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A Selected Probiotic Strain of *Lactobacillus fermentum* CM33 Isolated from Breast-Fed Infants as a Potential Source of β-Galactosidase for Prebiotic Oligosaccharide Synthesis

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Lactic acid bacteria from healthy breast-fed infants were isolated and screened for β-galactosidase production in MRS broth. Among 49 isolates that exhibited the yellow clear zone on MRS agar supplemented with bromocresol blue, the isolate CM33 was selected as being the highest β-galactosidase producer and was identified as *Lactobacillus fermentum* based on its morphological characteristics and 16S rDNA nucleotide sequence. *L. fermentum* CM33 exhibited a good survival rate under the simulated stomach passage model, comparable to known probiotic strains *L. gallinarum* JCM2011 and *L. agilis* JCM1187. *L. fermentum* CM33 was antagonistic to pathogenic bacteria *Listeria monocytogenes*, *Escherichia coli* 0157:H7, *Salmonella typhi*, and *Salmonella enteriditis*, using the well diffusion method. In addition, the selected lactobacilli exhibited a high growth rate when cultivated in modified MRS containing commercial galactooligosaccharide (GOS) as a sole carbon source, as well as in glucose. A preliminary study on the enzymatic synthesis of oligosaccharide using crude β-galactosidase revealed the capability for oligosaccharide synthesis by the transgalactosylation activity.

Keywords: β-galactosidase, lactic acid bacteria, *L. fermentum*, prebiotic oligosaccharide synthesis

Introduction

The lactose-hydrolyzing enzyme β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is accepted as an important enzyme in the dairy industry for the production of low-lactose food products to overcome the problems of the lactose intolerant consumer (Sani *et al.*, 1999), while it has also been used in the treatment of cheese whey for the reduction of environmental pollution caused by lactose (Rouwenhorst *et al.*, 1989). β-Galactosidases have been commercially produced from various sources, including plants, animal organs, bacteria, yeast, and fungi (Jurado *et al.*, 2002). However, the bacterial sources are preferable for various reasons including their simple process of fermentation, high levels of activity and good stability of the enzyme (Sani *et al.*, 1999). As the utilization of microbial β-galactosidase is mostly concerned with food products, particular attention has been paid to a special group of microorganisms, which are generally recognized as safe (GRAS). Lactic acid bacteria (LAB) are GRAS members that have been recognized as safe microbes and have been used in food-related products for a long time. LABs constitute a diverse group of lactococci and streptococci, within which lactobacilli have become a focus of scientific studies for three particular reasons. The first, lactose maldigesters may consume some fermented dairy products with little or no adverse effects. The second, the enzyme derived from GRAS members such as LAB can be used without extensive purification (Vasiljevic and Jelen, 2002). The last reason is that some strains have probiotic activity, such as an improvement of lactose digestion and microbial balance in the distal part of the gastro-intestinal system, which directly contributes specific benefits to the consumer (Vinderola and Reinheimer, 2003; Ljungh and Wadstrom, 2006).

Recent investigations of β-galactosidase have been widely aimed toward application of the transgalactosylation, or reverse hydrolysis reaction that leads to the formation of di-, tri-, or higher galactooligosaccharides (GOS) (Rabiu *et al.*, 2001; Hsu *et al.*, 2005; Kim *et al.*, 2006; Lu *et al.*, 2007). GOS have attracted particular interest because of their beneficial effects on human health as a prebiotic food ingredient, which promotes the growth of bifidobacteria and lactobacilli, the well-known health-promoting bacteria (Salminen *et al.*, 1995; Kim *et al.*, 2006; Splechtna *et al.*, 2006). The beneficial effects of GOS recently lead to the development of functional foods against colon cancer and also in the formulation of infant food to function as an immune moderator (Saulnier *et al.*, 2009). Although commercial prebiotic GOS is obtained by the enzymatic hydrolysis of galactose-containing polysaccharides, the GOS synthesized by β-galactosidase from the probiotic microbe is an alternative and believed to be higher in prebiotic efficiency (Rabiu *et al.*, 2001). In addition, most

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of the β-galactosidases used in the study of GOS synthesis have not yet been approved for application in food processing. Some are expensive and not available in sufficient quantities for industrial application (Albayrak and Yang, 2002; Hsu *et al.*, 2005).

In the present study, lactic acid bacteria were isolated from healthy-infant feces and their capability in β-galactosidase production was used as a criterion for selection. *In vitro* probiotic and antagonistic properties of the selected strain were also investigated. In addition, β-galactosidase produced by the selected strain was also preliminarily investigated for transgalactosylation activity involved in prebiotic oligosaccharide synthesis.

