Synergistic effect of *Aspergillus tubingensis* CTM 507 glucose oxidase in presence of ascorbic acid and alpha amylase on dough properties, baking quality and shelf life of bread

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Abstract The impact of *Aspergillus tubingensis* glucose oxidase (GOD) in combination with α -amylase and ascorbic acid on dough properties, qualities and shelf life of bread was investigated. Regression models of alveograph and texture parameters of dough and bread were adjusted. Indeed, the mixture of GOD (44 %) and ascorbic acid (56 %) on flour containing basal improver showed its potential as a corrective action to get better functional and rheological properties of dough and bread texture. Furthermore, wheat flour containing basal additives and enriched with GOD (63.8 %), ascorbic acid (32 %) and α -amylase (4.2 %) led to high technological bread making parameters, to decrease the crumb firmness and chewiness and to improve elasticity, adhesion, cohesion and specific volume of bread. In addition to that, the optimized formulation addition significantly reduced water activity and therefore decreased bread susceptibility to microbial spoilage. These findings demonstrated that GOD could partially

substitute not only ascorbic acid but also α -amylase. The generated models allowed to predict the behavior of wheat flour containing additives in the range of values tested and to define the additives formula that led to desired rheological and baking qualities of dough. This fact provides new perspectives to compensate flour quality deficiencies at the moment of selecting raw materials and technological parameters reducing the production costs and facilitating gluten free products development.

Keywords *A. tubingensis* glucose oxidase · Ascorbic acid · α -amylase · Mixture design · Bread quality · Bread shelf life

Introduction

Recently, the bread-making industry has undergone several changes in its productive processes. These changes were brought about by an increasing mechanization in its manufacturing operations. This fact led to an increase in the demand for strong wheat flours, yielding doughs with a high tolerance for mixing and an ability to remain stable during fermentation (Caballero et al. 2007a, b). Functional flour properties depend strongly on the gluten proteins. In fact, the quality of gluten is dependent on diverse factors such as growing conditions and wheat variety (Elpidio et al. 2005). For this reason, the capacity of some countries to produce high-flour quality is limited. Indeed, flour treatment with functional additives must be considered. There are several additives used in bakery to compensate flour quality deficiencies (Roccia et al. 2012).

In bread making, both chemical and enzymatic improvers are frequently used to enhance the technological properties of dough and sensory quality of bread, and to guarantee an industrial production with constant quality (Faccio et al. 2012).

Highlights The impact of glucose oxidase, ascorbic acid and α -amylase on bread-making was investigated.
A mixture design was applied to optimize additives formulation content. Two additives formulations were obtained for high bread quality. These optimized conditions allow a best dough behavior and bread quality. Optimized formulation led to reduce the bread microbial spoilage and increase bread shelf life.

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Chemical agents, such as ascorbic acid, are widely used as flour improvers in bread products. It is acknowledged that ascorbic acid strengthens the gluten matrix by increasing the density of disulfide bonds in gluten (Aamodt et al. 2003; Koehler 2003) which leads to higher loaf volumes (Decamps et al. 2014). Indeed, amylases are routinely added to wheat flours as standardizing and antistaling agents to retard crumb hardening caused by rearrangements in the starch network, to optimize the falling number and to change water distribution (Mäkinen and Arendt 2012). α -amylase (EC 3.2.1.1) hydrolyses α -1,4 links of starch producing a small dextrin allows the yeast to work continuously during fermentation and produces CO_2 gas serving to improve volume and crumb texture in the final bread product (Shafisoltani et al. 2014). The fermentable sugars liberated by these enzymes enhance Maillard reactions that are responsible for the browning of the crust and the development of an attractive flavor of the baked bread (Whitehurst and Oort 2010). For instant, glucose oxidase (GOD) has been claimed to improve dough handling, dough tolerance, crumb structure, and loaf volume as well as to promote the development of less extensible and more resistant dough when added to wheat flour (Bonet et al. 2006; Davidou et al. 2008; Steffolani et al. 2010; Piedra et al. 2010). Naturally, glucose oxidase enzyme (EC 1.1.3.4), in the presence of oxygen, catalyses the glucose oxidation to form gluconic acid and hydrogen peroxide (H_2O_2). Several researches reported that the liberated hydrogen peroxide was responsible for GOD action by promoting different reactions in wheat dough. H_2O_2 causes the oxidation of the free sulfhydryl units from gluten protein producing disulfide linkages which result in stronger dough and may also provoke cross-linking of arabinoxylans. It is worth highlighting that the effect of various additives is strongly dependent on the nature and quantity of the used additives. However, bread additives may also have a negative impact on baking quality if not correctly dosed (Mäkinen and Arendt 2012). The choice of additives level is an important key in the baking process. Thus, it is very difficult to predict the real effect of each additive on different formulations. The use of statistical mixture designs using an individual additive or formulations containing binary or ternary mixtures of various additives for the improvement of dough properties and bread quality has not yet been reported in the literature. Statistical methods were applied to different engineering problems for improving the performance and finding the optimum process variables. Statistical mixture designs are special class of response surface designs where the proportions of the components or factors are considered important. It involves the use of different combinations between the components for changing mixture composition and exploring how such changes will affect a specific response (Rao and Baral 2011; De Castro and Sato 2013).

