Operational stability and kinetics of lactose hydrolysis by β-galactosidase from *Kluyveromyces fragilis*

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ABSTRACT. The kinetic modeling of the lactose hydrolysis and the operational stability of the enzyme β-galactosidase of *Kluyveromyces fragilis* was determined using as substrate skimmed powdered milk, reconstituted in a way to supply two lactose concentrations: 5% and 10% (w/v). For the operational stability both lactose concentrations were studied in the presence and in the absence of the buffer pH 6.5. Every 40 minutes the residual activity was determined by the method of the initial velocities. The experimental results showed that the buffer leads to enzyme inactivation. However, for lactose solutions without buffer, the residual activity only declines 15% in 6 hours and later it decreases abruptly. In the kinetic modeling the hydrolysis reaction was led to 40ºC/7h, being used enzyme concentrations equivalent to 1500 and 8100 LAU/L. The adjusted model allowed us to conclude that it is necessary to use the quantity of 3450 LAU/L of the enzyme to obtain the hydrolysis from 70% to 80% of the milk lactose in two to three hours of reaction.

Key words: β-galactosidase, lactase, lactose, *Kluyveromyces fragilis,* operational stability, kinetics of lactose hydrolysis.

RESUMO. Estabilidade operacional e cinética de hidrólise da lactose pela β-galactosidase de *Kluyveromyces* fragilis. A modelagem cinética da hidrólise da lactose e a estabilidade operacional da enzima β-galactosidase de *Kluyveromyces fragilis* foi determinada utilizando como substrato leite em pó desengordurado e reconstituído de modo a fornecer duas concentrações de lactose: 5% e 10% (p/v). Para a estabilidade operacional, ambas concentrações de lactose foram estudadas na presença e na ausência de tampão pH 6,5. A cada 40 minutos, foi determinada a atividade residual pelo método das velocidades iniciais. Os resultados experimentais mostraram que o tampão provoca inativação da enzima. Para soluções de lactose em água, a atividade residual decaiu apenas 15% em 6 horas e depois decresceu bruscamente. Na modelagem cinética, a reação de hidrólise foi conduzida a 40ºC/7 h, empregando-se concentrações de enzima equivalentes a 1500 e 8100 LAU/L. O modelo ajustado permitiu concluir que é necessário utilizar a quantidade de 3450 LAU/L da enzima para se obter a hidrólise de 70% a 80% da lactose do leite em duas a três horas de reação.

Palavras-chave: β-galactosidase, lactase, lactose, *Kluyveromyces fragilis,* estabilidade operacional, cinética de hidrólise da lactose.

Introduction

Lactose is present in the milk of most, but not all mammals. At approximately 4.8% (w/v), lactose is the major carbohydrate in bovine milk and whey. The development of new uses for lactose, both as whey or whey permeates and as pure lactose is of great interest, as it has been for hundreds of years (Yang and Silva, 1995).

Various β-galactosidases (lactase) have been investigated for the preparation of low lactose milk and dairy products. Interest is mainly due to the occurrence of lactose intolerance in some populations. Also, whey utilization for the production of many valuable products has been extensively studied. One of the major obstacles to the whey utilization is lactose content, which causes crystallization at low temperatures, low sweetness and poor digestibility when used as food. Lactose is also less fermentable than others sugars. These problems can be solved if whey lactose is hydrolysed to glucose and galactose. The sweet syrup can be used as a sugar source or as a basis for further fermentation (Nijpels, 1981; Kim *et al.*, 1997; Maciunska *et al.*, 1998).

The cheese industry produces large amounts of lactose in the form of cheese whey and whey permeate. Many uses have been found for whey and lactose, but the demand is insufficient to use all available whey lactose. The result is a low market value for lactose; almost half of the whey produced each year remains unused and is a significant waste disposal problem (Yang and Silva, 1995).

Hydrolysis of lactose in milk is still an important problem in nutritional improvement, particularly for people with low intestinal β-galactosidase (Mozaffar *et al.*, 1985). Lactase activity is high in nearly all infants. However, after 3-5 years of age, the majority of the world's population experiences a 90-95% loss of intestinal lactase activity. This normal pattern of loss of lactase activity in the intestine is transmitted by a recessive gene and should not be considered a disease, but rather the common pattern in human physiology. Persistence of infant levels of lactase activity into adulthood occurs only in a few populations (Rosado *et al.*, 1994; Suarez and Savaiano, 1997).

