Effect of Signal Peptides on the Secretion of β -Cyclodextrin Glucanotransferase in *Lactococcus lactis* NZ9000

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Key Words

Cyclodextrin glucanotransferase • *Lactococcus lactis* •
Bacillus sp. G1 • Signal peptides • Nisin

Abstract

Cyclodextrin glucanotransferase (CGTase) is an extracellular enzyme which catalyzes the formation of cyclodextrin from starch. The production of CGTase using lactic acid bacterium is an attractive alternative and safer strategy to produce CGTase. In this study, we report the construction of genetically modified *Lactococcus lactis* strains harboring plasmids that secrete the *Bacillus* sp. G1 β -CGTase, with the aid of the signal peptides (SPs) SPK1, USP45 and native SP (NSP). Three constructed vectors, pNZ:NSP:CGT, pNZ:USP:CGT and pNZ:SPK1:CGT, were developed in this study. Each vector harbored a different SP fused to the CGTase. The formation of halo zones on starch plates indicated the production and secretion of β -CGTase by the recombinants. The expression of this enzyme is shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram analysis. A band size of ~75 kDa corresponding to β -CGTase is identified in the intracellular and the extracellular environments of the host after medium modification. The replacement of glucose by starch in the medium was shown to induce β -CGTase production in *L. lactis*. Although β -CGTase

production is comparatively low in NZ:SPK1:CGT, the SP SPK1 was shown to have higher secretion efficiency compared to the other SPs used in this study.

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Introduction

Cyclodextrin glucanotransferase (CGTase, 1,4- α -D-glucan: 1,4- α -D glucopyranosyl transferase EC 2.4.1.19) is a member of the α -amylase family (family 13) of glycosyl hydrolases. CGTase represents one of the most important microbial amylolytic enzymes and also one of the bacterial glycosyl transferase enzymes; it serves as a carbohydrate-converting enzyme. CGTase is capable of converting starch or any other polysaccharides into cyclodextrins (CDs). Apart from starch degradation, this enzyme is involved in synthesizing exopolysaccharides and various glycosylated antibiotics. CGTase can be grouped as α , β and γ based on the majority amount of CD (α -, β - and γ -CD, respectively) produced through the intramolecular transglycosylation reaction [Tonkova, 1998].

β -CGTase received a lot of attention because of the improved stability of guest molecules when an inclusion complex is formed with β -CD. β -CGTase is a 75-kDa extracellular protein mainly produced by *Bacillus subtilis*

Table 1. In silico analysis of signal peptides

Signal peptide	AA Sequence	Length	Net charge at pH 7	Isoelectric point	Hydrophilic/total, %	Signal peptide probability	Cleavage site	Protein GRAVY
USP45	MKKKIISAILMSTVILSAAAPLSG VYA	27	+3	10.6	26	1.000	VYA	1.174
SPK1	MKKILT LVFIFVISILTAT NVHA	23	+2	10.6	17	1.000	VHA	1.552
NSP	LNDLNDFLKTISLSFIFFLLLSLPT VAEA	29	-2	3.7	31	0.999	AEA	1.089

and also by *Klebsiella*, *Micrococcus*, and *Thermoanaerobacterium* [Gawande and Patkar, 1999; Tonkova, 1998]. CDs are ring-shaped molecules with a hydrophilic outer surface and a hydrophobic cavity [Baudin et al., 2000]. The large size of the non-polar cavity of β -CD is most suitable to accommodate molecules such as drugs and aromatics compared to α - and γ -CD. This gives rise to a higher demand of β -CGTase in various industries such as cosmetics, pharmaceuticals [Stella and Rajewski, 1997], food [Mabuchi and Nagoa, 2001] and others [Buschmann and Schollmeyer, 2002; Hirose and Yamamoto, 2001].

Due to the broad industrial application of β -CDs, a lot of interest is focused on improving the production of microbial β -CGTase. Production of CGTase in *Bacillus* and *Escherichia coli* eventually reduces the quality of the products with the presence of impurities such as proteases. Therefore, the production of a better quality β -CGTase can be enhanced by using the GRAS (Generally Regarded As Safe) lactococcal system for improved performance especially in the food and pharmaceutical industries. These bacteria do not produce lipopolysaccharides or other toxins, which will lead to safer usage of the products. In addition, purification of the protein of interest is simpler since fewer proteins are known to secrete to the extracellular region of the cell [Wells et al., 1996]. Therefore, *Lactococcus lactis* is an interesting alternative heterologous cell factory for the production and secretion of CGTase [Nouaille et al., 2003].

Signal peptides (SPs) navigate protein secretion into specific environment. To optimize production yield and protein secretion in *L. lactis*, the selection of SP plays a crucial role. A SP is usually 14–25 amino acids long, and consists of the amino (N-), hydrophobic (H-), and carboxy-terminal (C-)regions. A SP functions as a target and recognition signal for signal peptidases to cleave off the secretory protein and direct it to a specific location such as the intracellular or extracellular regions [Driessen and Nouwen, 2007; Natale et al., 2008]. In contrast to the cytoplasmic production, SPs aid secretion in extracellular production, thus increasing the production yield

of protein via the Sec-dependent pathway [Le Loir et al., 2005].

In this work, a nisin-inducible system was used to express the CGTase gene from *Bacillus* sp. G1 in *L. lactis*. CGTase was secreted to the extracellular environment of *L. lactis* with the aid of different SPs. Expression levels of these proteins are reported under different growth conditions. This study shows that heterologous CGTase can be produced and maintains its functionality when secreted by *L. lactis*.

