Full Length Research Paper

Comparison of kinetic characteristics of xylanases from *Aspergillus niger* and *Trichoderma* sp. with pH and temperature baking process parameters

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Arabinoxylans are the predominant non-starch polysaccharides of the cell walls of wheat grain, and can contribute up to 3% of the total polysaccharide content of the flour. Endo-(1-4)-β-xylanase is able to hydrolyze the glycosidic bonds between two xylose units in the xylan backbone during baking process. The use of xylanases in the baking process leads to changes in the rheology of the dough. The aim of this work was to establish the best enzymatic hydrolysis conditions. The results suggest that the pure xylanase from *Aspergillus niger* needs less substrate to achieve maximum velocity (V_{max}). However the xylanase from *Trichoderma* sp. has a higher V_{max} apparent which means that it needs less time to convert the substrate into products. The xylanase from *A. niger* presented 50°C as its optimum temperature, 40% residual activity at 25°C and 80% at 36°C. The xylanase from *Trichoderma* sp. presented 50°C as its optimum temperature, 20% residual activity at 25°C and 60% at 36°C, which could be evidence that this enzyme is less active than the xylanase from *A. niger*. Moreover, the xylanase from *Trichoderma* sp. presented 90% residual activity in the baking process pH range, indicating that this enzyme can be more effective in the dough making process. The catalytic reactions of both enzymes are endothermic due to positive enthalpy and they are favourable process because the both Gibbs free energy were negative.

Key words: Xylanase, baking, enzyme, *Trichoderma* sp., *Aspergillus niger*.

INTRODUCTION

Although starch is the main polysaccharide present in wheat flour, it also contains other non-starch polysaccharides such as pentosans or hemicelluloses, which contribute more than 3% of the total polysaccharides. The function of arabinoxylans in dough and in the process of bread making is well described, as is their ability to decrease viscosity, to hold water and their capacity for oxidative gelation (Redgwellf et al., 2001; Ahmed et al., 2009; Godin et al., 2011; Mohamed and Al-Hindi, 2012).

Hemicelluloses are heteropolysaccharides composed of D-xylose, L-arabinose, D-mannose, D-glucose and D-glucuronic acid, which are acetylated or methylated (Singh et al., 2003). The classification of this polymer is generally made in accordance with the sugar residues present in the hemicellulose main chain structure, such as D-galactans (galactose polymers) and D-xylan (xylose polymer) (Castro and Pereira, 2010; Magee and Kosaric, 1985).

The arabinoxylans are the major non-starch polysaccharides of the cell walls of wheat grain. They consist of a linear chain of units of β-D-xylanopyranosyl connected by α (1,4) bonds, which may be monosubstituted at the O-3

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or disubstituted at the O-3 and O-2 with units α-L-arabi-nofuranosil (Saunier and Ortiz-Ordaz, 2005). The hydro-lysis of hemicelluloses requires a set of extracellular enzymes, hemicellulases, due to its highly branched heteropolysaccharide structure. The two main hydrolases of the hemicelluloses are endo-1,4-β-D-xylanase and endo-1,4-β-D-mannanase (Singh et al., 2003). The hemi-cellulases are classified according to the substrate on which they operate, for example: the xylanases hydrolyze hemicelluloses of the xylan type and mannanases hydrolyze hemicellulose of the mannan type (Bastawde, 1992; Uenojo and Pastore, 2007; Simões et al., 2009; Yang et al., 2011; Ahmad et al., 2012). Hemicellulases obtained from various microorganisms can break down these polysaccharides. Fungi are the most common sources of industrial hemicellulases β-glucanases, xylanases, galactananases, mannanases, galactomanna-nases and pentosanases (Reed and Nagodawithana, 1993; Polizeli et al., 2005; Valeri et al., 2011).

Endo-(1-4)-β-xylanase (1,4-β-xylan xylohydrolase, EC 3.2.1.8) hydrolyze glucosidic bonds within the xylan, which results in a decrease in the degree of polymeriza-tion of the substrate (Reilly, 1981; Sunna and Antranikian, 1997; Mohamed and Al-Hindi, 2012). The use of xylana-ses in the baking process leads to changes in the rheology of the dough such as dough development time, consistency, extensibility and resistance to breakage. These changes can be visualized in the final product, the bread, as improved quality, especially in relation to the volume and crumb structure (Sorensen, 2003; Várnaia et al., 2010).

