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Effect of commercial lipase incorporation on technological properties of bread

[1]Santos, A. C., [1]*Morais, R. A., [1]Martins, G. A. S., [2]Carvalho, E. E. N., [2]Souza, A. R. M., [2]Miguel, K. O. R. and [1,2]Damiani, C.

[1]Department of Food Science and Technology, Federal University of Tocantins, 77001-090, Palmas, Brazil
[2]Department of Food Engineering, Federal University of Goiás, 74690-900, Goiânia, Brazil

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Abstract

Additives are used to improve bread quality; for this reason, lipase stands out, as it simulates the effect of emulsifiers. The present work aimed to verify the effect of commercial lipase addition on the physical and technological characteristics of the bread. The first stage used 3^3 factorial design namely three enzymes, five concentrations, and three fermentation times. The second stage used 2^3 factorial design, and stored over 28 days, with two formulations (control and with enzyme) and three storage temperatures. The PCA identified the variables with the most significance in PC_1: specific volume, volume, and variables related to texture and expansion. In the second stage (shelf life), the PCA revealed that the bread samples with enzymes in the formulation differed from the control samples. Based on the circle of correlations, enzyme samples were distinguished by lower chewiness, gumminess, and hardness. Microscopic analysis showed that the starch molecules were more uniform in the first 14 days. However, the starch molecules lost their conformational structure afterwards. Therefore, the incorporation of lipase improved the bread's technological parameters such as volume, texture, and structure.

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Introduction

Worldwide, bread is considered essential for human nutrition, providing macronutrients and micronutrients such as dietary fibres and vitamins (Lockyer and Spiro, 2020). Traditionally, bread is produced from wheat flour, water, salt, and yeast. However, due to the need to produce different types of bread with different functionalities, and the increase in mechanisation in the bakery industry, other components can be added to improve the characteristics of the dough during processing, and consequently, the quality of the final product. These additives include fats, sugars, emulsifiers, oxidising agents, and enzymes (Dahiya *et al*., 2020). For this purpose, the bakery industry has been considering the incorporation of commercial enzymes to improve the physical-chemical, thermal, and rheological properties of doughs in general. Bearing in mind that many of the existing chemical additives can harm health, the use of compounds generally recognized as safe (GRAS) in bakery products is a topic that arouses the interest of the population, producers, and researchers. In this sense, many enzymes are suitable for the bakery industry, especially amylases, cellulases, proteases, xylanases, and lipases (Dahiya *et al*., 2020; Haghighat-Kharazi *et al*., 2020; Both *et al*., 2021; Taddia and Tubio, 2022; Wu *et al*., 2022).

The functional and technological understanding of lipases has been continuously discussed. However, they are mainly based on the knowledge of the effects of lipids in baking (Dahiya *et al*., 2020). Lipases positively influence the physical characteristics of bread, particularly the specific volume, acting mainly on the lipid fraction, consequently increasing the number of molecules with emulsifying properties (Wang *et al*., 2018; Melis *et al*., 2019). As a result of the increase in bread volume, a more uniform, and therefore, softer crumb structure is obtained; they also modify the handling properties, increasing the stability and strength of the dough (Dahiya *et al*., 2020).

Lipase catalyses triglycerides into mono- or diglycerin, glycerol, and free fatty acids. Although

*Corresponding author.
Email: romuloitallo2505@gmail.com

the application of lipase in bread is a popular area of research in recent years, as well as other enzymes and emulsifiers that can help improve the characteristics of bakery products, it is worth emphasising that the concentration and number of enzymes added to bread reflect on the improvement of rheofermentative characteristics, water-holding capacity, and crumb structure (Huang *et al*., 2020). They also have the advantage of improving the processing of the dough, as well as the general quality of the bread, increasing the stability of the dough, resistance to extension and hardness, decreasing the viscosity of the dough, increasing the specific volume, improving the softness and structure of the crumb, and delaying retrogradation during storage (Melis *et al*., 2019). In this way, lipases make it possible to replace conventional emulsifiers, partially or fully in bakery products, promoting an improvement in the technological performance of bread in general. Until now, the use of lipases as bread improvers has been little explored. In this context, the main objective of the present work was to apply commercial lipases, and evaluate their effects on the technological characteristics of loaf bread during storage.

Materials and methods

Ingredients and reagents

Dona Benta® Type 1 wheat flour, Fleischmann® biological yeast, commercial granulated sugar, Cisne® refined salt, and ice water were used. All ingredients were purchased from local businesses in Goiânia, Brazil. The basic parameters of the flour used were moisture (14.59 g 100 g^{-1}), wet gluten (28 g 100 g^{-1}), ash (0.48 g 100 g^{-1}), colour parameter concerning brightness (92.64), and water absorption (57.50%). The enzymatic preparations (Lipopan F®, Lipopan XTRA®, and Lipopan Prime®) used as source of lipases were donated by the company Novozymes Latin América Ltd., and based on the manufacturer's information, they have enzymatic activities of 25 KLU g^{-1} (25 kg lipase units for each 1 g of enzyme), 7.2 KLU g^{-1} (7.2 kg lipase units for each 1 g of enzyme), and 10 KLU g^{-1} (10 kg lipase units for each 1 g of enzyme), respectively.

Bread preparation

All ingredients were weighed on an analytical scale (Marte®, model Ay220) to prepare the bread. Then, the enzymatic mixture (lipase, wheat, and sodium chloride), which was in the form of dry granules, was mixed with the other ingredients; first the dry ones followed by the wet ones. Next, the dough was homogenised in an automatic homogeniser (Master Bread NPF-53 Mondial 2970-01) for 10 min (a time defined by preliminary tests guaranteeing the shortest time and a homogeneous dough), and left to rest for another 10 min. Then, the dough was divided into three parts of approximately 220 g, shaped into an ellipse, and placed in an oven (Fanem®, model 515A) at 30°C with a relative humidity of 70% for the fermentation stage (1 h, 1 h 30 min, and 2 h 30 min). After this period, the doughs were baked in an electric oven (Layr®) preheated at 150°C for 23 min. After cooking and cooling, the bread was packed in low-density polyethylene plastic bags, and stored at 25°C until further analysis.

Experimental design for bread-making

In the first stage, the tests were conducted following the 3 × 5 × 3 factorial scheme, considering three types of enzymes (Lipopan F®, Lipopan XTRA®, and Lipopan Prime®), five enzyme concentrations (0.75, 1.5, 2.0, 2.25, 3.0, and 3.75 KLU g^{-1}), and three fermentation times (1 h, 1 h 30 min, and 2 h 30 min), and the treatments were compared with the control (without enzyme). Five repetitions were used for assessing the texture profile responses. Three repetitions were used for assessing the expansion index, dough fermentation, specific volume, colorimetric, number of alveoli, area, perimeter, and circularity.

