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ORIGINAL PAPER

Microbial Enzymes as Substitutes of Chemical Additives in Baking Wheat Flour—Part I: Individual Effects of Nine Enzymes on Flour Dough Rheology

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Abstract In this work, we evaluated the effects of nine commercial enzymes—fungal (AMY-F), bacterial (AMY-B), and maltogenic (AMY-M) α-amylases, fungal (XYL-F) and bacterial (XYL-B) xylanases, glucose oxidase (GOX), laccase (LAC), lipase (LIP), and transglutaminase (TG) on the rheological properties of common wheat flour. Falling Number (FN), farinography, and alveography analysess were carried out varying the enzyme concentrations from 25 to 833 U kg^{-1}, aiming to reach baking quality. α-Amylases affected mainly the farinographic properties, reducing the water absorption (WA) and stability time (ST). AMY-B was the most effective enzyme to adjust the FN, needing 150 U kg^{-1}, while for AMY-F and AMY-M, it was necessary 583 U kg^{-1}. In all tests, XYL-B was more efficient than XYL-F, but both improved the *W* value and *P*/*L* ratio. At 25 U kg^{-1}, GOX increased the development time (DT), as well as the ST and the *P*/*L* ratio. LAC, which is not a commonly used enzyme, significantly improved the ST and *W* values, being an interesting oxidant agent. Moreover, the ideal enzyme concentrations determined were compared with those suggested by the suppliers, with under and over dosages observed, especially for α-amylases and xylanases.

Keywords Enzymes · Wheat flour · Rheological analysis · Falling Number · Farinography · Alveography

Introduction

The growth of mechanization in the baking industry and the demand for a wide range of bread products are factors which have been considered for the necessity to modulate structure and viscoelastic properties of wheat flour dough, which are important to the quality of the final products (Dunnewind et al. 2002). The quality of wheat flour depends on several factors, such as wheat variety, climatic variations, cultivation, and storage conditions (Cauvain and Young 2007). The resulting flours have specific properties, measured by Falling Number (FN) value—also known as diastatic or α-amylase activity—farinographic and alveographic tests, which determine their potential to application (Dunnewind et al. 2002). Wheat flours for industrial bread making require specific rheological characteristics, as intermediate α-amylase activity, low development time, high water-absorption capacity, and stability to mechanical work. Besides, it is important to have resistance and malleability to extension, with good balance between elasticity and extensibility (Cauvain and Young 2007). These properties are used to estimate the behavior of dough during the bread-making process and the quality of the final products.

When these properties are not suitable for such purpose, rheological parameters should be corrected. Even strong flours often need correction of their rheological parameters, particularly FN, by the addition of commercial α-amylase (Barrera et al. 2015; Goesaert et al. 2009b). However, the standardization of the FN causes a negative distortion of the other rheological parameters, due to the hydrolysis of wheat starch, reducing the water absorption, viscosity, stability, and resistance,

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which characterizes the flour weakening (Barrera et al. 2015; Dunnewind et al. 2002; Leman et al. 2005). In order to minimize these side effects, the wheat-milling industry usually mixes different flours to correct rheological parameters (AACC 2005). However, this method, based on trial and error, is costly and inefficient, susceptible to waste a good raw material. Another usual alternative is the addition of chemical compounds, mainly oxidizing agents such as azodicarbonamide (ADA), for dough strengthening. However, the use of ADA has been widely associated with numerous health problems and allergies, and its use is banned in many countries whereby non-compliance is subjected to heavy penalties (Csáki 2011; Lerner and Matthias 2015; Noonan et al. 2008; Roberts et al. 2013; Ye et al. 2011).

In general, treatments with enzymes have been preferred instead of the addition of chemical additives, since they are natural, highly specific, and completely safe to health. Furthermore, enzymes do not remain active in the final product (Caballero et al. 2007). Among the enzymes, xylanases promote an improvement in the flour rheology by increasing the extensibility and malleability of the dough caused by hydrolysis of water-unextractable arabinoxylans (Ahmad et al. 2013). Oxidative enzymes, like glucose oxidase and laccase, have a strengthening impact on the dough by promotion of disulfide linkages in gluten protein and gelation of feruloylated arabinoxylans, affecting positively the rheology of dough, weakened by amylases (Goesaert et al. 2007). Additionally, transglutaminase has a strengthening effect in the dough rheology by the promotion of protein crosslinking, improving the gluten network (Collar and Bollain 2005). And, finally, lipase improves dough rheology by the hydrolysis of the wheat flour lipids, releasing fatty acids that have good emulsifier properties, promoting a lipid bind with the gluten proteins, stabilizing the gluten network (Gerits et al. 2014a).

Nevertheless, the main difficulty for using these enzymes in flour correction is the limited knowledge about their effects on the rheological parameters, specifically, as to what concentrations these enzymes improve flour quality. In general, the wheat-milling industry follows the dosages indicated by the enzyme suppliers; however, enzyme effects depend on the initial characteristics of the raw material, which can result in over or under dosage. Thus, in this work, we presented a comprehensive study about the effects of nine commercial enzymes—three types of α-amylases: fungal (AMY-F), bacterial (AMY-B), and maltogenic (AMY-M) amylases; two types of xylanases: fungal (XYL-F) and bacterial (XYL-B); glucose oxidase (GOX); laccase (LAC); lipase (LIP); and transglutaminase (TG)—on rheological parameters of baking flour. We tested each enzyme individually in wheat flour, without any other additive, measuring farinographic and alveographic parameters. Besides, we compared our results with the recommended indications of the manufactures, discussing on under or over dosages.

Material and Methods

Material

Enzymes

The enzymes were kindly provided by suppliers listed below and were selected under the condition of being pure, that is, without being mixed or complexed with other enzymes. For comparative purposes, the enzymes activities were determined and expressed as units per gram of commercial preparation.