An optimum dose of xylanase is defined as one that provides better performance to the properties of bread without causing sticky dough. Xylanase dosages provide more oven-rise and volume, the gluten is strengthened and the dough displays more elastic characteristics. With overdoses, the dough becomes stickier and the proper-ties of the gluten make the dough more viscous than elastic, which does not provide increased volume or oven - rise (Si, 1997; Okunowo et al., 2010). The addition of xylanase increases the extensibility of the dough in a dose-dependent manner (Primo-Martín et al., 2005).

Trichoderma and *Aspergillus* are the most efficient producers of xylanolytic enzymes of the fungi (Berlin et al., 2005; Romanowska et al., 2006). The aim of this work was to establish the best enzymatic hydrolysis conditions, such as substrate concentration, pH and temperature, and compare them to the baking process parameters.

MATERIALS AND METHODS

Commercial pure xylanase was derived from genetically modified microorganism *Aspergillus niger* by DSM (Delft, The Netherlands). In the case of xylanase from *Trichoderma* sp. that was not from genetically modified microorganism, it was from Shin Nihon Chemi-cal Co. Ltd. (Anjyo, Japan). Equipment for the analysis of enzymatic activity, were used according to the methods of enzymatic activity of the xylanases: i) Bain-marie, Fanem, model 147, capable of main-

taining a constant temperature of 50±0.1°C for incubating the enzymes; ii) pH meter, Orion, model 320, with precision of 0.01; iii) spectrophotometer, Femto, model 482, to 585 nm, iv) Magnetic stirrer, Tecnal, model TE085; v) Chronometer, Technos, model Quartz; vi) Vortex type, tube agitator, Phoenix, model AP-56, vii) Centrifuge, Celm, model Combate; viii) Test tubes. Reagents for analysis of enzymatic activity included: NaOH, distilled water, HCl, NaH_2PO_4. H_2O Merck art. n° 6346; $Na_2HPO_4.2H_2O$ Merck art. n° 6580; pure substrate 4-O-methyl-D-glucurono-D-xylan dyed with remazol Brilliant Blue R, Fluka; ethyl alcohol, glacial acetic acid, deionized water and Na_2CO_2; Birchwood Xylan.

Methods of enzymatic activity of the xylanases

The method with the substrate 4-O-methyl-D-glucurono-D-xylan dyed with Remazol Brilliant Blue R (RBBxylan) was used to analyze the enzymatic activity of the xylanase obtained from the culture of *Trichoderma* sp. The samples were incubated with the substrate in a bain-marie at a temperature of 50°C for 30 min, at a pH of 6.00; adjusted with the aid of $NaH_2PO_4.H_2O$ buffer. The reaction was stopped with the addition of a solution of hydrochloric acid (HCl) 0.014 N, diluted in ethanol PA. The substrate degradation into precipitated products is proportional to the endoxylanase activity. The blue coloration of the supernatant is due to non-precipitation of intact Remazol and is read in a spectrophotometer at 585 nm. One Unit of enzyme is defined as the sum of enzyme that releases 1 µmol of reducing sugar equivalent D-xylose from arabinoxylan at pH 6.00, per minute, at 50°C (Prozyn, 2008).

To analyze the enzymatic activity of xylanase derived from the culture of *Aspergillus niger,* the method with the substrate 4-O-methyl-D-glucurono-D-xylan dyed with Remazol Brilliant Blue R (RBBxylan) was used. The samples were incubated with the sub-strate, as previously mentioned, in a thermo-regulated device with temperature control at 50°C for 30 min, at a pH of 4.50; adjusted with the aid of glacial acetic acid buffer ($CH_3COONa.3H_2O$). The reaction was stopped with the addition of a solution of hydrochloric acid (HCl) 0.014 N, diluted in ethanol PA. The blue color of the supernatant is due to non-precipitation of intact Remazol and is read in a spectrophotometer at 585 nm. One Unit of enzyme is defined as the sum of enzyme that releases 1 µmol of reducing sugar equivalent D-xylose from arabinoxylan at pH 4.50, per minute, at 50°C (Prozyn, 2008).