Materials and Methods

Isolation and screening of β-galactosidase producer

Fecal samples were collected from healthy, breast-fed infants aged approximately 1-6 months and used as a source of lactic acid bacteria. One gram of fresh fecal sample was enriched in 10 ml MRS broth covered with 1 ml of sterile liquid paraffin at 37°C for 24 h. Then, serial decimal dilution was performed using 0.85% (w/v) NaCl solution as a diluent and the appropriate dilution was poured onto the MRS agar supplemented with 125 ppm bromocresol purple (Fisher Scientific, UK) and incubated at 37°C under anaerobic conditions for 24 h. Oval-shaped colonies surrounded by the yellow zone were selected and maintained on MRS agar slants. For screening of β-galactosidase producers, seed inoculants were prepared by transferring the individual colonies of newly isolated LABs into 10 ml MRS broth followed by incubation at 37°C for 12 h. A 1.0 ml inoculum, with a population of ca 10^9 CFU/ml was transferred to 50 ml sterile MRS broth in a 100-ml Duran bottle and incubated at 37°C for 24 h. The LAB cells were harvested by centrifugation at 4,200×g and resuspended in 5 ml of 0.1 M phosphate buffer pH 7.0, then disrupted by a sonicator. Cell debris was removed by centrifugation at 12,000×g at 4°C for 10 min and the supernatant was used as a crude enzyme solution.

Bacterial identification

Characterization and identification of the selected microorganisms were perfomed by Gram straining, gas production, catalase test and 16S rDNA sequence analysis. DNA was isolated from LAB cells grown in MRS broth and purified by the modified method of Marmur (1961). The 16S rRNA gene was sequenced and the nucleotide sequence obtained (1,418 bp) was aligned with the selected putative homolog sequences obtained from BLASTn searches of the GenBank/EMBL/DDBJ database by employing CLUSTAL_X (Thompson *et al.*, 1997). The phylogenetic tree was constructed by the Neighbor-Joining distance-based method (Saitou and Nei, 1987) in the MEGA program version 2.1. The confidence values of the phylogenetic tree branches were determined using bootstrap analyses based on 1,000 random resemblings. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of these strains is JF414736.

In vitro assessment of probiotic properties: studies on resistance to gastrointestinal conditions

Lactobacillus fermentum CM33 and two reference probiotic strains, *Lactobacillus gallinarum* JCM2011 and *Lactobacillus agilis* JCM1187, were cultured at 37°C overnight in MRS broth and harvested by centrifugation at 4,200×g for 15 min at 4°C. Cell pellets were washed once in sterile phosphate buffer and then resuspended in electrolyte solution containing 6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$, and 1.2 g/L $NaHCO_3$. A 1 ml aliquot was aseptic removed, serially diluted in 0.85% (w/v) NaCl and spread on MRS agar to determine the number of survival (CFU/ml) at time 0 as described by Vizoso-Pinto *et al.* (2006). To simulate the dilution and possible hydrolysis of bacteria in the human oral cavity, 1 ml of cell suspension was mixed with the same volume of sterile electrolyte solution, lysozyme (Wako Pure Chemical Industries LTD., Japan) was added to obtain a final concentration of 100 ppm, and the sample was then incubated for 5 min at 37°C. The sample was subsequently diluted 3:5 with an artificial gastric fluid consisting of 0.3% (w/v) pepsin (Fluka Biochemika, Germany) in the electrolyte solution, with the pH adjusted to 2.5. After 1 h of incubation at 37°C, a 1 ml aliquot was removed, prepared 10-fold serial dilutions in 0.85% (w/v) NaCl and spread on MRS agar. For simulation of the conditions in the small intestine, the remaining volume was diluted 1:4 using an artificial duodenal secretion (pH 7.2) consisting of 6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl, 0.5% (w/v) bile salts (Oxgall, Merck, Germany), and 0.1% (w/v) pancreatin (Fluka Biochemika, Germany). One milliliter aliquots were again removed after 2 and 3 h of incubation at 37°C, prepared 10-fold serial dilutions in 0.85% (w/v) NaCl and spread on MRS agar to determine the viable cell number. The differential value of bacterial survival after 3 h exposed to the simulated gastrointestinal conditions was determined and significance was tested by One-way ANOVA and Tukey's HSD test at the 5% ($P<0.05$) level.

Enzyme preparation and its activity assay

To prepare the enzyme solution, LAB cells in the culture were harvested by centrifugation at 4,200×g for 10 min at 4°C, washed twice with 0.1 M sodium phosphate buffer (pH 7.0), then resuspended in phosphate buffer pH 7.0. The cell suspension, maintained in an ice bath, was disrupted by sonication with 40% amplitude for 30 min (Vibra-Cell, SONICS) and then centrifuged at 12,000×g for 10 min. Precipitated cell debris was discarded and the supernatant was used as the enzyme solution. The β-galactosidase activity was assayed according to the method described by Hsu *et al.* (2005). The reaction mixture containing 0.15 ml of 4 mM ortho-nitrophenyl-β-D-galactopyronoside (ONPG) in 0.1 M sodium phosphate buffer, pH 7.0 and 0.15 ml of enzyme solution was mixed and incubated at 37°C for 30 min. The reaction was stopped by adding 0.7 ml of 0.5 M sodium carbonate solution (pH 10). The amount of *o*-nitrophenol released in the reaction mixture was measured by the absorbance at 420 nm using a spectrophotometer (Shimazu, Japan). One unit of the enzyme was defined as the amount of enzyme that released 1 micromole of *o*-nitrophenol from the sub-

strate per min.

