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Optimised amylases extraction from oat seeds and its impact on bread properties

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ABSTRACT

Statistical approaches were employed for the optimisation of the extraction of amylolytic activity from oat (*Avena sativa*) seeds. The application of the response surface methodology allows us to determine a set of optimal conditions (ratio seed weight/buffer volume 0.1, germination days 10 days, temperature 20 °C and pH 5.6). Experiments carried out under these conditions led to amylase production yield of 91 U/g. Its maximal activity was in the pH 5.6 and at 55 °C. Study of the incorporation of the optimised oat extract into the bread formulation revealed an improvement of the sensory quality and the textural properties of fresh and stored bread. Three-dimensional elaborations of Confocal Laser Scanning Microscopy (CLSM) images were performed on crumb of the different breads to evaluate the influence of amylase activity on microstructure. The result showed improved baking characteristics as well as overall microscopic and macroscopic appearance.

1. Introduction

The plants are used traditionally as natural reserves of a large number of products such as enzyme used in food. An increased consumer demand for healthy bread has led to considerable efforts to develop breads that combine health benefits with good sensory properties [1]. Amylase properties and action mechanisms determine starch structure in bread, and, hence, how amylopectin recrystallisation, starch network formation, and water redistribution occur during bread making and bread storage [2]. An enzyme with little impact on the outer, crystallisable amylopectin chains, such as a conventional bacterial alpha-amylase, can weaken the starch network in bread. However, it does not hinder amylopectin recrystallisation and the concomitant water immobilisation, which results in a decrease in plasticising water. Consequently, gluten mobility and resilience are reduced. In contrast, a mainly exo-acting enzyme, such as maltogenic alpha-amylase, limits amylopectin recrystallisation, and, hence, amylopectin network formation and consequent water immobilisation, ultimately resulting in a softer, more water-plasticised crumb with greater resilience [2].

The industrial production of enzymes is often made from bacterial or fungal strains. However, plants produce various types of enzymes that are more advantageous in food applications including amylolytic enzymes that are produced in large quantities primarily during seed germination. Oat (*Avena sativa* L.) is a member of the Poaceae family [3], being one of the crops cultivated by mankind for the longest time [4]; ranking seventh in world cereal production after wheat, maize, rice, barley, sorghum and millet. Since 2009–2013 the world production of oat was estimated to 21 millions of tons, with Russia being the greatest producer [5,6] and today it is an important and traditional agricultural crop produced in various regions of Europe and North America [7,8]. In Tunisia, Oats are the most important livestock feed [9,10].

Response surface designs such as Box–Behnken Design (BBD) are commonly selected for performing optimisation of plant extraction. The BBD method employs a spherical design with excellent predictability within the design space and it requires less experiment than the FFD or CCD with the same number of factors [11]. In addition, the BBD technique is rotatable or nearly rotatable regardless of the number of factors under consideration [12]. Over the last

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years, research interest in extractable seed components, such as storage carbohydrates, saponins and enzymes has increased [13]. However, to date, no studies have been reported concerning the extraction and the purification of a maltogenic amylase from oat seeds.

The aim of the present study was to investigate statistical approaches to maximise amylase yield extraction from oat seedling and further to evaluate the effectiveness of oat amylase application as an additive in wheat bread to improve the baking performance.

2. Materials and methods

2.1. Plant material and enzyme extraction

Oat seeds (*A. sativa*) were imbibed overnight in water at room temperature, and then placed to germinate in incubators at 98%RH at 25 °C in the dark for 5 days. The humidity was maintained at 98% to facilitate reduction in the drying of the sprouts by periodic watering [14]. On designated days after planting, seeds were ground using mortar and pestle with 0.02 M sodium acetate buffer (pH 5.6), filtered through two layers of cheese cloth to remove large particles and the supernatant obtained was centrifuged at 15,000 × *g* for 20 min [10,15]. All the preparations were carried out at 4 °C. The supernatant obtained was used as crude enzyme extract for amylase assay.

2.2. Amylase activity assays

The hydrolytic activity for soluble starch was measured in the standard reaction mixture consisting of 0.5% (w/v) soluble starch prepared in 0.1 M of sodium acetate buffer (pH 5.6) and the enzyme solution in a final volume of 1 ml. The reaction was started by adding substrate. The reducing sugar produced in 10 min at 55 °C was measured by DNS (3,5-dinitrosalicylate) method at 550 nm (T60U spectrometer, PG Instruments Ltd) by using glucose as standard reducing sugar [16]. One unit of amylase activity was defined as the amount of enzyme, required to produce reducing sugars equivalent to 1 μmol/min under these reaction conditions.

2.3. Effect of temperature and pH

The effect of temperature on the activity of the crude amylase extract was performed by its incubation at different temperatures ranging from 20 to 90 °C. The reaction was performed according to the method of amylase assays described above. The effects of pH on the activity of the crude extract were determined at pH ranging from 3.6 to 10.5 at 55 °C. The buffers used are: sodium acetate 0.1 M (pH 3.6–6.5), phosphate 0.1 M (pH 7–9) and glycine-NaOH 0.1 M (pH 9–10.5). The activities at optimal temperature and pH were defined as 100%.

2.4. Thermal stability

Amylases in sodium acetate buffer (100 mM, pH 5.6) were incubated at a temperature of 30 and 55 °C and then samples were withdrawn for enzyme assay at appropriate time intervals. The residual activity was estimated taking original activity as 100%.

2.5. Experimental design and statistical analysis

Optimisation of amylase production yield was achieved by using the response surface methodology (RSM). This approach explores the response surfaces covered in the experimental design, thus it makes the optimisation process more efficient and effective [17]. The most frequent designs in optimisation problems involving three or more factors are central composite designs, D-optimal designs, and others, such as Box–Behnken designs [18–20]. Central composite and Box–Behnken designs are the most appropriate to detect curvatures in a multidimensional space.