In this study, we aimed to investigate the individual effect of glucose oxidase as well as the interactions between GOD, Ascorbic acid and α -amylase, to optimize additives formulation content for maximizing dough properties and bread quality and to evaluate the optimized mixture on final product. This study aimed also to define the level at which GOD could substitute ascorbic acid.

Materials and methods

Material

Commercial French soft wheat flour used for all experiments was provided by a local milling company (STPA) with a relative humidity of 14 % and protein of 10.42 % (Kjeldahl method). Enzymes included active microbial *Aspergillus tubingensis* CTM 507 glucose oxidase 269.51 U/ml activity, commercial glucose oxidase (Gox, Gluzyme mono BG from Novozyme, Denmark) 100.000 U/g activity, commercial α -amylase (Fungamyl α -amylase CONC II, from Novozymes, Denmark) 10.000 skb activity and ascorbic acid (Cargill Food Ingredients Latin America) were used to prepare the additive formulation. The additive doses were utilized depending on the experimental design. Basal additives improver at 39 g/100 kg of flour contain 0.4 g α -amylase/100 kg of flour and 1.5 g xylanase/100 kg of flour. Wheat flour with basal improver was used as a control. All the reagent chemicals used were of analytical grade.

Fungal glucose oxidase preparation

The glucose oxidase evaluated in the present study was extracted from the culture of newly isolated fungi named *Aspergillus tubingensis* CTM 507. Following 32 h of cultivation, the extracellular enzyme was separated from the culture medium by filtration and centrifugation. The enzyme preparation was then partially purified by heat treatment (10 min at 50 °C) and 70 % of ammonium sulfate fractionation steps.

Alveograph testing

Alveograph measurements were obtained, under conditions of constant dough water content and mixing times, using the standard Method 54–30 (AACC 2000). The following alveograph parameters were automatically recorded by the Alveolink-NG computer software program developed by Chopin S. A. (Chopin, Tripette et Renaud, Villeneuve La Garenne, France), WA: maximum over-pressure needed to blow the dough bubble, P: index of resistance to extension, L: average abscissa at bubble rupture (index of dough extensibility), and W: deformation energy (index of dough strength). Two curves were considered for each sample and

the analyses were conducted in the Chopin model (MA 87, French) at the temperature of 18–25 °C.

Dough expansion capacity

The evolution of dough volume through the whole fermentation period was determined following the method described by IRAM (Instituto Argentino de Racionalización de Materiales) norms (method number 15865, IRAM 1991). Basic dough formulation (on flour basis) consisted of 2 % (w/w) salt, 1.8 % (w/w) compressed yeast, 0.05 % (w/w) additives and 50 % (w/v) water. The additives composition dissolved in distilled water was added to the bread formulation as described in Table 1. Ingredients were manually mixed for approximately 10 min until homogeneous dough was achieved. The resulting dough was allowed to rest for 5 min and the bulk dough was subsequently sheeted by hand. The dough was then divided into 100 g pieces and rounded.

To determine gas production, two pieces of dough were immediately put into 1000 ml calibrated graduated cylinders. Dough samples were pressed to achieve a smooth surface and the cylinders were left for 150 min in a water bath at 30 ± 0.5 °C. Dough rising was measured every 15 min for 120 min.

