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ORIGINAL ARTICLE

Use of alpha-amylase and amyloglucosidase combinations to minimize the bread quality problems caused by high levels of damaged starch

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Abstract The objective of this work was to investigate the contribution of α-amylase and amyloglucosidase to dough fermentation process and bread quality, as an alternative to reduce the negative effects caused by high damaged starch in flour. The dough properties during the proofing process were modified by higher damaged starch content. Higher damaged starch in flour resulted into breads with darker crusts and firmer crumbs. The enzymes reduced the negative influence of damaged starch, producing a positive effect on the maximum carbon dioxide pressure reached during fermentation and the carbon dioxide volume retained by dough. Incorporation of alpha-amylase reduced dimension ratio and crumb firmness attributes; however, progressive additions of this additive produced lower bread volume and red intensity, and higher crumb firmness. The amyloglucosidase additions produced higher bread volume and red intensity of the crust, and lower brightness crust and gas cell diameter. Incorporation of amyloglucosidase was beneficial in the presence of a suitable quantity of damaged starch. The results confirmed that the α-amylase and amyloglucosidase additions significantly improved bread quality. Incorporation of α-amylase and amyloglucosidase led to higher bread loaves and lower crumb firmness throughout the storage period, promoting a longer life of the finished product.

Keywords Damaged starch · Amylases · Fermentation process · Bread · Quality

Introduction

The bakery industry uses a large variety of enzymes to optimize dough properties and to improve the quality of baked products (Hoseney 1994). The α- and β-amylases are the most popular enzymes in breadmaking, albeit amyloglucosidase is also used (Wursch and Gumy 1994). The α-amylases are commonly employed in breadmaking to improve the textural properties of bread and to reduce elasticity (Barrera et al. 2015; Kim et al. 2006; Patel et al. 2012). In bread and Danish pastry production, amyloglucosidase is used to produce glucose from starch to enhance the fermentation process (Diler et al. 2015).

The starch granules can experience mechanical damage during wheat milling, producing damaged starch (DS). The level of damage is related to wheat hardness and milling conditions (Hoseney 1994). The granular integrity of starch is affected by mechanical damage, resulting in modifications of rheological behavior and functional properties of the starch systems (Barrera et al. 2012, 2013; Morrison et al. 1994; Tester et al. 1994). Damage of the starch granule facilitates swelling of granules (Tester 1997) and consequently, DS granules have the ability to absorb more water than native granules, and are more hydrolyzed by amylases (Hoseney 1994). The DS level of the regular wheat flour is between 5 and 13 %, (Sakhare et al. 2014;

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Colombo et al. 2008; Duyvejonck et al. 2011; Rehman et al. 2007). Damaged starch modified the physicochemical properties of wheat flour, causing higher water absorption capacity, which affects negatively the dough rheological behavior, as well as cookie and bread making quality (Ghodke et al. 2009; Jovanovich et al. 2003; Rao et al. 2010; El-Porai et al. 2013; Barrera et al. 2007, 2015). Amylases could help to reduce the technological problems produced by high DS levels, due to the greater susceptibility of DS to enzymatic hydrolysis. Taking into account of previous work (Barrera et al. 2015) where in beneficial effects of α-amylase and amyloglucosidase to attenuate the negative influence of high DS content on dough properties, the present objective of this work was to analyze the contribution of α-amylase and amyloglucosidase to flours with high DS levels in the breadmaking process. The effect of enzymes on the fermentation and bread quality was evaluated. Wheat flour samples with high level of DS were obtained from industrial milling; hence we determined the influence of combining these enzymes on dough proofing process and bread quality.

Materials and methods

Samples

Five bread wheat flours (WF1, WF2, WF3, WF4 and WF5) with high DS levels specially prepared by industrial milling (Molino Carlos Boero Romano SA (San Francisco, Córdoba, Argentina)) were used for the studies. The damaged starch content: (76–30A); protein content: (46–13); wet and dry gluten: (38–10); and α-amylase enzymatic activity: (56–81B) of flour were determined by methods of AACC (2000). The flour samples composition is shown in Table 1. The flour samples were produced from mixtures of different wheat bread grain lots. DS levels were selected with the purpose of covering a representative range of DS proportions in wheat flours. The samples of bread wheat flours used to the analyses mainly differed in DS content. The protein content and wet and dry gluten were similar, indicating that the protein ability to develop gluten network was similar between samples. In all cases *Falling number* values were >400 s, indicating low α-amylase activity.

Enzymes

The enzymes used for the analysis were α-amylase (AMY) (Fungamyl 4000BG, activity of 4000 FAU/g, NOVOZYMES) and amyloglucosidase (AMG) (AMG 3300BG, 3300 AGU/g, NOVOZYMES) and mixtures of them. For experimental testing, the doses used were the maximum dose and twice the minimum dose recommended by the producer company. Therefore, the enzymes doses used ranged from 0.0002 (AMY_{Min})−0.0006 (AMY_{Max}) g/100 g flour for α-amylase and 0.01 (AMG_{Min})−0.015 (AMG_{Max}) g/100 g flour for amyloglucosidase.