In this work, a Box–Behnken design [21–23] was set up to look for the best experimental conditions of four independent factors affecting the extraction of the amylase activity namely: the ratio seed weight (g)/buffer volume (ml) (X_1), germination days (X_2), temperature (X_3) and pH (X_4) [11].

The response (amylase yield) can be described by the following second order polynomial function for predicting the responses in the experimental region:

$$\eta = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4$$

where η: represents the theoretical response function; β_0, β_j, β_{jk} and β_{jj} are the true model coefficients; X_j: the coded variables of the system related to the natural variables $_{Uj}$ by the following equation:

$$X_j = \frac{(U_j - \text{Center(j)})}{\text{Step of variation}\,(j)}$$

where

$$\text{Center}\,(j) = \frac{(U_j,\ \text{high} - U_j,\ \text{low})}{2}$$

$$\text{Step of variation}\,(j) = \frac{(U_j,\ \text{high} + U_j,\ \text{low})}{2}$$

U_j, high and U_j, low: two extreme levels (high and low) given for each natural variable U_j.

The coded variables X_j are equal to −1 and +1 when the levels of natural variable U_j are U_j low and U_j high respectively.

The observed response y_i for the *i*th experiment is:

$$y_i = \eta_i + e_i \quad (e_i : \text{experimental error})$$

The model coefficients β_0, β_j, . . ., and β_{jj} are estimated by a least squares fitting of the model to the experimental results obtained in the 27 design points of the four-variable Box–Behnken design (Table 1). For the estimated values of these coefficients, the symbols b_0, b_j,. . ., and b_{jj} will be used. The computed values of the responses are designated by $\hat{y}$:

$$\hat{y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4$$

where $\hat{y}$ is the estimated response function; b_0, b_j, b_{jk} and b_{jj} are the estimated model coefficients.

The three replicates at the centre point (run nos 25–27) are carried out in order to estimate the pure error variance and the significance of the fitted model was tested by the mean of the analysis of variance (ANOVA) [17,19,23]. Thus, a statistical test of the model fit is made by comparing the variance due to the lack of fit to the pure error variance using the *F*-test. Model terms were selected or rejected based on the *p*-value (probability) with 95% confidence level [24]. The fitted model is considered adequate if the variance due to the lack of fit is not significantly different from the pure error variance [17,19,23]. The fitted model was used to study the relative sensitivity of the responses to the variables in the whole domain and to look for the optimal experimental conditions. The relationship between the response and the experimental variables is illustrated graphically by plotting the response surfaces and the isoresponse curves [25,26]. In this paper, the canonical analysis

Table 1
Experimental conditions of the Box–Behnken design in coded and natural variables and the corresponding experimental responses.

Run no.	X_1	X_2	X_3	X_4	Ratio (g/ml)	Germination days (days)	Temperature (°C)	pH –	Amylase yield (U/g)
1	1.0000	−1.0000	0.0000	0.0000	0.2	0	20	7	16.52
2	−1.0000	−1.0000	0.0000	0.0000	0.1	0	20	7	33.26
3	1.0000	1.0000	0.0000	0.0000	0.2	14	20	7	47.311
4	−1.0000	1.0000	0.0000	0.0000	0.1	14	20	7	93.414
5	1.0000	0.0000	−1.0000	0.0000	0.2	7	3	7	44.402
6	−1.0000	0.0000	−1.0000	0.0000	0.1	7	3	7	85.291
7	1.0000	0.0000	1.0000	0.0000	0.2	7	37	7	43.139
8	−1.0000	0.0000	1.0000	0.0000	0.1	7	37	7	73.106
9	1.0000	0.0000	0.0000	−1.0000	0.2	7	20	5	46.762
10	−1.0000	0.0000	0.0000	−1.0000	0.1	7	20	5	84.303
11	1.0000	0.0000	0.0000	1.0000	0.2	7	20	9	40.011
12	−1.0000	0.0000	0.0000	1.0000	0.1	7	20	9	63.337
13	0.0000	−1.0000	−1.0000	0.0000	0.15	0	3	7	17.051
14	0.0000	1.0000	−1.0000	0.0000	0.15	14	3	7	63.300
15	0.0000	−1.0000	1.0000	0.0000	0.15	0	37	7	19.832
16	0.0000	1.0000	1.0000	0.0000	0.15	14	37	7	61.325
17	0.0000	−1.0000	0.0000	−1.0000	0.15	0	20	5	7.903
18	0.0000	1.0000	0.0000	−1.0000	0.15	14	20	5	64.105
19	0.0000	−1.0000	0.0000	1.0000	0.15	0	20	9	23.857
20	0.0000	1.0000	0.0000	1.0000	0.15	14	20	9	58.983
21	0.0000	0.0000	−1.0000	−1.0000	0.15	7	3	5	57.373
22	0.0000	0.0000	1.0000	−1.0000	0.15	7	37	5	62.788
23	0.0000	0.0000	−1.0000	1.0000	0.15	7	3	9	56.056
24	0.0000	0.0000	1.0000	1.0000	0.15	7	37	9	59.495
25	0.0000	0.0000	0.0000	0.0000	0.15	7	20	7	53.289
26	0.0000	0.0000	0.0000	0.0000	0.15	7	20	7	56.187
27	0.0000	0.0000	0.0000	0.0000	0.15	7	20	7	56.745

is used also to find out the best experimental conditions, which permitted the maximisation of the amylase production yield. It consists in rewriting the fitted second-degree equation in a form in which it can be more readily understood by a rotation of axes that remove all cross-product terms b_{jk}, X_j, X_k while keeping the initial origin at the centre point [27]. This step is suitable when the stationary point is outside of the experimental domain.

In this study, the experimental design, regression and statistical analysis were performed by NemrodW® software [28].