Bread-making procedure

Bread was prepared according to a slightly modified AACC international (2000) method 10-10B. Dough was prepared following the formulation indicated previously (flour basis). The bulk dough was then moulded into a loaf shape and returned to the fermentation cabinet for 30 min. After this process, dough was baked at 215 °C for 24 min in a rotational gas oven (Ciclo Ingeniería, Buenos Aires, Argentina). Two

hours after baking, the bread obtained was evaluated as described below.

Crumb texture profile analysis

Texture profile analysis was performed by using a TA texture analyzer (Lloyd Instruments, Fareham, UK). A cylinder probe of 19-mm diameter was attached to a moving cross-head. Two hours after baking, the bread loaves were cut and two slices (2 cm thick) were subjected to a double cycle of compression under the following conditions: 1 mm/s speed test and 50 % 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 50 % of its initial thickness (Method 74-09; AACC International, American Association of Cereal Chemistry 2000).

Chewiness (firmness, springiness, cohesiveness) is defined as the force required to disintegrate the solid food until it is swallowed (Civille and Szczesniak 1973).

Loaf specific volume and density determination

The bread loaf specific volume was measured 2 h after baking using rapeseed displacement according to the AACC method 10-05.01 (AACC 2000). Specific volume corresponds to the ratio between volume and weight (cm³/g). The density is the inverse of the specific volume.

Improvement of bread specific volume was calculated as follows:

$$\text{Improvement} = [1 - (\text{specific volume of bread test}) / (\text{specific volume of control sample})] * 100$$

Triplicates were analyzed and results were expressed as mean values.

Microbiological analysis

For the microbiological analysis of bread, 10 g of crumb sample was diluted with 90 ml of sterile saline solution (9%) and serial decimal dilutions were prepared. Total aerobic mesophylic bacteria count was determined on plate count agar (PCA) medium after incubation at 30 °C for 72 h. Fungal flora were assayed on plate dextrose agar (PDA) medium, incubated at 25 °C for 3 to 7 days.

Plates with 30–300 colonies were selected and the average number of colonies was used to calculate the viable cell concentrations expressed in colony forming units (CFU)/g of

bread. Each assay was performed in triplicate and the mean of results was taken (Mnif et al. 2012).

Experimental mixture design

The effect of additives combination on dough properties (W index, P/L, Ie, hardness (N), chewiness (Nmm), cohesion and adhesion (N)) and bread quality (hardness, chewiness (Nmm), springiness (mm) and cohesion) was studied applying simplex-centroid mixture design. The following independent variables in the mixture design consisted of α -amylase (X1), ascorbic acid (X2) and *A. tubingensis* glucose oxidase (X3) (Table 1). All the components had the same range, between 0 and 1, and there were no constraints on the design space (Abdullah and Chin 2010). In the mixture experiments, the

Table 1 Experimental design “centroid simplex”

Mixtures	Proportion of each compound in the mixture (x_1, x_2, x_3)	Values of each compound (g) in 5 g of mixture		
		α -amylase	Ascorbic acid	Glucose oxidase
1	(1, 0, 0)	0.007	0.4	0.0005
2	(0, 1, 0)	0.004	1	0.0005
3	(0, 0, 1)	0.004	0.4	0.0015
4	(1/2, 1/2, 0)	0.0055	0.7	0.0005
5	(1/2, 0, 1/2)	0.0055	0.4	0.001
6	(0, 1/2, 1/2)	0.004	0.7	0.001
7	(1/3, 1/3, 1/3)	0.005	0.6	0.0008

sum of all the components in the blends was always 100 %. Enzyme levels were selected according to several bibliographical reports (Rasiah et al. 2005; Bonet et al. 2006; Caballero et al. 2007a, b; Steffolani et al. 2010).

The design consisted of seven experimental points was investigated in triplicate. These seven points consisted of three single-ingredient treatments, three two-ingredient mixtures and four three-ingredient mixtures. The data were analysed using Nemrodw software (Version 2007, software LPRAI, Marseille, France) and the response surface was generated using JMP software (version 3.2.2.; SAS Institute Inc., USA).

The fitted responses values were based on the cubic model:

$$Y = b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3$$

Where Y is the predictive dependent variable, b_i the equation coefficients; and X the proportions of pseudo-components.

The statistical equation significance was determined by variance analysis (ANOVA) at 5 %.