The following enzymes were tested: fungal α-amylase Veron M4 from *Aspergillus oryzae* (AB Enzymes), AMY-F; bacterial α-amylase Spring Alfa Bac 7500 from *Bacillus amyloliquefaciens* (Granolab), AMY-B; maltogenic α-amylase Veron Mac from *Bacillus stearothermophilus* (AB Enzymes), AMY-M; fungal xylanase Veron 191S from *Aspergillus niger* (AB Enzymes), XYL-F; bacterial xylanase Veron RL from *Bacillus subtilis* (AB Enzymes), XYL-B; glucose oxidase Spring Gluz from *B. subtilis* (Granolab), GOX; laccase Novoprime Base 268 from *A. oryzae* (Novozymes), LAC; lipase Enzymill FL from *Humicola* spp (Vallens Food Ingredients), LIP; and transglutaminase Veron TG from *Streptoverticilium mobarense* (AB Enzymes), TG.

Wheat Flour

The flour sample was provided by Tondo S.A. (Forqueta, RS) without blanching and any chemical or enzyme additives. The flour was stored (at 25 °C and 70 % of relative humidity, during 90 days) before use to stabilize the naturally maturing process. Falling Number, farinographic, and alveographic parameters were followed during the storage until a constant value was achieved, as stabilization end-point. The flour was composed of a mix of the Brazilian (50 %), American (10 %), and Argentine (40 %) wheat with an extraction of 70 %, without addition of Fe and folic acid and classified as type I, which according to the Brazilian regulation is categorized as commercially designed for baking.

The specifications of the flour, at time 0 and after 90 days of storage, were, respectively: moisture, 13.2 and 13.3 %; ash dry weight, 0.49 and 0.48 %; Falling Number, 395 and 420 s; gluten dry weight, 8.5 and 8.5 %; gluten index, 98.90 and 98.80; and color: L^* (whiteness or brightness/darkness), 92.04 and 91.95; a^* (redness/greenness), −0.66 and −0.53; and b^* (yellowness/blueness), 10.77 and 10.70. The farinographic and alveographic parameters were water absorption (WA), 56.0 and 57.1 %; development time (DT), 14.0 and 12.1 min; stability time (ST), 23.8 and 22.8 min; deformation energy (W), 299 and 221×10^{-4} J; and curve configuration (P/L ratio), 1.47 and 1.31.

Methods

All experiments are repeated three times, and the data represent the mean of these repetitions. Standard deviations were less than 5 % in all cases (data not shown).

Enzyme Activities

α-Amylases activities (AMY-F, AMY-B, and AMY-M) were determined by the starch hydrolysis by the release of reducing sugars proposed by Bernfeld (1955) with changes proposed by Santos et al. (2010), using maltose as standard. Reducing sugars were measured by 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). Soluble starch P.A. (Synth) was used as substrate (1 % (*w*/*v*), in 0.02 M sodium phosphate buffer, pH 6.9, and 0.006 M sodium chloride). An aliquot of 250 μL of the substrate solution was incubated at 37 °C for 10 min with 250 μL of enzyme solution, properly diluted. After the reaction, 500 μL of DNS was added and the mixture was boiled for 5 min. The reaction was cooled in an ice bath, added 1 mL of distilled water, and centrifuged at 5000×*g* for 5 min to precipitate the enzyme vehicle. Then, the absorbance of the supernatant was measured in a spectrophotometer at 540 nm. One α-amylase activity unit (U) was defined as the amount of enzyme capable of releasing 1 μmol of reducing sugars, expressed as maltose, per minute at 25 °C.

Xylanases activities (XYL-F and XYL-B) were determined by the amount of reducing sugars released from xylan "birchwood" (Sigma Aldrich), as described by Bailey et al. (1992), and modifications proposed by Palma (2003). Xylan solution (1 % (*w*/*v*) in 50 mM sodium acetate buffer, pH 5.0) was preheated at 50 °C for 5 min. Then, to complete 900 μL of substrate solution, 100 μL of enzyme solution was added, properly diluted, and incubated for 5 min. After, 1 mL of DNS was added and the mixture was boiled for 5 min and then cooled in an ice bath to room temperature. Then, it was diluted with 4 mL of distilled water and the absorbance was read in a spectrophotometer at 540 nm. One xylanase activity unit was defined as the amount of enzyme that releases 1 μmol of reducing sugar, expressed as xylose, per minute at 50 °C.

The GOX activity was determined by Ciucu and Patroescu's (1984) method by the benzoquinone (BQ) reduction to hydroquinone (HQ). In a cuvette, 1 mL of glucose solution 0.55 M (Merck), 500 μL of benzoquinone (Sigma Aldrich) 0.1 % (*w*/*v*) solubilized in citrate buffer (pH 5.0; 0.2 M), and 470 μL of citrate buffer (pH 5.0; 0.2 M) were added. The mixture was equilibrated for 5 min at 25 °C. Then, 30 μL of glucose oxidase solution, properly diluted, was added and stirred and the increase in the absorbance was followed for 2 min at 290 nm. All solutions were prepared using ultrapure water. Glucose oxidase activity was defined as the amount of enzyme which catalyzed the reduction of 1 μmol of BQ to HQ/min at 25 °C and expressed as units per gram of enzyme powder.

LAC activity was determined according to Leonowicz and Grzywnowicz's (1981) method using syringaldazine (Sigma Aldrich) as substrate with modifications suggested by Garcia (2006). The reaction started adding 100 μL of syringaldazine (1 mM, in absolute ethanol) to 840 μL of 50 mM sodium acetate buffer (pH 5.0) and 60 μL of enzyme previously diluted. The substrate oxidation was followed up from 5 to 10 min. The reaction was carried out under controlled temperature of 25 °C, measuring the substrate oxidation at 525 nm. The ε_{525} of the syringaldazine is 65,000 M^{-1} cm^{-1}. One laccase activity unit was defined as the amount of enzyme that oxidizes 1 μmol of syringaldazine/min at reaction conditions.

