Review Article

An extremophilic enzyme cocktail from Microbacterium Metallidurans TL13 as a cake-donut improver: a comparative study with commercial enzymes-based formulation optimized by a mixture design

Abstract

The use of hydrolytic enzymes from mesophilic micro-organisms in many food preparations is an ancient process. With recent advances in enzyme technologies and the discovery of extremozymes endowed with extraordinary properties and specificities, new fields of application continue to be explored. In this study, at the first stage, a mixture design approach was adopted to model and optimize enzyme-based cake-donuts improver's formula, with the specific volumes of donuts serving as responses and the proportions of three commercial microbial hydrolytic enzymes (amylase, cellulase and protease from mesophilic microbial strains) as variables. The selected polynomial model was validated by ANOVA with a 95% confidence and by comparing the regression coefficients of the predicted values to the experimentally measured ones ($R^2 = 0.97$ and Adj $R^2 = 0.92$). The postulated model was also investigated by the contour and three-dimensional surface plots. Results clearly indicated significant interactions and synergies between hydrolytic enzymes on donut quality. The maximum specific volume of cake-donuts of $10,71 cm^3/g$ was obtained in taking 55.85, 25.83, and 18.32% of amylase, protease and cellulase, respectively. This volume was considerably higher than that in cake-donuts prepared without enzymes addition ($2.43 cm^3/g$). At the second stage, this study demonstrated the performance of an extremophilic enzyme cocktail from Microbacterium metallidurans TL13 in improving donuts physical and sensory properties. The structural and biochemical properties of M. metallidurans extremozymes make them very attractive for a large range of food applications which need stable biocatalysts.

Keywords: cake-donuts, mixture design, extremozymes, actinobacterium, sensory evaluation, response surface methodology

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Introduction

Deep-fat fried donuts (doughnuts) are popular owing to their taste, distinctive flavor, aroma and crunchy texture, so they are served as breakfast, convenience food or even snacks.[1] They have attractive features such as a golden-brown exterior, a crispy crust and a core that resembles a baked product rather than a fried food.[2,3] Excepting cookies, no other fresh bakery product has as many possible varieties. It has been estimated that donuts may account for more than 10% of the total bakery products.[4] Donuts are very popular all over the world, requiring large daily production runs. Typically, bakeries purchase pre-assembled mixes containing all the basic ingredients, but if additional ingredients are required, they are added to the mix to avoid the time-consuming step scaling all the individual components of donut's formula. This trick also increases the uniformity of mixes and the quality of finished products.[5]

Using the conventional formula, a number of different types of wheat flour seem unable to produce satisfactory doughnuts. To solve this problem, the supplementation of improvers is a common practice for flour standardization but also as baking aids.[6] Enzymes are among the most widely used additives and improvers in bakeries, as they can effectively improve the color, texture and other qualities of finished products. Currently, the most used enzymes in bakery products are amylases, cellulases, xylanases and proteases. These enzymes can be obtained from cereal, fungal and bacterial sources. In fact, they

are involved in a number of improvement processes during mixing, fermentation, frying and other production operations. Microbial proteases, for example, help to control and reduce mixing time, ensure dough uniformity and regulate its strength and consistency, as well as controlling product texture and improving flavor. Microbial cellulases, xylanases and amylases may produce positive effects during donuts making process, such as improving handling properties, controlling rheological behavior of the dough matrix, increasing reducer sugars and accelerating yeast fermentation, and enhancing the quality of final products.[6]

Microbial enzymes can be added individually or in complex mixtures, which can act synergistically in the production of bakery products.[6–8] In this context, the production of cake-donuts represents a relatively new field of application for enzyme-based improvers. The optimization of enzyme mixtures based on statistical experimental designs, in particular mixture designs, saves a great deal of effort and time, leading to more concrete results than traditional optimization, which has many limitations.[9] These experimental designs have been successfully applied to many food preparations.[10–14] However, they have never been used to optimize donut formulations. Considering the industrial importance of the microbial enzymes as food improvers, several works have reported on the benefits of using low-cost processes for enzymes production, including the application of agro-industrial residues.[15–17] Recently, the production of multiple microbial

An extremophilic enzyme cocktail from Microbacterium Metallidurans TL13 as a cake-donut improver: a comparative study with commercial enzymes-based formulation optimized by a mixture design