Adults with low lactase activity maldigest lactose, thus allowing transit of lactose into the large intestine, where colonic bacteria ferment it. Excessive lactose in the colon may lead to flatulence and diarrhea, symptomatology commonly referred to as lactose intolerance. Factors that may affect the ability of a subject to tolerate lactose include lactase activity, gastrointestinal transit time, lactose load and colonic fermentation (Noh and Gilliland, 1994; Montes *et al.,* 1995; Jiang *et al.,* 1996; Suarez and Savaiano, 1997).

One method to reduce the lactose maldigestion is the addition of β-galactosidase to the milk. With this enzyme, the lactose in milk is hydrolyzed before consumption, resulting reduction or prevention of symptoms associated with lactose maldigestion following consumption of the treated milk. However, this hydrolyzed milk has a sweeter taste and is more costly than regular milk, so people may not readily accept it (Kotz *et al.*, 1994; Noh and Gilliland, 1994; Montes *et al.,* 1995; Jiang *et al.,* 1996; Burton and Tannock, 1997). It is considered satisfactory for the lactose intolerants a hydrolysis of 90% of the lactose, in a product that contains about 5g/L of it (Hernandez and Asenjo, 1982). In industrial conditions, Prenosil *et al.* (1987) considered hydrolysis of 75% to 85% as being outstanding.

Due to the quantity and gravity of the problems related to milk sugar, a number of β-galactosidases from various sources has been tested for hydrolyzing lactose in milk, with their properties such as optimum temperatures, optimum pH's, K_m values and types of inhibition (Mozaffar *et al.*, 1985). However, only a few papers have dealt with the operational stability and kinetics of lactose hydrolysis. The operational stability of the enzymes is one of the factors of great importance in the industry, due to the fact of some enzymes are unstable and quickly lose their activity (Dixon and Webb, 1979). The kinetic models proposed here to describe lactose hydrolysis by β-galactosidase are derived from the Michaelis-Menten model, modified with the incorporation of individual effects of inhibition by galactose, once the glucose does not affect the enzyme activity, and of the mutarotation of galactose (Scott *et al*., 1985; Griethuysen *et al*., 1988). This work's aim was to determine the operational stability of β-galactosidase from *Kluyveromyces fragilis*, as well as to model the kinetics of lactose hydrolysis by the enzyme.

Material and methods

Enzyme

β-galactosidase from *Kluyveromyces fragilis* (LACTOZYM 3000 LAU/L) was supplied by the NOVO NORDISK (Copenhagen, Denmark). The best concentration of the enzyme, determined by the Lowry's method (Lowry *et al.,* 1951), was 16.2189 protein mg/mL that corresponds to a dilution of 1:50 (Matioli *et al.,* 2001).

Substrate

Solution of lactose 5% w/v presents in the skimmed powdered milk from the make MOLICO® (Nestlé) with the following medium constitution: lipid 1.0%, protein 36.0%, lactose 52.0%, salts minerals 8.0% and water 3.0%.

Buffer solution

The solution was prepared with deionized water, 0.01% of sodium azide as preservative, and in such a way as to supply the chemical components in the concentration of minerals found naturally in the milk, according to recommendations of the NOVO (1979). The pH was adjusted with NaOH 4 N for pH 6.5; about 10% v/v of that preparation was used in each assay. The adjustment of pH of the solution below 6.5 was achieved by buffer disodium phosphate-citric acid 0.1 M. This solution was prepared according to method by Mcllvaine (Morita and Assumpção, 1972).

β-galactosidase activity assay

One unit of activity (U) corresponds to the amount of enzyme that liberates one micromol of glucose per minute in the reaction conditions. The activity assay conditions consisted of a lactose solution 5 and 10% w/v, both concentrations studied in the presence and in the absence of the buffer pH 6.5 and temperature of 40ºC. The specific activity was obtained dividing the activity by the mass of protein used in the assay.

Operational stability

The operational stability of the β-galactosidase enzyme was determined by the incubation of 4.0 mL of the enzyme LACTOZIM 3000 LAU/L in 196 mL of the substrate, up to the total precipitation of the milk proteins, which impeded the continuity of the tests. This was the enzyme stock solution. Every 40 minutes, the residual activity of the enzyme was determined by the initial velocities method (Dixon and Webb, 1979) in glass bath reactor (capacity of 50 mL each), containing the lactose solutions. It was added 1.0 mL of the enzyme stock solution and it samples of 0.5 mL are removed every 5 minutes, until completing the 30 minutes of reaction. The samples were boiled for 10 minutes for enzyme inactivation. The glucose formed in the reaction was determined by the GOD-PAP spectrophotometric method (Barham and Trinder, 1972), in which the reagent supplied by the BIOLAB-MERIEUX was used.