LIP activity was determined by titration of released fatty acids by the hydrolysis using olive oil as substrate, according to Soares (1999). Briefly, 1 mL of the substrate prepared by the emulsion of extra virgin olive oil with gum arabic 7 % (*w*/*v*; 1:1 oil/gum) was added to 0.8 mL of 0.1 M phosphate buffer (pH 7.0) and 0.2 mL of enzyme solution properly diluted. The reaction was incubated at 37 °C for 5 min under agitation, and adding 3 mL of 1:1 ethanol/acetone solution (*v*/*v*) was stopped. The free fatty acids released were titrated with 0.02 M KOH, using phenolphthalein as indicator. One lipase activity unit was defined as the amount of enzyme necessary to release 1 μmol of free fatty acid/min at reaction conditions.

The TG activity was determined by hydroxamate formation from N-CBZ-Gln-Gly substrate, using the method described by Grossowicz et al. (1950) with modifications proposed by Souza et al. (2008). To 400 μL of properly diluted enzyme solution, 400 μL of substrate solution (200 mM Tris–HCl buffer (pH 6.0), 100 mM hydroxylamine, 50 mM $CaCl_2$, 10 mM reduced glutathione, and 30 mM N-CBZ-Gln-Gly) was added. The reaction mixture was incubated at 37 °C for 10 min and then stopped by adding 400 μL of ferric chloride trichloroacetic acid reagent (1:1:1 (*v*/*v*/*v*) of 3 N HCl, 12 % trichloroacetic acid, 5 % $FeCl_3 \cdot 6H_2O$ diluted in 0.1 N HCl). After 5 min, the solution was centrifuged at 5000×*g*, and the absorbance of the supernatant was measured at 525 nm. One transglutaminase activity unit was defined as the amount of enzyme that catalyzes the formation of 1.0 μmol of L-glutamic acid γ-monohydroxamate/min at 37 °C.

Physical-Chemical Analysis

Moisture was measure by near-infrared spectral reflectance (NIR), method 44-15.02 (AACC 2009) in an infrared microanalyzer Gehaka IV 200 (Gehaka, Brazil). Gluten content and gluten index were determined by extracting gluten proteins, following the official methods, 38-12.01 and 38-12.02 (AACC 2009), respectively, using the Glutomatic System equipment (Perten, Sweden). Ash content was determined by incineration, method 08-01.01 (AACC 2009). The color

was determined by CIELAB scale, using a Colorimeter Minolta CR 310, method 14-22.01 (AACC 2000). These analyses were performed in the flour and flour with enzyme addition.

Rheological Analysis

The Falling Number was determined according to official Hagberg Falling Number by the method 56–81.03 (AACC 2009). Farinographic assays were performed in farinograph Brabender (Brabender Instruments, Germany) following the official method 54-21.02 (AACC 2009). The following parameters were studied: flour water absorption (%), dough development time (min), and dough stability time (min). The alveographic assays was carried out in an Alveograph NG Alveolink (Chopin Technologies, France) following the official method 54-30.02 (AACC 2009), measuring the deformation energy W ($\times 10^{-4}$ J), dough tenacity P, dough extensibility L, and their P/L ratio.

Effects of Enzymes Addition in Flour Rheology

The enzymes were added to the wheat flour, individually, varying their concentrations from 0 to 833 U kg^{-1} of flour. Previous experiments indicated that no changes were observed from concentrations above 833 U kg^{-1} of flour. After addition, all rheological parameters were measured and the effects of enzyme concentration in flour rheology were analyzed.

Statistical Analysis

The statistical analysis of results was carried out using Statistica 12.0 (Statsoft, USA), by analysis of variance (ANOVA). Data presented normal distribution and samples were compared by Tukey's test ($p \leq 0.05$).

Results and Discussion

Enzymatic Activities Characterization

Firstly, in order to compare the different enzymes, especially when more than one enzyme source was tested, all enzymatic activities were measured by standard methods commonly used in the literature. This was necessary because the suppliers do not mention the methods they used to determine the enzymatic activity, and more than this, they recommend the enzyme application based in the mass of enzyme preparation by mass of flour, as can be seen in Table 1. Thus, it is impossible to compare preparations from different sources or different suppliers. Therefore, the activity expressed in units per gram of each commercial preparation allows a fair comparison, minimizing the possible differences of each manufacturer. Moreover, it can be observed that the measured activities were very different from that provided by the manufactures. This is mainly due to different methods and reaction conditions for measuring the enzyme activities. In the following experiments, the activities were based in the measured activities presented in Table 1.

Individual Effects of Enzymes on Rheological Proprieties of Wheat Flour

Industrial wheat flours should have specific rheological parameters that will ensure the quality of the final products. If the final use is baking, the following parameters should be adjusted in specific ranges: FN, 250–320 s; WA, 56–57 %, DT, 4–10 min; and ST, >5 min. Values below or above these may result in changes in flour functionality and machinability (Cauvain and Young 2006; Pizzinatto et al. 2004). Additionally, alveographic parameters should range from 200 to 300 (10^{-4} J) for W and a P/L ratio above 0.88 (Pizzinatto et al. 2004).

In all studied concentrations, the enzymes did not presented any effect in the physical-chemical parameters (data not shown). Glucose oxidase and laccase only have bleaching action on flour, when associated with water and mechanical work, through hydrogen peroxide formation and carotenoid pigment oxidation (Bonet et al. 2006). Effects of enzymes, such as transglutaminase and glucose oxidase, on gluten are related with interaction forces between protein chains to improve the gluten quality, which is dependent of the initial content of gliadin and glutenin of the flour (Caballero et al. 2007; Collar and Bollain 2005).