Statistical analysis

The results related to the determination of the bread specific volume and CFU counts results were the average of 3 replicates of 3 separate tests. They were statistically analysed by SPSS software (version 10.0; SPSS Inc., Chicago, IL, USA) using Duncan test performed after analysis of variance (ANOVA).

Results and discussion

The interactions between *A. tubingensis* glucose oxidase, α -amylase and ascorbic acid on the dough properties and bread quality were studied in the seven experiments following a simplex centroid mixture design. Dough properties and bread quality using additives were determined at each experimental

point. The responses data were then modeled by multiple regression analysis and residuals plots were generated to check the goodness of model fit (Fig. 1). The *F*-test was used to identify the statistical significance of the models (Table 2). The predicted models present high determination coefficients (R^2) values indicating that they were relatively adequate for the prediction purpose. Based on these equations, the response behavior can be predicted within the experimental area and presented as a response surface.

Dough properties

The impact of *Aspergillus tubingensis* glucose oxidase and its combination with ascorbic acid and alpha amylase on dough properties were studied by alveographic and texture measures. Table 3 illustrates the relative responses of rheological and textural dough properties of each experimental point.

The experimental rheological measurements, which use large deformations, generally show good correlations with bread-making quality (Tronsmo et al. 2003). The alveograph can be considered the most appropriate for measuring rheological properties of dough (Dobraszczyk and Salmanowicz 2008). The finding showed that the extensibility of the dough (L), an indicator of its handling characteristics, was greatly reduced by the individual use of glucose oxidase. As a result of the glucose oxidase action on dough resistance and dough extensibility, the P/L ratio (which represents the balance of the elasticity and extensibility of flour dough) was augmented in dough containing glucose oxidase (0.004 %, w/w; Mix 3). The oxidant action of GOD led to a cross-link among proteins through disulfide and non-disulfide bonds into the gluten network, forming large protein aggregates (Steffolani et al. 2010). These results are in agreement with various studies (Primo-Martin et al. 2005; Bonet et al. 2006; Dagdelen and Gocmen 2007b) which reported that glucose oxidase effect on

Fig. 1 Mixture contour plots of the effects of additive on rheological and textural dough properties W (a), P/L (b), elasticity (c) masticability (d) and on textural bread quality: hardness (e), masticability (f), cohesion (g) and adhesion (h)

a **b** **c** **d** **e** **f** **g** **h**

Table 2 Regression coefficients and correlation for the adjusted model to experimental data of dough properties and textural bread in mixtures design

Coefficients	b ₁	b ₂	b ₃	b ₁₂	b ₁₃	b ₂₃	b ₁₂₃	R ²
Dough								
W index	260.37* ± 4.3	290.37* ± 4.3	260.26* ± 4.3	12.47 ± 19.81	32.47 ± 19.81	252.50* ± 19.81	-1469.47 ± 19.81	0.996
P/L	0.69* ± 0.02	0.66* ± 0.02	0.81* ± 0.02	-0.10 ± 0.11	-0.04 ± 0.11	-0.38 ± 0.11	0.82 ± 0.11	0.974
Ie	68.51 ± 5.89	85.52* ± 5.89	89.51* ± 5.89	-12.29 ± 27.07	-4.29 ± 27.07	29.71 ± 27.07	-482.86 ± 27.07	0.935
Hardness (N)	0.34* ± 0.01	0.44* ± 0.01	0.38* ± 0.01	-0.23 ± 0.05	0.13 ± 0.05	-0.58* ± 0.05	0.34 ± 0.05	0.995
Chewiness (Nmm)	0.84 ± 0.82	2.62 ± 0.82	3.30 ± 0.82	0.25 ± 3.75	0.62 ± 3.75	-8.87 ± 3.75	-7.57 ± 3.75	0.819
Cohesion	8.50* ± 0.44	11.69* ± 0.44	9.67* ± 0.44	-1.83 ± 2.00	5.74 ± 2.00	-7.77 ± 2.00	6.76 ± 2.00	0.974
Adhesion (N)	0.10 ± 0.08	0.22 ± 0.08	0.34 ± 0.08	0.10 ± 0.35	-0.02 ± 0.35	-0.65 ± 0.35	2.65 ± 0.35	0.867
Hardness	21.02 ± 3.19	27.61 ± 3.19	8.94 ± 3.19	-48.13 ± 14.67	-21.87 ± 14.67	-54.18 ± 14.67	110.55 ± 14.67	0.977
Chewiness (Nmm)	94.06 ± 13.33	210.80* ± 13.33	84.98 ± 13.33	-219.69 ± 61.31	-63.78 ± 61.31	-437.54 ± 61.31	461.54 ± 61.31	0.991
Springiness (mm)	12.35* ± 0.24	19.15* ± 0.24	13.29* ± 0.24	-4.32 ± 1.08	1.81 ± 1.08	-9.56 ± 1.08	8.11 ± 1.08	0.998
Cohesion	0.22 ± 0.10	0.42 ± 0.10	0.66 ± 0.10	0.61 ± 0.46	0.24 ± 0.46	0.17 ± 0.46	-3.49 ± 0.46	0.923

