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Combinations of glucose oxidase, α-amylase and xylanase affect dough properties and bread quality

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Summary The objective of this work was to study the effects of the combination of glucose oxidase (Gox), α-amylase (AM) and xylanase (Xyl) on dough properties and bread quality, applying response surface analysis. Gox improved dough stickiness and bread crumb uniformity, but had a negative effect on specific volume. A positive synergist effect was observed combining Xyl and AM on specific volume and crumb firmness but this synergist effect was negative on crumb uniformity. Using the 'desirability function', two optimal formulations for the baking process were obtained. In the first optimisation, the combination of Gox 0.0026, Xyl 0.016 and AM 0.01 g per 100 g of flour will allow to obtain breads with high specific volume and low firmness. Second optimisation (Gox 0.0037, Xyl 0.0089 and AM 0.0105 g per 100 g of flour) will allow to obtain breads with 37% higher specific volume than control and, additionally, with low dough stickiness.

Keywords Bread quality, glucose oxidase, response surface analysis, xylanase, α-amylase.

Introduction

Enzymes are used in bread-making with the objective of optimising dough properties and improving the quality and conservation of the end product. Enzymes from animal and vegetal sources can be utilised in food processing. In several foods, it is necessary to add exogenous enzymes to produce chemical modifications that may improve the quality of the product. Enzymes have great specificity of action; temperature and pH easily inactivate the enzymes when the desired reactions have already occurred, being then assimilated as the rest of proteins by the organism. The main flour components that could be modified by enzymes are proteins, lipids, pentosans and starch. The glucose oxidase (Gox) enzyme (EC 1.1.3.4), in the presence of oxygen, catalyses the oxidation of α-D-glucose to α- D-gluconolactone and H_2O_2; the H_2O_2 oxidises thiol groups of gluten proteins forming disulphide bonds (Haarasilta & Pullinen, 1992); it also produces dityrosine cross-linking (Rasiah *et al.*, 2005) and the gelation of water soluble pentosans (Vemulapalli *et al.*, 1998). Several research groups observed an increase in dough tenacity and elasticity and a decrease in dough extensibility upon Gox addition (Martinez-Anaya & Jimenez, 1998; Primo-Martin *et al.*, 2003; Rosell *et al.*, 2003; Caballero *et al.*, 2007; Davidou *et al.*, 2008; Steffolani *et al.*, 2010). The effect of Gox on specific volume of bread has been controversial; while Caballero *et al.* (2007) and Dagdelen & Gocmen (2007) reported an increase in bread volume, Rasiah *et al.* (2005) and Steffolani *et al.* (2010) found no or a negative effect in this parameter upon Gox addition, and they also reported an improving effect on crumb texture and strength.

The α-amylase (AM) (EC 3.2.1.1) hydrolyses α-1,4 links of starch producing low molecular weight α-dextrins (Bowles, 1996). Amylases are routinely used in baking and their effect is explained in terms of an increase in fermentable and reducing sugar content (Bowles, 1996). Fermentable sugars are substrate for yeasts, thus increasing CO_2 production; and promoting Maillard reaction, intensifying bread taste, aroma and colour (Drapron & Godon, 1987; Bowles, 1996). Finally, amylases reduce the hardening rate of crumb delaying bread staling; the α-dextrins, products of starch degradation, interfere in amylopectin re-association and retrogradation (León *et al.*, 1997; Min *et al.*, 1998; Defloor & Delcour, 1999).

Endo-β-1,4-xylanases (EC 3.2.1.8.), also referred to as pentosanases, find widespread use in bread-making applications. Endoxylanase hydrolyses water insoluble arabinoxylans causing a decrease in the molecular weight of these polymers and turning them water soluble. Water soluble arabinoxylans have a positive effect on dough properties and bread quality (Jiménez & Martínez-Anaya, 2001) because they stabilise gas cells,

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improving its expansion capacity during proofing and baking and consequently improving bread characteristics, such as specific volume and firmness (Gan *et al.*, 1995; Steffolani *et al.*, 2010). On the contrary, endoxylanase has little or no effect on bread quality when it hydrolyses water soluble arabinoxylans, reducing their molecular weight (Courtin & Delcour, 2001). Although the individual and synergistic effect of Gox, AM and xylanase (Xyl) – in combinations of two enzymes – on wheat dough and bread has been studied, as far as we know, there is no information about the effects of the three enzymes combined on dough and bread properties.

The objective of this work was to study the effect of the combination of three enzymes [Gox, AM and Xyl] on dough properties and bread quality and to determine the optimum combination to improve dough and bread quality.

Materials and methods

Materials

Commercial wheat flour used for all experiments was provided by a local milling company (Molinos Campodónico Ltda., La Plata, Argentina). Wheat flour alveograph parameters were deformation energy (W) = 323×0^{-4} J, tenacity (P) = 101 mm, extensibility (L) = 89 mm, P/L = 1.13 (Method 54-30A; AACC International, 2000), moisture = 13.1 ± 0.2% and protein = 10.9 ± 0.3% (Kjeldahl method, $N \times 5.7$). Enzymes included Gox (Gluzyme mono BG from Novozyme, Bagsvaerd, Denmark) 100 000 U g^{-1} activity (one unit of Gox is defined as the amount of enzyme that oxidises 1 μ mol^{-1} of *o*-dianisidine per min at 25 °C), endo-β-1,4-xylanase (Pentopan mono BG from Novozyme), 2500 fungal xylanase units per gram activity (FXU-S/g) and fungal α-amylase (Novozyme) 10 000 skb.

