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Lactose hydrolysis potential and thermal stability of commercial β-galactosidase in UHT and skimmed milk

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Abstract

The commercial enzyme (E.C. = 3.2.1.23) from *Kluyveromyces lactis* (liquid) and *Aspergillus oryzae* (lyophilized) was investigated for its hydrolysis potential in lactose substrate, UHT milk, and skimmed milk at different concentrations (0.7; 1.0 and 1.5%), pH values (5.0; 6.0; 6.5 and 7.0), and temperature (30; 35; 40 and 55 ºC). High hydrolysis rates were observed for the enzyme from *K. lactis* at pH 7.0 and 40 ºC, and from *A. oryzae* at pH 5.0 and 55 ºC. The enzyme from *K. lactis* showed significantly higher hydrolysis rates when compared to *A. oryzae*. The effect of temperature and β-galactosidase concentration on the lactose hydrolysis in UHT milk was higher than in skimmed milk, for all temperatures tested. With respect to the thermal stability, a decrease in hydrolysis rate was observed at pH 6.0 at 35 ºC for *K. lactis* enzyme, and at pH 6.0 at 55 ºC for the enzyme from *A. oryzae*. This study investigate the hydrolysis of β-galactosidase in UHT and skimmed milk. The knowledge about the characteristics of the β-galactosidase from *K. lactis* and *A. oryzae* enables to use it most efficiently to control the enzyme concentration, temperature, and pH in many industrial processes and product formulations.

Keywords: UHT and skimmed milk; β-galactosidase; enzymatic characterization; lactose intolerance; enzyme activity.

Practical Application: Lactose hydrolysis potential and thermal stability of commercial β-galactosidase.

1 Introduction

Milk is considered a high biological value and nutritional energy food, recommended for all age groups and essential for newborns and infants (Borges et al., 2000; Ferreira, 1997).

Lactose is found in milk in larger amounts (Carminatti, 2001; Hobman, 1984). In the human intestine, lactose is hydrolyzed by β-galactosidase, being absorbed as glucose and galactose, thus facilitating its absorption in the body (Bódalo et al., 1991; Zadow, 1984). In lactose-intolerant individuals, hydrolysis of lactose alleviates symptoms of gastrointestinal disorders (Manan et al., 1999; McSweeney & Fox, 2009). Lactose can be hydrolyzed using acid treatments (Jelen, 1983; Goursaud, 1985) or enzymatic catalysis by the enzyme β-galactosidase. Enzymatic hydrolysis can be performed in UHT milk and skimmed milk without prior processing, preserving the nutritional properties of the resulting products (Santos et al., 1998; Vitolo, 2001, Hronska et al., 2009, Pereira et al., 2012). Thus, the commercial importance of β-galactosidase is its application in the dairy industry to obtain low lactose products, ideal for intolerant consumers (Haider & Husain, 2008; Jurado et al., 2002; Kardel et al., 1995; Mahoney, 1997; Pivarnik et al., 1995).

However, the β-galactosidase activity can be affected by several factors, including temperature, pH, pressure, concentration of reactants (Evangelista, 1998), and presence of metal ions (Carminatti, 2001). Furthermore, depending on the source of extraction (vegetable, animal or microorganism), β-galactosidase may exhibit different properties with a variety of potential technological applications (Oliveira, 2005). According to Şener et al. (2006), enzymes extracted from filamentous fungi have higher activity at acidic pH, since the yeasts present better operating conditions at neutral pH and milder temperatures.

In this context, the knowledge about the best activity conditions of this enzyme extracted from different microorganisms is of great importance, aiming at more effective commercial applications, especially in the dairy and pharmaceutical industry. Therefore, the aim of this study was to evaluate the effect of the commercial enzyme β-galactosidase from the yeast *Kluyveromyces lactis* and the filamentous fungus *Aspergillus oryzae* on the hydrolysis of UHT milk and skimmed milk at different enzyme concentrations, temperature, and pH conditions.

2 Materials and methods

2.1 Enzymes

The enzymes used in the hydrolysis reactions were commercial β-galactosidase from microbial origin in liquid and lyophilized forms. The liquid enzyme MAXILACT® LX5000 (Sedim Cedex - France) was obtained from the yeast *Kluyveromyces lactis*, and the lyophilized enzyme Bio-Cat INC/USA was obtained from the fungus *Aspergillus oryzae*, according to information provided by the manufacturer. Both enzymes had lactase activity of 5000 ALU.

