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Effect of Enzyme Blends and Dough Strengthening Emulsifier on Extending the Shelf Life of Sandwich Bread Applying Response Surface Methodology

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Abstract - Nowadays enzyme use has been reconsidered and refined as contributing to additive reduction and product optimization. Different enzymes, such as lipases and xylanases are part of this effort. The present study aims at the investigation of the influence of amylase-xylanase, xylanase-lipase and the possible replacement of the emulsifier (DATEM) used in sandwich bread. The physical and thermo-physical properties of fresh and cold stored samples were investigated. Response surface methodology was used and different breads were prepared containing the above ingredients at various concentrations. A-amylase-xylanase influenced the crumb firmness, color and shape uniformity of cold stored breads. The impact of DATEM or xylanase-lipase on the quality characteristics of fresh and cold stored breads was less pronounced, with the main DATEM disadvantage being the whitish crumb color of stored loaves.

Index Terms – bread, enzymes, response-surface methodology

I. INTRODUCTION

Square-sliced sandwich bread is the most common commercial type of bread provided and distributed by super markets nowadays. Its convenience is closely related to the following factors: it is easy to find, versatile and its quality characteristics (e.g. freshness) can be maintained for a longer period compared to traditional loaf bread. In order to extend its shelf life, a number of ingredients have been tested and used including hydrocolloids, emulsifiers, shortenings, different flour mixtures and four enzymes in different storage conditions [1]-[3]. The main advantage of enzymes is that they can be used in very small quantities and can be inactivated after baking. For example, fungal α-amylase is inactivated at 75 ˚C in 10 min [4]. Although there are some naturally occurring types of enzymes found in wheat flour such as α- and β-amylases, proteases, and lipases, additional fortification is needed to achieve shelf life extension [5]. Furthermore, anti-staling agents, such as surfactants (complexing agents), α-amylase and hydrocolloids, are considered more effective additives [6].

The purpose of using enzymes such as amylases, lipases and xylanases in baking is to change the rheological properties of the dough, improve the quality of the final product (volume, color, structure of crumb, overall texture) and extend the shelf life of the bread i.e. retard its staling [7].In particular, amylases are widely used to increase the bread volume and reduce the staling rate of the crumb. According to Cauvain and Chamberlain [4] fungal α-amylase prolongs the period of dough expansion in the oven, increasing the maximum dough-piece height and loaf volume. However, it should be added with caution, because increased amount of it can cause dough stickiness and difficulties in dough handling [8]. The appropriate levels of fungal α-amylase have been reported to improve the crumb structure and texture of the final bread [9]-[11]. Some of the hydrolysis products of α-amylase are further fermented by the yeast, thus the increased bread volume may partly be due to the increased yeast activity [12]. The enzyme-induced changes in dough rheology are also a major reason for the increased bread volume. Owing to the softening of the dough it can expand more giving increased oven spring [13].

Amylases and xylanases improve bread characteristics and shelf life [14]. Xylanases are known to increase the specific volume and decrease bread firmness [15]. The degradation of xylans reduces the water absorption capacity of dough by releasing the water bound to xylans. This change causes softening but not stickiness of dough during processing. In addition, dough tolerance to fermentation, oven spring, and bread volume, shape and texture are improved. Combinations of xylanases with lipase reduce the softening effect and further improve volume, crumb texture and structure [9]. Commercial lipases for bread making are able to act on the low amount of triglycerides of the dough. Selected lipases are capable of hydrolyzing triglycerides into mono- and diglycerides. Monoglycerides form complexes with starch, which result in reducing of retro gradation and improved crumb softness [16]. Thus, it is possible to replace added emulsifiers such as monodiglycerides, DATEM partially or completely. A more recent study revealed that there was no significant difference between some lipase enzymes and DATEM as a bread volume improver [17]. In this way a product without any added chemical additive such as mono- or di-glycerides can be obtained.

DATEM, (Diacetyl Tartaric (Acid) Ester of Mono-glycerides) is an anionic emulsifier, which acts as a protein reactive ingredient used in crust breads to strengthen the dough by building a strong gluten network. Typically quantities from 0.375 to 0.5 % of the total flour

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weight are used in most commercial baked products [18] that promote a fine dispersion of the fat in the dough resulting in an increased volume of the finished product, regardless of the fat content. Using enzyme blends for bread making is not new. It is well known that xylanase used in combination with fungal amylase have synergistic effects. According to Si [7] a combination of xylanase and fungal amylase gives volume increase with better appearance without dough stickiness problem. A mixture of glycolipase and xylanase can improve the stability and dough handling and improve the structure of baked goods [16], [7]. Thus, the combined action of enzymes and emulsifiers is still of interest, as it can result in improved formulae. New enzyme types that influence both bread quality and staling process are found increasingly in the market. The purpose of this paper was to study the effect of some commercial enzymes (mixture of fungal amylase and xylanase, mixture of lipase-xylanase) and an emulsifier (DATEM) on the quality characteristics of the final quality of the sandwich bread by using Response-Surface Methodology (RSM).