Dough properties

Enzymes were added as described in Table 1. Basic dough formula (on flour basis) consisted of 1% (w/w) salt, 2.5% (w/w) sugar, 0.15% (w/w) sodium stearoyl-2-lactylate (SSL; Alpha Emulsionantes, Río Tercero, Argentina) and 58.5% (w/v) water (optimum level). Ingredients were mixed with a Philips HR 1495 high-speed mixer (Philips, Buenos Aires, Argentina) for 2 min and allowed to rest for 15 min in a fermentation cabinet at 30 °C and 70% rm (relative moisture). Three portions of rounded dough (10 g) were placed on a plate, pressed and rested for 45 min. Texture profile analysis (TPA) of three dough portions (15 mm thickness and 70 mm diameter, approximately) was performed using a TAXT2i Texture Analyser (Stable Micro Systems, Surrey, UK) equipped with a 25-kg load cell. A cylinder probe of 3.6 cm diameter was attached to a moving cross-head. The samples were subjected to a double cycle of compression under the following conditions: 1 mm s^{-1} cross-head speed and 60% maximum deformation. The texture profile parameters were determined using the Texture Expert 1.22 software (Stable Micro Systems). Dough hardness and stickiness were determined. Dough hardness is the maximum force of the first compression cycle from TPA curves, and stickiness (adhesiveness) is the negative force area of the first compression cycle or the work necessary to pull the plunger away from the sample.

Table 1 Rotatable central composite design for the optimisation of Gox, Xyl and AM addition

Assay	Gox (g per 100 g of flour)	Xyl (g per 100 g of flour)	AM (g per 100 g of flour)
1	0.0050	0.0180	0.0160
2	0.0027	0.0105	0.0000
3	0.0027	0.0231	0.0100
4	0.0050	0.0030	0.0040
5	0.0003	0.0180	0.0160
6	0.0027	0.0000	0.0100
7	0.0027	0.0105	0.0100
8	0.0003	0.0030	0.0040
9	0.0027	0.0105	0.0100
10	0.0000	0.0105	0.0100
11	0.0050	0.0030	0.0160
12	0.0066	0.0105	0.0100
13	0.0050	0.0180	0.0040
14	0.0003	0.0030	0.0160
15	0.0027	0.0105	0.0100
16	0.0003	0.0180	0.0040
17	0.0027	0.0105	0.0100
18	0.0027	0.0105	0.0201

Gox, glucose oxidase; Xyl, xylanase; AM, α-amylase.

Bread-making procedure

Bread-making tests were performed using the mould bread methodology used in wheat breeding programmes, according to IRAM 15858-1 (IRAM, 1996) with minimal modification in the baking time. Dough formulation was prepared following the formulation indicated previously (flour basis), with 3% (w/w) compressed yeast (Calsa, Lanús, Argentina). Ingredients were mixed 12 min in an Argental L-20 mixer (Argental, Rosario, Argentina). The resulting dough (~27 °C) was proofed (96% rm) for 80 min at 30 °C (first proof) with two intermediate punches (partial loss of gas to the atmosphere) at 45 min and at 60 min. The bulk dough was then sheeted in a Mi-Pan vf roller (Mi-Pan, Córdoba, Argentina) and divided into 130 g pieces, moulded into a loaf shape (Braesi MB 350, Brazil), panned (dimensions 15 × 8 × 6 cm) and returned to the

fermentation cabinet for 75 min (second proof). After this process, dough was baked at 215 °C for 24 min in a rotational gas oven (Ciclo Ingeniería, Buenos Aires, Argentina). Two hour after baking, the loaves were weighed and bread volume was determined according to AACC Approved Method 10-05 (AACC International, American Association of Cereal Chemistry, 2000) procedure. Specific volume of bread (SVB) was expressed as the volume/weight ratio of finished bread.

Crumb texture profile analysis

Texture profile analysis was performed by using a TA.XT2i Texture Analyser (Stable Micro Systems) equipped with a 25-kg load cell. A cylinder probe of 2.5 cm diameter was attached to a moving cross-head. Two hours after baking, the bread loaves were cut and two slices (2.5 cm thick) were subjected to a double cycle of compression under the following conditions: 1 mm s^{-1} cross-head speed and 40% maximum deformation. The texture profile parameters were determined using the Texture Expert 1.22 software (Stable Micro Systems). Bread crumb firmness and chewiness were determined. Firmness is defined as the force required to compress the bread slice to 40% of its initial thickness (Method 74-09; AACC International, American Association of Cereal Chemistry, 2000). Chewiness (firmness × cohesiveness × springiness) is defined as the force required to disintegrate the solid food until it is swallowed (Civille & Szczesniak, 1973).

Crust colour

Bread crust colour was determined according to AACC Approved Method 14-22 (AACC International, 2000) using a Minolta 508d spectrophotometer with 8-mm measurement aperture and D65 illuminant at 10° angle of observer. Crust colour was measured on the top of each bread sample. At least four readings were taken from each bread sample, and four bread pieces from each test point were analysed. Colour parameters were recorded as CIE-LAB, L^* (lightness) values.

