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SHORT COMMUNICATION

Lactulose biosynthesis by β-galactosidase from a newly isolated *Arthrobacter* sp.

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Abstract Lactulose, a ketose disaccharide, is used in both pharmaceutical and food industries. This study was undertaken to screen and isolate potent β-galactosidase-producing bacteria and to evaluate their enzymatic production of lactulose. Soil samples from fruit gardens were collected. One isolate designated LAS was identified whose cell extract could convert lactose and fructose into lactulose. The 16S rDNA gene analysis of LAS revealed its phylogenetic relatedness to *Arthrobacter* sp. The β-galactosidase produced by LAS was purified 15.7-fold by ammonium sulfate precipitation and subsequent Phenyl-Sepharose hydrophobic chromatography. The optimum pH and temperature for lactulose synthesis by this β-galactosidase were 6.0 and 20°C, respectively. The low optimum temperature of this enzyme compared to the currently used ones for lactulose production has the advantage of reducing the nonenzymatic browning in biotransformations. The results indicated that *Arthrobacter* could be used as a novel bacterial β-galactosidase source for lactulose production.

Keywords β-Galactosidase · Lactulose · *Arthrobacter* · Purification

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Introduction

Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) is a synthetic ketose disaccharide which cannot be decomposed by human digestive enzymes [15]. Lactulose has attracted attention due to its applications in the pharmaceutical and food industries. As a laxative, it is applied for the prevention and treatment of chronic constipation, portal systemic encephalopathy, and other intestinal or hepatic disorders [6]. As a prebiotic sugar, lactulose is applied to commercial infant formulas and various milk products because it specifically promotes the intestinal proliferation of Bifidobacterium, which is known to be a very important humanizing factor [14].

Lactulose can be produced by chemical synthesis or by microbial enzymatic transformation. Presently, for commercial utilization lactulose is exclusively produced by alkaline isomerization of lactose [1]. However, this method has several drawbacks, such as a high level of lactulose degradation, and considerable amounts of inorganic catalysts and colored by-products in the reaction mixture which lead to difficulty in waste management and product purification [17]. Therefore, a biocatalysis process is regarded as an alternative environmentally friendly strategy for lactulose production. In lactulose biosynthesis, β-galactosidase with both hydrolysis and transgalactosylation activities works as the catalyst [2]. The enzyme hydrolyzes lactose into D-glucose and D-galactose. The latter is then used as a donor in transgalactosylation when fructose is present as an acceptor. However, other oligosaccharides always coexist with the product lactulose in biotransformations because the transgalactosylation is a kinetically driven reversible reaction, which has an optimum at a certain conversion rate depending on reaction conditions and the intrinsic kinetic properties of the enzymes [10].

Although β-galactosidases from different sources either in the form of whole cells or purified enzymes are described, the promising ones are limited to those from *Kluyveromyces lactis*, *Sulfolobus solfataricus*, and *Pyrococcus furiosus* [8, 9, 11] whose optimum temperatures for lactulose production are relatively high (60, 80, and 75°C, respectively). High temperature with the high concentration of reducing sugars (lactose, fructose) induces non-enzymatic browning [10]. Therefore, novel microbial β-galactosidases suitable for the lactulose synthesis under mild conditions are desired.

The present study aimed at finding microbial strains which can produce β-galactosidase with new kinetic properties for lactulose biosynthesis. As a result, a newly isolated candidate was identified as an *Arthrobacter* sp. The optimum temperature for lactulose production by this enzyme is 20°C.

Materials and methods

Screening for β-galactosidase producers

Screenings were carried out by the spread-plate methods. Soil samples collected from fruit gardens, Wuxi, China, were suspended and serially diluted in sterile saline, and aliquots were spread onto the selective medium (2% lactose, 0.5% Bacto yeast extract, 1% Bacto peptone, 0.03% X-gal, and 2% agar). Plates were incubated for 3–7 days at 30°C. The colonies which exhibited blue colors were selected, and streaked on additional new plates until homogenous colonies were observed. The purified strains were stored at 4°C.

