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Isolation and Characterization of *Enterobacter* sp. Producing Galacto-oligosaccharides

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Enterobacter sp. producing β-galactosidase with high transgalactosylation activity was isolated from dairy wastewater. The isolate had common biochemical features to *E. aerogenes* and *E. cloacae*. Enzyme production increased as the cell mass increased with optimum enzyme activity of 0.21 Unit/mg-protein (o-nitrophenyl-β-D-galactoside (ONPG) as substrate) until 8 hr of culture. Whole cells permeabilized by toluene were used to produce galacto-oligosaccharide. Optimum toluene concentration, temperature and pH for β-galactosidase activity of permeabilized whole cells were 10% (v/v), 50°C and 6.0, respectively. A maximum of 38% (w/w) of galacto-oligosaccharide was obtained with lactose concentration of 20% (w/w) at 40°C and pH 6.0.

The hydrolysis of lactose in dairy products has both nutritional and industrial importance. One of the most attractive methods of hydrolyzing lactose to its constitutive monosaccharides, galactose and glucose, is to use β-galactosidase (β-D-galactoside galactohydrolase, EC 3. 2.1.23) (5, 6). Generally, β-galactosidase has been known to produce oligosaccharides, particularly galactooligosaccharide (GO) by transgalactosylation (5, 10, 15-17). GO is a major oligosaccharide in breast milk and is useful as a promoter in the growth of bifidobacterium known to play an important role in human intestines (2, 10). Although a variety of microorganisms producing β-galactosidase have been isolated to hydrolyze saccharides (11-13), only a few strains have been used to produce oligosaccharides as whole cell biocatalyst (21).

Whole cells can be used as biocatalyst because of the following advantages: 1) no need for enzyme extraction or purification, 2) potential increase in enzyme stability, and 3) low enzyme cost. Ohmiya *et al.* (14) reported the hydrolysis of lactose and skim milk using whole cells of *L. bulgaricus, E. coli,* and *K. lactis*. Recently, Hasal *et al.* (8) reported a continuous hydrolysis of sucrose using whole cells of yeast. While, Yun *et al.*(21) described a method for the production of panose from maltose using whole cells of *Aureobasidium pullulans*. However, GO production from whole cells has not been reported yet.

In this paper, a novel strain of *Enterobacter* sp. producing β-galactosidase with high transgalactosylation activity was isolated and identified. Characteristics of cell growth and enzyme production of the strain were examined and GO production was performed using toluene-permeabilized whole cell of *Enterobacter* sp.

MATERIALS AND METHODS

Materials

Lactose and other carbohydrates were purchased from Difco (Detroit, MI., USA) and TCI (Tokyo, Japan). o-Nitrophenyl-β-D-galactoside (ONPG) and other biochemical reagents were obtained from Janssen Chimical (Geel, Belgium) and Sigma (St.Louis, MO., USA). All other chemicals were of analytical grades.

Isolation and Identification of Microorganism

One gram of soil sample was added to 10 mL of sterile water and the supernatant liquid was spread onto an agar plate. A screening medium comprised 0.4% polypeptone, 0.4% yeast extract, 0.1% K_2HPO_4 0.1% KH_2PO_4, 0.2% $MgSO_4$, 0.05% NH_4Cl, 0.2% NaCl and 1% lactose (pH 6.8 with 20% Na_2CO_3). Na_2CO_3 and lactose solution were autoclaved separately and added to the medium. After incubation at 35°C for 2-3 day, well-grown colonies were transferred to fresh plates. All isolated strains were

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tested for the β-galactosidase activity and then the strain with high activity was selected. Microbiological characteristics of the selected strain were investigated according to the Manual for the Identification of Medical Bacteria (3). The identification was performed using Bergey's Manual of Systematic Bacteriology (18).

Culture of Microorganisms and Permeabilizing Condition

Isolated cells with β-galactosidase activities were aerobically grown in a medium containing 1% lactose, 1% peptone, 0.5% NaCl and 0.1% beef extract, which was adjusted to pH 6.8 with 0.2N HCl, for 12 hrs at 40°C. After incubation, cells were harvested in a refrigerated centrifuge at 5,000 rpm. Cell mass concentration was determined by measuring the optical density at 660 nm. The resulting cells were washed three times before being permeabilized with 10% (v/v) toluene at 40°C for 10 min.