Crumb structure

For each bread loaf, two slices were obtained from the central region and scanned (HP Scanjet G3010, Palo Alto CA, USA). JPEG images were analysed using image analyser software (Image J 1.38n; National Institute of Health, Bethesda, MD, USA). Colour images were converted to eight bits 256 grey level images. A single field of view (FOV) was evaluated for each image. The FOV captured the majority of the crumb area of each slice. Images were taken from the centre of the slice, and the rectangular FOV size was selected to exclude the crust and 1–1.3 cm of the crumb placed next to the crust. Crumb images were considered to contain grey level information from pixels of which the darkest individuals belong to cell and the brightest belong to cell wall. The segmentation method (conversion to a binary image) of the 256 grey level digital images was used to extract coherent information from raw image data. Each bread slice was evaluated by IsoData algorithm (software Image J 1.38n; National Institute of Health) and an optimum grey level threshold to divide images into regions of cells and surrounded cell wall material was obtained. The segmented images, where pixels with a grey level higher than the threshold value were associated with the air cells, while pixels with a grey level lower than the threshold value were associated with the wall cells, were analysed. The crumb cell features chosen were the total number of cells, total cell area, mean cell area and cell to total area ratio (cell fraction). Crumb uniformity (CU) was determined as the ratio of small to large cells counts (i.e. the ratio of the number of small cells to large ones, 4 mm^2); higher values indicate greater uniformity of crumb grain (Zghal *et al.*, 2001).

Experimental design

The effect of combination of enzymes on bread quality and dough properties was studied by means of a response surface regression method. The following independent variables were selected: Gox 0.0003–0.005 g per 100 g of flour, Xyl 0.003–0.018 g per 100 g of flour, AM 0.004–0.016 g per 100 g of flour. The enzyme levels were selected according to manufacturer recommendations and previous publications (Primo-Martin *et al.*, 2003; Rasiah *et al.*, 2005; Bonet *et al.*, 2006; Caballero *et al.*, 2007; Steffolani *et al.*, 2010). A rotatable central composite design was generated using Statgraphics plus 5.0 (Statpoint Technologies Inc., Warrenton, VA, USA); it was constituted by three factor combinations and five substitution levels of each factor. The experimental design is presented in Table 1. The experiment order was randomly selected to avoid the effect of a hidden variable. Four replicates at the central point made it possible to estimate the pure error of the analyses. The results were analysed by means of multiple regression method. The fitness model quality was evaluated by ANOVA (Statgraphics plus 5.0; Statpoint Technologies Inc.), and the determination coefficient R^2 was obtained to fit each model of experimental data. Multiple regression equation was developed only with significant coefficients ($P < 0.05$). Tri-dimensional response surface plots were generated by each quality parameter. The optimal enzymes combination for bread-making was determined using a multiple response method called 'desirability' (Ferreira *et al.*, 2007). This optimisation method incorporates desires and priorities for each of the variables. The desirability function approach is one of the most frequently used multi-

response optimisation techniques. The desirability lies between 0 and 1 and it represents the closeness of a response to its ideal value. If a response falls within the unacceptable intervals, the desirability is 0, and if a response falls within the ideal intervals or the response reaches its ideal value, the desirability is 1. The proposed desirability function transforms each response to a corresponding desirability value between 0 and 1. All the desirability can be combined to form a composite desirability function that converts a multi-response problem into a single-response one. The desirability function is a scale invariant index that enables quality characteristics to be compared with various units (Raissi & Eslami Farsani, 2009).

Results and discussion

Effect of combination of enzymes on bread dough properties

Experimental responses of dough rheological properties are presented in Table 2. The hardness is a measure of the resistance of dough to deformation. In general, dough with high levels of Gox (0.0050 and 0.0066 g per 100 g of flour) and low levels of Xyl (0.003 g per 100g of flour) and AM (0.004 and 0.010 g per 100 g of flour) presented high hardness. Gox had a linear positive effect on dough hardness, while Xyl and AM had a negative linear effect (R^2 = 0.87) (Table 3). This result is in agreement with Bonet *et al.* (2006) and Steffolani *et al.* (2010), who observed that high levels of Gox caused a high cross-linking of gluten proteins, so dough became strong and resistant to extension. On the contrary, a gradual decrease in hardness was observed when Xyl or AM increased but this effect was not significant when the enzymes were combined (Fig. 1). The dough softening effect of these enzymes could be positive, depending on bread-making conditions. The negative effect of Xyl on dough hardness could be due to the hydrolysis of

Table 3 Significant coefficients of the regression design fitting model for dough rheological properties

Factor	Hardness (g)	Stickiness (g s)
Constant	3538.00	3289.49
A	1.09E+05	−1.71E+05
B	−3.87E+04	3.45E+04
C	−8.44E+04	n.s.
AA	n.s.	n.s.
AB	n.s.	n.s.
AC	n.s.	n.s.
BB	n.s.	n.s.
BC	n.s.	n.s.
CC	n.s.	n.s.
R^2	0.87	0.94

n.s., no significant effect at level ($P < 0.05$); R^2, adjusted square coefficient of the fitting model (indicates the percentage of variability for which the model accounts); A, glucose oxidase; B, xylanase; C, α-amylase.

