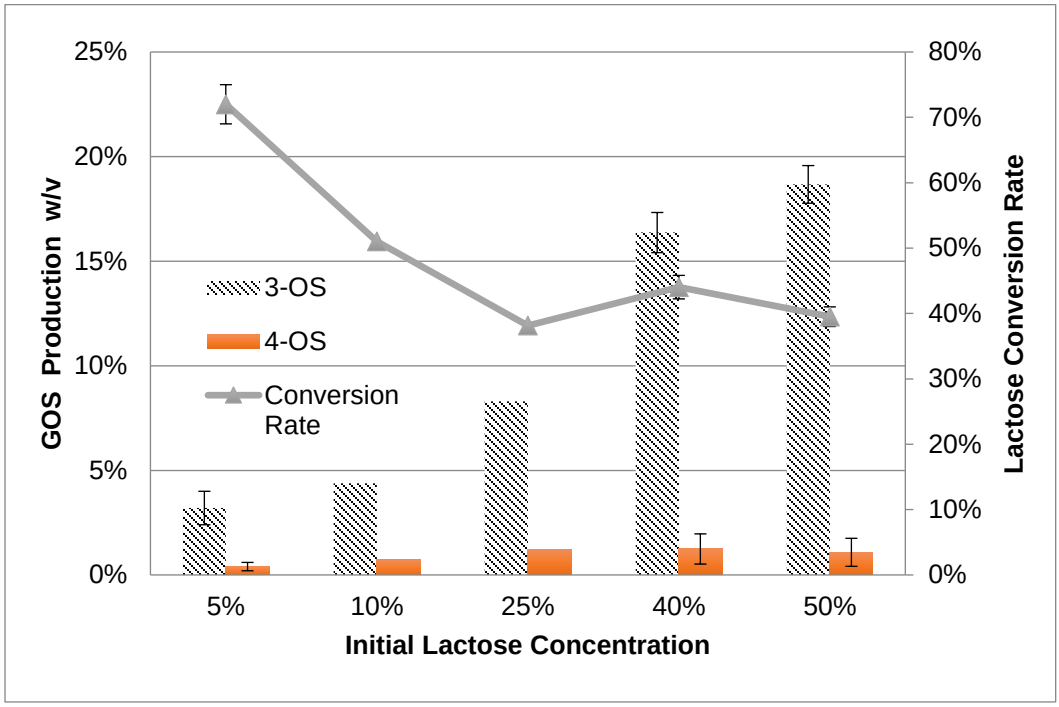


Figure 2. 8. Effect of initial lactose concentration on galactooligosaccharide (GOS) production. Activity was measured after incubation at pH 6 and 85°C for 25 h. 3-OS = trioligosaccharides; 4-OS = tetraoligosaccharides. The error bars indicate SD of duplicated samples.

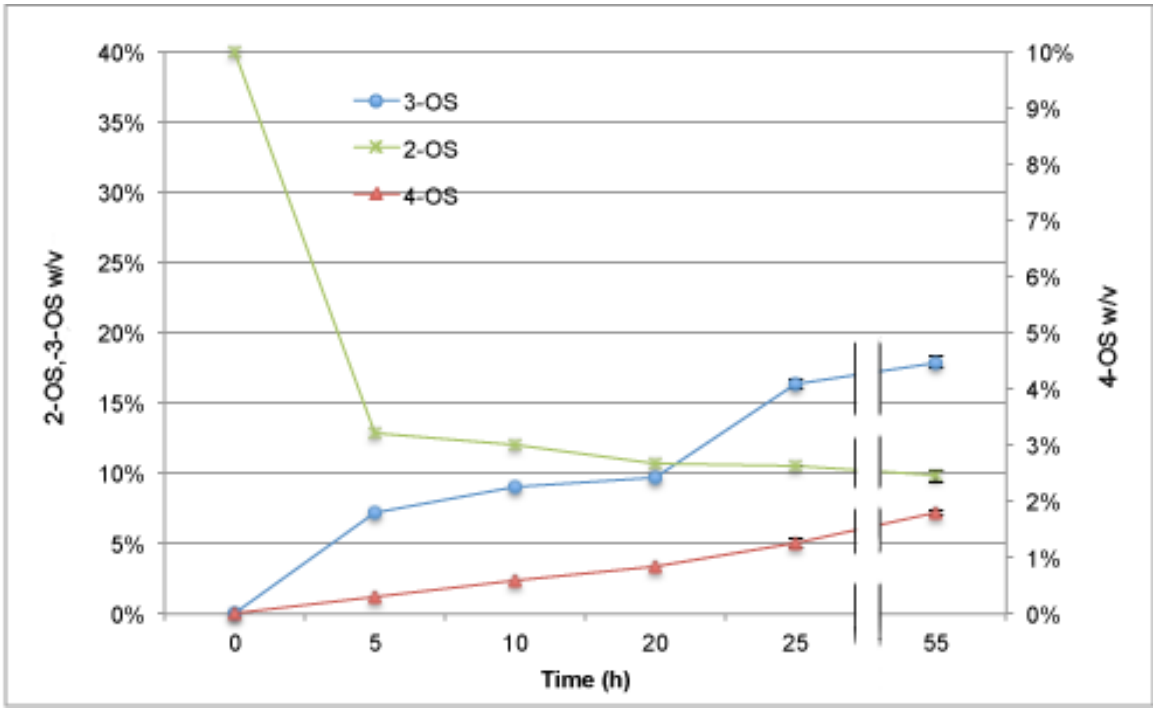


Figure 2. 9. Time courses of galactooligosaccharide (GOS) formation at 85°C and pH 6, with an initial lactose concentration of 40% (wt/ vol). 2-OS = dioligosaccharides; 3-OS = trioligosaccharides; 4-OS = tetraoligosaccharides. The error bars indicate SD of duplicated samples.

Effect of initial lactose concentration on GOS formation

The effect of initial lactose concentration was determined using 5 different initial lactose concentrations (Figure 2. 8). The production of GOS tri-saccharides increased linearly with increasing initial lactose concentrations, whereas GOS tetra-saccharide production maximized at 25% lactose, with no further increases with increasing initial lactose concentrations. When the initial concentration of lactose was 100 g/L, 50 g/L of total tri- and tetra-GOS could be detected. When the initial lactose concentration was increased to 500 g/L, the total of tri- and tetra-GOS produced increased to 197.5 g/L. The highest lactose conversion rate (72%) was obtained from 5% initial lactose and tended to decrease with increasing initial lactose concentrations up to 25% and somewhat stabilized after that.

Time course of GOS formation

The optimum incubation time for GOS production at pH 6 and 85°C with the 400 g/L initial lactose concentration was evaluated at various time points up to 55 h (Figure 2. 9). Disaccharides quickly decreased during the first 5 h and very little after that, suggesting that production of disaccharide GOS may have occurred by combining 1 glucose and 1 galactose unit. Both tri- and tetra-GOS production increased over time, with GOS trisaccharide production slowing after

25 h. A total amount of 197 g/L tri- and tetra-GOS was produced after 55 h of incubation, with trisaccharides as the major product.

Discussion

β-Galactosidase enzymes from different organisms have varying properties, such as optimum pH, temperature, spectrum of GOS products, and the total GOS yield. For example, the optimum temperature for GOS production using a β-galactosidase from *E. coli* was 37°C (Lee et al., 2011), whereas GOS production by a β-galactosidase from *Thermotoga maritima* maximized at 90°C (Ji et al., 2005). The majority of β-galactosidase enzymes function optimally only at pH 6 to 7.5, including those from *Bifidobacterium bifidum* (Tzortzis et al., 2005), *Kluyveromyces lactis* (Martínez-Villaluenga et al., 2008), and *Lactobacillus reuteri* (Splechtna et al., 2006). However, a β-galactosidase enzyme from *A. oryzae* continued to produce GOS optimally even when the pH was as low as 4.5 (Albayrak and Yang, 2002). Seven different types of di- and trisaccharide GOS products were detected using a β-galactosidase from *Lactobacillus plantarum* (Iqbal et al., 2010), whereas more than 20 different types of di-, tri-, tetra-, and pentasaccharides were identified in GOS samples catalyzed by β-galactosidases from *A. oryzae* and *Strep. thermophilus*. (Kimura et al., 1995). The β-galactosidase enzyme from *Bifidobacterium longum* BCRC 15708 produced 13.0 g of GOS products/L from 40% initial lactose (Hsu et al., 2007). In comparison, GOS production was much higher with the thermostable β-

galactosidase from *S. solfataricus*, in which 315 g of GOS/L was obtained from 60% initial lactose (Park et al., 2008).

In this study, a synthetic biology approach was used to express a modified gene encoding the hyperthermostable β-galactosidase enzyme (*lacSt*) from *S. solfataricus* that was codon optimized for *Lc. lactis*. The nisin-induced expression system used enabled the production of *Lc. lactis* cells containing high levels of this enzyme and the subsequent permeabilization of these cells enabled them to be used directly. The optimum pH and temperature for GOS production and ONPG hydrolysis catalyzed by these whole cells was consistent with the purified hyperthermostable β-galactosidase enzyme from *S. solfataricus* (Park et al., 2008; Wu et al., 2013). In addition, the final lactose conversion rate into tri- and tetrasaccharides (49.25%) was similar and much higher than that achieved using β-galactosidase enzymes from nonthermophiles (Torres et al., 2010). Given that GOS products can also consist of non-lactose disaccharides, which could not be differentiated from lactose by the MS approach used in the current study, the actual yield of GOS obtained here is likely much higher. Recently, it was shown that up to 40% of total GOS products obtained using a β-galactosidase enzyme from *A. oryzae* consisted of disaccharides (Urrutia et al., 2013). Therefore, the disaccharide peak shown in Figure 5a likely contains non-lactose disaccharides with different glycosidic linkages, such as allolactose [β-dgalactopyranosyl (1→6)-d-glucose]. The large trisaccharide peak area in Figure 5b likely also contains more than 1 individual trisaccharide component, such as β-d-Gal-

(1→3)-β-d-Gal-(1→4)-d-Glu and β-d-Gal-(1→6)-β-d-Gal-(1→4)-d-Glu. The double shoulder in this peak area substantiates this. These 2 trisaccharides were also found to be the dominant GOS products produced by the purified β-galactosidase from *S. solfataricus*, with a specificity for the formation of β-(1→6), β-(1→3) glycosidic bonds (Petzelbauer et al., 2000). The double shoulder in the tetrasaccharide peak area (Figure 2. 5c) likely also results from these different linkages.

The small peak at 3.1 min retention time (Figure 2. 5c) most likely consists of disaccharide dimers, as it has the same retention time as disaccharides. The molecular weight of a disaccharide monomer is 341 Da and the mass of a disaccharide dimer is consistent with its predicted molecular weight (683 Da). Disaccharide dimers have previously been reported for lactose (Kapková, 2009). Galactooligosaccharide was produced by transgalactosylation during the hydrolytic activity of lactose. In the current study, the optimum temperature and pH for hydrolysis was almost the same as those for transgalactosylation activity. The final GOS concentration increased dramatically with a high concentration of lactose substrate (Figure 2. 8). This is consistent with previous observations (Nakkharat and Haltrich, 2006; Park et al., 2008). However, the GOS conversion rate from lactose generally decreased with increasing lactose concentrations. Interestingly, the highest lactose conversion rate [72% (wt/wt) of tri- and tetrasaccharides] was found in a low lactose concentration (5%). This provides

opportunities for producing highly GOS-enriched foods from low lactose-containing products, such as whey and its derivatives.

Conclusions

In this study, we used whole-cell technology to generate a high yield of GOS products. The permeabilized cells containing hyperthermostable β-galactosidase were easier to prepare and less expensive than the purified enzyme. In addition, the refrigerated samples retained full enzyme activity for at least 6 mo. The use of whole cells to protect enzymes from environmental stresses has previously been noted (de Carvalho, 2011). Using this whole-cell approach, the cells can be further immobilized in porous beads, such as alginate. With these advantages, a convenient and cost-effective method can be developed for producing GOS-enriched food products.

Acknowledgements

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Chapter 3. Immobilization of Whole Cells of *Lactococcus lactis* Containing High Levels of a Hyperthermostable β-Galactosidase Enzyme in Chitosan Beads for Efficient Galactooligosaccharide Production

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Galacto-Oligosaccharides (GOS) are prebiotic food ingredients that are proposed to stimulate the growth of beneficial gut microorganisms, particularly the bifidobacteria. Previously, we developed a method for efficient GOS production using whole cells of *Lactococcus lactis* containing high levels of a hyper-thermostable β-galactosidase enzyme from *Sulfolobus solfataricus*. In this study, a recombinant DNA removal and whole cell enzyme immobilization process was developed to produce GOS from lactose before removal of the immobilized whole cell enzyme, which could be reused for subsequent applications. Chitosan was found to be a superior immobilization material than alginate, as it retained its bead structure during the high temperature (90°C) used here for GOS production. Prior to immobilization, the recombinant DNA was degraded in the whole cells using UV treatment, resulting in an immobilized whole cell enzyme that was free of recombinant DNA and with minimum effect on the efficiency of the enzyme. The optimum pH and temperature for GOS synthesis using the chitosan beads was pH = 5.5 and 90°C. The highest GOS production using the chitosan beads occurred with 40% initial lactose resulting in 150 g/L of GOS (3-OS and 4-OS) in addition to 2-OS GOS products that were not quantified. Notably, the highest lactose conversion rate was found using lower starting lactose concentrations, with more than 60% conversion into 3-OS and 4-OS. The immobilized enzyme retained ~ 50% activity after 2 cycles of GOS production. In conclusion, the chitosan immobilized whole cells enzyme can be used for efficient GOS production that is free of the whole cell enzyme as well as and detectable recombinant DNA.