II. MATERIALS AND METHODS

A. Materials and Baking Process

A high-gluten containing flour (coming from the milling of Hungarian winter hard wheat, Loulis Mills S.A., Sourpi, Greece), with protein content 13.5 % (d.b.), gluten content 34 %, moisture content 13.1 % and ash content 0.52 %) was used to prepare the dough mixture of bread loaves. The basic dough formula contained: vegetable shortening (Elais S.A., Athens, Greece), crystal sugar (Hellenic Sugar Industry S.A., Greece), compressed yeast (Zanae S.A., Greece), salt (Kalas S.A., Greece) and ascorbic acid (Danisco, USA, Inc.). The composition of the bread loaves' dough based on 100 g flour content was: vegetable shortening 2 g, yeast 2 g, salt 2g, sugar 2.5 g, ascorbic acid 3 mg, α-amylase-xylanase 6.5-9.0 mg/100g flour (60-90 ppm), xylanase-lipase 0-8.0 mg/100g flour (0-80 ppm), DATEM 0-0.8 g according to the RSM. A quantity of 2 kg of flour was used per batch. The amount of water of 64 g/100g of flour was used in all recipes according to farinograph consistency of 500 BU (Brabender GmbH & Co, Duisburg, Germany at 60 rpm, 30^{o}C). Depending on the formula DATEM (Panodan 526, Danisco, Denmark) was used as an emulsifier, as well as two commercial-type bakery enzymes: α-amylase-xylanase mixture (Veron SX, AB Enzymes, Darmstadt Germany) and a xylanase-lipase mixture (Grindamyl Power Bake 4100, Danisco, Denmark).

The bread making procedure followed consisted of 5 different phases. After the weighing, all ingredients, except for water, were blended together with a velocity of 60 rpm for 5min. Then water was added and mixing continued at 60 rpm for 3min and then at 80 rpm for another 6 min. The dough was left to relax at room temperature for 10min in order for the gluten strings to reach their final length. After relaxation, the dough was weighed, hand-moulded in loaves and put in rectangular (12x12x34cm) pans with a closed top and the pans were left in an environmentally controlled chamber at 35 ^{o}C and 75 % RH for 80min for proofing. Finally, the pans were brought to a commercial oven (Bongard S.A.S, Holtzheim, France) where loaves were baked at 200 ^{o}C for 60 min. The freshly baked loaves were left to cool in ambient conditions and sealed in polyethylene bags. Four bread loaves were prepared for each formula. The samples were divided in halves and the first half of each sample was measured the following day. Their characterisation as "fresh" is arbitrary, since in industrial bread making there is a period of several hours until sandwich bread is finally placed on the market shelf. Bread loaves halves remaining after the "fresh" halves were measured, were stored in a refrigerated incubator model FOC 225 (VELP Scientific, Milan, Italy) at 5 ^{o}C. Stored samples properties were measured after a 7day period.

B. Experimental Design

Statgraphics System V.2.1 (Stagraphics, MD, USA) software was used for experimental design implementation and a Response-Surface Methodology (RSM) was chosen with three independent variables taken into account.

Control samples without any emulsifier or enzyme were used in preliminary experiments. The addition of an emulsifier or α-amylase-xylane improved its physical characteristics, thus an experimental design in which the concentration of DATEM and/or two enzyme combinations has been built.

The three independent variables (experimental factors), were amylase mixture, emulsifier DATEM and the xylanase-glucolipase mixture added. The use of a Box-Behnken design model with three center points resulted in 15 design points (bread formulas, 13 combinations and 2 further replications of the center point). The combination of three factors (enzyme mixtures and emulsifier) studied in the RSM and their actual range amounts in accordance to the flour quantity are shown in Table 2. The design was created to find out the combined effect of these variables to the final product and not to compare them with a control sample without added enzymes or DATEM.

C. Baking Yield

Baking yield (BY) was obtained according to Eq. (1) right after baking and cooling in ambient temperature for an hour. A quantity of 655 g of dough (Wd) was used and the loaf was weighed again after baking and cooling (Wl). Baking yield represents the percentage of the weight of the final loaf after the loss of water occurring when baking is carried out. Thus, higher values of BY indicate a smaller water loss due to evaporation and consequently heavier bread loaves. An average value for each recipe was determined out of four loaves.

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$BY = W_1/W_d \times 100$ (1)

Where, W_d is the weight of the dough before baking (g) and W_1 the weight of the loaf after baking and cooling (g).

D. Moisture Content

Moisture content was calculated according to AACC 44-15A method [19], for both fresh and stored loaves, using an oven (Memmert GmbH, Model U, Schwabach, Germany).

E. Crumb Firmness

The firmness of the bread crumb was evaluated according to AACC 74-09 method [20]. Experiments were performed using a Universal Testing Machine (Instron 1011, Massachusetts, USA) and a cylindrical probe with a die of 40mm diameter was used. Four specimens (2.5 cm slices) from each formula (fresh and stored loaves) were tested and the maximum force during compression was recorded representing the value for firmness. Central slices from bread loaves were used.