Investigation of antagonistic activity against pathogenic bacteria

The well diffusion test as described by Mayr-Harting *et al.* (1972) was used to study the antagonistic activity of the cell-free culture supernatants (CFCS) obtained from culture of the selected strain in MRS broth at 37°C for 24 h. The supernatant obtained after cell removal by 12,000×g centrifugation at 4°C for 10 min was used as CFCS. The antagonistic activity was investigated against a variety of gastrointestinal pathogenic strains including *Salmonella typhi*, *Salmonella enteritidis*, *Shigella dysenteriae*, and *Escherichia coli* 0157:H7. The food-borne pathogen *Listeria monocytogenes* was also investigated. The clinical strains of *S. typhi* and *S. dysenteriae* were kindly provided from the Division of Clinical Microbiology, Faculty of Associated Medical Science, Chiang Mai University. *E. coli* 0157:H7 and *S. enteriditis* DMST17368 and *L. monocytogenes* DMST10373 were obtained from the Department of Medical Science Type Culture Collection, Thailand (DMST). The neutralized CFCS was prepared by adjusting the pH to 7.0 by sterile 5 M NaOH.

Antagonistic property of *L. fermentum* CM33 grown in mixed culture was also investigated. A cell suspension of 10^6 CFU was co-cultivated with the pathogenic gastrointestinal bacteria *E. coli* 0157:H7 and *S. typhi* in 100 ml MRS broth containing 0.5% (w/v) glucose as a sole carbon source, at 37°C. Culture sampling was carried out at 3-h intervals and viable cells were determined using MRS, Salmonella-Shigella and EMB agar for *L. fermentum* CM33, *S. typhi*, and *E. coli* 0157:H7, respectively.

Effect of various carbon sources on enzyme production

Seed inoculum was prepared by culturing *L. fermentum* CM33 in 50 ml MRS broth at 37°C for 24 h. Bacterial cells were harvested by centrifugation at 4,200×g and washed twice with sterile 50 mM phosphate buffer, pH 7.0. To reduce the effect of glucose suppression that might occur, bacterial cells were aseptically re-suspended in sterile glucose-deleted MRS and cultivation was continued at 37°C for 6 h. Cells were harvested and washed again with the same buffer, then, approximately 10^8 CFU were inoculated into 100 ml of enzyme production medium containing 10 g carbon source, 10 g peptone, 10 g yeast extract, 5.0 g $(NH_4)_2SO_4$, 3.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, and 0.3 g L-cysteine per L (Onishi and Tanaka, 1997) and were statically cultivated at 37°C for 24 h. The populations of viable LAB cells and β-galactosidase activity produced by the selected strain were then determined as described previously. The carbon sources investigated in this experiment were glucose, galactose, mannose, lactose, maltose, cellobiose, lactitol, and sorbitol.

Growth of the selected LAB on prebiotic oligosaccharides

Glucose-free inoculum was prepared as described previously by culturing *L. fermentum* CM33 in 10 ml MRS broth at 37°C for 24 h. Cells were harvested by centrifugation at 4,200×g at 4°C for 5 min and washed twice with 10 ml of sterile 50 mM phosphate, pH 7.0. Then cells were resuspended into appropriate volumes of the same buffer. A glucose-free inoculum of approximately 10^8 CFU was transferred into modified MRS medium containing 1% (w/v) of fructooligosaccharides (FOS) (Wako Pure Chemical Industries, Japan), or galactooligosaccharide (GOS) (Wako Pure Chemical Industries, Japan) as a sole carbon source. MRS broths with either 1% (w/v) glucose or no carbon source added were used as control treatments. Bacterial growth was determined at 3-h intervals as described previously. Enzyme activity of β-galactosidase at the final step was also determined.

Enzymatic synthesis of oligosaccharides

Oligosaccharide synthesis by the transgalactosylation activity of β-galactosidase was investigated according to the method described by Moller *et al.* (2001). Crude β-galactosidase (10 units) was incubated with 0.4 M lactose in 0.1 M phosphate buffer, pH 7.0, in a reaction volume of 1 ml at 40°C for 20 h. The synthesized product formed was analyzed by thin layer chromatography using butanol: ethanol: water in the ratio of 5:3:2 as a mobile phase. The migrated component was visualized by dipping the TLC plate into 5% (v/v) H_2SO_4 in methanol and heating it at 150°C for 5 min (Kitaoka *et al.*, 2005).

