Research Article

Formation of galacto-oligosaccharides during lactose hydrolysis by a novel β-galactosidase from the moderately thermophilic fungus *Talaromyces thermophilus*

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Discontinuous and continuous processes of lactose hydrolysis and concomitant galacto-oligosaccharide (GalOS) formation were studied. To this end a wide experimental range of the main variables was evaluated, including the initial lactose concentration, the degree of lactose conversion, the pH value and the temperature for discontinuous transformations, while the initial lactose concentration and the feed rate were varied for the continuous process. For both processes a high-initial lactose concentration proved to be advantageous for the formation of GalOS. The maximum amount of GalOS (100 g/L, corresponding to a yield of approximately 50% based on the initially employed lactose) was obtained after 8 h of incubation when using 200 g/L lactose as substrate and 90% lactose hydrolysis was observed. GalOS productivity in the continuous process (g/L·h) was enhanced by an increase of the flow rate. The maximum GalOS productivity of 70 g/L·h was obtained at a flow rate of 24 mL/h when using a reactor with a total working volume of 21 mL. As was evident from these experiments, this β-galactosidase from a moderately thermophilic fungus showed a strong transgalactosylation activity and can be used for the formation of GalOS, sugars that are of considerable interest for functional food applications because of their presumed health-promoting effects.

Keywords: Continuous production · β-Galactosidase · Galacto-oligosaccharides · Lactose hydrolysis · Prebiotics

1 Introduction

Galacto-oligosaccharides (GalOS), also known as 'bifidus growth factor' since they promote the growth of desirable intestinal microflora, are nondigestible carbohydrates comprised of di-, tri-, tetra- or pentasaccharides that mainly consist of galactose and glucose units joined through various glycosidic bonds. GalOS are now widely used as low calorie sweetener, food ingredients, pharmaceuticals and other biologically active compounds. Especially the use of GalOS in various foods have lately attracted increasing attention as evidence is accumulating which shows that the consumption of these prebiotic oligosaccharides can be beneficial to human health [1]. GalOS can be synthesized by chemical synthesis, but their preferred mode of synthesis on a preparative scale is by enzymatic catalysis from lactose using an appropriate β-galactosidase. The formation of GalOS by β-galactosidases from yeast, fungi and bacteria has been reported. The structure and product ratio of GalOS obtained by

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Abbreviations: CE, capillary electrophoresis; **GalOS,** galacto-oligosaccharide

transgalactosylation reactions depend on the enzyme as well as on the conditions employed in the transformation reaction [1–5].

Various reactor designs and configurations have been reported for lactose hydrolysis and GalOS formation, including the batch reactor, continuous stirred-tank reactor (CSTR), CSTR coupled with crossflow filtration, hollow fibre membrane reactor, fixed-bed and fluidized-bed reactor [6–11]. In the batch process, the enzyme initially added to the reaction mixture is obviously lost at the end of the reaction. The aim of continuous processes has been chiefly to reduce the enzyme cost by reusing the enzyme for the transformation of fresh substrate. This can be achieved by immobilizing the enzyme on a carrier or by retaining the soluble enzyme in the reactor by using an ultrafiltration membrane.

In the present study, we focus on the use of both a discontinuous stirred tank and a recycle membrane reactor, consisting of a reactor coupled to a crossflow ultrafiltration membrane, for the production of GalOS using a novel intracellular β-galactosidase from the moderately thermophilic ascomycete *Talaromyces thermophilus*. This enzyme was found to be fairly thermostable with a half-life time of activity of approximately 200 h at 40°C. It is characterized by a relatively low Michaelis constant of 18 mM for lactose, which compares favorably with kinetic constants for β-galactosidases from other organisms. Furthermore, this enzyme is only moderately inhibited by its end product galactose (K_i = 420 mM) which is in contrast to many other microbial β-galactosidases which are severely inhibited by this sugar [12]. These properties make the β-galactosidase from *T. thermophilus* attractive for technological applications such as the production of GalOS.