Table 2 Experimental responses of bread quality and dough parameters

	Bread quality parameters					Dough parameters	
Assay	SVB ($cm^3\ g^{-1}$)	Firmness (g)	Chewiness (g)	*L**	CU	Hardness (g)	Stickiness (g s)
1	5.59	147	180	49.9	6.51	2703	3250
2	5.50	162	185	59.1	5.85	3573	3533
3	5.97	135	166	49.0	8.81	2350	5640
4	4.68	207	198	54.2	7.37	4060	1814
5	6.38	108	138	49.2	5.74	2577	4821
6	5.71	127	165	54.7	6.30	4015	2446
7	6.33	102	138	52.3	7.65	2760	3073
8	5.76	130	171	55.5	6.13	3263	2895
9	6.32	101	138	51.6	7.65	2784	3100
10	6.43	103	141	50.2	6.77	2676	4518
11	4.64	261	174	54.8	10.48	4543	1563
12	4.50	302	199	56.2	9.72	3743	1399
13	4.23	338	216	56.0	12.87	4096	2459
14	5.56	131	179	51.1	5.53	2659	3479
15	6.23	112	160	52.4	7.40	2777	3018
16	5.55	140	191	54.4	6.73	3189	4965
17	6.25	109	151	51.6	7.02	2793	3015
18	5.90	151	198	48.3	6.44	2784	3760

SVB, specific volume of bread; texture parameters of bread crumb, firmness and chewiness; *L**, Bread crust luminosity (CIE-LAB); CU, uniformity (relationship between number of cells smaller than 0.04 mm^2 and the number of cells larger than 0.04 mm^2); hardness, dough hardness; stickiness, dough stickiness.

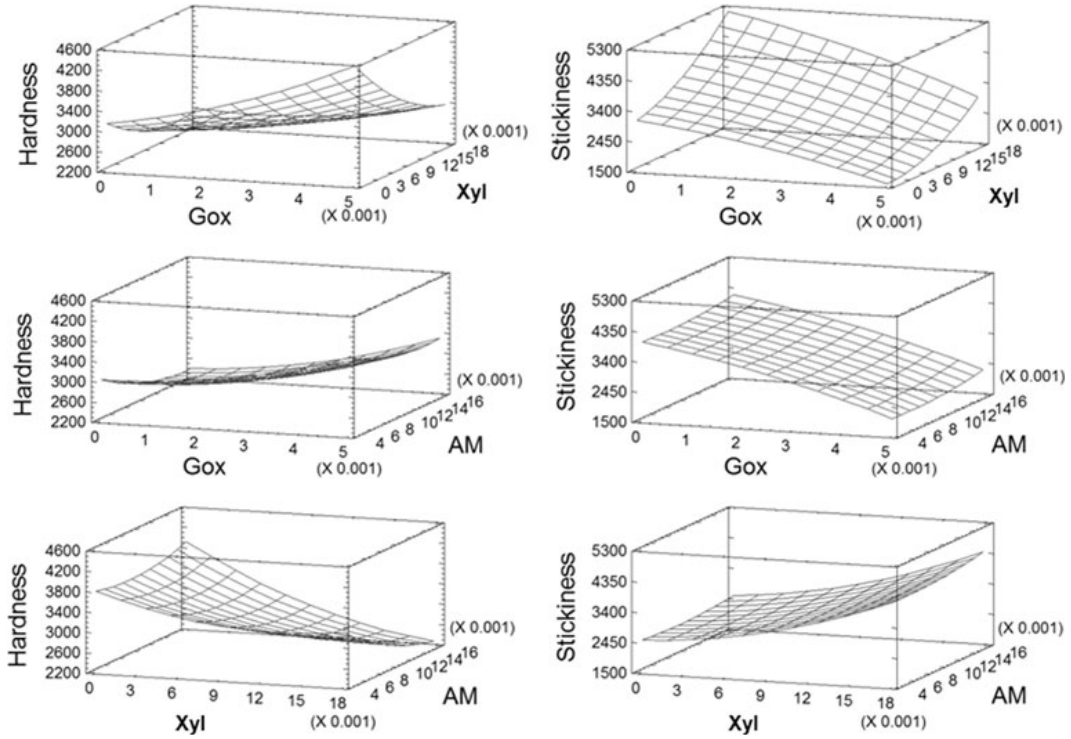

Figure 1 Response surface plots of dough textural parameters. Hardness (g) and stickiness (g. s). Gox, glucose oxidase; Xyl, xylanase; AM, α-amylase.

water insoluble pentosans, reducing the steric impediment, thus modifying the interaction among proteins, water and pentosans. Likewise, the AM negative effect could be due to the increase in starch hydrolysis by enzyme action.

The high stickiness causes malleability problems during the dough developing process. However, dough with very low stickiness can be very dry and hard. Therefore, bread dough must have an appropriate stickiness providing good performance during the baking process, particularly in automated bread-making processes. Xyl had a linear positive effect on dough stickiness, whereas Gox had a negative linear effect (R^2 = 0.94) (Table 3). In the testing where the formulation contained a high Xyl content, the resulting dough showed high stickiness value (Fig. 1). High levels of Xyl led to an excessive pentosan hydrolysis (water soluble and insoluble) causing negative effects on bread dough (Courtin *et al*., 1999; Courtin *et al.,* 2001). On the contrary, high Gox levels noticeably reduced stickiness resulting in hard and dry dough (Vemulapalli *et al*., 1998) because of the oxidative gelation of soluble pentosans. Dough with intermediate stickiness values (between 2500 and 3000 g s) were obtained by combining different Xyl and Gox levels independently of AM level (Table 2). No significant increase in dough stickiness was observed by AM presence (Fig. 1). This result could be due to the presence, in the same formulation, of Gox, which has an opposite effect to AM.