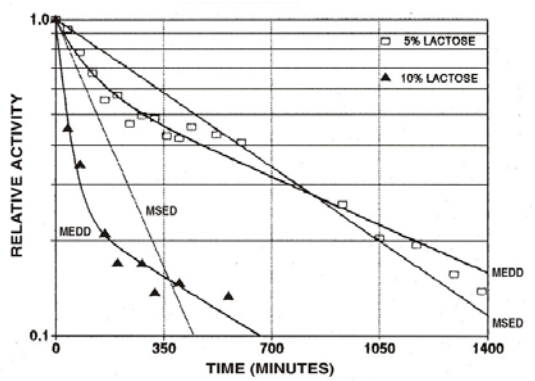
Lactose conversion

The tests of lactose conversion in glucose and galactose as a function of the reaction time were accomplished in glass bath reactor of 500mL, and the reaction volume of 200mL. The reactions were accompanied during a period of 7 hours, and the samples were taken each 5 and 10 minutes in the reaction first hour, respectively for the enzyme concentrations of 8100 and 1500 LAU/L, and after this time, each 30 minutes. The lactose conversion assays were accomplished with substrate concentration of 5 and 10% (w/v) in lactose. For the concentration of 1500 LAU/L was used a total of enzyme of 1.6219mg/mL solution, and for 8100 LAU/L of 8.7582mg/mL of solution. The enzyme presented a specific activity of 132.6858 U/mg. The tests were performed at 40ºC and pH 6.5. The samples were boiled in warm-bath for 10 minutes for enzyme inactivation and maintained at 4ºC for later glucose determination. The constants kinetic K_m, K_s and V_{max} were obtained for the initial velocities method using 50mL of the substrate in the concentrations from 0.025 to 15% (w/v), pH 6.5 and 40ºC (Matioli, 1991).

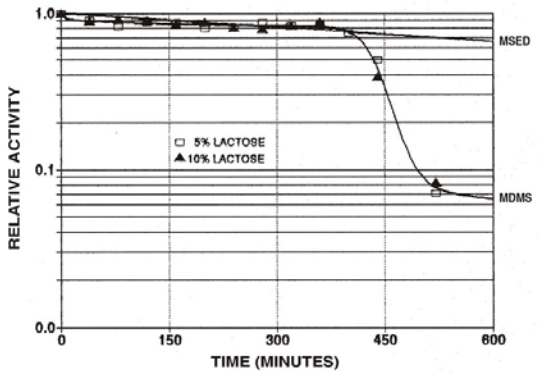
Results and discussion

Operational stability

The assay results of operational stability of the β-galactosidase enzyme from *Kluyveromyces fragilis* are demonstrated in Figure 1. The data comparison in the presence and in the absence of the buffer reveals a strong influence of this buffer in the enzyme inactivation.



a) Assay with buffer



b) Assay without buffer

Figure 1. Operational stability of β-glactosidase at 40ºC and pH 6.5. Solution of lactose 5% and 10% w/v presents in the skimmed powdered milk. Models of decline adjusted to the experimental data: Model of simple exponential decline (MSED), Model of exponential double decline (MEDD) and Model of decline modified sigmoidal (MDMS).

The Figure 1b demonstrates that for the lactose solutions without buffer, the β-galactosidase enzyme stability is greater than for the solutions with buffer, providing the time of reactor operation occurs up to 6 hours. In this period, the relative residual enzyme activity declines approximately 15%, however, from this point on, the activity decreases abruptly due to the milk proteins precipitation. The lactose

concentration variation between 5% and 10% did not have a significant influence in this case and the data contained for these two concentrations were better adjusted for a sigmoidal type equation, which was called Model of Decline Modified Sigmoidal (MDMS):
$y = a + b / \{1 + \exp [(x - c) / d] \} + e\, x^f$, where *a* to *f* are adjustable constants. The half-life time of the enzyme calculated with the adjusted equation it corresponds at 7.24 hours.

For the lactose solutions with buffer, Figure 1a, the enzymatic activity decline is more accentuated, being regular with the time. Besides, it is observed that the increase of lactose concentration from 5 to 10%, reduces the enzyme stability, contrarily to the rule usually verified that the substrate concentration increase protects the enzymes. This effect must be the consequence of a complex interaction among the buffer ions, the milk enzyme and proteins that are denatured during the treatment. It is observed that the buffer and a higher proteins concentration, as in the case of the lactose solution 10%, favored a faster flocculation. This way, the observed enzymatic inactivation is, in fact, the sum of two processes: the normal thermal inactivation and the β-galactosidase capture of the solution by the occlusion in the protein flakes. In this case, the inactivation can be represented satisfactorily by a function formed by the sum of two exponential decreases that was called Model of Exponential Double Decline (MEDD):
$y = [a \exp (- b\, x) + c \exp (- d\, x)] / (a + c)$, where *a* to *d* adjustable constants. From the adjusted equations it was obtained the half-life time for the lactose solutions 5 and 10%, in other words, 4.73 and 0.62 hours, respectively.