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2.2 Effect of β-galactosidase concentration, temperature, and pH on the hydrolysis of lactose substrate, UHT milk and skimmed milk

The hydrolysis reactions were performed using 5% (w/v) lactose in potassium phosphate monobasic buffer for the enzyme from *K. lactis* and sodium acetate buffer for the enzyme from *A. oryzae*. The variables tested were enzyme concentration (0.7, 1.0, and 1.5%), temperature (30, 35, 40, and 55 ºC) and pH (5.0, 6.0, 6.5, and 7.0).

For lactose hydrolysis, 100 mL of UHT milk and skimmed milk (Cativa – Londrina, Parana, Brazil) were used under the same conditions described above. All analyses were performed in triplicate.

2.3 Thermal stability

The optimum enzyme concentration, temperature, and pH for each enzyme were defined according to the trials performed in Section 2.2. To evaluate the thermal stability, 1.5% of each enzyme was mixed with 100 mL of buffer solution in Erlenmeyer flasks of 250 mL. The flasks were incubated in water baths at various temperatures, as shown in Table 1. Every 30 minutes during 300 minutes, a flask was removed from water bath, 5% lactose was added, and the flask was placed again in the water bath for 15 minutes for lactose hydrolysis. Then, the analysis was performed according to the analytical determinations described in section 2.4.

2.4 Analytical determination

To determine the degree of hydrolysis, 2 mL sample was collected and placed in boiling water bath for 5 minutes, and then on ice-bath for 3 minutes to inactivate the enzyme. Glucose was determined by the glucose-oxidase method using the kit Glucose PP (Analysa®). Lactose was determined in milk by the phenol-sulfuric method, adapted by DuBois et al. (1956).

2.5 Statistical analysis

Data were analyzed by Statistic software 10.0 (Statsoft, 2007). Differences between means were analyzed by Tukey`s test at a significance level of 5 % ($P < 0.05$).

3 Results and discussion

3.1 Effect of β-galactosidase concentration, temperature, and pH on the hydrolysis of the lactose substrate

The commercial β-galactosidase from *K. lactis* and *A. oryzae* exhibited optimum activities under different reaction conditions, and lactose hydrolysis increased with the increase in enzyme concentration. Therefore, only the concentration of 1.5% was used for both enzymes, once the highest enzyme activity was observed at this concentration. At 30, 35, and 40 ºC and pH 6.0, 6.5, and 7.0, significant lactose hydrolysis rates ($p < 0.05$) were observed for the enzyme from *K. lactis* when compared to *A. oryzae*, with no activity at pH 5.0, demonstrating its sensitivity at low pH values (Figure 1A). At pH 7.0 and 40 ºC, high hydrolysis rate (97.9%) was observed for the enzyme from *K. lactis*, as shown in Figure 1D. In general, the enzyme from *K. lactis* was sensitive to temperatures above 40 ºC, with complete inactivation at 55 ºC at all pH ranges studied.

Table 1. Experimental conditions to evaluate the thermal stability of the enzymes from *K. lactis* and *A. oryzae*.

	K. lactis	*A. oryzae*
Buffer	Potassium phosphate	Sodium acetate
Enzyme concentration (%)	1.5	1.5
Ph	6.0	5.0
Temperature (ºC)	30, 35, 40	50, 55, 60

High hydrolysis rates were observed for the enzyme from *A. oryzae* at pH 5.0, with percentages of 42.9, 46.8, 52.1, and 67.4% at 30, 35, 40, and 55 ºC, respectively (Figure 1A). Similar hydrolysis profiles were observed at pH 6.0 and 6.5, but with lower percentages of hydrolysis (Figure 1B and C). In contrast, lower rates (20%) were observed at pH 7.0 for the enzyme from *A. oryzae*, as shown in Figure 1D.