Fermentation process of dough

The changes in dough properties during the proofing process were evaluated using a rheofermentometer (Chopin rheofermentometer F3, Chopin, France). From the *dough development curve*, the following parameters were obtained: maximum dough height (mm) reached (Hm), time (min) reached at the maximum height (T1), dough height (mm) at the end of the fermentation process (h), and dough development speed (DDS) between 10 and 40 min of fermentation. From the *gaseous release curve*, we obtained: maximum height (mm) of carbon dioxide production (Hm′) related to the maximum pressure of the system reached during the process as a result of carbon dioxide production, time (min) required to reach maximum pressure (T1′), time (min) when dough becomes permeable as a consequence of its expansion (Tx), carbon dioxide production rate (mm/min) during the first 10 min of the fermentation process (GPR), and carbon dioxide volume (mL) retained by dough (Vr). Additionally, we determined dough height (mm) at the time of maximum pressure (H at T1′) and dough height (mm) at the time when porosity appears (H at Tx).

Table 1 Samples composition

Sample	Damaged starch (%)	Protein (%)	Wet gluten (%)	Dry gluten (%)	Falling Number (s)
WF1	8.90[a]	9.43[a]	30.10[a]	10.68[a]	490[b]
WF2	10.13[b]	10.55[b]	29.60[a]	10.96[a]	407[a]
WF3	11.07[c]	9.99[a]	28.42[a]	10.43[a]	416[a]
WF4	14.30[d]	9.71[a]	29.63[a]	10.76[a]	489[b]
WF5	15.03[d]	9.53[a]	28.56[a]	10.42[a]	453[ab]

Different letters indicate significant differences ($p \leq 0.05$; ANOVA)

The dough samples from WF2 and WF5 flours with and without the addition of enzymes were evaluated according to Chopin protocol. The minimum and maximum doses of enzymes ranges mentioned previously were used for the fermentation test. Dough samples were prepared as follows: firstly, dry ingredients (200 g flour, 4 g salt, 2.4 g dry yeast) were mixed for 2 min, using a Kitchen Aid mixer (Kitchen Aid 525 watts, USA). Afterwards, water (120 g) was added, with the same mixing time (9 min at constant speed) for all samples. A piece of dough (200 g) was used for the fermentation test according to the following conditions: 2 kg resistance weight, at controlled temperature of 28.5 °C during 3 h. The flour samples were hydrated according to its moisture content (WF2 = 13.15 % and WF5 = 13.13 %). Analyses were performed in duplicate.

Breadmaking procedure

Breadmaking tests were performed using the mould bread methodology applied in wheat breeding programs, according to IRAM 15858-1 (IRAM 1996) with minimal modification in baking time. Dough formulation: 100 % wheat flour, 3 % compressed yeast, 1 % salt and 58.5 % water. Compressed yeast and salt were pre-dissolved in water, separately. The ingredients were mixed and kneaded for 10 min (MPZ, Argentina). The resulting dough (27 °C) was proofed (Pauna-Cst, Argentina) for 80 min (30 °C and 98 % RH) (first proof), with two intermediate punches (partial loss of gas) at 45 and 60 min. The bulk dough was then sheeted in a Mi-Pan vf roller (Mi-Pan, Cordoba, Argentina) and divided into 150 g pieces, molded into a loaf shape (Braesa MB 350, Brazil) and returned to the fermentation cabinet for 75 min at 30 °C and 98 % RH (second proof). Finally, the pieces were baked at 215 °C for 20 min in a forced convection oven (Pauna-Cst, Argentina). The baked loaves were cooled at room temperature for 2 h and then were stored in polyethylene bags at 23 ± 2 °C until analysis.

Bread quality parameters

Bread quality parameters were evaluated after 24 h of baking. Loaves were weighed and bread volume was determined according to AACC-method 10-05 (AACC 2000). Specific volume of bread (SVB) was expressed as volume/weight ratio. The dimension ratio (DR) of each bread piece was calculated as the ratio between the height and width of each piece. Bread crust color was determined according to AACC-method 14-22 (AACC 2000) using a portable reflectance spectrophotometer (CM-700d/600d Konica Minolta, Ramsey, USA). Color parameters were recorded as CIE-LAB (C.I.E 1976): L* = lightness, a* = position between red and green and b* = position between yellow and blue. Bread crumb firmness (F) was evaluated using an INSTRON (Universal Testing Machine, 3342, USA) (74-09; AACC 2000). Bread slices (25 mm thick) were subjected to a compression test with a 25 mm cylinder probe. Test conditions were as follows: compression cell of 5 kg; speed 100 mm/min; 40 % maximum strain; grip dimension 36 mm. The force required to compress the crumb (crumb firmness) was determined by Instron Bluehill 2.27 software. The crumb structure was evaluated by image analysis using software Image J v 1.45 s (National Institutes Health, Bethesda, MD, USA). For each loaf, two slices from the central pieces were obtained and scanned (HP Scanjet G3010, USA). Color images were converted into 8-bit images, grayscale and then segmented from a gray value in order to create a binary image. The gray threshold value for image segmentation was automatically performed with the software. From this, all pixels with gray level higher than the threshold value were white and associated with wall cells, while pixels with gray level lower than the threshold value were black and considered empty areas (gas cells). In each image, object category was assigned (gas cell) to areas of the gray intensity between zero and threshold value. The parameters obtained from image analysis were: area fraction (AF), the fraction of area occupied by gas cell in relation to the total area, and Feret Diameter (FD), the major diameter average of gas cells.