In addition, the Figure 1 shows that the Model of Simple Exponential Decline (MSED) is inadequate in the presence and in the absence of the buffer.

The effects here observed with the milk did not happen in the work of Mahoney and Whitaker (1977) that used pure artificial substrate. This demonstrates the importance of the enzymatic parameters determination in conditions similar to those of the industrial applications.

Kinetic modelling

Considering that the kinetic models proposed to describe the lactose hydrolysis are derived from the Michaelis-Menten model for the reactions that present inhibition by the substrate, as well as by the substrate and product, the integrated kinetic equation presents the same form, however the parameters are differently defined. The equation in the case of the bath reactor is represented by Eq. 1. The parameters α and β are defined for Eq. 2 and 3 for reactions that only present inhibition by the substrate, and for Eq. 5 and 6 when the inhibition effects are considered by substrate and product. The lactose conversion equation in glucose and galactose (X_A) was deduced with base in the equation of Hernandez and Asenjo (1982).

$$\tau_b = \alpha X_A - \beta \ln (1 - X_A) - \gamma X_A^2 \quad (1)$$

$$\alpha = (1 + S_0 / K_S) A_T S_0 / V_{max} \quad (2)$$

$$\beta = A_T K_m / V_{max} \quad (3)$$

$$\alpha = (A_T S_0 / V_{max}) (1 - K_m / K_i + S_0 / K_S) \quad (4)$$

$$\beta = (A_T K_m / V_{max}) (1 + C_{p0} / K_i + S / K_i) \quad (5)$$

$$\gamma = A_T S_0^2 / 2 K_S V_{max} \quad (6)$$

$$\tau_b = A_T t / V_R \; ; \; A_T = MP A_c \quad (7)$$

$$X_A = 2[(C_g - C_{gb})/C_{li}][M_l/(M_l/M_g + M_{ga})]100 \quad (8)$$

In this work, the kinetic model applied was also derived from Michaelis-Menten model, incorporating the effects of the inhibition by the substrate and of the inhibition by the substrate and product (Eqs 1 to 7). The model adjustment parameter was the constant of the inhibition by the product (K_i), and, once chosen the value of K_i, the parameters α and β of Eqs. 4 and 5 are determined.

This way, the value of the inhibition constant by the product (K_i, mol/L) that better is adjusted to the obtained experimental data is presented in the adjusted curves, as for the case of inhibition by the substrate, as for the inhibition by the substrate and product (Figures 2 and 3). It is observed that the model with inhibition only by the substrate did not adjust to the experimental data, however, the model with inhibition by the substrate and product presented a satisfactory adjustment up to the constant conversion. The factors that can contribute to the deviation between the model and the experimental data can be: the reaction reversibility, the mutarotation, the transgalactosidation, the inhibition by the milk and buffer constituents, the thermal enzyme inactivation.

The value of the inhibition constant (K_i), obtained according to the best adjustment of the integrated kinetic equation with inhibition by the product (galactose) and substrate is not the same for all the accomplished assays, showing that the kinetics of hydrolysis reaction is more complex than priory here assumed. The K_i value can group, besides the galactose inhibition, other factors previously mentioned, which were not included in the model.

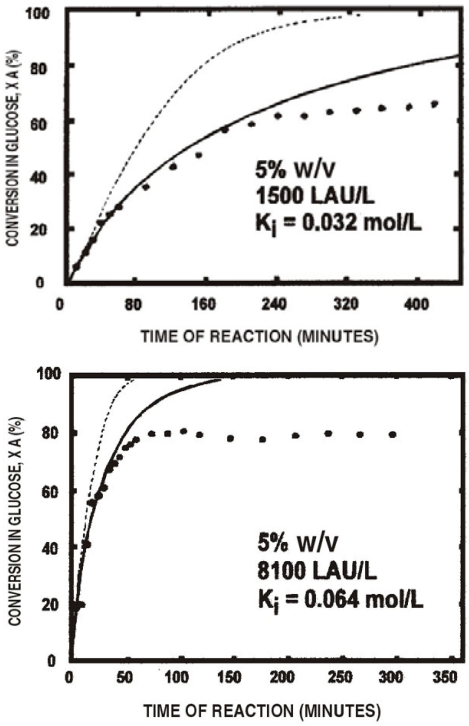


Figure 2. Conversion of lactose 5% (w/v) in function of time: (•) experimental data; (---) inhibition by the substrate; (—) inhibition by the substrate and product.