Introduction

Prebiotics are generally short chain dietary fibers, which can be fermented by some gut bacteria and have the potential to specifically modulate the gut microflora depending on the type of prebiotic. Previous studies have demonstrated that the dietary intake of Galacto-oligosaccharides (GOS) specifically promoted the growth of bifidobacteria and can therefore be referred to as 'bifidogenic' food ingredients (Depeint et al., 2008; Davis et al., 2010; Veereman-Wauters et al., 2011). It was also shown to reduce infection from *Salmonella enterica* serotype *Typhimurium* in a mouse model (Tzortzis et al., 2005; Searle et al., 2009). Consequently, GOS is becoming a very desirable prebiotic food ingredient.

GOS can be produced from lactose using different β-galactosidase enzymes by transgalactosylation activity (Torres et al., 2010). These enzymes have been obtained from different organisms, such as *Aspergillus oryzae*, *Bifidobacterium longum*, *Lactobacillus reuteri*, *Bacillus circulans*, *Kluyveromyces lactis*, and *Thermotoga maritime*. (Tanaka and Horiuchi, 1975; Kim et al., 2004; Chockchaisawasdee et al., 2005; Nguyen et al., 2006; Hsu et al., 2007; Li et al., 2010; Yin et al., 2017). The efficiency of GOS production using these enzymes varies depending on the source. Generally, GOS synthesis is more efficient at higher temperatures in part due to the better solubility of lactose. Therefore, β-galactosidase enzymes from thermophiles are good candidates for efficient GOS production. A yield of 52.5% (w/w) GOS was obtained from an initial lactose

concentration of 600 g/L using a β-galactosidase enzyme from the extreme thermophile, *Sulfolobus solfataricus* (Park et al., 2008), which was the highest concentration of GOS yield reported at that time.

To facilitate efficient GOS production, enzyme immobilization by entrapment, covalent binding or adsorption, have been used. Various materials have been use, such as cellulose, chitosan, alginate and cotton cloth. (Albayrak and Yang, 2002; Sakai et al., 2008; Lu et al., 2012; Chen and Duan, 2015). Most immobilized β-galactosidase enzymes function at a range of temperatures between 30-50^{o}C. However, hyperthermostable enzymes with optimal temperatures greater than 70^{o}C would require materials for immobilization with a high thermostability.

The first reported immobilized thermophilic enzyme was a protease from *Thermus aquaticus*, which was immobilized using three different materials, Sepharose 4B, CM-cellulose, and controlled pore glass (CPG), with Sepharose exhibiting the greatest thermostability (Cowan and Daniel, 1982). However, this enzyme immobilization process requires activation of Sephorose with cyanogen bromide and is therefore not suitable for food applications (Wilchek and Miron, 2002). Other immobilization techniques have now been developed for thermophilic enzymes, such as agarose and chitosan (Filho et al., 2008). Both materials demonstrated efficient activity at high temperatures, but glutaraldehyde was used as a cross-linker in both cases, to physically connect the

immobilization materials to the enzyme itself. However, glutaraldehyde is a biocide and exposure to it has been linked to respiratory and skin irritation (Nayebzadeh, 2007).

Previously, we developed an efficient GOS production system using the hyperthermostable β-galactosidase enzyme from *S. solfataricus* overexpressed in *Lactococcus lactis* using a synthetic biology approach. Rather than using purified enzyme, whole, permeabilized cells of *L. lactis* containing this enzyme were used for GOS production at 85°C (Yu and O'Sullivan, 2014). While efficient and cost effective, removal of the whole cells from the GOS preparation following synthesis was cumbersome, due to the need for centrifugation and filtration. The objective of this study was to remove the recombinant DNA from the enzyme containing whole cells, and develop a suitable thermostable immobilization system to facilitate removal of the whole cells following GOS synthesis.

Methods

Bacteria, media and culture conditions

Lactococcus lactis LM0230 (pDOLY105, pDOC23) (Chandrapati and O'Sullivan, 1999; Yu and O'Sullivan, 2014) was inoculated in M17 (Becton, San Jose, CA) broth media with 0.5% glucose and grown at 30^{o}C without shaking. Both erythromycin and chloramphenicol were used at a level of 3 µg/ml each for *L. lactis*.

Whole cell enzyme preparation

L. lactis LM0230 (pDOLY105, pDOC23) was grown until an $OD_{600\ nm}$ of 0.7 before nisin (Sigma-Aldrich, St. Louis, MO) was added at a concentration of 1.0 IU/mL to induce enzyme production for 2.5 hours. The induced cell pellet was then washed with the same volume of ice-cold water and resuspended with a half volume of POM buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, and 1 mM $MgCl_2$, pH 7.4). Another half volume of 100% ethanol was subsequently added to kill and permeabilize the cells. The whole cells were further incubated at room temperature for 25 min and centrifuged (10,000 × *g* for 15 min at 4°C). The cells were then washed using 1 volume of POM buffer prior to resuspension in one third volume of molecular biology grade water.

Removal and detection of recombinant DNA

An aliquot of 5 mL of permeabilized cells were placed in an uncovered petri dish and exposed to UV irradiation for 30, 60 and 90 seconds in a UV Stratalinker 2400 (LabX, Midland, Canada). Subsequently, the UV treated cells were pelleted by centrifugation and the pellets were resuspended in 500 µl molecular biology grade water, before disruption with 250 µl of glass beads (< 600 µm) (Sigma-Aldrich, St. Louis, MO) using a bead-beater (BioSpec, Bartlesville, OK) for 1 min at maximum speed. An aliquot of 1 µl was taken from the mixture for PCR detection to determine the efficiency of UV treatment.

The *lacSt* gene for hyperthermostable β-galactosidase was detected by PCR using Taq DNA polymerase (New England Biolabs, Ipswich, MA). Two oligonucleotides, *LacSt* F 5'-GCGAATGCATCTAGATCCCC-3', *LacSt2* R 5'-CAGCTATGACCATGATTACGCC-3', were designed and synthesized for use as primers. The PCR reaction was performed using a PTC-200 thermocycle (MJ Research, Ramsey, MN) with cycling conditions consisting of an initial denaturation at 95^{o}C for 5 min, before 35 cycles of denaturation at 95^{o}C for 20 s, annealing at 50^{o}C for 30 s and extension at 68^{o}C for 90 s, and one final extension at 68^{o}C for 5 min. The PCR reaction consisted of a buffer (10 mM Tris-HCl, 50 mM KCl and 1.5 mM $MgCl_2$, pH 8.3), 200 µM dNTPs, 0.2 µM of each primer, 1µl template, 0.75 units Taq DNA polymerase and molecular grade water to bring the total volume to 25 µl.

Immobilization of whole cells

Whole cells (30 mL) were resuspeneded in 10 ml of 2% low molecular weight chitosan (molecular weight 50 -190 kDa; 75 – 85% deacethylated) (Sigma-Aldrich, St. Louis, MO) in 1% acetic acid. The mixture was then added drop wisely into 1 L of 1.5% sodium triphosphate (Na-TPP) (Thermo Fisher Scientific, Cleveland, OH) using a micropipette p1000 with continuous stirring. The suspension was magnetically stirred for 1 hour at room temperature for bead formation. The chitosan beads were washed with distilled water before use.

Alginate beads were prepared by mixing 100 mL of 2% (w/v) alginate (Sigma-Aldrich, St. Louis, MO) with 30 mL of whole cells. This mixture was added drop wisely through a micropipette (p1000) into 100 mL of 2 % (w/v) $CaCl_2$ solution with continuous stirring. The suspension was magnetically stirred for 1 hour at room temperature. The alginate beads were washed with distilled water before use.

β-Galactosidase measurements

β-Galactosidase activity was measured by hydrolysis of ortho-nitrophenyl-β-galactoside (ONPG). ONPG is composed of galactose and ortho-nitrophenol, and the latter compound turns a yellow color when cleaved from galactose and quantitatively reflects enzyme activity. Both immobilized cells and free whole cell enzymes were assayed at 85^{o}C, in 1 mL of 0.05 M sodium acetate buffer (pH 6). An aliquot of 200 µl of ONPG 4 mg/mL was added to the reaction mixture. The reaction was stopped by adding 500 µl of a 1 M Na_2CO_3 solution at room temperature when sufficient yellow color was developed and the number of minutes for the reaction was recorded. The color was measured spectrophotometrically at 420 nm by the released ortho-Nitrophenol. Units of enzyme activity was calculated using the following equation: [Units = 1,000 × ($OD_{420\ nm}$ − 1.75 × $OD_{550\ nm}$)/(volume of cells used × time of reaction × $OD_{600\ nm}$ of cells used), as proposed by Miller (Miller, 1972).

GOS synthesis and detection

An aliquot of 5 beads containing whole cells of thermostable β-galactosidase were added to a reaction mixture, in which 5% lactose was dissolved in 0.05 M sodium acetate buffer (pH 6). The reaction mixture was incubated at 85^{o}C for 25 hours. The reaction was terminated by incubating the reaction mixture at 4^{o}C. The sample was filtered through a 0.22 μm membrane and diluted 4,000-fold using sterile ammonium acetate buffer (pH 8.4) for further analysis. An aliquot of 10 μl of sample was used for UPLC-MS analysis. The conditions for the UPLC-MS were as previously described (Yu and O'Sullivan, 2014). The transgalactosylation activity was determined by the production of trisaccharides (3-OS) and tetrasaccharides (4-OS). The lactose conversion rate is (tri-oligosaccharides +tetra-oligosaccharides) concentration/initial lactose concentration.

Thermostability of the encapsulated whole cell enzyme

The themostability for both free whole cell and immobilized whole cell enzymes was analyzed by incubating 1 mL samples in eppendorf tubes at 100^{o}C. Samples were withdrawn for analysis every 3 minutes and β-galactosidase activity was quantified at 85^{o}C, pH 6.

Reusability of chitosan encapsulated beads

The immobilized β-galactosidase beads were used for successive batches of GOS production. After one cycle, the reaction media was discarded and

separated from the immobilized enzyme. The immobilized enzyme was then incubated again with fresh media to start another cycle for GOS production. Each cycle lasted for 24 hours. Both hydrolysis and transgalactosylation activities were measured to determine the reusability.

Scanning electron microscopy (SEM)

The topology of the chitosan beads was evaluated by scanning electron microscopy. Before using SEM, chitosan beads were dried using different concentrations of ethanol for moisture removal. Beads were immersed in an increasing ethanol/water mixtures ranging from 30 to 100% ethanol. The beads were left for 10 minutes in each solution and then dried at 30^{o}C for 5 hours. Dried beads were coated with electronic conductive materials, consisting of 60% gold and 40% palladium, prior to observation. Micrographs were taken using a 4700 Scanning Electron Microscope (Hitachi, Krefeld, Germany).

Results

Immobilization of whole cells of *Lactococcus lactis* containing a hyperthermostable β-Galactosidase

In a previous study, we developed a cell-based technology to overproduce GOS using a hyperthermostable β-galactosidase enzyme (Yu and O'Sullivan, 2014). To facilitate easy separation from the reaction mixture, the whole cell enzyme needed to be encapsulated in a thermostable and food-grade material. Both alginate and chitosan have been used for food applications as coating

materials, dietary fiber and food stabilizers (Gennadios et al., 1997; Shahidi et al., 1999; Brownlee et al., 2005; Rinaudo, 2006). In addition, previous studies have successfully immobilized thermostable enzymes, such as α-amylase and β-glycosidase, for applications up to 95^{o}C, using these two materials (D'Auria et al., 1996; Tee and Kaletunç, 2009). Therefore, both alginate and chitosan were evaluated to immobilize the hyperthermostable β-galactosidase enzyme using $CaCl_2$ and tripolyphosphate as crosslinkers, respectively. The entrapped whole cell enzyme beads were circular in shape with a diameter of ~ 2.5 mm radius.