2 Materials and methods

2.1 Materials

Talaromyces thermophilus CBS 236.58 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) was used as the source of β-galactosidase. β-Galactosidase solutions were produced by the moderately thermophilic fungus and subsequently purified as recently reported [12]. β-Galactosidase activity was measured by using lactose as the substrate [12]. Lactose was purchased from Sigma (Deisenhofen, Germany); all other chemicals were of the highest grade available and were from Merck (Darmstadt, Germany). The commercial GalOS product Elix'or was obtained from Borculo (Zwolle, The Netherlands).

2.2 Methods

2.2.1 *Batch conversions*

Discontinuous conversions were performed in a beaker using 10 mL of lactose solution (5–20% w/v lactose in 50 mM sodium phosphate buffer, pH 6.5, containing 10 mM $MgCl_2$). This substrate solution was incubated with β-galactosidase (25 U) in a shaker incubator under constant agitation of 130 rpm at 40°C for 24 h. Aliquots were taken at various times and the enzymatic reaction stopped by a 10-min incubation at 95°C. The sugars were analysed by capillary electrophoresis (CE) as previously described [13]. The effect of pH on the conversion of lactose and GalOS formation was examined in the pH range of 5.5–6.5 using an initial lactose concentration of 10% w/v. The effect of temperature on GalOS formation was examined at both 30 and 40°C at pH 6.5 and an initial lactose concentration of 10% w/v. Experiments were repeated at least twice, and the SD of corresponding experiments was always less than 5%.

2.2.2 *Continuous conversions*

A reactor with a total volume of 21 mL was used with an external crossflow ultrafiltration unit (Amicon) with a cut-off of 10 kDa and a filtration surface of 50 cm^2 (Fig. 1). The enzyme was retained in the reactor while a solution containing the relatively low-molecular weight products (GalOS, glucose and galactose) and substrate (lactose) passed through the membrane. The enzyme reactor was operated at 40°C and using lactose solutions (initial concentration 10 and 20% w/v) dissolved in 50 mM phosphate buffer (pH 6.5) containing 10 mM $MgCl_2$ and β-galactosidase (210 U total activity). A feed solution (10 and 20% w/v lactose in 50 mM phosphate buffer, pH 6.5, containing 10 mM $MgCl_2$) was continuously fed to the reactor at the flow rates indicated until equilibrium of the reaction was achieved. No leakage of enzyme was observed as determined by the enzyme activity in the permeate. No permeate was recycled. Given experimental results for the production of GalOS are always for steady state conditions. Experiments were repeated at least twice, and the SD of corresponding experiments was always less than 5%.

3 Results and discussion

3.1 GalOS formation

Formation of notable amounts of byproducts during the β-galactosidase-catalyzed hydrolysis of lactose, in addition to the two primary reaction products galactose and glucose, has been described for a number of β-galactosidases from various sources [1], primarily for the retaining enzymes of glycosyl hydrolase family 2 or 1, such as the *Escherichia coli* lacZ β-galactosidase. A strong transgalac-

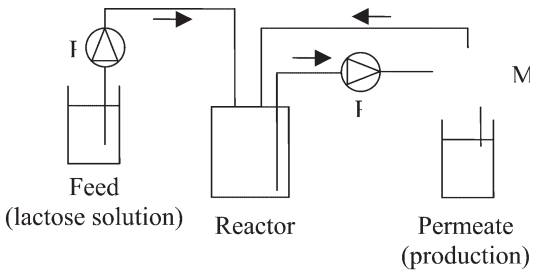

Figure 1. Scheme of the continuous laboratory scale reactor with an external crossflow ultrafiltration unit. P, peristaltic pump; M, UF membrane.

tosylation activity was also found for the novel β-galactosidase from *T. thermophilus* both in discontinuous and continuous transformation experiments. As was analysed by CE, not only the main hydrolysis products glucose and galactose but also GalOS were formed during incubation of the enzyme with lactose. Analysis of product mixtures using CE revealed the formation of several GalOS, with different nonlactose disaccharides and trisaccharides being the main reaction products (Fig. 2).