*Significant at 0.05 level

dough properties was thought to be the result of increased S-S formation and/or arabinoxylan cross-linking. A small increase in the deformation energy (W) index, used to estimate the dough behavior during the baking process, was noted when excessive levels of additives were added. Indeed, the addition of glucose oxidase (Mix 3) or alpha amylase (Mix1) presents insignificant increase of W (260.10^{-4} j) when compared to the control test (250.10^{-4} j). However, the binary combinations (Mix 6) of glucose oxidase and ascorbic acid significantly increased the deformation energy levels (340.10^{-4} J) indicating a positive interaction between the two oxidant additives. A synergism among glucose oxidase, alpha amylase and ascorbic acid was observed in the ternary combinations (Mix 7), resulting in a decrease in the rheological dough properties (W, P/L, Ie) compared to the binary mixture of glucose oxidase and ascorbic acid and low level of α -amylase. This result further demonstrated the enhancing effect of GOD when combined with the ascorbic acid, which brought about improving rheological dough properties. With regard to these results we can conclude that the individual use of glucose oxidase as oxidant agent cannot substitute the effect of ascorbic acid in wheat flour and the association of ascorbic acid and GOD is beneficial to dough rheological properties.

Accordingly, texture evaluation allowed to note that additives incorporation had a significant effect in wheat dough properties ($p \leq 0.05$). Indeed, when compared to the control test, the treatment by individual oxidant (mix 1 and 2) showed a significant increase in the hardness, chewiness, cohesion and adhesion while the binary combinations of glucose oxidase and ascorbic acid considerably improved the texture profile of dough. In fact, a notable decrease in textural parameters values was observed for dough containing oxidant agent (mix 6). The textural results showed greater correlation with the alveographic reports, which means that the mixed oxidative system improved dough properties.

Bread properties

The results of crumb texture are displayed in Table 4. The findings revealed that additives incorporation in wheat flour entailed a significant modification of bread texture. The only GOD addition in wheat flour produced a significant decrease in crumb hardness, chewiness and adhesion (Mix 3), suggesting an antistaling effect of this enzyme. These results were expected since previous studies had confirmed the beneficial effect of GOD addition. Bonet et al. (2006) stated that the addition of GOD in dough stimulated the incorporation of pentosans into the insoluble glutenin protein matrix which led to an increase in the quantity of the total pentosans associated with glutenin

Table 3 Dough rheological and textural properties

Mixture	W (10^{-4} J)	P (mm)	L (mm)	P/L	Ie	Hardness (N)	Chewiness (Nmm)	Cohesion	Adhesion (N)
1	260 ± 0.013	66 ± 0.017	96 ± 0.020	0.68 ± 0.016	68 ± 0.013	0.34 ± 0.009	0.92 ± 0.022	0.31 ± 0.018	0.11 ± 0.015
2	290 ± 0.009	66 ± 0.011	100 ± 0.002	0.66 ± 0.005	85 ± 0.007	0.44 ± 0.003	2.69 ± 0.005	0.52 ± 0.008	0.23 ± 0.007
3	260 ± 0.012	66.27 ± 0.006	82 ± 0.011	0.81 ± 0.014	89 ± 0.020	0.38 ± 0.018	3.37 ± 0.016	0.94 ± 0.012	0.35 ± 0.015
4	280 ± 0.015	64.9 ± 0.013	101 ± 0.022	0.64 ± 0.009	76 ± 0.013	0.33 ± 0.015	1.51 ± 0.017	0.49 ± 0.014	0.16 ± 0.009
5	270 ± 0.009	65.45 ± 0.016	89 ± 0.014	0.73 ± 0.011	80 ± 0.008	0.33 ± 0.012	1.95 ± 0.015	0.51 ± 0.018	0.19 ± 0.012
6	340 ± 0.003	65.45 ± 0.007	103 ± 0.012	0.63 ± 0.004	97 ± 0.006	0.26 ± 0.009	0.78 ± 0.013	0.31 ± 0.012	0.09 ± 0.008
7	260 ± 0.004	64.16 ± 0.003	94 ± 0.001	0.68 ± 0.002	78 ± 0.004	0.29 ± 0.011	2.15 ± 0.014	0.63 ± 0.009	0.22 ± 0.012
Control	300 ± 0.002	56.1 ± 0.005	127 ± 0.008	0.44 ± 0.001	66 ± 0.005	0.31 ± 0.009	1.75 ± 0.012	0.51 ± 0.008	0.19 ± 0.013