Shelf life

For the evaluation of shelf life, a 2 × 3 experimental design was adopted over 28 days with evaluation every seven days (0, 7, 14, 21, and 28 days), with two types of formulations (control and with enzyme), and at three different storage temperatures (15, 25, and 35°C, stored in a biological oxygen demand (B.O.D.) incubator to maintain stable temperatures). Shelf-life data were subjected to analysis of variance, and then, the test of mean comparison to compare the storage times of the treatments. A comparison was also made between the means of the treatments, performing a multivariate analysis using the principal component analysis (PCA) to identify patterns of dispersion and similarity between the treatments based on the response variables.

Dough analysis

Expansion rate and specific volume

The expansion index and specific volume were determined according to Mudgil *et al*. (2016). First, the bread expansion rate (IE) was calculated using Eq. 1. Then, the quotient established the specific volume between the volume (cm^3) and the dough (g) of each sample supplied, with results expressed in $cm^3\ g^{-1}$ (Eq. 2):

$$Expansion\ rate\ (IE) = \frac{(dp+hp)}{2\frac{(dm=+hm)}{2}} \quad \text{(Eq. 1)}$$

$$VE = \frac{V}{m} \quad \text{(Eq. 2)}$$

where, dp and hp = diameter and height of bread after baking (cm); dm and hm = diameter and height of moulded doughs (cm); VE = specific volume ($cm^3\ g^{-1}$); V = volume (cm^3); and m = dough (g).

Number of alveoli and perimeter

The bread crumb structures were evaluated using digital images according to Juárez *et al*. (2008). The images were obtained by digitalisation at a resolution of 550 DPI in an HP ScanJet 2400 scanner in the central area of the crumb with a resolution of 900 × 900 pixels. The images were analysed using the ImageJ® 1.47v software (National Institute of Health, USA). These, in turn, were saved as files in JPEG format, and cropped for a field of view of 900 × 900 mm. Finally, colour images were converted to 8-bit grayscale, and the Otsu thresholding algorithm was applied. From this, it was possible to obtain the values of the number of alveoli and the perimeter.

Analysis of bread during shelf life

Texture profile

The texture profile of the bread was determined by the instrumental method in a benchtop texturometer (Texture Analyzer, TA-XT Plus, Surrey, England). Ten slices of bread were cut 15 mm thick for each treatment, and the external slices on both sides were discarded. For sample compression, an aluminium probe with a diameter of 35 mm and the following parameters were used: test velocity, 2.0 mm s^{-1}; pre-test velocity, 5.0 mm s^{-1}; post-test velocity, 5.0 mm s^{-1}; compression rate, 40% deformation; and an interval of 5 s between compression cycles, according to Carr and Tadini (2003). Each slice of bread was compressed twice to obtain texture parameters (hardness, elasticity, cohesiveness, gumminess, chewiness, and resilience). The analysis was performed in quintuplicate at a temperature of 25°C.

Colorimetric analysis

The colorimetry of the bread crumb was determined using Colorquest® Colorimeter, a standard of the International Commission on Illumination (CIE), calibrated with a standard white plate, following the manufacturer's instructions. Colour measurements were expressed numerically using the coordinates L*, a*, and b*, where L* is brightness, a* is the red-to-green coordinate, and b* is the yellow-to-blue coordinate. According to Minolta, the chromaticity value, C*, and hue angle, or H°, are called the CIELAB colour system. Colour measurements were expressed numerically using the chromaticity coordinates or C* and the hue angle or H°. The determinations of all colorimetric coordinates were performed in triplicates.

Chemical analysis

The moisture content was determined by the gravimetric test, and expressed in g by 100 g of sample (g 100 g^{-1}). The water activity was determined at an average temperature of 25°C in the Aqualab® equipment model (Decagon Devices, Inc. Pullman Washington). Determination of hydrogen ion potential (pH) was performed using a digital potentiometer. The titratable acidity was determined by titration, and the results expressed in g of citrus acid per 100 g sample (g 100 g^{-1}). All methodologies were performed in triplicate, and followed the protocols AOAC (2012).

Scanning electron microscopy (SEM)

The samples were initially prepared by removing the crumb portion, followed by drying at 75°C for 6 h. After this period, they were placed in a beaker, and ethyl ether was added until the samples were covered and stirred in four rotations for 50 min. Next, they were taken to an oven at the same temperature until complete evaporation of the solvent. Next, the bread was subjected to scanning electron microscopy (JSM 6610 model, Jeol, Tokyo, Japan), equipped with EDS (Thermo Scientific NSS Spectral Imaging).

Differential scanning calorimetry (DSC)

The stored bread's enthalpy of retrogradation (△H) was analysed using a Q100 differential

scanning calorimeter (DSC, TA Instruments, New Castle, DE, USA). The samples (4 mg) were weighed, mixed with deionised water (1:3), and sealed in a DSC aluminium pan. Samples were considered and packed in a DSC tray without loss of weight or moisture, followed by heating from 10 to 120°C at a heating rate of 10°C min^{-1} (Bosmans *et al*., 2013).

Statistical analysis

A factorial analysis of variance was performed to identify the significance of factors and their interactions. The data were then submitted to a multivariate analysis using the principal component analysis (PCA) *via* a covariance matrix to identify treatments with an ideal profile. With the treatments identified, the Tukey's test was applied to compare the means ($p < 0.05$). Finally, statistical analyses were performed in the R software (R Development Core Team 2021).

Results and discussion

Experimental design for bread-making

First, principal component analysis was performed to understand the data set better, and reduce the multivariate dimensionality, thus looking for correlated treatments (Figure 1) (Morais *et al*., 2024). The first component (PC1) corresponded to 49.32% of the total data variation, and the second component (PC2) was 19.32%, totalling 68.64% of the variance. The variables with the greatest weight in PC1 were hardness, gumminess, chewiness, and hue angle, and variables related to texture and expansion. Among the response variables, the microstructure and colorimetric data (number of alveoli, perimeter, area, circularity, and hue angle) least explained the variation of data in PC1 (Figure 1B). The variables with the most significant weight in PC1 distributed the scores horizontally. This indicated that the Time 1 scores on the left side of the graph (dotted circle) had higher hardness, gumminess, and chewiness indexes, and lower volume indexes (Figure 1A). The desirable characteristics of loaf bread are those with greater volume and lower levels of chewiness, gumminess, and hardness.