Experimental design

The effect of the combination of DS, α-amylase and amyloglucosidase on bread quality was evaluated by *Response Surface Methodology*. The independent variables were: damaged starch range: 8.9–14.3 % DS; α-amylase range: 0.0002–0.0006 g/100 g flour; amyloglucosidase range: 0.01–0.015 g/100 g. The combined effect of the three factors (DS and both enzymes) on bread quality parameters was evaluated from 16 breadmaking assays defined by *Box-Behnken* design. The experimental design is shown in Table 2. Preliminary tests were performed with flour samples with and without enzymes using different water proportions. Results indicated that the water proportion to make dough with suitable consistency to be machined during the breadmaking process was 58.5 %. WF1, WF3 and WF4 flour samples were used in the experimental procedure. The quality of bread pieces without enzymes was evaluated. In agreement with previous studies, the specific bread volume (WF1 = 3.8 cm^3/g; WF3 = 3.6 cm^3/g; WF4 = 2.5 cm^3/g) and the crumb firmness (WF1 = 12.23 N; WF3 = 16.06 N; WF4 = 19.13 N) gradually decreased and increased, respectively ($p \leq 0.05$), when damaged starch content rose. Taking into account that flour samples came from

Table 2 Experimental design and responses of bread quality parameters

Experimental design				Responses variables							
Assay	AMY*	AMG*	DS (%)	SVB (cm^3/g)	DR	L*	A*	B*	F (N)	AF (%)	FD (mm)
1	0.0002	0.0125	8.90	5.26	1.09	71.56	7.81	28.89	5.27	36.90	0.135
2	0.0004	0.01	8.90	4.34	1.04	73.58	6.90	26.82	5.64	36.14	0.142
3	0.0004	0.015	8.90	5.77	1.07	69.91	8.65	29.38	5.05	36.49	0.137
4	0.0006	0.0125	8.90	4.12	1.01	71.82	7.72	28.02	6.89	36.97	0.139
5	0.0002	0.015	11.07	3.79	0.99	62.46	11.78	32.89	5.62	35.44	0.134
6	0.0002	0.01	11.07	3.81	0.99	63.67	11.12	32.41	7.62	35.19	0.158
7	0.0004	0.0125	11.07	4.35	0.87	61.16	13.15	34.20	5.59	35.29	0.141
8	0.0004	0.0125	11.07	4.17	0.90	57.56	13.77	31.93	5.34	35.86	0.141
9	0.0004	0.0125	11.07	4.49	0.87	56.94	14.73	34.48	5.50	33.94	0.134
10	0.0004	0.0125	11.07	4.29	0.92	59.75	13.88	32.23	4.93	35.35	0.136
11	0.0006	0.01	11.07	3.53	0.95	61.52	12.57	32.41	8.97	35.61	0.131
12	0.0006	0.015	11.07	3.80	0.82	57.93	14.40	34.08	7.48	35.29	0.140
13	0.0002	0.0125	14.30	3.15	0.93	59.77	12.53	31.28	9.82	32.99	0.133
14	0.0004	0.01	14.30	3.25	0.84	61.75	12.64	32.81	7.19	33.44	0.131
15	0.0004	0.015	14.30	3.40	0.85	56.61	15.14	35.08	9.96	32.36	0.123
16	0.0006	0.0125	14.30	3.08	0.89	60.69	12.67	31.99	11.05	31.91	0.127

* Enzyme doses (enzyme g/100 g flour)

AMY α-amylase, *AMG* amyloglucosidase, *DS* damaged starch, *SVB* specific bread volume, *DR* dimension ratio, texture parameters of bread crumb (*AF* area fraction and *FD* Feret diameter), *F* firmness and bread crust color (L* a* b*)

consecutive batch of grains, the same mill company, and they have similar protein and gluten proportions, it was assumed that the bread quality changes were mainly related to the differences in the damaged starch content. From the experimental design, regression equations and response surface plots were obtained for the quality parameters.

The predictive equations were validated by two experiments applying *Multiple Response* method: *1-Experiment*: α-amylase, amyloglucosidase and DS levels were determined in order to maximize SVB and DR, minimize F, and keep crust color within L* = 65; a* = 11 and b* = 31 values (intermediate color values defined from the range of values experimentally obtained); *2-Experiment*: α-amylase and amyloglucosidase levels were calculated in order to obtain a bread piece from flour with 14.3 % DS, maximizing SVB and DR but minimizing F and keeping crust color within L* = 65; a* = 11 and b* = 31 values (color values defined from the range of values experimentally obtained). Baking tests were performed from the optimum variable levels. Bread quality parameters (SVB, DR, crust color and F) were determined in order to confirm the predicted values of regression equations. WF2 flour sample was used in the *1-Experiment* and WF4 flour sample in the *2-Experiment*.

Statistical analysis

Data were statistically treated by variance analysis (ANOVA). The means were compared by LSD-Fisher test at a significance level of 0.05; the relationship between measured parameters was assessed by Pearson-test (significant levels at $p \leq 0.05$) using Infostat Statistical Software (Di Rienzo et al. 2011).

The effect of variable combination was evaluated by *Box-Behnken* experimental design and *Multiple Regression* process of *Response Surface Methodology* (Statgraphics plus 5.0). The optimum variable combinations was determined using *Multiple Response Method* (Statgraphics plus 5.0). The *Box-Behnken* design is a second-order rotatable design. The experiment order was completely randomized to avoid hidden variable effects. Four replicates of the central point were made to allow estimation of pure error by sums of squares. Results were analyzed by *Multiple Regression Method*. The quality of the fitted models was evaluated by ANOVA (Statgraphics plus 5.0). From the *p value* of each factor or variable, the significant relationship between the variables and the responses was determined. For each model fitted, the determination coefficient R^2 was determined. Optimum variable combination for breadmaking was determined from the desirability function (Ferreira et al. 2007).

