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Lactose Hydrolysis and Formation of Galactooligosaccharides by a Novel Immobilized β-Galactosidase From the Thermophilic Fungus *Talaromyces thermophilus*

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Abstract

β-Galactosidase from the fungus *Talaromyces thermophilus* CBS 236.58 was immobilized by covalent attachment onto the insoluble carrier Eupergit C with a high binding efficiency of 95%. Immobilization increased both activity and stability at higher pH values and temperature when compared with the free enzyme. Especially the effect of immobilization on thermostability is notable. This is expressed by the half-lifetime of the activity at 50°C, which was determined to be 8 and 27 h for the free and immobilized enzymes, respectively. Although immobilization did not significantly change kinetic parameters for the substrate lactose, a considerable decrease in the maximum reaction velocity V_{max} was observed for the artificial substrate o-nitrophenyl-β-D-galactopyranoside (oNPG). The hydrolysis of both oNPG and lactose is competitively inhibited by the end products glucose and galactose. However, this inhibition is only very moderate as judged from kinetic analysis with glucose exerting a more pronounced inhibitory effect. It was evident from bioconversion experiments with 20% lactose as substrate, that the immobilized enzyme showed a strong transgalactosylation reaction, resulting in the formation of galactooligosaccharides (GalOS). The maximum yield of GalOS of 34% was obtained when the degree of lactose conversion was roughly 80%. Hence, this immobilized enzyme can be useful both for the cleavage of lactose at elevated temperatures, and the formation of GalOS, prebiotic sugars that have a number of interesting properties for food applications.

Index Entries: Immobilization; Eupergit; β-glycosidase; lactase; transgalactosylation.

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Introduction

The enzyme β-D-galactoside galactohydrolase (EC. 2.2.1.23), commonly known as β-galactosidase or lactase, is a commercially important enzyme with a well-established use in the dairy and food industries. Its occurrence in nature is quite diverse and it has been found in plants, animals, and microorganisms *(1)*. β-Galactosidase hydrolyzes β-D-galactopyranosides such as lactose, but it also catalyzes transgalactosylation reactions: for example, lactose can serve both as galactosyl donor and as acceptor to yield di-, tri-, or higher oligosaccharides *(2)*. Lactose hydrolysis products, lactose-reduced or lactose-free dairy products, have the advantage of improved quality as they are more easily digested by customers who are lactose intolerant. In whey, lactose hydrolysis offers additional product utilization of this abundant renewable resource which is still considered a waste and discarded frequently. The resulting sweeter product (lactose syrup) has numerous applications in the food and dairy industry. In addition, the undesirable crystallization of lactose in certain products such as ice cream is avoided by its hydrolysis *(1,3,4)*.

Oligosaccharides produced by the reaction of transferase activity of β-galactosidases during lactose hydrolysis, the so-called galactooligosaccharides (GalOS), were already reported in the early 1950s when they were considered undesired byproducts *(5)*. These GalOS typically contain a galactosyl-galactose chain with a terminal glucose residue. They are currently used as a low caloric sweetener in food applications, or as pharmaceutical compounds. More recently, GalOS, also known as "Bifidus growth factor," have been used as a food ingredient beneficial to human health *(3,6)*.

The application of β-galactosidases was described both for soluble enzymes, which are normally used in batch processes, or for immobilized preparations, which predominate in continuous processes. Many different solid supports and immobilization strategies have been developed, including entrapment, crosslinking, adsorption, and covalent attachment. The major industrial application of immobilized β-galactosidase has been for the hydrolysis of lactose or for the production of GalOS, thereby improving the functional properties of dairy products. Immobilization is an attractive tool for obtaining an enzyme preparation that can be easily recovered and used in continuous operations, thus making possible the efficient use of an enzyme in an industrial application *(1,7)*.

This work describes the characterization of the moderately thermophilic β-galactosidase from *Talaromyces thermophilus* immobilized onto Eupergit C and its comparison with the free enzyme. We also investigated the performance of this enzyme preparation for lactose hydrolysis and GalOS formation.