The correlation circle showed negative correlations between variables related to bread volume and chewiness, gumminess, and hardness, making it possible to identify treatments with desirable characteristics. It was possible to observe that the treatments A 2.25 and A 3.75 at time 3 (2 h 30 min of fermentation); B 2.25 and B 3.0 at time 2 (1 h 30 min of fermentation), B 3.75 at time 1 (1 h of fermentation), C 2.25 and C 3.0 at time 2 (1 h 30 min fermentation), and C 3.75 at time 3 (2 h 30 min of fermentation) stood out concomitantly for higher rates of specific volume, volume, resilience, elasticity, cohesiveness, and expansion. The multivariate technique allows for selecting treatments by simultaneously viewing the variables. These treatments were superior in proceeding with a more detailed univariate analysis, and looking for significant differences between their means.

The data presented in Table 1 show very subtle differences between the chosen treatments based on the multivariate analysis of principal components (PCA). However, treatment C 3.75 was eliminated since it presented a value greater than $p < 0.05$ of hardness. According to Serventi *et al*. (2016), both the sensory and instrumental analyses correlated very well in the description of bread volume, crumb hardness, and pore size; so, it was decided to discard it since greater hardness would compromise technological parameters. Therefore, the analysis of principal components significantly correlated with the following variables: hue angle, hardness, gumminess, chewability, expansion, and cohesiveness values. Sample C 3.75 presented the best results compared to the others, which was positive for loaf bread since low values indicate that the bread would disintegrate more quickly. Furthermore, statistical averages ($p < 0.05$) were found in several tests for the other parameters. In addition to analysing the standards, the PCA was used again, and it inferred that B 3.75 behaved better, considering the objectives of the studies.

Based on the correlations found, the samples with enzymes were distinguished by having less chewiness, gumminess, and hardness, indicatives of a softer bread. The results agreed with Giannone *et al*. (2016), in which, when applying lipase and amylase in loaf bread, they observed that during the entire storage period, the samples containing the enzyme were markedly softer than the control, demonstrating the effectiveness in delaying retrogradation in all stages of enzymatic formulations assessed. Furthermore, the results showed that the tested enzymes promoted positive effects in wheat bread, and showed synergistic interactions in preventing aging, showing a more marked effect in delaying staling and chewiness during storage.

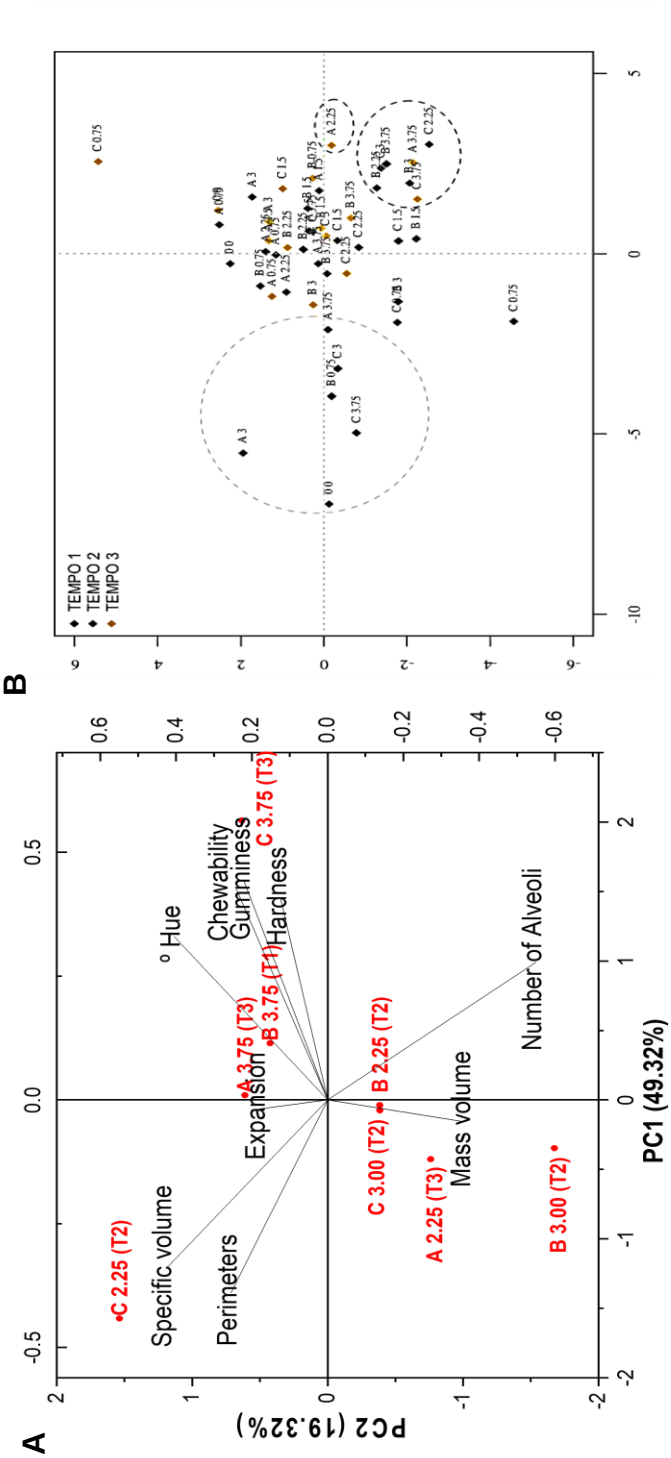

Figure 1. Principal component analysis (PCA) and scatter plot (PC1 *vs.* PC2) of variables studied as control sample (without enzyme) and enzymatic sample at times studied with projection in factorial plane. In graph B, A: Lipopan F®; B: Lipopan XTRA®; C: Lipopan Prime®.

Table 1. Treatments giving best behaviour in analysing principal components (PCA) of produced breads.