Results

DNA Sequence of the His-Tagged β -CGTase Gene

The β -CGTase secreted by *Bacillus* sp. G1 consists of a secretory SP (29 aa) and the gene coding for the matured region of β -CGTase (2,021 bp). The 2,021-bp gene coding for β -CGTase was cloned into the expression vector pNZ8048. Additional sequences which encoded for the His-tag comprising six histidines were added at the C-terminus of the β -CGTase, preceded by its native SP (NSP). The total size of these was 2,126 bp and yielded the pNZ:NSP:CGT vector. For the pNZ:USP:CGT vector, the gene coding for the matured region of CGTase was ligated with the lactococcal SP USP45 (27 aa), which resulted in a 2,102-bp cassette. The fusion of SPK1 (23 aa) with the gene coding for β -CGTase without the NSP resulted in a 2,090-bp cassette and yielded the pNZ:SPK1:CGT vector. As with the pNZ:NSP:CGT vector, both pNZ:USP:CGT and pNZ:SPK1:CGT also contained the His-tag sequence.

SP Analysis

The computational analysis results of NSP, SPK1 and USP45 (table 1) predicted that SPK1 has the highest potential to target the proteins through the Sec compartment pathway compared to other SPs in this study. From the prediction, SP SPK1 showed highest level of protein GRAVY and isoelectric point and lower level of hydrophilicity compared to the other two SPs.

Fig. 1. Growth profile of *L. lactis* NZ9000 harboring pNZ8048 (×) as a control, NZ:NSP:CGT (◇), NZ:USP:CGT (□) and NZ:SPK1:CGT (▲).

Fig. 2. Iodine plate test for extracellular CGTase expression. **a** Overnight cultures on a nisin-containing plate before iodine test. **b** Overnight cultures on a nisin-containing plate after iodine test. Halo zone around culture indicates CGTase expression into extracellular region. A: *L. lactis* NZ9000 harboring pNZ8048 as a control; B: NZ:NSP:CGT; C: NZ:USP:CGT; D: NZ:SPK1:CGT.

Plasmid Stability Test

The doubling time of NZ:NSP:CGT, NZ:USP:CGT and NZ:SPK1:CGT was found to be 58.5, 59 and 59.5 min, respectively (fig. 1). The doubling time was used to calculate the time needed to reach 100 generations. The stability of these recombinants is important to ensure that the plasmids can be maintained in the strain. In this study, all recombinant plasmids proved to be 100% stable when cultured in medium with chloramphenicol (Cm), up to at least 100 generations.

Starch Plate Assay

Iodine was poured onto overnight cultures of NZ:NSP:CGT, NZ:USP:CGT and NZ:SPK1:CGT on

starch plate supplemented with 7.5 µg/ml Cm. A concentration of 20 ng/ml nisin was spread onto the plate prior to culturing. Formation of halo zones around the colonies indicated the ability of these clones to secrete CGTase into the extracellular environment (fig. 2).

Nisin Induced β -CGTase Production in *L. lactis* NZ9000

A series of induction experiments were carried out to determine the optimum conditions for protein expression. Preliminary studies were carried out in different medium, at different induction times (3, 6 and 9 h) and with different nisin concentrations (10, 20, 30 and 40 ng/ml) using NZ:NSP:CGT.

Fig. 3. a Optimization of medium for β -CGTase expression. Lane M: PageRuler™ Unstained Protein Ladder (Fermentas). Lanes 1–8: intracellular protein after sonication. Lanes 9–16: extracellular protein after protein precipitation. Lanes 1, 2, 9, and 10: induction using GM17 broth. Lanes 3, 4, 11 and 12: M17 supplemented with 0.5% starch. Lanes 5, 6, 13 and 14: M17 supplemented with 0.5% sucrose. Lanes 7, 8, 15 and 16: GM17 supplemented with 0.5% starch. In M17 medium with starch, β -CGTase was successfully secreted to the extracellular environment as indicated

by the arrow which shows the 75-kDa band corresponding to this protein. **b** Carbon influence on CGTase enzyme production. EA S indicates enzyme activity (U/ml) in the presence of glucose (0.5% W/V); EA G indicates enzyme activity (U/ml) in the presence of soluble starch (0.5% W/V). Enzyme activity increases with the increase in soluble starch concentration. Maximum enzyme activity was obtained in the presence of 0.5% soluble starch, in the absence of glucose as shown by the arrow.

The expression of this gene was not observed in transformants grown in GM17 medium, which is a well-known and favorable medium for the growth of *L. lactis*. The medium was modified to enhance the production of CGTase in *L. lactis*. Glucose (0.5%) was replaced with starch or other carbon sources to facilitate the expression. After several induction conditions using GM17, M17 supplemented with 0.5% starch or 0.5% sucrose or GM17 supplemented with 0.5% starch, a band corresponding to CGTase (75 kDa) was observed for NZ:NSP:CGT, using 0.5% starch as a carbon source. Based on the SDS-PAGE result, the samples from the intracellular fraction did not indicate any differences in the production of the enzyme. However, the production and secretion of this enzyme is

clearly visible in the extracellular samples, as shown by the arrow in figure 3a. Thus, CGTase secretion to the extracellular fraction is only detectable in M17 supplemented with 0.5% starch.

Nisin concentration is another parameter which influences the expression of genes cloned in pNZ8048 downstream of the nisin promoter PnisA. Using higher nisin amount might lead to product inhibition and is costly when applied in large-scale production. Thus, preliminary study was conducted at different concentrations (10, 20, 30 and 40 ng/ml) using NZ:NSP:CGT to determine the optimum nisin concentration. There were no noticeable differences in the intracellular CGTase when different nisin concentrations were used. However, a band cor-

Fig. 4. Optimization of nisin concentration for β -CGTase expression. Lane M: PageRuler Unstained Protein Ladder (Fermentas). Lanes 1–4: intracellular protein after sonication. Lanes 5–8: extracellular protein after protein precipitation. Lanes 1 and 5: induction at 10 ng/ml nisin. Lanes 2 and 6: induction at 20 ng/ml nisin. Lanes 3 and 7: induction at 30 ng/ml nisin. Lanes 4 and 8: induction at 40 ng/ml nisin. In M17 medium with starch, at 20 ng/ml nisin concentration, β -CGTase was successfully secreted to the extracellular environment as indicated by the arrow which shows the 75-kDa band corresponding to this protein.

responding to 75 kDa was visible using the extracellular samples at 20, 30 and 40 ng/ml of nisin (fig. 4). Since the protein was able to be induced at 20–40 ng/ml nisin, the lowest concentration of 20 ng/ml nisin was used for the rest of this study.