F. Color

Hunter Lab parameters were measured using a Minolta colorimeter (CR-200, Minolta Company, Ramsey, NJ, USA). Four specimens (bread slices) from each formula were used to calculate the L/b ratio and color difference dEab of the crumb.

$$dE_{ab} = \sqrt{(dL)^2 + (da)^2 + (db)^2} \quad (2)$$

The dL, da και db, parameters represent the differences between the specimen L, a, b values and the white calibration ceramic plate of the Minolta colorimeter with the following characteristics: L=93.4, a=-1.8 και b=4.4. Where a represents redness (positive values) versus greenness (negative values), is positive for yellow and negative for blue color and L is a correlate of lightness scaled between 0 (black) and 100 (white). Measurements were performed for both fresh and stored loaves and the L, a, b parameters represent the average of five different points on the bread crumb slice out of four slices per formula.

G. Shape and Crumb Grain Uniformity

Scanned sliced bread high quality images (24 bit, 600ppi) were obtained with a scanner (Hewlett Packard Scanjet 4370) device. Four specimens from the centre of the loaves (1cm thick slices) were used from every formula and crumb grain uniformity [21] as well as the height/width ratio (H/W) were calculated using image analysis software (Image Pro Plus V. 1.6, Media cybernetics, MD). Crumb grain uniformity calculated as the ratio of number of small to large cells using a cell area threshold of 4.00 mm2, larger values denote a more uniform cellular structure. The cell area of 1mm2 was set as minimum for measurements, which means that a cell smaller than 1mm2 was not taken into account. Four specimens were used from every formula for both fresh and stored loaves.

H. Thermal Analysis

Samples of the bread crumb core were pressed and portions of approximately 9 mg were weighted in a hermetically sealed aluminium pan and analysed with a calorimeter (DSC Q100, TA Instruments, California, USA). The DSC was calibrated using mercury, distilled water and indium. An empty pan was used as reference. Samples were fast cooled from 25 to -40oC, held at this temperature for 5 min and heated at 5oC/min from -40 to 150oC. Dry nitrogen gas flow of 20 ml/min was used to minimize water condensation in the measuring cell.

The initial (Tstart), onset (Tonset), peak (Tmax) and conclusion (Tstop) temperatures and the transition enthalpy (J/g) of ice melting were calculated. Freezable water (FW) weight fraction was obtained from the relationship between the enthalpy of ice melting and latent heat of ice melting (334 J/g) [22] using Eq.(3) . Each measurement was performed at least three times.

FW (g/100g of total water) = [Peak enthalpy x (latent heat of fusion of ice)-1 x (g total water /g sample)-1] x 100. (3)

J. Statistical Analysis

Statistical analysis was performed using the Statgraphics Statistical Graphics System, V. Centurion XV (Statgraphics, Rockville, Md., USA). Fisher's LSD was used to determine significant differences between samples. A multiple variable analysis was performed in order to create 3D surface plots. A p-value of less than 0.05 was considered significant.

III. RESULTS AND DISCUSSION

A. Box-Behnken Design Model Coefficients

The significant coefficients of the multiple regression equations of the design used are presented on Table 1 for fresh and stored samples respectively. The factors of the Box-Behnken design model were the three independent variables (enzymes and DATEM) and the response variables the properties investigated (baking yield, crumb moisture content, firmness, color, shape uniformity and crumb grain uniformity). Significant coefficients ($p<0.05$) can be seen only for baking yield, firmness, color and shape uniformity, while data and surface plots for each quality characteristics separately are presented thereafter. The greatest effects on the quality characteristics of the breads were observed when α-amylase-xylanase was used, as significant coefficients of first-order, quadratic and second-order interaction factors can be seen. Furthermore, only the color from the quality characteristics measured was affected by all variables investigated.

B. Baking Yield

Baking yield was in the range of 84-89.9% and was significantly affected by DATEM, xylanase-lipase

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concentration and their second-order interactions (Table 1). As shown in Table 2, the formulation that contained only α-amylase-xylanase, at 6.5 mg/100g, presented the highest baking yield value ($p<0.05$). Increasing the xylanase-lipase mixture quantity or DATEM at the same α-amylase-xylanase level (65 ppm) resulted in a decrease in baking yield, although variations among the most of the combinations were slight. A possible explanation for the baking yield reduction in the presence of increased amount of xylanase could be an over-hydrolysis of both soluble and insoluble araboxylan that results in a loss of water holding capacity [23]. This is consistent with the reduced values of moisture content of samples that contained an increase amount of xylanase (Table 2).

C. Moisture Content

Moisture content of fresh samples was between 38.6-41.9% (Table 2). Differences in values were slight, and statistical differences among samples of different composition were noticed in only few cases ($p<0.05$). Furthermore, no significant effect of design factors (enzymes or DATEM) on moisture content of final baked breads was found (Table 1). Due to cold storage, moisture content decreased from 1.2-7.6%. The slightest differences between fresh and stored samples were found in samples containing the three design factors at moderate concentrations. The addition of amylase-xylanase at low concentration (40ppm) combined either with DATEM or xylanase-lipase, again at low concentrations (0.4 % and 4.0 mg/100g respectively), resulted in the greatest water loss values during storage (e.g. 7.4 or 7.6%).