Methods

Materials

T. 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 according to Nakkharat *(8)*. Eupergit C was obtained from Röhm (Darmstadt, Germany). o-Nitrophenyl-β-D-galactopyranoside (oNPG) and lactose were purchased from Sigma (Deisenhofen, Germany).

Immobilization of β*-Galactosidase*

The immobilization of β-galactosidase was carried out at room temperature (approx 25°C) for 96 h. The soluble enzyme (70 U), which previously had been purified to apparent homogeneity of a specific activity of 68 U/mg, was incubated with 580 mg of Eupergit C in a total volume of 3 mL. After the selected time, both the supernatant and the support were analyzed for enzyme activity. The immobilized enzyme preparation was used without further treatment and stored at 4°C. The amount of enzyme that binds to the matrix (U_{bound}) was calculated as the difference of the total activity (U_{tot}) used for immobilization and the remaining activity measured in the supernatant (U_{sup}) after immobilization. The recovery of activity is calculated as the ratio of $(U_{tot}–U_{sup})/U_{tot}$. The binding efficiency, η, relates the measured value of the immobilized preparation (U_{imm}) to the value expected from the difference in the activity of the free enzyme before and after the immobilization (U_{imm}/U_{bound}).

Enzyme Assay

The activity of β-galactosidase was determined by using the artificial substrate oNPG. An appropriate amount of soluble or immobilized enzyme (approx 1 mg) was mixed with 22 m*M* oNPG dissolved in 500 µL of 50 m*M* Na-phosphate buffer, pH 6.5. After incubation at 40°C for 15 min, the reaction was stopped by adding 750 µL of 0.4 *M* Na_2CO_3. The absorbance was measured at 420 nm and the concentration of o-nitrophenol (oNP) released was calculated using a standard curve. One unit of β-galactosidase activity is defined as the amount of enzyme that releases 1 µmol of oNP from oNPG/min under the experimental conditions described above. When using lactose as a substrate, 600 m*M* lactose in 50 m*M* Na-phosphate buffer, pH 6.5 was incubated under the same condition as above, and the reaction was stopped by heating at 95°C for 10 min. Lactose hydrolysis was monitored by measuring the amount of glucose released using the glucose oxidase assay *(9)*. One unit of β-galactosidase activity refers to 1 µmol of glucose released per minute under the reaction conditions selected.

Determination of Optimum Temperature, pH, and Kinetic Parameters

Optimum temperature and pH were determined by changing individually the condition of the standard β-galactosidase assay (varying the temperature from 35°C to 60°C, and the pH from 4.5 to 8.0). The same amount of free and immobilized enzyme (in terms of protein) was used to determine the kinetic parameters by measuring the enzyme activity with varying substrate concentrations. Kinetic constants were calculated using Lineweaver-Burk plots and nonlinear regression (SigmaPlot 2000; SPSS, Chicago, IL).

Stability

The immobilized enzyme was incubated at various temperatures ranging from 35°C to 60°C. The half-lifetime of the activity was estimated using plots of $\ln(a/a_o)$ as functions of time at the respective temperature, where, a and a_o are the activity at time t and initial activity of the enzyme, respectively.

Lactose Hydrolysis

An immobilized enzyme preparation (2 $U_{lactose}$) was incubated with 20% lactose in Na-phosphate buffer pH 6.5 at 40°C and 1200 rpm using an Eppendorf Thermomixer (Munich, Germany). Aliquots were taken at various times and the enzymatic reaction stopped by a 10-min incubation at 95°C. The sugars were analyzed by capillary electrophoresis (CE) as previously described *(9)*.

Results

Immobilization of β-Galactosidase

Eupergit C consists of macromolecular beads of an acrylic polymer and is a suitable carrier for the immobilization of industrial enzymes by covalent attachment. Because of its mechanical stability, which was described as "highly reactor-compatible," it can be employed in most commonly used reactor types, including stirred tank or fixed bed reactors *(10)*. β-Galactosidase from *T. thermophilus* was covalently attached to Eupergit C with a high binding efficiency η of 95% and 75% recovery of the enzyme activity. In long-term stability tests of this immobilized enzyme preparation, enzyme activity was completely retained when stored for 16 d at 4°C.

