Hydrolysis of lactose in milk by *Kluyveromyces lactis* β-galactosidase immobilized in polyvinylalcohol gel

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Summary

β-galactosidase was immobilized to polyvinylalcohol hydrogel lens-shaped capsules, LentiKats®. After immobilization, the enzyme retained 20% of the free-enzyme activity. We found that 0.1 mol·l^{-1} Na_2SO_4, which is usually used for hardening of LentiKats®, caused the inhibition of β-galactosidase enzyme activity (after 60 min, it decreased to less than 1% of the initial value). Therefore, we successfully used 0.1 mol·l^{-1} K_2SO_4 for this crucial step. The pH optimum for both immobilized and free forms of the enzyme was 6.0–6.5, optimum temperature was 48 °C. The immobilized enzyme was used for lactose hydrolysis in repeated batch mode in ultra-heat treated milk (4.7% w/v lactose) at 15, 30 and 45 °C. The entrapped enzyme retained 80% of the inicial activity (59 μmol·min^{-1}g^{-1}) after 10 repeated batches at 30 °C without any change in the mechanical stability of LentiKats®. During milk hydrolysis, production of galactooligosaccharides was also observed.

Keywords

β-galactosidase; immobilization; lactose; hydrolysis; polyvinylalcohol gel

Hydrolysis of lactose with β-galactosidase (EC 3.2.1.23) is a well-established process used in the food industry. The enzyme is utilized for development of new hydrolysed-lactose products suitable for nutrition of lactose-intolerant people, for improvement of technological properties of non-fermented milk products and for removing of lactose from whey. Lactose is naturally found at high concentrations only in milk and milk products (cows' milk contains 4.5–5% lactose). This disaccharide fulfils important biological functions in human body such as stimulating of bifidobacteria growth and supplying galactose, an essential nutrient for the formation of galacto-oligosaccharides and cerebral galactolipids [1]. Lactose cannot be digested as a disaccharide, but it must be hydrolysed into its component saccharides by the action of intestinal ß-galactosidase. A significant portion of the adult population (almost 75%) is unable to digest lactose. Additionally, some individuals suffer from inborn metabolic lactose intolerance or lactase deficiency.

The enzymatic hydrolysis of milk lactose has two alternatives, free or immobilized ß-galactosidase may be used. Immobilization of ß-galactosidase is a promising method to decrease the cost of the lactose hydrolysis process because it facilitates multiplied repetitive use of the enzyme for the bioprocesses. The immobilized enzyme may be used for lactose hydrolysis in a batch format or continuously [2, 3].

On the basis of previous excellent results of whey hydrolysis with ß-galactosidase immobilized into LentiKats® [4], we carried out new experiments, hydrolysis of lactose in phosphate buffer and milk by immobilized yeast ß-galactosidase at neutral pH.

MATERIALS AND METHODS

Reagents

Kluyveromyces lactis β-galactosidase LH 3000 (Lactozym®, liquid form, 3997 U·ml^{-1}) was obtained from Novozymes (Bagsvaerd, Denmark). Polyvinylalcohol (PVA 17-99), polyethylene glycol was provided by Lentikat (Praha, Czech Republic). Milk was obtained from a local supermarket. Other chemicals were of analytical grade.

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Immobilization of β-galactosidase

The immobilization was performed on a pilot scale by Lentikat [5, 6]. A volume of 50 ml of β-galactosidase was mixed with 1 l of polyvinylalcohol gel (10%, w/v) and LentiKats® were prepared by the technique described in the previous work [4]. The immobilized enzyme were stored at 4 °C in phosphate buffer (0.1 mol·l^{-1}, pH 6.5) containing $MgCl_2$ (2 mmol·l^{-1}) and ethanol (6%, v/v).