Moisture loss and specifically moisture migration from bread crumb to crust can increase the bread staling. Furthermore, a redistribution of water is often correlated to a change in free water content. There is a correlation between dehydration and texture firmness increase and it has been noticed that a reduction of dehydration rate is more effective in preventing staling than the increase in the initial moisture [24].

D. Crumb Firmness

Firmness values with respect to composition can be seen in Fig. 1a. Firmness values ranged from 1.0 N-4.7 N in fresh samples to 3.0-11.5 N in stored samples, thus a significant increase in firmness (threefold greater values approximately) of stored breads was observed. This increase was much greater than the moisture decrease in storage, suggesting that apart from dehydration and water redistribution a staling process was evident.

There is a quadratic effect of amylase-xylanase on firmness (see also Table 1), which can be seen in the following surface plots (Fig. 1b, 1c). It can be seen that this quadratic effect of α-amylase-xylanase on bread texture is observed in both fresh and stored samples, but in stored samples there is also a linear effect of α-amylase-xylanase on bread firmness (Table 1). For both fresh and stored samples there is a maximum in firmness values at a concentration of 65 ppm amylase-xylanase and then a marked decrease in their values for greater concentrations (9.0 mg/100g of amylase-xylanase), suggesting that bread softness can be changed significantly by changing the amount of α-amylase-xylanase, regardless of storage. DATEM and xylanase-lipase mixture do not seem to affect bread firmness compared to α-amylase-xylanase effect.

A-amylase is well known to be an effective anti-stalling agent. In recent studies a mixture of α-amylase with other enzymes (xylanase and lipase) has proved to be effective in reducing bread staling in high-fibre bread [25]. A phase separation between amylopectin and amylose can occur with the use of α-amylase and its mixing with other enzymes. Thus, cross-links and entanglements between amylose and amylopectin are avoided. Furthermore, α-amylase reduces the connectivity between the crystallites in the continuous starch phase, in spite of the fact that crystallinity increases during storage. This is an anti-firming action, although there is the risk of structure collapse. In the presence of α-amylase, this risk is reduced by the formation of kinetically stabilized starch networks that reduce crystallization rate [26]-[27]).

It is also known that xylanases promote dough softening by breaking down soluble pentosans [28], and improve the final bread texture, resulting mainly in softer samples [8], [29]-[31]). Xylanases are also effective as anti-staling agents as they decrease crumb-firming rate during storage [30], [32]. However, the combined addition of amylase and xylanase did not always result in further bread quality improvement [33]. In the present study it is the amylase-xylanase amount in the concentrations investigated that predominates and mainly controls the staling process.

E. Color Parameters

Color difference values (dEab) for fresh and stored samples can be seen in Fig. 2a. As shown in Fig. 2b and Table I, in fresh samples, color difference dEab was affected mostly by DATEM and amylase-xylanase content as well as by the single effect of xylanase-lipase. Thus, color (dEab) is influenced by all ingredients used in a more complicated way than other attributes. As α-amylase-xylanase amount increased, the color difference (dEab) of bread crumb in fresh loaves increased also, due to its quadratic effect on color values and on the second order interactions with DATEM. As a result, breads containing high α-amylase-xylanase amount appeared to be less white in comparison to those with less α-amylase-xylanase. On the other hand, a moderate amount of DATEM resulted in bread crumb with lower dEab, in other words in a whiter crumb. Thus, α-amylase-xylanase and DATEM resulted in more or less yellowish samples respectively and their combination at low α-amylase-xylanase and medium DATEM amount resulted in the most whitish breads (Fig. 2b). This has also been observed in other studies, when DATEM addition contributes to preserving the whiteness of breads [34], [35]. However, increasing DATEM concentration

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resulted in dEab increase as well, but in a lower extend than by increasing α-amylase-xylanase concentration.

In stored samples dE_{ab} was not significantly influenced by the factors investigated. However, the parameter L/b representing the yellowish appearance of the crumb was significantly affected by α-amylase-xylanase addition in a linear way (Fig. 2c). Xylanase-lipase mixture or DATEM did not contribute to this effect. As a result, formulas that contained more α-amylase-xylanase still had a yellowish hue after storage, suggesting that α-amylase-xylanase can preserve color during storage.

F. Grains' Crumb and Shape Uniformity

Crumb uniformity differed depending on the composition, as it can be seen for fresh samples in Fig. 3a. The lowest values were noticed in the case of samples that had a high α-amylase and DATEM content (90 ppm and 0.8%) and medium xylanase (4.0 mg/100g) while the highest values were noticed in samples with moderate concentrations of the three variables or those which had high α-amylase-xylanase and low xylanase content. According to researchers the combination of amylase and xylanase enhanced the specific volume of breads and xylanase alone improved bread shape [29], [36].