pH and Temperature Dependence of Enzyme Activity

The activity-pH profiles of the free and the immobilized enzyme were determined for both substrates, oNPG and lactose (Fig. 1). The optimum pH of the free and the immobilized enzyme were found to be 5.5 and 6.0, respectively, when using lactose as a substrate, and 6.0 for oNPG hydrolysis. Figure 2 shows the temperature optimum determined for both preparations

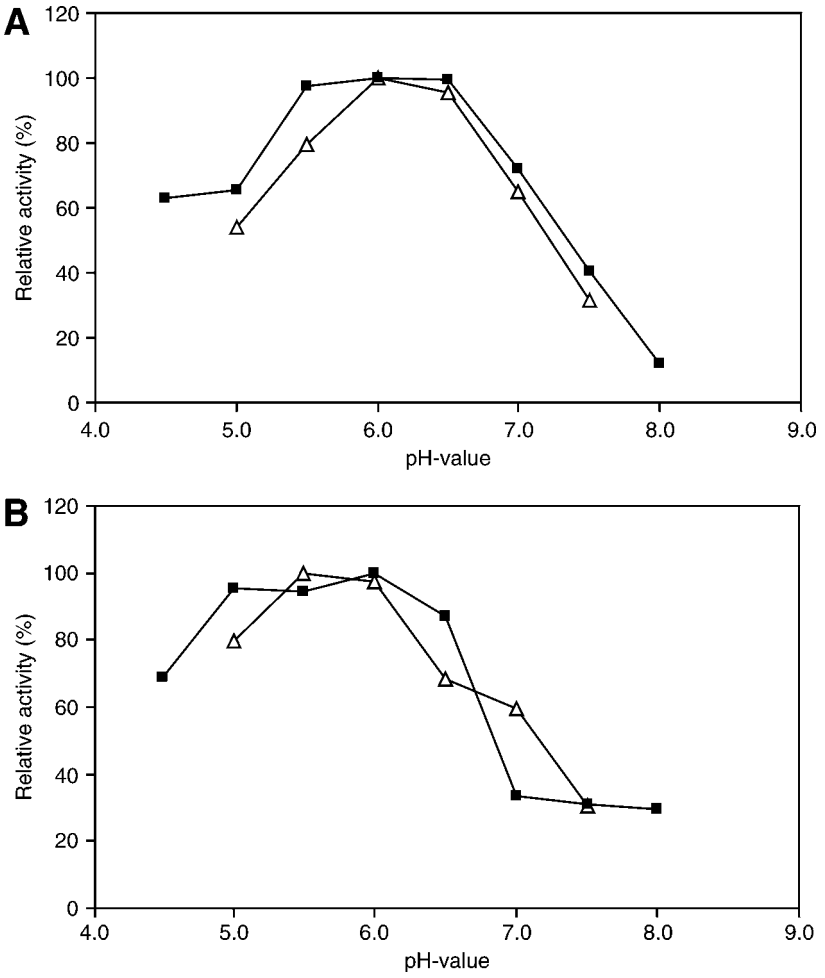

Fig. 1. pH optimum of free (Δ) and immobilized (■) β-galactosidase from *T. thermophilus*. **(A)** oNPG hydrolysis; **(B)** lactose hydrolysis. Activity tests were carried out in 50 m*M* Na phosphate buffer using the following reaction condition: reaction time, 15 min; *T*, 40°C.

for the 15-min assay. Immobilizing the enzyme increases its temperature optimum from 50°C to 60°C and from 45°C to 50°C for lactose and oNPG as the substrate, respectively. The thermostability of β-galactosidase for both the free and the immobilized preparation was evaluated by determining the half-lifetime of the activity at various temperatures. The plot of half-lifetime as a function of temperature is shown in Fig. 3. The half-lifetimes of activity for the free and immobilized enzyme at 50°C were estimated to be approx 8 and 27 h, respectively.

Kinetic Parameters

The kinetic parameters K_m and V_{max} of the free and immobilized β-galactosidase were determined for the two substrates, oNPG and lactose.