Protein and β-Galactosidase Activity Assay

Protein concentration was determined by the bicinchoninic acid (BCA) assay kit supplied by Sigma Co. Bovine serum albumin was used as the standard protein. *In situ* enzyme activity of whole cell was estimated using ONPG as a substrate. A mixture containing 1 mL of 5 mM ONPG in 0.1M phosphate buffer (pH 6.0) and 1 mL of permeabilized cell solution (OD 2.0 at 660 nm) was incubated at 40°C for 15 min. 1 mL of the reaction mixture was mixed with 1 mL of 10% sodium carbonate solution to stop the reaction, and then diluted to 10 mL with water. The *o*-nitrophenol released was determined by measuring the absorbance at 420 nm. Intracellular enzyme activity of the cell was measured by the method of Nagano *et al.* (12). One unit is the quantity of enzyme that will liberate 1mmol of o-nitrophenol per min under the assay condition.

Carbohydrate Assay

All saccharides produced from the reactions were analyzed with Waters HPLC systems with Phenomenex IB-Sil NH_2 column (250 × 4.6 mm) maintained at ambient temperature. A mixture of acetonitrile-water (3:1) was used as mobile phase with a flow rate of 1.8 mL/min (9).

RESULTS AND DISCUSSION

Morphology and Biochemical Characteristics of Isolated Strains

About 10 strains producing β-galactosidase were isolated from dairy wastewater streams at Gong-Ju province in Korea. Among them, a strain of KN-1 that exhibited a high β-galactosidase activity was selected for further studies. The isolate was rod shaped, motile, nonsporulating, facultatively aerobic, and Gram negative. Fig. 1

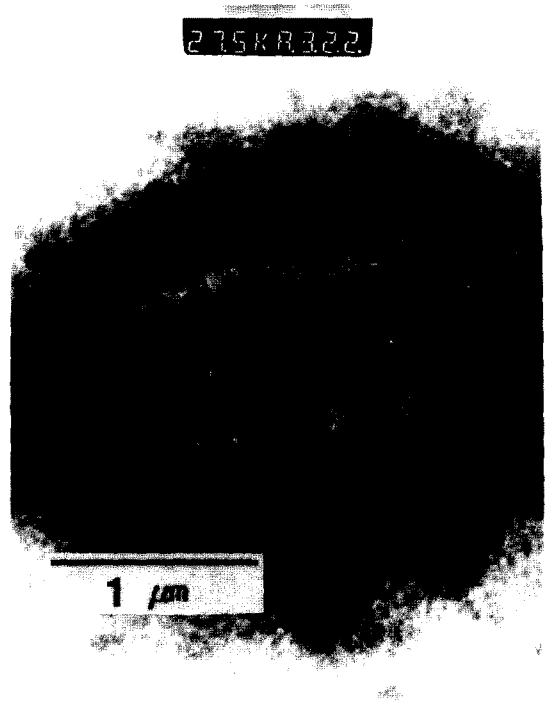

Fig. 1. Electron micrograph of the isolate KN-1.
Scanning electron micrograph of 12hrs old culture were taken after the sample was coated by gold. Bar indicates 1 μm.

shows that the cell size was about 2 μm and the cell wall somewhat wrinkled. In addition, the cell was found to be encapsulated by the extracellular polymer. Isolated KN-1 had many common characteristics to *E. aerogenes* and *E. cloacae* with a few differences such as methyl red, arginine decarboxylase, and yellow pigment test. From the results summarized in Table 1, it is thought to be an *Enterobacter* sp.

Characteristics of Growth and Enzyme Production

Batch cultivations were carried out in 250 mL Erlenmeyer flask containing 100 mL of medium (pH 6.8). The cultivation temperature was 40°C, and rotating speed of the shaker was 160 rpm. The maximum cell growth was obtained with 1% lactose as a sole carbon source. Enzyme production increased with the cell growth and maximum specific activity was attained after 8 hr of culture (Fig. 2). The decrease of pH in culture broth was due to the production of acidic metabolites. Substrate inhibition phenomena appeared at the concentration over 10% lactose. Aeration had not much effect on cell growth in flask culture. pH-stat culture (pH 6.8) in 2.5L fermentor showed no increase in specific enzyme activity.