Study of the curve of pH and temperature of enzyme activity

Using the enzymatic activity methods described above, the curves of optimum pH and temperature for the action of each enzyme studied were drawn. For the pH curves, the temperature was set at the value of the methods (50°C), and readings of enzyme activity were made at pH 4.00, 4.50, 5.00, 5.50, 6.00, 6.50 and 7.00. For the temperature curves, the pH was set at the values of the met-hods and the reading of enzyme activity was carried out at tempe-ratures 20, 30, 35, 40, 50, 60 and 70°C. The pH and temperature conditions of the baking process were measured in the mixture of dough at the initiation and termination of proofing. The values of enzyme activity were collected at these pH and temperature condi-tions measured in accordance as described above (Prozyn, 2008).

Kinetic and thermodynamic studies of the xylanases

The kinetic constants K_M (Michaelis constant) and V_{max} (maximum velocity) were calculated using the Lineweaver-Burk method for xylanase from *A. niger*. In case of the xylanase from *Trichoderma* sp, as this enzyme has residual activity, the thermodynamic para-meters adopted for K_M and V_{max} were considered as apparent. The activation energy (E_a) was determined by the Arrhenius method and
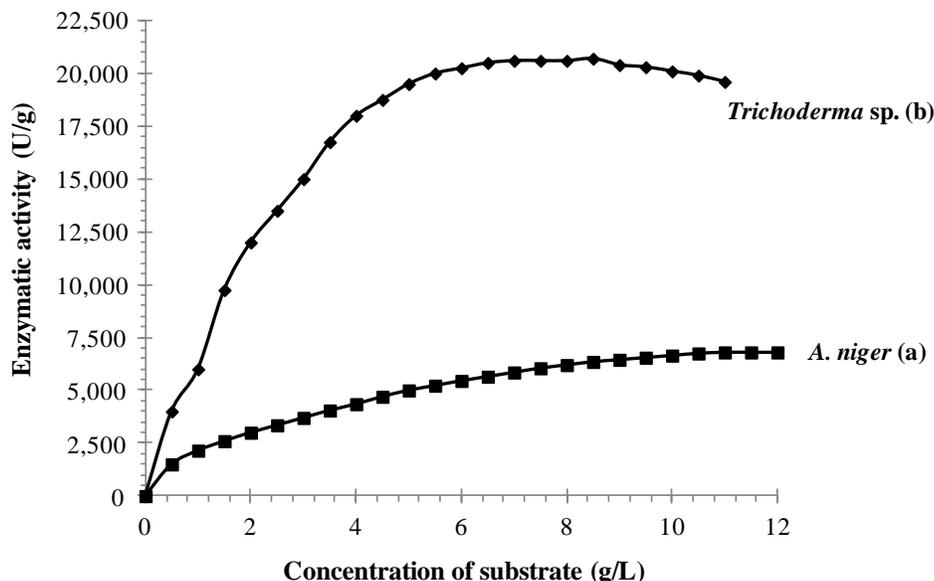
Figure 1. Enzymatic activity (U/g) per minute: the endoxylanase obtained from the fermentation of *Aspergillus niger* as a function of substrate concentration (g/L) at pH 4.5 and 50°C (a) and from *Trichoderma* sp. at pH 6.0 and 50°C (b), respectively.

Table 1. Kinetic and thermodynamic studies of the xylanases.

Xylanase	K_M (g/L)	V_{max} (U/g)	Ea (J/mol)	ΔG (J/mol)	ΔH (J/mol)	ΔS (J/mol K)
Aspergillus niger	2.077	6,993	29,318	- 6,016	26,632	109
Trichoderma sp.	3.310*	29,869*	54,951	-10,856	52,266	204

*Apparent values.

served to calculate the thermodynamic parameters ΔG (free energy change), ΔH (enthalpy change) and ΔS (entropy change) corresponding to the optimum reaction temperature, through conventional thermodynamic relationships (Castellan, 1986).