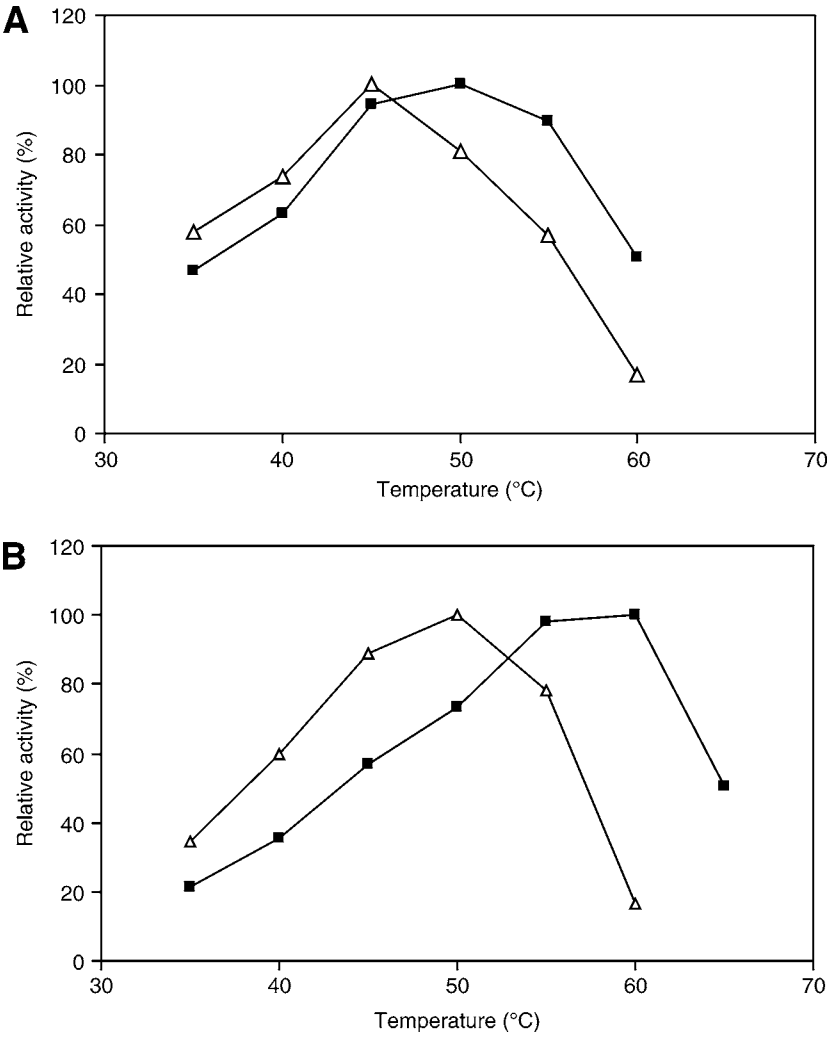

Fig. 2. Temperature optimum of free (Δ) and immobilized (■) β-galactosidase from *T. thermophilus*. **(A)** oNPG hydrolysis; **(B)** lactose hydrolysis. Activity tests were carried out in 50 m*M* Na phosphate buffer using the following reaction condition: reaction time, 15 min; pH, 6.5.

In addition, different concentrations of the end products, glucose and galactose, were tested for a possible inhibiting effect. Results are shown in Table 1. When measuring kinetic constants for β-galactosidase immobilized onto Eupergit C, it was found that V_{max} was lowered to approx 150 µmol/min/mg for oNPG as a substrate whereas the K_m-value was decreased approximately threefold to 10 m*M*. In contrast, the K_m for lactose remained almost unaltered whereas V_{max} even increased to some extent on immobilization. As was evident from this kinetic analysis, the hydrolysis of both oNPG and lactose is competitively inhibited by glucose and galactose for both enzyme preparations. Glucose was found to be a stronger competitive

