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Effect of a *Thermoascus aurantiacus* thermostable enzyme cocktail on wheat bread qualitiy

D.S. Oliveira [a], J. Telis-Romero [a], R. Da-Silva [b], C.M.L. Franco [a,*]

[a] Laboratory of Cereals, Roots, and Tubers, UNESP – São Paulo State University, Rua Cristovão Colombo, 2265 São José do Rio Preto, São Paulo CEP 15054-000, Brazil
[b] Laboratory of Biochemistry and Applied Microbiology, UNESP – São Paulo State University, Rua Cristovão Colombo, 2265 São José do Rio Preto, São Paulo CEP 15054-000, Brazil

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ABSTRACT

Thermophilic fungus *Thermoascus aurantiacus* (CBMAI 756) on solid-state fermentation using corncob as a nutrient source produces an enzyme pool with the potential to be used in bread making. In this paper, the use of this enzyme cocktail as a wheat bread improver was reported. Both products released by flour arabinoxylan degradation and bread quality were investigated. The main product released through enzyme activity after prolonged incubation was xylose indicating the presence of xylanase; however, a small amount of xylobiose and arabinose also confirmed the presence of xylosidase and α-L-arabinofuranosidase, respectively. Enzyme mixture "*in vitro*" mainly attacked water-unextractable arabinoxylan contributing to beneficial effect in bread making. The use of an optimal enzyme concentration (35 U xylanase/100 g of flour) increased specific volume (22%), reduced crumb firmness (25%), and reduced amylopectin retrogradation (17%) during bread storage. In conclusion, the enzyme cocktail produced by *T. aurantiacus* CBMAI 756 can improve wheat bread quality.

1. Introduction

Bread staling is a complex phenomenon that happens during storage, and it is largely caused by water migration and transformations that occur in the starch. Alterations related to this phenomenon include an increase in moisture in the crust (loss of crispiness), an increase in crystallinity in the starch granule, an increase in crumb firmness, a loss of organoleptic properties in the loaves, and the crumb's loss of water-holding capacity (Gray & BeMiller, 2003; Ribotta & Le Bail, 2007). For years, it was believed that amylopectin retrogradation was the most important phenomenon responsible for the increase in crumb firmness during bread storage. Nevertheless, bread staling does not occur only because of amylopectin retrogradation (Gray & Bemiller, 2003; Martin, Zeleznak, & Hoseney, 1991). Gluten-starch interactions and moisture transfer also seems to be involved in bread staling (Gray & Bemiller, 2003).

In most countries, the bread is made from wheat flour. The main constituents of flour are starch and proteins, although minor compounds, such as lipids and non-starch polysaccharides (NSP) (including arabinoxylan (AX) and β-glucans), also influence the process and quality of the final product. Among NSPs, wheat AX has been reported to be the most important. Cereal AX is classified into water-extractable arabinoxylan (WE-AX) and water-unextractable arabinoxylan (WU-AX). AX concentration in wheat flour varies between 1.5% and 2.5%, among which 0.4–0.6% is WE-AX (Courtin & Delcour, 2002).

In cereals, these polymers are comprised of a principal chain made up of β-D-xylopyranosyl units, linked through 1,4 glycosidic linkages that can be substituted in various degrees by α-L-arabinofuranosyl residues at positions C-2 and/or C-3 of the xylose (Kulkarni, Shendye, & Rao, 1999). Other substituents, such as glucuronic acid, D-galactose, and phenol groups (ferulic acid, and *p*-coumaric acid), may also be present (Subramaniyan & Prema, 2002). Due to the great complexity of AX structure, a cocktail of enzymes containing endo-1,4-β-xylanase (EC 3.2.1.8), 1,4-β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), acetylxylan esterase (EC 3.1.1.72), feruloyl esterase (EC 3.1.1.73), and *p*-coumaric esterase (EC 3.1.1.-), which synergistically act to hydrolyze this heteropolysaccharide, is required for its complete degradation (Subramaniyan & Prema, 2002; Waters, Murray, Ryan, Arendt, & Tuohy, 2010).