Effect of combination of enzymes on bread quality parameters

It is widely accepted that the main quality parameters of mould bread are a high specific volume and a soft and uniform crumb structure. Therefore, the objective was to maximise the response of specific volume and crumb uniform of bread and to minimise the response of crumb firmness and chewiness. For each group of response, a quadratic equation was generated with significant coefficients ($P < 0.05$) to obtain a R^2 value as high as possible. Based on these equations, the response behaviour can be predicted and presented as a response surface.

Bread quality parameters are presented in Table 2. Figures 2 and 3 show response surface plots of specific volume and crumb firmness, chewiness and uniformity. The determination coefficient, R^2, explained the 97.2%, 91.8%, 83.5% and 74.9% of data variability, respectively. In the present study, the specific volume of control bread (without enzyme addition) was 4.38 $cm^3\ g^{-1}$. Almost all tested enzyme combinations presented higher specific volume than control bread, however, assays no. 11, 12 and 13 (including high Gox

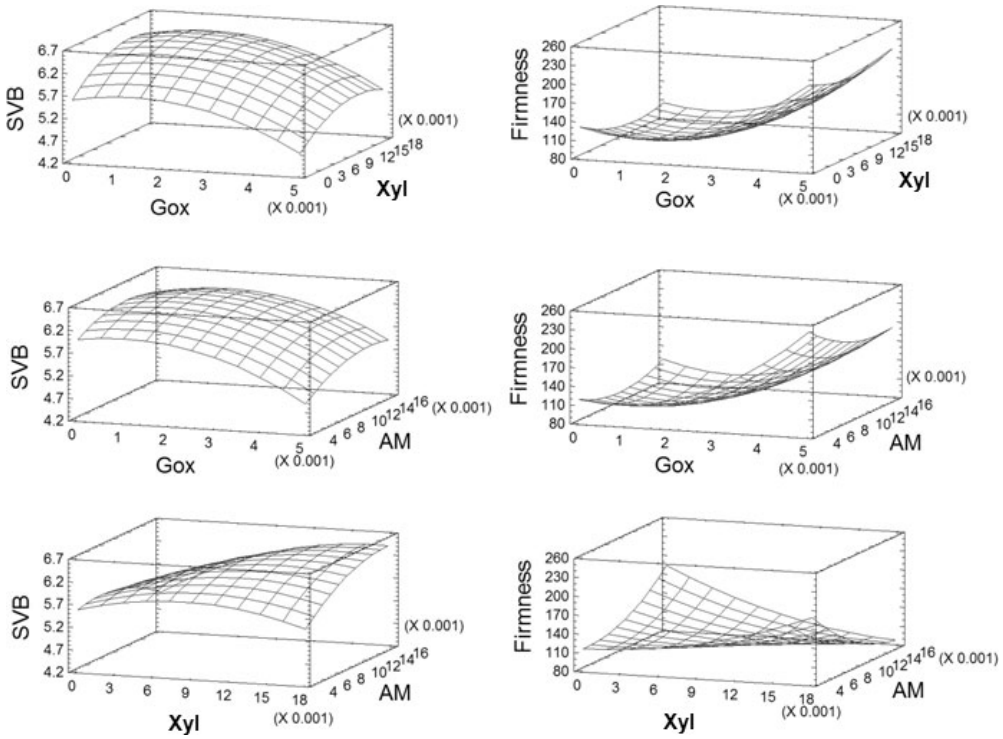

Figure 2 Response surface plots of specific volume of bread (SVB, $cm^3\ g^{-1}$) and crumb firmness (g). Gox, glucose oxidase; Xyl, xylanase; AM, α-amylase.

content), presented similar specific volume to control bread. It has been reported that Gox increases dough strength, which is useful when using poor quality flour. Nevertheless, when good quality flour is used – such as those found in Argentina – an over-reinforcement of dough is observed after Gox addition. This effect hampers expansion during proofing and, consequently, negatively affects bread volume (Rosell *et al*., 2003; Steffolani *et al*., 2010). On the other hand, low Gox content combined with other enzymes, such as Xyl and AM, allowed the development of high specific volume of bread (assays no. 3, 5 and 7). These results are similar to those presented by Caballero *et al*. (2007); these authors informed significant synergist effect on bread quality when Gox and Xyl were combined. Although gelation of water soluble arabinoxylans promoted by Gox may negatively affect bread quality, the generation of small ferulic acid containing arabinoxylan fragments by Xyl and the subsequent interference action of those in the formation of new arabinoxylan cross-links by Gox has been recently proposed as a theory for justifying this synergistic effect (Primo-Martin *et al*., 2005). The significant regression coefficients of different variables are presented in Table 4. All enzymes had a positive linear effect and negative quadratic effect on specific volume of bread. Moreover, a positive contribution of the interaction between Xyl and AM was observed ($R^2 = 0.97$). These results indicated that although the rise of each enzyme content increased specific volume of bread, this increase was up to an optimum level (of each enzyme) (Fig. 2), because over these levels, the specific volume decreased.