RESULTS AND DISCUSSION

Kinetic studies

Both enzymes followed the Michaelis-Menten model, in which, with low concentrations of substrate, the reaction rate doubles with the substrate concentration, while, with high concentrations of substrate, the rate approached a maximum (Figure 1).

The kinetic constants K_M and V_{max} were determined for the fungal xylanase in the study (Table 1). According to the results shown, we can infer that the xylanase obtained from *A. niger* requires a lower quantity of substrate to achieve its maximum velocity when compared to the xylanase obtained from the fermentation of *Trichoderma* sp. Although the latter has a higher maximum velocity apparent (V_{max} = 29,869 U/g), it transforms more substrate into products in the same reaction time for the conditions tested. Moreover, the lower K_M value also

suggests that the substrate becomes saturated more quickly for the xylanase from *A. niger*, or has a higher affinity for the substrate. The determination of thermodynamic parameters Gibbs free energy change (ΔG), enthalpy (ΔH) and entropy change (ΔS) were carried out by measuring the enzymatic activity of xylanases at different temperatures: 20, 30, 40 and 50°C. After this procedure, the parameters were calculated using the Arrhenius and Eyring equation (Owusu et al., 1992). Table 1 shows the thermodynamic parameters of xylanases studied.

The Gibbs free energy change (ΔG) indicates the spontaneity of the reaction catalyzed under the conditions of temperature and pressure employed. In this study, both values were negative indicating that the processes were exergonic, that is they are energetically favourable and occur with the release of energy from the system (Yaws, 1999).

The enthalpy (ΔH) change in the system corresponds to the heat released or absorbed in the transformation (constant pressure). In this study, the positive value of this parameter indicates that the catalytic reaction is endothermic that the heat of reaction is extracted from the surroundings in the transformation of the substrates
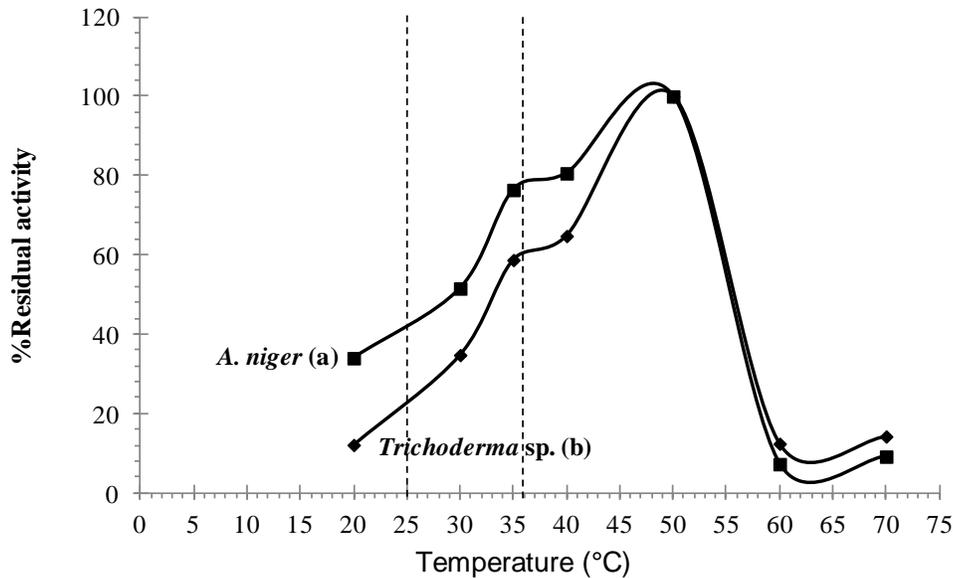
Figure 2. Residual activity as a function of temperature at pH of 4.5 of the xylanase obtained from the fermentation of *Aspergillus niger* (a) and from *Trichoderma* sp. at pH 6.0 (b), respectively. The lines are mean temperatures in the environment and inside a proofing chamber, of the proofing process in a bakery.

into products under conditions of constant temperature and pressure (Yaws, 1999).

