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Production of Galactooligosaccharide by β-Galactosidase from *Kluyveromyces maxianus* var *lactis* OE-20

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Abstract A galactooligosaccharide(GalOS)-producing yeast. OE-20 was selected from forty seven strains of yeast growing in Korean traditional *Meju* (cooked soybean) and the yeast was tentatively identified as *Kluyveromyces maxianus* var *lactis* by its morphology and fermentation profile. A maximum yield of 25.1%(w/w) GalOS, which corresponds to 25.1 g of GalOS per liter, was obtained from the reaction of 100 g per liter of lactose solution at 30°C, pH 7.0 for 18 h with an intracellular crude β-galactosidase. Glucose and galactose were found to inhibit GalOS formation. The GalOS that were purified by active carbon and celite 545 column chromatography were supplemented in MRS media and a stimulated growth was observed of some intestinal bacteria. In particular the growth rate of *Bifidobacterium infantis* in the GalOS containing MRS broth increased up to 12.5% compared to that of the MRS-glucose broth during a 48 h incubation period.

Keywords : galactooligosaccharides, β-galactosidase, *K. maxinus* var *lactis* OE-20

INTRODUCTION

β-Galactosidase(Lactase, β-D-galactoside galactohydrolase EC 3,2,1,23) is known to catalyze not only the hydrolysis of the β-D-galactoside linkage to D-glucose and D-galactose but also the transgalactosylation of galactooligosaccharides. Both reactions have many applications in the food industry. It is well established that hydrolyzed lactose milk can reduce the lactose intolerance problem that occurs in some infents. Lactose that is hydrolyzed in whey and whey permeates are excellent natural sweeteners and can be utilized in frozen desserts, confectionery, bakery, fermentation products, and fermented beverages.

The galactooligosaccharides can also be employed as probiotics (bifidogenic growth factors), humectants, and emulsifiers, etc [1].

Galactooligosaccharides(GalOS) are (galactosyl)n-lactose oligomers, where the may vary from 2 to 4. They have useful health effects such as the reduction of toxic metabolites and constipation, protection of liver functions, blood pressure, and they also have anticancer effects [2,3]. GalOS are also currently being used as food ingredients and cosmetic additives in several Asian and European countries. The commercial product is a mixture of tetrasaccharide (Gal-Gal-Gal-Glc), trisaccharide (Gal-Gal-Glc), lactose, glucose, and galactose. The structural elucidation of transgalactosylation products shows a predominant formation of β-(1,6) linkages. The synthesis reaction can also form other linkages, such as a β-(1,4) galactosyl linkage, but the structures of the oligosccharides produced depends upon the source of the enzymes[1-5].

GalOS can be produced either by enzymes or by fermentation. Lactose serves both as a galactosyl donor and an acceptor to yield di-, tri-, or higher oligosaccharides. The enzymatic process has advantages over the fermentation process in that a high substrate concentration. Can be used, and the reaction conditions and process control are relatively easy to maintain. Though the enzymatic synthesis of GalOS from lactose has been reported by many investigators and GalOS are being commercially produced by bacteria (*Bacillus circulans*) and fungi (*Aspergillus oryzae, Cryptococcus laurentii*) with β-galactosidase, using lactose as substrate, the productivity is rather low. Therefore, most efficient more efficient and inexpensive method for GalOS production is highly desirable [5,6].

The objectives of this study were to select the GalOS-producing yeast growing in Korean traditional *Meju* and to optimize the production conditions of GalOS.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. Acotonitrile and water for the HPLC analysis were obtained from J. T. Baker (USA). Yeast extracts, MRS, Bactopeptone and Bacto-agar were purchased from Difco Lab (Detroit, Michigan, USA) and lactose was obtained from Sigma Chemical Co. (St. Louis, Missouri, USA).

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Selection and Identification of the GalOS-producing Yeast

Forty seven strains of yeasts that were isolated from Korean traditional *Meju* [7] were cultured in LY broth containing 1.0%(W/V) lactose and 0.6%(W/V) yeast extracts at 30°C for 2 days. The yeast which showed the highest β-galactosidase activity and GalOS productivity was chosen for further studies from these strains. Morphological, biochemical and cultural characteristics of the selected strain were studied according to taxonomy and methods for the identification of microorganisms and yeasts [8,9].

Production and Purification of the GalOS

After the *K. maxianus* var *lactis* OE-20 was grown in LY media at 30°C for 2 days, the cells were harvested by centrifugation (8,000 rpm; 10 min), resuspended in 10mM phosphate buffer (pH 7.0) and disintegrated by glass beads (2:1) for 60 sec. The crude extract was separated from the cell debris by centrifugation (15,000 rpm; 10 min).