Effects of α-Amylases Concentration

Although the flour presented good rheological properties, the α-amylase activity was low. Low α-amylase activity (FN >320 s) results in opaque breads, with dried crisp crumb and low volume (Cauvain and Young 2007; Goesaert et al. 2009b). On the other hand, excessive α-amylase activity (FN <250 s) produces a high saccharification of starch molecules during the baking process, resulting in gummy low-volume breads with a dark crust (Caballero et al. 2007; Van der Maarel et al. 2002).

AMY-B showed superior performance than AMY-F and AMY-M in the FN reduction. As shown in Fig. 1, with a concentration of 150 U kg^{-1}, AMY-B reduced the FN from 420 to 306 s, while for AMY-F and AMY-M, 583 U kg^{-1} was necessary to obtain the same result. AMY-F and AMY-M presented the same effect in the FN ($p > 0.05$), and both were different from AMY-B. The difference in the activities of AMY-B, AMY-F, and AMY-M can be explained because bacterial amylases have an inactivation temperature of about 90 °C, while

Table 1 Enzymes characteristics and measured activities

Enzyme	Code	Organism	Measured activity (U g^{-1})	Activity declared by the manufacturer
Fungal α-amylase	AMY-F	*Aspergillus oryzae*	2400	1728 AZ g^{-1}
Bacterial α-amylase	AMY-B	*Bacillus amyloliquefaciens*	1800	7500 UAM g^{-1}
Maltogenic α-amylase	AMY-M	*Bacillus stearothermophilus*	2079	Not declared
Fungal xylanase	XYL-F	*Aspergillus niger*	2194	1700 XylH g^{1}
Bacterial xylanase	XYL-B	*Bacillus subtilis*	2077	568 XylH g^{1}
Glucose oxidase	GOX	*B. subtilis*	2376	10000 GODU g^{-1}
Laccase	LAC	*A. oryzae*	260	800 LAMU g^{-1}
Lipase	LIP	*Humicola* spp	5700	9000 U g^{-1}
Transglutaminase	TG	*Streptoverticilium mobarense*	1400	100 TGU g^{-1}

AMY-F and AMY-M start to be inactivated at 60 and 70 °C, respectively, requiring higher concentrations to present the same activity of AMY-B (Cauvain and Young 2007). Thus, to adjust the FN, the ideal values were 150 U kg^{-1} of AMY-B and 583 U kg^{-1} of AMY-F and AMY-M.

According to Cauvain and Young (2007), WA around 56–57 % is ideal for dough development during baking process; values below or above this may result in changes in its functionality and machinability. As it is shown in Table 2, in increasing the AMY-F and AMY-B concentrations, a decrease in the WA of flour was observed, from 57.1 % (control) to 53.8 % (AMY-F) and 53.3 % (AMY-B) at the highest enzyme concentration (833 U kg^{-1}). AMY-M did not affect the WA even at high concentration (833 U kg^{-1}). Thus, to maintain a proper WA, a minimal concentration for AMY-F and AMY-B was needed.

The starch hydrolysis promotes the reduction of WA capacity. According to Leman et al. (2005), endo-amylases, such as AMY-F and AMY-B, attack the internal α-1,4-linked chains of amylose and amylopectin, releasing α-dextrin limit (low molecular weight). Some reports suggested that AMY-M may act as endo- but preferably as exo-amylase, attacking the external chains of amylopectin and releasing intermediate molecular weight dextrins (maltose, maltotriose, and maltotetraose), without requiring a non-reducing end (Goesaert et al. 2009a, 2010; Leman et al. 2005). Thus, the external chains of amylopectin become very short to crystalize, inhibiting the formation of crystalline junction area, limiting the recrystallization of amylopectin, and, consequently, immobilizing the water in the starch granule (Goesaert et al. 2007, 2009b; Hu et al. 2012; Van der Maarel et al. 2002).

AMY-F and AMY-B reduced the DT, while AMY-M increased this parameter (Table 2). The major starch hydrolysis promoted by AMY-B led to a reduction in the sample viscosity, decreasing the DT. On the other hand, the smaller starch hydrolysis promoted by AMY-M indicates that more mechanical work is required to achieve the optimal development of the dough. Although AMY-F presented similar FN than AMY-M, its effect on DT was different. The same effects were

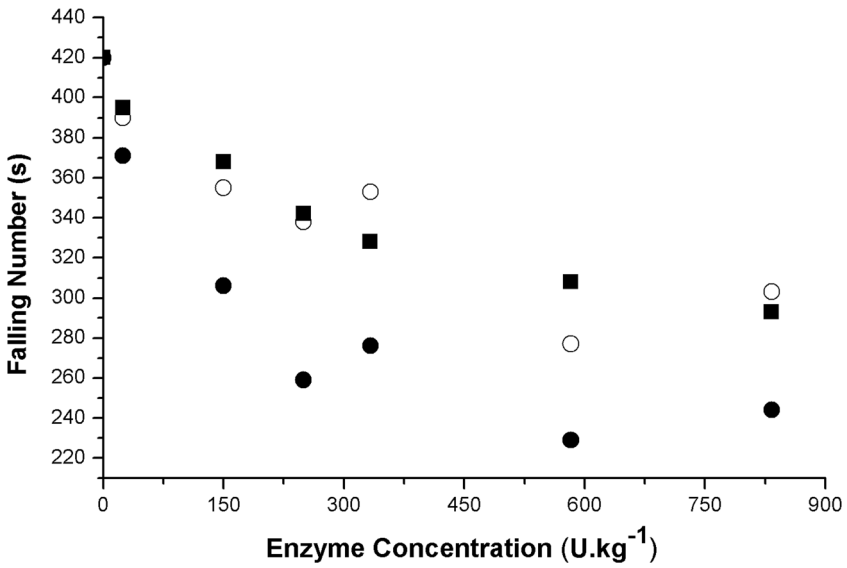

Fig. 1 Effect of α-amylases concentration on Falling Number. *Filled cricles*, AMY-B; *filled squres*, AMY-F; *empty circles*, AMY-M