macropolymers (GMP). This effect linked to the ability of pentosans to retain high amounts of water through inter-chain associations involving oxidative coupling and chain entanglements might be responsible for the reduced hardness increase (Gujral and Rosell 2004). In contrast, Steffolani et al. (2012) noted that GOD promoted a higher interaction among gluten protein that contributed to the formation of a closed and dense bread crumb. However, the individual use of ascorbic acid led to a significant ($p \leq 0.05$) crumb hardness, chewiness and springiness (Mix 2). The combination of GOD and ascorbic acid presented the lowest hardness (3.61) and chewiness (33.86). Thus, the interaction between these oxidants had a negative effect on textural bread characteristics. These results showed that oxidizing agents differently affected the protein fractions (glutenins, gliadins, albumins or globulins) depending on their particular action mechanism (Caballero et al. 2007a, b). Indeed, oxidant agents with different biochemical activities could induce synergistic effects on bread behavior or product quality. The most dramatic effect of additives on textural crumb bread was observed when a mix of glucose oxidase, α -amylase and ascorbic acid were added. A positive interaction between these three additives was noted on textural responses of bread crumb. The blend of additives (Mix 7) exercised a significant effect on textural parameters which resulted in an improvement of the bread rheological behavior and the quality of the final product. From these results, we can conclude that the association of different gluten

modifying oxidants with α -amylase could be an excellent option to improve overall quality of baked products.

Optimization

The optimization calculations were performed to find optimum mixture proportions for enhancing dough properties and bread quality. Two optimal formulations which gave the highest composite desirability were chosen.

The optimal mixture recorded for rheological and textural dough properties (M_1) was obtained with 0.44 % of GOD, 0.56 % of ascorbic acid and 0 % of alpha amylase supplement. This formulation allowed to maximize the three responses (Elasticity index, P/L and W index) and to minimize textural parameters (hardness, chewiness and adhesion), simultaneously. Applying the optimal conditions, the rheological and the textural dough properties were significantly improved (Table 5). In fact, W (10^{-4} J), P/L, Ie index, elasticity and cohesion were absolutely more important than those of the control test. Moreover, hardness and adhesion values were less than the control dough sample.

The effect of optimized formulation (M_1) addition on gas retention capacity of fermented dough was also evaluated. The obtained results show that dough with added formulation presented the best profile of gas retention overall in the control test (without supplement additives) and with commercial GOD. Indeed, we can report that mixture addition led to more

Table 4 Textural properties of crumb bread

Mixture	Hardness (N)	Chewiness (Nmm)	Springiness (mm)	Cohesion	Adhesion (N)
1	21.30 ± 0.002	95.23 ± 0.014	12.37 ± 0.007	0.21 ± 0.001	9.55 ± 0.005
2	27.88 ± 0.006	211.97 ± 0.012	19.18 ± 0.003	0.41 ± 0.001	10.49 ± 0.002
3	9.21 ± 0.004	86.15 ± 0.010	13.31 ± 0.008	0.65 ± 0.003	6.47 ± 0.003
4	11.16 ± 0.001	92.85 ± 0.013	14.59 ± 0.005	0.50 ± 0.002	6.36 ± 0.003
5	8.39 ± 0.003	68.93 ± 0.007	13.94 ± 0.009	0.53 ± 0.001	4.94 ± 0.001
6	3.60 ± 0.006	33.86 ± 0.004	13.75 ± 0.002	0.61 ± 0.002	2.46 ± 0.007
7	7.90 ± 0.008	60.34 ± 0.009	14.10 ± 0.001	0.46 ± 0.003	4.27 ± 0.004
Control	25.93 ± 0.005	198.55 ± 0.017	12.92 ± 0.004	0.55 ± 0.004	15.35 ± 0.002