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extremozymes with direct potential application in the food sector has resulted in an increased enzyme market.[18] The demand for enzymes from extremophiles may increase in the future because of their activity under harsh industrial conditions.[19–22]

In this context, the present investigation adopted a mixing design and response surface methodology as a reasonable and effective strategy to determine the optimal amounts of three commercial hydrolytic enzymes that maximize donut's specific volume. It was aimed also at studying un extremophilic α-amylase, protease and cellulase produced by *M. metallidurans* TL13 on an agro-industrial residue as an inducer substrate. The performance of the enzyme preparation from TL13 strain was tested in the donut-making process and compared to optimized formulation with commercial enzymes.

Material and methods

Chemical and raw materials

α-Amylase from *Bacillus licheniformis* (0.33U/mg), Cellulase from *Aspergillus niger* (0.8 U/mg) and protease from *Streptomyces griseus* (4 U/mg) were purchased from Sigma-Aldrich. Extremophilic plant growth-promoting bacterium *Microbacterium metallidurans* TL13, isolated from the wastewater of TMM Tunisian leather industry[23–25] was used for amylase, protease and cellulase production. Its genome shotgun project was deposited at DDBJ/ENA/GenBank under the accession SZZQ00000000.[23] Low quality palm dates (Kentichi variety) purchased from a local market was used as a substrate for enzymes production by *M. metallidurans* TL13. Donut ingredients were purchased from a local supermarket. All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich or Merck unless otherwise specified.

Donut's preparation and specific volume measurement

The cake-donuts were prepared according to the method described by Nouri et al [1]. With slight modifications. Briefly, the wheat flour was mixed with other powder ingredients (sugar, salt, chemical yeast, vanilla, and active dried yeast). After that, water and liquid ingredients (Milk, vegetable oil, warm water and egg) were putted and blended for 3 minutes at low speed. The butter was finally added and the mixture was kneaded at high speed for 7min. The enzyme-treated and enzyme non-treated samples were incubated at 37 °C for 10 minutes to ferment. After fermentation, the donuts dough were rolled out to a thickness of 1.5 cm ± 2 mm, shaped with a 7 cm diameter cookie cutter and fried at 180 °C for 2 minutes. Finally, donuts were cooled down to room temperature for 45–60 min and stored in polyethylene bags at ambient temperature before uses.

Specific volume of donuts measured based on the AACC method 72–10.[26] The weight of donuts was determined and then the specific volume was calculated as bellow:

Specific volume (cm³/g) = sample volume (cm³)/sample weight (g).

Optimization of enzyme-based donut improver's formulation using a simplex centroid mixture design

A mixture design is a statistical approach and a variant of response surface methodology based on regression analysis, which translates the relationship between the response and the factors studied.[10–13] Mixture designs, such as the simplex-lattice design and the simplex-centroid design, have proved highly effective for optimizing formulations, particularly in the food and pharmaceutical sectors.[10–13] The simplex centroid mixture model is characterized by several underlying assumptions that guide its application and interpretation. The response variable is assumed to depend on the relative proportions of the mixture components rather than their absolute amounts. The response surface is assumed to be a continuous function of the component proportions. The design requires that the sum of all component proportions equals one. It is typically assumed that the errors in the response measurements are independent and identically distributed with a normal distribution, which allows for valid statistical inference about the effects of component proportions on the response. In a simplex centroid design for three components, the design space is represented as an equilateral triangle. The vertices of this triangle correspond to pure components, while points along the edges represent binary mixtures, and points within the triangle represent ternary mixtures.[27,28] 14-point simplex centroid mixture design was used in this study to modelize the response (specific volume of cake-donuts) as a function of all possible combinations of three microbial hydrolytic enzymes (amylase, protease and cellulase) (Table 1). The first three runs represent pure compositions of each enzyme. The next three runs represent binary enzyme mixtures. The seventh run represents an equal mixture of all three enzymes. The last three runs are weighted enzymatic mixtures that provide additional exploration within the design space. The last runs are replicate points used to estimate the error variance.[27,28]

Table 1 Experimental mixture design showing the doses of the commercial enzymes used in donut's formula and the corresponding experimental and theoretical responses.