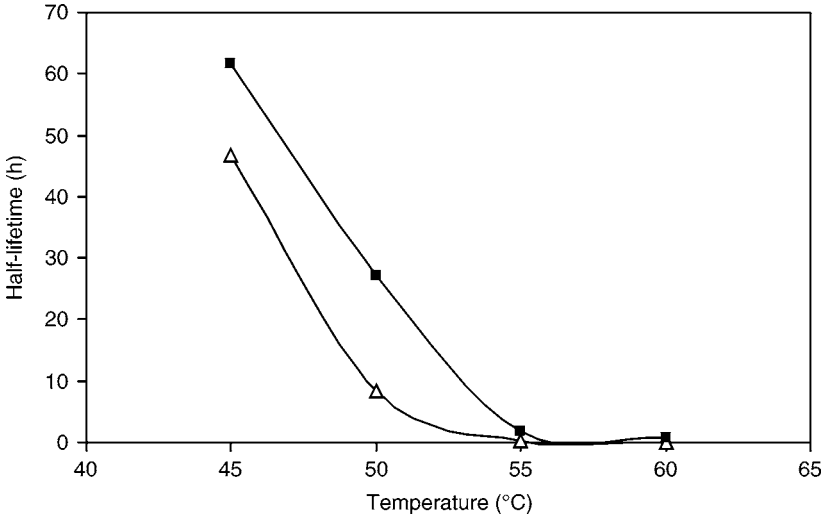

Fig. 3. Half-lifetime of activity of the free (Δ) and immobilized (■) β-galactosidase from *T. thermophilus*. Activity tests were carried out in 50 m*M* Na phosphate buffer using the following reaction condition: reaction time, 15 min; *T*, 40°C; pH, 6.5.

Table 1
Kinetic Parameters of Free and Immobilized β-Galactosidase

			Kinetic constant		
	Substrate	Inhibitor	V_{max} (μmol/min/mg)	K_m (m*M*)	K_i (m*M*)
Free	oNPG	D-glucose	450	35	66
		D-galactose	400	30	370
	lactose	D-galactose	95	19	420
Immobilized	oNPG	D-glucose	150	11	58
		D-galactose	146	10	251
	lactose	D-galactose	145	21	355

Activity tests were carried out in 50 m*M* Na phosphate buffer using the following reaction condition: reaction time, 15 min; *T*, 40°C; pH, 6.5.

inhibitor of oNPG and lactose hydrolysis for both enzyme preparations than galactose, yet based on the kinetic analysis and the inhibition constants K_i calculated, β-galactosidase is only moderately inhibited by both reaction end products.

Lactose Hydrolysis and GalOS Formation

Immobilized β-galactosidase from *T. thermophilus* was able to catalyze transgalactosylation reactions with lactose as substrate. As was analyzed by CE, not only the main hydrolysis products glucose and galactose but