Apart from medium modification and inducer concentration, induction interval was analyzed in this study. After an OD₆₀₀ of 0.5 was reached, 20 ng/ml nisin was used to induce the enzyme expression for 3, 6 and 9 h. β -CGTase secretion was visible extracellularly at 3, 6 and 9 h after induction (fig. 5). The shortest time (3 h) was selected as the best induction time throughout the experiment.

Zymogram

Following electrophoretic separation, enzyme activity of CGTase was detected through formation of a clearing zone on the native gel where the substrate was hydrolyzed. Figure 6b shows formation of halo zone in both intra- and extracellular fractions.

CGTase Assay

From the intracellular samples, the highest amount of enzyme was produced at 3 h after induction by NZ:USP:CGT, yielding 5.55 U/ml of CGTase. NZ:NSP:CGT and NZ:SPK1:CGT were shown to produce 4.25 and

Fig. 5. Optimization of induction time for β -CGTase expression. Lane M: PageRuler Unstained Protein Ladder (Fermentas). Lanes 1–4: intracellular protein after sonication. Lanes 5–8: extracellular protein after protein precipitation. Lanes 1 and 4: induction at 3 h. Lanes 2 and 5: induction at 6 h. Lanes 3 and 6: induction at 9 h. In M17 medium with starch, at 20 ng/ml nisin concentration, β -CGTase was successfully secreted to the extracellular environment at 6 and 9 h after induction as indicated by the arrows which show 75-kDa bands corresponding to this protein.

1.95 U/ml enzymes, respectively. From the supernatant (sample) at 3 h after induction, the enzyme activity reached 0.5 U/ml in all the transformants. The enzyme activities in the cellular fractions were approximately 3- to 10-fold higher than the extracellular one. This result suggests that secretion of CGTase in *L. lactis* is inefficient and that a large amount of the protein was still maintained in the cells even with the presence of SPs (NSP from *Bacillus* sp. G1, USP45 or SPK1). Although NZ:SPK1:CGT produced the lowest total amount of CGTase compared to the other strains in this study, it gave the highest secretion efficiency (SE) of 21% at 3 h after induction (table 2). SE is measured by the ratio of mature protein secreted in the supernatant as a fraction of total protein expressed intracellularly and extracellularly. A 6-hour induction was carried out to study the effect of secretion with longer induction time where higher enzyme activity was found at extracellular regions when compared to that of 3-hour samples (fig. 6a). At this time, about 49% of the expressed recombinant β -CGTase was shown to be secreted out into the extracellular medium by NZ:SPK1:CGT. This result suggests that increased induction time may be needed to translocate the proteins more efficiently and that the SP SPK1 seems to work well in the *L. lactis* host used to express the β -CGTase gene.

Fig. 6. a Protein induction (6 h) for β -CGTase expression. Lane M: PageRuler Unstained Protein Ladder (Fermentas). Lanes 1, 2 and 3: intracellular protein for pNZ8048 in *L. lactis*, NZ:NSP:CGT, NZ:USP:CGT and NZ:SPK1:CGT strains, respectively. Lanes 4, 5 and 6: extracellular protein after protein precipitation for pNZ8048 in *L. lactis*, NZ:NSP:CGT, NZ:USP:CGT and NZ:SPK1:CGT strains, respectively. Arrows show 75-kDa bands corresponding to β -CGTase. **b** CGTase zymogram by recombinant strains. Lanes 1, 2 and 3: intracellular protein for pNZ8048 in *L. lactis*, NZ:NSP:CGT, NZ:USP:CGT and NZ:SPK1:CGT strains, respectively. Lanes 4, 5 and 6: extracellular protein after protein precipitation for pNZ8048 in *L. lactis*, NZ:NSP:CGT, NZ:USP:CGT and NZ:SPK1:CGT strains, respectively. The arrow shows β -CGTase activity as indicated by the clearing zones around the protein.

Table 2. Distribution of CGTase activity in different fractions of *L. lactis* harboring the CGTase gene from *Bacillus* sp. G1

Cell fraction	NZ:NSP:CGT		NZ:USP:CGT		NZ:SPK1:CGT	
	I	E	I	E	I	E
<i>3 hours</i>						
CGTase activity, U/ml	4.245	0.633	5.556	0.551	1.947	0.539
Fractions of total activity, %	87.0	13.0	91.0	9.0	78.3	21.7
<i>6 hours</i>						
CGTase activity, U/ml	1.384	1.220	4.022	2.029	1.489	1.431
Fractions of total activity, %	53.1	46.9	66.5	33.5	51.0	49.0

I = Intracellular fraction; E = extracellular fraction. Figures in italics indicate higher secretion efficiency at 3- and 6-hour intervals.

Recombinant CGTase Production in Batch Fermentation

The fermentation profile is shown in figure 7. CGTase was produced from 2 h after addition of 20 ng/ml of nisin. From 2 to 8 h, CGTase production increased rapidly with concurrent rapid consumption of soluble starch until the 11th h where CGTase production begins to reduce slowly and soluble starch reaches almost 0 g/l. This indicates that the recombinant strains were able to utilize the soluble starch. The presence of the SPs permits CGTase secretion to the culture medium, and the starch is rapidly converted to CD by the enzyme in the medium. In the batch fermentation, maximum CGTase activity of 4.23 U/ml was ob-

tained by NZ:SPK1:CGT at 8 h after induction, whereby NZ:NSP:CGT and NZ:USP:CGT recorded a maximum enzyme activity of 4.03 and 3.7 U/ml at 8 and 7 h after induction, respectively. CGTase productivity was calculated by dividing maximum enzyme activity over production time for each recombinant strains. Highest CGTase productivity was achieved by recombinant strains NZ:USP:CGT and NZ:SPK1:CGT (0.53 U/ml·h, respectively) compared to NZ:NSP:CGT (0.5 U/ml·h). Maximum CGTase yield was obtained by NZ:USP:CGT (6.21 U/g of starch) when soluble starch was supplemented as carbon source, whereas strains NZ:NSP:CGT and NZ:SPK1:CGT give yields of 2.36 and 3.45 U/g of starch, respectively.