Preparation of cell extracts

Cells grown in a shake flask containing the liquid medium (2% lactose, 0.5% Bacto yeast extract, 1% Bacto peptone) were collected and concentrated by centrifugation at 8,000 × *g* for 10 min. The supernatant was discarded, and the cells were washed with one volume of 100 mM potassium phosphate buffer (pH 7.0) and then centrifuged again. The pellets were suspended in one volume of the above buffer, broken by sonication, and centrifuged at 12,000 × *g* for 10 min. The supernatant was obtained for β-galactosidase analysis or purification.

Assay of β-galactosidase activity

β-Galactosidase activity was assayed in 100 mM potassium phosphate (pH 7.0) using the chromogenic substance *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The reaction mixture was incubated at 37°C for 10 min. The enzyme reaction was stopped by adding 1 M Na_2CO_3. The increase in absorbance at 420 nm due to the liberation of *o*-nitrophenol (ONP) was measured spectrophotometrically. One unit of β-galactosidase activity was defined as the amount of enzyme that produced 1 μmol of ONP per minute.

Identification and phylogenetic analysis of isolate LAS

One isolate designated LAS, the β-galactosidase from which could convert lactose and fructose into lactulose, was identified. The detection of physiological and biochemical characteristics was performed based on *Bergey's Manual of Determinative Bacteriology* [7]. Morphological properties were examined by using a scanning electron microscope Quanta 200 (Fei, Eindhoven, Netherlands) with an accelerating voltage of 10.0 kV.

Sequence analysis of the 16S rDNA was performed by amplifying the 16S rDNA of the LAS with PCR technique according to Weisburg et al. [16]. The obtained 16S rDNA sequence (1,413 bp) was aligned to sequences in GenBank databases using the basic local alignment search tool (BLASTN) program [3]. The analyzed sequences were compiled in an alignment with the CLC Sequence Viewer 6.3 program using a progressive alignment algorithm (http://www.clcbio.com), and the tree was then constructed with the neighbor-joining algorithm. The bootstrap values for constructed tree were based on 100 replicates.

Purification of β-galactosidase

Solid ammonium sulfate was added into the cell extract of isolate LAS. The precipitate formed in the range of 30–60% of saturated ammonium sulfate was collected and dissolved in 20 mM phosphate buffer (pH 6.8), containing 1 M ammonium sulfate. The solution was applied to a Phenyl-Sepharose column (1.6 × 10 cm) equilibrated with the same buffer. The enzyme was eluted in a linear gradient from 1 to 0 M ammonium sulfate in 140 ml, at a flow rate of 0.8 ml/min. The eluate containing active β-galactosidase was concentrated by using a YM-10 membrane with a molecular weight cutoff of 10,000 (Amicon, Danvers, USA).

Lactulose biosynthesis and identification

The mixture of 40% (w/v) lactose, 20% (w/v) fructose, and cell extracts from each isolate was incubated at 37°C for 6 h during the microbial screening process. The reaction was stopped by boiling the mixture for 10 min. Lactulose produced was monitored by HPLC analysis. The HPLC system (Agilent HP 1100, Agilent Technologies, USA) was equipped with a RID-10A detector (Shimadzu, Japan). A SHODEX SH1011 column (7 μm, 300 × 8 mm,