Recombinant DNA removal from the whole cell enzyme

Plasmid pDOLY105 with the recombinant *lacSt* gene was previously transformed into *L. lactis* for hyperthermostable β-galactosidase enzyme production. Given only the enzyme is needed for GOS production, UV exposure was used to eliminate the recombinant DNA below the level of detection by PCR. The synthetic *lacSt* gene was not detected after 30s, 60s and 90s UV exposure (Fig.3. 1). Therefore, 30 s UV treatment was found to be adequate to degrade the recombinant DNA below the level of detection by PCR. It was also found that the treated enzyme retained more than 97% of β–galactosidase activity. This indicated that UV treatment had minimum effect on the β-galactosidase enzyme activity and the UV treated whole cells were suitable for immobilization.

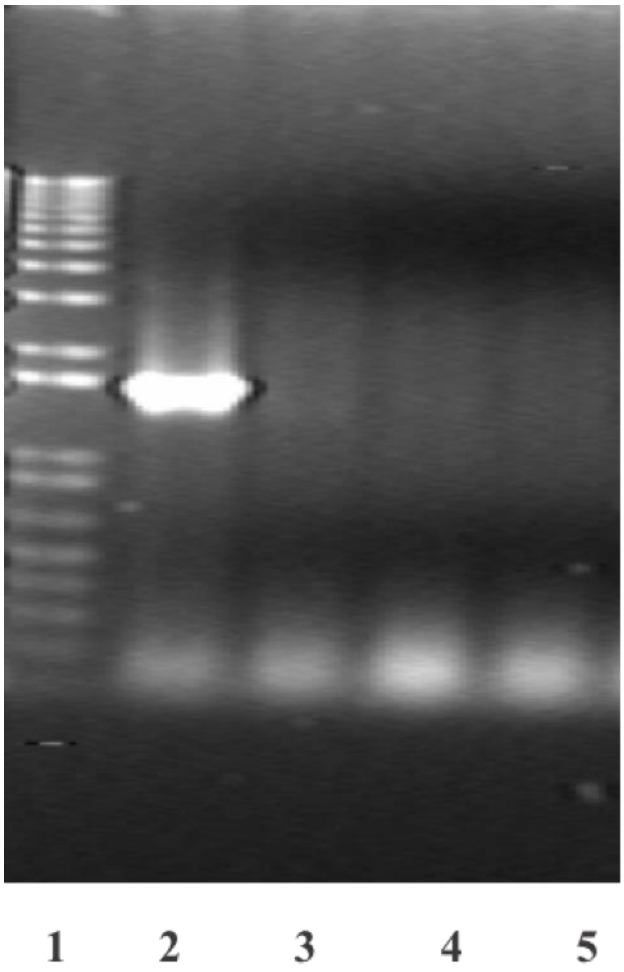

Figure 3. 1. Effect of UV on genetically modified genes. Lanes: 1, 1-kb ladder; 2, PCR products using the whole cells without UV treatment as a template; 3, PCR products using the whole cells with 30-s UV treatment as a template; 4, PCR products using the whole cells with 60-s UV treatment as a template; 5, PCR products using the whole cells with 90-s UV treatment as a template.

Effect of temperature and pH on β-Galactosidase hydrolysis and transgalactosylation using both free and immobilized whole cell enzymes

The effect of temperature on β-galactosidase hydrolysis was determined by assaying both free whole cells and immobilized whole cells (in alginate and chitosan) in sodium acetate buffer (pH 6) at temperatures ranging from 70 - 95^{o}C, while the optimum pH (5-7) was evaluated in the same sodium acetate buffer at 85^{o}C. Entrapped enzymes (both alginate and chitosan beads) exhibited optimum hydrolysis activities at similar conditions (pH 6 and 90^{o}C) to the free whole cell enzyme (Fig 3. 2). There was no significant difference over the temperature range between free whole cells and entrapped cells, but at the lowest pH tested (5.0), the relative hydrolysis activity was lower for the free whole cells.

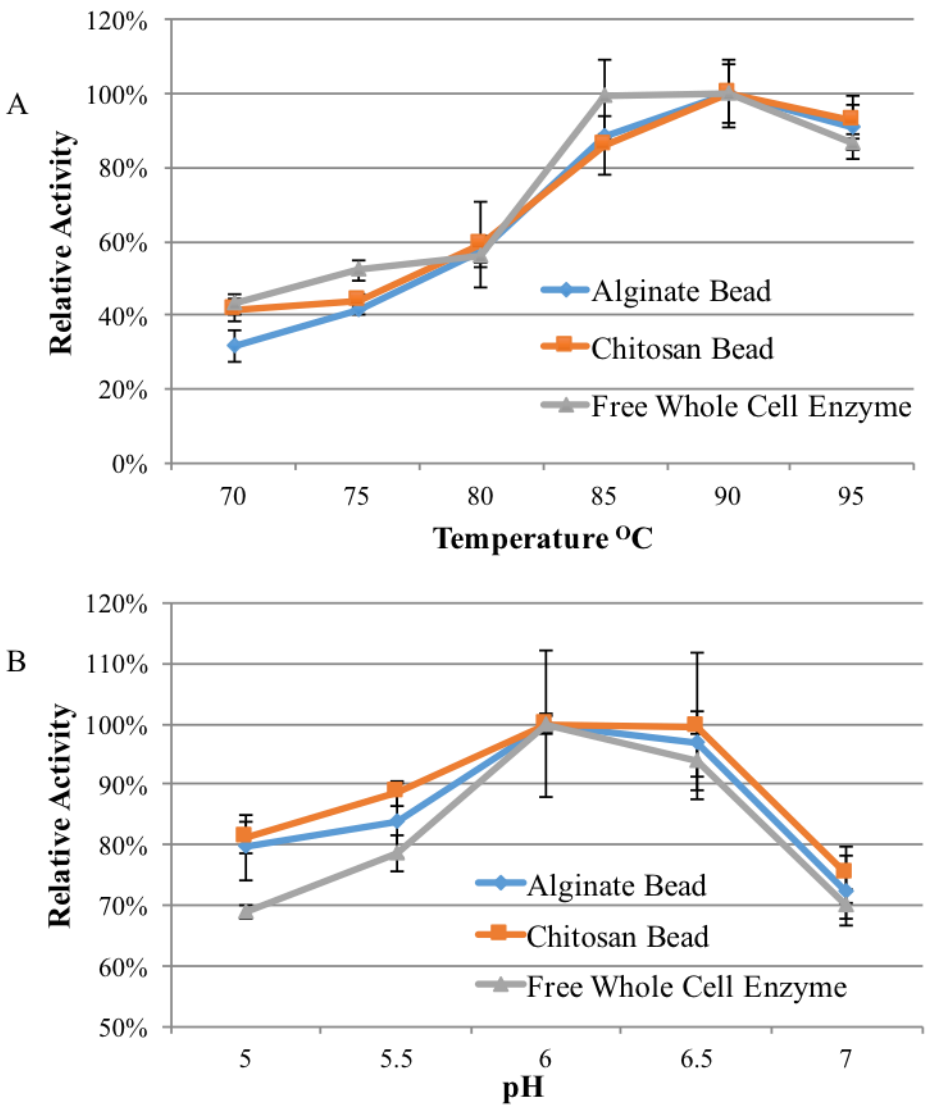

Figure 3. 2. Effect of temperature and pH on β-galactosidase activity using both free whole cell enzyme and immobilized enzyme. The error bars indicate SD (N=3). Relative activities are the ratio of β-galactosidase activity under different conditions to the highest β-galactosidase activities for free whole cell enzyme and chitosan immobilized enzyme, respectively.

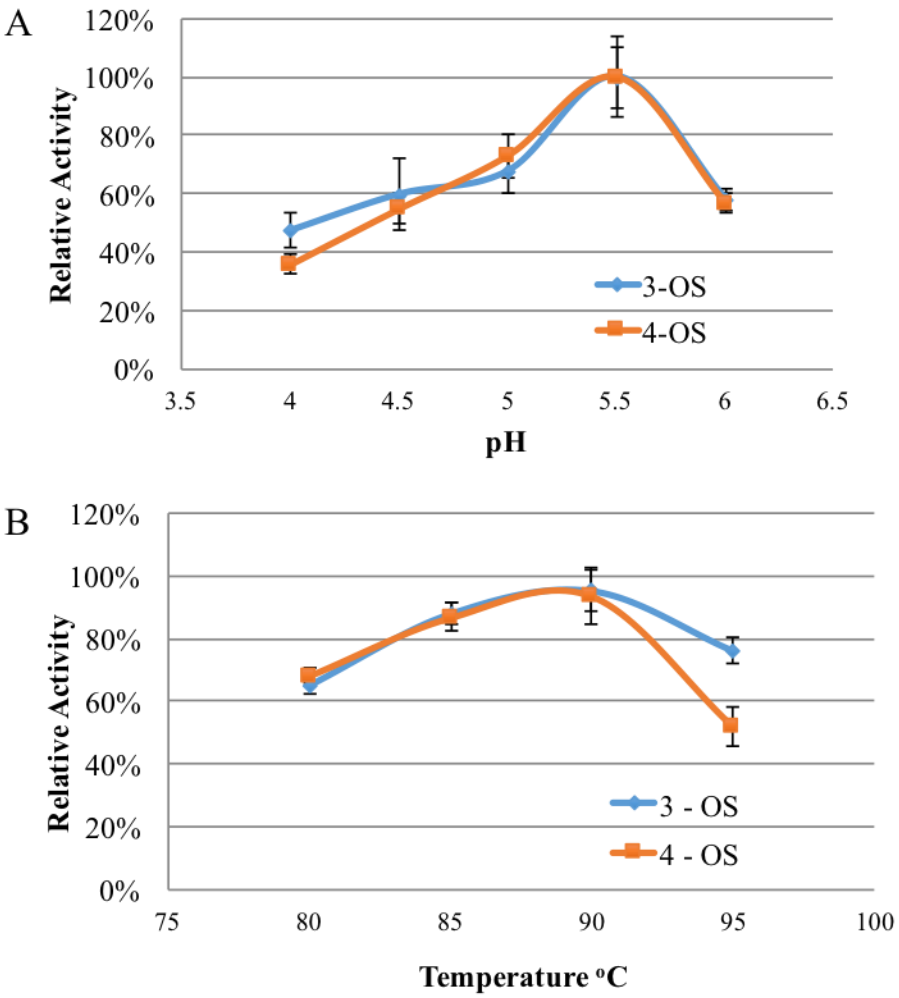

Figure 3. 3. Effect of pH and temperature on GOS synthesis using immobilized enzyme. The effect of pH on GOS production was investigated in a range from 4-6 with 5% lactose as substrate at 85ºC for 25 h. The effect of temperature on GOS production was investigated in a range from 80-95oC (80, 85, 90, 95) with 5% lactose as substrate at pH 6 for 25 h. 3-OS = trioligosaccharides; 4-OS = tetraoligosaccharides. The error bars indicated SD(n=2).

As the alginate beads swelled dramatically during prolonged exposure to these high temperatures, only the chitosan beads were evaluated for GOS production (transgalactosylation activity) at 80 - 95°C and various pH values (4-6) using a 5% lactose solution over 25 hours. As Fig. 3. 3 illustrates, the optimum pH for GOS synthesis using the immobilized enzymes was 5.5 and the transgalactosylation activity dropped significantly at pH 6. In addition, the optimum temperature for GOS synthesis using the immobilized enzymes was 90^{o}C, with a noticeable drop off at 95°C.

Effect of Initial lactose concentration on tri and tetra GOS production

GOS production was evaluated using lactose concentration from 5 to 50% at 90°C and pH 6 for 25 h. The total GOS production increased with increasing lactose concentrations up to a 40% initial concentration, resulting in ~ 150 g/L of GOS, not including disaccharide GOS moieties which were not distinguishable from lactose by the MS methodology used in this study (Fig 3. 4). However, the lactose conversion rate into GOS was highest for the lowest starting lactose of 5%, which resulted in almost 60% conversion into tri and tetra GOS. Specifically, 3 g/100 mL of total tri- and tetra-GOS was produced from 5 g/100 mL of initial lactose.