Treatment	Hardness (N)	Elasticity (m)	Cohesiveness (N)	Gumminess (N)	Chewability (N)	Resilience	Dough volume
A 2.25 (T3)	384.90 ± 36.86^{b}	0.95 ± 0.01^{a}	0.76 ± 0.00^{ab}	275.422 ± 19.35^{bc}	259.28 ± 23.51^{bc}	0.38 ± 0.00^{a}	32.25 ± 2.25^{a}
A 3.75 (T3)	390.32 ± 16.06^{b}	0.95 ± 0.00^{a}	0.75 ± 0.00^{bc}	287.88 ± 12.15^{bc}	269.78 ± 11.41^{bc}	0.33 ± 0.00^{bc}	29.93 ± 0.11^{ab}
B 2.25 (T2)	394.74 ± 6.10^{b}	0.95 ± 0.01^{a}	0.76 ± 0.01^{ab}	295.444 ± 15.27^{bc}	281.16 ± 17.07^{bc}	0.33 ± 0.01^{bc}	30.00 ± 0.50^{ab}
B 3.00 (T2)	346.60 ± 11.28^{b}	0.95 ± 0.02^{a}	0.73 ± 0.00^{d}	251.72 ± 11.32^{c}	241.34 ± 9.74^{c}	0.30 ± 0.02^{c}	31.10 ± 1.10^{a}
B 3.75 (T1)	344.64 ± 9.06^{b}	0.96 ± 0.00^{a}	0.77 ± 0.00^{a}	309.42 ± 37.47^{ab}	300.64 ± 26.20^{ab}	0.35 ± 0.00^{ab}	27.73 ± 0.75^{b}
C 2.25 (T2)	333.54 ± 24.45^{b}	0.94 ± 0.00^{a}	0.76 ± 0.00^{ab}	252.24 ± 24.90^{c}	246.85 ± 26.20^{c}	0.33 ± 0.02^{bc}	30.00 ± 1.00^{ab}
C 3.00 (T2)	374.98 ± 21.56^{b}	0.97 ± 0.01^{a}	0.75 ± 0.00^{c}	278.33 ± 12.66^{bc}	275.10 ± 8.92^{bc}	0.33 ± 0.01^{bc}	30.50 ± 0.50^{ab}
C 3.75 (T3)	560.20 ± 21.56^{a}	0.95 ± 0.01^{a}	0.75 ± 0.00^{bc}	354.14 ± 35.37^{a}	332.99 ± 31.93^{a}	0.34 ± 0.01^{b}	30.73 ± 0.25^{a}

Treatment	Specific volume ($cm^3\ g^{-1}$)	Expansion ($cm^3\ g^{-1}$)	H°	Number of alveoli	Perimeters	Circularity
A 2.25 (T3)	4.02 ± 0.18^{ab}	1.49 ± 0.07^{b}	95.50 ± 0.13^{bc}	751 ± 87.15^{a}	6.88 ± 0.58^{b}	0.79 ± 0.01^{a}
A 3.75 (T3)	3.88 ± 0.07^{b}	1.87 ± 0.11^{a}	96.47 ± 0.38^{b}	672 ± 9.29^{a}	6.11 ± 0.23^{b}	0.80 ± 0.01^{a}
B 2.25 (T2)	3.88 ± 0.13^{b}	1.36 ± 0.11^{b}	94.82 ± 0.46^{c}	707 ± 28.00^{a}	6.49 ± 0.32^{b}	0.79 ± 0.02^{a}
B 3.00 (T2)	3.77 ± 0.08^{b}	1.48 ± 0.04^{b}	95.44 ± 0.60^{bc}	774 ± 85.49^{a}	6.45 ± 0.40^{b}	0.79 ± 0.01^{a}
B 3.75 (T1)	3.93 ± 0.06^{ab}	1.44 ± 0.03^{b}	96.27 ± 0.34^{b}	742 ± 51.73^{a}	6.16 ± 0.04^{b}	0.79 ± 0.00^{a}
C 2.25 (T2)	4.24 ± 0.13^{a}	1.48 ± 0.03^{b}	96.20 ± 0.25^{b}	623 ± 29.70^{a}	8.19 ± 0.38^{a}	0.77 ± 0.01^{a}
C 3.00 (T2)	3.90 ± 0.14^{b}	1.46 ± 0.04^{b}	95.85 ± 0.42^{bc}	726 ± 63.22^{a}	6.47 ± 0.47^{b}	0.77 ± 0.00^{a}
C 3.75 (T3)	3.79 ± 0.05^{b}	1.46 ± 0.03^{b}	98.55 ± 0.74^{a}	759 ± 77.09^{a}	5.94 ± 0.15^{b}	0.79 ± 0.00^{a}

Means followed by different lowercase superscripts in the same column differ statistically using the Tukey's test ($p < 0.05$). T1: 1 h of fermentation; T2: 1 h and 30 min of fermentation; T3: 2 h and 30 min of fermentation; A: Lipopan F®; B: Lipopan XTRA®; and C: Lipopan Prime®. Enzyme concentrations are expressed in KLU g^{-1}: 2.25, 3.00, 3.75.

Considering the correlation circle of the variables, it can be suggested that the acidity, area, volume, and the number of alveoli were directly correlated with bread that had commercial enzymes in their composition, that is, breads that had in their composition enzymes tended to show higher values of acidity, volume, and number of alveoli when compared to control treatments. This indicated that adding Lipopan to the formulations brought positive effects. This was mainly related to generated lipids, which could indirectly stabilise gas cells in the dough. This is an important characteristic, as the gas retention capacity of the dough is one of the characteristics, if not the most important, in bread-making, as it is associated with crumb structure and high specific volume (Gerits *et al*., 2014).

Based on PCA results, it can be suggested that the variables were directly correlated with the bread with commercial enzymes in their composition compared to the controls. The data showed very subtle differences between the chosen treatments, which were based on multivariate analysis (PCA). However, C 3.75 was initially eliminated as it presented a hardness value greater than $p < 0.05$. On the other hand, B 3.75 presented higher cohesiveness values, which is positive for loaf bread, since very low values indicate that the bread would disintegrate more easily. Regarding the other parameters, statistically equal means ($p < 0.05$) were found in several tests. Therefore, B 3.75 showed better behaviour, taking into account the objectives of the study, and was therefore chosen for the subsequent stages (B 3.75, Lipopan XTRA®, 1 h of fermentation, and enzyme concentrations expressed in 2.25 KLU g^{-1}). This result indicated that adding Lipopan XTRA® to the formulations brought positive effects. This enzyme was then used to estimate shelf life for 28 days at two different temperatures (25 and 35°C).

Shelf life

Texture profile

The TPA results, obtained through double-cycle compressions at 40% depth, showed the structural alterations that affected the bread samples added with Lipopan XTRA® and in the control tests during storage.

