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Production of galactooligosaccharides using a hyperthermophilic β-galactosidase in permeabilized whole cells of *Lactococcus lactis*

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ABSTRACT

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.

Key words: galactooligosaccharide, prebiotics, *Sulfolobus*, extremophile

INTRODUCTION

Prebiotics are non- 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* (Rodriguez-Colinas 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

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Table 1. Bacterial strains and plasmids

Strain/plasmid	Features[1]	Source or reference[2]
Bacterial strain		
Escherichia coli DH5α	Plasmid free cloning host	Invitrogen
E. coli ER2925	Plasmid free cloning host; Dam^-	NEB
Lactococcus lactis LM0230	Plasmid cured derivative of *Lc. lactis* C2	DCC
Plasmid		
pUC57	*E. coli* cloning vector; Amp^r	GenScript
pUC105	*E. coli* cloning vector containing *lacSt*; Amp^r	This study
pDOC99	Transcription fusion of *nisA* promoter to *lacZ* gene from *Streptococcus thermophilus*; 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).

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.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

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

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 5′-GCGAATGCATC-TAGATCCCC-3′ and LacSt R 5′-GGTGGTGTCGAC-CAGCTATGACCATGATTACGCC-3′, 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 μ*M* deoxyribonucleotide triphosphates (dNTP), 0.5 μ*M* concentration of each primer, 100 ng of template