Enzyme assay

Lactose hydrolysis was performed in 30 ml of 0.1 mol·l^{-1} phosphate buffer (pH 6.5) containing 2 mmol·l^{-1} $MgCl_2$ and 10% (w/v) of lactose with stirring at 30 °C. The reaction mixture contained 4 g immobilized or 0.1 ml of free ß-galactosidase. Samples (0.5 ml was taken 10 min after the initiation of the enzyme reaction) with free enzyme were boiled for 5 min to inactivate the enzyme. Concentration of lactose, glucose and galactose were determined by HPLC. One unit (U) of enzyme activity was defined as the amount of enzyme able to form 1 μmol of glucose per min from lactose at 30 °C and pH 6.5. The specific enzyme activity was defined as the activity per 1 ml of free enzyme (U·ml^{-1}) or per 1 g of the immobilized enzyme (1 U·g^{-1}). The relative activity was calculated as the ratio of actual and maximum activity.

Temperature and pH profile

The optimum temperature for free and immobilized forms of the enzyme was studied in the range from 20 °C to 70 °C. The pH optimum was assayed at different pH values, ranging from 3.7 to 7.9 using 0.1 mol·l^{-1} acetate (3.7–5.0) and phosphate (5.0–7.9) buffers with 2 mmol·l^{-1} $MgCl_2$.

Milk hydrolysis

Batch procedure

Immobilized β-galactosidase (4 g) was incubated with stirring at 30 °C or 45 °C with 80 ml (or 60 ml) of milk. Aliquots of 0.8 ml were taken at regular intervals to measure the amount of glucose formed. Before the determination of saccharides by HPLC, proteins and lipids were removed from each sample by the following method: 0.4 ml of the sample was transferred to a 25 ml flask and mixed with 1 ml of Carrez solution I (15 g $K_2Fe(CN)_6.3\,H_2O$ in 100 ml of distilled water) and 10 ml of distilled water. A volume of 1 ml of Carrez solution II (30 g $ZnSO_4.7H_2O$ in 100 ml of distilled water) was dropped into the prepared agitated mixture and the volume was adjusted to 25 ml. The sample was filtered and the extract was used for determination of saccharides by HPLC [7]. After each batch, LentiKats® were separated, washed with phosphate buffer (0.1 mol·l^{-1}, pH 6.5) with 2 mmol·l^{-1} $MgCl_2$ and transferred into fresh milk for the next hydrolysis run.

Reactor procedure

An amount of 2 g of the immobilized enzyme was added to 80 ml (or 60 ml) of fresh UHT milk. Repeated hydrolyses were carried out in a reactor with stirring at 15 °C. When the lactose hydrolysis reached 90–98%, the hydrolysed milk was separated and fresh UHT milk was added into the reactor. After removing proteins and lipids, the concentration of saccharides was determined by HPLC.

HPLC analysis

Concentrations of glucose, galactose and lactose were determined by HPLC with refractive index detector K-2301 (Knauer, Berlin, Germany), Ionex column (Watrex 250 mm × 8 mm, polymer IEX 8 μm H form; Watrex, Praha, Czech Republic) with 9 mmol·l^{-1} H_2SO_4 as the mobile phase at a flow of 0.7 ml·min^{-1}, at 50 °C.

RESULTS AND DISCUSSION

Commercial enzyme preparation of ß-galactosidase was immobilized into the highly elastic and stable polyvinylalcohol hydrogel lens-shaped capsules. This matrix was chosen because of its several benefits such as low cost, simple preparation and separation from the reaction mixture [8]. Protocol of LentiKats® preparation involves their hardening in 0.1 mol·l^{-1} Na_2SO_4 [6]. This step is necessary and it cannot be omitted. We found that after 15 min of stabilization with this solution, the specific enzyme activity decreased to 5% of the initial value (197 U·ml^{-1}) and after 60 min, it was 33 U·ml^{-1} (less than 1% of the initial enzyme activity). In order to avoid such loss in activity, 0.1 mol·l^{-1} K_2SO_4 was tested as an alternative stabilizing solution. Using this solution for stabilization of LentiKats®, no change in the activity of ß-galactosidase was observed during 60 min. Based on this, 0.1 mol·l^{-1} K_2SO_4 was used for stabilization of the immobilized enzyme in further experiments.