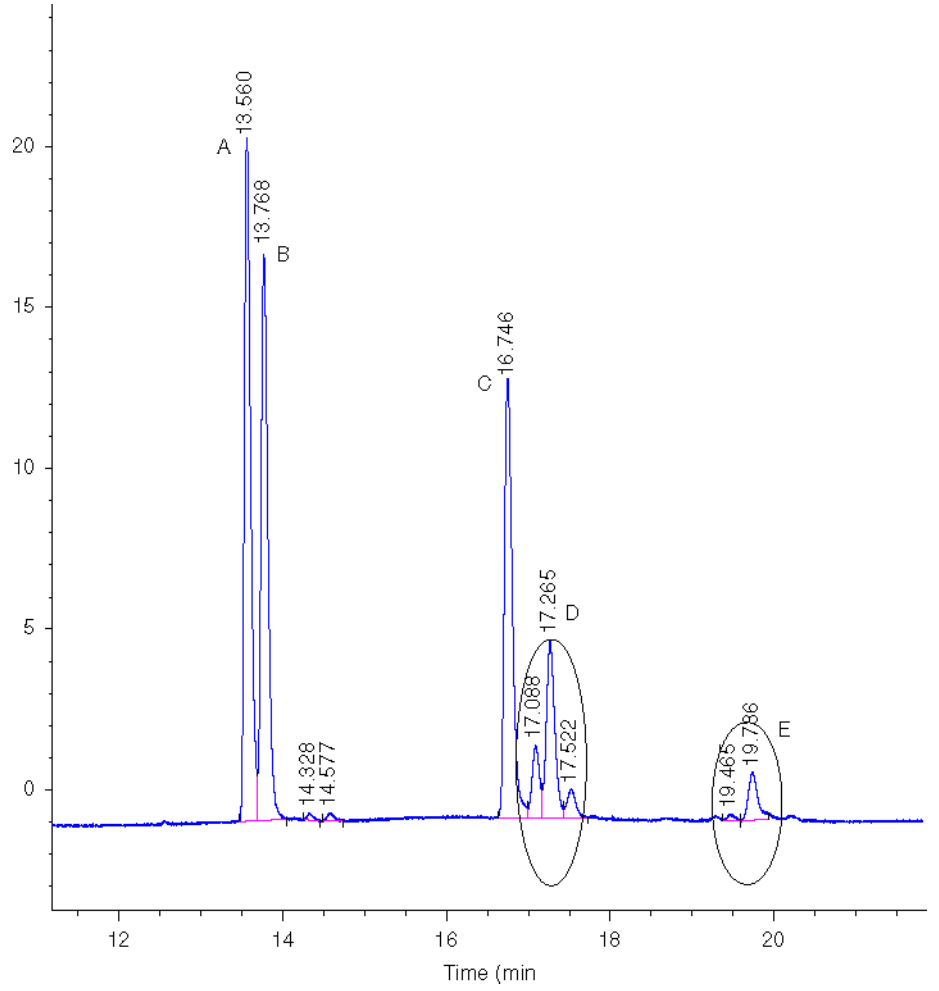

Fig. 4. CE chromatogram of lactose converted by immobilized β-galactosidase from *T. thermophilus* on Eupergit C: glucose **(A)**, galactose **(B)**, lactose **(C)**, disaccharides **(D),** and trisaccharides **(E)**. Reaction condition for the transformation: 20% initial lactose condition, immobilized β-galactosidase activity 2 $U_{lactose}$, 70% lactose conversion.

also GalOS are formed during incubation of the enzyme with increased concentrations of lactose. Analysis of product mixtures obtained at a lactose conversion of 70–80% revealed the formation of several GalOS with different nonlactose disaccharides and trisaccharides being the main reaction products (Fig. 4). Figure 5 shows the time-course of lactose hydrolysis and GalOS formation by immobilized enzyme when using 20% lactose as the initial substrate concentration. The maximum amount of GalOS obtained after 24 h incubation was approx 60 mg/mL, and this amount was maintained until the end of the incubation period. Approximately 80% of the lactose was hydrolyzed when the GalOS yield reached its maximum.

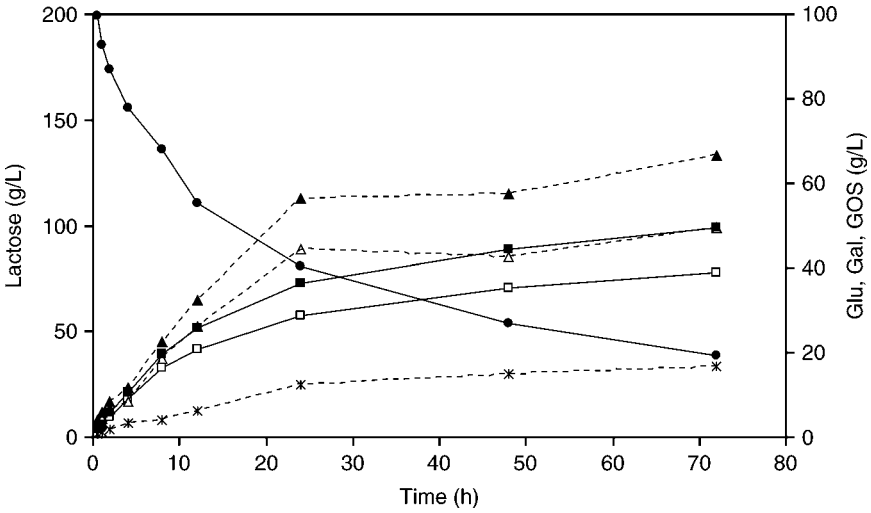

Fig. 5. Time-course of lactose hydrolysis as analyzed by CE: lactose (•), glucose (Glu) (■), galactose (Gal) (□), and total GalOS (▲) including disaccharides (Δ) and trisaccharides (*). Reaction condition for the transformation: 20% initial lactose condition, reaction time 72 h, immobilized β-galactosidase activity 2 U_{lactose}.