2.6. Baking and bread characterisation

Wheat bread was baked with crude oat extract additions at concentrations 0.12 U/g, 0.24 U/g and 0.48 U/g. The formulation used consisted of 50 g wheat flour, 0.5 g of salt, 1 g of dried brewer yeast and the optimal water absorption. The yeast first and then the enzyme solution were added to the remaining ingredients prior to mixing [29]. The dough was prepared by manually mixing the flour and water for approximately 10 min until homogeneous dough was achieved [30]. Assays were performed in triplicate. After fermentation (at 30 °C and a relative humidity of 85% for 30 min), the loaves were baked at 190 °C top and bottom heat for 45 min in a deck oven. The three composite loaves for each replacement level were used as replicates for further analyses. All recipes were baked twice and in random order. After baking, the loaves were depanned and cooled for 90 min on cooling racks at room temperature before analysis. Breads were also submitted to different storage times (2 h, 6 h, 24 h and 30 h) at room temperature in sealed plastic bags to prevent moisture loss for analysis of the extent of amylopectin retrogradation [31].

A Texture Procedure Analysis (TPA) test was performed using a Texture Analyser (Texture Analyser: LLOYD instruments, England) equipped with a 1000 (N) load cell, 0.05 (N) detection range [32]. Sample of dough was transferred into a moulded Nalgene polypropylene tube (5 cm height) that was placed in a fixture to hold it in place under the Texture Analyzer. An acrylic cylindrical probe was used to compress the semolina sample by 50% of its original height (40 mm) at a speed of 10 mm/s. The Texture Analyzer was interfaced with a computer, which controls the instruments and analyses the data, using the software supplied by Texture Technologies Corp. Hardness (peak force of first compression cycle), stickiness (distance of the detected height of the product on the second compression divided by the original compression distance), cohesiveness (ratio of positive areas of second cycle to area of first cycle), adhesiveness (negative force area of the first byte represented the work necessary to pull the compressing plunger away from the sample), were determined.

2.7. Confocal laser scanning microscopy

CLSM is an efficient tool for a deeper understanding of the microstructure of cereal products due to its ability to produce optical sections through a three-dimensional specimen and to select and differentiate particular structures in the food system through staining procedures [29]. For these reasons CLSM was applied in order to visualise the formation of gas cells and glycoprotein networks in the oat extract supplemented breads. Indeed, the breads for microscopy were prepared as described for the baking tests. Fresh crumb samples were cut from the centre of the loaves and immersed in a 2% agar solution. After the agar solution had solidified, thin crumb slices were cut from the agar and placed on a welled slide. To stain glycoproteins, a solution of 0.02% Fuchsin acid (Sigma–Aldrich) in 1% acetic acid was added to the sample and kept for 15 min. After staining, excess of dye was removed by rinsing the sample with deionised water for 30 min. When the staining procedure was completed a glass coverslip was placed on the sample [29]. A LSM 510 META confocal laser-scanning system mounted on an upright microscope (Zeiss S.A.S, Germany) with a 40 oil immersion objective was used. Fluorescence images (excitation = 543 nm, emission = 600–640 nm) of a number of optical sections were acquired by scanning the sample along the optical axis. A micrograph was taken of the projection of the layers.

2.8. Sensory evaluation

The organoleptic characteristics of breads were carried out by 36 panelists. The panelists were asked to evaluate the products for product colour, odour, texture, taste, touch and overall quality [33]. The ratings were on 4-point hedonic scale ranging from 4 (like extremely) to 1 (dislike extremely) for each organoleptic characteristic.

3. Results and discussion

3.1. Extraction optimisation of oat amylases

3.1.1. Estimated model

In order to select the optimal experimental conditions, a second order model is built to analyse the relation between the four factors (ratio, germination days, temperature and pH) and the response (amylase yield). Indeed, 27 experiments have been carried out and the experimental conditions were arranged according to the four variable Box–Behnken design and the corresponding observed values of the amylase extraction yield are indicated in the last column of Table 1. Results of experiments of the Box–Behnken design were used to compute the model coefficients (without using the check points) using the least square method. The resulting estimated model, expressed in coded variables is:

$$\hat{y} = 55.4 - 16.213X_1 + 22,501X_2 - 0.317X_3 - 1.792X_4 + 5.201X_1^2 - 7.34X_1X_2 + 2.73X_1X_3 + 3.555X_1X_4 - 15.298X_2^2 - 1.190X_2X_3 - 5.268X_2X_4 + 1.737X_3^2 - 0.492X_3X_4 - 0.537X_4^2$$

3.1.2. Statistical analysis and validation of the model

The analysis of variance for the fitted model shows that the regression sum of squares is statistically significant at the level 99.9% (their p-value is less than 0.01) and the lack of fit is not significant. Thus, we can conclude that the models correlate well with the measured data [17,18,23].

3.1.3. Interpretation of the response surface model

We can observe that the second-order polynomial model is a conic function that has a stationary point S where the partial derivative of predicted response with respect to each of the variables is zero $\left(\partial y/\partial X_1 = 0;\ \partial y/\partial X_2 = 0;\ \partial y/\partial X_3 = 0;\ \partial y/\partial X_4 = 0\right)$. When this point is situated outside the experimental domain, the canonical analysis requires only a rotation of the X_j axes in such a way that they become parallel to the principal axes Z_j of the contour system [27]. Under these conditions, the canonical model is of the form:

$$\hat{y} = y_s + \sum_{j=1}^{4} b_j Z_j + \sum_{j=1}^{4} \lambda_{jj} Z_j^2$$

The λ_j (j = 1,2,3,4) and b_j will describe the curvature of the response and the slope of the ridge in the corresponding direction, respectively, while the constant y_s is the calculated response value at the stationary point. The interpretation can be made by analysing each response along every Z_j-axis separately. Using the variable transformation equations:

$$X_1 = 0.908Z_1 - 0.156Z_2 - 0.357Z_3 + 0.153Z_4 - 0.087$$

$$X_2 = -0.190Z_1 + 0.048Z_2 - 0.084Z_3 + 0.977Z_4 + 0.430$$

$$X_3 = 0.250Z_1 + 0.939Z_2 + 0.235Z_3 + 0.023Z_4 - 0.902$$

$$X_4 = 0.277Z_1 - 0.303Z_2 + 0.900Z_3 + 0.146Z_4 + 1.393$$

We obtained the following canonical form of the model:

$$\hat{y} = 59.835 - 19.574Z_1 + 0.000Z_2 + 0.000Z_3 + 0.000Z_4 + 6.884Z_1^2 + 1.560Z_2^2 - 1.058Z_3^2 - 16.283Z_4^2$$