Results and discussion

Effect of damaged starch and enzymes on dough proofing behavior

Dough fermentation properties were modified by DS levels ($p \leq 0.05$) (Table 3). WF5 dough showed a proofing performance lower than that of WF2 dough. The *dough development curve* showed that high DS level decreased Hm and h; and increased T1 ($p \leq 0.05$). However, DDS during the early stage of fermentation process was not influenced.

In relation to the *gaseous release curve,* high DS level decreased Hm' and increased T1′, albeit WF5 dough height at the time of maximum pressure (H at T1′) was higher ($p \leq 0.05$; WF2 = 9.7 mm; WF5 = 13.5 mm). Tx was not modified as a consequence of DS increment, which indicated that WF2 and WF5 dough samples became permeable at the same time. However, the dough with high DS content showed lower ($p \leq 0.05$) height at the time when the dough became permeable (H at Tx) (WF2 = 18.0 mm; WF5 = 14.7 mm). Gas production rate (GPR) and volume retained by dough (Vr) decreased as DS level increased.

Higher dough height during fermentation suggests a good combination between the gas produced and the structure of the system. In previous studies, Barrera et al. (2015), Rao et al. (2010) and El-Porai et al. (2013) have proved that dough with higher DS levels became more viscous and resistant to deformation as well as less elastic and extensible, which can explain the lower maximum dough heights and the increase in the time required to reach it. Limited dough expansion during fermentation process could be related to a change in the gluten network, probably due to less developed gluten and a more open gluten network arrangement or a combination of them as a result of modifications in water distribution during network formation and the large size of the swollen DS granules (Barrera et al. 2015).

The fermentation behavior of WF2 and WF5 dough was modified by enzymatic incorporations (Table 3). The

Table 3 Fermentation parameters of the dough samples

Sample	Dough development attributes				Gaseous release attributes				
	Hm (mm)	T1 (min)	H (mm)	DDS	Hm′ (mm)	T1′ (min)	Tx (min)	Vr (ml)	GPR (mm/min)
WF2	19.4^{b}	78.8^{a}	20.6^{a}	0.28^{a}	34.6^{b}	43.5^{a}	101.3^{a}	750.5^{b}	1.39^{b}
WF5	16.0^{a}	180.0^{b}	16.0^{b}	0.22^{a}	27.9^{a}	67.5^{b}	101.2^{a}	647.5^{a}	1.05^{a}
WF2	19.4^{b}	78.8^{a}	20.6^{bcd}	0.28^{c}	34.6^{a}	43.5^{a}	101.3^{bc}	750.5^{b}	1.39^{e}
WF2 + AMY_{Max}	22.6^{cd}	145.5^{d}	16.5^{abc}	0.21^{ab}	31.4^{a}	70.5^{ef}	102^{bc}	717.1^{ab}	1.26^{cd}
WF2 + AMY_{Min}	16.3^{a}	96.0^{b}	16.3^{ab}	0.22^{abc}	31.3^{a}	58.5^{c}	88.5^{abc}	702.0^{a}	1.21^{cd}
WF2 + AMG_{Max}	20.5^{bc}	180^{g}	20.5^{bcd}	0.23^{abc}	44.1^{c}	76.5^{g}	78.0^{ab}	952.1^{d}	1.26^{cd}
WF2 + AMG_{Min}	20.6^{bc}	180^{g}	20.6^{bcd}	0.25^{bc}	40.0^{b}	64.5^{d}	72.0^{a}	885.2^{c}	1.16^{abc}
WF2 + AMY_{Min} + AMG_{Min}	23.6^{de}	150^{d}	22.2^{de}	0.27^{bc}	42.5^{bc}	69.0^{e}	70.5^{a}	928.0^{cd}	1.32^{de}
WF2 + AMY_{Max} + AMG_{Min}	24.0^{de}	130.5^{c}	20.8^{cd}	0.22^{abc}	42.0^{bc}	72.0^{f}	75.1^{a}	934.0^{d}	1.19^{bc}
WF2 + AMY_{Min} + AMG_{Max}	23.4^{d}	168.0^{f}	20.9^{de}	0.18^{a}	40.0^{b}	75.1^{g}	78.0^{ab}	913.1^{cd}	1.08^{ab}
WF2 + AMY_{Max} + AMG_{Max}	25.9^{e}	159.0^{e}	25.2^{e}	0.18^{a}	42.2^{bc}	84.0^{h}	85.5^{abc}	942.0^{d}	1.05^{a}
WF5	16.0^{a}	180.0^{a}	16.0^{a}	0.22^{bc}	27.9^{a}	67.5^{c}	101.2^{cd}	647.5^{a}	1.05^{e}
WF5 + AMY_{Max}	23.1^{cde}	180.0^{a}	23.1^{cde}	0.16^{ab}	26.0^{a}	75.0^{e}	117.0^{d}	613.0^{a}	0.67^{a}
WF5 + AMY_{Min}	20.6^{b}	180.0^{a}	20.6^{bcd}	0.19^{abc}	26.0^{a}	64.5^{b}	70.5^{ab}	604.0^{a}	0.71^{ab}
WF5 + AMG_{Max}	20.9^{bc}	180.0^{a}	20.9^{bcd}	0.25^{c}	38.4^{c}	61.5^{a}	63.0^{a}	869.0^{d}	0.86^{cd}
WF5 + AMG_{Min}	19.6^{b}	180.0^{a}	19.6^{abc}	0.22^{bc}	36.6^{bc}	76.5^{e}	79.5^{abc}	806.0^{c}	0.96^{de}
WF5 + AMY_{Min} + AMG_{Min}	23.3^{def}	180.0^{a}	23.3^{cde}	0.25^{c}	37.8^{c}	66.0^{bc}	69.0^{ab}	843.0^{cd}	0.82^{bc}
WF5 + AMY_{Max} + AMG_{Min}	25.6^{f}	180.0^{a}	25.6^{e}	0.21^{bc}	37.1^{c}	75.0^{e}	76.5^{abc}	839.0^{cd}	0.94^{de}
WF5 + AMY_{Min} + AMG_{Max}	21.9^{bcd}	180.0^{a}	21.9^{cde}	0.14^{a}	33.5^{b}	88.5^{f}	93.0^{bcd}	746.0^{d}	0.60^{a}
WF5 + AMY_{Max} + AMG_{Max}	24.7^{ef}	180.2^{a}	24.7^{de}	0.18^{ab}	35.3^{bc}	72.8^{d}	76.5^{abc}	814.0^{c}	0.67^{a}