Higher hydrolysis rate (4.54% / min) occurred at pH 6.0 and 40 ºC for the enzyme from *K. lactis* in the first 15 minutes of reaction, while lower rate (2.30%/min) was observed at similar pH at 35 ºC. Trindade et al. (2004) found higher hydrolysis rates at 40 ºC and pH 6.6 using the enzyme from *K. lactis* in free form, similar to the conditions obtained in this experiment. For the enzyme from *A. oryzae,* no differences were observed in the rate of hydrolysis, with high enzyme activity at pH 5.0 and 55 ºC (2.20%/min). Guidini et al. (2010) evaluated the immobilized β-galactosidase from *A. oryzae* and found similar results of this study. Ribeiro et al. (2005) observed maximum activity at pH from 4.5 to 4.8, which decreased at different pH values, with stability at pH 3.0 to 6.0. Regarding the temperature, the same authors observed higher enzyme activity at 55 ºC, followed by gradual thermal inactivation up to complete loss of activity at 70 ºC.

3.2 Effects of temperature and β-galactosidase concentration on the hydrolysis of UHT milk and skimmed milk

Lactose hydrolysis in UHT milk was higher than in skimmed milk for both enzymes, probably due to the higher fat content in UHT milk.

For the enzyme from *K. lactis*, a significant difference ($P < 0.05$) was observed between milk samples, once 100% hydrolysis was observed in UHT milk at 35 and 40 ºC, against 92.49% in skimmed milk at 40 ºC (Table 2). Campos et al. (2009) found 90% hydrolysis in UHT milk after 5 hours of reaction, at temperatures ranging from 30 to 40 ºC. In general, lower hydrolysis rates were observed for the enzyme from *A. oryzae* with values of 41.34% and 32.7% in UHT milk and skimmed milk at 55 ºC, respectively (Table 2), probably due to the pH 6.5, once the enzyme activity is optimized at pH 5.0, and decreases at pH above 6.0. Guidini et al. (2011) evaluated the β-galactosidase from *A. oryzae* in free and immobilized forms with glutaraldehyde, and found a decrease of 56.42% at pH 1.0 to 5.0, and 13.46% at pH 5.0 to 8.0 for the enzyme in free form, while a great stability was observed for the immobilized enzyme at all pH values (1.0 to 8.0), evidencing that the immobilization process is effective to maintain the enzyme stability.