Fig. 7. CGTase production and soluble starch consumption by recombinant strains. Filled symbols indicate soluble starch consumption and unfilled symbols indicate CGTase production. □ = NZ:SPK1:CGT; △ = NZ:NSP:CGT; ○ = NZ:USP:CGT.

Discussion

In the present study, we describe and compare the expression of the β -CGTase gene of *Bacillus* sp. G1 by the GRAS microorganism, *L. lactis* NZ9000. *L. lactis* was chosen for CGTase production because of the restrictions found in *Bacillus* and *E. coli* as the producers of pharmaceutically important proteins. The *Bacillus* system secretes a large number of hydrolytic enzymes including proteases into the medium that often affect the stability of products [Simonen and Palva, 1993], while *E. coli* is expensive to use and have problematic downstream purification processes as most proteins are commonly targeted to the intracellular (in the periplasm and cytoplasm). In addition, endotoxins or lipopolysaccharides from *E. coli* should be removed from proteins for human administration [Morello et al., 2008].

In this study, a lactic acid bacterium (LAB) was used as a host to express and secrete CGTase extracellularly. The advantages of *L. lactis* include their GRAS status, lack of endotoxins and high protein secretion capacities [Glenting et al., 2007]. Three SPs, the NSP of CGTase, USP45 from *L. lactis* MG1363, and SPK1 from *Pediococcus pentosaceus* K1, were analyzed to study their efficiency in CGTase secretion in the *L. lactis* host.

Plasmids that could not be stably maintained in a host will prevent or decrease the production of the recombinant proteins. Therefore, it is important to use stable plasmids in downstream processes or in large scale protein production. A 100% stability of the three plasmids in *L.*

lactis NZ9000 recombinants for up to 100 generations suggested the potential usage of these recombinant strains for large-scale CGTase production in the future.

The Nisin Controlled Expression system was used where the PnisA promoter was induced with nisin to express downstream genes. Nisin is a bioactive peptide which is synthesized by certain strains of *L. lactis* [de Vuyst et al., 1990] and is used as an inducer to induce protein expression. The nisA gene from the promoter sequence efficiently controls the transcription initiation process and is dependent on the extracellular concentration of the antimicrobial peptide nisin. PnisA has been used for the expression of many heterologous proteins in *L. lactis* such as the *Staphylococcus aureus* nuclease (Nuc) as a secretion reporter [Le Loir et al., 1994], the *Staphylococcus hyicus* lipase [Drouault et al., 2000; van Asseldonk et al., 1990], bovine rotavirus nonstructural protein 4, NSP4 [Ball et al., 1996], human papillomavirus antigen E7 [Bermudez-Humaran et al., 2002], and *Bruceella abortus* antigen L7/L12 [Oliveira and Splitter, 1994; Ribeiro et al., 2002].

The growth medium of a cell plays an essential role in permitting certain enzyme production [Nishida et al., 1999, 2007]. Even though GM17 is known as an optimum growth medium for LAB, it was recently reported that the presence of glucose in the medium initiates repression of CGTase production [Kuo et al., 2009]. Thus, in this study, glucose was substituted with other carbon sources such as starch and sucrose to determine the effect on CGTase production. Whilst Heng et al. [2005] showed that sucrose was able to induce the expression of α -amylase in *E. coli*, in this work CGTase (also a member of the α -amylase family) production could not be similarly initiated in LAB using sucrose. However, the presence of starch influenced the production of CGTase in *L. lactis*. Similar observations were reported by Kuo et al. [2009] and Nishida et al. [1999, 2007] in their studies on CGTase expression in *E. coli* and *Bacillus*. Starch is required at the posttranscriptional level to allow the full-length mRNA transcription to continue through the inverted repeat structure to produce this enzyme, while the presence of glucose represses the transcription process [Kuo et al., 2009; Nishida et al., 1999, 2007]. When CGTase production was examined in the presence of both glucose and starch at equal concentrations, a band size of 75 kDa which corresponded to CGTase was not visible. To further analyze carbon influence, CGTase production was examined under a combination of different glucose and starch concentrations. At constant starch level (0.5%), CGTase production decreased rapidly with an increase

of glucose concentration, while at constant glucose level (0.5%), CGTase production is increased with an increase in starch concentration (fig. 3b). These observations suggest that the production of CGTase in *L. lactis* under the control of PnisA required two different inducers. The first inducer nisin controlled the transcription initiation process [de Ruyter et al., 1996] and the second inducer starch induces posttranscriptional processes. Therefore, M17 supplemented with soluble starch was shown to be an optimum medium to enhance the CGTase production and its secretion in *L. lactis*.

Under these conditions, the active CGTase enzyme was highly expressed and secreted out of the *L. lactis* cells efficiently as shown by the clearing zones on the agar plates (fig. 2). Apart from the formation of halo zones, SDS-PAGE results also indicated that CGTase was secreted into the extracellular environment. *L. lactis* NZ9000 carrying pNZ:NSP:CGT produced CGTase, and at 3 h after induction, the protein was produced intracellularly and was secreted to the extracellular region. The same results were also obtained for both NZ:USP:CGT and NZ:SPK1:CGT, which suggested the ability of these SPs to transport the *B. subtilis* β -CGTase in *L. lactis* comparatively to the NSP. The band at 75 kDa was identified to contain CGTase activity as shown by the clearing zone on the native gel (fig. 6b). The secretion of CGTase to the extracellular environment was possible with the aid of SPs, where the SPs are cleaved off and the mature protein is translocated to the extracellular region.