Bread-making industries have used diverse agents to reduce bread staling, including enzymes, which have received special attention. Amylases, proteases and hemicellulases have been reported as having a direct influence on starch retrogradation and final product quality (Goesaert, Slade, Levine, & Delcour, 2009; Verjans, Dornez, Delcour, & Courtin, 2010; Waters, Ryan, Murray, Arendt, & Tuohy, 2011).

* Corresponding author. Tel.: +55 17 3221 2267; fax: +55 17 3221 2299.
E-mail addresses: denisefronteira@gmail.com (D.S. Oliveira), javier@ibilce.unesp.br (J. Telis-Romero), dasilva@ibilce.unesp.br (R. Da-Silva), celia@ibilce.unesp.br (C.M.L. Franco).

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Because of the hydrolytic action on non-starch polysaccharides, xylanase has received special attention, and this enzyme is commonly found in formulations for bread improvement. It has beneficial effects on bread quality – it improves rheological properties of dough, the specific volume of bread, the crumb texture, and the bread staling rate (Damen et al., 2012; Laurikainen, Härkönen, Autio, & Poutanen, 1998; Martinez-Anaya & Jiménez, 1997; Shah, Shah, & Madamwar, 2006; Waters et al., 2011).

According to Courtin and Delcour (2001, 2002), xylanases that preferentially attack the WU-AX positively impact both the properties of the dough and the volume of the bread, because they affect moisture redistribution in the system and break glycosidic linkages with consequent water liberation and reduction in WU-AX size. Thus, the significant challenge today is to obtain xylanases that will act on WU-AX, modifying its structure and functionality.

Most studies have shown mesophilic xylanase activity, but little can be found regarding the use of thermophilic xylanases. The advantage of using heat-stable enzymes comes from a longer activity during the baking period. Studies using recombinant thermophilic endoxylanase in breads showed an increase in specific volume, and a reduction in crumb firmness and less amylopectin retrogadation during the storage of these products (Jiang, Bail, & Wu, 2008; Jiang, Li, Yang, Li, & Tan, 2005). According to Jiang et al. (2005), thermophilic enzyme can maintain activity during great part of baking period, enhancing the breakdown of AX at increasing temperature resulting in more oven spring during baking. However, for hyperthermophilic enzymes, such as those active after 100 °C, a caution should be taken since an excessive degradation of AX can negatively alter dough properties and bread quality.

Recently, thermophilic fungi have attracted growing attention in baking, and enzyme cocktails from *Talaromyces emersonii* have been successfully applied to bread making (Waters et al., 2010, 2011). The thermophilic fungus *Thermoascus aurantiacus*, a not toxin-producing microorganism (Kongbuntad, Saenphet, Khanongnuch, & Lumyong, 2006; Oliveira, Meherb-Dini, Franco, Gomes, & Da-Silva, 2010), has been used to produce xylanase on solid-state fermentation (SSF) (Da-Silva et al., 2005). In a recent study in which corncob was used as a substrate, *T. aurantiacus* expressed a cocktail of hemicellulolitic enzymes that contained high xylanase and low protease and amylase activities. This enzyme profile fits well into the baking industry, because it resulted in low degradation of starch and gluten, but showed high solubilization of xylan (Oliveira et al., 2010).

The effect of xylanases on the bread staling rate is still not completely known; thus, the goal of this study was to investigate the influence of the enzyme cocktail with xylanolytic activity from *T. aurantiacus* fungus CBMAI 756 on bread quality and the staling process, as well as to identify the specific products released through its activity on wheat flour arabinoxylans. To the authors' knowledge, this is the first report dealing with *T. aurantiacus* enzymes as a bread improver.

2. Materials and methods

2.1. Material

Wheat flour with 72% extraction rate was kindly donated by the Sete Irmãos Mill (Uberlândia, Brazil). The wheat flour contained 0.48% ash, 1.43% fat, 11.78% protein, and 3.50% total dietary fiber, which were determined according to the American Association of Cereal Chemists methods (AACC, 2000). Farinograph and extensograph parameters of the wheat flour, determined according to the AACC methods 54–21 and 54–10 (AACC, 2000), respectively, were: water absorption capacity (63.9%), stability time (10 min), mixture tolerance index (30 BU), extension resistance (520 BU), extensibility (135 mm), and proportional number (3.8).