Evaluating each analysed variable separately, after 21 days of storage, a significant increase in the hardness was observed in the formulations with enzyme submitted to refrigeration at 15 and 25°C. Furthermore, it was observed that the overall mean hardness with the use of the enzyme was significantly lower ($p < 0.05$) than the control. This result corroborated that observed in the multivariate analysis. These changes could be attributed to the more excellent gas retention by the dough treated with lipase, since according to Dahiya *et al*. (2020), the addition of lipases leads to an increase in bread volume, resulting in an improved and highly uniform crumb, and therefore, greater firmness. This also corroborated Serventi *et al*. (2016) who, when evaluating the addition of different enzymes to improve the sensory quality of bread, found that formulations with lipase incorporation (Lipopan XTRA®) showed an increase in the hardness. In this sense, Soares *et al*. (2017) stated that sucrose increases the glass transition temperature *Tg*, consequently reducing the retrogradation of starch, making the bread harder over the days of storage.

The elasticity indicated a decrease in its values over the storage periods, both for the control bread and those with the addition of enzyme (Lipopan XTRA®). However, in samples with enzyme, only those stored at room temperature (35 ± 2°C) and at 25°C differed statistically ($p < 0.05$). It was also observed that the mean of elasticity did not present a significant difference ($p > 0.05$) between the control bread and those with lipase, taking into account the same storage conditions. Therefore, it was impossible to predict lipase's effect on texture. These results agreed with those found by Becker *et al*. (2009) who found that adding different enzymes intensified the distending properties of the dough (extensibility), and minimised the elasticity of the bread. Furthermore, this decrease in the elasticity value was inversely proportional to the hardness value found in the bread analysed during storage; bread with higher hardness values tended to show a decreasing behaviour for the elasticity during storage (Esteller, 2014).

At 28 days, bread with enzyme addition, regardless of storage temperature, had statistically lower cohesiveness ($p < 0.05$) than the days before it. It was also observed that the overall average did not present a significant difference ($p > 0.05$), considering the control treatment and enzyme addition at the same temperature. This parameter refers to the deformation force before failure. It is understood that higher values of this attribute indicate bread with greater freshness. This behaviour can be explained by the fact that during the kneading process (beating), water is incorporated into the flour, and gluten develops. Disulphide bridges and ionic bonds

(addition of salt) maintain cohesiveness, and ensure the retention of volatiles during baking. However, during the storage period, changes in these bonds (water migration, starch crystallisation, fat hydrolysis) lead to a gradual disarrangement of the structure, which consequently tends to decrease the cohesiveness values (Esteller, 2014).

The gumminess of the bread samples showed a trend similar to hardness. The gumminess increased gradually and significantly over the days of storage, regardless of the temperature at which the bread was stored compared to the control. However, the treatments that used enzymes, regardless of storage temperature, obtained lower overall averages ($p < 0.05$). In the loaf bread prepared here, it was verified that the application of Lipopan XTRA® (3.75 KLU) could reduce the gumminess. The results agreed with Serventi *et al*. (2016) who, when studying the addition of enzymes to improve the sensory quality of wheat-cassava compound bread, found that the addition of lipase (Lipopan XTRA®) promoted a reduction in crumb gumminess, and associated this behaviour with increased volume. In this sense, the decrease in gumminess levels can be explained due to the decrease in the content of soluble solids present in the samples (López and Goldner, 2015).

After 21 days, as occurred for hardness, there was a significant increase in chewability ($p < 0.05$) in the formulations with the addition of the enzyme subjected to study temperatures of 15 and 25°C. It was also observed that the tests with the incorporation of Lipopan XTRA® (3.75 KLU) presented lower overall averages ($p < 0.05$) for chewiness under the three storage conditions analysed. This result confirmed what was observed in the analysis of principal components. Therefore, the application of commercial lipase was promising in reducing the chewiness of the breadcrumbs studied. The same behaviour was verified in the study by Barret *et al*. (2002) when producing bread with oxidising gums, stored at temperatures of 4, 21, and 38°C and stored for 0, 2, 6, and 12 weeks. The authors found an increase in hardness and chewiness values, and a decrease in elasticity and cohesiveness during storage compared to a control without additives.

Bread resilience decreased with storage time regardless of treatment. These results could be attributed to water loss during storage, and water molecules' migration to the crust. These agreed with De La Hera *et al*. (2014), finding that resilience measures were affected by the water content in bread. The treatments with the addition of Lipopan XTRA® (3.75 KLU) showed lower resilience values ($p < 0.05$) within the same temperature, except under 15°C, in which the means of the control and with the addition of enzyme were statistically equal. For this parameter, higher values indicate the bread's quality. Therefore, Lipopan XTRA® appeared ineffective under the study's conditions.

Colorimetric analysis

Colour evaluation may indicate failures during processing, which can be perceived by the crust colour being too light or too dark. Table 2 shows the chromaticity and hue angle values for the bread of control treatment and those with the addition of enzyme (Lipopan XTRA®), where the results showed a tendency for the values to decrease in different proportions, depending on the storage conditions, that is, it made the crumb colour lighter, with a more opaque colour, tending more towards cream.

However, when analysing the control bread (without the addition of enzyme), it was possible to verify that they presented the opposite behaviour; that is, their chromaticity tended to increase over the days of storage. As for the overall average, it was found that only the bread with enzyme (Lipopan XTRA®) at 25°C obtained a lower value ($p < 0.05$) compared to the control within the same storage conditions. Almeida *et al*. (2013) found a similar behaviour when studying dietary fibre sources in frozen pre-baked bread and their influence on technological quality.

Therefore, it can be inferred that the control bread (without the addition of enzyme) tended to have a colour tending to red/brown at the end of storage, as they showed an increasing behaviour (from 11.02 on day 0 to 13.85 on day 28 at room temperature). On the other hand, the bread added with the enzyme (Lipopan XTRA®) at the end of storage tended to become more yellow and lighter, as their behaviour shows decreasing results over the days of storage. In this sense, Dube *et al*. (2020) stated that crumb colour is related to the type of flour and the structure of the crumb's air cell, affecting how light reflects on a piece of bread. Emulsifiers, *e.g*., lipases, change the structure of the crumb, and make the air cells smaller and more evenly distributed, reducing the crumb's blackness.

When evaluating the hue angle (Table 2), it was observed that the bread stored at room temperature presented the same decreasing trend over the days of storage. However, all the samples were

found close to the 90° axis, confirming the tendency towards a more yellowish colour, a typical colour tone of breadcrumbs. Similar results were reported by Almeida *et al*. (2013) when verifying new sources of dietary fibre in frozen pre-baked bread and their influence on functional and technological quality, obtaining results very similar to those reported in the present work about the tonality of the crumb.