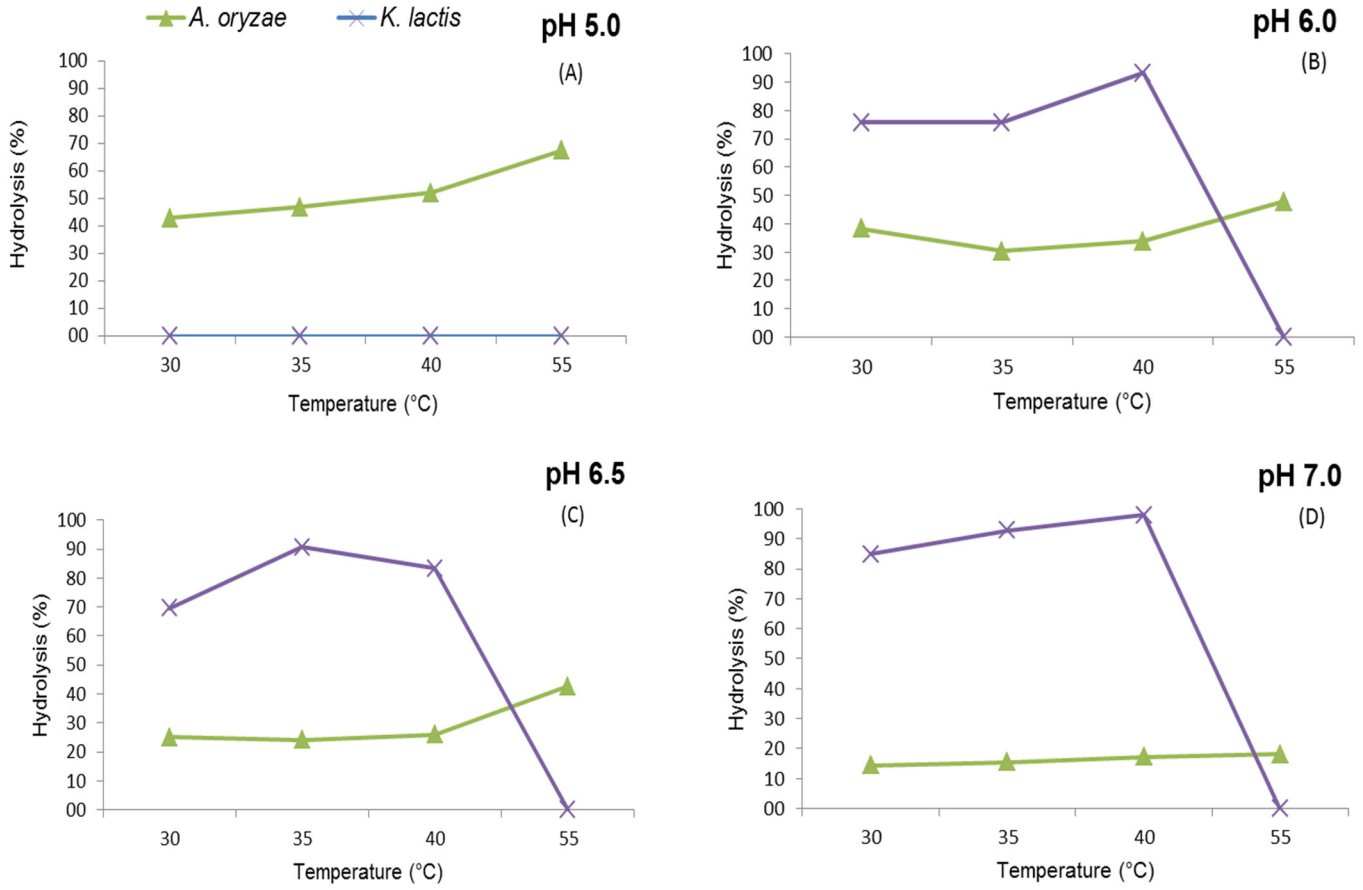

Figure 1. Lactose hydrolysis by the enzymes from *K. lactis* and *A. oryzae* at different temperatures and pH 5.0 (A), 6.0 (B), 6.5 (C), and 7.0 (D).

Table 2. Lactose hydrolysis by the enzymes from *K. lactis* and *A. oryzae* in UHT milk and skimmed milk at different temperatures.

Temperature	Hydrolysis (%)*			
	UHT milk		Skimmed milk	
	K. lactis	*A.oryzae*	*K. lactis*	*A.oryzae*
30 °C	86.42 ± 1.029 ^{cA}	28.89 ± 0.674 ^{cC}	78.85 ± 0.998 ^{cB}	16.28 ± 0.496 ^{dD}
35 °C	100.0 ± 1.666 ^{aA}	26.99 ± 0.190 ^{dC}	86.43 ± 1.001 ^{bB}	19.14 ± 0.421 ^{cD}
40 °C	100.0 ± 0.425 ^{bA}	32.91 ± 0.577 ^{bC}	92.49 ± 0.669 ^{aB}	21.12 ± 0.257 ^{bD}
55 °C	40.61 ± 0.220 ^{dB}	41.34 ± 0.421 ^{aA}	18.60 ± 0.060 ^{dD}	32.70 ± 0.651 ^{aC}

*Means followed by the same uppercase letter on the same line, and lowercase letter in the same column do not differ significantly among treatments, by Tukey's test ($P < 0.05$).

The higher hydrolysis rates for the enzyme from *K. lactis* were observed in UHT milk and skimmed milk at 40 °C, with 4.29%/min and 3.28%/min, respectively, in the first 15 minutes of reaction. For the enzyme from *A. oryzae,* higher rates were observed in UHT and skim milk at 55 °C, with 1.72%/min and 1.08%/min, respectively (Figure 2).