The other ingredients that were used to prepare the loaves were obtained from a local market (São José do Rio Preto, Brazil). Chromatographic degree standards, arabinose (A), and xylose (X_1) were acquired from Fluka (Madrid, Spain), and glucose (G_1) was obtained from Sigma Aldrich (St. Louis, USA). Xylooligosaccharide standards with DP 2–6 (X_2–X_6) were acquired from Megazyme (Bray, Ireland). The α-amylase enzyme from *Aspergillus oryzae* was kindly supplied by Granotec (Curitiba, Brazil), and the amyloglucosidase was isolated from genetically modified *Saccharomyces cerevisiae* in the Laboratory of Biochemistry and Applied Microbiology of São Paulo State University (São José do Rio Preto, Brazil). All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Microorganism, culture conditions and enzyme extract

Microorganism and enzyme production was carried out as previously described by Oliveira et al. (2010). Briefly, the fungus *T. aurantiacus* CBMAI 756 was cultivated at 50 °C and kept in test tubes with slanting Sabouraud dextrose agar (Oxoid Ltd., Basingstoke, Hampshire, England) at room temperature. Mycelial suspension in mineral solution was used to inoculate 250-mL Erlenmeyer flasks containing 10 g of corncob as the nutrient source for SSF (67% moisture) at 50 °C for 6 days. The crude enzyme solution was produced by adding 40 mL of distilled water to the fermented material, and it was concentrated with 75% ethanol at 4 °C overnight. The precipitates were separated using centrifuge at 13,700g for 10 min and were then dissolved in a small amount (4 mL) of distilled water.

2.2.2. Enzyme activities

All the enzyme reactions and their controls were run in triplicate. The activities of xylanolitic enzymes (endo-1,4-β-xylanase – EC 3.2.1.8 and 1,4-β-xylosidase – EC 3.2.1.37), endoglucanase or CMCase (EC 3.2.1.4), avicelase (EC 3.2.1.91) and α-amylase (EC 3.2.1.1) were determined by the incubation of 0.1 mL of enzymatic solution (appropriately diluted) with 0.9 mL of acetate buffer 0.10 mol/L (pH 5.0) The solution contained 0.5% Birchwood xylan (Sigma) in the case of the xylanase and xylosidase combination; it contained 2% carboxymethylcellulose (CMC-Sigma C5768) in the case of endoglucanase; it contained 1% cellulose (avicel-Sigma) in the case of avicelase, and it contained 0.5% soluble starch (Mallinckrodt) in the case of α-amylase. After incubating the reaction mixture at 60 °C for 10 min, 1 mL of dinitrosalicylic acid (DNS) was added to interrupt the reaction. The reducing sugars released from the reaction were determined according to Miller (1959). Controls were prepared by adding the enzyme after the DNS. One International Unit (IU) of enzymatic activity was defined as one micromole of reducing substances expressed as xylose (xylanase and xylosidase) or glucose (CMCase, avicelase, α-amylase) released per minute under the aforementioned test conditions using standard curves of either xylose or glucose. The activity of amyloglucosidase was determined by the peroxidase/glucose-oxidase enzymatic method (Bergmeyer & Bernt, 1974).

The β-glucosidase activity (EC 3.2.1.21) was determined by incubating 50 μL of enzymatic extract in 250 μL of acetate buffer 0.1 mol/L (pH 5.0) and 250 μL of *p*-nitrophenyl-β-D-glucopyranoside 0.004 mol/L (PNPG, Sigma) (Palma-Fernandez, Gomes, & Da-Silva, 2002). The reaction mixture was kept at 60 °C for 10 min. The enzymatic reaction was stopped by the addition of 2 mL of sodium carbonate 2 mol/L, and the released nitrophenol was quantified using a spectrophotometer (Jasco, V-639 Bio, Brazil) at 410 nm. One unit of enzymatic activity was defined as the quantity of the enzyme needed to release 1 μmol of nitrophenol