Table 2. Means of chroma and hue angle of breads stored at three temperatures over 28 days.

Chroma (C)						
Day	**Ambient (35°C)**		**15°C**		**25°C**	
	Control	**Enzyme bread**	**Control**	**Enzyme bread**	**Control**	**Enzyme bread**
0	11.02 ± 0.47^{b}	12.78 ± 2.24^{a}	-	-	-	-
7	11.99 ± 1.07^{b}	10.14 ± 0.07^{ab}	11.92 ± 2.55^{a}	10.73 ± 0.17^{a}	11.61 ± 1.12^{a}	11.50 ± 0.56^{a}
14	12.05 ± 0.02^{b}	10.48 ± 0.77^{ab}	11.20 ± 0.40^{a}	10.19 ± 0.67^{a}	11.46 ± 0.85^{a}	11.10 ± 0.42^{ab}
21	11.98 ± 0.62^{b}	9.14 ± 0.57^{b}	11.7 ± 0.60^{a}	9.99 ± 1.27^{a}	11.39 ± 0.59^{a}	10.30 ± 1.27^{ab}
28	13.85 ± 0.27^{a}	12.56 ± 0.27^{a}	12.09 ± 0.60^{a}	10.48 ± 0.74^{a}	12.12 ± 0.54^{a}	9.87 ± 0.78^{b}
Mean	12.17^{A}	11.29^{ABC}	11.73^{AB}	10.34^{C}	11.64^{AB}	10.69^{BC}
Hue angle (H°)						
Day	**Control**	**Enzyme bread**	**Control**	**Enzyme bread**	**Control**	**Enzyme bread**
0	95.21 ± 0.37^{a}	94.16 ± 1.18^{a}	-	-	-	-
7	94.68 ± 1.07^{a}	95.45 ± 0.32^{ab}	94.73 ± 0.79^{a}	95.38 ± 0.08^{ab}	94.93 ± 0.55^{a}	94.69 ± 0.63^{a}
14	$93.79 \pm 0.0.2^{a}$	93.96 ± 0.19^{ab}	94.45 ± 0.41^{a}	94.36 ± 0.27^{b}	95.35 ± 0.07^{a}	94.52 ± 0.21^{a}
21	93.64 ± 0.62^{a}	94.5 ± 0.35^{a}	94.97 ± 0.32^{a}	95.95 ± 0.08^{a}	94.71 ± 0.63^{a}	94.33 ± 0.16^{a}
28	93.07 ± 0.27^{a}	92.94 ± 0.05^{a}	94.13 ± 0.06^{a}	95.17 ± 0.36^{ab}	95.41 ± 0.61^{a}	94.23 ± 0.46^{a}
Mean	94.08^{B}	94.21^{B}	94.57^{AB}	95.22^{A}	94.45^{AB}	95.10^{A}

Means followed by different lowercase superscripts in the same column differ statistically by Tukey's test ($p < 0.05$). Means followed by different uppercase superscripts in the same row differ statistically by Tukey's test ($p < 0.05$).

Chemical analysis

Water activity is one of the most critical factors in the food industry. It quantifies the water available for the growth of microorganisms,a and the biochemical and technological reactions that can alter food quality. Results ranged from 0.89 to 0.94 a_w for control treatments and 0.91 to 0.95 a_w for treatments with lipase incorporation (3.75 KLU). An increase ($p < 0.05$) was observed from the twenty-first day of storage onwards for treatments with the addition of enzyme at a temperature of 15°C, whose average was 0.94 a_w. In general, there were no significant differences between the control and the formulations with added enzyme (Lipopan XTRA®) at any of the temperatures evaluated ($p > 0.05$).

The moisture content tended to increase in all treatments kept under refrigeration (25 and 15°C) over storage. However, after seven days, a lower moisture content ($p < 0.05$) was observed. This behaviour during bread storage is directly related to bread staling, caused by changes in starch and water migration. Because of this, bread freshness loss occurs due to increased moisture in the crust, increased starch crystallinity, and crumb firmness (Dahiya *et al*., 2020). However, a different behaviour was observed in tests at room temperature, in which the means were higher than in the first days of storage, and decreased at the end of shelf life ($p < 0.05$). This phenomenon could have been attributed to the storage temperature (35 ± 2°C) which favoured the evaporation of water molecules. Following the legislation in force in the country (Brazil, 2000), the humidity of loaf bread must have a maximum of 38%; therefore, all treatments were within the humidity parameters prescribed by the legislation in force.

As for the pH, the doughs with the addition of Lipopan XTRA® (3.75 KLU) showed lower overall pH means ($p < 0.05$) when compared to the control samples under the same temperature conditions, but still within the normal range for loaves of bread. According to Debonne *et al*. (2020), the pH value is directly correlated with the ingredients present in the bread formulation. So, most studies conducted with bread mention the pH reduction as responsible for technological and nutritional changes, and bread conservation. In the evaluation of the titratable

acidity, the control samples presented a variation of 1.30 - 1.05 g 100 g^{-1}, whereas the samples with the addition of lipase (Lipopan XTRA®) presented an acidity of 1.39 - 1.00 g 100 g^{-1}. Until the end of storage, there was an increase in titratable acidity values in all samples, reflecting the drop observed in pH values. In general, among the samples, those added with lipase showed significantly ($p < 0.05$) higher acidity than the control samples. The intense acidifying activity of yeasts is mainly responsible for this pronounced acidity. In addition, the presence of lactic acid bacteria, responsible for producing organic acids, also promotes this behaviour (Sharma and Gujral, 2019).

Scanning electron microscopy (SEM)

In Figure 2, the micrographs of the bread stored at time 0 are shown, being categorised as a control sample and with the addition of the enzyme lipase (Lipopan XTRA®), obtained at 500× magnification. It consisted of water and wheat fractions, such as starch, protein, and bran. All bread samples contained small and large starch granules of spherical and lenticular shapes distributed over the protein matrix. This observation was similar to reports by Aponte *et al*. (2014) on baked bread during the first day of storage.

Still, on the micrographs at time 0, it was possible to observe that the starch had oval, spherical, or polygonal shape, as previously reported in the literature, without any cavities or fissures, with a smooth surface (Deckardt *et al*., 2014; Liu *et al*., 2015). By analysing the two micrographs (A and B), no significant differences were observed between the control bread and those with the addition of enzyme, with a clear division of starch molecules and protein networks and their cavities. As it was time 0, no damaged zones were found since the physical and chemical variables such as humidity, water activity, pH, acidity, and the technological variables such as hardness and cohesiveness had not undergone changes that would justify the appearance of damaged zones that would compromise the integrity of the sample. Damaged areas indicate deformation of the gluten network and consequent release of small starch granules. However, this aspect may be related to sample preparation. Depending on the preparation method, there may be changes in its morphology. Therefore, the sample must not undergo excessive preparation, which can distort the structure and provide alteration between the components (Sharma and Gujral, 2019).