0.2 mL of the crude extract (1.0 U/mL) [1] was added to 1 mL of phosphate buffer containing 5% lactose and reacted at different temperatures. After sulfosalicylic acid (50 μL) was added to the reaction mixture, it was incubated for 3 h and the mixture was then centrifuged to remove insoluble proteins. Monosaccharides in the reaction solution were absorbed through a carbon and celite 545 column, and washed with distilled water. The residual lactose was washed with 30% ethanol, and the galactooligosaccharides were eluted by 50% (v/v) ethanol.

Determination of GalOS

The GalOS that were produced in the reaction mixture were centrifuged, filtered through a 0.22 μL membrane and analyzed by HPLC [2]. The reaction products were monitored using a Waters 600 HPLC system. Analyses were performed isocratically on a 300 mm × 3.9 mm μBondapak NH_2 column and an RI detector. The flow rate was 1.5 mL/min with acetone:H_2O (84:16) as the mobile phase.

Intestinal Bacteria and Effects of GalOS on the Growth

Intestinal bacteria such as *Bifidobacterium bifidum, Bifidobacterium infantis, Lactobacills casei, Lactobacillus acidophilus,* and *Lactobacillus rhamnosus* were obtained from stock cultures in the Food Biotechnology laboratory at the Macdonald Campus of McGill University (Montreal, Quebec, Canada) and were used for the assimilation of galactoogosaccharides. Cells were grown in MRS-glucose (1%) medium at 37°C for 48 h anaerobically [10].

To test the effects of the GalOS on growth, 0.5% (w/v) of the GalOS was added top the MRS medium, inoculated with 10^7-10^8/mL of bacteria and incubated at 37°C for 48 h anaerobically. Growth of the intestinal bacteria was determined spectrophotometrically as absorbance of 660 nm and calculated from their relative growth (RG) as follow [6,10].

$$RG(\%) = (OD_{oligo}-OD_{media})/(OD_{glucose}-OD_{media}) \times 100$$

OD_{oligo}: absorbance at 660 nm on growth in the GalOS containing media
OD_{media} : absorbance at 660 nm on growth in glucose-free media
$OD_{glucose}$: absorbance at 660 nm on growth in glucose containing media

RESULTS AND DISCUSSION

Selection and Identification of the GalOS-producing Yeast

Among the 47 strains of yeasts that were isolated from Korean traditional *Meju* [7], The yeast OE-20 strain showed the highest GalOS production therefore this strain was selected for the production of GalOS. Morphological and physiological characteristics of the OE-20 strain are summarized in Table 1 and 2. The OE-20 strain is an ovoid-ellipsoidal shaped yeast (2.5 × 2.0 μm) that is formed as an ascospore. In broth cultures of YM media, the strain forms a pellicle, has no urease activity and also cannot assimilate KNO_3. A pink color was shown in the TTC colorization test and the G+C content was 41.6 mol%. Although the strain assimilated most of the sugars, only glucose and lactose become fermented.

The strain was tentatively identified to be *Kluyveromyces maxianus* var *lactis* by a yeast taxonometrie study [9].

Table 1. Morphological and physiological characteristics of the yeast strain OE-20

Classification	Characteristics*
Cell shape	ovoidal-ellipsoidal
Cell size (μm)	2.5 × 2.0
Vegetative reproduction	budding
Ascospore	present (spheroidal, 1-4)
Growth : 50% glucose/YEA	+
20% (5%) NaCl/YEPD	−(+)
1% acetic acid	−
Culture in YM : pellicle	present
ring	present
Colour on growth of YM agar	creamy
Vitamin requirement	+
Resistance : cycloheximide (1,000 ppm)	+
ethanol (10%)	+
KNO_3 assimilation	−
Urease activity	−
TTC colorization	pink
G+C content (mol%)	41.6

* + or − means positive or negative, respectively.

Table 2. Assimilation and fermentability of carbon sources by the yeast strain OE-20

Carbon source	Assimilation*	Fermentability*
Glucose	+	+
Galactose	+	−
Fructose	+	−
Sucrose	+	−
Lactose	+	+
Maltose	+	−
Raffinose	+	−
Soluble starch	+	−
Xylose	+	−
Ribose	+	−
Sobitol	+	−
Inositol	−	−

* + ; good and − ; not assimilable or fermentable

Reaction Conditions for GalOS Production

Various parameters such as the source of the enzyme, substrate concentration, pH, and temperature can influence the equilibrium of the enzyme reaction catalysis in the sysnthesis of the desired GalOS.

The effects of temperature on the production of GalOS were examined in the range of 25°C to 40°C for 12 h using a 5% (w/v) lactose solution. The GalOS production reached a maximum of 15 g/L from 50 g/L lactose at 30°C (Table 3). The results are similar to those of mesophilic microbes (30-40°C) [3,6], lower than those of thermophilic *Thermus aquaticus* YT-1 [2] but higher than those of psychroptrophic *Bacillus subtilis* KL88 [4].