3.2 Batch conversions

The effect of various reaction conditions, namely the pH value (5.5, 6.0 and 6.5), temperature (30 and 40°C) and lactose concentration (5, 10 and 20% w/v) on the formation of GalOS by β-galactosidase from *T. thermophilus* was studied in discontinuous bioconversion experiments. The pH value exerted a slight effect on GalOS formation with pH 5.5 being optimal and GalOS formation decreasing somewhat with increasing pH (Fig. 3). Increasing the temperature from 30 to 40°C accelerated lactose hydrolysis as well as GalOS formation (Fig. 4). Similar results were reported for the enzyme of *Bifidobacterium infantis* HL96

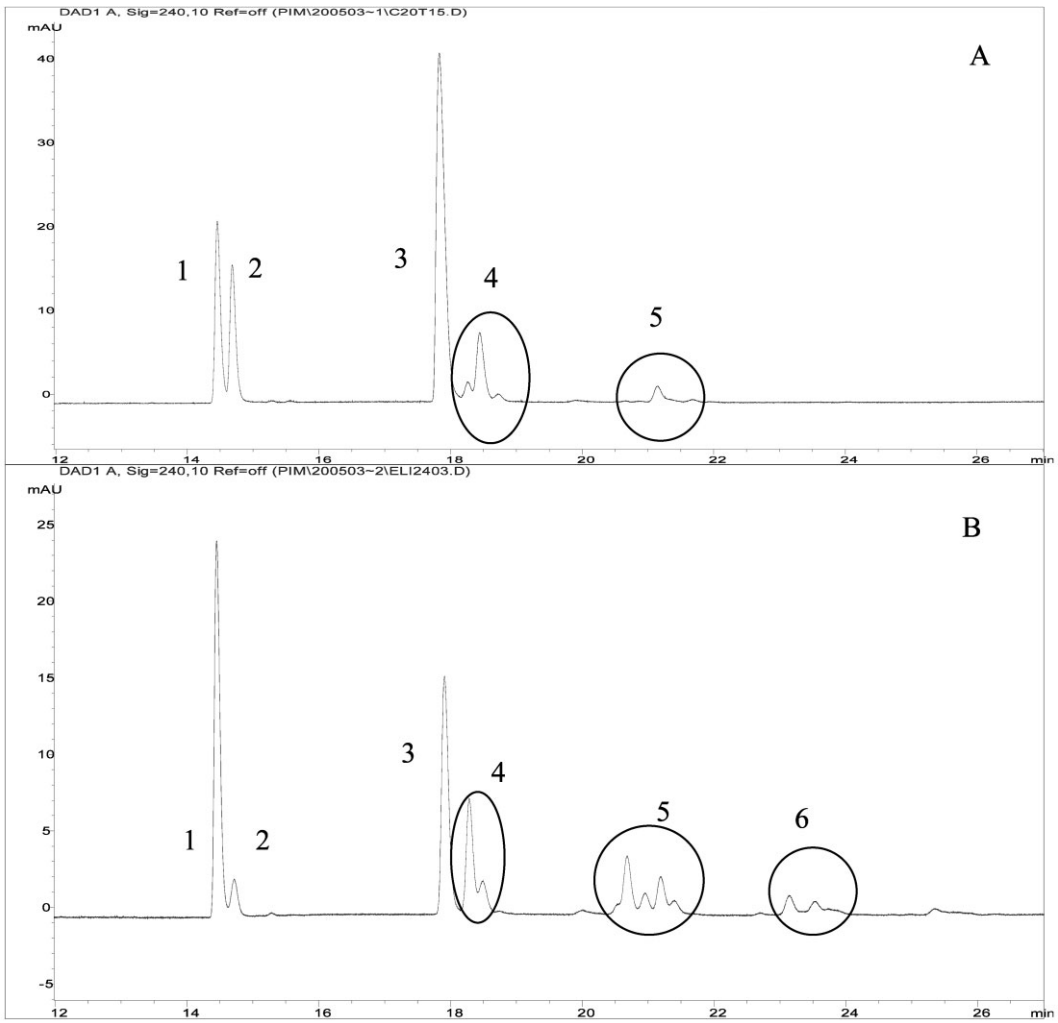

Figure 2. CE analysis of products from continuous lactose conversion by (A) β-galactosidase from *T. thermophilus* and (B) the commercial product Elix'or: glucose (1), galactose (2), lactose (3), disaccharides (4), trisaccharides (5) and tetrasaccharides (6). Reaction conditions for the transformation in (A): 20% w/v initial lactose concentration, reaction time 8 h with 90% lactose conversion.