The micrographs were separated into control bread (without enzyme addition) and bread with enzyme addition (Lipopan XTRA®) over 28 days of storage to obtain more precise and cohesive results (Figure 2). It could be inferred that the results obtained for the micrographs of the control samples ratified the results of the physical, chemical, and technological analyses since at room temperature (35 ± 2°C), on the fourteenth day, damaged zones (DZ) began to appear, a fact mainly stemming from the decrease in water activity, which consequently increased the hardness, making the bread more brittle and with easily identifiable damaged areas. On the other hand, the opposite behaviour was observed for temperatures of 15 and 25°C, where humidity and water activity showed a slight increase, thus ensuring a more homogeneous and less crumbly bread (< hardness).

By evaluating the micrographs obtained from the bread with the addition of the enzyme lipase (Lipopan XTRA®) (Figure 2), it was possible to notice a change in the conformation of the starch molecules, where visually they were different over the days of storage; the same behaviour could also be observed in the control bread samples. According to Deckardt *et al*. (2014), when evaluating the effects of applying organic acids in starch granules, the authors showed that their addition led to the modification of the starch granules. Furthermore, they found that changes in starch granules underwent slight surface deformations with increasing acidity. This same behaviour could be observed in the micrographs of the present work; in the first fourteen days of storage, the starch molecules were uniform, smooth, circular, and defined. However, over the days of storage, we noticed that such molecules began to lose their structural shape, becoming less circular and more oval, or even losing their conformational structure completely.

The scanning electron microscopy data also showed significant modifications on the surface of the starch granules between the fourteenth and twenty-eighth days. Severe changes and deformation in the appearance of the surface, especially on the twenty-eighth day at temperatures of 25 and 15°C, are also visible in Figure 2, in line with the higher acidity values for this bread, which would contribute to the conformational derangement of the structure. At the

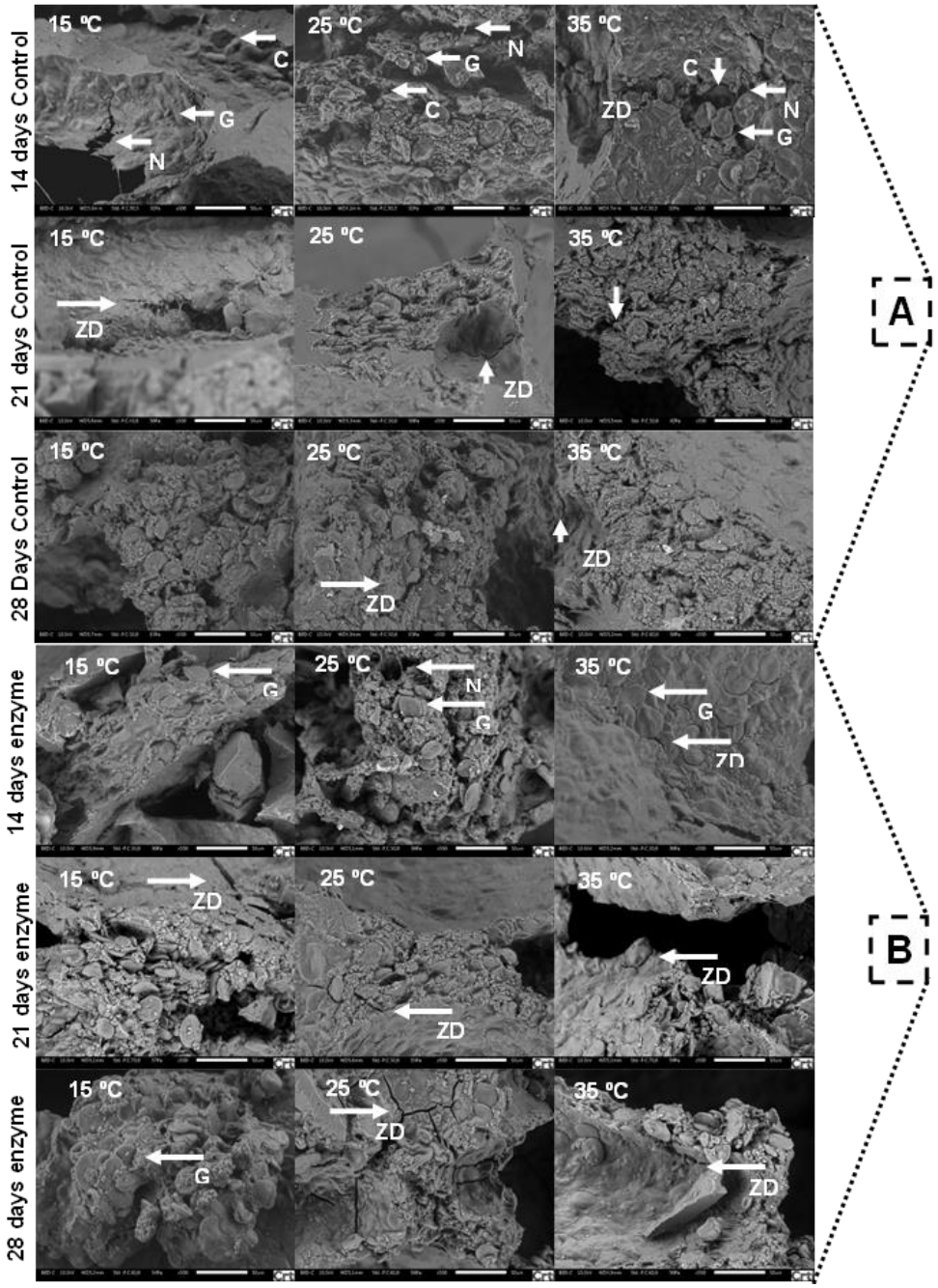

Figure 2. Micrographs of control bread **(A)** and enzymatic bread **(B)**, with 500× magnification at 14, 21, and 28 days of storage, at temperatures 15, 25, and 35°C ± 2. Arrows show granules of starch (G), cavities (C), protein networks (N), and damaged areas (ZD).

end of storage, the starch granules appeared trapped inside the compact protein matrix, especially those subjected to 25 and 35°C (Figure 2). This visually detected interaction between starch granules and matrix could have decreased the enzymatic hydrolysis of starch, and increased the amount of resistant starch in the final product (Deckardt *et al*., 2014; Hayta and Hendek Ertop, 2017).