3.3 Thermal stability of the enzymes

The higher inactivation rate of β-galactosidase from *K. lactis* was directly proportional to the raise in temperature, especially from 40 °C. The enzyme from *A. oryzae* proved to be more resistant to higher temperatures, reinforcing the results obtained in the previous tests. A negative linear correlation was observed between the reaction time and the rate of hydrolysis for the enzyme from *K. lactis* at 30 to 35 °C, while a strong negative correlation was observed for the enzyme from *A. oryzae* at all temperatures (Figure 3 A and B). At 30 °C, the enzyme from *K. lactis* showed inactivation rate of 0.12% /min and activity of 46.2% at the end of 300 minutes, while at 35 °C, the inactivation rate was 0.18%/min and 35.4%. At 40 °C, the enzyme was inactivated almost completely (94.8%) in the first 60 minutes of reaction, with inactivation rate of 1.45%/min. A significant loss of enzyme activity was observed for the enzyme from *K. lactis* in the first 60 minutes for all temperature conditions (Figure 3). For the enzyme from *A. oryzae*, the reduction of activity during storage was less intense when compared to the enzyme from *K. lactis*. When subjected to 50 °C, the enzyme activity reduced 17.7% after 300 minutes, which is equivalent to

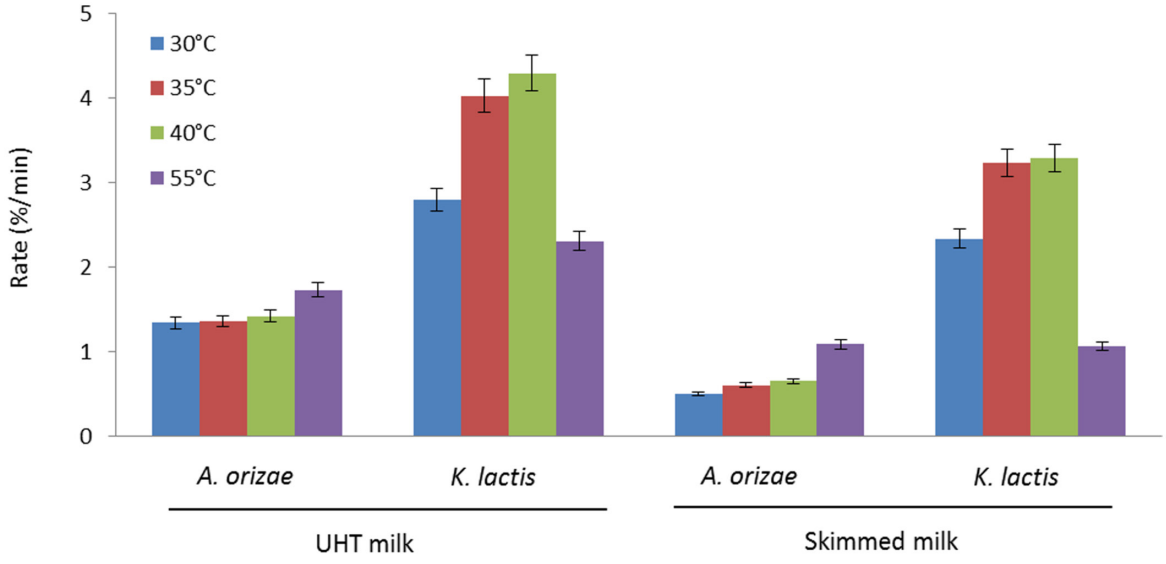

Figure 2. Rate of lactose hydrolysis (in the first 15 min of reaction) in UHT milk and skimmed milk subjected to different temperatures.