Mixtures	Coded values			Uncoded values			Y_{exp}: SV (cm³/g)	Y_{cal}: SV (cm³/g)
	A	P	C	Amylase (U/ml)	Protease (U/ml)	Cellulase (U/ml)		
M1	1	0	0	6	0	0	7.96	8.2
M2	0	1	0	0	6	0	5.17	4.29
M3	0	0	1	0	0	6	9.65	9.76
M4	0	0.5	0.5	0	3	3	1.08	0.95
M5	0.5	0	0.5	3	0	3	9.62	9.49
M6	0.5	0.5	0	3	3	0	6.94	7.33
M7	0.67	0.17	0.17	4	1	1	10.26	10.64
M8	0.17	0.67	0.17	1	4	1	5.17	5.56
M9	0.17	0.17	0.67	1	1	4	4.4	4.78
M10	0.33	0.33	0.33	2	2	2	8.14	7.57
M11	1	0	0	6	0	0	8.5	8.2
M12	0	1	0	0	6	0	0.107	4.29
M13	0	0	1	0	0	6	9.94	9.76
M14	0,50	0.5	0	3	3	0	7.84	7.33

Abbreviations: M, mixture; A, amylase; P, protease; C, cellulase; Y_{exp}:Ycal: SV, specific volume

An extremophilic enzyme cocktail from Microbacterium Metallidurans TL13 as a cake-donut improver: a comparative study with commercial enzymes-based formulation optimized by a mixture design

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The effects of the enzymes on the donut's specific volumes were evaluated by changing their concentrations simultaneously and keeping their total concentration constante. These enzymes and their final concentration were chosen on the basis of preliminary experiments, carried out prior to implementation of the experimental design (data not shown). The results obtained from the selected experiments were fitted to a polynomial model using the method of least squares to calculate the coefficients of the postulated model:[10–13]

$$Y\ (SV) = b_1\ A + b_2\ P + b_3\ C + b_{12}\ AP + b_{13}\ AC + b_{23}\ PC + b_{123}\ APC;$$

Where, Y is the response (Specific volume of donuts); A, P and C the enzymes amylase, protease and cellulase, respectively; b_1, b_2 and b_3 are the linear coefficients and b_{12}, b_{13}, b_{23} and $b1_{23}$ are the interaction coefficients.

Design-Expert software was chosen to run the experimental program, perform statistical and graphical analyses, and select the best combination of independent variable levels.

In silico characterization of TL13 enzymes

In silico physico-chemical properties of TL13 amylase (NCBI accession number: WP_077049650.1), protease (NCBI accession number: WP_077051805.1), and cellulase (NCBI accession number: WP_238592314.1) were computed using ExPASy ProtParam tool (https://web.expasy.org/protparam/).[29]

Secondary structures of PR proteins were predicted with online Phyre2 tool (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).[30] This tool considers sequence-based predictions of disordered regions as well as secondary structures such as percentages of α-helices and β-sheets. The three-dimensional (3-D) structures of TL 13 amylase, protease and cellulase were acquired by homology modelling using SWISS-MODEL (http://swissmodel.expasy.org). [31] The enzymes structures were validated with Ramachandran plots and statistics.[32]

Enzymes production, activities assays and stability tests

Amylase, cellulase and protease were produced by *M. metallidurans* under submerged fermentation [24,25] using the powder of palm dates as an economical substrate.[25]

Cellulase and amylase were determined by measuring the release of reducing sugars by the dinitrosalicylic acid method as described previously.[24,25] The Protease was measured following the Casine hydrolysis method, as described previously.[33] Enzyme activities were expressed in international units (the amount of enzyme required to release 1 μmole of product from the used substrate per mL per min under the standard assay conditions).