Texture evaluation is very important because it is directly associated with consumer acceptance. Therefore, all factors that contribute to bread texture have an impact on sensorial perception (or at the moment of consumption). Generally, crust and crumb texture is analysed. In fresh bread, the crust is generally hard but brittle, so it has to be crispy (when masticated) (Cauvain & Young, 2000). On the contrary, bread crumb have a distinct aerated porous structure, and all air cells (alveolus) are interconnected by means of a solid matrix constituted by gluten and starch (Cauvain & Young, 2000). The fresh bread crumb are characterised as spongy and soft. The results obtained in 18 assays of crumb texture profile analysis are presented in Table 2. The control bread (without enzyme) presented high firmness and chewiness (328 and 277 g, respectively). The enzymes incorporation significantly modified crumb texture. Considering all studied bread formulation, the combination of Gox 0.0027, Xyl 0.0105 and AM 0.01 g per 100 g of flour presented the lowest crumb firmness and chewiness. These intermediate levels of the three enzymes (Gox, Xyl

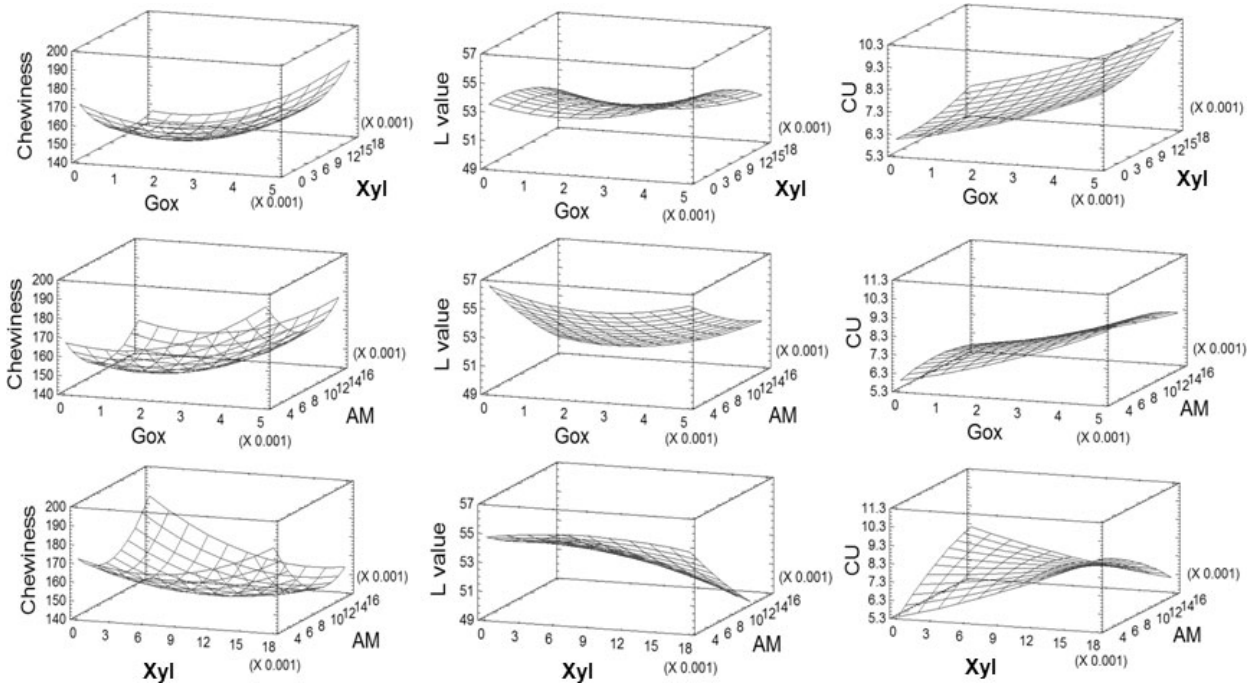

Figure 3 Response surface plots of bread crumb chewiness and uniformity (CU, relationship between number of cells smaller than 0.04 mm^2 and number of cells larger than 0.04 mm^2) and crust bread luminosity (L^* values). Gox, glucose oxidase, Xyl, xylanase; AM, α-amylase.

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

Factor	SVB (cm^3 g^{-1})	Firmness (g)	Chewiness (g)	L^*	CU
Constant	5.42	101.76	201.81	57.81	2.23
A	137.79	−1.04E+04	−7205.86	n.s.	442.64
B	51.88	n.s.	n.s.	216.79	n.s.
C	89.98	n.s.	n.s.	−536.33	n.s.
AA	−7.52E+04	7.55E+06	1.92E+06	1.79E+05	n.s.
AB	n.s.	n.s.	n.s.	n.s.	n.s.
AC	n.s.	n.s.	n.s.	n.s.	n.s.
BB	−4641.86	n.s.	n.s.	n.s.	n.s.
BC	6750.00	−7.76E+05	n.s.	n.s.	−2.74E+04
CC	−7234.29	5.49E+05	4.50E+05	n.s.	n.s.
R^2	0.97	0.92	0.84	0.88	0.75

n.s., no significant effect at level ($P < 0.05$); R^2, adjusted square coefficient of the fitting model (indicates the percentage of variability for which the model accounts); SVB, specific volume of bread; texture parameters of crumb bread, firmness and chewiness; L^*, bread crust luminosity (CIE-LAB); CU, uniformity (relationship between number of cells smaller than 0.04 mm^2 and the number of cells larger than 0.04 mm^2); A, glucose oxidase; B, xylanase; C, α-amylase.

and AM) were a good combination to improve bread texture.

