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Study of the transgalactosylation activity of β-galactosidase from a new strain *Kluyveromyces lactis* 3

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ABSTRACT

Beta-galactosidase (EC.3.2.1.23) is an important enzyme industrially used for the hydrolysis of lactose from milk and milk whey for several applications. Lately, the importance of this enzyme was enhanced by its galactosyltransferase activity, which is responsible for synthesis of transgalactosylated oligosaccharides that act as prebiotics with several beneficial effects on the consumers. ß-Galactosidase production by *Kluyveromyces lactis* 3 was studied in shake flask culture. The highest enzymatic activity was obtained at 10-th hour of the fermentation. The optimum temperature for transferase activity was 50°C. When incubated with 30% lactose in 50 mM phosphate buffer (pH 6.0) the enzyme can synthesize up to 41% galacto-oligosaccharides (GalOS). β-Galactosidase from strain *Kluyveromyces lactis* 3 produces mainly oligosaccharides with degree of polymerization (DP) 6 at 40°C and with DP 3 at 50°C.

Key words: beta-galactosidase, galacto-oligosaccharides, *Kluyveromyces lactis*, prebiotics

Introduction

Galacto-oligosaccharides (GalOS) occur naturally in breast and cow milk, honey and a variety of fruits and vegetables, but only in trace amounts (Angus et al., 2005). Currently, galacto-oligosaccharides are commercially produced from transgalactosylation reaction of ß-galactosidase from lactose. Also, the price of ß-galactosidase is rather high and, due to the low value of the substrate, the direct addition of the enzyme is economically unacceptable (Manning & Gibson, 2004). GalOS provide several health benefits, which make their use as prebiotics. Due to their β-configuration, galacto-oligosaccharides are resistant to hydrolysis by human saliva and gastric enzymes (Mussatto & Mancilha, 2007). GalOS are used as non-digestible, carbohydrate-based food ingredients in human and animal nutrition. Most of the studies are focused on the microorganisms that produce β-galactosidases with improved quality for production of GalOS (Hsu et al., 2007; Splechtna et al., 2007; Lu et al., 2009; Asraf & Gunasekaran, 2010).

β-Galactosidase based medical and industrial applications include cleavage of blood types A and B, biosensor for specific lactose determination in milk and disease diagnosis, treatment of lactose malabsorption, production of lactose hydrolyzed milk (Asraf & Gunasekaran, 2010). These enzymes catalyze the hydrolysis of lactose into glucose and galactose and transgalactosylation reactions with lactose as acceptor of galactose units giving rise to galacto-oligosaccharides of different glycosidic linkages and molecular weights (Otieno, 2010; Hernández-Hernández et al., 2011). The production of galacto-oligosaccharides from lactose was first studied with β-galactosidases from different strains of *Aspergillus oryzae*, *Bacillus circulans*, and *Kluveromyces lactis.* Enzymes from different sources have different characteristics, such as pH optima, temperature optima, Km for lactose and type and distribution of galacto-oligosaccharides. Amount and structures of produced galacto-oligosaccharides are dependent on the source of the enzyme (Mahoney, 1998). Hydrolysis rates and transgalactosylation of GalOS are different and related to the enzyme source, substrate concentration and reaction conditions (Rabiu et al., 2001; Cardelle-Cobas et al., 2008).

Different linkages between galactose and the reducing glucose unit have been identified, namely, β-D-Gal-(1-2)-D-Gl, β-D-Gal-(1-3)-D-Gl and β-D-Gal-(1-4)-D-Gl (Smart, 1991; Onishi & Tanaka, 1997). Also, branched glucose

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residues occur, whereas oligogalactose fragments contain mainly 1-4 or 1-6 linkages (Zárate & López-Leiva, 1990; Smart, 1993).

The degree of polymerization (DP), the type of linkage and the monosaccharide composition have a key role in the biological effects of GalOS (Cardelle-Cobas et al., 2011). There is, therefore, potential to study varying selectivities upon fermentation of these products.

The aim of this work was to study the transgalactosilation reaction of β-galactosidase from a new strain *Kluyveromyces lactis* isolated from Bulgarian traditional dairy products and to characterize synthesized galacto-oligosaccharides.