Results and Discussion

Isolation of lactic acid bacteria and screening for β-galactosidase producers

Forty-nine LABs were isolated from the feces collected from 10 breast-fed infants based on the appearance of yellow zones around the colonies on MRS agar supplemented with 125 ppm bromocresol purple. Morphological study by Gram staining revealed that 38 isolates were Gram-positive rods and that the other 11 isolates were Gram-positive cocci. The number of bifidobacteria and lactic acid bacteria in the stool of breast-fed infants was reported to be higher than those of bottle-fed children (Hopkins *et al.*, 2005). In addition, breast milk was assumed to be a natural source of LAB for new born, acquired through breast-feeding (Solis *et al.*, 2010). Although the isolation of lactic acid bacteria from either adult or infant fecal samples is not a new idea, bacteria with glycosidase producing capability are still attracting interest because of their transglycosylation activity in the synthesis of oligosaccharides, which are useful for synbiotic formulation in functional food development (Rastall and Maitin, 2002). For this study, the β-galactosidase produced by the newly isolated LAB is our main objective. In order to screen for β-galactosidase producers, pre-activated LABs were inoculated into 10 ml of MRS broth and incubated at 37°C for 24 h. The results indicated that among 49 bacterial isolates, the isolate CM33 produced the highest β-galactosidase activity, up to 0.42 units per 100 ml culture broth, and it was selected for further studies.

Strain CM33 identification

Staining and preliminary observation of cell morphology under a scanning electron microscope revealed that the se-

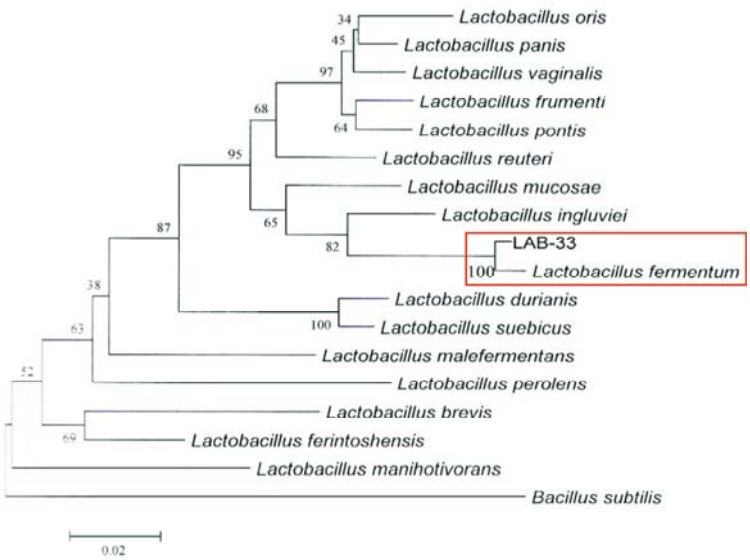

Fig. 1. Phylogenetic tree of *L. fermentum* CM33 and related species.

lected strain was a Gram-positive, rod-shaped lactic acid bacterium. The catalase test was negative, but the isolate was positive for gas production. Based on the analysis of 1,418 bp 16S rDNA nucleotide sequence, the isolate CM33 showed 100% identity to *L. fermentum* strain KLDS 1.0602 (Accession no. EU419589). The results from the phylogenetic tree construction (Fig. 1) confirmed that the selected strain was correctly identified and assigned as *L. fermentum* CM33. It is reasonable that the new isolate was *L. fermentum*, as some previous reports concluded that the most common species of lactobacilli and bifidobacteria isolated from GI tracts are *L. plantarum*, *L. fermentum*, *L. salivarius*, *B. bifidum*, *B. longum*, and *B. infantis* (Vlkova *et al.*, 2005; Slover and Danziger, 2008). However, changes in diet, climate, aging, medication, illness, stress or infection can lead to an increase or decrease of lactic acid bacteria (Mitsuoka, 1992). Generally, lactobacilli have been reported to have a lower content of

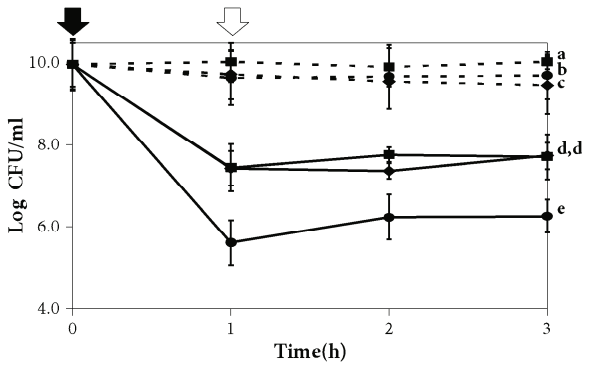

Fig. 2. Effect of simulated gastric and intestinal transit on viability (log10 CFU/ml). *L. fermentum* CM33 (■), *L. agilis* JCM1187 (●), *L. gallinarum* JCM2011 (◆). Dashed lines represent control conditions at pH 7. Black and white arrows indicate addition of simulated gastric juice after 1 h and simulated duodenal juice after 2 and 3 h, respectively. Each point represents the mean±standard deviation (SD) of three replicates. The letters a, b, c, d, and e are a statistical comparison between groups using ANOVA and Tukey's HSD test.