These data allow us to determine the features of the response surface in each direction of the experimental domain. The corresponding curves are represented in Fig. 1A and B. From these curves and the variable transformation equations, we can conclude that the maximisation of the amylase yield requires low level of X_1 ($X_1 = -1$), medium to high level of X_2 (X_2 = 0–1) and medium to low level of X_3 and X_4 (X_3 or $X_4 = -1$ to 0). This corresponds to the following settings of the natural variables: ratio = 0.1 g/ml, germination days = 7 to 14 days, temperature = 3 to 20 °C and pH 5–7.

The relationship between the response and the experimental variables can be illustrated graphically by plotting three-dimensional response surface plots and their respective contour plots (Fig. 1C). In these plots, the two factors not represented by the two axes were fixed at their 0 coded level. Such plots are helpful in studying the effects of the variation of the factors in the domain studied and consequently, in determining the optimal experimental conditions [26]. With oat amylase activity as the response, the response surface (3D) was shown in Fig. 1C. The obvious trough in the response surfaces indicates that the optimal conditions were exactly located inside the design boundary. Fig. 1C illustrates graphically the evolution of the amylase yield versus ratio (X_1 = 0.1–0.2 g/ml) and germination days (X_2 = 0–14 days) at fixed levels of temperature (X_3 = 20 °C) and pH (X_4 = 7). We have observed that the enzyme yield enhances essentially by decreasing the ratio. It is also clear from Fig. 1C that there is a gradual increase in the enzyme yields upon increasing the days of germination. Thus, it was implied that an increasing of germination days (7–14) was favourable for the production of amylase from oat. At 20 °C and pH 7, the amylase yield can reach more than 80 U/g when we use a ratio of 0.1 and germination days upper than 7 days. These results were in agreement with others, for example, Uno-Okamura et al. [15], who reported an increase in amylase activity of oat coleoptile or leaf by almost 3-fold during germination. Or, Khemakhem et al. [11] have shown that extraction of amylase from fenugreek was significantly increased over 7 days. The results presented above showed that the ratio and germination days were the more relevant factors for the amylase yield.

3.1.4. Optimisation

As the results of the canonical analysis agree with those of the contour plot study, we can conclude that there is no a masked optimum: the one predicted by few sections of contour plot analysis represents a real optimum for the whole experimental domain [27]. Therefore, in order to select optimal conditions, we fixed the temperature and pH at 20 °C and 5.6 respectively and we plot ratio versus germination days (Fig. 1B and C). The optimal conditions selected were: ratio 0.1, germination days 10 days, temperature 20 °C and pH 5.6. Under these conditions, the expected value of the maximum amylase yield was 91 U/g. A supplementary experiment was carried out under the selected optimal conditions. It led to an experimental yield of amylase equal to 89 U/g which is in close agreement with the expected value (91 U/g).

3.2. Optimum temperature and thermal stability

The amylase activity measured as a function of temperature in a temperature range from 20 to 90 °C showed that the enzyme preserved more then 80% of its activity over a range of 40–70 °C with an optimum at 55 °C (Fig. 2A). The optimum temperature of the amylase from oat seedling was higher than any other plant