Different letters in each group of samples indicate significant differences ($p \leq 0.05$) (ANOVA)

Hm maximum dough height, *T1* time necessary to reach Hm, *h* height at the end of fermentation, *DDS* development speed, *Hm′*: maximum carbon dioxide pressure, *T1′* time necessary to reach Hm′, *Tx* time when dough becomes permeable, *Vr* carbon dioxide volume retained, *GPR* carbon dioxide production rate, *AMY* α-amylase, *AMG* amyloglucosidase

enzymes in WF2 dough sample increased Hm and T1 ($p \leq 0.05$); however, h and DDS parameters were not modified, except for $AMY_{Max} + AMG_{Max}$ incorporation which increased h parameter, and AMY_{Max} and mixture of AMY and AMG additions which reduced DDS parameter. In relation to maximum dough height, AMG additions produced no effect. Regarding WF5 dough sample, results indicate that enzymes increase Hm and h parameters ($p \leq 0.05$), especially $AMY_{Max} + AMG_{Min}$ and $AMY_{Max} + AMG_{Max}$, causing greater developments. However, the enzymatic additions did not modify T1 and DDS, except for the decrease caused by $AMY_{Min} + AMG_{Max}$ in DDS. Results indicated that α-amylase and amyloglucosidase addition had a significant positive effect on dough development during proofing. The enzymatic additives improved the fermentation behavior of the dough with higher DS levels. In general, the dough development profile of the WF2 and WF5 dough samples were similar as a result of the enzyme action. Similar α-amylase positive effects were recorded by Sanz Penella et al. (2008). Dough strengthening improvements of α-amylase and amyloglucosidase combinations (higher Hm and h parameters) could be attributed to the hydrolysis of DS granules by enzymes, which probably produced a change in the dough structure plasticity, as a consequence of water released from DS hydrolyzed, and a better gluten hydration.

The enzymatic activity increased ($p \leq 0.05$) Hm′ and T1′ in WF2 dough; however, AMY incorporation did not show a significantly effect on the maximum carbon dioxide pressure. Tx parameter was influenced by AMG_{Min}, $AMY_{Min} + AMG_{Min}$ and $AMY_{Max} + AMG_{Min}$; the dough became permeable in a shorter time as a consequence of the incorporation of these enzymes. Vr parameter increased ($p \leq 0.05$) and GPR decreased ($p \leq 0.05$) by enzymatic additions. The dough height at the time of maximum pressure (H at T1′) was higher ($p \leq 0.05$) than the control dough in all cases. The height at the time when the dough samples became permeable (H at Tx) was not influenced as a consequence of the enzyme action (data not shown).

In relation to WF5 sample, the presence of enzymes increased Hm′ ($p \leq 0.05$), although AMY incorporation did not cause significantly modifications. T1′ parameter increased ($p \leq 0.05$) due to the effect of enzymes. The incorporation of enzymes did not change Tx, except AMY_{Min}, AMG_{Max} and $AMY_{Min} + AMG_{Min}$, which decreased ($p \leq 0.05$) this parameter. Vr parameter increased ($p \leq 0.05$) by the effect of enzymes, excepting the AMY incorporation. AMG_{Max} and $AMY_{Min} + AMG_{Min}$ were the most important incorporations in relation to volume of gas retained. GPR decreased ($p \leq 0.05$) as a result of enzymatic additions, even though AMG_{Min} and $AMY_{Min} + AMG_{Min}$ did not change this parameter. The dough height at the time of maximum pressure (H at T1′) was not modified by enzymes, excepting the $AMY_{Max} + AMG_{Min}$ incorporation, which produced an increment ($p \leq 0.05$) in this parameter (data not shown). The height at the time when dough samples became permeable (H at Tx) was not influenced as a consequence of the enzyme action (data not shown).