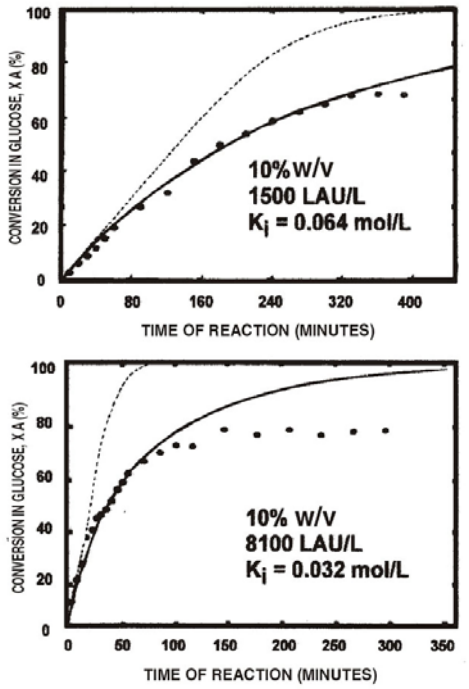


Figure 3. Conversion of lactose 10% (w/v) in function of time: (•) experimental data; (---) inhibition by the substrate; (—) inhibition by the substrate and product.

The milk for consumption contains lactose 5% approximately. Therefore, based on the adjusted model, it was considered that to obtain a hydrolysis from 70% to 80%, from the substrate containing lactose 5% (w/v), pH 6.5, 40ºC, at 2 - 3 hours of reaction, it would be necessary to use enzyme of 3450 LAU/L.

Conclusion

The experimental results demonstrated that the used buffer interferes with the operational stability of the enzyme, causing accentuated inactivation, with does not follow the model of exponential inactivation. The solutions of lactose 5 and 10% (w/v) with buffer followed the same tendency, but the second presented a larger inactivation, demonstrating that in the presence of the buffer there is a complex interaction among its ions, the enzyme and the milk proteins that flake more quickly and contribute to the reduction of the enzymatic activity. For the solutions of lactose 5 and 10% (w/v) without buffer, the activity only declines 15% in 6 hours and later it decreases abruptly. Therefore, the employment of the β-galactosidase of *Kluyveromyces fragilis* is not viable for a period above 6 hours due to the milk proteins inactivation and possible microbial contamination 40ºC.

The kinetic modeling for the inhibition by the substrate and product represented well the experimental data of conversion in function of the time up to the region where the conversion practically reached its maximum value. The kinetics of the lactose hydrolysis reaction is more complex than that assumed priory here, therefore the value of the inhibition constant (K_i) is not the same for all the accomplished assays. The concentration of lactose 5% (w/v) is the most appropriate to the process, due to the substrate and energy economy, once the conversion reaches its maximum more quickly when the substrate concentration is lower. The enzyme quantity to obtain a conversion of 70%, from substrate 5% (w/v), pH 6.5, 40ºC, in 2 hours is 3450 LAU/L.

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Notation

Ae	specific activity of the enzyme (U/mg)
AT	total activity of the enzyme presents in the reactor
Cg	glucose concentration presents in the sample in one time "t" (mg/mL)
Cgb	glucose concentration presents in the white (mg/mL)
Cli	initial concentration of lactose (mg/mL)
Cp0	initial concentration of the inhibition product
Ki	inhibition constant by the product (galactose)
Km	Michaelis-Menten constant(0.2294 mol/L)
KS	inhibition constant by the substrate (0.1986 mol/L)
Mg	glucose molecular weigh (180 g/gmol)
Mga	galactose molecular weigh of the (180 g/gmol)
ML	lactose molecular weigh (342 g/gmol)
MP	protein used in the assay (mg)
S	substrate concentration in a determinate time
S0	initial concentration of the substrate
T	reaction time
U	micromoles of the product / minute
Vmax	maximum velocities of the reaction (0.1693 mol glucose / h L solution)
VR	reaction volume in one time "t"
XA	lactose conversion in glucose and galactose
α, β, γ	parameters of Eq. 1, defined for Eqs. 2 to 6
τb	time of residence normalized, defined for Eq. 7

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