Protein secretion increased when induction time was increased. This suggests that a longer time is needed to translocate proteins to the extracellular region of *L. lactis*. At 6-hour induction, 46.9% SE was recorded using the native *B. subtilis* SP (NSP), while the use of USP45 and SPK1 demonstrated 33.5 and 49% SE, respectively (table 2). This result indicates that SPK1 is the best SP to secrete CGTase into the supernatant compared to other SPs used in this study, which is in agreement with the bioinformatic analysis results (table 1). Bermudez-Humaran et al. [2006] reported that SE of Nuc (18 kDa) and IFN- ω reached 70% in *L. lactis* after 1-hour induction, whereas a lower secretion of 40% Nuc protein with the fusion of USP45 SP in *L. lactis* was reported by Nouaille et al. [2003]. Relative to these two reports, 49% secretion of CGTase recorded in this study is within the 40–70% range. The remaining CGTase protein precursor did not undergo secretion and remained stacked in the cell, probably associated with the cell envelope. A similar effect was also observed in *L. lactis* strain engineered to secrete NSP4 [Ball et al., 1996] and GroEL protein [Miyoshi et al., 2006]. Even though a low secretion

of protein was observed, *L. lactis* was able to produce and target a significant amount of active CGTase to the extracellular medium. This opens up new possibilities of using SPK1 as a potential SP for protein secretion in *L. lactis* to produce and secrete important enzymes with high molecular weight such as CGTase for pharmaceutical and industrial purposes.

This study pioneered increased CGTase production and secretion by a food grade bacterium using genetic manipulation. The performance of recombinant strains in batch fermentation was further tested. In the batch fermentation, agitation was maintained at 80 rpm to allow mixing of the whole culture to ensure even nutrient distribution and effective nisin distribution for the induction purpose. Almost similar CGTase productivity was observed for the recombinant strains NZ:USP:CGT and NZ:SPK1:CGT at 0.53 U/ml·h, at 7 and 8 h after induction, respectively, while NZ:NSP:CGT exhibited slightly lower enzyme productivity (0.5 U/ml·h). As reported by Ong et al. [2008], the *Bacillus* sp. G1 CGTase productivity was three times higher (1.48 U/ml·h) in the *E. coli* strain after 24 h of cultivation. Generally, the levels of recombinant proteins produced by *L. lactis* and other LAB are low when compared with that produced by *E. coli* [Jana and Deb, 2005] and *B. subtilis* expression hosts [Schallmeyer et al., 2004].

Conclusion

In this study, the recombinant β -CGTase was successfully expressed in the food grade bacterium *L. lactis* NZ9000. SPK1 was shown to be the best SP to secrete β -CGTase efficiently compared to the other SPs used in this study. Starch acts as an inducer and enables the expression of this protein in the absence of glucose. This result suggests a potential usage of *L. lactis* as a host for β -CGTase production in food and pharmaceutical industry and SPK1 as a potential heterologous SP for secretion of proteins in *L. lactis*.

Experimental Procedures

Culture Media, Antibiotics and Incubation Conditions

For *L. lactis*, GM17 broth (M17 broth; Merck, Germany) supplemented with 0.5% glucose) was used as a standard culture medium. A volume of 0.5% glucose was substituted with soluble starch (0.5%) in the protein induction medium. *L. lactis* cultures were incubated statically at 30°C. When required, 7.5 μ g/ml of Cm was added.

Table 3. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid used	Relevant features	References
<i>L. lactis</i> NZ9000	Plasmid-free strain, with the chromosomal gene R and K needed for nisin introduction; host strain for nisin-inducible vector	De Ruyter et al. [1996]
<i>L. lactis</i> MG1363	Wild type, plasmid free	Gasson [1983]
<i>P. pentosaceus</i> (K1)	Isolated from pandan, a local herbal plant in Malaysia	Baradaran et al. [2012]
pNZ8048	3.3 kb, Cm-inducible expression vector carrying PnisA	Kuipers et al. [1998]
pET-21a (+)	5.4 kb, resistant to ampicillin, T7 promoter, carries an N-terminal T7; Tag sequence plus an optional C-terminal His Tag sequence	Novagen, USA
pNZ:NSP:CGT	Modified pNZ8048 containing PnisA promoter upstream His-tagged CGTase gene, Cm	This study
pNZ:USP:CGT	Modified pNZ8048 containing PnisA promoter with upstream of His-tagged CGTase gene without native signal peptide, Cm; native signal peptide of CGTase gene substituted with USP45	This study
pNZ:SPK1:CGT	Modified pNZ8048 containing PnisA promoter upstream of the His-tagged matured region of CGTase gene, Cm; native signal peptide of CGTase gene substituted with SP	This study

Table 4. Oligonucleotide primers used in this study

Primer code number	Primer name	Primer sequence (5'–3')
1	CGT FP	GCCGG <u>GCATGC</u> CG TTG AAC GAT TTA AAT GAT TTT TTG AAA
2	CGT RP	AAA <u>TCTAGA</u> GTG GTG GTG GTG GTG GTG CCA ATT AAT CAT
3	mCGTS FP	CCCG <u>GGATCC</u> ATG GAC GTA ACA AAC AAA GTC AAT TAC TCA
4	SPK1FP	GGCAGT <u>GCA TGC</u> CC ATG AAA AAA ATA TTG ACG
5	SPK1 RP	CCCG <u>GGATCC</u> TCG TAC ATG TAA GCA GCG TCA
6	mCGTU FP	CGG <u>AAG CTT</u> GAC GTA ACA AAC AAA GTC AAT TAC TCA AAA
7	USP45FP	GCCGG <u>GCA TGC</u> AA ATG AAA AAA AAG
8	USP45RP	CGC <u>AAG CTT</u> AGC GTA AAC ACC TGA CAA

Restriction enzyme sites are italicized and underlined. (GCATGC encodes for *Sph*I, TCTAGA encodes for *Xba*I, GGATCC encodes for *Bam*HI and AAG CTT encodes for *Hind*III).