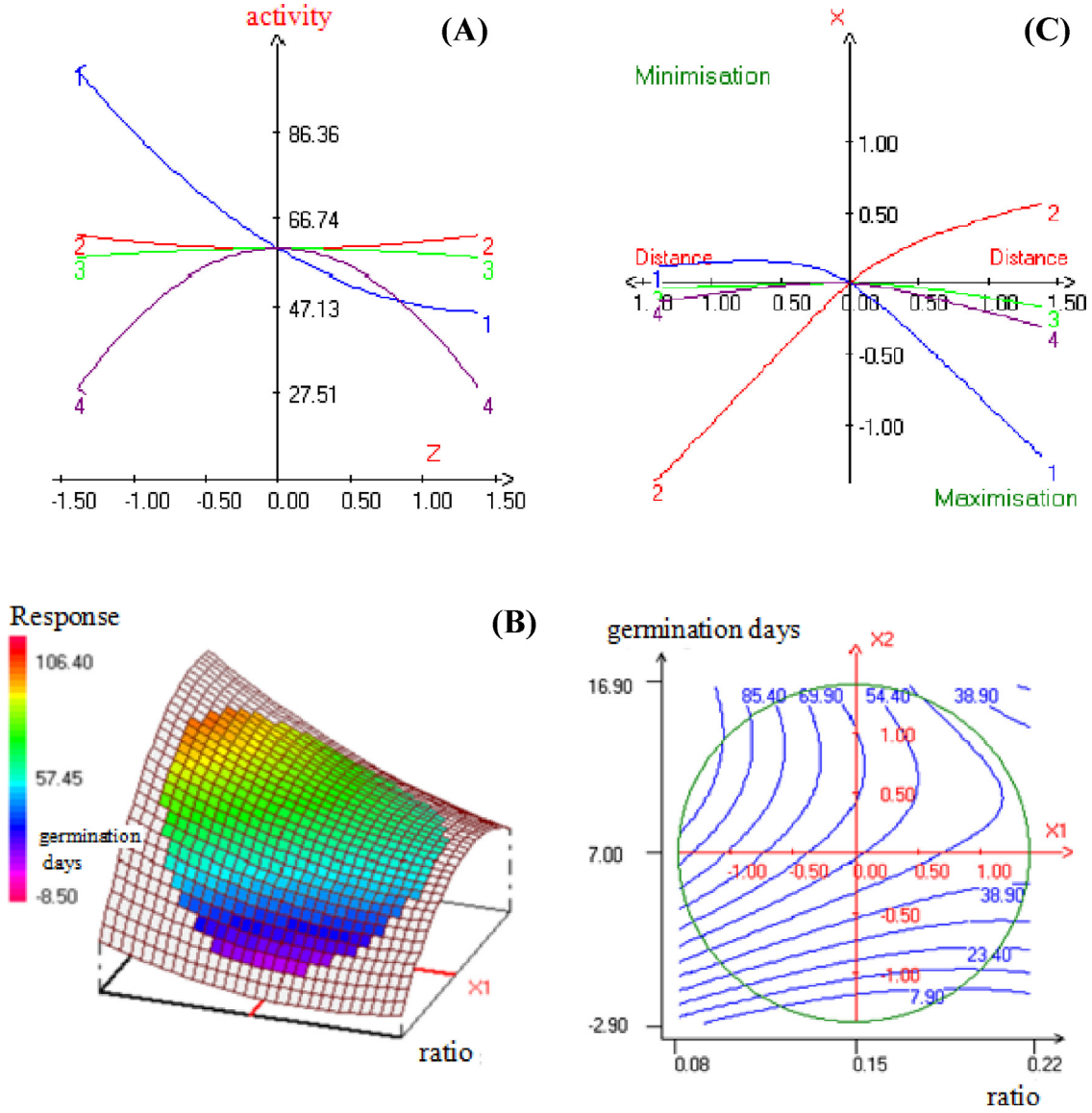

Fig. 1. (A) Curvature of amylase yield response versus Z_j (j = 1, 2, 3 and 4); (B) study of optimal way for response; (C) contour plots and response surface plot showing the effect of the ratio seed weight (g)/buffer volume (ml), germination days and their mutual interaction on the amylase yield with temperature and pH fixed respectively at 20 °C and 7. Amylase activity is expressed in U/g.

amylase reported so far. Indeed, this optimum is 40 °C and 37 °C for α-amylases from wheat and pericarp of *Borassus indica* respectively [34,35]. Moreover, this optimum is comparable to the amylase from safflower (*Carthamus tinctorius* L.) germinated seeds and from *Opuntia ficus-indica* seeds which have an optimum of temperature of 55 °C and 60 °C respectively [36,37]. Only the α-amylase of Korean pine seed [38] has optimum temperature of 65 °C.

The study of the thermostability reveals that the enzyme preserve about 75% of its maximum of activity within 15 min of incubation at 55 °C and then was rapidly decreased and was totally inactivated at 55 °C within 120 min in the absence of the substrate (Fig. 2B). However, the thermostability of oat amylase remained constant at 30 °C during 120 min.

3.3. Optimum pH

α-Amylases are generally active in the pH range from 5.5 to 8, but exceptions exist on both sides, mainly for enzymes of microbial origin [11,39]. In this study, the amylase activity was measured at different pHs ranged from 3.6 to 10.5. The results in Fig. 2C showed that amylolytic activity of germinated seeds from oat (*A. sativa*) preserve more than 70% of its maximum activity in the range of pH of 5–7 with an optimum observed at pH 5.6. In the other hand, the same optimum pH was described for amylase extracted from the peel of *Citrus sinensis* [39].

3.4. Baking tests

The addition of three level of amylolytic activity from oat extract was examined (0.12 U/g; 0.24 U/g and 0.48 U/g). Standard baking tests were conducted on three breads (n = 3) from each enzyme level. The overall effect of oat extract application on the macrostructure of breads is clearly showed by the appearance of the bread slices (Fig. 3). Crumb structure appears improved after oat extract treatment. As shown in Fig. 3, increasing the level of enzyme (i.e. 0.24 U/g and 0.48 U/g) was darkened the bread crust, and resulted in a further increase of crumb stickiness which was probably associated with dextrins produced as a result of alpha amylase activity [40,41]. Moreover, the addition of oat extract led to a more open crumb grain and a more cohesive dough and internal resistant