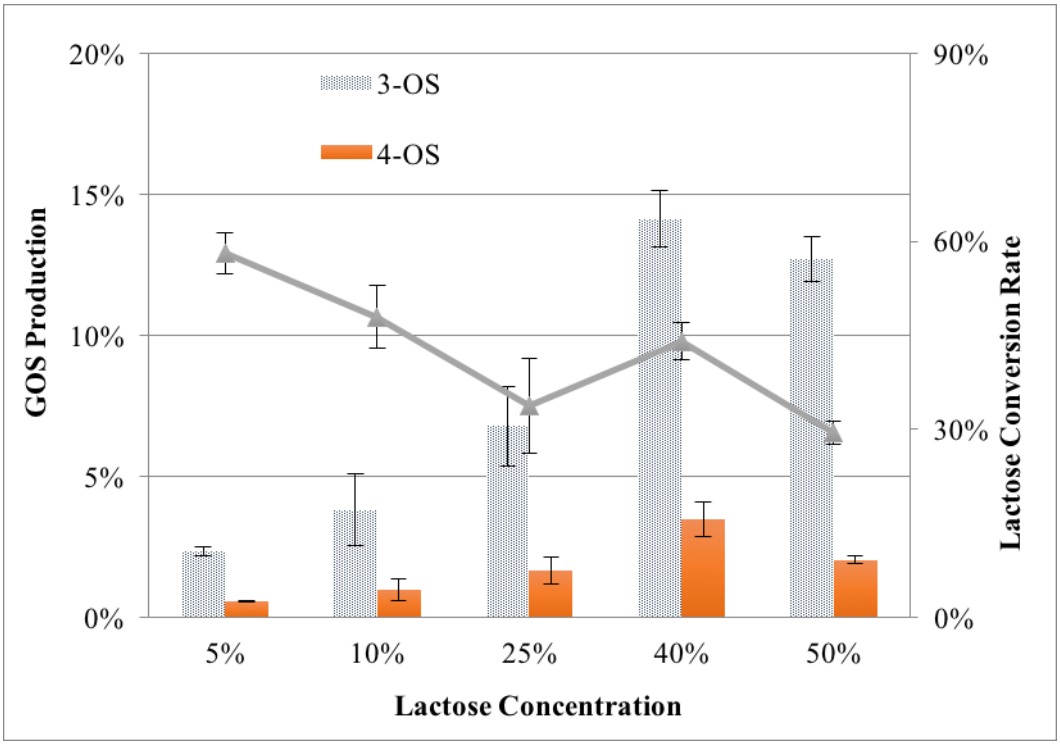

Figure 3. 4. Effect of initial lactose concentration using immobilized enzyme. The effect of initial lactose on GOS production was investigated in a range 5%, 10%, 25%, 40%, 50% lactose as substrate at 90°C for 25 h. 3-OS = trioligosaccharides; 4 -OS = tetraoligosaccharides. The error bars represent SD (n=2).

Thermostability and reusability of the chitosan immobilized whole cell hyperthermostable β-Galactosidase enzyme

The thermostability of the immobilized whole cell enzyme was determined by investigating the effect of incubation at 100^{o}C on its subsequent hydrolysis activity. The immobilized enzyme showed a higher thermostability at 100^{o}C than the free whole cell enzyme (Fig. 3. 5). After 18 mins incubation at 100°C, the immobilized whole cell enzyme still retained ~ 50% activity, while the free whole cell enzyme lost more than 90% of its β-galactosidase activity.

The reusability of the immobilized whole cell enzyme was examined using both a β-galactosidase assay for hydrolysis activity and GOS synthesis for transgalactosylation activity. Both methods showed similar profiles for the recycling efficiency (Fig. 3. 6). The immobilized enzyme beads retained ~ 50% activity with 40% of initial lactose after 2 cycles of GOS production and exhibited ~ 20% of both hydrolysis and transgalactosylation activities after 6 cycles of reuse.

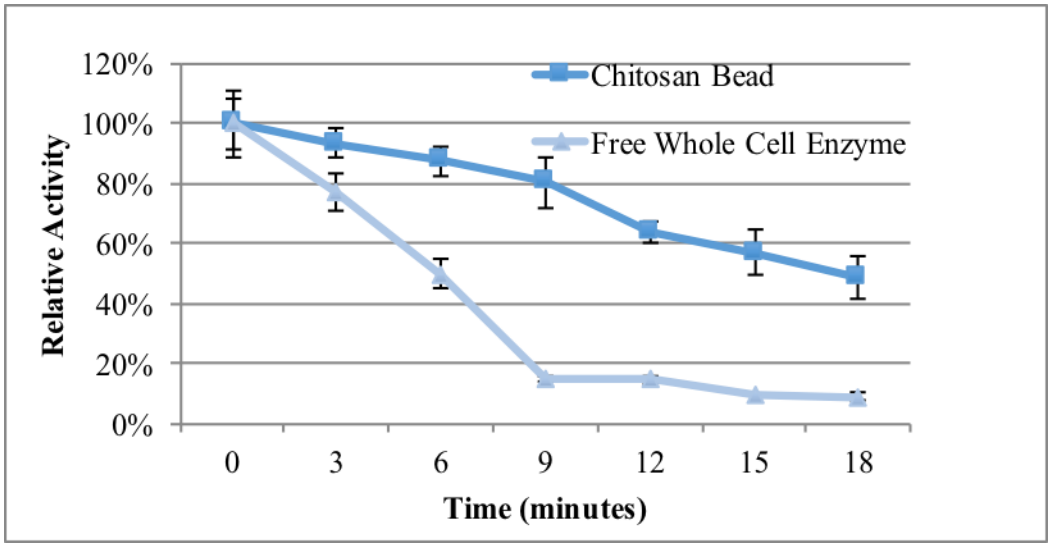

Figure 3. 5. Effect of immobilization of the thermostability. The thermostability of the immobilized enzyme was determined by high temperature (100^{o}C) incubation and β -galactosidase assay. The error bars represent SD (n=3).

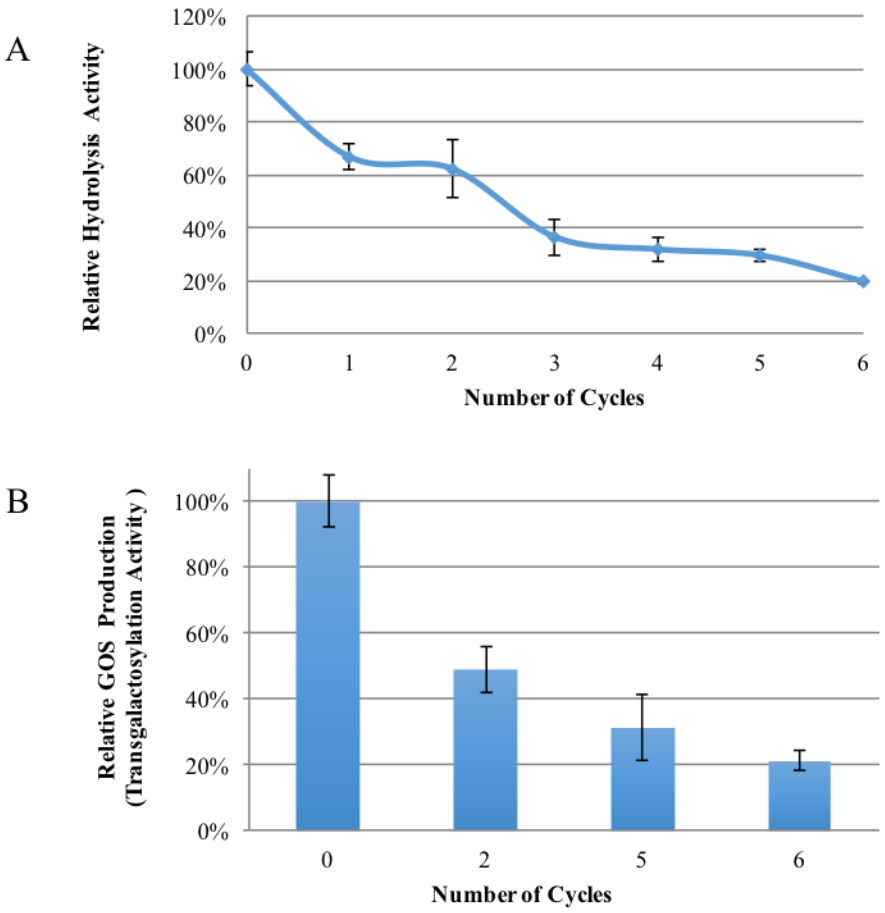

Figure 3. 6. Effect of immobilization on reusability. The reusability of immobilized enzyme was examined using both β -galactosidase assay for hydrolysis activity (A) and for transgalactosylation activity using an initial lactose concentration of 40% for GOS synthesis (B). The maximum (100%) amount of GOS produced was 150 g/L of tri and tetra GOS moieties.

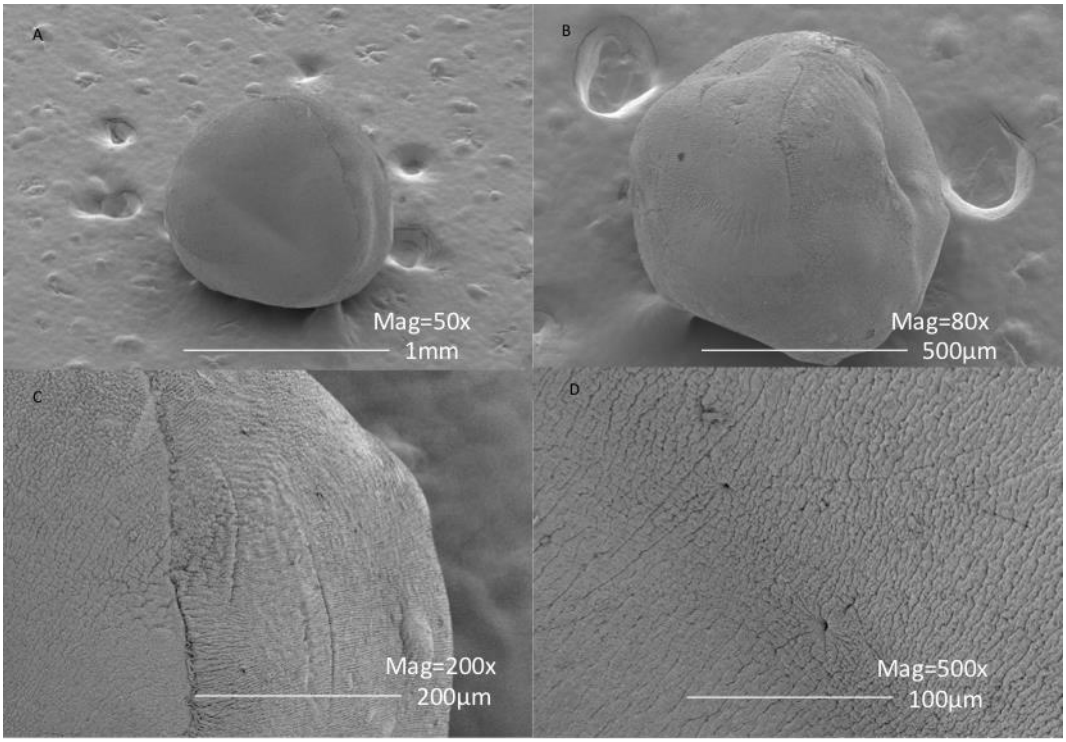

Figure 3. 7. Scanning electron microscopy (SEM) micro-graphs. (A), (B), (C) and (D) illustrate the surface of chitosan immobilized enzyme. Each picture was observed under different magnifications 50x, 80x, 200x and 500x.

Surface characterization of the chitosan beads

The topology of the chitosan bead surface was investigated using scanning electron microscope (SEM). The surface of the beads was rough with small holes visible on the surface (Fig. 3. 7). The average diameter for each bead was around 1 mm, with the reduced size occurring due to a dehydration process during SEM preparation.

Discussion

Lactococcus lactis is one of the most utilized food grade lactic acid bacterium in food fermentations worldwide (Leroy and De Vuyst, 2004). The advent of synthetic biology has now enabled approaches to be designed that can enable exotic enzymes to be overexpressed in this host. We have previously overexpressed a β-galactosidase enzyme from an extreme hyperthermophilic organism for efficient GOS production and in this study we have converted this into a food grade and reusable technology for GOS production. This involved immobilizing the whole cells containing this enzyme in chitosan, which is a thermostable matrix that can be readily removed following GOS production. While immobilization techniques have previously been developed for GOS production none of them produce GOS under extreme high temperatures in a food grade approach (Zheng et al., 2006; Hsu et al., 2007; Sakai et al., 2008; Neri et al., 2009; Chen and Duan, 2015). In this study, the chitosan immobilized whole cell enzyme was utilized at 90^{o}C and pH 5.5 for 25 hours to convert

solutions of various concentrations of lactose into GOS. The use of alginate-based beads for this purpose resulted in a loss of part of the gel network under these conditions indicating that this material, which is commonly used for immobilization purposes, is not suitable for very high temperature uses.

The different performances of these two immobilization materials can be explained by their chemical structures which react differently under the conditions used for GOS production. Alginate is composed of β-D-mannuronate (M) and α-L-guluronate (G) residues, which are [1→4] linked. The G component is involved in gel formation with divalent ions, such as Ca^{2+} in the bead formation conditions used in this study (Lee and Mooney, 2012). Alginate can be easily gelled when the Ca^{2+} binds to different G blocks resulting in a gel network, but can be disrupted when the divalent ions are replaced by monovalent metal ions (Fig. 3. 8). This results in uptake of water and bead swelling (Bajpai and Sharma, 2004). In this study, GOS was synthesized in sodium acetate buffer, in which the Na^{+} ions can interact with the alginate gel network and gradually replace the Ca^{2+} ions reducing the ionic strength causing loss of structure and swelling. However, the chitosan beads behaved differently under these conditions. Chitosan is composed of β-[1→4]-linked *N*-acetyl-D-glucosamine (N) and D-glucosamine (G) residues. It is produced from chitin via deacetylation and fragmentation into different sizes. The gel-network of chitosan was generated via ionic interaction with the cross-linking agent, TPP. Chitosan becomes soluble in acetic acid (low pH) resulting in the ammonia group of the G residue becoming ionized, which

then replaces the sodium ions of sodium TPP to form a gel (Fig. 3. 8). This explains why the chitosan beads showed a higher stability during the GOS synthesis conditions used in this study.