Stability tests of crude enzymes against pH, temperature and salinity (% of NaCl) were performed as described previously.[33]

Sensory analysis

Three frying donut's groups (formulation without enzymes, formulation with optimized commercial enzymes and formulation with *M. metallidurans* extremozymes) were evaluated for sensory attributes, flavor, aroma, texture, part finition, elevation, color, taste, lightness of dough and overall acceptability. A hedonic nine-point hedonic scale (7 like very much and 1 dislike very much) was carried out with a sensory panel. Samples were presented on a plastic dish assigned with a random three-digit number, and served at room temperature under normal lighting conditions. Non-trained panelists evaluated the acceptability of donuts.[34]

Statistical analyses

The mixture design SV responses were statistically analyzed by means of variance analysis (ANOVA), including Fisher's and Student's t-tests, with a significant level (α) of 0.05.[9] All statistical analyses were conducted using Design-Expert 7.1.5 (Stat-Ease Inc.) software. For sensory analyses, one-way ANOVA and Tukey's post hoc tests were conducted. In order to evaluate the relationship between different products and analyzed parameters, Principal Component Analysis (PCA) was carried out on different donut's samples. The rationale for employing PCA in sensory analysis is multifaceted, primarily focusing on its ability to simplify complex datasets, enhance interpretability, and reveal underlying structures within the data. Unlike methods that may require assumptions about data distribution or linearity, PCA is a non-parametric technique that can effectively summarize complex relationships without imposing strict model requirements. Additionally, PCA's ability to visualize data in reduced dimensions makes it particularly useful for exploratory analysis and hypothesis generation. PCA reduces the dimensionality of sensory data while retaining most of the variability present in the dataset. By transforming the original variables into a smaller set of uncorrelated principal components (PCs), PCA helps to filter out noise and highlight significant patterns. PCA aids in identifying key sensory attributes that differentiate products. The first few principal components often capture the majority of variance in the data, which corresponds to the most important sensory dimensions. This allows researchers to focus on those attributes that have the greatest impact on sensory evaluations, facilitating a better understanding of consumer preferences and product characteristics.[35] All sensory analyses were performed with R software (version 12) using the SensMap package.

Results and discussion

A three-component mixture design was implemented to study the individual and combinatorial effects of commercial mesophilic hydrolytic enzymes, namely amylase, protease and cellulase on the specific volume of cake-donuts. 14 experiments were carried out according to the matrix obtained using Design Expert software as shown in Table 1. The coefficients of the postulated mathematical model were determined via the least-squares method and the predicted values of the specific volumes of the donuts were calculated by the statistical software Design Expert. The ANOVA test suggested that the model is valid with a significant regression model and a non-significant lack-of-fit (Table 2), indicating that almost all of the interindividual variability in the responses can be efficiently predicted by the mixture design model ($R^2 = 0.97$ and adj $R^2 = 0.92$). The statistical significance of the differences between mean values of specific volumes of cake-donuts as the function of enzymes mixtures was assessed by the Student's *t*-test. A big *t*, with a small *p*-value, means that the regression coefficient is significant. It can be easily observed from Table 2 that the three enzymes A, P and C and their interactions were statistically significant and that the highest significant effect on the response was observed for amylase enzyme. The response surface and contour plots representing the effect of the three enzymes (amylase, protease and cellulase) on the response (specific volume of cake-donuts) were presented in Figure 1. The highest specific volume of donuts (10.71 ± 0.50 cm³/g) was obtained with a ternary mixture (amylase 55.85%, protease 25.83%, and cellulase 18.32%) and it was in close agreement with the predicted value 10.41 cm³/g, thus indicating the suitability of the postulated model and the success of RSM in optimizing cake-donut's formula. The optimum donut's specific volume obtained was significantly greater (P<0.01) than that obtained by the control formulation without the addition of enzymes (2.43 ± 0.20). It should also be noted that, an increase of the fermentation time by a factor of

An extremophilic enzyme cocktail from Microbacterium Metallidurans TL13 as a cake-donut improver: a comparative study with commercial enzymes-based formulation optimized by a mixture design

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10 for the control formulation leads to a similar specific volume with optimal formulation (data not shown). Our findings are in agreement with the study of Mohd Thani et al.[36] who reported a positive effect of enzyme (amylase) on the quality of donuts. Indeed, thanks to high amounts of carbohydrates and sugars in its formulation, donuts are suitable sources for recovery of total reducing sugar through enzymatic hydrolysis.

Table 2 Coefficients of the mixture design regression model and their level of significance determined by *p*-values.