The height/width ratio is considered as a criterion for bread crumb elasticity and cohesiveness and its values may be higher than one, as also in breads studied [37]. The loaves were baked in top square pans, thus the width of the pan should not change. Significant differences between samples were not evident ($p<0.05$), especially in fresh samples. The height/width ratio of stored samples is affected by DATEM and amylase-xylanase content, while xylanase-lipase mixture presents no significant contribution to this effect ($p>0.05$) (Table I, Fig. 3b). Amylase-xylanase has a quadratic effect on H/W. Specifically, up to a concentration of 65 ppm of α-amylase-xylanase a positive relation of amylase-xylanase concentration to H/W can be seen (Fig.3b), a fact that was also noticed by other researchers as well [37].

G. Thermal Analysis

Thermal analysis experiments using DSC were performed with samples that contained α-amylase-xylanase at moderate concentration (6.5 mg/100g), that found to be a critical concentration for quality characteristics of bread, alone or combined either to DATEM (0.8%) or to xylanase-lipase (8.0 mg/100g). The coded numbers of formulas used were 15, 14 and 7 respectively. The FW for these samples was calculated using Eq. (4) and it was also calculated in a sample weight basis. The initial temperature of ice-melting (T_{start}), onset temperature (T_{onset}) and peak temperature (T_{max}) are shown in Table III as well. Differences in temperature values were slight among samples (no statistical differences at $p<0.05$). Differences due to storage were also slight. In order to observe significant differences a longer storage period might be required [38]. The FW represents the water amount that can be phase separated within the crumb matrix and can form detectable crystals when cooled [39]. It may therefore be considered as the available water for chemical reactions and microbial growth that lead to product deterioration. The FW determined for the samples investigated ranged from 49.0-57.3 for fresh to 50.2-56.0 (g/100g total water) for stored samples. FW decreases over storage time in bread samples, suggesting that water in bread becomes more bound or immobilized during staling[40], [41]. However, in some cases, due to crumb matrix weakening during storage, which makes water re-available, FW increases [42]. A decrease in freezable water was observed in samples that contained only α-amylase-xylanase ($p<0.05$). In the other samples the amount of freezable water was almost constant ($p<0.05$), suggesting that the combined effect of two different enzymes or of an enzyme with an emulsifier can be quite effective in keeping FW constant during this short storage period.

IV. CONCLUSION

In sandwich breads containing a mixture of enzymes and DATEM, α-amylase-xylanase mixture predominated in affecting most of the quality parameters of samples investigated. It affected firmness, loaf shape uniformity, and color of both fresh and stored samples, whereas xylanase-lipase and DATEM had a lower contribution to the above properties. Concerning color, DATEM addition resulted in whitish samples. In firmness values its contribution was negligible, thus a reduction in the amount used can be proposed. A-amylase-xylanase alone resulted in breads with a decreased amount of freezable water, a fact that can be ascribed to a staling process during storage. On the contrary, its combination with DATEM and especially with xylanase-lipase resulted in constant freezable water contents before and after storage. Further research is required for different additives combinations in narrow amylase-xylanase concentrations of 6.5 mg/100g.

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APPENDIX

TABLE I: Significant Coefficients of the Design Factors (Independent Variables) of the Box-Behnken Stepwise Fitting Model for Dependent Quality Characteristics of the Produced Fresh and Cold Stored Breads[a]

	Fresh samples			Cold stored samples		
Factors	Baking yield (%)	Firmness (N)	Colour (dE_{ab})	Firmness (N)	Colour (L/b)	Shape uniformity (H/W)
Constant	0.89	-4.41	31.89	-8.34	4.94	0.85
AX[b]			-0.20**	0.55*	0.02*	
DA[b]	$-7.8\cdot10^{-2}$**					$6.6\cdot10^{-2}$*
XL[b]	$-2.6\cdot10^{-4}$*		$-1.3\cdot10^{-2}$**			
AX^2		$-1.8\cdot10^{-3}$*	$1.2\cdot10^{-3}$*	$-4\cdot10^{-3}$*		$-3.06\cdot10^{-5}$*
AX•DA			0.15*			
AX•XL						
DA^2			9.74**			
DA•XL	$1.25\cdot10^{-3}$ ***					
XL^2						
R^2	95.89	84.48	97.27	87.20	70.85	85.54
SEE[c]	$4.9\cdot10^{-3}$	0.61	0.47	1.38	0.13	$4.9\cdot10^{-3}$

[a] *significant at p<0.05, ** significant at p<0.01, ***significant at p<0.001

[b] AX: amylase-xylanase mixture, DA: DATEM, XL: xylanase-lipase mixture

[c] SEE: the standard error of the estimate as a measure of the accuracy of the predictions

TABLE II: Baking Yield, Moisture Content of Fresh and Cold Stored Bread Crumb and Water Loss (%) as Affected by Storage[a,b,c]