Influence of pH and temperature

To find the optimal conditions of lactose hydrolysis, effects of pH and temperature on ß-galactosidase activity was tested. The effect of pH was determined in experiments in the range between 3.7 and 7.9. The maximum of the enzyme activity for both free and immobilized forms was reached at pH 6.5, the entrapped enzyme being

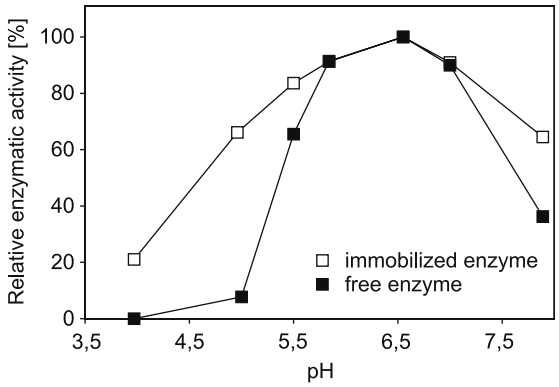

Fig. 1. pH optimum of free and immobilized β-galactosidase at 30 °C.
Conditions of hydrolysis: 30 ml of 10% (w/v) lactose in 0.1 mol·l^{-1} acetate or phosphate buffers (pH ranging from 3.7 to 7.9) with 2 mmol·l^{-1} $MgCl_2$, temperature 30 °C, stirring, 0.1 ml of free ß-galactosidase or 4 g of immobilized enzyme.

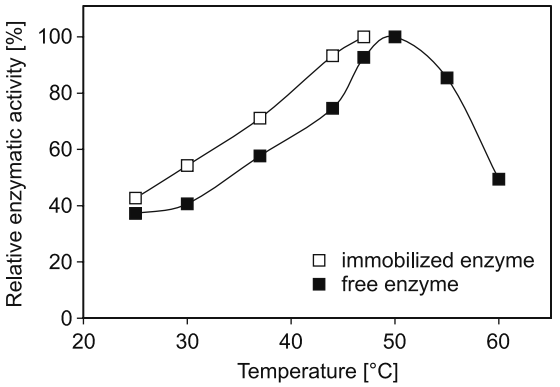

Fig. 2. Effect of temperature on ß-galactosidase activity at pH 6.5.
Conditions of hydrolysis: 30 ml of 10% (w/v) lactose in phosphate buffer (pH 6.5) with 2 mmol·l^{-1} $MgCl_2$, temperature 20–70 °C, stirring, 0.1 ml of free ß-galactosidase or 4 g of immobilized enzyme.

more stable at lower and higher pH than the free enzyme (Fig. 1).

This value of pH was also optimal for storage stability of the immobilized β-galactosidase. No change in enzyme activity was observed after 150 days of storage of the enzyme in 0.1 mol·l^{-1} phosphate buffer (pH 6.5) with 2 mmol·l^{-1} $MgCl_2$ and 6% (v/v) of ethanol at 4 °C. Ethanol was added to the phosphate buffer for prevention of microbial contamination, which might have caused a decrease in pH with a subsequent negative effect on the enzyme activity during storage.

Regarding temperature optimum of free and immobilized ß-galactosidase, 48 °C was found to be an optimum for the immobilized enzyme, as LentiKats® were found mechanically unstable at temperatures above 50 °C (Fig. 2) [8]. The optimal temperature for the free enzyme was 50 °C, higher temperatures caused a decrease in the relative enzyme activity, e g. to 40% at 60 °C.

Lactose hydrolysis in phosphate buffer and milk

Immobilization of ß-galactosidase is a promising method to decrease the costs of the enzyme catalyst in food industry because of the potential of its repetitive use. In order to obtain information on this aspect, LentiKats® prepared by the method described above were applied batchwise to lactose hydrolysis processes using 10% (w/v) lactose buffered solutions. Hydrolysis was carried at 30 °C with stirring and the degree of hydrolysis was maintained between 80% and 100%. After 30 repeated runs, the immobilized enzyme was found to have retained more than 78% of its initial activity (Fig. 3).