At the end of storage, the starch-protein matrix that formed the microstructure of stored bread was more discontinuous, and appeared weaker than in fresh bread. This behaviour, as mentioned, could have been due to increased crumb firmness (hardness and gumminess) and decreased elasticity (elasticity and cohesiveness) for all breads during storage. Similar behaviour was observed by Hayta and Hendek Ertop (2017) when studying bread stored for ten days. This change in the microstructure of the stored bread also indicated that the starch-starch interaction resulted from retrogradation, and not from the starch-gluten interaction.

Differential scanning calorimetry (DSC)

The results obtained through differential scanning calorimetry (DSC) analysis are enthalpy variation (*ΔH*), heat capacity of materials, and temperature of thermal events (Table 3). From the thermograms obtained in this analysis, it was possible to determine the T0 (initial temperature of the starch retrogradation process), *Tp* (peak temperature), and ΔH_1 (enthalpy of retrogradation). It can be seen that starch retrogradation started at a temperature of 138.60°C (T0). The other analysed times were below 173.00°C since starch is mainly composed of amylopectin, which, according to Zobel *et al*. (1988), is one of the components responsible for the crystalline part of the granule; this compound being one of the main factors in the process of starch recrystallisation or retrogradation.

It was observed that the retrogradation process reached its maximum point when the peak temperature was measured (*Tp*), showing that, despite being subjected to different temperatures and during 28 days of storage, substantial fluctuations in their temperatures were not observed (Table 3). It was also noted that the enthalpy of retrogradation (*ΔH*) values, as observed in hardness, increased with storage time. The increase in *ΔH* indicates the presence of less amorphous and more crystalline starch regions (Ou *et al*., 2022). This change does not result from a single effect but includes the reorganisation of polymers within the amorphous region, loss of moisture content, water content distribution between the amorphous and crystalline zones, and the macroscopic structure of the crumb (Ozkoc *et al*., 2009). Similar behaviour was observed by Ma *et al*. (2022) when evaluating the effects of sugars on the retrogradation of starch gels during storage, who observed that the enthalpy of fusion (*ΔH*) of all samples increased significantly with increasing storage time.

Table 3. Differential scanning calorimetry (DSC) results of breads stored at three temperatures over 28 days.

Time		Control				Enzyme			
		T0 (°C)	***Tp* (°C)**	**Tp - T0 (°C)**	**ΔH_1 (J g^{-1})**	**T0 (°C)**	***Tp* (°C)**	**Tp - T0 (°C)**	**ΔH_1 (J g^{-1})**
Ambient	0	138.60^{gB}	147.90^{fA}	9.30^{cA}	812^{bA}	139.60^{eA}	147.90^{eA}	8.3^{dB}	251^{mB}
	7	121.50^{kB}	122.20^{mB}	0.70^{kA}	625^{gA}	126.00^{jA}	126.70^{lA}	0.70^{hA}	593^{kB}
	14	148.50^{dB}	157.30^{cB}	8.80^{dA}	628^{fA}	160.90^{aA}	164.60^{aA}	3.70^{eB}	627^{iB}
	21	149.40^{cA}	150.50^{eA}	1.10^{iB}	389^{kB}	138.10^{hB}	147.70^{fB}	9.60^{cA}	653^{fA}
	28	130.70^{iB}	144.40^{kA}	13.70^{bA}	384^{lB}	138.70^{fA}	139.30^{jB}	0.60^{iB}	691^{eA}
15°C	0	138.60^{gB}	147.90^{fA}	9.30^{cA}	812^{bA}	139.60^{eA}	147.90^{eA}	8.3^{dB}	251^{mB}
	7	137.80^{hB}	146.00^{gB}	8.20^{eA}	231^{mB}	148.50^{bA}	149.50^{cA}	1.00^{gB}	706^{bA}
	14	155.10^{bA}	157.50^{bA}	2.40^{gA}	605^{hB}	138.60^{gB}	139.20^{kB}	0.60^{iB}	730^{aA}
	21	124.00^{jB}	143.40^{lA}	19.40^{aA}	743^{cA}	141.70^{dA}	142.80^{iB}	1.10^{fB}	626^{jB}
	28	144.30^{fA}	145.10^{iB}	0.80^{jB}	481^{jB}	134.70^{iB}	151.70^{bA}	17.00^{bA}	636^{hA}
25°C	0	138.60^{gB}	147.90^{fA}	9.30^{cA}	812^{bA}	139.60^{eA}	147.90^{eA}	8.3^{dB}	251^{mB}
	7	144.30^{fA}	144.90^{jA}	0.60^{lB}	697^{dB}	123.30^{kB}	144.20^{hA}	20.90^{aA}	700^{dA}
	14	173.00^{aA}	175.70^{aA}	2.70^{fA}	3465^{aA}	148.50^{bB}	149.30^{dB}	0.80^{hB}	701^{cB}
	21	155.10^{bA}	157.00^{dA}	1.90^{hA}	664^{eA}	144.00^{cB}	145.00^{gB}	1.00^{gB}	637^{gB}
	28	144.70^{eA}	145.40^{hA}	0.70^{kA}	547^{iA}	119.60^{lB}	120.20^{mB}	0.60^{iA}	498^{lB}

Means followed by different lowercase superscripts in the same column differ statistically by Tukey's test ($p < 0.05$). Means followed by different uppercase superscripts in the same row differ statistically by *t*-test ($p < 0.05$).

Conclusion

Among the formulations proposed by the experimental design, all showed slight differences between the chosen treatments based on the multivariate analysis (PCA). The variables with the most significant weight in PC1 were hardness, gumminess, specific volume, and chewiness, and variables related to texture and expansion, since they are technological parameters. The means of hardness, gumminess, and chewiness were significantly lower ($p < 0.05$) with the use of enzymes in the formulation

at all storage temperatures. The hardness, elasticity, cohesiveness, gumminess, chewiness, and resilience showed significant differences ($p < 0.05$) between the samples during storage. Despite the impairment of some variables during storage, incorporating lipase (Lipopan XTRA®) was promising for further studies and subsequent commercialisation. In this sense, further research should be conducted to verify the sensory acceptance profile, and application in fibre-rich products, such as products enriched with vegetable extracts, ensuring that such bread becomes technologically and economically viable.

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