Run	Amylase-xylanase mixture (ppm)	DATEM (% flour based)	Xylanase-lipase mixture (ppm)	Baking yield (%)	Fresh samples (gH_2O/100g product, wet basis)	Cold stored samples (gH_2O/100g product, wet basis)	Water loss (gH_2O/100g bread)
1	40	0.4	0	0.861 (0.011)^{cde}	39.5 (1.4)^{abc}	36.5 (1.0)^{ab}	7.6*
2	40	0	40	0.871 (0.011)^{e}	40.3 (1.3)^{bcd}	37.3 (0.7)^{abc}	7.4*
3	40	0.8	40	0.859 (0.010)^{cde}	39.9 (0.4)^{abcd}	38.5 (1.0)^{cd}	3.6
	90	0.4	80	0.857 (0.009) ^{cd}	39.1 (0.6)^{ab}	37.9 (1.6)^{bcd}	3.2
5	90	0.8	40	0.865 (0.009)^{cde}	39.9 (1.3)^{abcd}	38.7 (0.5)^{cde}	3.0
6	65	0.4	40	0.857 (0.007)^{cd}	38.6 (1.5)^{a}	36.5 (0.1)^{a}	5.4*
7	65	0	80	0.844 (0.004)^{ab}	39.5 (0.4)^{abc}	36.7 (0.6)^{ab}	7.1*
8	65	0.4	40	0.868 (0.009)^{de}	40.7 (0.4)^{cde}	40.2 (0.4)^{f}	1.2
9	65	0.4	40	0.868 (0.008)^{de}	40.6 (0.4)^{cde}	40.1 (0.4)^{ef}	1.2
10	90	0	40	0.886 (0.000)^{f}	40.8 (0.4)^{de}	39.0 (0.9)^{def}	4.6*
11	90	0.4	0	0.868 (0.004)^{de}	41.9 (0.7)^{e}	39.0 (1.2)^{def}	6.9*
12	65	0.8	80	0.865 (0.011)^{cde}	40.3 (0.5)^{bcd}	38.8 (1.5)^{cde}	3.6
13	40	0.4	80	0.855 (0.009)^{bc}	39.3 (0.4)^{ab}	38.1 (1.3)^{cd}	2.9
14	65	0.8	0	0.840 (0.011)^{a}	39.6 (1.6)^{abcd}	37.9 (0.4)^{abcd}	4.1
15	65	0	0	0.899 (0.007)^{g}	40.2 (0.4)^{bcd}	39.0 (0.9)^{def}	3.0

[a] In parentheses standard deviation values.
[b] Samples within the same column with different letters differ significantly (p<0.05), runs 8,9: central points
[c] * difference between initial and final moisture content. Samples with an asterisk had a significant lower moisture content value after storage compared to the initial one

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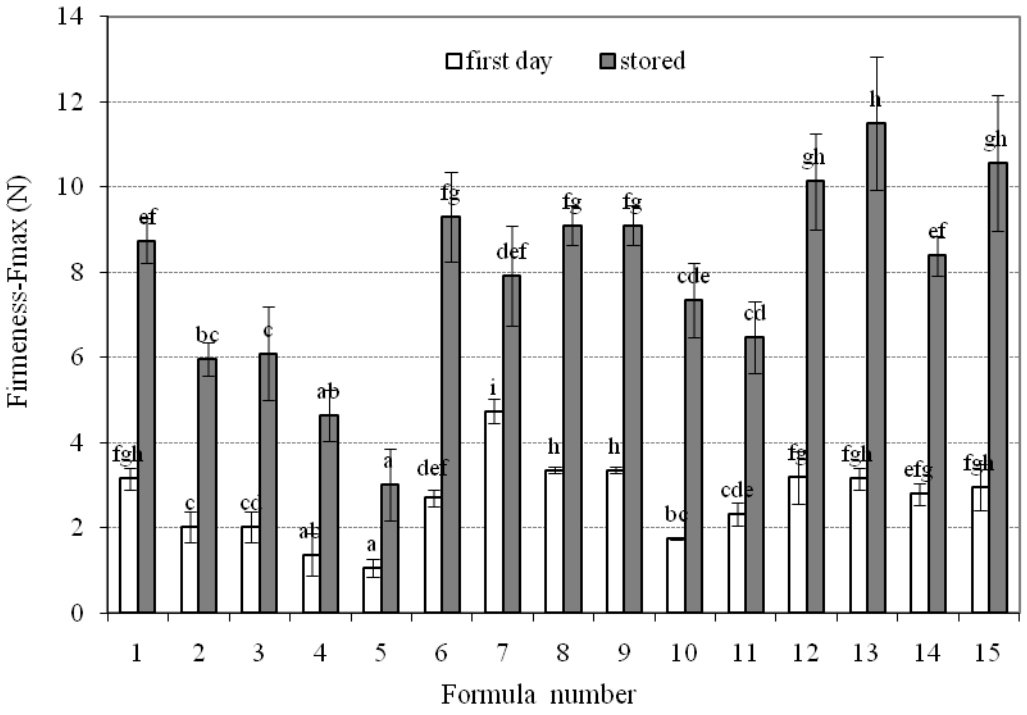
TABLE III: Thermal Characteristics of Fresh and Cold Stored Sandwich Breads that Contained α-Amylase-Xylanase Mixture Combined with Xylanase-Lipase Mixture or DATEM[a]