The dough properties were improved by enzymes as was expected, due to the starch hydrolysis products which increased the carbon dioxide production. Results showed that the enzymes brought an improvement in the proofing properties of dough with higher DS levels. WF2 and WF5 dough profiles with enzymes were similar. Enzymes improved the maximum carbon dioxide pressure produced and the carbon dioxide volume retain during fermentation in dough high DS levels. $AMY_{Max} + AMG_{Min}$ and $AMY_{Max} + AMG_{Max}$ incorporations improved dough volume, while AMG_{Max} and $AMY_{Min} + AMG_{Min}$ increased carbon dioxide volume. However, $AMY_{Min} + AMG_{Min}$ addition decreased the time when the dough became permeable. The higher gas production associated to the α-amylase and amyloglucosidase additions can be explained by the higher fermentable sugar proportions (maltose and glucose) produced by enzymes from higher DS granules (Barrera et al. 2015; Diler et al. 2015), considering the synergistic effect between these enzymes reported by Fujii et al. (1988).

Contribution of damage starch and enzyme combination to bread quality

Table 2 shows the bread quality attributes resulting from 16 baking tests and Table 4 exhibits the significant ($p \leq 0.05$) coefficients of the regression equations for each quality attribute.

Relative contribution of damaged starch

DS content had a positive linear effect and a negative cross interaction (AMG*DS) on SVB parameter, which resulted in a negative impact on the same (Fig. 1). Damaged starch had linear negative and quadratic positive effects on DR and F attributes (Fig. 1). In relation to FD parameter, positive linear and negative quadratic effects were recorded. The negative contribution of high DS levels to DR, F and FD was greater than the positive influences recorded. These results are related to lower bread pieces and compact crumb structures. In addition, progressive increases in DS proportions had a positive linear effect on AF parameter. In relation to color, linear negative and quadratic positive effects were produced on L* and positive linear and negative quadratic influences were observed for a^* and b^*. Reduction in brightness and increase in red and yellow

Table 4 Significant coefficients of the design of the regression fitting model for bread quality parameters

Coefficients	SVB (cm^3/g)	DR	L*	*a**	*b**	F (N)	AF (%)	FD (mm)
Constant	−2.7	2.8	259.4	−78.5	−36.0	64.38	20.3	0.11
A:AMY	Ns	−421	Ns	Ns	Ns	−3.1E+04	Ns	Ns
B:AMG	1339	Ns	−3553	1255	Ns	Ns	Ns	−11.5
C:DS	0.086	−0.27	−27.7	13.0	12.5	−6.59	2.91	0.029
AA	−1.1E+07	Ns	Ns	−2.6E+07	Ns	4.3E+07	Ns	Ns
AB	Ns	Ns	Ns	Ns	Ns	Ns	Ns	1.7E+04
AC	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
BB	Ns	Ns	Ns	Ns	Ns	Ns	Ns	Ns
BC	−58.2	Ns	Ns	Ns	Ns	Ns	Ns	Ns
CC	Ns	0.011	1.2	−0.55	−0.53	0.25	Ns	−0.0012
R^2	94.7	91.7	95.4	95.8	88.5	91.7	92.2	89.2

Ns no significant effect at level ($p \leq 0.05$), R^2: adjusted square coefficient of the fitting model (data variability percentage which explains the regression equation in each case), *DS* damaged starch, *AMY* α-amylase, *AMG* amyloglucosidase, *SVB* specific bread volume, *DR* dimension ratio, texture parameters of bread crumb (*AF* area fraction and *FD* Feret diameter), *F* firmness and bread crust color (L* *a** *b**)