The values of ΔH and ΔS obtained in this study were greater than zero, which means that the reaction was spontaneous at the temperature used. If there is an increase of enthalpy ($\Delta H>0$), which is opposite to the process, this only occurs if the entropy change is positive ($\Delta S>0$), which is verified in this work.

The two enzymes presented in Table 1 had low activation energy (E_a) values, which means that this enzymatic reaction occurs easily, that is requires little energy to reach the transition state. In fact, as it is a biological catalyst, it is expected that severe conditions are not required for the reaction to occur, since biological reactions occur in mild pH (4.5 to 6) and temperature conditions (50 to 60°C). However, the xylanase from *Trichoderma* sp. had higher activation energy apparent (54.951 J/mol), than that of *A. niger* (29.318 J/mol) requiring a little more energy to reach the transition state, that is it occurre less easily than that of *A. niger*.

Effect of temperature on the activity of the xylanases compared to the conditions of the baking process

The curves of activity as a function of temperature were compared to mean, minimum and maximum temperatures of the proofing process in a Brazilian bakery. Proofing in the baking process can occur at room temperature, approximately at 25°C, or at 36°C when it is carried out in a proofing chamber, with controlled temperature and humidity (Camacho and Aguilar, 2003).

The curve of optimum temperature for the xylanase from *A. niger* was superimposed on the proofing temperature range of the baking process (Figure 2; curve a). The optimum temperature of activity of the xylanase from *A. niger* supplied by the manufacturer was confirmed by practical readings, as being 50°C at pH 4.50. The temperature range in which the proofing takes place in baking was less than the optimum temperature of the enzyme and the residual activity was approximately 40% at 25°C and 80% at 36°C. This suggests that the enzyme operates with lower efficiency under baking process conditions. However, because the enzyme is used in baking, it may be that adjusting the dosage corrects this low efficiency. Moreover, these readings were made at a pH of 4.50, slightly below the process in which the enzyme is applied that is 5.5 to 4.80 (pH values of the dough measured at the initiation and termination of the proofing process respectively). These data may also indicate that the action of this enzyme is better, that has 80% residual activity when used in proofing chamber processes. However, in chambers at maximum 36°C, the breads are fermented for less time, for about 1.5 h, while at room temperature, proofing can last up to an average of 12 h when using long proofing (Camacho and Aguilar, 2003).

The fermentation of xylanase from *Trichoderma* sp. also had optimum temperature of action of 50°C at a pH of 6.00, but showed lower residual activity in the range of temperature of the proofing process in bread making, being about 20% at 25°C and 60% at 36°C (Figure 2; curve b). These data suggests that the xylanase from *Trichoderma* sp. is less efficient than that of *A. niger* at the temperatures of the baking process. However, the
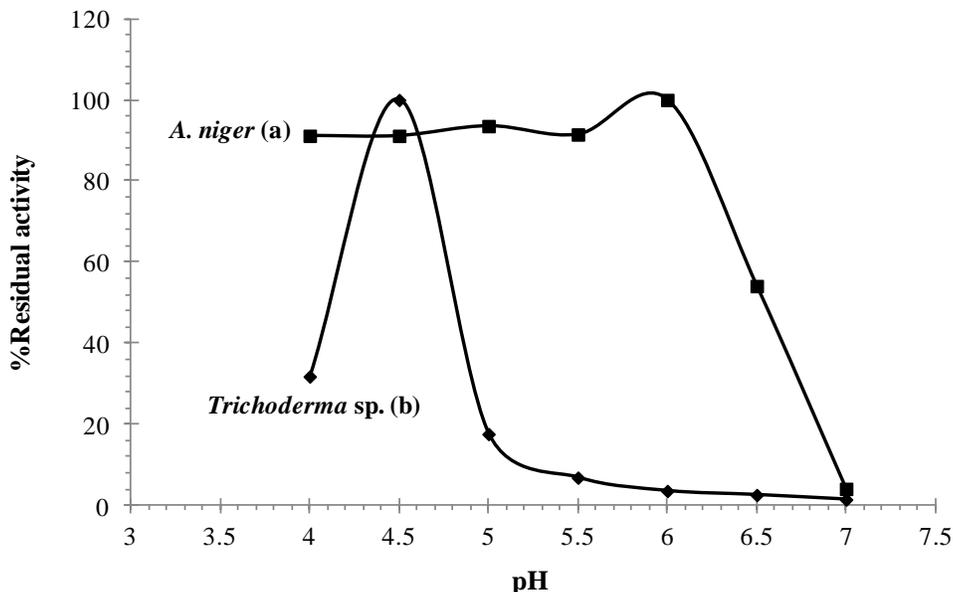
Figure 3. Residual activity of the xylanase obtained from the fermentation of *Aspergillus niger* as a function of pH at 50°C (a) and from *Trichoderma* sp. as a function of pH at 50°C (b), respectively.