Table 2 Effects of amylases on rheological parameters of flour

Enzyme	Enzyme concentration (U kg^{-1} flour)	Farinographic parameters			Alveographic parameters	
		WA (%)	DT (min)	ST (min)	W (10^{-4} J)	*P/L*
AMY-F	0	57.1 a	12.1 a	22.8 b	221 c	1.31 a
	25	56.2 b	12.0 a	23.7 a	221 c	1.29 a
	150	55.2 c	7.0 e	18.8 e	204 d	0.96 b
	250	54.9 c	10.1 c	21.1 c	199 e	1.15 ab
	333	54.5 d	8.5 d	16.7 f	166 f	1.15 ab
	583	54.1 e	10.2 c	16.6 f	253 a	0.85 b
	833	53.8 e	10.7 b	19.2 d	235 b	0.95 b
AMY-B	0	57.1 a	12.1 a	22.8 g	221 c	1.31 b
	25	55.8 b	10.9 b	20.9 a	189 e	1.86 a
	150	54.7 c	10.3 c	19.5 b	237 b	1.27 b
	250	54.3 d	10.1 cd	17.9 e	255 a	1.05 b
	333	56.4 e	8.0 f	20.8 a	222 c	1.24 b
	583	53.8 f	9.4 e	18.8 c	238 b	1.01 b
	833	53.3 g	9.8 d	16.4 f	200 d	1.20 b
AMY-M	0	57.1 a	12.1 e	22.8 a	221 b	1.31 abc
	25	57.1 a	15.7 b	19,9 d	223 b	1.42 ab
	150	57.0 ab	15.9 ab	22.9 a	230 a	1.38 ac
	250	56.8 ab	14.1 c	20.7 c	223 b	1.42 a
	333	57.1 a	16.1 a	20.0 d	198 d	1.47 a
	583	56.8 ab	14.1 c	19.0 e	229 a	1.10 c
	833	56.7 b	13.7 d	22.0 b	207 c	1.11 bc

Same letters in the same column, for each enzyme, are statistically equal by Tukey's test ($p < 0.05$)

observed on flour stability, where AMY-F and AMY-B presented stronger effect than AMY-M reducing the ST. A short DT and a high ST is desired for baking flour. A fast development and good stability to mechanical process, could minimize the losing during the industrial and automated bread production (Cauvain and Young 2006).

Thus, considering the farinographic parameters, AMY-F (250 U kg^{-1}) promoted a desirable reduction on DT, with a minimal interference on the ST, while for AMY-B, a minimum concentration of 25 U kg^{-1} promoted the same results. In the case of AMY-M, even at the highest concentration used, it was not possible to reduce the DT, although the ST was undesirably reduced at all concentrations, with the exception of 150 U kg^{-1}, which was not statistically different ($p > 0.05$) from the control.

As can be seen, α-amylases presented smaller interferences on alveographic parameters than to the FN and farinographic tests. The results showed that at 150, 583, and 833 U kg^{-1}, AMY-F reduced the *P/L* ratio compared with the control. This means that the dough tenacity was reduced while its extensibility increased, demonstrating a high degree of depolymerization of the wheat starch. However, it was possible to observe that the effects in *W* values were independent of enzyme concentration. Same result were found by Caballero et al. (2005), where polysaccharide degrading enzymes did not present significant effects on alveographic properties compared with gluten crosslinking and gluten-degrading enzymes, probably because of the gluten network influence on rheological behavior of dough.

Most of flours designed to baking industry require adjustments in the FN to ideal ranges (250–320 s), by the addition of commercial α-amylases. According to our results, a concentration of 150 U kg^{-1} of AMY-B or 583 U kg^{-1} of AMY-F and AMY-M is necessary. Nevertheless, these corrections resulted in negative changes in the other rheological parameters.

Effects of Xylanases Concentration

Wheat flour arabinoxylans (AX) are the principal components of the non-starch polysaccharide cell-wall material and present a high water-binding capacity, which are technologically important for the baking. Xylanase attacks the backbone of AX breaking it in smaller units, suxh as xylose and arabinose, releasing one molecule of water and modifying its functionality, improving the bread texture and volume (Moers et al. 2005).

As can be seen in Table 3, XYL-F and XYL-B presented a small influence on FN compared with the control, although both enzymes did not present any starch-degradation action. Falling Number is a viscosimetric method, and a reduction of suspension viscosity by xylanases activity, decreases the

Table 3 Effects of fungal and bacterial xylanases on rheological parameters of flour

Enzyme	Enzyme concentration (U kg^{-1} flour)	FN (s)	Farinographic parameters			Alveographic parameters	
			WA (%)	DT (min)	ST (min)	W (10^{-4} J)	*P/L*
XYL-F	0	420 a	57.1 cd	12.1 f	22.8 c	221 c	1.31 ab
	25	406 d	57.0 d	10.8 g	22.7 c	240 a	1.50 a
	150	400 e	57.1 cd	14.3 b	21.7 d	232 b	1.40 a
	250	398 e	57.4 ab	14.3 b	18.9 e	213 d	0.80 c
	333	416 b	57.3 bc	12.9 c	24.0 a	206 e	1.05 bc
	583	412 c	57.6 a	14.6 a	23.7 b	206 e	0.68 c
	833	400 e	57.3 bc	12.4 e	17.9 f	176 f	0.67 c
XYL-B	0	420 a	57.1 b	12.1 b	22.8 a	221 a	1.31 ab
	25	420 a	57.2 b	13.4 a	18.3 d	207 b	1.06 bc
	150	394 d	57.5 a	12.1 b	22.0 b	162 e	1.01 c
	250	399 c	57.3 ab	12.1 b	17.5 e	192 c	0.89 c
	333	413 b	57.1 b	12.2 b	20.4 c	141 g	1.47 a
	583	390 e	56.4 c	11.5 c	16.5 f	172 d	1.07 bc
	833	400 c	55.7 d	8.5 d	15.3 g	156 f	1.0 c

Same letters in the same column, for each enzyme, are statistically equal by Tukey's test ($p < 0.05$)

water-binding capacity, resulting in a false effect on the FN. Moreover, AX is linked in the starch structure, and steric hindrances make it difficult to access natural amylases presented in the flour. When the AXs are hydrolyzed by xylanases, the liberation of these fractions of starch molecule, facilitating the access to the natural amylases, even at low concentration, reducing the FN occurs.