When the free whole cell β-galactosidase enzyme was used for GOS production, the optimum conditions were found to be pH 6 and 85^{o}C (Yu and O'Sullivan, 2014). While using chitosan entrapped whole cell enzyme, the optimum conditions had changed slightly to pH 5.5 and 90^{o}C. This change is consistent with some previous studies that found the optimum temperature and pH of immobilized enzymes were different from the free enzymes (Zhou and Chen, 2001). The thermostability of the immobilized enzyme was also increased (Fig. 3. 5) illustrating the protective effect of the chitosan for the whole cell enzyme. This increase in thermostability for immobilized enzymes has also been found in previous studies (Ladero et al., 2003, Rhimi et al., 2010).

In this study, a maximum GOS production of 150 g/L was obtained from 40% starting lactose using the immobilized whole cells containing β-galactosidase from *S. solfataricus*. While this is somewhat less than the 197 g/L we previous reported for the free whole cells of this enzyme, the reduction may be due to less whole cells present in the beads compared to the free whole cells used. Other studies using encapsulated β-galactosidase enzymes from different organisms also produced similar yields (Table 3.1).

Table 3. 1. GOS production using different immobilized β-galactosidase enzymes

Enzyme original host	Immobilization support	Optimized condition	GOS produced	GOS products detected for quantification	Initial lactose	Reference
Aspergillus oryzae	Polyvinyl alcohol (PVA)	pH 4.5 and 40 °C	117 g/L	3-OS and bigger	40%	(Jovanovic-Malinovska et al., 2012)
A. oryzae	Cotton cloth	pH 4.5 and 40 °C	106 g/L	3-OS and bigger	40%	(Albayrak and Yang, 2002)
Kluyveromyces lactis	Polystyrene nanofibers	pH 7.2 and 37 °C	110 g/L	None[a]	40%	(Misson et al., 2016)
K. lactis	Chitosan	pH 6.5 and 45 °C	186 g/L	3-OS and bigger	40%	(Klein et al., 2013)
Lactobacillus bulgaricus L3	Microcrystalline cellulose	pH 7.6 and 45 °C	157 g/L	3-OS and bigger	40%	(Lu et al., 2012)
Sulfolobus solfataricus[b]	Chitosan	pH 5.5 and 90 °C	150 g/L	3-OS and bigger	40%	This study

[a], estimated by subtracting final lactose, glucose and galactose amounts from starting lactose;

[b], encapsulated whole cells of *Lactococcus lactis* containing the enzyme

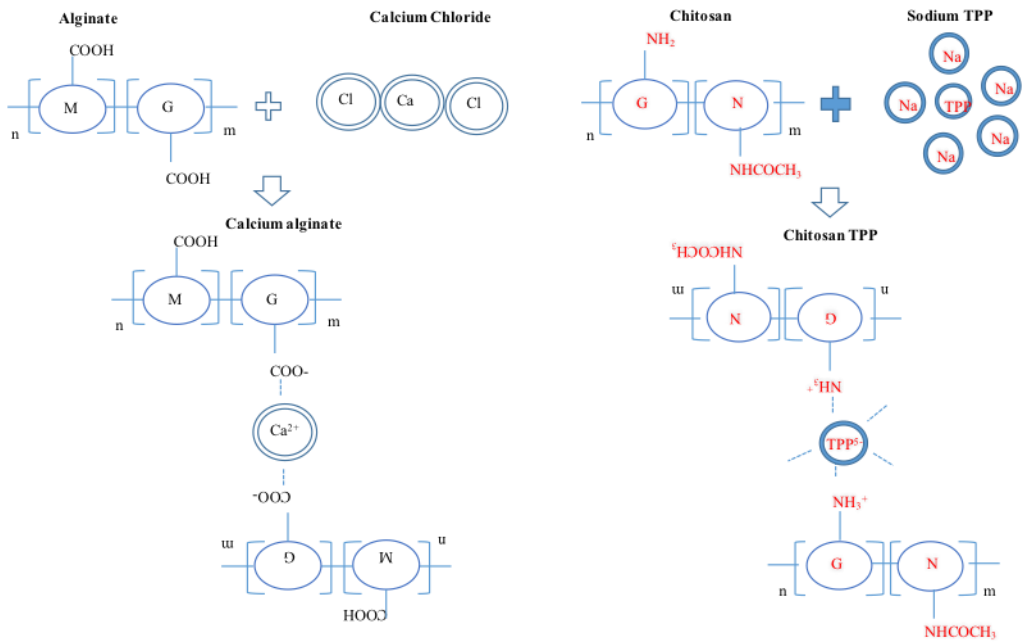

Figure 3. 8. Gel network formation process by alginate and calcium chloride, chitosan and Sodium TPP.

When GOS was produced with the immobilized whole cell enzyme using different initial concentrations of lactose (5 – 50%), the highest production of tri and tetra GOS was found with 40% initial lactose, which was consistent with our previous study using the whole cell enzyme (Yu and O'Sullivan, 2014). A similar optimum initial lactose concentration was observed for GOS production using other β-galactosidase enzymes from *B. longum* and *Penicillium expansum* (Hsu et al., 2007). However, GOS production using the β-galactosidase enzyme from *Aspergillus oryzae* showed a higher amount of GOS using 50% initial lactose illustrating that enzymes from different sources can have different substrate saturation levels.

Recombinant DNA technology can be utilized to produce natural food ingredients that are free of any recombinant DNA. Chymosin, from the abomasum of a suckling young bovine, is a well-known example that is produced via recombinant DNA technology in *E. coli*, yeast and fungi and is used worldwide in commercial cheese manufacture (Mohanty et al., 1999). This was the first food ingredient produced via recombinant DNA technology to be approved by the Food and Drug Administration in 1990 and its use in food production does not constitute a GM process under current labeling guidelines as the enzyme does not contain any genetically modified materials. In this study the recombinant DNA in the whole cell enzyme was degraded by UV to below the level detectable by PCR, which is the standard currently used in foods to differentiate a GM food from and GM-free food.

In conclusion, a chitosan based immobilized hyperthermostable β–galactosidase enzyme was developed and this enabled efficient GOS production for food grade use. This technology enabled more than 60% of the lactose in a 5% initial lactose solution, which is similar to cheese whey, to be converted into tri and tetra GOS products in addition to dimer GOS products that were not quantified. Upon removal of the beads following GOS production, the final GOS enriched product was free of any recombinant material and therefore suitable for food grade applications.

Acknowledgements

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Chapter 4. Use of *Lactococcus lactis* as a Production System for Peptides and Enzymes Encoded by a Lantibiotic Gene Cluster from *Bifidobacterium longum*

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Bifidobacterium longum DJO10A was previously demonstrated to be able to produce a broad-spectrum lantibiotic, but production in media was very limited and only on solid media periodically. Given the difficulty of obtaining these lantibiotic peptides using *B. longum* DJO10A due to its tightly controlled production, genes predicted to be needed for its production and immunity were designed and codon optimized according to the preferred codon used by *L. lactis*. Since the *lanR1* gene within this lantibiotic gene cluster was the only one without a characterized analogue from other lantibiotic gene clusters, its annotation was re-examined as it was previously suggested to be a regulatory protein. Lack of DNA binding motifs did not support this and a current analysis suggested a high likely hood of it interacting with LanD. Therefore, gene *lanR1* together with *lanADMIT* were codon optimized and synthesized. Those genes were then cloned into an efficient dual-plasmid nisin controlled expression system in *L. lactis.* The addition of the *lanR1* gene exhibited toxicity in *E. coli*, specifically causing a shorter cell size as observed by SEM. No toxicity was observed in *L. lactis*. While this production system did not result in producing a bioactive lantibiotic by *L. lactis*, it did successfully produce all the peptides and enzymes encoded by the original lantibiotic gene cluster from *B. longum*, as confirmed by LC-MS. This will now facilitate efforts into determining the proper conditions required for these enzymes to produce a bioactive lantibiotic.

Introduction

Lantibiotics are lanthionine-containing, anti-microbial peptides, which are produced by some gram-positive bacteria from the Firmicutes and Actinobacteria phyla. The name lantibiotic is derived from “lanthionine-containing antibiotic” and their synthesis includes post-translational modifications. Specifically, lantibiotics undergo dehydration of some serine and threonine residues, resulting in the unusual amino acids, 2,3 didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb), respectively. Subsequently, thioether ring structures are generated by linking these amino acids to cysteine residues [1–4]. These special residues and structures can facilitate an increased resistance to environmental conditions and a broad-spectrum of anti-microbial activity. Therefore, the production of lantibiotics has drawn significant attention. While most of the lantibiotics currently identified are produced by the bacteria from the Firmicutes phylum, and increasing number of lantibiotcs from different members of the Actinobacteria phylum exhibiting some novel post translational modifications have been described [5]. For example, microbisporicin, which is produced by *Microbispora coralline*, contains the novel post translational modifications of chlorotryptophan and dihydroxyproline [6].

Previously in our laboratory, a genomic analysis of a minimally cultured, human intestinal isolate, *Bifidobacterium longum* DJO10A, revealed a cluster of genes predicted to encode a novel lantibiotic. Repeated culturing of this isolate in broth media resulted in loss of the gene cluster, resulting in the mutant strain *B.*

longum DJO10A-JH1. A comparison of the competitive abilities of this mutant with the parent strain during growth experiments in a simulated anaerobic fecal medium demonstrated that the parent strain impeded the growth of newly isolated *E. coli* and *Clostridium difficile* strains, significantly more than the lantibiotic deletion mutant. This substantiated that the production of this lantibiotic, termed bisin, was involved in the control of clostridia and *E. coli* in vivo [7]. Repeated attempts to detect the lantibiotic in broth media were not successful, but some production was demonstrated on agar media periodically [7]. It was then hypothesized that the formation of a bioactive lantibiotic from *B. longum* required unknown, but specific conditions for proper functioning of the modification enzymes, as periodically bioactive lantibiotic could be detected on agar media but never in broth media.

Expression of the lantibiotic genes by *B. longum* DJO10A in broth media was found to be transcriptionally repressed. However, addition of a crude lantibiotic preparation from agar media resulted in switching on transcription of the gene cluster via a two-component regulatory system, encoded by *lanR2* and *lanK* [8]. However, this did not result in production of a bioactive lantibiotic, presumably due to the lack of correct conditions for functioning of the modification enzymes. Designing an efficient production system for the peptides and enzymes involved in biosysthesis of this lantibiotic would facilitate their characterization and uncovering their optimum conditions for producing a bioactive lantibiotic. Given the tight transcription control of these genes in *B.*

longum, designing a more efficient and controlled system for their production would greatly facilitate this objective.

We have previously developed a dual plasmid system for *Lactococcus lactis* for high level gene expression. This system utilizes the regulatory signals from the lactococcal nisin lantibiotic gene cluster (*nisRK*) as well as the *nisA* promoter and have been used in our laboratory for high level expression of different heterologous genes in *L. lactis* [9,10]. It was therefore the objective of this study to engineer this system to produce all the peptides and enzymes encoded by the gene cluster in *B. longum*.

Methods

Bacteria, plasmids and growth conditions

The *Lactococcus*, *Bifidobacterium*, *Micrococcus* and *E. coli* strains and plasmids used in this study are described in Table 1. *L. lactis* strains were inoculated in M17 broth (Becton, Dickinson and Co, Franklin Lakes, NJ) supplemented with 0.5% glucose (Sigma-Aldrich, St. Louis, MO) at 30^{o}C, without shaking. Bifidobacteria were grown anaerobically in de Man, Rogosa and Sharpe (MRS) medium containing 0.05% L-cysteine HCl (Sigma-Aldrich) or Bifdobacteria Low-Iron Medium with Iron (BLIM+Fe) agar plates (2.0% proteose peptone, 0.15 % K_2HPO_4, 0.5 % glucose, 0.15 % $MgSO_4$ ($7H_2O$), 0.05 mM $FeCl_3$, 100 mM PIPES and 1.8 % agar, pH = 7.0) at 37^{o}C. *E. coli* strains were grown in LB medium or Brain Heart Infusion (BHI) medium with shaking at 37^{o}C. *Micrococcus*

luteus was grown with shaking at 30^{o}C in LB medium. When needed, erythromycin was supplemented at a level of 150 µg/mL for *E. coli* (in BHI medium) and 3 µg/mL for *L. lactis*. Chloramphenicol was added at a level of 20 µg/mL for *E. coli* and 3 µg/mL for *L. lactis*. Ampicillin was used at 150 µg/mL for *E. coli*. CopyCutter induction solution was used according the manufacture's recommendation to increase the copy number of plasmid in *E. coli* EPI 400 cells (Epicentre, Madison, WI).