Construction of Plasmid Vector for Expression of CGTase in *L. lactis* NZ9000

Bacterial strains and vectors used in the present study are described in table 3, while PCR primers are summarized in table 4. Taq DNA polymerase, restriction enzymes and T4 DNA ligase were purchased from Fermentas, Canada. The gene encoding CGTase from *Bacillus* sp. G1 (accession: AY770576) was PCR amplified with the SP coding sequence and His Taq coding sequence using primers 1 and 2. The resulting PCR product was digested with *Sph*I (GCATGC) and *Xba*I (TCTAGA), respectively. Ligation to a similarly digested lactococcal vector pNZ8048 was carried out at 16°C, overnight, yielding pNZ:NSP:CGT. The ligation reaction mixture was transformed into competent *L. lactis* NZ9000 [Holo and Nes, 1989], using Gene Pulser (Biorad, USA) with the

field strength of 2.3 kV, 25 µF capacitance and 200 Ω resistance and plated onto SGM17 (M17 agar added with 0.5% glucose and 0.5 M sucrose) agar containing 7.5 µg/ml Cm, yielding NZ:NSP:CGT. For other clones with USP45 or SPK1 SPs, the gene encoding CGTase was PCR amplified without the gene sequence coding for its NSP using primers 3 and 2 (with *Hind*III site) or primers 6 and 2 (*Bam*HI site). USP45 [van Oort et al., 1989] was amplified from the *L. lactis* MG1363 genome using primers 7 and 8, while SPK1 was amplified from *P. pentosaceus*, K1 [Baradan, 2010] (obtained from Microbial Biotech Laboratory, UPM, accession number: UPMC15) using primers 4 and 5. Cassettes of SPs fused with the gene coding for CGTase (without the NSP) were double digested and ligated with similarly digested pNZ8048, yielding pNZ:USP:CGT and pNZ:SPK1:CGT. The ligation mix-

Fig. 8. Schematic design of CGTase and SP cassettes for the production and secretion by *L. lactis*. The diagram shows the nisin-inducible promoter PnisA, His protein sequence His, and SPs (NSP of the CGTase gene, USP45 SP of the usp45 gene, and SPK1 from *P. pentosaceus*).

tures were electro-transformed into competent *L. lactis* NZ9000 and plated onto SGM17 agar containing 7.5 µg/ml Cm, yielding NZ:USP:CGT and NZ:SPK1:CGT. Figure 8 shows the construction of the CGTase and SP cassettes. The resulting plasmids had a C-terminus six-histidine tagged CGTase gene, and DNA sequencing confirmed the nucleotide sequence of the gene.

Signal Peptide Analysis

SP sequences of NSP, USP45 and SPK1 were analyzed based on online tools SignalP3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) and ExPASy (ExPASy Bioinformatics Resource Portal, <http://expasy.org>).

Iodine Plate Assay

β-CGTase activity was detected using the plate assay method. NZ:NSP:CGT, NZ:USP:CGT and NZ:SPK1:CGT were streaked on GM17 agar plate containing 0.5% starch, 7.5 µg/ml Cm and supplemented with 10 ng/ml of nisin as an inducer. The cultured plate was incubated overnight at 30°C. Iodine test was performed by pouring 2 ml of 0.01 M KI₂ solution onto the plates. The formation of halo zone would indicate positive CGTase activity in the extracellular location.

Growth Study

Overnight cultures of *L. lactis* NZ:NSP:CGT, NZ:USP:CGT or NZ:SPK1:CGT were diluted in GM17 broth (1:100). One milliliter of inoculum was used to determine the optical density at 600 nm (OD₆₀₀) using a spectrophotometer (Thermo Scientific, USA). Growth curves were constructed (OD₆₀₀ vs. time) and the doubling time was calculated from the log phase.

Plasmid Stability Test

Plasmid stability test was carried out based on the method by Imanaka and Aiba [1981] with minor modification. NZ:NSP:CGT, NZ:USP:CGT or NZ:SPK1:CGT were grown overnight in GM17 broth supplemented with 7.5 µg/ml Cm at 30°C. Overnight cul-

tures were diluted into fresh 100 ml GM17 broth (without antibiotic) and grown at 30°C. Every 6 h, 100 µl of each culture was inoculated into a fresh 100 ml GM17 broth to maintain the cells in exponential growth. At 100 generation, 100 µl of culture was serially diluted with fresh GM17 broth and spread onto GM17 agar plate without the selection pressure. 100 colonies were randomly picked and cultured on GM17 agar plates with the presence of antibiotic. After 12 h at 30°C, the number of colonies was calculated. Plasmid stability represents the percentage of number of colonies grown on antibiotic plate to the total number of transferred colonies (100):

Plasmid stability =

$$\frac{\text{Number of colonies grown on GM17 plate with Cm}}{\text{Number of colonies transferred}} \times 100\%$$

Protein Induction and Extraction

Overnight cultures of NZ:NSP:CGT, NZ:USP:CGT and NZ:SPK1:CGT were sub-cultured into fresh M17 broth supplemented with 0.5% soluble starch and incubated statically at 30°C with the presence of 7.5 µg/ml Cm. Nisin was added to the medium once the optical density (OD₆₀₀) reached 0.5. Induction was conducted at 30°C for 3 h after which cells were pelleted (16,000 g for 5 min at 4°C) and washed once with distilled water followed by PBS (phosphate-buffered saline). Pellets were resuspended with 500 µl PBS and sonicated using Omni Ruptor 4000 sonicator (Omni International, USA) for 10 s followed by a 30-second pause. This was repeated 10 times. The supernatant was mixed with an equal volume of 2× sample buffer and heated in boiling water for 5 min. The samples were kept at -20°C prior to SDS-PAGE or protein purification. Intracellular proteins were purified using His-Tag purification column procedures following the manufacturer's instructions (GE Healthcare, UK). Extracellular proteins were precipitated using 1/10 of the culture volume of 100% TCA (trichloroacetic acid). The mixtures were vortexed for 15 s and kept on ice for 2 h. The proteins were pelleted down by centrifugation (14,000 g for 10 min). The pellets were then washed twice using acetone and dried. Equal volumes of PBS and 2× sample buffer were added. The proteins were denatured for 3 min at 95°C by boiling in water bath.