XYL-F did not change the WA, while XYL-B promoted an undesirable reduction, proportional to the increase of its concentration. Ahmad et al. (2013) showed that for an untreated flour (control) with a maximum WA of 57.6 %, the addition of fungal xylanase at the concentrations of 200 and 400 U kg^{-1} decreases the WA to 56.4 and 55.7 %, respectively. The most important property of arabinoxylan is its water-binding potential, where 1 g of arabinoxylan can bind up to 15 g of water (Courtin and Delcour 2001). According to Ahmad et al. (2013), xylanase breaks larger molecules of carbohydrates into smaller ones having low water-binding capacities, resulting in reduced WA.

XYL-F at 25 U kg^{-1} reduced the DT and at 583 U kg^{-1} increased it. Other concentrations were not significantly different ($p > 0.05$). In contrast, XYL-B presented important effects in the reduction of DT and ST (Table 3). According to Courtin and Delcour (2001), XYL-F has the best activity during acid conditioning (pH 3.5–5.0), while XYL-B is better near neutrality (pH 6.0–7.0) and under alkaline conditions. Since in farinograph tests, the sample is not fermented, the pH of the dough is near neutrality, which justifies the best performance of XYL-B (Bataillon et al. 2000; Polizeli et al. 2005).

Fungal and bacterial xylanases have different substrate selectivity (Courtin and Delcour 2001; Figueroa-Espinoza et al. 2004). Whereas XYL-B has a preference towards water unextractable arabinoxylan (WU-AX), XYL-F has an affinity to water-extractable arabinoxylan (WE-AX) fraction (Courtin and Delcour 2001; Moers et al. 2005). The best activity of XYL-B compared with XYL-F in DT and ST can be explained because the wheat flour is composed of 2–3 % arabinoxylans and the major part is WU-AX (Goesaert et al. 2007).

Additionally, the lower WA caused by the AX depolymerization by xylanases activities, also affected the reduction of the DT and ST by softening the dough structure. Ahmad et al. (2013) studied the effects of xylanases on the rheology of wheat flour, obtaining similar results. The authors verified a decrease in the farinographic values by increasing the enzyme concentration.

At the concentrations of 25 and 150 U kg^{-1}, XYL-F had a positive effect on the *W* value and *P/L* ratio. However, concentrations of XYL-F higher than 250 U kg^{-1} and XYL-B at all concentrations reduced the alveographic parameters compared with the control. XYL-B effects on alveographic parameters were more pronounced than XYL-F, similarly as what occurred for the farinographic tests. The reduction of the *P/L* ratio indicates a decrease of the tenacity and an increase on extensibility, due to the breaking of the AX and the softening of the dough, consequently also reducing the *W*.

The tenacity reduction (*P/L* ratio) promoted an improvement in the *W* value, which is the main objective to use of the xylanases. According to our results, a minimal concentration of both xylanases (25 U kg^{-1}) when used individually, to promote a beneficial effects in the most rheology parameters with minimal prejudicial interferences in the remaining sets is necessary.

Effects of Oxidoreductases Concentration

Oxidative enzymes, such as GOX and LAC have high potential to industry as bread improvers. GOX catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide, promoting inter- and intramolecular disulfide linkages in the gluten network and the gelation of WE-AX (Joye et al. 2009). LAC catalyzes the polymerization of feruloylated arabinoxylans by dimerization of their ferulic esters and oxide, the sulfhydryl groups of wheat flour, resulting in disulfide bonds (Minussi et al. 2002; Selinheimo et al. 2007).

As can be seen in Table 4, GOX presented positive effects on WA at 250, 583, and 833 U kg^{-1} without statistical difference ($p > 0.05$). At these concentrations, GOX increased the WA from 57.1 % (control) to around 58.1 %, while at 333 U kg^{-1}, caused an undesirable decrease in this parameter. Bonet et al. (2006) and Steffolani et al. (2010) found that the addition of GOX did not modify significantly the WA and promoted an increase in dough stability, while Roccia et al. (2012) found an increase in water absorption proportional to the increase of the GOX concentration.

GOX also increased the DT until 583 U kg^{-1} as well as improved ST from 150 to 333 U kg^{-1}. This is in agreement with Steffolani et al. (2010) and Roccia et al. (2012), where the addition of GOX promoted an increase on ST at the highest enzyme concentrations. The most important effect for GOX was on *P*/*L* ratio, which increased with the enzyme concentration. This indicates a strengthening of the dough by increasing the tenacity in detriment of the extensibility, which has been related by the formation of additional protein crosslinks via disulfide linkages (Degrand et al. 2015). This strengthening of the dough was also observed in the *W* value, where with the increase in the enzyme concentration, a reduction in the *W* value is shown, except at 25 and 833 U kg^{-1}, when the concentration of GOX was not enough to rigidify the gluten network or was excessive.

Laccase is a multi-copper enzyme that catalyzes a variety of aromatic and non-aromatic compounds, with concomitant reduction of molecular oxygen to water (Claus 2004; Selinheimo et al. 2006). In baking, LAC has a strengthening effect that is mainly due to the crosslinking of the esterified ferulic acid on the AX fraction of wheat dough resulting in a strong AX network (Labat et al. 2001). LAC can also oxidize the amino acids, tyrosine and cysteine, to catalyze peptide polymerization, and to crosslink certain proteins, promoting an S–S network (Mattinen et al. 2006; Selinheimo et al. 2008).