Table 5 Comparison of rheological and textural parameters between the control and the enriched dough and bread by optimized formulation

	Parameters	Control	Optimized formulation with synthetic GOD	Optimized formulation with commercial GOD
Dough	W (10^{-4} J)	250.00 $\pm$ 0.002	310.00 $\pm$ 0.002	320.00 $\pm$ 0.011
	P/L	0.44 $\pm$ 0.009	0.69 $\pm$ 0.011	0.66 $\pm$ 0.003
	Ie	66.00 $\pm$ 0.005	97.00 $\pm$ 0.007	97.00 $\pm$ 0.001
	Hardness (gf)	52.98 $\pm$ 0.001	41.00 $\pm$ 0.003	33.77 $\pm$ 0.002
	Elasticity (mm)	9.59 $\pm$ 0.002	11.00 $\pm$ 0.001	11.18 $\pm$ 0.012
	Cohesion	0.47 $\pm$ 0.012	0.58 $\pm$ 0.002	0.62 $\pm$ 0.008
Bread	Hardness (gf)	636.10 $\pm$ 0.004	402.42 $\pm$ 0.016	398.00 $\pm$ 0.002
	Elasticity (mm)	11.07 $\pm$ 0.003	15.00 $\pm$ 0.014	15.22 $\pm$ 0.001
	Cohesion	0.48 $\pm$ 0.014	0.68 $\pm$ 0.009	0.72 $\pm$ 0.018

cohesive dough and internal resistant structure resulting in a better retention gas capacity under fermentation and consequently in a higher specific volume.

The mixture of glucose oxidase and ascorbic acid showed its potential to modify functional dough which led to the improvement of not only dough behavior but also manufacturing conditions. Dough is made more ‘machine-friendly’ as it does not stick to the machinery parts (Butt et al. 2008).

In the second optimization, we tried to minimize crumb firmness and chewiness and to maximize bread elasticity. The optimal mixture proportions (M_2) that met the bread quality desirability included 0.638 % of GOD, 0.32 % of ascorbic acid and 0.042 % of alpha amylase. This formulation showed a great improvement of textural properties of bread (Table 5).

The findings revealed that there were no huge differences between the optimized additives mixture contents. Both optimized formulation showed that alpha amylase supplement had a negligible effect on dough and bread quality. Interestingly, we can note that adding a combination of glucose oxidase and ascorbic acid to wheat flour for bread dough can be used as a corrective action to reduce problems in dough caused by the use of alpha amylase. Indeed, the addition of α -amylase to flour leads to a sharp decrease in dough stability and this makes it weaker and results in dough with a sticky texture.

Sticky dough causes handling problems and affects its capacity to rise, which may lead to the rejection of a batch. These changes do not allow dough to retain the gases fermentation (Shafisoltani et al. 2014). The investigated oxidative system might be applicable as an alternative to modify functional properties of food proteins.

Evaluation of the effect of optimized formulation (M_2) on the bread quality

Baking test results

Loaf specific volume provides a quantitative measurement of baking performance. It is therefore one of the most important visual characteristics of bread, strongly influencing consumer’s choice. Hence, it is a key parameter to look at when evaluating bread quality (Hager and Arendt 2013). The effect of optimized bread additives formulation (M_2) on specific volume is summarized in Table 6. The obtained results showed that the addition of the optimized additives formulation significantly increased the specific volume and consequently decreased the bread density. Specific volume was enhanced reaching up to 3.19 cm³/g. It was, approximately, two folds higher than the control test bread (1.64 cm³/g). Indeed,

Table 6 Appreciation of bread quality prepared with and without optimized additives formulation