enzyme of *Trichoderma* sp. showed higher activity by weight in grams, a parameter that can correct this flaw in efficiency at temperatures lower than its optimum. Moreover, in this study, the curves were produced at the optimum pH indicated by the manufacturers and not at the baking pH, which ranges from 5.50 to 4.80.

Effect of pH on the activity of the xylanases compared to the conditions of the baking process

The curves of activity as a function of pH were compared to the pH of a baking process. We collected the pH values of the dough of Brazilian French bread in three different processes, and values ranged from 5.40 at the initiation of proofing to a minimum of 4.80 at the termination of proofing.

The reaction rates of this enzyme within the pH range found in a baking process were low (Figure 3; curve a). That is, at the initiation of the process, the residual activity was less than 20% and as the pH drops, the reaction rate increased, reaching approximately 60% of residual activity at a pH of 4.88.

From these data, it can be inferred that the enzyme from the fermentation of *A. niger* operates more efficiently in a process with 3% yeast and 1.5 h of proofing and works less efficiently, with less reaction velocity, in long proofing processes with a low quantity of yeast.

The enzyme obtained from the fermentation of *Trichoderma* sp. presents approximately 90% of its maximum velocity at the initial and final pH of the baking process, indicating the possibility of reducing the dosage of enzyme compared to that of *A. niger* (Figure 3; curve b).

Its optimum pH of 6.00, as supplied by the manufacturer was confirmed. From the data of pH and temperature, it can be inferred that despite the enzyme from *Trichoderma* sp. being less efficient at the temperatures of the baking process when compared to the enzyme from *A. niger*, its efficiency in relation to the pH of the process was much higher (about 80% higher at the initiation of the process and 50% at the termination) and with less variation in activity with pH variations. This suggests that the xylanase obtained from *Trichoderma* sp. is more active in baking processes.

Conclusions

From the characterization analyses of the enzymes, it can be concluded that the two xylanases follow the Michaelis-Menten hyperbolic trend model, in which, with low concentrations of substrate, the reaction rate doubles with the substrate concentration, while with high concentrations of substrate the rate approaches a maximum. However, the kinetic constants of the enzymes are different. The xylanase from *A. niger* had a lower K_M value of 2.077 g/L compared to 3.310 g/L (apparent value) of *Trichoderma* sp., which signifies that it had more affinity for the substrate, but was saturated more quickly. As the maximum velocity apparent (V_{max}) for the xylanase from *Trichoderma* sp. was greater, 29,869 U/g compared with 6,993 U/g, it transformed more substrate into products within the same time interval. Consequently, to increase the reaction velocity for larger quantities of substrate, it is necessary to increase the concentration of the enzyme produced by *A. niger*, while that of *Trichoderma* sp. will

still act under the same conditions without the necessity to increase the dosage. Importantly, this observation has significant practical implications in the manufacture of bread.

The xylanase obtained from *Trichoderma* sp. has a wider range of pH activity, which includes the actual measured values of the industrial baking process, maintaining 90% activity during the process. This value is about 80% higher than the residual activity of the xylanase from *A. niger* at the initiation of the process of bread manufacturing and 50% higher in the termination. It can be inferred that the xylanase obtained from the fermentation of *Trichoderma* sp. studied, is more active than that of *A. niger* under the conditions of pH and temperature of the bread manufacturing process as both enzymes had low residual activity in the temperature range of the process, about 20 to 60% for *Trichoderma* sp. and 40 to 80% of *A. niger*.

ACKNOWLEDGEMENTS

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