The properties of β-galactosidase from yeasts (optimum pH 6.5, good stability at lower temperatures and good commercial accessibility) suggest that yeasts β-galactosidase may be preferred in the delactation process of milk and milk products [3, 9]. Therefore, in this work, β-galactosidase immobilized into polyvinylalcohol was also used for lactose hydrolysis in commercial UHT milk. The diagram presented in Fig. 4 illustrates the drop in the content of lactose in milk. As can be seen, the content of lactose in UHT milk was approximately 6% after 4 h. During hydrolysis, formation of galactooligosaccharides was also observed. These compounds are known as prebiotics, i.e. food ingredients that are able to support the metabolic activity of probiotic acidic bacteria [1]. After milk

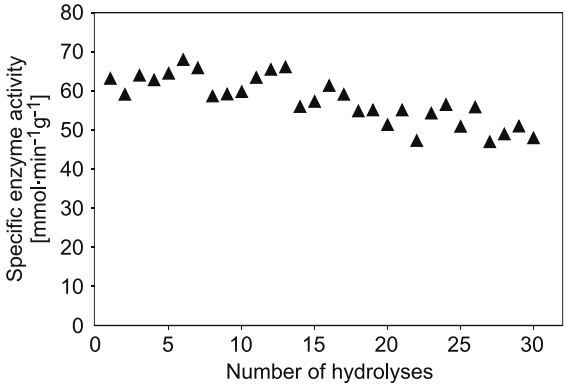

Fig. 3. Specific activity of immobilized ß-galactosidase during the repeated batch hydrolysis.
Conditions of hydrolysis: 30 ml of 10% (w/v) lactose in phosphate buffer (pH 6.5) with 2 mmol·l^{-1} $MgCl_2$, temperature 30 °C, stirring, 4 g of immobilized enzyme.

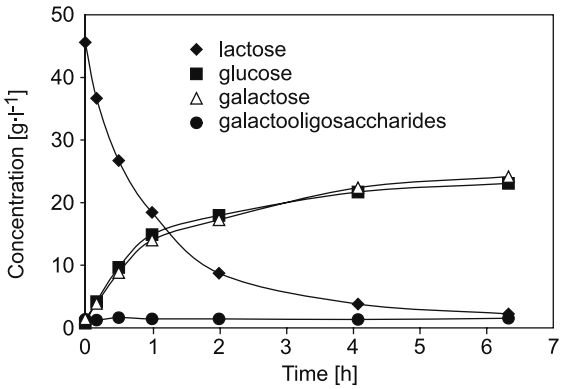

Fig. 4. Hydrolysis of milk by immobilized β-galactosidase.
Conditions: 60 ml of milk (lactose, 4.7% w/v), temperature 30 °C, stirring, 4 g of immobilized enzyme.

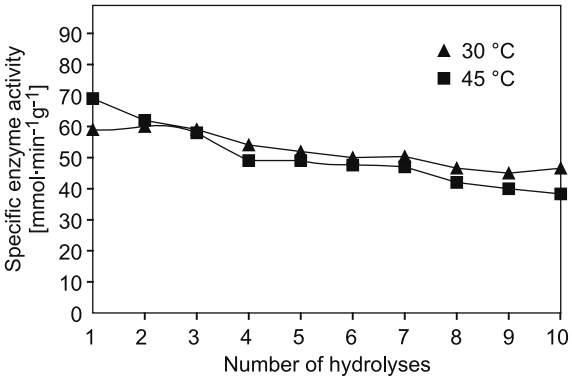

Fig. 5. Specific acitivity of immobilized β-galactosidase during repeated batch hydrolysis in UHT milk at temperatures 30 °C and 45 °C.
Conditions: 60 ml UHT milk, stirring, 4 g of immobilized enzyme.

hydrolysis, their concentration was minimal because their production strongly depends on the lactose concentration in solution, which was only 4.7% (w/v). Formation of galactooligosaccharides during milk hydrolysis and their importance were also described by Novalin et al. [10] and Neri et al. [11].