β-galactosidase than bifidobacteria (IDF, 1999); however, the highest activity of β-galactosidase was found in lactobacilli, such as *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus*, for which the activity was higher than that in bifidobacteria (Vinderola and Reinheimer, 2003).

Studies on resistance to gastrointestinal conditions

L. fermentum CM33 was investigated for probiotic properties. An *in vitro* model was used for the investigation of CM33's probiotic features, along with two reference strains (*L. gallinarum* JCM2011 and *L. agilis* JCM1187). The effect of artificial saliva, and gastric and duodenal juices on the viability of *L. fermentum* CM33 is presented in Fig. 2. The statistical comparison of the viability for each species at the the end of the treatments revealed that the surviving capability of *L. fermentum* CM33 against the artificial gastrointestinal conditions was clearly comparable to *L. gallinarum* JCM2011, while it was better than that of *L. agilis* JCM1187. *L. fermentum* is considered a normal inhabitant of the human intestinal tract, with some strains able to metabolize cholesterol and colonization seems to be an age-dependent process (Marika and Mihkel, 2009). There are many reports that have revealed the probiotic properties of various strains of this bacterium. Marika and Mihkel (2009) reported the ability of *L. fermentum* ME-3 to protect the host from various diseases induced by pathogenic bacteria such as *Salmonella* spp. and *Shigella* spp., and also against inflammation and oxidation stress. Furthermore, these lactic acid bacteria showed unique properties among *Lactobacillus* spp., having both antimicrobial and physiologically effective antioxidative properties. Oral administration of *L. fermentum* 5716 for 2 weeks significantly reduced the colonic damage score, i.e. the extent of colonic necrosis and/or inflammation, in the TNBS model of rat colitis (Peran *et al.*, 2006). *L. fermentum* AD1, *L. fermentum* RC-14, and *L. fermentum* isolated from Thai traditional fermented foods have also been reported to possess probiotic properties due to their tolerance of GI tract conditions and their adhesion to various

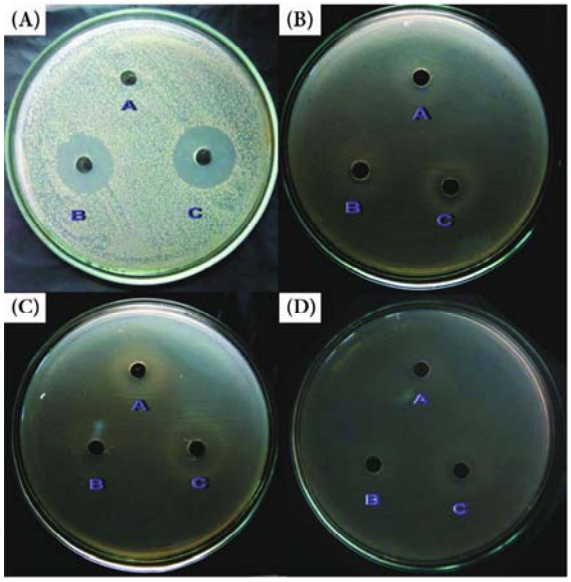

Fig. 3. Agar well diffusion assay of cell-free culture supernatant (CFCS), obtained from *L. fermentum* CM33 culture in MRS broth, against *L. monocytogenes* (A), *E. coli* 0157:H7 (B), *S. typhi* (C) and *S. enteritidis* (D); A, control (MRS broth); B, neutralized CFCS (pH 7.0); and C, unneutralized CSCF (pH 4.3)

intestinal cells (Reid *et al.*, 1993; Klayraung *et al.*, 2008). Based on the resistance of *L. fermentum* CM33 to simulated GI conditions, and previous reports concerned with this bacterial species, we are quite confident that *L. fermentum* CM33 can be used in food and food-related applications as an effective probiotic strain.