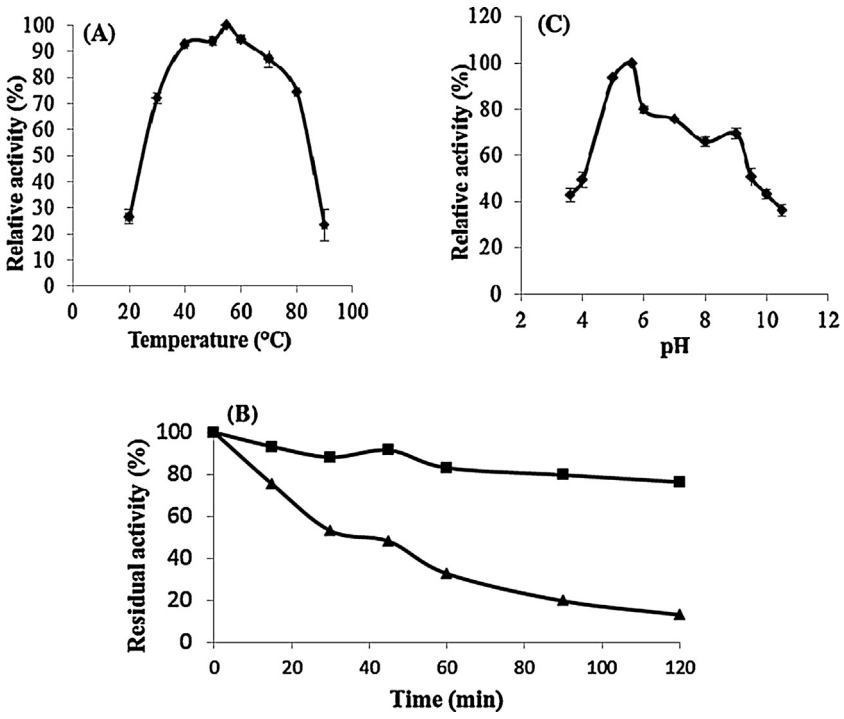

Fig. 2. (A) Temperature effect on the activity of amylase from oat (*Avena sativa*) seeds. The temperature profiles, determined in 100 mM acetate buffer (pH 5.6), were ranging from 20 to 90 °C. Relative activity is expressed as a percentage of the maximum temperature activity of the enzyme; (B) thermostability of amylase from oat (*Avena sativa*) seeds. The residual activity was determined after incubation in absence of substrate at 55 °C (▲) and 30 °C (■) at pH 5.6; (C) effect of pH on the activity of amylase from oat (*Avena sativa*) seeds. The pH profiles, determined at 55 °C, were ranging from 3.6 to 10.5. Relative activity is expressed as a percentage of the maximum pH activity of the enzyme.

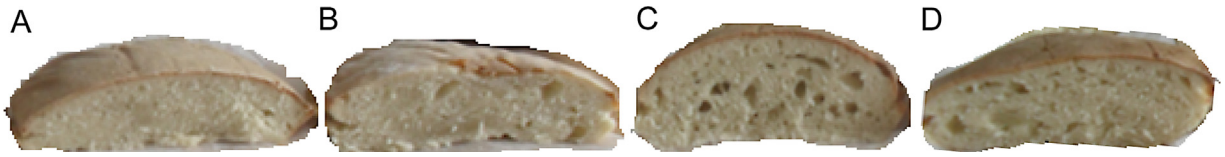

Fig. 3. Bread slices treated with different levels of amylolytic activity from crude oat extract. A: Control bread (0 U/g); B: bread made with 0.12 U/g; C: bread made with 0.24 U/g; D: bread made with 0.48 U/g.

structure, resulting in a better retention gas capacity under fermentation and consequently a higher specific volume. Thus, the loaf volume increase significantly due to the increase of the number of holes (gas cells) produced within fermentation. The enhancement of gas retention capacity of fermented dough could favour a better texture and volume of bread. It also permits a decrease in use of yeast and time requirements for dough fermentation [30]. Fig. 3 shows that dough with added 0.24 U/g of amylolytic activity (C) presented a better profile of gas retention overall than in the control test (A). In fact, it was demonstrated that the added amylases increase the level of fermentable and reducing sugars in flour and dough, thus promoting yeast fermentation and the formation of Maillard reaction products, which, in their turn, intensify bread flavor and crust colour. However, amylase functionality may also be related to the reduction of dough viscosity during starch gelatinisation, thus prolonging oven rise and resulting in an increased loaf volume [2].

3.5. Texture profile analysis

Amylases are routinely added to wheat flours to optimise the falling number, as well as antistaling agents to retard crumb hardening caused by rearrangements in the starch network and changes in water distribution [41]. Mechanisms of action include the overall weakening of the starch networks by endo-acting amylases and the prevention of amylopectin recrystallisation side chain cleavage by exo-acting amylases [2]. The release of fermentable sugars increases the yeast activity and produces precursors for the Maillard reaction [42]. In addition to the many functions of amylases, also proteases, lipases and oxidative enzymes which can be present in the crude oat extract improve bread volume and crumb grain by the modification of bulk and surface rheological properties [42,43].

The results of TPA analysis are displayed in Fig. 4A. In fact, we note an increase of the upper crust hardness values, from 3.4 N for the control bread to 4.5 N for the amylase treated bread with the concentration of 0.48 U/g. This increase can be explained by two phenomena: the caramelisation and the Maillard reaction [44]. Indeed, after several minutes of cooking, the surface of the dough dries up to 130–160 °C and the two phenomena of browning appear and therefore enhanced by the release of reducing sugars by the amylase activity from the oat extract leading to the crust hardness increasing. On the other side, slightly decrease in crumb hardness was observed after crude oat application. The results for elasticity showed no significant difference between the different types of bread at the crust and the crumb of the samples (Fig. 4A) suggesting that our oat extract does not alter the textural properties of wheat flour. During storage time, crumb and crust hardness increases, but the addition of the enzyme extract could affect positively the bread shelf life (Fig. 4B). Indeed, after 30 h of storage, the control bread recorded the highest crumb hardness. When bread is stored, water