The effect of carbon and nitrogen sources on growth and production of the enzyme was examined under the conditions described above. As seen in Table 2, the enzyme was proved to be inducible and peptone was

Table 1. Biochemical characteristics of the isolate KN-1.

Characteristics	Strain		
	Isolated strain	*Enterobacter aerogenes**	*Enterobacter cloacae**
Methyl red	+	−	−
Voges-Proskauler	+	+	+
Catalase	+	+	+
Oxidase	−	−	−
Citrate as C source	+	+	+
Indole	−	−	−
Gelatin liquefaction	+	d	+
Nitrate reduced	+	NL	+
Urase	−	−/w	−
Arginine decarboxylase	+	−	+
Lysine decarboxylase	+	+	−
Omitine decarboxylase	+	+	+
Aesculin hydrolysis	+	+	NL
Acid from Adonitol	+	+	d
Arabinose	+	+	+
Dulcitol	−	d	d
Glucose	+	+	+
Inositol	+	+	−
Lactose	+	+	+
Maltose	+	+	+
Raffinose	+	+	+
Rhamnose	+	+	+
Sorbitol	+	+	+
Sucrose	+	+	+
Trehalose	+	+	+
Xylose	+	+	+
Yellow pigment	+	+	−

* : data taken from Bergey's Manual, +: positive, −: negative, d: differs among strains, NL: not listed, w: weakly

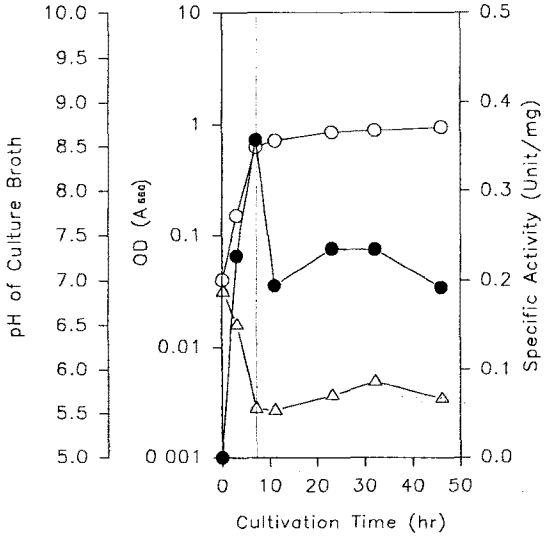

Fig. 2. Growth and enzyme production of *Enterobacter* sp. KN-1.
○; optical density at 660 nm, ●; specific activity (Unit/mg-protein), △; pH of culture broth.

considered to be a good nitrogen source. Yeast extract had no effect on the cell growth or the enzyme production. The isolate produced a thin layer of extracellular polysaccharide by the addition of yeast extract. This resulted problems in cell harvesting, so yeast extract was excluded from the medium throughout the experiment. From data presented above, the optimum medium was composed of 1% lactose, 1% peptone, 0.5% NaCl, and 0.1% beef extract, which was adjusted to pH 6.8 and the culture temperature was 40°C. The strain has been deposited in the Korean Collection for Type Cultures (KCTC number 2701)

β-Galactosidase Activity of Permeabilized Cells

Bacteria and yeast can be effectively permeabilized by surfactant and organic solvents such as ethanol, toluene, and chloroform (4, 19). In this work, toluene was chosen as the appropriate permeabilizing agent by preliminary study. As shown in Fig. 3, the enzyme activity of toluene treated cells increased three times higher than that of untreated cells. However, Somkuit and Steinbery (19) reported that Triton X-100 treated *Streptococcus thermophilus* displayed 15 times higher level of β-galactosidase activity than untreated cell. Flores *et al.* (4) used ethanol and chloroform to increase β-galactosidase activity of *Kluyvermyces lactis* by 90-folds. The difference in enzyme activity of *Enterobacter* sp. with the strains mentioned above arises from the variations in the membrane permeability and culture time.

Table 2. The effect of carbon and nitrogen sources on β-galactosidase production of KN-1.