Materials and Methods

Microorganisms, culture media and culture conditions

Strains *Kluyveromyces lactis* 3 and *Kluyveromyces lactis* 546 were obtained from SIBIO'93 LTD, Plovdiv, Bulgaria. Yeasts were maintained on YEPD (1% yeast extract, 2% bacto-peptone, 2% glucose) agar slopes. One liter of the used basic medium for enzyme production consisted of 10 g yeast extract; 20 g bacto-peptone; 10 g lactose; 3.0 g KH_2PO_4 and 3.0 g $MgSO_4.7H_2O$ at the initial pH value 6.0.

In shake-flask experiments, cultures were grown at 30°C using 250 ml Erlenmeyer flasks, containing 50 ml medium. The flasks were permanently shaken at 200 rpm on a New Brunswick rotary shaker (New Brunswick Scientific, USA).

Analytical methods

Bacterial growth was measured by a turbidimetric method at 650 nm and calibrated against cell dry-weight measurements as previously described (Iliev et al., 2003). Cell growth was monitored by measuring the optical density at 650 nm (OD_{650}) during a fermentation experiment. Yeasts were harvested by centrifugation at 10000g for 20 min at 4°C. The cells were washed twice, resuspended in 0.05 M phosphate buffer (pH 6.0), and subsequently disrupted by ultrasonic desintegrator. β-Galactosidase activity was determined by hydrolysis of o-nitrophenyl β-D-galactopyranoside (ONPG, Sigma-Aldrich) to o-nitrophenol. Liberated o-nitrophenol was measured spectrophotometrically at 420 nm (molar absorbance coefficient of 4500 $L.mol^{-1}.cm^{-1}$) (Inchaurrondo et al., 1994). One unit of enzyme activity was defined as the enzyme quantity that liberated 1 μmol of o-nitrophenol per minute under the assay conditions. Protein concentration was determined according to the procedure of Bradford (Bradford, 1976) using bovine serum albumin as a standard. The total carbohydrate concentration was determined using the phenol-sulfuric acid method (Dubois et.al., 1956) and glucose as a standard.

Oligosaccharide synthesis and analysis

Oligosaccharides were synthesized by incubating of 1 U per milliliter of β-galactosidase in 0.05 M phosphate buffer (pH 6.0) containing from 5% to 30 % lactose at 40, 50 and 60°C with shaking (100 rpm). Samples were taken at intervals of 1 hour, and the reaction was stopped by heating for 5 min at 100°C. The oligosaccharides were analyzed by HPLC using a Symmetry C_{18} column (4.6 x 150 mm) and a Waters 1525 Binary HPLC Pump (Waters, Milford, MA, USA). Oligosaccharides were detected by using a Waters 2414 refractive index detector. The products were identified in the chromatograms as described by Remaud-Simeon et al. (1994).

Sugars (residual glucose, fructose, galactose and oligosaccharides in fermentation broth after fermentation) were determined by HPLC, using Zorbax carbohydrate column (4.6 x 150 mm; Agilent, Santa Clara, CA, USA), analytical guard column Zorbax NH2 (4.6 x 12.5 mm), and a mobile phase of 75/25 (v/v) acetonitrile/water. Breeze Chromatography Manager Software (Waters) was used for analysis of the data.

Results and Discussion

The effect of the initial concentration of lactose on the production of β-galactosidase from strains *Kluyveromyces lactis* 3 and *Kluyveromyces lactis* 546 was studied. The strains showed high activity of produced β-galactosidase in basic medium with 1% lactose after preliminary performed screening of different strains *Kluyveromyces lactis*. Enzyme production was conducted for 48 hours under various concentrations of lactose and glucose. The maximum activities (32.2 U/ml and 22.0 U/ml) were measured at the 10-th hour after cultivation in media with 5% lactose for *Kluyveromyces lactis* 3 and *Kluyveromyces lactis* 546, respectively (Figure 1). No activity was detected after cultivation of the studied strains in glucose-containing medium. Probably, β-galactosidase from this strains is mainly inducible. The detected activity of β-galactosidase produced from strain *Kluyveromyces lactis* 3 was ten times higher than the measured activity of the enzyme produced by strain *Kluyveromyces lactis* 546.

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The produced β-galactosidase from *Kluyveromyces lactis* 3 was used for synthesis of galacto-oligosaccharides at varying concentrations of lactose.