Variables	Sum of squares	df	mean square	F-value	*p*-value	Significance
Model	96.43	8	12.05	21.02	0.002	S
[1]Linear Mixture	47.18	2	23.59	41.14	0.001	S
AP	1.57	1	1.57	2.73	0.16	NS
AC	0.21	1	0.21	0.36	0.57	NS
PC	29.79	1	29.79	5195	0.001	S
APC	4.6	1	4.6	8.02	0.03	S
Residual	2.87	5	0.57			
Lack of fit	0.83	1	0.83	1.62	0.27	NS
R²	0.9711					
R² adjusted	0.9249					

Model equation : Donut's SV = 8.20 A + 4.29 P + 9.76 C + 4.33 AP + 2.03 AC - 24.30 PC - 214.93 APC

Abbreviations of the levels of statistical significance: S, significant at 5% level of significance (p<0.05), NS, not significant.
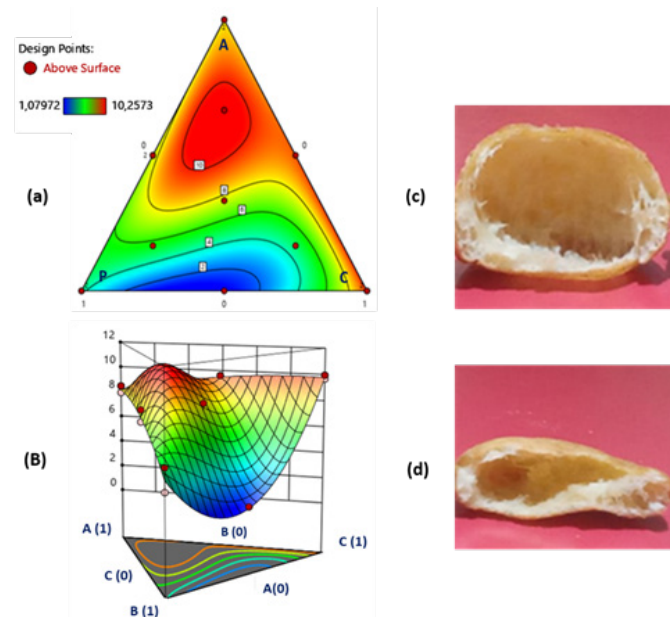

Figure 1 (a) Contour plot, and (b) 3D surface plot of the effect of the three commercial enzymes on donut's SV response. (c) donut prepared with optimal enzyme formulation (SV= 10.41 cm³/g) and (d) control donut prepared without enzymes addition (SV= 2.43 cm³/g).

Enzymes from extremophilic microorganisms are extensively explored for several industrial and basic applications, due to their high catalytic efficiency and thermal stability. Both the discovery of new extremophiles and the explosion of genomic data provide a route to new extremozymes, which are stable at extreme environmental conditions.[19–22] The use of enzyme cocktail from extremophiles to improve quality of baked goods have been deeply investigated

in the last decade.[33,37–40] In this study, the genome mining of TL13 strain identified genes encoding alpha amylase, CMCase and serine protease.