		Control test	Optimized formulation with synthetic GOD	Optimized formulation with commercial GOD
Bread characteristic	Volume (cm ³)	146.00 $\pm$ 0.002	255.63 $\pm$ 0.003	240.95 $\pm$ 0.005
	Specific volume (cm ³ /g)	1.64 $\pm$ 0.001	3.19 $\pm$ 0.004	2.94 $\pm$ 0.002
	Density (g/cm ³)	0.60 $\pm$ 0.003	0.31 $\pm$ 0.001	0.33 $\pm$ 0.002
	Improvement of bread Specific volume (%)	100.00 $\pm$ 0.001	194.40 $\pm$ 0.003	179.08 $\pm$ 0.003
Water activity	Crust	30.00 $\pm$ 0.120	27.20 $\pm$ 0.040	28.80 $\pm$ 0.010
	Crumb	39.30 $\pm$ 0.160	35.60 $\pm$ 0.090	35.90 $\pm$ 0.020
Microbiological analysis	Mold (CFU/g of bread)	8.10 ⁴ $\pm$ 0.24.10 ⁴	2.10 ⁴ $\pm$ 0.20.10 ⁴	3.10 ⁴ $\pm$ 0.18.10 ⁴
	Bacteria (CFU/g of bread)	13.10 ⁴ $\pm$ 0.37.10 ⁴	6.10 ⁴ $\pm$ 0.15.10 ⁴	7.10 ⁴ $\pm$ 0.52.10 ⁴

Fig. 2 Crumb structure aspect of loaf prepared with optimized additive formulation: *A. tubingensis* GOD (a), commercial GOD (b) and control (c)

the improvement of bread specific volume by a simple formula prepared by synthetic GOD was more important than commercial GOD. The beneficial effect of additive formulation (M₂) on bread appearance and crumb structure was illustrated in Fig. 2. It was clear that the supplementation of additive at their optimum levels resulted in a better crumb quality with larger bread slice area. The crumb structure was more regular with homogeneous small cells. Indeed, additives allow a finer and closer grain, brighter crumb, high uniformity in cell size and improved slicing characteristics of bread.

Effects of optimized mixture additions on bread microbiological characteristics: reduction of water activity and susceptibility to spoilage

The decline of bread quality is generally affected by two fundamental processes: staling and microorganisms spoilage. Microorganisms might proliferate during the commercial life of loaf, could damage the texture, sensorial and nutritional properties of bread, and also could cause multiple diseases by the synthesis and liberation of amylolytic and proteolytic enzymes and toxins (Viedma et al. 2008). Hence, the control of microorganism's germination in bread after cooking is an essential tool for assessing the level of contamination of food and the nature of microflora (Mnif et al. 2012). In this study, bread enriched by the optimized formulation (M₂) was examined for stability and microbiological changes throughout 30-day storage at room temperature. Our findings showed good microbial stability of optimized-formula enriched bread. Indeed, the first microbiological changes occurred only after 15 days of storage. Until this time, the product was still acceptable. No microbiological changes were observed proving that optimized formulation effectively decreased the bread susceptibility to microbial contamination. Thereafter, especially after 24 days of storage, the presence of moulds and bacterial growth were detected (Table 6). The inhibitory effect of fungi and bacteria spoilage could be attributed to the significant lower free water of bread with added optimized formula in comparison to the control test which results an important decrease of bread water activity (Table 6). Indeed, optimized formula was recorded to be significantly more effective

in decreasing water activity and consequently susceptibility of spoilage during storage. The decrease of the relative humidity could be associated with the ability of pentazans to absorb large amounts of water in the process of gelation under the influence of glucose oxidase (Vukic et al. 2013). Dagdelen and Gocmen (2007a, b) observed that the addition of GOD increases water absorption by dough.

Conclusion

In this study, statistical methods were applied to improve the bread-making performance and find the optimum dough and bread additives formula. The findings revealed that the additive incorporation in wheat flour modified dough behavior, evidenced by the different rheological and textural properties. As a whole, these modifications resulted in bread with high technological quality. In fact, the combination of the two oxidative agents, glucose oxidase and ascorbic acid improved the Elasticity, P/L and W index and declined dough hardness, chewiness and adhesion. Additionally, the simultaneous presence of GOD, ascorbic acid and α -amylase led to a significant increase in the specific volume and in the bread shelf life. These results were of huge interest for further investigation of *A. tubingensis* CTM 507 glucose oxidase as an additive in bakery product formulations. They could lead to bread with excellent quality and aid in preserving dough quality under storage.

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