Investigation of antagonistic activity

A well diffusion method was used to investigate the antagonistic activity of *Lactobacillus* isolates from infant feces. CFCS from *L. fermentum* CM33 demonstrated an inhibitory effect against all investigated pathogenic bacteria (Fig. 3). The most strongly inhibitory effect was observed against *Listeria monocytogenes* in a non-pH-adjusted sample (Fig. 3A), while the neutralized CFCS was slightly less active. *E. coli* 0157:H7, *S. thypi* and *S. enteriditis* were also sensitive to CFCS of *L. fermentum* CM33, however, inhibition was found only for unneutralized CFCS. This indicates that *E. coli* 0157:H7 and *S. thypi* and *S. enteriditis* were inhibited by the acidic condition of CFCS from the *L. fermentum* CM33 culture, which corresponded to the final pH of 4.3 when measured after cultivation at 37°C for 24 h. The inhibitory effect on *L. monocytogenes* appears to have resulted mainly from bacteriocin and/or bacteriocin-like metabolites produced by *L. fermentum* CM33. Generally, an unneutralized CFCS always shows an inhibitory effect because of the acidic pH, which is not suitable for the growth of most pathogenic bacteria, whereas the inhibitory properties found in neutralized CFCS are commonly caused by bacteriocin or bacteriosin-like metabolites (Montville and Kaiser, 1993). Our findings corresponded to the report of Arici *et al.* (2004) who found that four isolates of *L. fermentum* isolated from infant feces showed antimicrobial activity against pathogenic bacteria by the production of bacteriocin and/or bacteriocin-like materials.

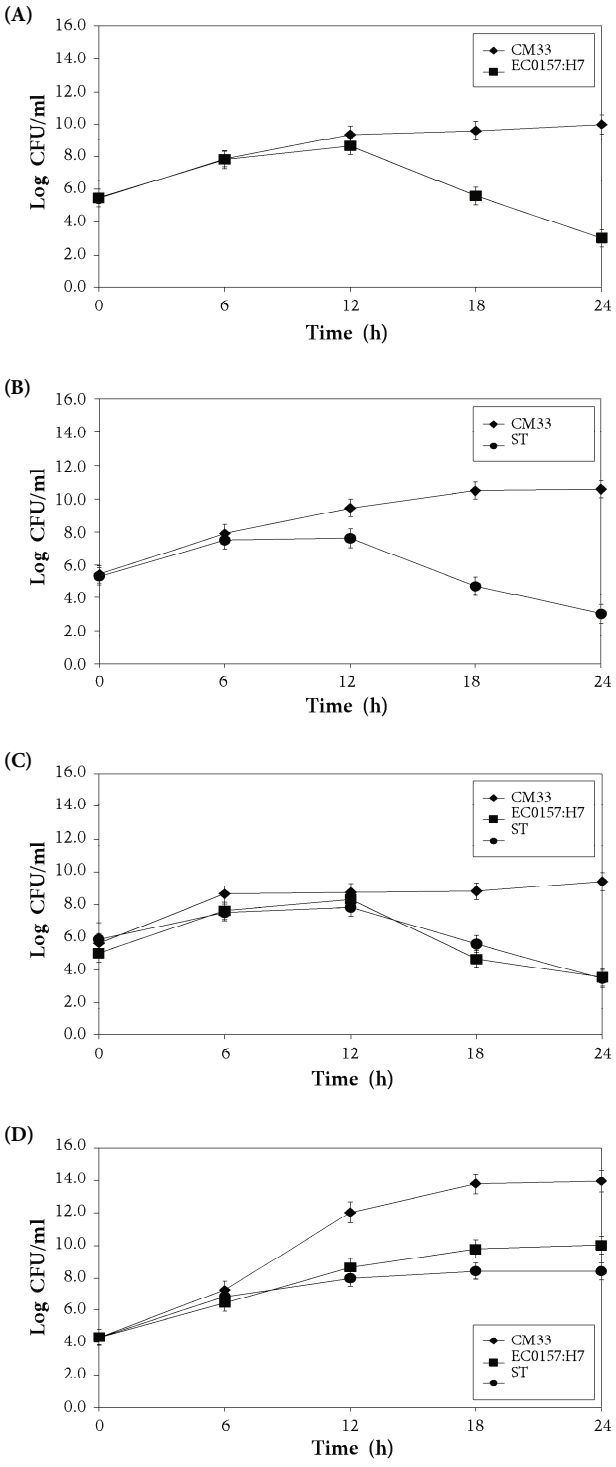

Fig. 4. The inhibitory effect of *L. fermentum* CM33 cultivated in MRS broth against *E. coli* 0157:H7 (A), *S. typhi* (B), mixed culture of *E. coli* 0157:H7, and *S. typhi* (C) and for comparison, axenic cultures of each strain grown separately in MRS broth (D); CM33, *L. fermentum* CM33; EC0157:H7, *E. coli* 0157:H7 and ST, *S. typhi*. Each point represents the mean±SD of three replicates.