Quantification of Recombinant Proteins

The concentration of recombinant proteins in this study were measured based on Bradford method [Bradford, 1976] using the Pro-Pure™ Proteomics Grade Protein assay kit according to the manufacturer's instruction (AMRESCO, USA) at 595 nm.

SDS-PAGE

SDS-PAGE was performed as described by Sambrook [1989]. For SDS-PAGE, 10% SDS-polyacrylamide separating gel and 4% stacking gel were used, and the gels were stained with Coomassie blue stain followed by destaining and gel imaging.

Zymogram

Zymography of CGTases was performed according to Lacks and Springhorn [1980] with slight modifications. The enzyme samples were applied to 10% native PAGE. After gel electrophoresis, the gel was washed twice with distilled water. Then, the gel was immersed in 0.1 M sodium phosphate buffer (pH 7.0) which

contains 4% soluble starch. The gel was incubated for 30 min at 60°C. The gel was washed prior to adding a mixture of 0.3% of KI and 0.1% I₂. Formation of halo zone around the band indicates positive CGTase activity.

Quantification of CGTase

Extracellular CGTase was quantified using a phenolphthalein assay [Kaneko et al., 1987]. The reaction mixture containing 1 ml of 0.04 g starch in 0.1 M sodium phosphate buffer (pH 7.0) and 0.1 ml enzyme solution was incubated at 60°C for 10 min in a water bath. The reaction was stopped by adding 3.5 ml of 0.03 M NaOH solution. A volume of 0.5 ml of 0.02% (w/v) phenolphthalein in 0.005 M Na₂CO₃ was then added to the reaction mixture. After 15 min at room temperature, the decrease in color intensity was measured at 550 nm. One unit of enzyme activity was defined as the amount of enzyme that forms 1 μmol β-CD/min.

Cultivation and Analytical Methods

The recombinant strains were grown in M17 medium containing 0.5% soluble starch supplemented with 7.5 μg/ml Cm. The medium (100 ml in a 250 ml shake flask) was inoculated with 10% (v/v) inoculum and incubated at 30°C with slow agitation at 80 rpm. 20 ng/ml nisin was added to the broth to induce recombinant CGTase production once the culture reached OD₆₀₀ 0.5.

Soluble Starch Analysis

The residual starch content was determined by measuring the light absorption of the iodine-starch complex color [Nakamura, 1981]. Assay mixtures contained 1 ml of appropriately diluted sample, 0.1 ml of iodine solution (0.2 g of I₂ and 2.0 g of KI in 100 ml of distilled water), and sufficient distilled water to bring the volume to 10 ml. The color intensity was measured spectrometrically at 590 nm.

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Disclosure Statement

The authors declare that they have no competing interests.

References

- Ball JM, Tian P, Zeng CQ, Morris AP, Estes MK: Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. *Science* 1996;272:101–104.
- Baradaran A: Development of secretory *Lactococcus lactis* vectors with characterized heterologous signal peptide from *Pediococcus pentosaceus*; thesis, Universiti Putra Malaysia, 2010.
- Baradaran A, Sieo CC, Foo HL, Ilias MR, Khatijah Y, Raha AR: Cloning and in silico characterization of two signal peptides from *Pediococcus pentosaceus* and their function for the secretion of heterologous protein in *Lactococcus lactis*. *Biotechnology Letters*, 2012.10.1007/s10529-012-1059-4.
- Baudin C, Pean C, Perly B, Goselin P: Inclusion of organic pollutants in cyclodextrin and derivatives. *Int J Env Anal Chem* 2000;77:233–242.
- Bermudez-Humaran LG, Langella P, Commissaire J, Gilbert S, Le Loir Y, L'Haridon R, Corthier G: Controlled intra or extracellular production of staphylococcal nuclease and ovine omega interferon in *Lactococcus lactis*. *FEMS Microbiol Lett* 2006;224:307–313.
- Bermudez-Humaran LG, Langella P, Miyoshi A, Gruss A, Guerra RT, Montes de Oca-Luna R, Le Loir Y: Production of human papillomavirus type 16 E7 protein in *Lactococcus lactis*. *Appl Environ Microbiol* 2002;68:917–922.
- Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254.
- Buschmann HJ, Schollmeyer E: Applications of cyclodextrins in cosmetic products a review. *J Cosmet Sci* 2002;53:185–191.
- de Ruyter PG, Kuipers OP, de Vos WM: Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl Environ Microbiol* 1996;62:3662–3667.
- de Vuyst L, Mulders J, de Vos WM, Vandsamme EJ: Phenotypic and genotypic characterization of nisin-producing *Lactococcus lactis* subsp. *Lactis* strains; in Abstract Book of the 6th International Symposium on Genetics of Industrial Microorganisms, Strasbourg, August 1990, p 186, D-62.
- Driessen AJM, Nouwen N: Protein translocation across the bacterial cytoplasmic membrane. *Annu Rev Biochem* 2007;77:643–667.
- Drouault S, Corthier G, Ehrlich SD, Renault P: Expression of the *Staphylococcus hyicus* lipase in *Lactococcus lactis*. *Appl Environ Microbiol* 2000;66:588–598.
- Gasson MJ: Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic acid streptococci after protoplast-induced curing. *J Bacteriol* 1983;154:1–9.
- Gawande BN, Patkar AY: Application of factorial designs for optimization of cyclodextrin glycosyltransferase production from *Klebsiella pneumoniae* AS-22. *Biotechnol Bioeng* 1999;64:168–173.
- Glenting J, Poulsen LK, Kato K, Madsen SM, Frokiaer H, Wendt C, Sorensen HW: Production of recombinant peanut allergen Ara h 2 using *Lactococcus lactis*. *Microb Cell Fact* 2007;6:28.
- Heng C, Chen Z, Du L, Lu F: Expression and secretion of an acid-stable α-amylase gene in *Bacillus subtilis* by SacB promoter and signal peptide. *Biotechnol Lett* 2005;27:1731–1736.
- Hirose T, Yamamoto Y: Hinokitol containing cyclo olefin polymer compositions and their molding with excellent antimicrobial and gas barrier properties. Japanese Patent 2001, JP 55480.
- Holo H, Nes IF: High frequency transformation by electroporation of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl Environ Microbiol* 1989;55:3119–3123.
- Imanaka T, Aiba S: A perspective on the application of genetic engineering: stability of recombinant plasmid. *Ann New York Acad Sci* 1981;369:1–14.
- Jana S, Deb JK: Strategies for efficient production of heterologous proteins in *Escherichia coli*. *Appl Microbiol Biotechnol* 2005;67:289–298.