The physicochemical properties of these enzymes like molecular weight (MW), theoretical isoelectric point (pI), instability index (II), aliphatic index (AI) and grand average hydropathicity (GRAVY) were calculated using ProtParam software from ExPASy (Table 3). The calculated pI values of the enzymes of strain TL13 (pI <7) indicate that these enzymes are acidic in nature and can be purified by the isoelectric focusing method using an adequate buffer system. Based on the II values, the ExPASy protparam classifies the TL13 strain enzymes as stable proteins (II < 40). The high AI values of the enzymes from strain TL13 suggest that they may be stable over a wide temperature range.[33] Finally, the low GRAVY indices of TL13 enzymes indicate and illustrate the hydrophilic nature of these enzymes. The *in silico* study of the physicochemical properties of the TL13 enzymes also shows dominance of negatively charged amino acids compared to positively charged amino acids, which indicates and testifies to the thermal and saline stability of the enzymes of strain TL13.[33] It has been described that the negative charges of enzymes are involved in maintaining the tertiary structure thus facilitating protein folding and preventing protein aggregation by stabilizing bonds with water and/or ions. The secondary structures of TL13 enzymes predicted by the Phyre2 software highlighted flexible conformations of TL13 enzymes which enhance their activity and stability under stressful conditions. The higher percentages of amino acids forming α-helices indicates the temperature stability of these enzymes [33]. The 3D structural models of TL13 enzymes were built using SWISS-MODEL. The structures of the alpha-amylase II of *Thermoactinomyces vulgaris* R-47 (PDB id: 1wzk.1), the serine protease of *Microbacterium enclense* (A0A443JH58_9MICO) and the endoglucanase Cel6 from *Mycobacterium tuberculosis* (PDB id: 1uoz), : having 25.54, 91.82 and 37.32% sequence identities with TL13 amylase, protease and cellulase respectively, were selected as a templates to generate the 3D models (Figure 2). The Global Model Quality Estimate (GMQE) scores expressed as numbers between 0 and 1were used to validate the stereochemical quality of generated 3D models. The GMQE of the generated models were 0.66, 0.89 and 0.72 for TL13 amylase, protease and cellulase, respectively indicating good quality predicted 3D models. The modeled structures were also validated by predicting the Ramachandran plot which indicated 90.63, 96, 66 and 90.75% were in the favored region (Figure 2). MolProbity scores for TL13 amylase, protease and cellulase were 1.90, 1.20 and 1.40 and respectively.
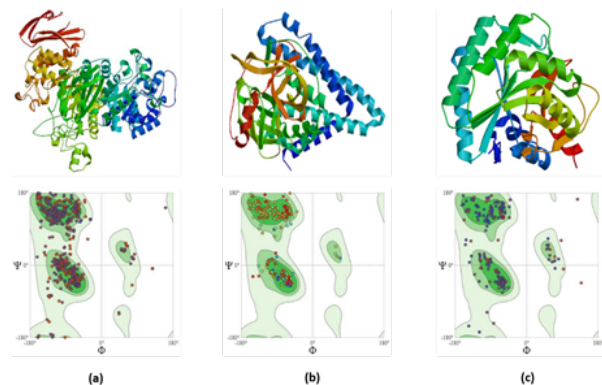

Figure 2 3D models and their corresponding Ramachandran plots of *M. metallidurans* extremozymes, generated from the SWISS-MODEL tool. (a) alpha amylase (WP_077049650.1); (b) serine protease (WP_077051805.1) and (c) cellulase (WP_238592314.1).

An extremophilic enzyme cocktail from Microbacterium Metallidurans TL13 as a cake-donut improver: a comparative study with commercial enzymes-based formulation optimized by a mixture design

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Table 3 Physicochemical properties and secondary structure forms of *M. metallidurans* extremozymes computed using Expasy's ProtParam tool.

	Alpha-amylase (pullulinase) (WP_077049650.1)	Putative Serine protease (WP_077051805.1)	Possible CMCase (WP_238592314.1)
Physicochemical properties			
MW (Da)	46968.03	39349.47	33424.13
pI	4.94	4.48	4.50
II	31.15	27.10	24.68
AI	97.79	120.79	87.54
GRAVY	0.010	0.690	-0.152
-R / +R	64/35	32/20	44/23
Secondary structure forms			
Alpha helix (%)	26	33	37
Disordered (%)	0	30	5
Beta stand (%)	16	22	12
TM helix (%)	4	21	0

Abbreviations: MW, molecular weight; pI, theoretical isoelectric point; II, instability index; AI, aliphatic index; GRAVY, grand average hydropathicity; R, number of negative residues; +R: number of positive residues