Table 3 shows the effect of the reaction time on the production of GalOS from 50 g/L lactose with β-galactosidase of the OE-20 at 30°C and pH 7.0. The GalOS production reached a maximum level of 16.2 g/L after 18 h, but the yield of GalOS eventually decreased as a function of the reaction time. This is in agreement with those of mesophilic microbes [3,6], where the GalOS eventually decreased with the hydrolysis time, but showed a different pattern when compared with those of *B. singularis* [5] and other thermophilic microbes.

The effect of lactose concentration on the production of GalOS were investigated with the different initial lactose concentration a 10 g to 200 g/L. The maximum yield, 26.4 g/L of GalOS, was obtained using a lactose concentration of 20% (Table 3).

It is known that the production rate of GalOS increases with an increasing lactose concentration. Rahim and Lee [4] reported that the highest GalOS was formed at a lactose concentration of 29% (Type I), 16% (Type II) and 35%(Type II) using the *B. subtilis* enzyme respectively, whereas Greenberg [11] reported that GalOS could formed in a 5% lactose solution using *Streptococcus thermophilus* β-galactosidase at 37°C at maximum yield.

The effect of glucose and galactose on GalOS production was studied. The amount of GalOS production decreased from 26.5 g/L to 9.6 g/L and 12.0 g/L, respectively as the addition amount of glucose and galactose increased to 30 g/L (data not shown). These results were similar to those of *B. singularis* [5] and *T. aquaticus* [2], which indicated the by-products of reaction, glucose and galactose inhibited GalOS production functioning as β-galactosidase inhibitors that, competed with lactose for the active site of the enzyme [18].

Though the yield of GalOS produced by the OE-20 β-galactosidase was lower than those of the other microbes such as *C. laurentii* [14], *S. rectivirgula* [17] *T. aquaticus* [2], *S. elvae* [19], *A. oryzae* [20], *S. magnum* [21] and *B. singularis* [5], considerable interest has developed in the production of GalOS from a industrial yeast which

Table 3. Effect of reaction temperature and time, lactose concentration on the production of galactoolioosaccharide

	Reaction temp.(°C)[1]				Reaction time (h)[2]				Lactose conc. (%, w/v)[3]				
	25	30	37	40	8	12	18	24	1	3	5	10	20
Galactooligo-saccharide (g/L)	9.1	15.0	11.5	4.2	5.1	14.0	16.2	11.8	2.5	11.0	19.1	25.1	26.4

[1] 5.0% lactose was reacted with β-galactosidase from *K. maxianus* var *lactis* OE-20 for 12 h at the indicated temperature.
[2] 5.0% lactose was reacted with β-galactosidase from *K. maxianus* var *lactis* OE-20 for the indicated time at 30°C, pH 7.0.
[3] The indicated concentration of lactose was reacted with β-galactosidase from *K. maxianus* var *lactis* OE-20 at 30°C for 18 h.

Table 4. Comparison of GalOS production by β-galactosidase from several microorganisms

Enzyme source	Reaction condition	Productivity (g L^{-1}·h^{-1})	Reference
C. laurentii	2.5% lactose at 50°C	0.35	[14]
S. rectivirgula	60% lactose at 70°C	12.3	[17]
T. aquaticus	1.6% lactose at 70°C	2.3	[2]
S. elvae	20% lactose at 60°C	3.25	[19]
A. oryzae	38% lactose at 40°C	24.8	[20]
S. magnum	20% lactose at 60°C	3.0	[21]
B. singularis	30% lactose at 45°C	4.8	[5]
K. maxianus var *lactis* OE-20	10% lactose at 30°C	1.39	This work

Table 5. Effect of the GalOS on the growth of intestinal bacteria

Compounds	*L. casei*[1)]	*L. acidophilus*	*L. rhamnosus*	*B. bifidum*	*B. infantis*
Glucose	+++[2)]	++	+++	+++	+++
Galactooligo-saccharides	++[3)] (62.5%)	+ (83.3%)	++ (87.0%)	+++ (101.2%)	+++ (112.5%)

[1)] Intestinal bacteria were incubated in each MRS media containing glucose (1.0%) or GalOS (0.5%)
[2)] Growth ; +++(strong), ++(moderate), +(weak)
[3)] Relative growth (%, Material and Methods)

can grow easily in more inexpensive media. Futhemore, the GalOS was produced from a much lower concentration of lactose and in a shorter reaction time than those of other microbes (Table 4).

To increase the GalOS yield, further studies are required which include enzyme concentration and the effects of precursors, etc.

Growth Stimulation by the GalOS

The effect of the purified GalOS on the growth of some intestinal bacteria were investigated using GalOS (0.5%) containing MRS broth and compared with that of glucose (1.0%) containing MRS broth.

As shown in Table 5, the purified GalOS was assimilated by some of the lactic acid bacteria. In particular, relative rate of *Bifidobacterium infantis* in the GalOS containing MRS broth was about 12.5% higher than that of the MRS- glucose broth.

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