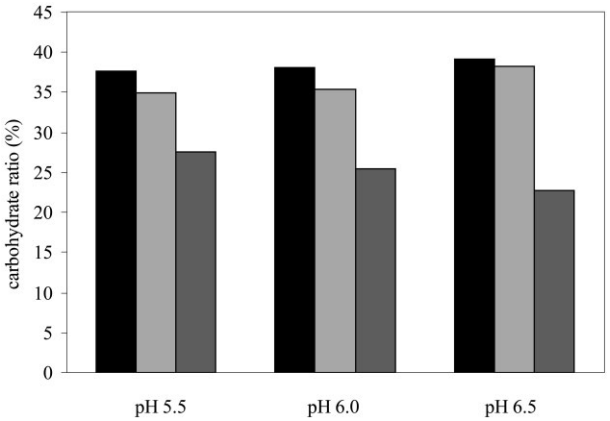

Figure 3. Effect of the pH value on the hydrolysis of lactose and product formation when using an initial lactose concentration of 10% (reaction conditions: 40°C, 24 h reaction time, β-galactosidase activity 2.5 U/mL). Sugars are given as the percentage of total sugar content in the mixture. ■, Glu; ■, Gal; ■, GalOS.

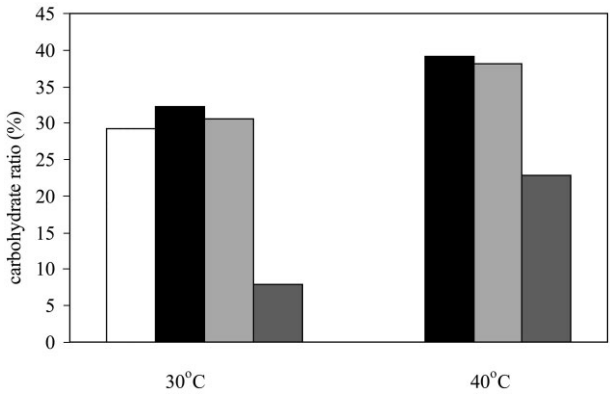

Figure 4. Effect of temperature on the hydrolysis of lactose and product formation when using the initial lactose concentration 10% (reaction conditions: pH 6.5, 24 h reaction time, β-galactosidase activity 2.5 U/mL). Sugars are given as the percentage of total sugar content in the mixture. □, Lac; ■, Glu; ■, Gal; ■, GalOS.

[14] and it was also suggested that temperature increase is correlated with an increased transgalactosylation activity [1, 15]. A further increase in temperature was found to be disadvantageous when using the *T. thermophilus* enzyme since it is rapidly inactivated at temperatures above 50°C [12].

One of the most important factors to increase the yields of GalOS products is the substrate concentration, which should be as high as possible to shift the reaction more towards transglycosylation [1]. The formation of GalOS by *T. thermophilus* β-galactosidase showed a marked dependence on the initial substrate concentration. As is shown in Fig. 5, at initial substrate concentrations of 5, 10 and 20% w/v lactose, a maximum yield of 28, 32 and 50% GalOS was obtained, respectively. The time course of lactose hydrolysis and product formation was investigated in more detail when using 20% w/v lactose as the initial substrate concentration (Fig. 6). A maximum concentration of approximately 100 g/L GalOS was observed when 90% of the lactose was hydrolyzed. Since these various oligosaccharides are, however, also substrates of β-galactosidase and lactose is continuously depleted from the reaction mixture, they are only formed transiently and are in turn hydrolyzed when the reaction proceeds. At the beginning of the reaction with this initially high lactose concentration, transgalactosylation seems to proceed faster than hydrolysis and the first major reaction products are various trisaccharides, which accumulate to up to 30% of the total sugars in the mixture (60 g trisaccharides *per* liter) when lactose conversion is