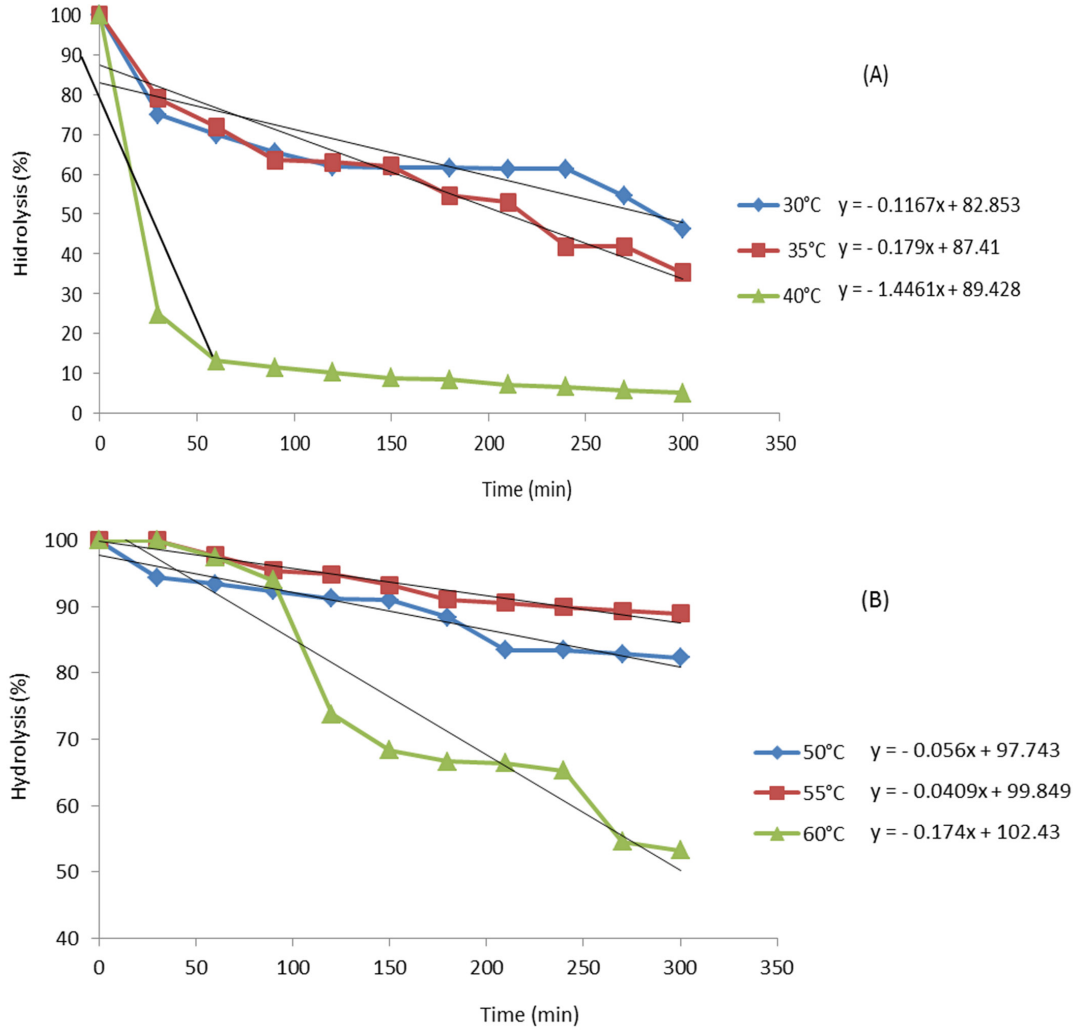

Figure 3. Rate of lactose hydrolysis by the enzymes from *K. lactis* (A) and *A. oryzae* (B) at different temperatures, and loss of enzyme activity during storage. No linear correlation was observed for the enzyme from *K. lactis* at 40 °C.

Table 3. Lactose hydrolysis by the enzyme from *K. lactis* as a function of time and temperature of storage.

Time (minutes)	30 °C	35 °C	40 °C
0	68.476 ± 0.621 a*	68.476 ± 0.621 a	68.476 ± 0.621 a
30	51.304 ± 1.243 b	54.061 ± 1.422 b	17.058 ± 0.264 b
60	47.938 ± 0.298 c	49.259 ± 0.257c	9.058 ± 0.387 c
90	45.306 ± 1.234 d	43.472 ± 0.128d	7.915 ± 0.146 d
120	42.372 ± 0.298 e	43.129 ± 1,047 d	7.026 ± 0.073 e
150	42.286 ± 0.197 e	42.443 ± 0,771 d	6.137 ± 0.073 f
180	42.200 ± 0.224 e	37.427 ± 0,385 e	5.798 ± 0.146 f
210	42.027 ± 0.325 e	36.312 ± 1.479 e	4.994 ± 0.146 g
240	41.984 ± 0.523 e	28.681 ± 0.680 f	4.571 ± 0.219 gh
270	37.324 ± 1.608 f	28.681 ± 0,000 f	4.021 ± 0.073 hi
300	31.671 ± 0.197 g	24.222 ± 0.267 g	3.555 ± 0.127i

*Means followed by the same letter in the same column do not differ significantly by Tukey's test (P > 0.05).

Table 4. Lactose hydrolysis by the enzyme from *A. oryzae* as a function of time and temperature of storage.