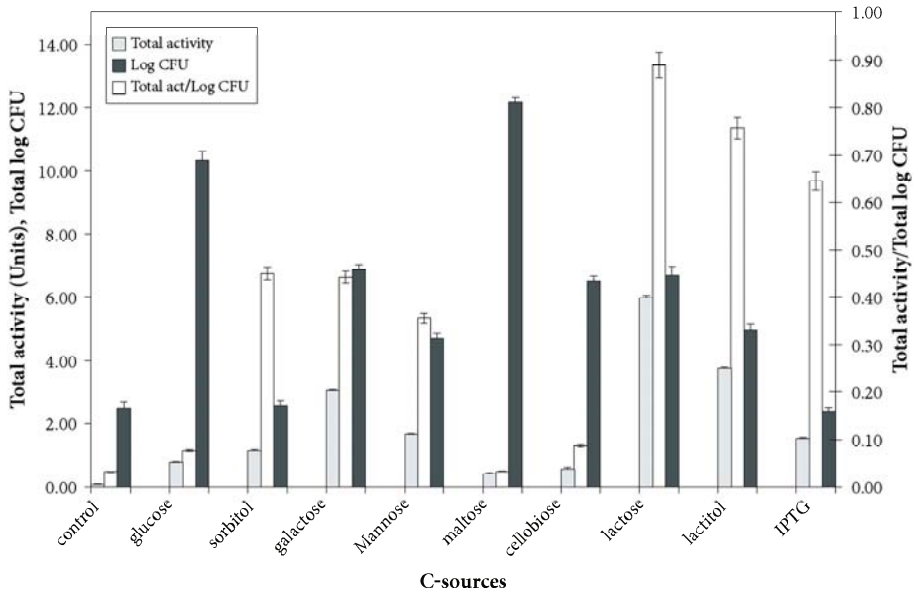

Fig. 5. Effect of various carbon sources on β-galactosidase production and the growth of *L. fermentum* CM33 at 37°C in MRS medium containing 1% (w/v) of each carbohydrate as a sole carbon source. Each bar represents the mean± SD of three replicates.

Antagonistic activity in mixed culture

Because pathogenic bacteria can cause serious gastrointestinal diseases in humans, 2 pathogenic strains, *E. coli* 0157:H7 and *S. typhi*, were selected to investigate the effectiveness of *L. fermentum* CM33 during mixed culture. In mixed cultures, *L. fermentum* CM33 clearly demonstrated antagonistic activity against these two important gastrointestinal pathogens. The number of *E. coli* 0157:H7 clearly decreased after 12 h of mixed cultivation with *L. fermentum* CM33 in MRS broth containing 1% (w/v) glucose (Figs. 4A and C), while the same decreasing pattern was also observed with *S. typhi* (Figs. 4B and C). Although MRS broth is not the most suitable medium for *E. coli* and *S. typhi*, both were capable of growth in single culture of each strain (Fig. 4D) and this supports that *L. fermentum* CM33 inhibited their growth, either directly or through its metabolites generated during growth in mixed culture. Our result supports the idea that *L. fermentum* CM33 could be used as a probiotic strain to help ward off pathogenic bacteria in the GI tract through development of a synbiotic neutraceutical.

Enzyme production using various carbon sources

The result of β-galactosidase production in media containing various sugars as a sole carbon source is shown in Fig. 5. With consideration of either total activity of β-galactosidase or the ratio of total activity to cell number (CFU), lactose was the best inducer when compared to the other carbon sources investigated. Lactose was followed by lactitol, a sugar alcohol produced by the hydrogenation of lactose, and by galactose. Poor induction was observed for other carbon sources, including glucose, mannose and sorbitol. The poorest carbon sources for β-galactosidase production were found to be cellobiose and maltose.

The result of carbon source induction found in *L. fermentum* CM33 corresponded to the previous reports on the induction of β-galactosidase production in various microbes. Our results agree with the previous reports as the result clearly indicated that lactose is the best carbon source to induce the maximum synthesis of β-galactosidase, while glucose is a poor carbon source for β-galactosidase production. Hsu *et al.* (2005) studied the effect of carbon source on the production of β-galactosidase of *B. longum* CCRC15708 in culture medium supplemented with lactose, glucose or galactose as the sole carbon source. The highest level of β-galactosidase at 5.44 U/ml was obtained with 1% (w/v) lactose. Becerra and Siso (1996) reported that the inexpensive and easily available materials, such as milk-whey containing lactose, can be effectively used as the substrate for the production of β-galactosidase by *Kluyvermyces lactis*. In addition, Nakkharat and Haltrich (2006) also reported that lactose were necessary for increasing the β-galactosidase activity produced by *T. thermophilus* using bioreactor cultivation for 50 h of fermentation (3.8 U/ml). However, glucose, mannose and sorbitol were unsuitable carbon sources for β-galactosidase production as a significant decline in β-galactosidase activity of several *Lactobacillus bulgaricus* strains was observed upon the addition of small amounts of glucose to a growing culture (Hickey *et al.*, 1986).