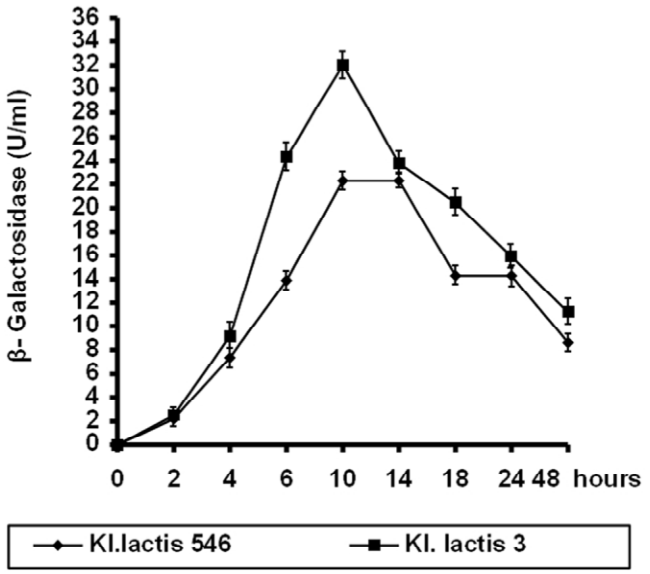

Figure 1. *Fermentation profiles of β-galactosidase production from Kluyveromices lactis 546 and Kluyveromices lactis 3 cultivated in synthetic medium with 5% lactose.*

The synthesis of galacto-oligosaccharides was performed at 20%, 25% and 30% of lactose and with an enzyme concentration of 1 U/ml. As illustrated in Figures 2 and 3, the maximum yield (47%) in total galacto-oligosaccharides was observed when 25% lactose was used. β-Galactosidase from strain *Kluyveromyces lactis* 3 produces mainly oligosaccharides with DP 6 at 40°C and with DP 3 at 50°C (Figure 2 and Figure 3). The results demonstrate that the β-galactosidase from *Kluyveromyces lactis* 3 efficiently synthesized oligosaccharides with DP 6 overall yield of 29% using 25% initial lactose concentration at 40°C and only 14% using 25% initial lactose concentration at 50°C. The yield of the GalOS with DP 3 using 30% initial lactose concentration is higher by increasing of the reaction temperature (50°C). The values reported here are slightly higher than those reported by Dumortier et al. (1994).

As shown on the HPLC chromatogram of transgalactosylated reaction, products synthesized with β-galactosidase from studied strain displayed two mainly peaks for DP 3 and DP 6 (Figure 4). The oligosaccharides synthesized by the studied enzyme show the same retention time as those obtained with the Amano β-galactosidase (data not shown). Among the GalOS, only tri- and tetra-saccharides were synthesized and longer chain oligosaccharides than DP 6 were not found at any time during the course of the reaction. This observation confirms the results described by Smart et al. (1993), according to which high lactose concentrations favor the synthesis of short chain oligosaccharides. It was observed that complete inhibition of oligosaccharide synthesis occurred when the monosaccharides content of the reaction medium reached the total concentration of GalOS.

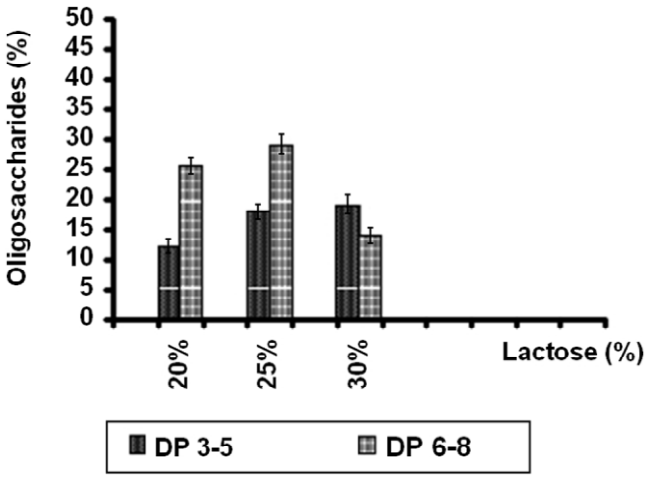

Figure 2. *Oligosaccharide synthesis using β-galactosidase from Kluyveromyces lactis 3 at 40°C and different initial concentration of lactose (20%, 25% and 30%).*