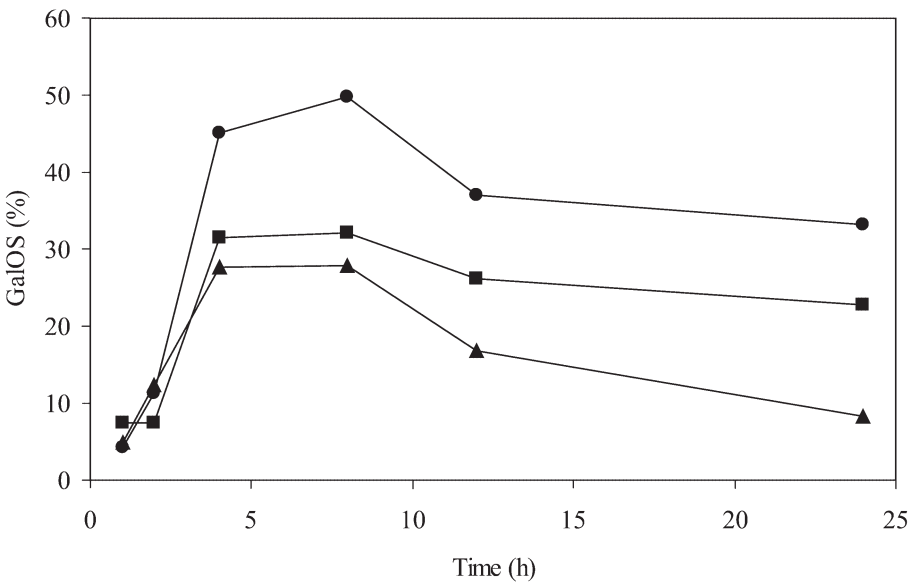

Figure 5. Time course of lactose hydrolysis in enzymatic batch reaction when using different initial concentrations of lactose: (▲) 5%, (■) 10% and (●) 20% (reaction conditions: 40°C, pH 6.5; β-galactosidase activity 2.5 U/mL).

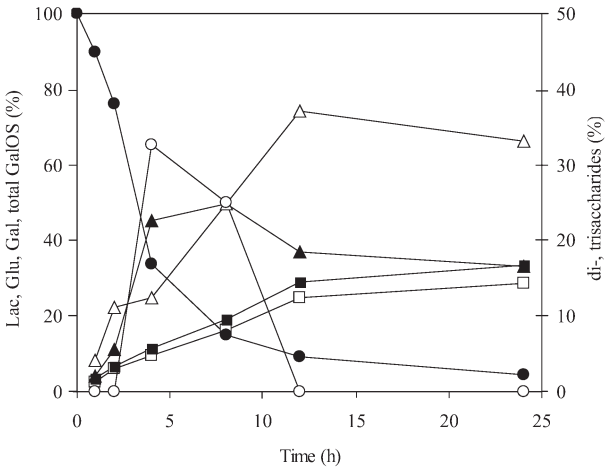

Figure 6. Time course of lactose hydrolysis in enzymatic batch reaction when using 20% initial concentration of lactose: Lactose (●), glucose (Glu) (■), galactose (Gal) (□), total GalOS (▲) including nonlactose disaccharides (△) and trisaccharides (○) as analysed by CE.

approximately 50%. These trisaccharides, however, are rapidly degraded with the progress of the reaction. With the increased availability of the primary reaction products galactose and glucose, various nonlactose disaccharides are then formed as major transgalactosylation products. In contrast to the trisaccharides, these are more resistant to hydrolysis by *T. thermophilus* β-galactosidase. Hence, depending on the reaction time or degree of lactose conversion, a different range of products can be obtained in these reactions. The maximum amount of GalOS was obtained after 8 h of incubation when 90% lactose hydrolysis was observed. This maximum yield (gram GalOS produced *per* gram of total lactose employed) of approximately 50% compares very favorably to data published in the literature for other microbial β-galactosidases [2]. It has been stated that highest levels of GalOS obtained (of around 40%) are associated with neutral pH β-galactosidases from bacteria and yeast rather than acid pH enzymes from fungi [16]. The acidic β-galactosidase from *T. thermophilus* (pH optimum for lactose hydrolysis of 5.5–6.0) clearly does not confirm this trend as it catalyses the formation of very high yields of total GalOS (nonlactose disaccharides and higher oligosaccharides).