Laccase promoted an increase in all parameters evaluated. For the tested LAC concentrations, there were no statistical differences on WA ($p > 0.05$), but its addition increased the WA 2.9 % compared with the control, from 57.1 to 58.8 %. LAC addition increased the DT by 28 %, from 12.1 (control) to 17.7 min. For dough ST, there was an improvement from 22.8 to 26.8 min at 25 U kg^{-1}, while at 333 U kg^{-1}, it decreased to 19 min. All other concentrations did not show significant differences to the control.

The *W* (10^{-4} J) value and the *P*/*L* ratio increased with all LAC concentrations in relation to the control. The *P*/*L* ratio varied from 1.37 using 25 U kg^{-1}, to 2.35 using 833 U kg^{-1}. It is known that some increase in the *W* value could be good, although for LAC, there was also an increase in the *P*/*L* ratio, which is not good for bread making, being an indication of excessive oxidation of the flour proteins. A high *P*/*L* ratio

Table 4 Effects of glucose oxidase and laccase on rheological parameters of flour

Enzyme	Enzyme concentration (U kg^{-1} flour)	FN (s)	Farinographic parameters			Alveographic parameters	
			WA (%)	DT (min)	ST (min)	W (10^{-4} J)	*P*/*L*
GOX	0	420 a	57.1 a	12.1 cd	22.8 c	221 c	1.31 f
	25	407 d	57.2 a	13.8 bc	20.1 d	255 a	1.32 f
	150	411 c	57.7 a	17.1 a	27.0 a	207 e	1.94 e
	250	407 d	58.1 a	16.8 a	23.2 bc	216 d	2.33 d
	333	415 b	56.8 a	17.1 a	25.1 ab	189 f	2.85 c
	583	408 d	58.1 a	14.6 b	20.1 d	187 f	5.0 a
	833	409 cd	58.3 a	11.0 d	15.6 e	224 b	4.44 b
LAC	0	420 c	57.1 c	12.1 f	22.8 e	221 f	1.31 c
	25	411 e	58.8 a	17.7 a	26.8 a	253 d	1.37 c
	150	415 d	58.4 b	16.2 e	24.7 b	244 e	1.72 b
	250	431 a	58.4 b	16.3 de	24.4 c	262 b	1.59 bc
	333	412 e	58.3 b	16.8 c	19.0 f	243 e	1.86 b
	583	424 b	58.2 b	17.2 b	23.3 d	268 a	1.72 b
	833	415 d	58.2 b	16.5 d	24.7 b	258 c	2.35 a

Same letters in the same column, for each enzyme, are statistically equal by Tukey's test ($p < 0.05$)

value results in low-volume breads, because the gas produced by fermentation is not strong enough to compete with the dough tenacity. Additionally, Selinheimo et al. (2007) found that laccase-added doughs were harder and less extensible and the effects increased in function of the enzyme concentration.

Thus, the addition of 25 U kg^{-1} of LAC had significant difference than the control and promoted good improvements in the ST and *W* values, being an interesting alternative for the correction of weak flours, as an oxidizing agent.

Effects of Lipase and Transglutaminase Concentration

Lipases act hydrolyzing the lipids of the wheat flour releasing fatty acids, mainly mono- and diglycerides, which have good emulsifying properties (Gerits et al. 2014b). Wheat flours usually have from 2.0 to 2.5 % of lipids that have been subdivided into starch lipids (40 %), which are located within the starch granule, and non-starch lipids (60 %). According to Gerits et al. (2014a), during mixing, part of the free lipid fraction is transferred to the bound lipid fraction (lipid binding) interacting with the gluten network, due to their amphiphilic nature and ionic interactions. These lipids promote a gluten aggregation by decreasing the electrostatic repulsion between the gluten polymers. The same authors suggested that the polar lipids stabilize the gas cell on the dough by the interactions with the gluten proteins, stabilizing also the gluten network of the dough, characterizing an emulsifier effect.

As can be seen in Table 5, LIP did not present any significant effect on FN, WA, and *P*/*L* ratio ($p > 0.05$), except at 25 and 833 U kg^{-1}, which enhanced the *P*/*L* ratio. The DT was undesirably increased at 333 and 833 U kg^{-1} from 12.1 (control) to 14.6 and 16.8 min, respectively. On the other hand, all other LIP concentrations decreased the DT, where at 150 U kg^{-1}, the highest reduction from 12.1 to 5.3 min was observed but with low interference on stability time, which decreased from 22.8 to 21.9 min. Furthermore, there was a significant improvement ($p < 0.05$) on *W* (10^{-4} J) value, from 221 (control) to 247, without statistical changes in the *P*/*L* ratio. According to Pareyt et al. (2011), the binding of free lipids with gluten proteins at the interface of gas cells provides suitable elasticity and extensibility and decreasing stickiness. This effect offers resistance and malleability to the dough, allowing greater expansion of the bubble in the alveographic tests, justifying the good improvement in the *W* value.

Thus, LIP, at a concentration of 150 U kg^{-1}, was able to promote a desirable decrease in the development time and an important enhancement in the *W* value, with minimal interferences in the other rheological parameters.