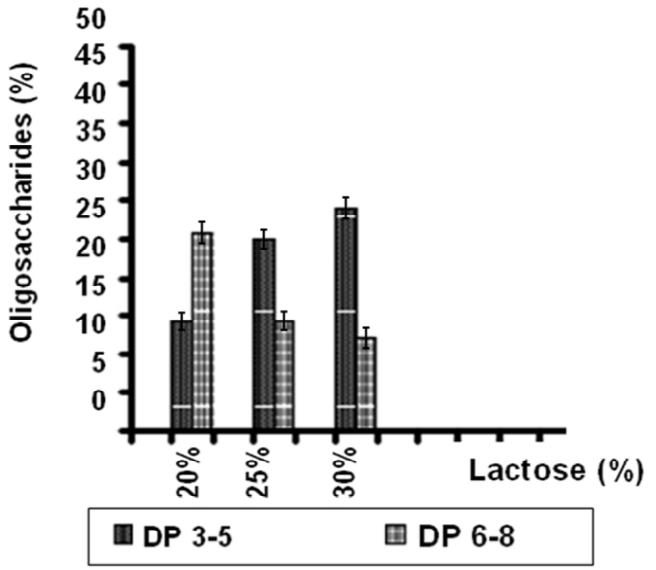

Figure 3. *Oligosaccharide synthesis using β-galactosidase from Kuyveromyces lactis 3 at 50°C and different initial concentration of lactose (20%, 25% and 30%).*

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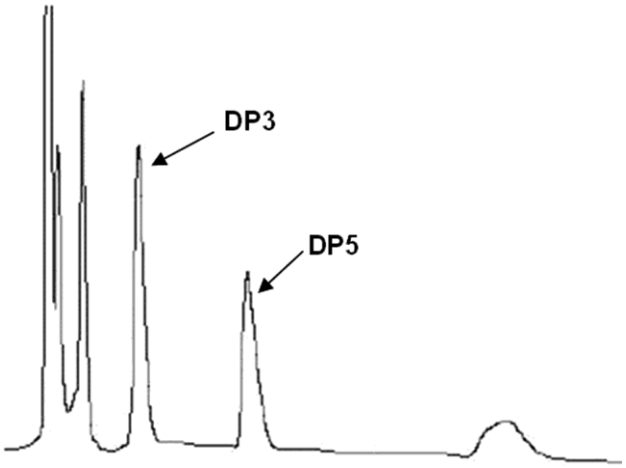

Figure 4. *Chromatogram of the oligosaccharides synthesized in the presence of 25% lactose by β-galactosidase from Kluyveromyces lactis 3.*

Our results are comparable with results by other authors investigating the synthesis of galacto-oligosaccharides by β-galactosidase received from different microorganisms. The synthesis of GalOS with a high yield of 55% from 275 g/L lactose at 50°C for 12 h have been performed using transglycosylating β-galactosidase produced from *E. cloacae* (Lu et al., 2009). Splechtna et al. (2007) have been optimized the synthesis of GalOS from lactose using β-galactosidase from *Lactobacillus* sp. It has been proved the beneficial effect of directly applied crude cell-free enzyme extract for the conversion, since similar GalOS yields and composition have been obtained as when using the pure enzyme preparation (Splechtna et al., 2007). Hsu et al. (2007) have been studied the production of GalOS by transgalactosylation using ß-galactosidase from *B. longum* BCRC 15708. Two types of GalOSs, tri- and tetrasaccharides, have been formed after ß-galactosidase action on 40% lactose. A highest yield of 32.5 % (w/w) GalOSs have been achieved from 40% lactose solution at 45°C, pH 6.8. It has been found that an increase of the initial lactose concentration in the reaction mixture leads to a higher GalOS production (Hsu et al., 2007).

All these examples confirm that the hydrolysis rates and transgalactosylation are different and related to the enzyme source, substrate concentration and reaction conditions (Rabiu et al., 2001; Cardelle-Cobas et al., 2008).

Conclusion

We describe the production of β-galactosidases from new strains *Kluyveromices lactis* 546 and *Kluyveromices lactis* 3, isolated from Bulgarian traditional dairy foods. The maximum activity of β-galactosidase of 32.2 U/ml was detected at the 10-th hour after cultivation of *Kluyveromices lactis* 3 on medium with 5% lactose.

The studied β-galactosidase from *Kluyveromices lactis 3* is a very efficient enzyme for the synthesis of transgalacto-oligosaccharides because of its high product yields and transfer rates. The enzyme from *Kluyveromices lactis* 3 showed higher effectivity during synthesis for low molecules transgalacto-oligosaccharides. The enzyme synthesized mainly oligosaccharides with DP 6 at 40°C in the presence of 25% lactose and oligosaccharides with DP 3 at the higher lactose concentration of 30% and higher temperature (50°C). These results allow further studies, related to prebiotic properties of the galacto-oligosaccharides synthesized by β-galactosidase from the strain *Kluyveromices lactis* 3.

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