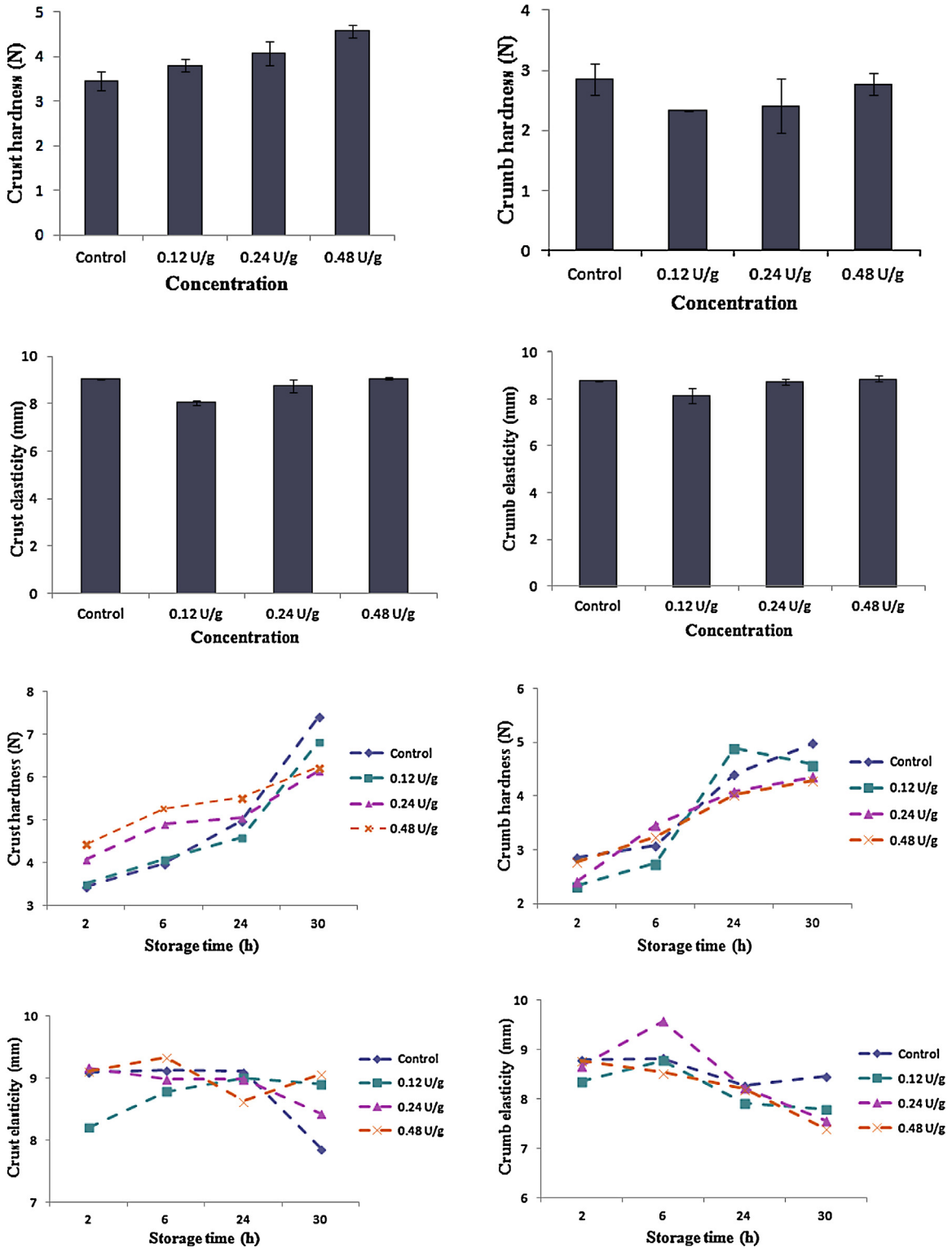

Fig. 4. (A) Crumb and crust texture profile analysis of crude oat extract treated breads; (B) crumb and crust hardness and elasticity during storage time of crude oat extract treated breads.

migrates from crumb to crust, leading to an additional reduction of the crumb moisture content. As such, the local moisture content of the gluten network can drop below the critical point for gluten to be fully plasticised. The resulting increased stiffness of the gluten network also contributes to the increase in crumb firmness during storage [31]. In fact, during this period, the aromatic compounds of the crumb will migrate to the crust and inversely the specific compounds formed in the crust are found in the crumb. During storage, starch tries to regain its original form (retrogradation of the starch) which hardens the crumb. Furthermore, the water migrates toward the crust which causes desiccation and hardness of the crumb and therefore it loses flexibility and elasticity, which worst the appearance and the taste of bread [44]. The important role of amylase consists of slowing the water exchange

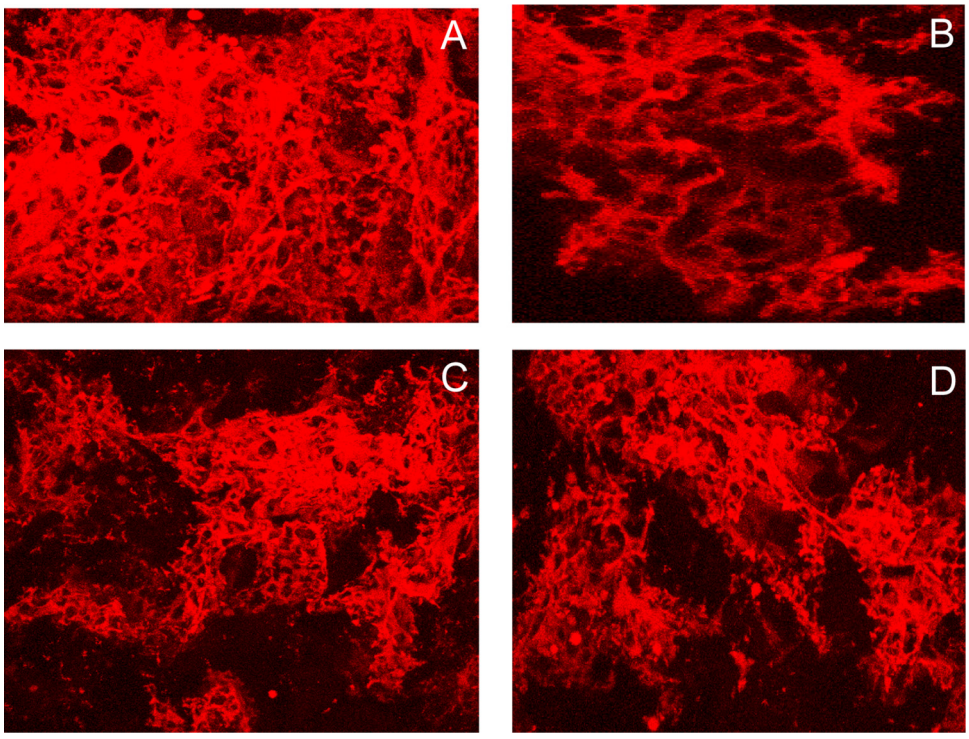

Fig. 5. 3D elaboration of CLSM images of wheat bread crumb (40× magnification): (A) control bread (0 U of enzyme); (B) 0.12 U of oat extract enzymes/g; (C) 0.24 U of oat extract enzymes/g; (D) 0.48 U oat extract enzymes/g. Proteins are stained red together with yeast cells which appear round shaped. The control bread shows a continuous protein network, characteristic only of wheat breads. The addition of the enzymes results in a crumb with a large number of cavities produced by the carbon dioxide released by the yeast.