- Kaneko T, Kato T, Nakamura N, Horikoshi K: Spectrophotometric determination of cyclization activity of β -cyclodextrin-forming cyclomaltodextrin glucanotransferase. *J Jpn Soc Starch Sci* 1987;34:45–48.
- Kuipers, OP, de Ruyter PG, Kleerebezem M, de Vos WM: Quorum sensing-controlled gene expression in lactic acid bacteria. *J Biotechnol* 1998;64:15–21.
- Kuo CC, Lin CA, Chen JY, Lin MT, Duan KJ: Production of cyclodextrin glucanotransferase from an alkalophilic *Bacillus* sp. by pH-stat fed-batch fermentation. *Biotechnol Lett* 2009;31:1723–1727.
- Lacks SA, Springhorn SS: Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. *J Biol Chem* 1980;255:7467–7473.
- Le Loir Y, Azevedo LV, Oliveira SC, Freitas DA, Miyoshi A, Bermudez-Humaran LG, Nouaille S, Ribeiro LA, Leclercq S, Gabriel JE, Guimaraes VD, Oliveira MN, Charlier C, Gautier M, Langella P: Protein secretion in *Lactococcus lactis*: an efficient way to increase the overall heterologous protein production. *Microb Cell Fact* 2005;4:2.
- Le Loir Y, Gruss A, Ehrlich SD, Langella P: Direct screening of recombinants in gram-positive bacteria using the secreted staphylococcal nuclease as a reporter. *J Bacteriol* 1994;176:5135–5139.
- Mabuchi N, Nagao M: Controlled release powdered flavour preparations and confectioneries containing preparations. Japanese Patent 2001, JP 128638.
- Miyoshi A, Bermudez-Humaran LG, Ribeiro LA, Le Loir Y, Oliveira SC, Langella P, Azevedo V: Heterologous expression of *Brucella abortus* GroEL heat-shock protein in *Lactococcus lactis*. *Microb Cell Fact* 2006;5:14.
- Morello E, Bermudez-Humaran LG, Llull D, Sole V, Miraglio N, Langella P, Poquet I: *Lactococcus lactis*, an efficient cell factory for recombinant protein production and secretion. *J Mol Microbiol Biotechnol* 2008;14:48–58.
- Nakamura LK: *Lactobacillus amylovorus*, a new starch-hydrolyzing species from cattle waste-corn fermentations. *Int J Syst Bacteriol* 1981;31:56–63.
- Natale P, Brüsser T, Driessen AJ: Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane-distinct translocases and mechanisms. *Biochim Biophys Acta* 2008;1778:1735–1756.
- Nishida T, Nakamura A, Masaki H, Uozumi T: Transcriptional regulation of *Bacillus ohbensis* cyclodextrin glucanotransferase gene in *B. subtilis*. *Biosci Biotechnol Biochem* 1999;63:1902–1909.
- Nishida T, Nakamura A, Masaki H, Uozumi T: Regulation of cyclodextrin glucanotransferase synthesis in *Bacillus ohbensis*. *FEMS Microbiol Lett* 2007;149:221–226.
- Nouaille S, Ribeiro LA, Miyoshi A, Pontes D, Le Loir Y, Oliveira SC, Langella P, Azevedo V: Heterologous protein production and delivery systems for *Lactococcus lactis*. *Genet Mol Res* 2003;2:102–111.
- Oliveira SC, Splitter GA: Subcloning and expression of the *Brucella abortus* L7/L12 ribosomal gene and T-lymphocyte recognition of the recombinant protein. *Infect Immun* 1994;62:5201–5204.
- Ong RM, Goh KM, Mahadi NM, Hassan O, Raja Noor Zaliha RAR, Rosli MI: Cloning, extra-cellular expression and characterization of a predominant β -CGTase from *Bacillus* sp. G1 in *E. coli*. *J Ind Microbiol Biotechnol* 2008;35:1705–1714.
- Ribeiro LA, Azevedo V, Le Loir Y, Oliveira SC, Dieye Y, Piard JC, Gruss A, Langella P: Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Appl Environ Microbiol* 2002;68:910–916.
- Sambrook J: EFTM: Molecular Cloning: A Laboratory Manual, ed 2. New York, Cold Spring Harbor Laboratory Press, 1989.
- Schallmeyer M, Singh A, Ward OP: Developments in the use of *Bacillus* species for industrial production. *Can J Microbiol* 2004;50:1–17.
- Simonen M, Palva I: Protein secretion in *Bacillus* species. *Microbiol Rev* 1993;57:109–137.
- Stella VJ, Rajewski RA: Cyclodextrins: their future in drug formulation and delivery. *Pharm Res* 1997;14:556–567.
- Tonkova A: Bacterial cyclodextrin glucanotransferase. *Enzyme Microb Technol* 1998;22:678–686.
- van Asseldonk M, Rutten G, Oteman M, Siezen RJ, de Vos WM, Simons G: Cloning of *usp45*, a gene encoding a secreted protein from *Lactococcus lactis* subsp. *lactis* MG1363. *Gene* 1990;95:155–160.
- van Oort, MG, Deveer AM, Dijkman R, Tjeenk ML, Verheij HM, de Haas GH, Wenzig E, Gotz F: Purification and substrate specificity of *Staphylococcus hyicus* lipase. *Biochemistry* 1989;28:9278–9285.
- Wells JM, Robinson K, Chamberlain LM, Schofield KM, Le Page RW: Lactic acid bacteria as vaccine delivery vehicles. *Antonie van Leeuwenhoek* 1996;70:317–330.