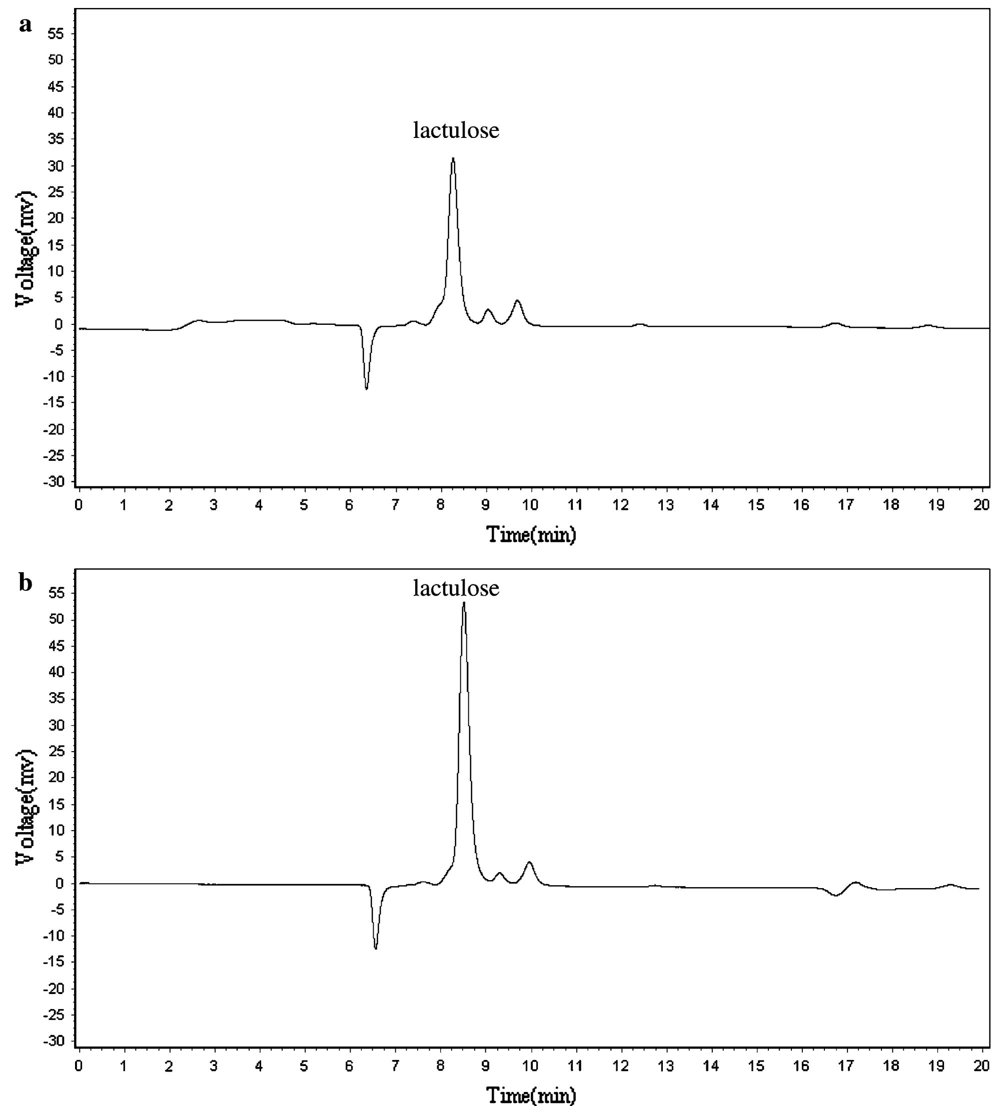

Fig. 1 HPLC monitoring of sugars in the reaction mixture of substrates and β-galactosidase from *Arthrobacter* sp. LAS (**a**), and with authentic lactulose added as internal standard (**b**)

Waters, USA) was used for detection of sugars. The column was eluted at 50°C with 0.01 mol/l sulfuric acid at a flow rate of 0.8 ml/min. The sugars were identified by comparison with the retention time of the corresponding standards. To search for the optimum reaction pH and temperature for lactulose production by the partially purified LAS β-galactosidase, the pH was varied from 5.5 to 7.0, and temperature was varied in the range of 10–30°C.

Results and discussion

Screening for bacterial strains producing β-galactosidase with both hydrolysis and transgalactosylation activities

Production of lactulose by bioconversion needs a β-galactosidase which can catalyze not only the lactose hydrolysis but also transgalactosylation reaction. Therefore, in this study, we first screened the dark blue colonies, β-galactosidase producers, on the selective medium, and analyzed the hydrolysis activity of each isolate using the ONPG as substrate. Isolates with higher hydrolysis activities were chosen and their transgalactosylation abilities were then assessed by using lactose and fructose as the substrates.