Carbon source[1] (1%)	Specific activity* (Unit/mg)	Nitrogen source[2] 0.1%	Nitrogen source[2] 1%	Specific activity* (Unit/mg)
None	0.04	none	none	0.00
Arabinose	0.04	none	peptone	0.17
Xylose	0.02	beef extract	none	0.16
Glucose	0.06	beef extract	peptone	0.21
Galactose	0.02	beef extract	asparagine	0.05
Fructose	0.02	beef extract	casitone	0.05
Sucrose	0.03	beef extract	$(NH_4)_2SO_4$	0.06
Lactose	0.21	beef extract	$(NH_4)_2HPO_4$	0.11
Maltose	0.03	beef extract	$Ca(NO_3)_2$	0.09
Raffinose	0.04	beef extract	KNO_3	0.08
Starch	0.05	beef extract	NH_4NO_3	0.03
Inositol	0.02	yeast extract	peptone	0.02
Sorbitol	0.02	malt extract	peptone	0.04

[1] Nitrogen sources were fixed (0.1% beef extract and 1% peptone). [2] Carbon source was fixed (1% lactose). * Calculated by the method in 'Materials and Methods'

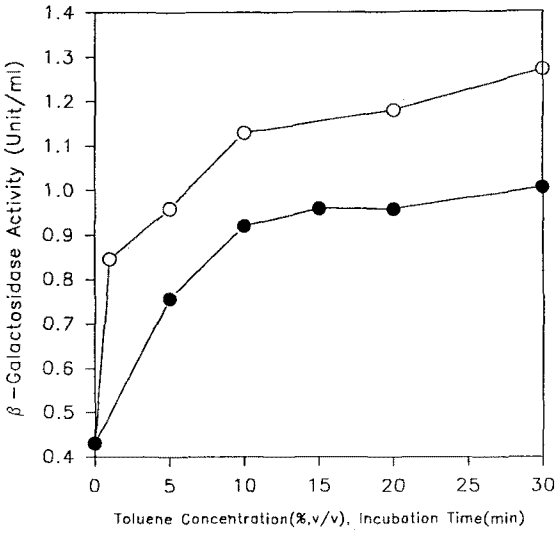

Fig. 3. Effect of toluene concentration and incubation time on the permeabilization of *Enterobacter* sp. KN-1, expressed as total activity (Unit/ml).
Cells were suspended in potassium phosphate buffer (pH 6.0, 0.1 M) and the OD is 2.0 at 660 nm. ○; toluene concentration (incubation time was 10 min), ●; incubation time (toluene concentration was 5%).

Incubation of cell suspension in 10% toluene for 10 min at 40°C was enough to use the cell as biocatalyst (Fig. 3). Toluene-permeabilized whole cell showed its optimum enzyme activity at 50°C and pH 6.0 (Fig. 4A), and retained its activity for 120 min at 45°C (Fig. 4B). Generally, permeabilization efficiency evaluated as the enzyme activity is related with the loss of viability of cell suspensions (4, 19). In our case, the use of toluene rendered all cells nonviable, but cell viability was not important in GO production.

Galacto-oligosaccharide Production

GO production was performed in 50 mL temperature-controlled vessel with phosphate buffer (pH 6.0, 0.1M) at 40°C. Fig. 5 is a time profile of saccharides concentration during hydrolysis of 20% (w/w) lactose with toluene-permeabilized cells of KN-1. A maximum of 38% GO including 23% of trisaccharide was produced after 40 hr reaction by 67% conversion of 20% (w/w) lactose, which is much more than that of purified enzymes of *Saccharomyces lactis* and *Streptococcus thermophilus* (1, 7, 17). Produced GO were totally hydrolyzed into its monomers, galactose and glucose. Thus, thermodynamic equilibrium concentration of GO was not reached.

β-Galactosidase from *Bacillus circulans* produced 41% of GO from 4.5% (w/v) lactose solution at 40°C and pH 6.0 (11). Recently, an extracellular β-galactosidase from *Sacchropolyspora rectivirgula* was reported to produce 41% of GO with 60% (w/v) lactose at 70°C and pH 7.0 (13). The yield obtained in this work, 38% (w/w), was one of the highest values reported so far. Typically, it consumed more time to produce GO by whole cells (40~60 hr) than by purified enzyme (10~20 hr). Taking into account that whole cell is a very economical enzyme, toluene-permeabilized whole cell could be used for GO production economically without further purification of the enzyme. The amount of oligosaccharide increased with the initial lactose concentrations and whole cell concentrations. The main products of trisaccharides were considered to be 6'-galactosyllactose according to the R_f values of thin layer chromatography and HPLC retention time (data not shown).