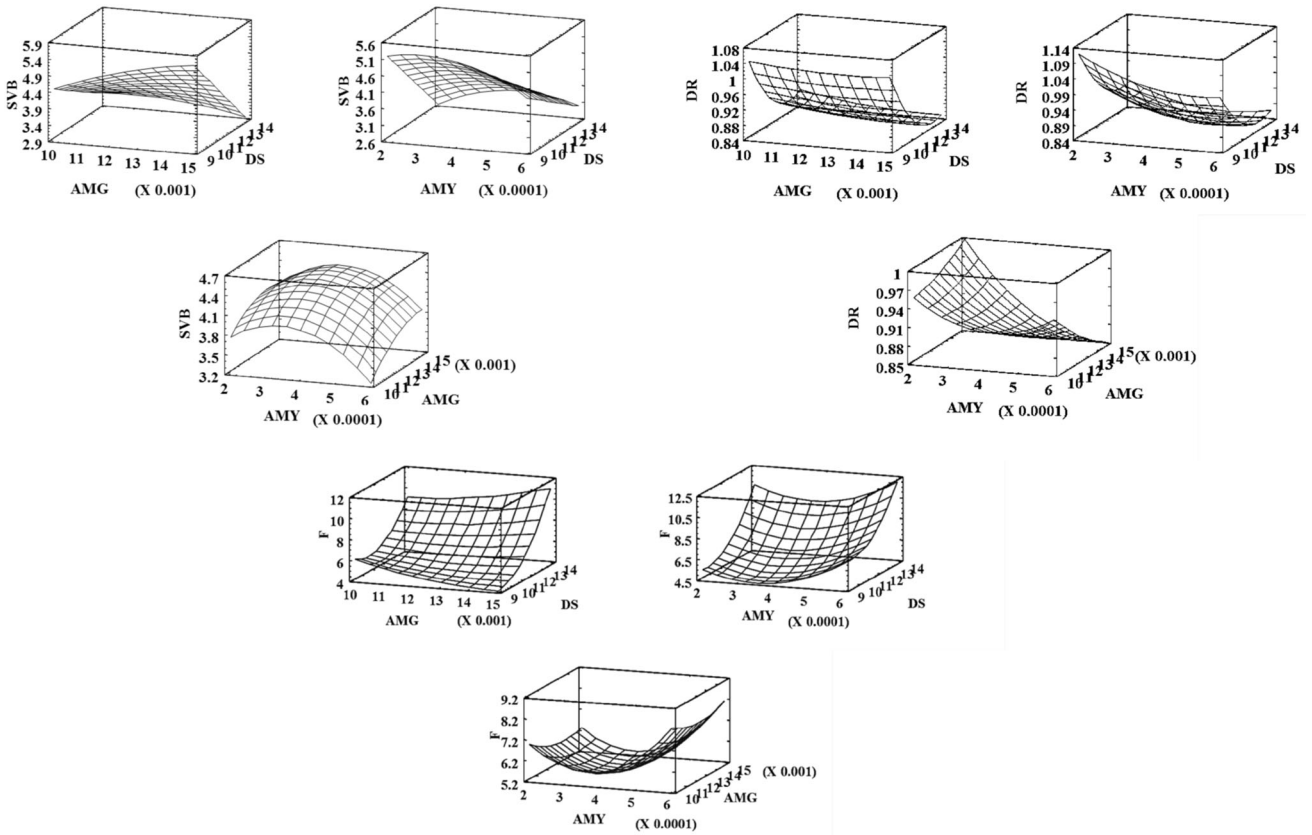

Fig. 1 Response surface plots of bread quality parameters. *DS* damaged starch, *AMY* α-amylase, *AMG* amyloglucosidase, *SVB* specific bread volume, *DR* dimension ratio, *F* firmness

intensity indicated that progressive increments in DS produced darker crusts. In relation to the bread crumb, higher DS proportions produced firmer crumbs, despite the increments recorded in the crumb area fraction occupied by gas cell.

As previously indicated, high DS levels modified the dough fermentation performance, which resulted in smaller bread pieces and firmer crumbs indicating lower backing performance. The bread darker crust (Maillard-reactions) observed could be associated to higher concentrations of

reducing sugars produced by enzymatic hydrolysis of the gelatinized DS granules, which have the ability to gelatinize spontaneously in cold water (Barrera et al. 2013, 2015; Tester 1997; Morrison et al. 1994). DS content significantly modified the response of most attributes of bread quality as expected. In the predictive equations, the contribution of DS variable had a greater impact on quality parameters compared to the effect of enzymes.

Relative contribution of enzymes

The α-amylase incorporation to bread formulation had a negative linear effect on DR and F and a positive quadratic influence on F (Fig. 1). Moreover, this enzyme showed negative quadratic influence on SVB (Fig. 1) and a* intensity parameter. These results suggested that gradual α-amylase increments reduced DR and crumb F attributes. The progressive addition of α-amylase was convenient until its concentration reached a critical level from which successive increases led to lower bread volume and red intensity and higher crumb firmness. At low α-amylase levels, DR and crumb F reductions could be associated to a larger production of fermentable sugars, mainly maltose and glucose (Barrera et al. 2015) derived from DS granules hydrolyzed, which is related to larger gas production during fermentation process and dough development (Kragh 2002; Linko et al. 1997). In addition, it is possible to suggest that granular degradation by α-amylase promoted a change in gluten hydration and plasticization during dough development, improving the elastic properties and carbon dioxide retention of dough (Martinez-Anaya and Jimenez 1997; Barrera et al. 2015). The reason behind the improvement observed from the use of this additive is not clear yet. Probably, it is attributed to large gas production during fermentation process or to changes in the dough rheological properties. Patel et al. (2012) have reported that fungal α-amylase incorporation in minimum dose is beneficial since it improves dough handling and reduces dough adhesiveness properties, leading to good quality bakery products. High α-amylase proportions had a negative impact on bread volume and crumb structure, probably due to excessive DS degradation caused by enzymatic action (Bowles 1996). In this study, the negative impact on bread volume and crumb structure was related to a combination of high DS and high α-amylase levels. It is suggested that the water released, as a consequence of a much higher number of DS granules hydrolyzed, probably promotes a change in the plasticization of the system, affecting negatively dough rheological properties (Barrera et al. 2015) and thus the product quality. Bread crust color was not significantly increased at high α-amylase levels despite the higher reducing sugars proportions in the system. Crust color is promoted by high temperatures (Maillard-reactions) and by dehydration (caramelization-reactions). Since the coloring rate is moisture dependent, possibly, dough pieces with lower development during fermentation and oven-spring might keep surface wetness producing less coloration.

Regarding the incorporation of amyloglucosidase, the result showed that it had a positive lineal influence on SVB and a* intensity parameter. However, a negative linear effect was recorded on L* and FD values. Thus, gradual amyloglucosidase additions produced higher bread volume and red intensity and darker crust and lower gas cell diameter. AMY*AMG interaction showed positive contribution to FD, and DS*AMG interaction had negative contribution to SVB. The improvement of bread volume as a consequent of gradual addition of amyloglucosidase could be related to its ability to produce glucose as a main product in DS degradation (Barrera et al. 2015; Diler et al. 2015). High glucose concentrations during fermentation process promote a better dough development, resulting in higher bread volumes. Regarding crust color, the darker crust obtained by higher levels of amyloglucosidase could be associated to high proportions of reducing sugars and more developed bread pieces. AMY*AMG positive interaction suggests that amyloglucosidase hydrolyzed the maltose produced by α-amylase (Fujii et al. 1988; Diler et al. 2015). Therefore, it is likely that part of the glucose and other reducing sugars are used during fermentation and consequently the promotion of a larger gas cell diameter was produced. The negative cross interaction between AMG*DS in bread volume suggests that the incorporation of amyloglucosidase is beneficial only in the presence of a limited quantity of DS in the flour. Likely, an excessive sugar production led to a deep and negative change in the dough rheological properties, as was described in a previous study (Barrera et al. 2015).