The HPLC analysis showed that one isolate, designated LAS, exhibited lactulose productivity (Fig. 1a). The product was identified by comparison of its retention time with that of standard lactulose. In addition, this standard was added into the reaction mixture, and HPLC was performed again. No new peaks appeared and the peak of lactulose was enhanced (Fig. 1b), which further confirmed that the lactulose was synthesized by β-galactosidase produced by the isolate LAS. Although several microbial sources of β-galactosidase were reportedly able to convert

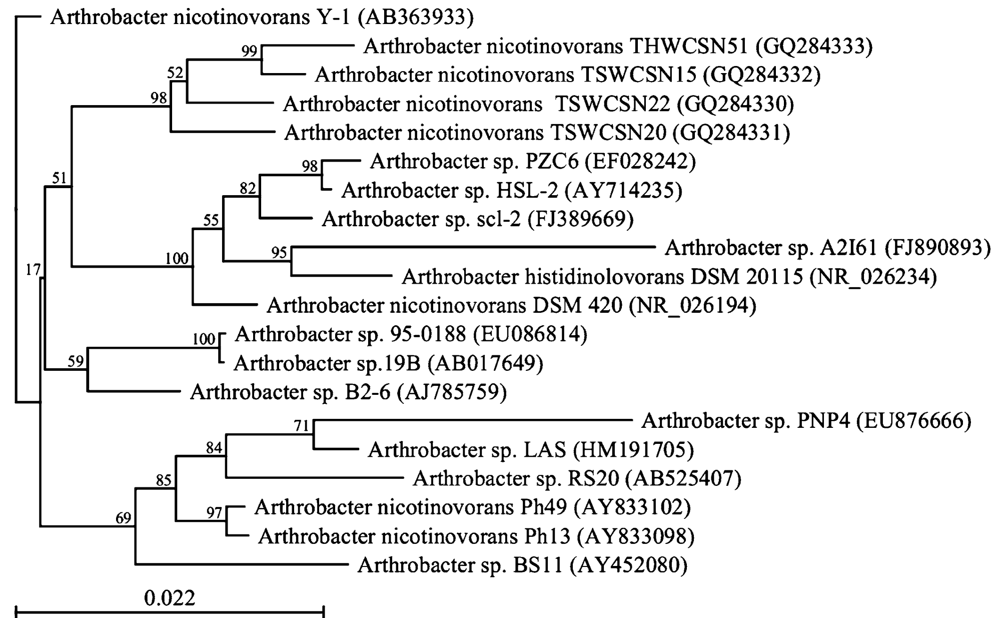

Fig. 2 Phylogenetic tree based on maximum-parsimony analysis of the 16S rDNA gene of *Arthrobacter* sp. LAS and closely related species. The *numbers in parentheses* are GenBank accession numbers

Table 1 Purification of β-galactosidase from isolate *Arthrobacter* sp. LAS

Fraction and purification step	Total protein (mg)	Specific activity (U/mg protein)	Total activity (U)	Purification (fold)	Recovery (%)
Cell extract	500	0.63	315	1	100
$(NH_4)_2SO_4$	140	1.38	192.5	2.2	61.1
Phenyl-Sepharose	7.8	9.9	77.5	15.7	24.6

lactose and fructose into lactulose, the lactulose productivities varied significantly because of the enzymes' different transgalactosylation activities, i.e., the ability to transfer the galactosyl group to fructose [9]. This was also true in the present work, as with all the isolated β-galactosidase producers tested, only LAS produced the β-galactosidase with lactulose-synthesizing ability; this observation suggested that the mechanism of hydrolysis and transgalactosylation action might be different between various microbial origins.

Identification of the strain LAS

Because of the good transgalactosylation ability of the β-galactosidase from LAS, this strain might be a potential microorganism for lactulose production; therefore, identification of the strain was carried out. Electron microscopy of LAS cells cultured on a slant for 24 h showed that the cells were irregular rods, V-shaped, and with clubbed ends. The strain was Gram-positive, aerobic, and non-sporing, and predominantly formed smooth-looking yellow colonies after 2–3 days of incubation. It was unable to ferment glucose, sucrose, or fructose.