Interestingly, in the case of maltose, bacterial cells could utilize maltose as a carbon source and gave the highest growth when compared to others, but the activity of β-galactosidase was found to be at very low levels. This indicates poor induction of β-galactosidase synthesis during the utilization of this disaccharide. There is the maltose assimilation pathway of lactic acid bacteria in *L. fermentum* Ogi E1, proposed by Santoyo *et al.* (2003), where maltose was taken up by the bacterial cell and hydrolyzed by intracellular α-glucosidase, yielding 1 molecule of glucose and 1 molecule of glucose-6-phosphate. As glucose is a poor carbon source for the induction of β-galactosidase production (Fig. 5), it is reasonable to get the low level of β-galactosidase by *L. fermentum* CM33. However, the assimilation pathway of cellobiose by lactic acid bacteria has not been described. It might be suggested that the low-level induction effect of cellobiose resulted from the similar mechanism as that found in the case of maltose.

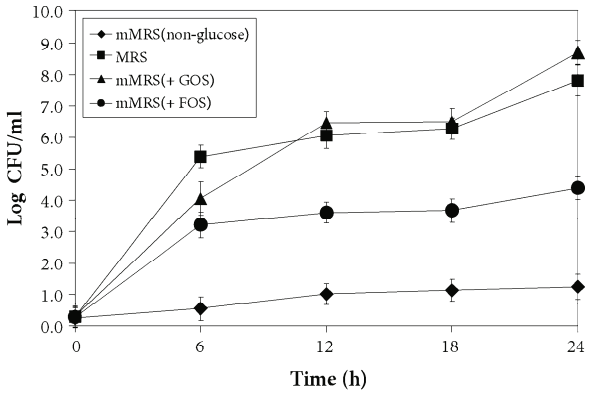

Fig. 6. Utilization of prebiotic oligosaccharides as a sole carbon source by *L. fermentum* CM33; (◆) modified MRS without glucose, (■) MRS, (▲) modified MRS with 1% (w/v) commercial GOS, and (●) modified MRS with 1% (w/v) commercial FOS. Each point represents the mean± SD of three replicates.

In addition, induction of β-galactosidase production by simple carbon sources, such as glucose, galactose and lactose in combination with isopropyl β-D-1-thiogalactopyranoside (IPTG), demonstrated that the IPTG inductive efficacy was decreased (data not shown), which is similar to the IPTG induction mechanism of the β-galactosidase gene in *E. coli* (Hansen *et al.*, 1998).

Utilization of prebiotic oligosaccharides as a carbon source

Time course of microbial growth obtained by culturing *L. fermentum* CM33 in modified MRS broth containing 1.0% (w/v) commercial galactooligosaccharide (GOS) and fructooligosaccharide (FOS) as a sole carbon source is shown in Fig. 6. *L. fermentum* CM33 demonstrated its ability to use GOS as a sole carbon source and grew up as well as the positive control containing glucose as a sole carbon source. Although the experiment started with the same size inoculum (10^6 CFU), growth on FOS was only half that observed with GOS and glucose, while the negative control (MRS without any carbon source) had the lowest level of growth. These results indicated that the GOS utilizing capability was at the same level as for general carbon sources such as glucose and that GOS supported the catalytic function of the β-galactosidase, which was produced in high levels and functioned efficiently in *L. fermentum* CM33. The low level of FOS utilization might be due to the low level of inulinase or levan sucrase produced by the strain CM33. Considering the probiotic properties of *L. fermentum* CM33, including its resistance to gastrointestinal conditions, inhibition of undesirable bacteria and also its capability in GOS utilization, this lactic bacterium has potential for application in the formulation of functional synbiotic neutraceutical.

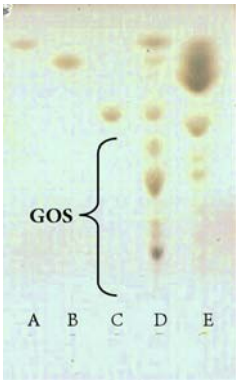

Fig. 7. Thin layer chromatogram of oligosaccharide formed after incubating lactose with crude β-galactosidase from *L. fermentum* CM33. Lanes: A, glucose; B, galactose; C, lactose; D, commercial GOS; and E, oligosaccharides from the reaction mix.

Enzymatic synthesis of oligosaccharides

The preliminary study on enzymatic synthesis of oligosaccharide was conducted using lactose as substrate for crude β-galactosidase from *L. fermentum* CM33. The reaction mixture was incubated at 40°C for 20 h, and then analyzed by TLC. The TLC chromatogram (Fig. 7) demonstrated the transgalactosylation capability of crude β-galactosidase, as the group of sugars appeared at the position corresponding to oligosaccharides. Interestingly, a different pattern of sugar was observed as compared to the commercial GOS that was being used as a standard. This may be caused by a different structure of the transgalactosylated oligosaccharide products. However, the transgalactosylated oligosaccharide product synthesized by the purified *L. fermentum* CM33 β-galactosidase is necessary to confirm the difference. Furthermore, the optimal conditions for achieving a high yield of GOS from transgalactosylation activity have to be further investigated.

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