between the crumb and crust. Bosmans et al. [31] also demonstrated that crumb firmness was largely impacted by formation of amylopectin crystals during storage. This was reflected in the linear relation between crumb firmness and amylopectin retrogradation. Moreover, Goesaert et al. [2] reported a coherent view on the bread crumb firming during storage and amylase anti-staling properties. During bread storage, the gelatinised starch (amylopectin) network, present in soft, fresh bread, is gradually transformed into an extensive, partially crystalline, permanent amylopectin network, with amylopectin crystallites acting as junction zones. This network increasingly accounts for the bulk rheological behaviour of aging bread crumb. Furthermore, as amylopectin retrogradation proceeds, moisture migration within the crumb structure occurs, and more and more water is immobilised within amylopectin crystallites. The crystalline hydrate water can no longer plasticise the different networks, which goes hand in hand with increased crumb firmness and decreased crumb resilience, due to a less flexible gluten network.

The elasticity was investigated during 30 h. The results obtained show that during the first 2 hours elasticity is almost constant with different enzyme concentrations at the crust. However, after 30 h of storage, it increases to record a maximum value of 9.07 mm in 0.48 U/g oat amylase added bread, while in the control bread, crust elasticity was lesser than the treated breads. Otherwise, no significant differences were recorded for the crumb elasticity in the different breads and it decreased during storage (Fig. 4B). Goesaert et al. [2] reported also that the efficiency of anti-staling amylases can be related to the extent they limit the formation and the strength of the permanent amylopectin network, and the water immobilisation. Conventional alpha-amylases weaken the amylopectin network by cutting the long polymer chains connecting the crystalline regions, but have little effect on amylopectin recrystallisation. In contrast, maltogenic alpha-amylase primarily shortens the amylopectin side chains, thus hindering amylopectin recrystallisation, and the concomitant network formation and water immobilisation.

3.6. Confocal laser scanning microscopy

To gain a deeper insight on the effect of crude oat extract on the functionality of the wheat flour, CLSM was used to investigate the microstructure of the bread crumbs. The glycoprotein networks of the crumbs appear red in the micrographs (Fig. 5). Analysis of bread crumb CLSM images revealed clear differences for oat amylase-treated breads and the control bread (Fig. 5). The micrographs of breads prepared with increased levels of oat amylase looked very similar with a fibrous, continuous protein network and a high number of holes (Fig. 5C and D). In control bread, glycoproteins appeared to be distributed in aggregates with lesser holes (gas cells) (Fig. 5A). We can conclude that our extract containing the amylolytic activity helped to liberate simple sugars (e.g. glucose) which are more easily assimilated by yeast and in turn may stabilise gas cells and may therefore be the reason for the higher bread volume of the oat extract breads observed in this study. The macrostructure observation of the slices of breads (Fig. 3) is with accordance of the microstructure one (Fig. 5) that shows the presence of a larger number of gas cells in the crumb of bread with increased concentration relative to the control bread. These cells are produced by the carbon dioxide released by yeasts within fermentation that expands the dough by creating multiple cells and gives it its soft properties.

3.7. Sensorial evaluation

Consumer's acceptability studies of breads enriched with different concentrations of crude oat extract preparations are very important for the introduction of these products to the market. This acceptability could be evaluated by several parameters such as colour, odour, texture, taste, touch and overall acceptability.

Results of sensorial evaluation of breads (0 U/g, 0.12 U/g, 0.24 U/g and 0.48 U/g) were investigated. From this result we can classify the breads by ascending order based on consumer's preference. Indeed, globally (based on the overall acceptability) panelists prefer breads with 0.24 U/g and 0.48 U/g.

4. Conclusion

The cultivated oat (*A. sativa* L.) which is one of the crops grown by mankind for the longest time as a source of food and feed is abundant all over the world especially in Europe and America and in Tunisia is very used in agriculture. Butt et al. [45] illustrate that the incorporation of whole oat grains or extract from oat in the food products improves not only the nutrition but also a therapy against various diseases. The data presented in the study of Sørensen et al. [46] demonstrate the application of oat seed extracts for the preservation of rye bread.

This work has revealed that the response surface methodology was a useful tool to determine the optimal experimental conditions for the extraction of amylase activity from oat. The ratio seed weight/buffer volume proved to be the principal factor that affects the yield of the amylase extraction. The selected optimal conditions (ratio 0.1, germination days 10 days, temperature 20 °C and pH 5.6) have been checked and confirmed by supplementary experiments using these optimal conditions. The experimental response value (89.82 U/g) was found to be in good agreement with the predicted one (91.88 U/g). Enhanced extraction of oat amylase by using the statistical methodology outlined in this paper will help in various biotechnological applications at industrial levels. The extracted oat amylase with maximal activity recorded in the pH 5.6 and at 55 °C, ameliorates the bread quality as evidenced by the sensory evaluation. In fact, it enhanced the textural properties of fresh and stored breads. Results are of much interest for further investigation of crude oat extract as an additive in bakery product formulations.

Aqueous seed extracts from oat meet absolutely no restrictions and the use of oat seed extract would be very interesting so oat is ecological, cheaper than other plants and don't require many step of purification. Therefore it is recommended to using oat extract in food industries.

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