Glucose oxidase had a negative linear effect and positive quadratic effect on firmness and chewiness of bread crumb ($R^2 = 0.92$ and 0.84, respectively) (Table 4). These results could be indicating that when low levels of Gox are added, the textural properties of crumb are improved, while high levels of Gox lead to an increase in firmness and chewiness (Figs 2 and 3). Changes in gluten protein, cross-linking and polymerisation modified crumb structure and texture (Oates, 2001). Gox promoted a higher interaction among gluten protein that contributed to the formation of a closed and dense bread crumb.

α-Amylase had a quadratic positive effect on crumb firmness, while the results also showed an interaction between AM and Xyl (significant synergist effect, $R^2 = 0.92$) (Table 4). At a given Gox concentration, crumb firmness decreased when Xyl and AM levels increased simultaneously (Fig. 2). The crumb characteristic of fresh bread is related to crumb moisture content after baking and amylose retrogradation (Eliasson & Larsson, 1993). During baking, starch gelatinisation and pasting and heat-setting of gluten proteins occur, resulting in the typical solid foam structure of baked bread (Hoseney *et al*., 2008). Therefore, the bread crumb are composed of two networks: the continuous and permanent gluten network, which constitutes a matrix between gelatinised and swelled starch granules; and the polymer network of gelatinised starch. The crumb firmness is strongly related to the structure of the gas cell walls and depends mainly on the number and size of the cells. The high firmness of samples with Gox could result from the presence of a higher number of gas cells (approximately 65 cell cm^{-2}) of smaller size (approximately 1.5 mm^2) surrounded by a thick solid matrix with little aeration.

The AM and Xyl combination had a negative effect on crumb firmness ($R^2 = 0.92$) (Table 4). In Fig. 2, it can be observed that at a given Gox concentration, when the levels of AM and Xyl are increased simultaneously in the bread formulation, crumb firmness decreases. The presence of AM produced reducing sugars that promote a higher CO_2 production by yeast and, in consequence, the high gas pressure caused cell expansion. Smaller pentosans produced by Xyl are located around cell walls reinforcing them, and thus avoiding gas loss (Gan *et al*., 1995; Jiménez & Martínez-Anaya, 2001). Therefore, the combination of AM with Xyl allowed a large dough expansion during proofing without CO_2 loss and highly aerated breads, with large air cell and soft crumb.

Samples with high Xyl and AM contents and low Gox level content had the best textural characteristics. The AM and Xyl enzymes are known to decrease crumb firmness (León *et al*., 1997, 2002; Courtin *et al*., 1999; Courtin *et al.,* 2001). However, improved crumb chewiness and firmness is related to the increase in specific volume of bread. Previous work (Every *et al*., 1998) showed an inverse relationship between specific volume and crumb firmness of bread. In this work, similar results were found, as the Pearson's correlation coefficient between SVB and firmness was $r = -0.92$ ($P < 0.05$).

Crust colour is an important attribute of bread that influences consumer preference. In general, bread crust is characterised by low moisture and dark colour. The colour is produced by chemical reactions, such as Maillard and caramelisation reactions. Breads formulated with high AM content had low L^* values, indicating the formation of darker crust. AM had a negative linear effect ($R^2 = 0.88$) (Table 4) on bread crust colour, indicating that the enzyme promoted the formation of sugars available for Maillard reactions; in consequence, breads turned out to be darker with increasing enzyme level in formulation (Fig. 3). Usually, the consumer prefers breads with an intermediate colour crust, between light and dark brown. However, the effect of AM on crust colour is usually compensated by improving the aroma and taste, as a result of Maillard reaction products. Figure 3 shows that the intense brown colour caused by AM increased when a high Xyl content was also present and decreased with high Gox content; nevertheless, this tendency was not significant because enzymes interaction was not observed for L^*. In the first case, Xyl hydrolysed pentosans releasing reducing sugars that are used as substrates in the Maillard reactions. Additionally, the Xyl decreased pentosan size in dough and produced water redistribution. This turned dough surface sticky (Steffolani *et al*., 2011) and led to a drier surface during baking, favouring Maillard reactions. On the contrary, Gox strengthened the gluten network promoting water retention during baking, which is unfavourable for Maillard reactions. In addition, Gox had positive quadratic effect on L^* indicating that Gox decreased the crust luminosity up to a certain optimal value and then began to increase.

Glucose oxidase had a linear positive effect on crumb uniformity ($R^2 = 0.75$) (Table 4), while it had a negative effect on SVB, which indicates that as Gox level increased, crumb uniformity was higher (CU = 10.48 to assay no. 11) and specific volume of bread, lower (SVB = 4.64 g cm^{-3} to assay no. 11). Gox addition to dough produced from strong flours, as used in this study, promoted a positive effect on crumb uniformity, while a negative one on bread volume. Ribotta *et al*. (2008) observed similar result, but instead of Gox, these authors used transglutaminase (TG) as dough strengthening enzyme in their work. The effects caused by Gox and TG on gluten network justified the information provided by Zghal *et al*. (2000), who stated that excessive breakdown of cell walls and gas cell coalescence, associated with weaker dough, leads to coarse and nonuniform crumb grain.