3.3 Continuous conversion

For the steady state condition in a continuous reactor, significant product (GalOS) concentrations can be achieved in the permeate. Figure 7 shows the effect of both the substrate concentration and the flow rate on GalOS production. In accordance with the discontinuous experiments, the maximum GalOS concentration obtained depends on the concentration of the substrate in the feed. The GalOS productivity (g/L·h) was also enhanced by an increase of the flow rate. The maximum GalOS productivity of 70 g/L·h was obtained with a flow rate of 24 mL/h. Under these optimized conditions (20% w/v lactose, flow rate 24 mL/h, 40°C, pH 6.5), approximately 50% of the lactose was converted. In contrast to the discontinuous transformation, where trisaccharides were the main products at approximately 50% lactose conversion, the main products under a continuous mode of operation were disaccharides (25% of total sugars in the reaction mixture), while trisaccharides were formed only to a lesser extent (7% of total sugars in the reaction mixture). The productivity of 70 g/L·h obtained under these reaction conditions, which corresponds to a specific productivity of 7.0 mg

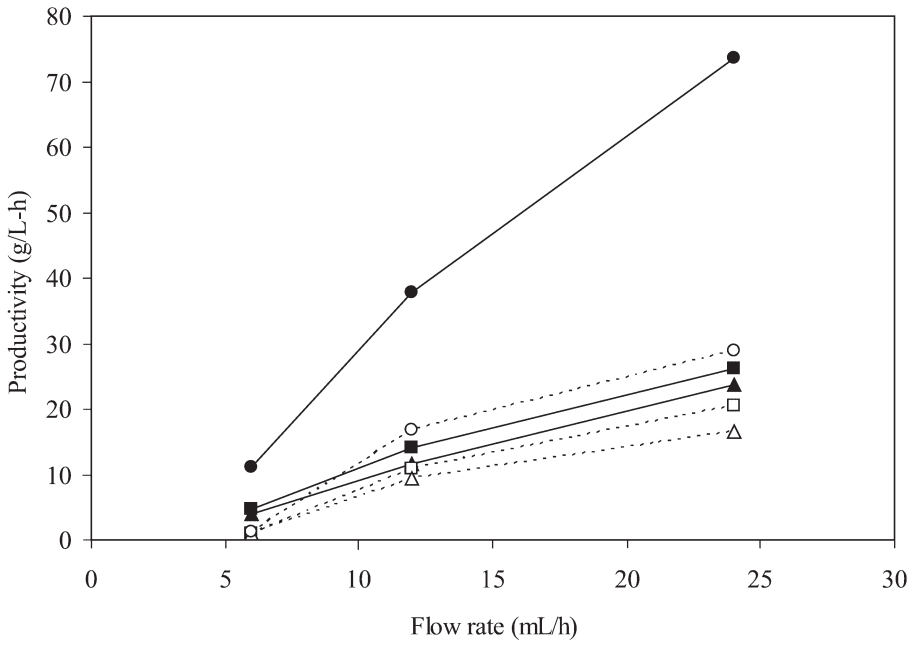

Figure 7. Formation of GalOS in a continuous reactor: Effect of both the substrate concentration and flow rate on the productivity; GalOS (cycles), Glu (squares) and Gal (triangles). Experiments were carried out using an initial lactose concentration of 10% (opened symbols) and 20% (closed symbols).

GalOS/U·h, is in excellent agreement with other reports on continuous systems using free enzymes that are retained by ultrafiltration membranes, *e.g.* a value of 6.4 mg GalOS/U·h was obtained when using the β-galactosidase from the yeast *Kluyveromyces lactis* [17].

4 Concluding remarks

Our results presented in this work suggest that β-galactosidase from the thermophilic fungus *T. thermophilus* cannot only be used for lactose hydrolysis but also for the efficient formation of prebiotic GalOS, compounds that are of great interest for food and feed applications because of proven and presumed beneficial effects on health and well-being [1]. This study also recommends that high lactose concentrations and high temperature are desirable for GalOS synthesis; high temperature favored both increased reaction velocity and substrate solubility, while also helping to prevent unwanted microbial contamination during a long time process. Both discontinuous and continuous modes of operation are possible for attaining significant GalOS formation. Interestingly, the mode of operation had a clear effect on the composition of the product mixture with a larger fraction of trisaccharides being formed in batch production. Further studies, however, need to be carried out to investigate and evaluate the effects of these different GalOS structures on fermentation properties of various intestinal bacteria and thus on their prebiotic effect.

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