Formula variables				Thermal characteristics					
Amylase-xylanase mixture (ppm)	DATEM (%)	Xylanase-lipase mixture (ppm)		T_{start} (◦C)	T_{onset} (◦C)	T_{max} (◦C)	T_{stop} (◦C)	FW (g/100g of total water)	FW (g/100g of sample)
Fresh samples									
65	0	0	-22.10 (1.10)^{ab}	-9.54 (0.60)	-4.11 (0.13)	-0.65 (0.63)	57.3 (4.8)^{b}	23	
65	0.8	0	-22.59 (0.30)^{a}	-10.15 (0.60)	-4.51 (0.18)	-1.39 (0.30)	52.0 (3.1)^{ab}	20.59	
65	0	80	-20.40 (1.10)^{bc}	-10.90 (1.20)	-4.50 (0.65)	-1.29 (0.68)	49.0 (2.5)^{a}	19.35	
Cold stored samples									
65	0	0	-20.10 (0.21)^{c}	-10.43 (0.09)	-4.44 (1.39)	-1.39 (0.42)	50.9 (1.8)^{a}	19.85	
65	0.8	0	-20.49 (1.26)^{bc}	- 9.48 (0.01)	-4.23 (0.06)	-0.79 (0.01)	56.0 (2.9)^{ab}	21.22	
65	0	80	-21.70 (0.80)^{abc}	-10.00 (0.50)	-4.36 (0.05)	-1.84 (0.21)	50.2 (0.05)^{a}	18.42	

[a]Samples with different letters in the same column differ significantly at $p<0.05$

Fig. 1. Influence of composition variables on crumb firmness A) raw data b) fresh samples using RSM plot c) cold stored samples using RSM plot

A



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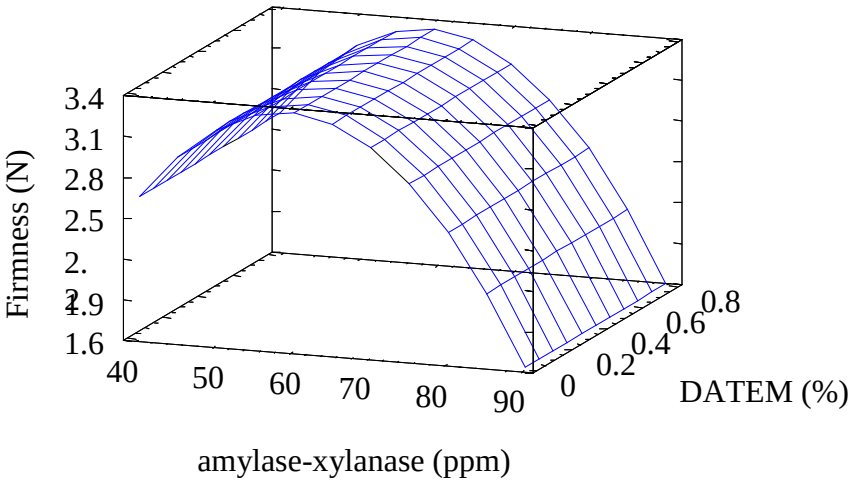