The extremozyme cocktail of *M. metallidurans* TL13 strain was produced by submerged fermentation using the powder of palm dates as a low-cost substrate (Figure 3). The TL13 crude enzyme extracted at 13th days of fermentation presented the following optimal activities (in U/gds) : cellulase (CMCase) 49.5, amylase 96.1 and protease 4.11. Experimental biochemical characterization results represented showed interesting features like good temperature stability (40-60 °C), pH stability (6.0-9.0), and saline stability (0.1-1.0 M of NaCl) (data not shown). This behavior was in line with the computational characterization and predictive information obtained by ProtParam tool. The TL13 extremozyme cocktail was applied in the production of cake-donuts and compared with optimized formulation with commercial enzymes. It can be clearly seen from the Figure 4 that the donut produced with extremozymes was the most appreciated with a significant difference in the texture, flavor, taste, lightness of dough and overall acceptance. In the other hand, it was noted that the aroma of donut with enzymes (commercial enzymes and TL13 extremozymes) was significantly more appreciated than the negative control one (the donut produced without enzyme addition). For the rest of attributes, the difference in all products were not significant. The results of the PCA and ANOVA showed that the donut samples in different groups (control donuts without enzyme addition, donuts with commercial enzymes and donuts with TL13 extremozymes) were clearly distinguished between the two principal components, and the different samples in the same group clustered well (Figure 4). The donuts prepared with TL13 extremozymes were highly perceived in terms of organoleptic attributes, based on hedonic sensory responses to color, texture, flavor, taste, lightness of dough, aroma and overall acceptance (Figure 4). The enzymes derived from the extremophilic actinobacterium *M. metallidurans* performed better than commercial mesophilic enzymes thanks to their high thermal stability and high resistance to harsh conditions. The secreted enzyme cocktail could be used as an efficient additive in baked donuts to improve product volume and sensory characteristics.
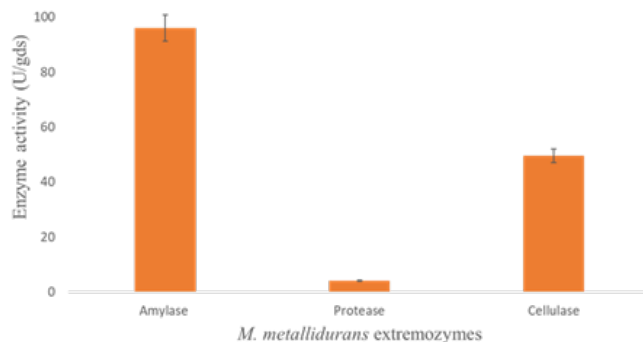
Figure 3 Amylase, protease and cellulase activities obtained at 13th day of *M. metallidurans* cultivation on palm dates based medium.
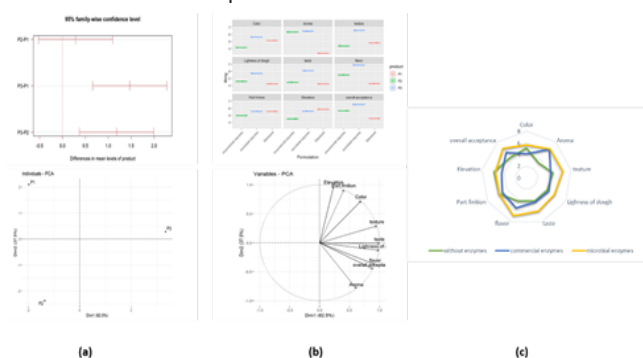
Figure 4 Sensory analyses of control donuts (without enzyme addition), donuts prepared with commercial enzymes and donuts prepared with TL13 extremozymes: statistical analysis of variance (ANOVA), (b) principal component analysis (PCA) and (c) Spider chart representing sensory attributes of donuts samples.

Conclusion

In conclusion, in this study, a three-factor mixture design was applied to study the individual and combinatorial effects of three commercial microbial enzymes, namely amylase, cellulase and protease, on the specific volume of donuts and to determine their optimal levels resulting in better product quality and customer satisfaction. This approach succeeded in gathering the maximum information in minimum time at the lowest cost. The quality of the postulated mathematical model was verified using ANOVA, Fisher's test, the significance of the model regression coefficients and the insignificance of the lack of fit of the model. The predictive quality of the mixture design model was also checked using the multilinear regression coefficients. In addition, the graphical exploitation of the model allowed to elucidate the significant interactions between the three variables (commercial enzymes) and to determine an optimal enzymes mixture leading to donuts with desired physical and sensory properties. Furthermore, our results prove the advantage of *M. metallidurans* hydrolytic extremozymes cocktail on the quality of baked donuts compared to commercial enzymes. To the best of our knowledge, this is the first report on applying mixtures of hydrolytic enzymes to improve donut's properties and qualities. Future work will be focused on testing *M. metallidurans* extremozymes in other food formula, particularly in the baking and pastry fields.

Acknowledgments

An extremophilic enzyme cocktail from Microbacterium Metallidurans TL13 as a cake-donut improver: a comparative study with commercial enzymes-based formulation optimized by a mixture design

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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