An alignment of the 16S rDNA gene sequence of the isolate LAS (GenBank accession number HM191705) with the sequences available in the GenBank databases at NCBI demonstrated that the isolate LAS should be classified as an *Arthrobacter* sp. (Fig. 2).

Purification of β-galactosidase

The β-galactosidase was purified to 15.7-fold and the recovery of total activity was 24.6% by ammonium sulfate precipitation and Phenyl-Sepharose chromatography (Table 1). Further purification was attempted; however, the recovery reduced dramatically because of loss of the enzyme activity (data not shown). Therefore, the active enzyme after hydrophobic chromatography was used in the following study to obtain preliminary characteristics.

Effect of pH and temperature on β-galactosidase activity from *Arthrobacter* sp. LAS for lactulose production

The enzyme activity of the β-galactosidase from *Arthrobacter* sp. LAS for lactulose production was examined at

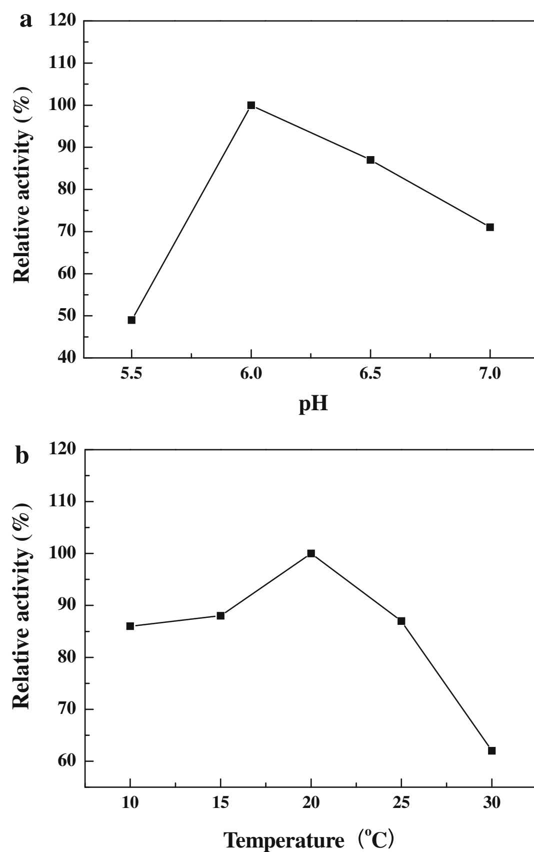

Fig. 3 Effects of pH (**a**) and temperature (**b**) on β-galactosidase activity from *Arthrobacter* sp. LAS for lactulose production

pH ranging from 5.5 to 7.0 (Fig. 3a). The optimum pH for partially purified enzyme activity was determined to be 6.0, lower than that of 7.0 from *Kluyveromyces lactis* [9]. The effect of temperature on the enzyme activity for lactulose production is shown in Fig. 3b. Maximum activity was recorded at 20°C, which is the lowest temperature compared with those reported for β-galactosidases used in lactulose production. Although there are several studies on β-galactosidase production by cold-adapted *Athrobacter* sp., the studies only focused on the hydrolysis activities and the potential application of such enzymes in dairy industry to hydrolyze lactose in refrigerated milk for lactose-intolerant consumers [4, 5, 12, 13]. The optimum temperature and pH for hydrolysis by β-galactosidase from *Arthrobacter psychrolactophilus* strain F2 were 10°C and 8.0 [12, 13], whereas those from *Athrobacter* sp. 20B were 6.0–8.0 and 25°C [4], respectively. No attention was paid to their lactulose biosynthesis abilities under low temperature. Therefore, whether the optimum conditions for hydrolysis were the same as those for transgalactosylation remains unclear.

Since β-galactosidase genes have been successfully expressed in recombinant *E. coli* and the purified enzymes have been used in lactulose production [8, 11], the expressing of β-galactosidases genes from other microbes such as *Arthrobacter* sp. LAS in *E. coli* might change the condition for lactulose production and minimize the by-products.

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