B
c

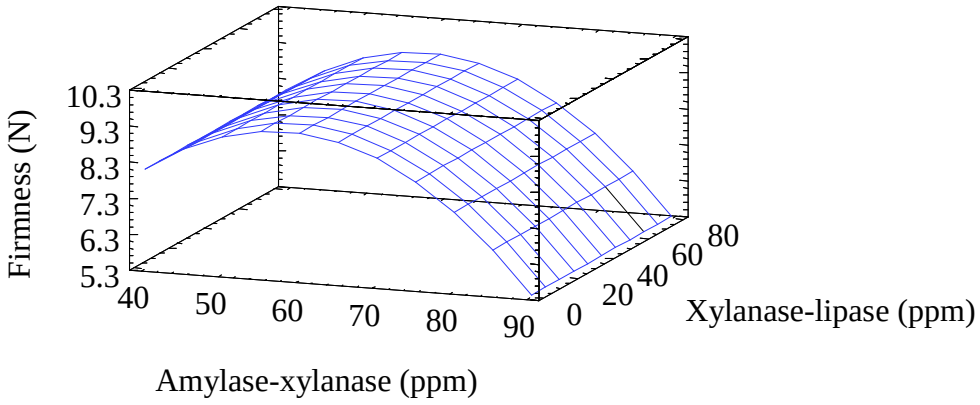

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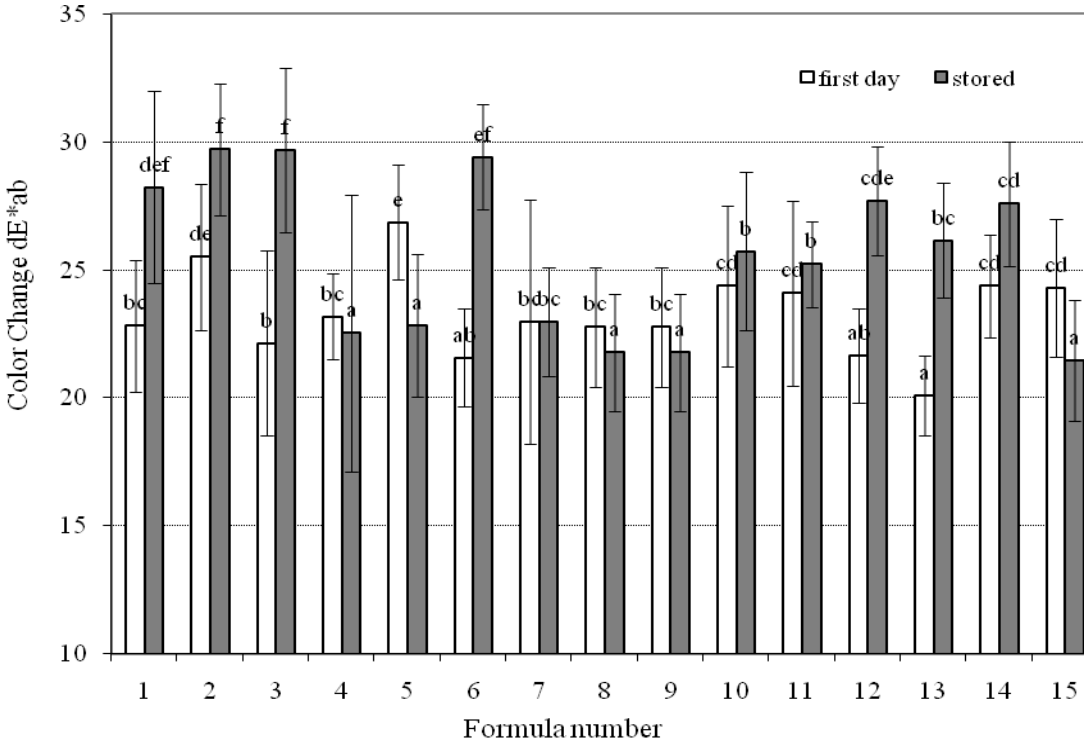
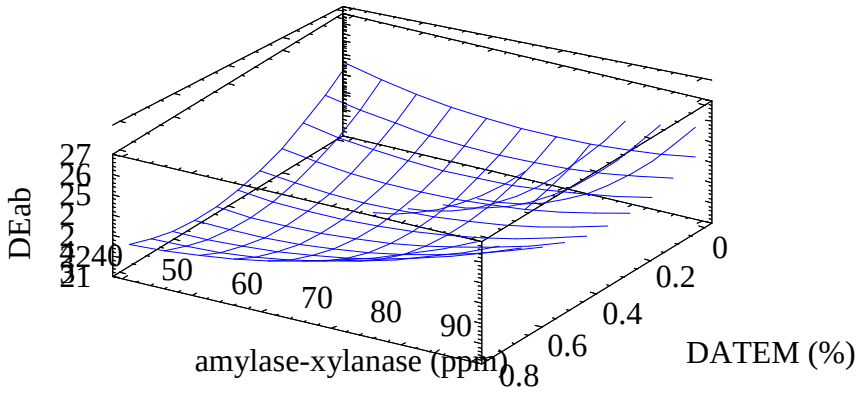
Fig. 2. Influence of composition variables on sandwich bread crumb color of A) raw data B) dE_{ab} of fresh samples using RSM plot c) L/b of cold stored samples using RSM plot

A



B



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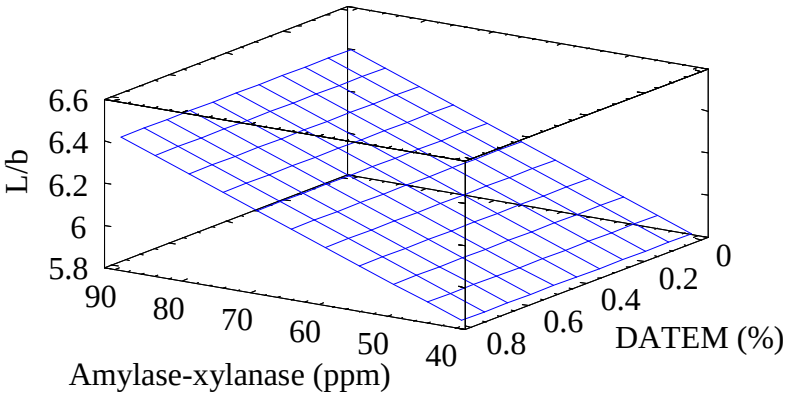

Fig. 3. A) Crumb grain uniformity of fresh sandwich bread B) Shape uniformity of fresh and cold stored samples Coded names as inTable 2. Samples in the same column with different letters differed significantly ($p<0.05$)

A