Table 4. 1. Strains and plasmids used in this study

Strain/ plasmid	Relevant characteristics *	Source or reference †
Lactococcus lactis LM0230	Plasmid cured derivative of *L. lactis* C2	† DCC
Bifidobacterium longum DJO10A	Human gut isolate	[7,8]
Micrococcus luteus ATCC 4698	Indicator for bioassay	ATCC
E. coli DH5α	Plasmid free cloning host	Invitrogen
E. coli EPI400	Plasmid free cloning host, which reduces the copy number of pUC and pET-type vectors plasmid	Epicentre
pUC57	*E. coli* cloning vector; Amp^r	GenScript
pDOC99	Transcription fusion of *nisA* promoter to *lacZ* gene from *Streptococcus thermophilus*; Em^r	[9]
pDOC23	*E. coli*-lactococcal shuttle vector with *nisR* and *nisK* genes; Cm^r	[9]
pDOLXD7	Expression vector containing synthetically engineered *lanADM* genes; Em^r;	This study
pDOLXD7-R1	Expression vector containing synthetically engineered *lanAR1DM* genes; Em^r;	This study
pDOLY6235	Expression vector containing synthetically engineered *lanIT* genes; Cm^r	This study
pDOJHR-WD6	Expression vector for *B. longum*; Cm^r	[25]
pDOJHR-WD6-YL	Expression vector for *B. longum* containing *lanAR1DMIT* genes; Cm^r	This study

* Amp^r, ampicillin resistance; Em^r, Erythromycin resistance; Cm^r, chloramphenicol resistance † DCC, Dairy Culture Collection (University of Minnesota, St. Paul); ATCC, American Type Culture Collection); Invitrogen, Invitrogen Corp (Grand Island, NY); Epicentre, Epicentre® (an Illumina company) (Madison, WN); GenScript, GenScript USA Inc (Piscataway, NJ);

Molecular techniques

Plasmids were isolated from *E. coli* using QIAprep Spin Miniprep Kits (QIAGEN, Germantown, MD, USA) according to the manufacturer's methodology. Plasmids were isolated from lactococci and bifidobacteria as previous described [11]. Restriction enzyme and T4 DNA ligase (New England Biolabs, Ipswich, MA) reactions were conducted according to the manufacturer's recommendations in 20 µl reactions. Two oligonucleotides, F-lan F 5'-GGTGGTGCGGCCGCAAGGAGGCCTATA TGAGCATCAATGAGAAGTCC-3' and R-lan R 5'-ACCACCACTAGTTCACTCCTGCCG GTACAGCCGCGAATACAATCCGCCG -3', were designed as primers to amplify the lantibiotic gene cluster. The underlined letters represent restriction sites for *Not*I and *Spe*I, respectively. PCR was conducted using Phusion DNA polymerase (NEB) in 50 µl reactions in a PTC-200 thermocycler (BioRad, Hercules, CA). Routine PCR was conducted using Taq DNA polymerase (Thermo Fisher Scientific, Rockford, IL) in 25 µl reaction. DNA fragments were separated and analyzed by gel electrophoresis, using 0.9% agarose in TAE buffer (Thermo Fisher Scientific). DNA fragments were further cleaned by QIAquick Gel Extraction Kits (QIAGEN) or DNA Clean & Concentrator™-5 kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. Commercial competent cells, *E. coli* DH5α (Invitrogen, Carlsbad, CA) and *E. coli* EPI400 (Epicentre, Madison, WI), were used for transformation according to the manufacturer's protocols. All newly generated plasmids were sequenced to confirm insert

orientation and integrity. Sequencing was conducted at the Biomedical Genomics Center, University of Minnesota.

Bioassay for detecting bisin production

L. lactis strains were grown to mid-log phase and then induced with nisin (Sigma-Aldrich) at 1.0 IU/mL at 30^{o}C for 2 hours. *B. longum* strains were grown into mid-log phase in BLIM+Fe broth media. The cell pellets were separated from supernatant via centrifuge. Prior to bioassay, cell pellets were resuspended in 500 µl H_2O and were disrupted with 50 µl glass beads (< 106 µm) (Sigma-Aldrich) for 1 min using a bead-beater at maximum speed (BioSpec Products). Aliquots (100 µl) of both cell pellet mixture and supernatant were transferred into well in a BLIM+Fe agar plates. After diffusion at room temperature, the plates were overlayed with molten BLIM+Fe agar (0.5%) seeded with 1.0% of indicator strains (*M. luteus* or *E. coli*).

MS detection of peptides from the lantibiotic production proteins in *L. lactis*

Following induction with nisin, cells were isolated by centrifugation, resuspended in phosphate buffer (pH 6.8) and disrupted using glass beads as described above. The mixture was left on ice for 5 minutes for the glass beads to settle. An SDS loading dye was added to the mixture and heated at 95°C for 5 minutes. The total proteins were separated by SDS-PAGE using a 4-20% precast gel (BioRad). Precision PlusProtein Unstained Standards (BioRad) was used as

a protein size marker. The gel bands corresponding to the predicted sizes of lantibiotic peptides were excised from the gel, followed by in-gel digestion with trypsin (Promega, Madison, WI, USA) in buffer (50 mM NH_4HCO_3 and 5 mM $CaCl_2$) [12]. The peptides were subsequently desalted with a C18 resin STAGE tip made with SDB-XC reversed-phase material (3M, Maplewood, MN, USA) [13]. The peptide fractions were injected into a LC-MS/(Waters TQD UPLC/Triple Quadrupole-MS) and analyzed at the Mass Spectrometry and Proteomics Center at the University of Minnesota, as described previously [14].

Scanning electron microscopy (SEM)

Morphological changes of *E. coli* cells were evaluated by SEM. Prior to SEM, *E. coli* EPI400 (pDOLXD7-R1) were grown overnight to an $OD_{600\ nm}$ of > 1.0, before inducing with induction solution to increase the copy number of the plasmid for 2h. The cells were pelleted and washed with sterile PBS, pelleted again and dried using different concentrations of ethanol for moisture removal, using an increasing concentration of ethanol/water mixtures ranging from 30 to 100% ethanol, and then with hexamethyldisilazane (HMDS). After drying, cells were coated with a layer of gold particles. Micrographs were taken using a 4,700 Scanning Electron Microscope (Hitachi, Krefeld, Germany).

Results

A lantibiotic gene cluster from Bifidobacterium longum DJO10A was previously shown to be involved in production of a potentially broad spectrum lantibiotic [7]. The genes were organized in two apparent transcriptional units, one (lanR2K) encoding a two-component regulatory system, and the other (lanAR1DMIT) encoding lantibotic production and immunity [8]. All of these genes encoded proteins with analogs from other lantibiotic gene clusters that had characterized functions except for lanR1. The original annotation for this gene was a likely transcriptional regulator primarily based on its size. Based on its location this prediction was reexamined to determine its likely role.

Re-examination of the predicted function for LanR1

To determine if current databases contained any analogs for *lanR1*, a re-annotation was conducted. No similarities to any known proteins were found and suggesting *lanR1* encoded a hypothetical protein with unknown function. Given transcriptional regulators should contain a DNA binding motif, LanR1 was extensively analyzed for DNA binding motifs without any being found. However, a motif search using MotifFinder at the GenomeNet Database Resources website did suggest a possible ABC-2 family transporter protein from the Pfam database, but no DNA binding motifs. These new data indicated that LanR1 was less likely to be a regulatory protein and may be involved in the biosysthesis of the lantibiotic. To further investigate this possibility, the sequence of LanR1 was analyzed using the UniProt software package to predict its likelihood of protein-

protein interactions with the other Lan proteins. It was found that the predicted LanR1 peptide was most likely to interact with LanD (Fig 4. 1), substantiating its likely role in production of the lantibiotic.

Synthesis of codon optimized, synthetic genes for the *lanAR1DMIT* genes for *L. lactis*

Given the difference in codon usage between the high G/C bifidobacteria and the low G/C lactococci, it was necessary to codon optimize these genes to ensure adequate expression in *L. lactis*. A codon usage Table for the preferred codons for *L. lactis* was utilized for this process [10]. These six genes, containing an optimized ribosomal binding site for *L. lactis* [15], were chemically synthesized (Supplemental Figure S1) and cloned into the *E. coli* plasmid vector, pUC57. Finally, An optimized terminator for *L. lactis*, that we previously developed, was added downstream of the *lanM* and *lanT* genes [10].

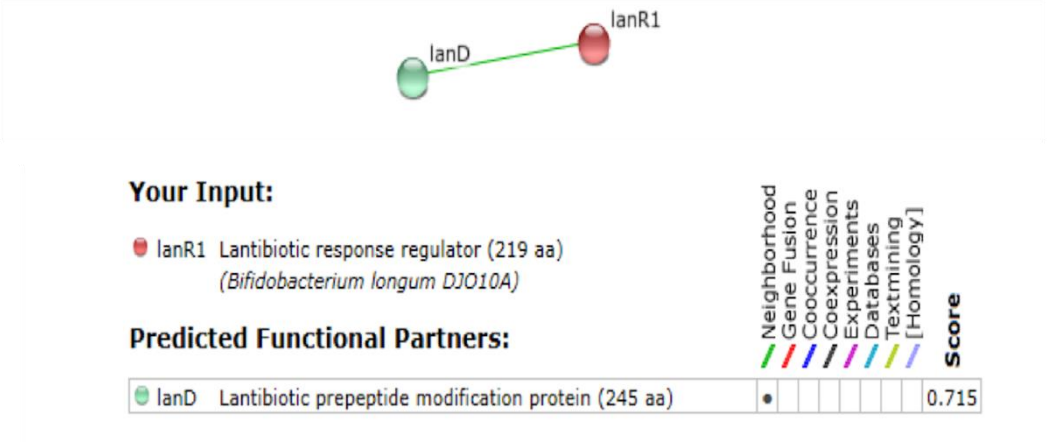


Figure 4. 1. UniProt prediction for the interaction of LanR1 with LanD.

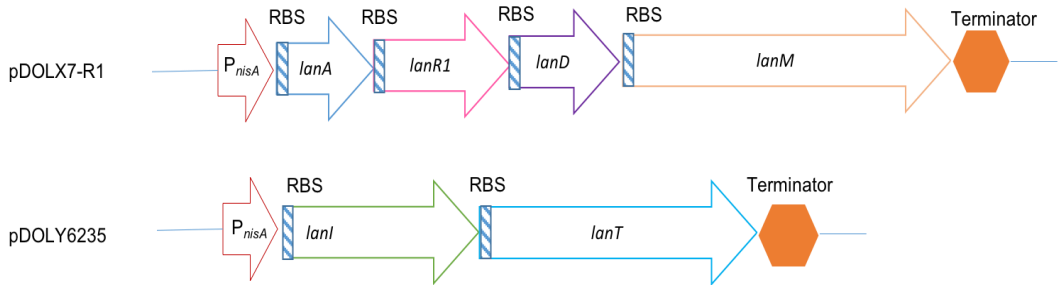
Cloning of the *lanAR1DMIT* genes and into a nisin-inducible expression system for *L. lactis.*

To facilitate expression of all these genes in *L. lactis*, they were organized in two cassettes as illustrated in Fig 4. 2. Both of these cassettes were assembled in two compatible plasmids, pDOLXD7-R1 (Fig 4. 3) and pDOLY6235 (Fig 4. 4), based on our original two plasmid nisin expression system. It was noted that *lanR1* could only be cloned in *E. coli* using the plasmid copy-cutter *E. coli* EPI400 cells for transformation. Subsequently, plasmid pDOLXD7-R1 was introduced into *L. lactis* without any toxicity issues.

Cloning of the *lanAR1DMIT* genes into an expression vector for *B. longum*

To understand the conditions for active lantibiotic production, the 8-kb lantibiotic gene cluster was amplified using PCR and cloned downstream of a 16s promoter in an expression vector, as illustrated in Fig 4. 5. The cloned insert was sequenced to ensure no PCR sequence errors occurred. The resulting plasmid was designated as pDOJHR-WD6-YL. Subsequently, plasmid pDOJHR-WD6-YL was introduced into *B. longum* via conjugation using the procedure previously described by Dominguez and O'Sullivan [16].

a



b

Figure 4. 2. (a) Organization of the codon optimized synthetic cassettes designed for the dual plasmid production system for *L. lactis*. P *nisA*, *nisA* promoter; RBS, ribosomal binding site (2a). (b) Native gene order of original lantibiotic cluster in *B. longum*.