Xylanase and AM combination had a negative effect on crumb uniformity ($R^2 = 0.75$) (Table 4). Figure 3 shows that, when AM and Xyl were combined in the formulation (at a given concentration of Gox), uniformity only increased up to a certain optimal value and then began to decrease.

Kamman (1970) postulated that the visual and physical structures of bread crumb are quality factors related to each other and should be regarded as a single entity. In addition, the author supported that the nature of bread crumb is largely determined by wall thickness, size and uniformity of gas cell. Results obtained in this work

showed that crumb uniformity positively correlated with crumb firmness ($r = 0.81$; $P < 0.05$). Also, specific volume of bread correlated inversely with uniformity ($r = -0.65$; $P < 0.05$). The enzyme combination that produced breads with high volume and low firmness also led to lower crumb uniformity; so the development of high bread volumes was detrimental for crumb uniformity. Gox mainly affected crumb uniformity; enzyme activity caused a high cross-linking degree between dough proteins, leading to the formation of crumbs with small, uniformly distributed gas cells, with thick walls; these breadcrumbs characteristics led to an increase in firmness.

Figure 4 presents bread slices images of control (without enzyme) and most significant assays (no. 9, 10, 14 and 16). It can be observed from Fig. 4 that control bread presented cells uniformly distributed and thick cell walls, which could be related to its low SVB. Assays no. 14, 16, 9 and 10 show that as the enzyme is added in the formulation, bread volume increased and crumb had larger cells which were not uniformly distributed, so crumb structure was more aerated and the cell walls were thinner.

Optimisation

In the first optimisation, we tried to maximise specific volume and crumb uniformity and to minimise crumb firmness and chewiness, simultaneously. Results indicated that the combination of Gox 0.0026, Xyl 0.016 and AM 0.01 g per 100 g of flour will allow to obtain a bread with 43% higher specific volume than control (without enzyme) and with a uniform and soft crumb (Table 5). The synergist effect of AM and Xyl would mainly contribute to an increase in specific volume and a decrease in crumb firmness, while Gox would be responsible for improving crumb uniformity.

At the industrial level, mould bread is mainly made through a line of fully automated processes. Therefore, high dough stickiness is a disadvantage for bread preparation. Thus, in the second optimisation, we tried to maximise specific volume and to minimise crumb firmness, chewiness and dough stickiness. The combination of Gox 0.0037, Xyl 0.0089 and AM 0.0105 g per 100 g of flour will result in doughs with low stickiness and a bread with 37% higher specific volume than control (Table 5). In this case, it was found that by adding lower contents of Xyl and higher contents of Gox, breads with slightly lower volume than the first optimisation but with convenient dough stickiness for process development, could be obtained.

Table 5 Expected response values for each optimisation

Optimisation 1		Optimisation 2	
Response variable	**Expected value**	**Response variable**	**Expected value**
SVB ($cm^3\ g^{-1}$)	6.27	SVB ($cm^3\ g^{-1}$)	6.00
Firmness (g)	114.20	Firmness (g)	135.45
Chewiness (g)	150.74	Chewiness (g)	153.06
CU	7.91	CU	7.95
		Stickiness (g s)	2583.29

SVB, specific volume of bread; texture parameters of bread crumbs, firmness and chewiness; CU, uniformity (relationship between number of cells smaller than 0.04 mm^2 and the number of cells larger than 0.04 mm^2); stickiness, dough stickiness (g s).

Conclusion

The effects of combination of Gox, AM and Xyl on dough properties and bread quality were studied using response surface methodology. The prediction models developed with multiple regression method had high R^2 (above 0.70) for all dependent variables. Addition of Gox significantly decreased specific volume and increased both firmness and chewiness. However, Gox had a positive effect on uniformity of bread crumb. The AM negatively affected dough hardness and bread crust luminosity, but it increased specific bread volume. Xylanase was the factor which most improved specific bread volume and crumb firmness, although it greatly increased dough stickiness. Therefore, in practice, appropriate combinations levels of Gox – AM – Xyl can be used as improvers in breadmaking. In conclusion, formulation optimisations showed that combination of intermediate levels of the three enzymes results in dough with low stickiness and a

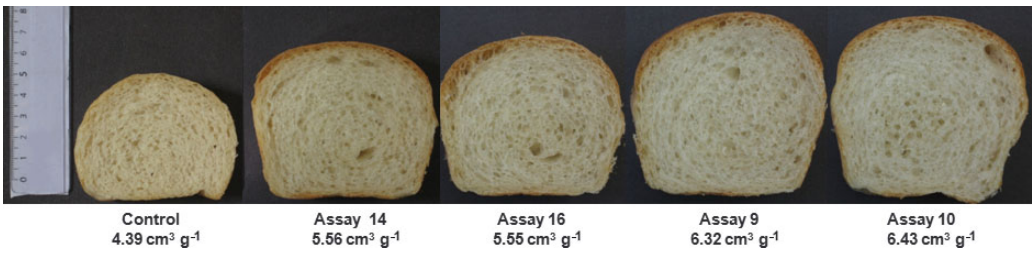

Figure 4 Representative images of bread slices. Presented values correspond to specific volume of bread ($cm^3\ g^{-1}$). Control: without enzyme. Assay number corresponds to the numbers presented in Table 1.

bread with ≈40% higher specific volume than control (without enzyme) and with a uniform and soft crumb but consumer acceptance test will be needed to confirm the desirable bread quality.

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