Repetitive use of polyvinylalcohol hydrogel lens-shaped capsules was also performed in batchwise reactions using UHT milk as the substrate. In the experiments focused on testing the stability of the immobilized biocatalyser, the degree of hydrolysis was maintained between 90% and 98% (Fig. 5). Ten batch operations of milk treatment were carried out at two temperatures, 30 °C and 45 °C. At both temperatures, a slight decrease in β-galactosidase activity was observed. However, at a temperature of 30 °C, the decrease of enzyme activity was slightly lower, from 59 μmol·min^{-1}g^{-1} to 47 μmol·min^{-1}g^{-1}, compared to the loss in the enzyme activity at 45 °C, which was from 69 μmol·min^{-1}g^{-1} to 38 μmol·min^{-1}g^{-1}. This could be caused by lower stability of the immobilized yeast β-galactosidase at a temperatures higher than 37 °C. In a similar study, Neri et al. [11] reported an approximately 50% decrease in the initial enzymatic activity after 20 batch cycles of milk hydrolysis with β-galactosidase covalently bound to magnetic polysiloxane-polyvinyl alcohol.

Experiments in a bioreactor

Because a lower temperature of lactose hydrolysis could reduce the industrial production costs as well as the risk of microbial contamination, performance of the immobilized enzyme was tested at lactose hydrolysis in commercial milk

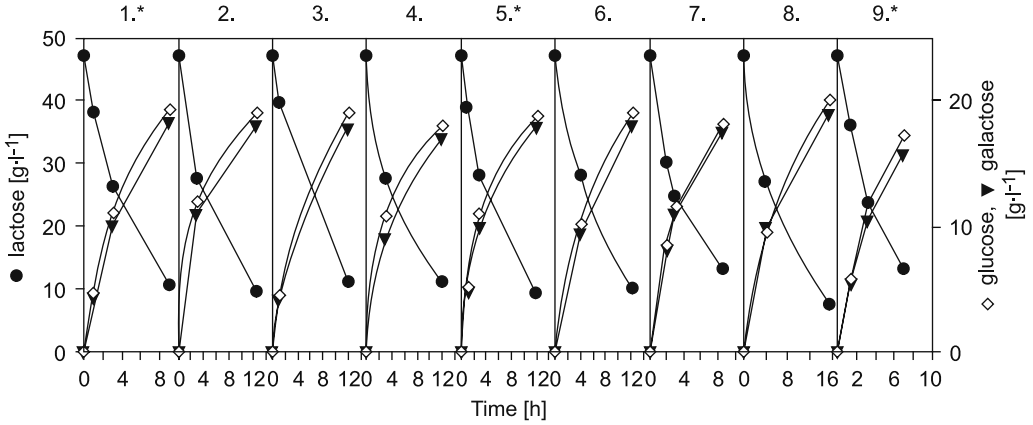

Fig. 6. Repeated batch hydrolysis of UHT milk by immobilized β-galactosidase at 15 °C.
Conditions: bioreactor, 80 ml of milk (* – 60 ml of milk), stirring, 2 g of immobilized enzyme.

at a temperature of 15 °C. The experiments were performed on a laboratory-scale bioreactor packed with 2 g of LentiKats®, and 80 ml or 60 ml of cold milk. The initial enzyme specific activity was 15 μmol·min^{-1}g^{-1}. The immobilized β-galactosidase retained more than 86% of its initial activity after 9 uses which took 100 h (Fig. 6). Total volume of the hydrolysed milk was 620 ml.

During lactose hydrolysis in milk, blockage of matrix pores was observed. This might have been caused by certain milk components, as milk proteins and lipids have been previously found to interfere with lactose hydrolysis [9, 10]. The blocking effects of proteins and/or lipids on the surface of LentiKats® might have increased the diffusion barrier to the substrate and the products. Subsequently, during repeated hydrolysis of milk, the enzyme activity would decreases.

CONCLUSION

In this study, a new method of β-galactosidase immobilization into polyvinylalcohol gel capsules is described. The immobilized enzyme was successfuly used for lactose hydrolysis in phosphate buffer and also in commercial UHT milk. Effects of pH and temperature on the enzymatic activity and lactose hydrolysis were investigated. In addition, we showed that LentiKats® could be used repeatedly at various temperatures for lactose hydrolysis in milk with good mechanical and operational stability. These results demonstrate the potential of this new type of immobilizate, which is a simple system for lactose hydrolysis at neutral pH.

Acknowledgement

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