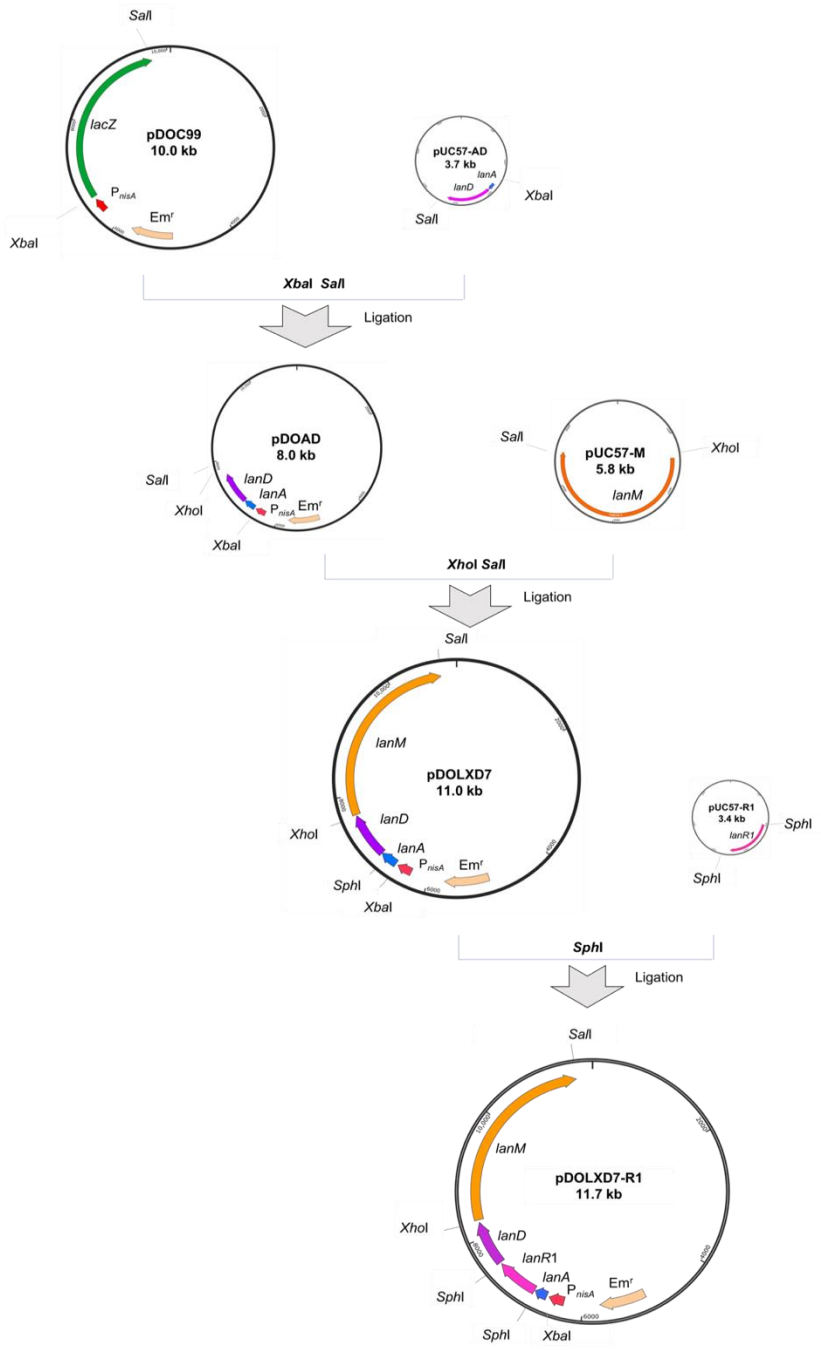


Figure 4. 3. Construction strategy including relevant restriction enzyme sites of the expression vector pDOLXD7-R1. P *nisA*, *nisA* promoter.

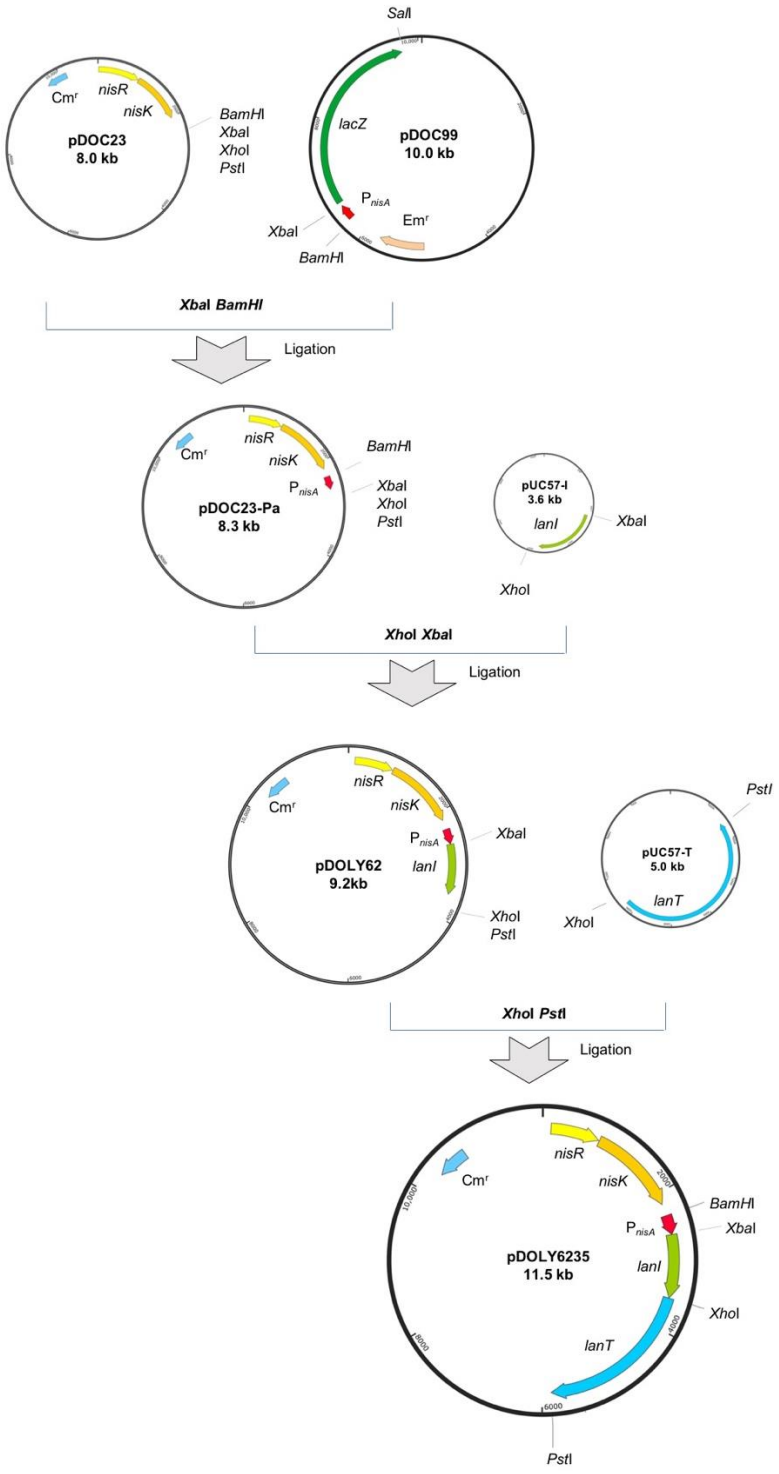


Figure 4. 4. Construction strategy including relevant restriction enzyme sites of the expression vector pDOLY6235. P *nisA*, *nisA* promoter.

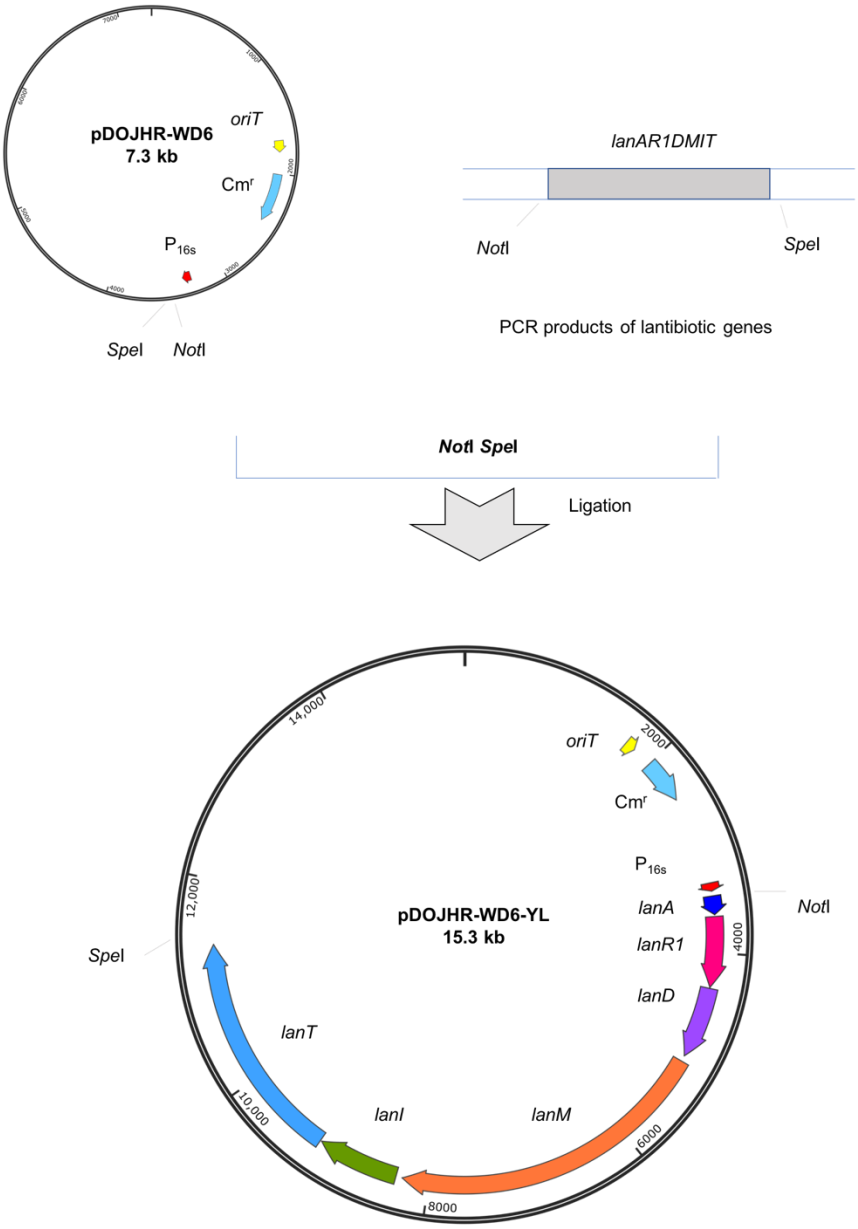


Figure 4. 5. Construction strategy including relevant restriction enzyme sites of the expression vector pDOJHR-WD6-YL. P 16S, 16s promoter

Surface characterization of *E. coli* cells containing lanAR1DM

The *E. coli* EPI400 cells containing pDOLXD7-R1 following induction of the plasmid copy number were observed to have a decreased cell size. Almost all of the un-induced cells have a size larger than 5 µm, consistent with the plasmid less strain, while almost all of the plasmid copy number induced cells have a size less than 3 µm (Fig 4. 6).