Validation of bread-quality predictive equations

Two new experiments were performed to analyze the predictive capacity of bread-quality equations. Each experiment was calculated considering simultaneously more than one quality attribute. The aim of *1-Experiment* was to determine DS and enzyme levels necessary to obtain the highest quality bread. On the other hand, the objective of *2-Experiment* was to determine the concentration of enzymes required to produce the highest quality bread from flour with high DS content (WF4 flour). The optimum variable levels are shown in Table 5. The Taking account of the variable ranges used for the analysis, results of *1-Experiment* suggested that the combination of flour with 9.8 % DS (WF2 flour), and the mixture of half AMY_{Max} doses and AMG_{Max} doses was the most convenient condition to obtain good quality bread. With regard to

Table 5 Predictive capacity of the regression equations of the quality parameters

Optimum variable combination			
Variables	Experiment		
	1	2	
DS (%)	9.8	14.4	
AMY*	0.0003	0.0004	
AMG*	0.014	0.0125	
Quality parameters	PV	EV	(EV−PV)/PV (%)
1-Experiment			
SVB (cm^3/g)	5.1	4.3	−16
DR	1.0	0.9	−12
L*	65.1	57.7	−11
a*	10.9	13.8	27
b*	31.2	34.5	11
F (N)	4.38	6.17	41
2-Experiment			
SVB (cm^3/g)	3.5	3.7	4
DR	0.9	1.0	12
L*	58.4	57.6	−1
a*	14.0	15.4	11
b*	32.9	36.0	9
F (N)	8.46	4.49	−47

* Enzymes: (enzyme g/100 g flour)

DS damaged starch, *AMY* α-amylase, *AMG* amyloglucosidase, *PV* predicted values, *EV* experimental values, *SVB* specific bread volume, *DR* dimension ratio, *F* firmness and bread crust color (L* a* b*, CIE-LAB)

2-*Experiment*, results indicated that the best combination of enzymes to attenuate the quality impairment caused by high DS proportions is a mixture of the intermediate concentration of AMY and AMG, according to the dose ranges established in each case. In both cases, baking tests were performed. The results demonstrate that the regression equations of the quality parameters evaluated allowed successfully prediction of bread quality attributes in both cases (Table 5). The crumb firmness was not efficaciously predicted (Table 5).

The combination of variables calculated from *1-Experiment* resulted in loaves pieces with higher SVB (∼28 %) and DR (∼18 %), lower F (∼31 %) and darker crust, comparing to the bread samples prepared from WF2 flour without enzymes (SVB = 3.7 cm^3/g; DR = 0.82; L* = 72.45; a* = 5.9; b* = 29.6; F = 14.13 N). Regarding to the *2-Experiment*, the combination of variables calculated produced bread pieces with higher SVB (∼29 %), similar DR (∼3 %), lower F (∼44 %) and darker crust, compared to the product obtained from WF4 flour without enzymes (SVB = 2.5 cm^3/g; DR = 0.93; L* = 72.0; a* = 5.5; b* = 29.4; F = 19.13 N). The finished products confirmed that the addition of α-amylase and amyloglucosidase significantly improved the quality of products made from flour with high DS level.

The storage effect on breads made following *2-Experiment* evaluated by crumb firmness as previously described. The crumb hardness of bread pieces stored was measured after 0, 1, 2, 3 and 8 days. During the storage period, crumb hardness increased ($p \leq 0.05$) in the control bread (0 days = 7.4 N and 8 days = 37.1 N) as well as in the bread made using and amyloglucosidase (0 days = 4.5 N and 8 days = 22.8 N), as expected. However, bread loaves without additives showed higher crumb firmness than that of the samples with enzymes. The addition of α-amylase and amyloglucosidase showed lower crumb firmness ($p \leq 0.05$) throughout the storage period, promoting a longer life of the finished product. In baked products it is well known that amylase addition retards the aging effect of products (Goesaert et al. 2005), in agreement with the results obtained. The mechanism by which these enzymes produce this beneficial effect is not clear yet, since this phenomenon has been attributed to dextrins derived from starch hydrolysis, which interfere with the re-association of amylose and amylopectin polymers; in other cases it has been associated with molecular changes of the starch polymers derived from enzymatic degradation (Goesaert et al. 2005).

Conclusion

Enzymes were beneficial to attenuate the negative effects caused by DS. The combination of α-amylase and amyloglucosidase improved dough performance during fermentation process and bread quality attributes. Lower α-amylase and higher amyloglucosidase doses showed better impact on bread volume and crumb structure. In general, the maximum amyloglucosidase concentration of 0.015 g/100 g flour showed favorable effects, whereas α-amylase concentrations lower than the average (0.0004 g/100 g flour) were more appropriate.

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