As well as for lipase, TG did not present any effects on FN, WA, and *P*/*L* ratio compared with the control ($p > 0.05$). The addition of TG increased the DT by 28 %, from 12.1 (control) to 17.7 min, but there are no statistical differences between all the tested concentrations ($p > 0.05$). For ST, except for 333 and 583 U kg^{-1}, all studied concentrations were better than the control. The concentrations from 25 to 250 U kg^{-1} were statistically equal ($p > 0.05$) and provided an increased from 22.8 min (control) to around 28 min. According to Steffolani et al. (2010), TG catalyzes acyl-transfer reactions, introducing covalent crosslinks in proteins between lysine residues and glutamine residues to form an ε-(γ-Glu)-Lys bonds, strengthening the dough and improving its stability. Moreover, for *W* values, all concentrations were statistically different ($p < 0.05$)

Table 5 Effects of lipase and transglutaminase on rheological parameters of flour

Enzyme	Enzyme concentration (U kg^{-1} flour)	FN (s)	Farinographic parameters			Alveographic parameters	
			WA (%)	DT (min)	ST (min)	W (10^{-4} J)	*P*/*L*
LIP	0	420 a	57.1 a	12.1c	22.8 c	221 f	1.31 b
	25	411 a	56.9 a	6.4 f	20.2 e	223 f	1.52 a
	150	415 a	57.4 a	5.3 g	21.9 d	247 b	1.18 b
	250	431 a	57.4 a	7.0 e	16.5 g	249 ab	1.22 b
	333	412 a	57.1 a	14.6 b	23.3 b	244 c	1.28 b
	583	424 a	57.3 a	9.2 d	19.1 f	240 e	1.23 b
	833	415 a	56.9 a	16.8 a	25.1 a	251 a	1.64 a
TG	0	420 a	57.1 a	12.1 b	22.8 f	221 f	1.31 a
	25	424 a	57.1 a	17.7 a	29.2 a	266 c	1.20 a
	150	425 a	56.9 a	16.9 a	28.0 b	290 b	1.24 a
	250	424 a	56.9 a	16.1 a	27.1 c	268 c	1.24 a
	333	433 a	57.8 a	16.5 a	23.3 e	262 d	1.22 a
	583	422 a	56.8 a	16.8 a	23.5 e	257 e	1.21 a
	833	418 a	57.6 a	16.7 a	25.6 d	302 a	1.26 a

Same letters in the same column, for each enzyme, are statistically equal by Tukey's test ($p < 0.05$)

and better than the control, similarly as that found by Steffolani et al. (2010). Moreover, the concentrations of 150 and 833 U kg^{-1} provided an increased from 221×10^{-4} to 290×10^{-4} and 302×10^{-4} J, respectively, without significant effects on *P*/*L* ratio.

Although, at 25 U kg^{-1} TG promoted good results on flour rheology, considering its individual effects and its high costs compared with the other studied enzymes, its use in correction of flours to baking is not justified. Moreover, GOX promoted similar effects at low concentrations and, usually, at lowest prices. More studies considering its use in association with other enzymes, as identification of the possible cooperative effects would be necessary.

Ideal Enzyme Concentrations Measured Compared with the Manufacturers' Recommendations

Table 6 presents the comparison between the results obtained in the present work, based on the effects of each enzyme in the flour rheological parameters and the recommended dosages from the enzyme suppliers. Considering the FN, only AMY-B was able to adjust this parameter in the concentration range recommended by the supplier, while for AMY-F and AMY-M, concentrations much higher than that specified by the manufacturer were required. Therefore, by just following the manufacturers' recommendations, without the knowledge of the flour characteristics and the behavior of each amylase, it is not possible to ensure the standardization of the FN.

The concentrations required to adjust FN by AMY-F and AMY-M are economically unfeasible because at high concentrations, the costs do not justify their use in industry. Considering that AMY-B was able to correct the FN at low concentration, however, more studies are necessary to verify possible cooperation effects, when the three amylases are used together.

Table 6 Comparison between ideal studied concentrations and supplier dosage recommended to each enzyme

Enzyme	Measured ideal concentration		Dosage recommended by the suppliers
	U kg^{-1}	ppm	ppm
AMY-F	583	243	10–20
AMY-B	150	83	50–150
AMY-M	583	280	10–100
XYL-F	25	11	30–50
XYL-B	25	12	20–50
GOX	25	10	2–50
LAC	25	96	Not declared
LIP	150	26	5–50
TG	25	18	10–30

For xylanases, in using a concentration much lower than that recommended by the manufacturers, it was possible to improve the flour rheology, mainly by reducing the tenacity, by the increase of *W* and the *P*/*L* ratio. Thus, the use of concentrations suggested by the suppliers could lead to excessive dosages, being necessary to know previously the characteristics of the flour and how the xylanases will affect them.

The dosage range suggested to GOX is very ample to ensure functionality in several types of flour. Therefore, for strong flours, such as the one used in the present study, low concentrations were enough to obtain good improvements. Moreover, the rheological parameters were sensible to GOX concentration and excessive dosages could be more prejudicial than beneficial, as shown in Table 4. LAC is not a usual enzyme employed in baking flour correction. However, even at a low concentration, LAC proved to be a good alternative, as well as GOX, to substitute the chemical oxidants, mainly by its strengthening effects, evidenced by the improvement on ST and *W* values.

LIP showed interesting effects on flour rheology, mainly by the improvements on the *W* value and *P*/*L* ratio. The LIP concentrations employed depend largely on the wheat lipid composition. Thus, the decision about the lipase concentration should always consider its impact on flour rheology, mainly in DT reduction and improvement of *W* value.

According to the effects presented in this work, the use of TG for flour correctionis not recommended, since TG is an expensive enzyme. To flour strengthening, GOX and LAC showed to be better alternatives than TG.

Conclusion

Wheat flours used for baking generally require correction of α-amylase activity. However, this adjustment resulted in changes in other rheological parameters implying the flour quality and its bread making potential. Different enzymes can act on different wheat flour fractions, according to their individual mechanisms, and thus improve the rheological properties of wheat flour in different ways.

Based on the present work, it can be concluded that XYL-B and LIP reduced the DT and *P*/*L* ratio (suitable for baking), confirming their potential for correction of flours for baking. The increase in the ST and *W* values, caused by GOX and LAC, showed that these enzymes could be good alternatives to adjust the rheological parameters, after the FN correction by α-amylase. Furthermore, it is important to know the effect of each enzyme on flour rheology for correct use, avoiding over dosages, such as those specified by the manufacturers. Moreover, further studies using the combination of these enzymes should be performed in order to identify the cooperative and antagonist interactions between the enzymes when

used together, as substitutes of oxidant agents, such as azodicarbonamide.

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