Time (minutes)	50 °C	55 °C	60 °C
0	32.991 ± 0.125 a*	32.991 ± 0.125 bcd	32.991 ± 0.125 a
30	30.730 ± 0.683 b	34.888 ± 0.708 a	33.470 ± 1.267 a
60	30.808 ± 0.136 b	34.073 ± 0.642 ab	32.625 ± 0.445 a
90	30.4539 ± 0.3615 b	33.3022 ± 0.0000 abc	31.4421 ± 0.5071 a
120	30.0988 ± 0.1808 b	33.0879 ± 0.1964 abcd	24.6804 ± 0.4075 b
150	30.0199 ± 1.4119 b	32.5307 ± 1.1572 bcde	22.8632 ± 1.1077 bc
180	29.1521 ± 0.1808 bc	31.7593 ± 1.0042 cde	22.3138 ± 0.7607 c
210	27.5347 ± 1.0932 cd	31.5878 ± 0.7750 cde	22.2293 ± 0.2639 c
240	27.5347 ± 0.1367 cd	31.3735 ± 0.2572 cde	21.8489 ± 1.1983 c
270	27.3375 ± 0.3131 d	31.1592 ± 0.5196 e	18.2567 ± 0.3803 d
300	27.1402 ± 0.2464 d	31.0306 ± 0.1485 e	17.8341 ± 0.3191d

*Means followed by the same letter in the same column do not differ significantly (P > 0.05) by Tukey's test.

an inactivation rate of 0.06%/min. At 55 °C, the reduction was 11.1% with inactivation rate of 0.04%/min. Finally, at 60 °C, the enzyme activity reduced 25% after 90 minutes of storage, with inactivation rate of 0.17%/min (Figure 3B).

A significant decrease in the enzyme activity from 68.47% to 42.37% was observed for *K. lactis* at 30 °C within 120 minutes, while no significant losses were observed from 120 to 240 minutes, which ranged from 42.37% to 41.98% (Table 3). Similar behavior was observed at 35 °C. However, a high reduction in enzyme activity was observed at 40 °C within 90 minutes of storage, with a reduction from 68.47% to 7.91% (Table 3). Other studies have shown that the increase in temperature leads to losses in enzyme activity of *K. lactis* (Cavaille-Lefebvre & Combes, 1998; Zhou & Chen, 2001).

Small variations in enzyme stability were observed for the enzyme from *A. oryzae*, at 50 °C and 55 °C during 300 minutes of storage. At 60 °C, no significant losses were found within 90 minutes, with a significant reduction from 31.4% to 18.2% from 90 to 270 minutes (Table 4). Several authors (Ateş & Mehmetoğlu, 1997; Dhaked et al., 2005; Zhou & Chen, 2001) have reported that the increase in temperature directly affects the enzyme activity, i.e., the higher the temperature the faster the enzyme inactivation. However, in this study, the enzyme from *A. oryzae* was stable with increasing temperature to 60 °C, even after 5 hours, differing from the results obtained by Haider & Husain (2007), who found that the enzyme from *A. oryzae* lost about 60% of activity after 6 hours at 60 °C. Additional tests were carried out to evaluate the thermal stability of the enzyme from *K. lactis* and *A. oryzae* at 70 and 80 °C, and no enzyme activity was observed for both enzymes.

4 Conclusions

Significant differences were observed for the enzyme activity from both the yeast *K. lactis* and the filamentous fungus *A. oryzae* in lactose hydrolysis. The variables enzyme concentration, temperature, and pH significantly affect hydrolysis reactions. The highest activities were observed at 40 °C and pH 7.0 for the enzyme from *K. lactis*, and at 55 °C and pH 5.0 for the enzyme from *A. oryzae* when tested in lactose substrate. Higher enzyme activity was observed in UHT milk when compared to skimmed milk, for both enzymes. The enzyme from *A. oryzae* was not effective in the target substrates due the differences between pH of milk and the optimum pH range for enzyme activity. However, with respect to thermal stability, it was observed that the enzyme from *A. oryzae* had higher heat resistance when compared to that from *K. lactis*. The knowledge about the characteristics of β-galactosidase from *K. lactis* and *A. oryzae* allows using it more efficiently in many industrial processes and product formulations, and as guidelines for direct consumption by lactose intolerant individuals.

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