Discussion

The aim of the present study was to determine properties of a novel β-galactosidase from the moderately thermophilic fungus *T. thermophilus* when covalently immobilized onto Eupergit C, and to compare these properties to those of the free enzyme, for example, in terms of pH and temperature optimum, thermal stability and kinetic parameters. Recently, soluble β-galactosidase from *T. thermophilus* was characterized and found to exhibit some properties that make this novel enzyme attractive for applications in food technology *(8)*. The immobilized enzyme exhibited a shift of the optimal pH by about 0.5 units toward the alkaline side when compared with soluble β-galactosidase. Similar results were also reported for the immobilization of cyclodextrin glucosyltransferase on Eupergit C *(11)*. The immobilized β-galactosidase also showed a slightly broader pH optimum profile for both substrates as compared to the free enzyme. In addition, the covalent immobilization of β-galactosidase on Eupergit C significantly improves both the temperature optimum and stability as is expressed by the increase in the half-lifetime of activity at 50°C from 8 to 27 h. This improved stability is important for long-term applications of the enzyme, for example, when using it in continuous reactor configurations.

The comparison of the kinetic parameters for oNPG and lactose hydrolysis catalyzed by immobilized and soluble enzyme preparations revealed different effects on substrate turnover and binding as expressed by the values V_{max} and K_{m}. When using oNPG as the substrate, V_{max} was lowered for the immobilized enzyme. Furthermore, K_{m} also decreased

significantly when oNPG was the substrate indicating improved binding in the active site. In contrast, the K_m values for lactose hydrolysis by free and immobilized β-galactosidase are identical, whereas V_{max} was slightly increased. These results indicate that accessibility to the active site of the enzyme is probably not limited by the covalent attachment to the carrier, for example, by increased diffusional resistance. A possible explanation for these altered kinetic constants could be that immobilization results in slight changes of the conformation of the enzyme and the active site, which causes these differences in binding and/or turnover of the two different β-galactosidase substrates. It is well known that the end products of lactose hydrolysis, glucose and galactose, can have a severe inhibitory effect on β-galactosidases from various sources. Kinetic analysis of oNPG and lactose hydrolysis showed that *T. thermophilus* β-galactosidase is inhibited by both of these products, albeit this inhibition is only moderate as is obvious from the high values of K_i/K_m calculated for the immobilized enzyme preparations. These values were found to be 5.3 and 25 for oNPG hydrolysis, and glucose and galactose, respectively. These values indicate that lactose hydrolysis by β-galactosidase from *T. thermophilus* is only negligibly inhibited by its reaction products under operational conditions.

Eupergit C-immobilized β-galactosidase could be conveniently used for lactose hydrolysis as well as for GalOS formation. The maximum yield of GalOS of approx 34% was obtained when the degree of lactose conversion was approx 80%. From the time-course of hydrolysis (Fig. 5) it is obvious that the galactose concentration is constantly lower than that of glucose during conversion experiments. The glucose-to-galactose ratio during lactose hydrolysis can be used as a good measurement for GalOS formation as galactose moieties are transferred to a suitable acceptor such as another sugar molecule including lactose or even the reaction product galactose instead of being released as the free monosaccharide *(9)*. Maximum values for the glucose-to-galactose ratio of 1.26 were found over a broad range of substrate conversion (50–80%). The main oligosaccharide products that were qualitatively identified by CE are disaccharides and trisaccharides (Fig. 4). This together with the high glucose-to-galactose ratio indicates that galactosyl moieties are not only transferred to lactose, resulting in the formation of galactosyl-lactose, but also to other galactose molecules, and that the main transgalactosylation products are various positional isomers of galactosyl-galactose.

In conclusion, immobilization of *T. thermophilus* β-galactosidase onto Eupergit C is characterized by a high binding efficiency and a significant improvement of the properties of this enzyme with the most notable effect being on thermostability. Because of this increased thermostability the immobilized enzyme preparation can be used more efficiently than the soluble enzyme, for example, when using different continuous reactor configurations. The immobilized enzyme cannot only be used for lactose hydrolysis but also for the 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 *(3)*.

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