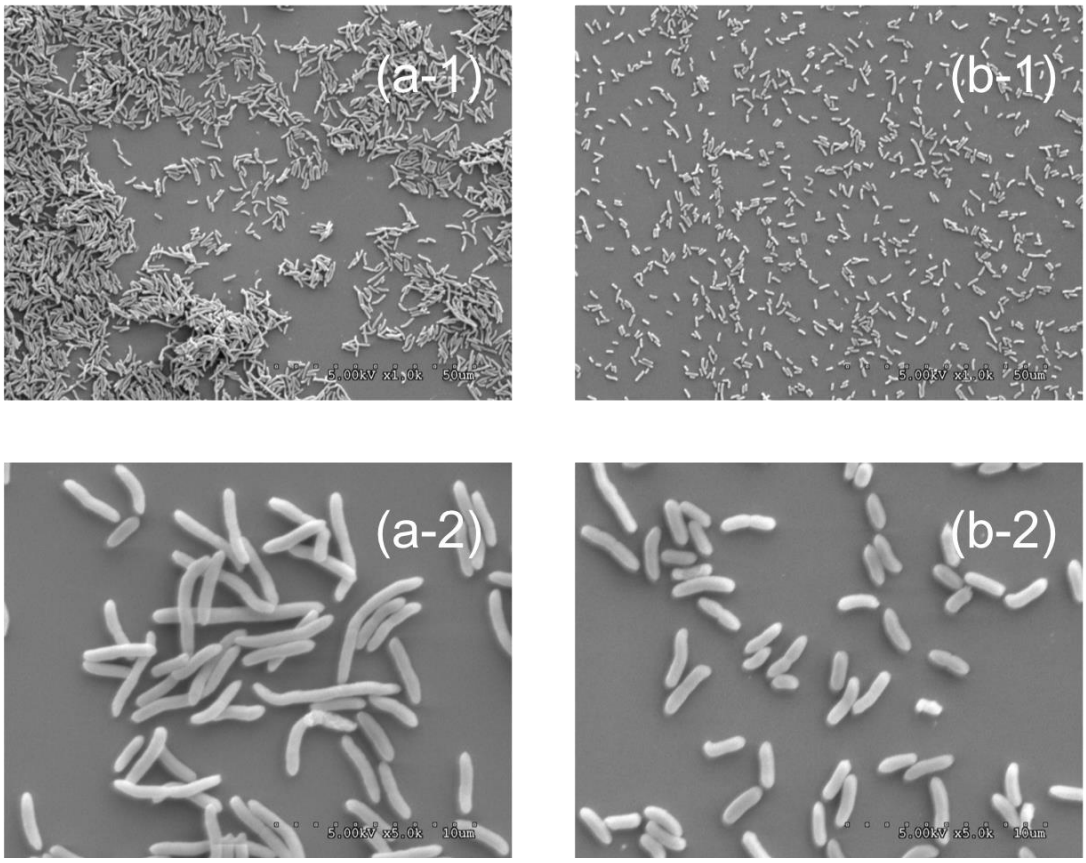


Figure 4. 6. Morphological examination of *E. coli* cells without (a) and with plasmid copy number induction (b). (a1) SEM micrograph of *E. coli* cells containing pDOLXD7-R1 at 1,000 X magnification; (a2) SEM micrograph of *E. coli* cells containing pDOLXD7-R1 at 5,000 X magnification; (b1) SEM micrograph of *E. coli* cells containing pDOLXD7-R1 following induction of plasmid copy number at 1,000 X magnification; (b2) SEM micrograph of *E. coli* cells containing pDOLXD7-R1 following induction of plasmid copy number at 5,000 X magnification.

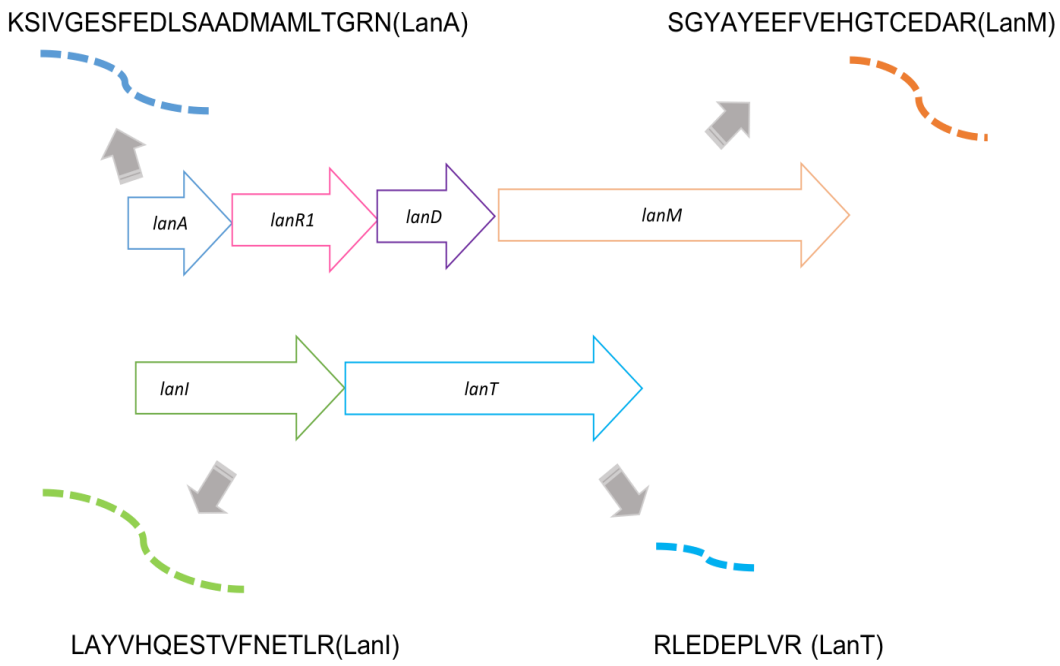


Figure 4. 7. Four representative peptide fragments from four predicted proteins encoded by the lantibiotic production genes in the expression cassette of the engineered *L. lactis* strain, as identified by LC-MS. A total of 33 peptides from the predicted proteins required for lantibiotic production confirmed the production system was functioning as designed to produce the required peptides and enzymes.

Expression of predictive lantibiotic production genes in the engineered strains of *L. lactis*

To evaluate if the production system resulted in a bioactive lantibiotic, the induced cultures of the engineered *L. lactis* strain and a negative control *L. lactis* LM0230 (pDOC99/pDOC23), were analyzed for antimicrobial activity. Both the disrupted cell pellets and the supernatants were evaluated for antimicrobial activity using a well-diffusion bioassay with *Micrococcus luteus* as the indicator culture. No antimicrobial activity was detected in any of engineered *L. lactis* strains using this bioassay consistent with the use of the parent *B. longum* culture in broth media.

Expression of predictive lantibiotic production genes in the engineered strains of *B. longum*

The engineered *B. longum* strain and a negative control *B. longum* DJO10A-JH1 were grown in broth media and subsequently analyzed for antimicrobial activity. Both the disrupted cell pellets and the supernatants were evaluated for antimicrobial activity using a well-diffusion bioassay with *Micrococcus luteus* as the indicator culture. No antimicrobial activity was detected in any of engineered *B. longum* strains.

Detection of the lantibiotic peptides in the engineered strains of *L. lactis*

To investigate if expression of predictive lantibiotic genes in the engineered strain of *L. lactis* resulted in the production of the encoded gene products, LC-MS/MS was used to identify the expected peptides in the induced culture while the un-induced culture was used as a negative control. Thirty three peptide fragments from predicted peptides and enzymes spanning both these cassettes were detected with ≥ 95% confidence, while none were present in the un-induced negative control (Fig. 7). These results confirmed that the expression system was functioning as designed and all peptides and enzymes associated with the lantibiotic were successfully expressed.

Discussion

Previous studies with *B. longum* DJO10A established that the lantibiotic gene cluster was essential for its ability to control the number of wild-type *E. coli* and *Clostridium difficile* in a fecal environment supporting the antimicrobial potential of this lantibiotic in vivo [7]. Although this lantibiotic could be produced from *B. longum* DJO10A, the production level was low and only found sometimes in agar media, which was originally presumed to be solely due to a tightly controlled regulatory system. Therefore, *L. lactis* was used to overcome the problem by expression of all the production genes in this host without the regulatory systems that are functioning in the parent strain. A similar strategy was previously used to enable *L. lactis* to produce the enterocin bacteriocin from *Enterococcus faecium* [17].

It was initially proposed during the original genome annotation of *B. longum* DJO10A that *lanR1* encoded a putative transcription regulator [7]. In addition, an extended 5' untranslated region (284 bp) of mRNA upstream from the *lanA* structural gene supported the involvement of a transcriptional repressor [8]. A reinvestigation of this annotation for *lanR1* does not find any similarities to transcription regulators and BLAST searches indicated it was a putative protein of unknown function. As a transcription regulator should have a DNA binding motif, LanR1 was extensively analyzed for any such motif and none were found. In addition, a UniProt analysis indicated a high probability for its interaction with LanD. Therefore, *lanR1* was also codon optimized, synthesized and cloned into

the nisin expression system in *L. lactis*. It should be noted that cloning of *lanR1* was quite challenging, when *E. coli* DH5α was used as a shuttle vector. Retarded growth of *E. coli* containing the plasmid with *lanR1*(pDOLXD7-R1) had been observed (data not shown), suggesting toxicity of the additional gene *lanR1*. Toxicity of this gene in *E. coli* was previously noted substantiating this phenotype [18].

A reduced size of *E. coli* EPI400 cells containing pDOLXD7-R1 was observed after induction of the plasmid copy number via SEM. This might be a result of some expression of the *lanR1* gene due to the high AT nucleotide content in this codon optimized gene for *L. lactis*. Membrane targeting antimicrobials such as phenyl lactic acid (PLA) and peptoids have previously been shown to affect the size of *E. coli* cells [19,20]. Expression of LanR1 in *L. lactis* did not result in any detectable toxicity or cell morphological changes as determined by SEM (data not shown). However, induction of the *lanR1* gene in *L. lactis* coincided with induction of the immunity gene (*lanI*), which may have protected the cell.

To produce a bioactive lantibiotic, the first step is to have an efficient production system for all the peptides and enzymes involved. The production system developed in this study was confirmed to achieve this as evidenced by the detection of multiple peptides using LC-MS representing the predicted proteins from the lantibiotic gene cluster (Fig 4. 7). This will now facilitate studies

into the correct conditions needed for proper functioning of the enzymes involved for production of a bioactive lantibiotic. Given the conditions in the human colon are very different than our current in vitro conditions, the enzymes involved may require similar conditions.

Other enzymes have shown requirements for specific conditions besides pH and temperature. For example, oxidative stress has been shown to reduce the activity of some enzymes, such as glucose-6-phosphate dehydrogenase [21]. Production of antimicrobial peptides in different systems has also been shown to have specific requirements for antimicrobial activity. For example, the lantibiotic NAI-107 produced by strains of *Actinoallomurus* and *Microbispora*, also from the phylum Actinobacteria, have been shown to require bromide in the production medium for optimum antimicrobial activity [22]. In addition, this lantibiotic modification dehydratase enzyme requires glutamyl-tRNA(Glu) for Ser/Thr dehydratase activation [23]. The lantibiotic gallidermin produced by *Staphylococcus gallinarum* Delta P requires four vitamins and three metal ions for its production in vitro [24].

Given that this engineered *L. lactis* strain was able to express all the genes within the lantibiotic gene cluster of *B. longum* DJO10A, it can now be used as a production system to further investigate what is required for the production of the bioactive lantibiotic from *B. longum* DJO10A.

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Chapter 5. General conclusion

Current status of *Lactococcus lactis* as a host strain for enzyme production

The bacterium we now know as *Lactococcus lactis* was first described by Louis Pasteur in 1857 and its subsequent use in dairy fermentations followed [1]. The culture has been well researched throughout the last century, characterized and has demonstrated a remarkable ability as a host culture either for food fermentations or heterologous gene expression. A number of expression systems have been developed for *L. lactis* using both constitutive promoters and regulated promoters. One of the most important one is a nisin controlled expression system, which is composed of a two-component regulatory system [2]. Various enzymes important for food ingredients as well as proteins that are associated with antigens for vaccine delivery were successfully produced by *L. lactis* using this efficient system [3]. However, only few studies have been conducted for novel enzyme production using synthetic biology and little has been discussed for its use in the food industry for production of ingredients that are free of GMO materials.

Contributions to the field

This thesis has developed a food grade technology for overexpression of a hyper-thermostable beta-galactosidase enzyme using a nisin controlled expression system in *L. lactis*. This enabled inactivated and permeabilized cells to be directly utilized as a whole cell enzyme for efficient production of a prebiotic

food ingredient (galactooligosaccharides: GOS) at very high temperatures. This production technology was efficient and cost effective for GOS production.
In order to make the hyper-thermostable beta-galactosidase enzyme more applicable for the food industry, the whole cell enzyme was further immobilized in chitosan beads following DNA removal using UV treatment. As a food grade material, chitosan has showed the ability to be stable at a high temperature during the process for GOS synthesis. In addition, UV treatment helped to degrade the recombinant DNA in the cell with little effect on the enzyme, resulting in a GM free product. The technology enabled a high production of GOS and made the enzyme suitable for food grade applications. This is also the first use of chitosan as an immobilization material at high temperature conditions for GOS production.

In addition to novel enzyme production, *L. lactis* can also be used to produce lantibiotic peptides and enzymes that can then be used to understand the conditions necessary for production of bioactive lantibiotics. In this thesis synthetic genes from the lantibiotic gene cluster of *Bifidobacterium longum* DJO10A were designed and cloned into a *L. lactis* expression system. This successfully enabled all the peptides and enzymes encoded by this gene cluster to be produced. While no bioactive lantibiotic was detected it now provides a means to investigate what essential components or conditions are required for bioactive lantibiotic production.

Future research

Genetically modified technology can be used to produce various food ingredients without genetically modified DNA being part of the final product. However, currently in the food industry, it is only limited to chymosin, an enzyme used for cheese production. In this study, novel enzymes and peptides are successfully expressed and the recombinant DNA has been degraded below the PCR detection level. PCR detection is the standard that is currently used to distinguish the GM from GM-free products. This study opens a door to production of food ingredients containing no detectable genetically modified DNA using recombinant DNA technology.

In addition, the use of chitosan as a heat stable immobilization material could be an interesting area for food processing at high temperature conditions. The gene expression system could also be used for expression of other novel proteins, such as CAS-CRISPR, which could be further used to expand our understanding of lactococci and bifidobacteria strains.

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