Some β-galactosidase have been known to produce disaccharides from lactose as the main products (1, 7, 20). In our investigations, large amounts of disaccharides (ca. 20%) were also observed. These disaccharides could be used as bifidobacterium growth factors (2).

In conclusion, the isolated *Enterobacter* sp. KN-1 produced β-galactosidase with high transgalactosylation

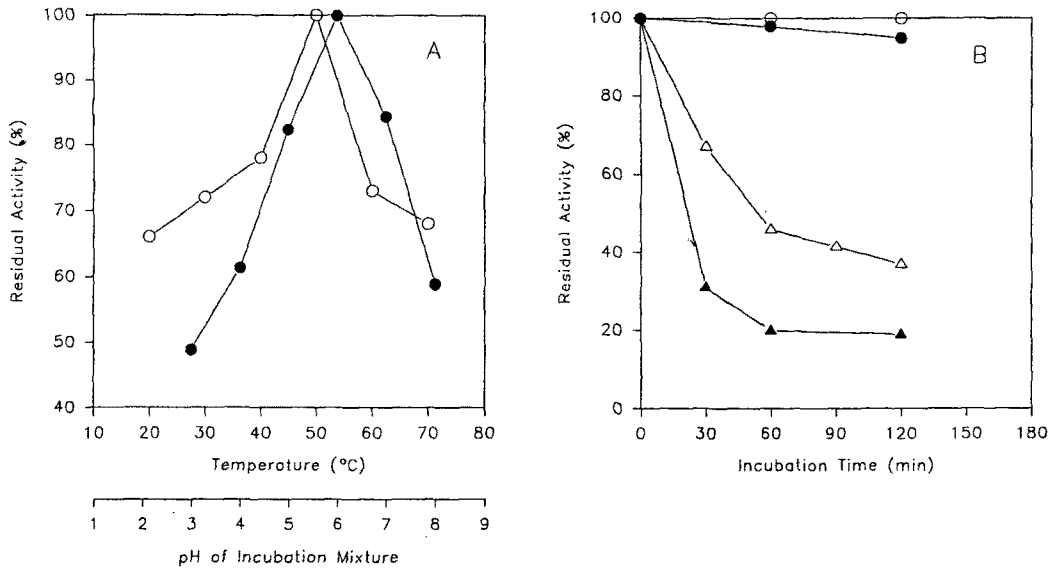

Fig 4. (A) Effect of incubation temperature and pH of buffer on β-galactosidase activity of permeabilized KN-1.
Acetate buffer (0.1 M) was used in the range of pH from 3 to 5 and phosphate buffer (0.1 M) from 6 to 8. ○; incubation temperature, ●; pH of buffer.
(B) Effect of incubation time on β-galactosidase activity of permeabilized KN-1.
Incubation temperature: ○; 40°C, ●; 45°C, △;50°C, ▲; 55°C.

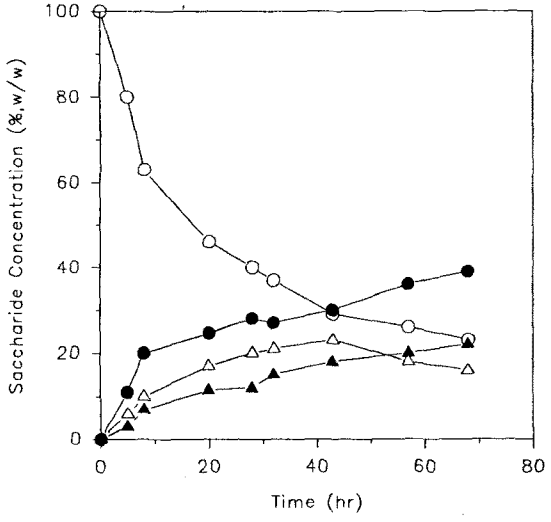

Fig. 5. Time course of lactose hydrolysis.
Initial lactose concentration was 20% (w/w) after adding 5 ml of permeabilized cell suspension to the reaction vessel. Reaction was performed in potassium phosphate buffer (pH 6.0, 0.1 M). ○; lactose, ●; sum of glucose and galactose, △; trisaccharide, ▲; disaccharide.

activity for GO production. This novel method using permeabilized whole cells could give great practical advantages for GO production at optimum conditions.

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