

**Effect of lactase, transglutaminase and temperature on ice cream crystal by a
response surface methodology approach**

**Efeito da lactase, transglutaminase e temperatura nos cristais do sorvete considerando
uma abordagem de metodologia de superfície de resposta**

**Efecto de la lactasa, transglutaminasa y temperatura en cristales de helado
considerando un enfoque de metodología de superficie de respuesta**

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Abstract

This study aimed to evaluate the ice cream crystal content considering the addition of enzymes lactase (0.3% to 0.9%) and transglutaminase (0.6% to 7.4%), employing different incubation temperatures (13 to 47 °C) through a 2³ central composite rotatable design (DCCR). The crystals content was estimated by ice cream scattering in blades and the crystals images were obtained with a bright field optical microscope for counting and determining the crystals size using Image J software. All ice cream treatments prepared at 40 °C (T2, T6, and T8) and TA2 treatment (T2 treatment similar formulation) showed small content of crystals if compared with the temperatures of 20 and 30 °C; it was probably associated with a large presence of air bubbles, fat globules and probably some casein micelles, making them ideal for small crystals agglomeration that form a firmer, smooth and cohesive texture. Moreover, the combined use of lactase and transglutaminase enzymes in the ice cream is viable, efficient and an easy technology for ice cream production. Furthermore, the use of response surface

methodology was effective in selecting the best formulation in relation to desirability features ensuring its use in the ice cream development.

Keywords: Central composite rotatable design; Crystal size; Enzymes; Microscopy; Strawberry ice cream.

Resumo

Este estudo teve como objetivo avaliar os cristais de sorvete considerando a adição das enzimas lactase (0,3% a 0,9%) e transglutaminase (0,6% a 7,4%), empregando diferentes temperaturas de incubação (13 a 47 °C) através de um Delineamento Composto Central Rotacional 2³ (CCRD). O conteúdo de cristais foi estimado pelo espalhamento de sorvete em lâminas e as imagens dos cristais foram obtidas em um microscópio óptico de campo claro para contagem e determinação do tamanho dos cristais usando o software Image J. Todos os sorvetes preparados a 40 °C (T2, T6 e T8) e o tratamento TA2 (formulação semelhante ao tratamento T2) apresentaram pequeno teor de cristais se comparados às temperaturas de 20 e 30 °C; provavelmente foi associado a uma extensa presença de bolhas de ar, glóbulos de gordura e algumas micelas de caseína, favorecendo a aglomeração de pequenos cristais que formam uma textura mais firme, lisa e coesa. Além disso, o uso combinado das enzimas lactase e transglutaminase no sorvete é uma estratégia viável, eficiente e possível para a produção de sorvetes. Além disso, o uso da metodologia de superfície de resposta foi eficaz na seleção da melhor formulação em relação às características de desejabilidade para o sorvete.

Palavras-chave: Delineamento composto central rotacional; Tamanho de cristal; Enzimas; Microscopia; Sorvete de morango.

Resumen

Este estudio tuvo como objetivo evaluar los cristales de helado considerando la adición de las enzimas lactasa (0.3% a 0.9%) y transglutaminasa (0.6% a 7.4%), utilizando diferentes temperaturas de incubación (13 a 47 °C) através de un diseño compuesto central y rotativo 2³ (CCRD). El contenido de cristales se estimó extendiendo helado en hoja de vidrio y las imágenes de los cristales se obtuvieron en un microscopio óptico de campo de luz para contar y determinar el tamaño de los cristales utilizando el software Image J. Todo helado preparado a 40 °C (T2 , T6 y T8) y el tratamiento TA2 (formulación similar al tratamiento T2) tuvo un contenido de cristales pequeño en comparación con temperaturas de 20 y 30 °C; probablemente se asoció a una presencia extensa de burbujas de aire, glóbulos grasos y

algunas micelas de caseína, favoreciendo la aglomeración de pequeños cristales que forman una textura más firme, lisa y cohesiva. Además, el uso combinado de enzimas lactasa y transglutaminasa en helados es una estrategia viable, eficaz y posible para la producción de helados. Además, el uso de la metodología de superficie de respuesta fue eficaz para seleccionar la mejor formulación en relación con las características deseables para el helado.

Palabras clave: Diseño compuesto central y rotativo; Tamaño de cristal; Enzimas; Microscopía; Helado de fresa.

1. Introduction

Ice cream is one of the most appreciated milk derivatives (Tsuchiya et al., 2017). It is considered a colloidal system (1nm to 1 μ m in size particle system), consisting of crystals, air bubbles with crystalized fat and water in a highly concentrated sugar solution, containing hydrocolloids, casein micelles and other proteins (Adhikari et al., 2020; Aloglu et al., 2018; Homayouni et al., 2018). It is important to point out that ice cream chemical composition determines several structural and quality parameters of the final product.

Enzymes are used in a wide variety of applications in the food industry. Lactase (β -galactosidase enzyme) (EC. 3.2.1.23) hydrolyzes lactose into its monosaccharides. The lactose hydrolysis is becoming increasingly important for food use as it modifies lactose solubility, sweetness, reducing power and milk products fermentability, overall allowing an increased digestibility of these foods by lactose-intolerant consumers (Tsuchiya et al., 2017). Lactase enzyme incorporation is a promising process, as it enables the development of new lactose-free products, prevents lactose crystallization in ice cream production, fermented products such as yoghurt, condensed milk, and *dulce de leche* (milk jam) (Dekker et al., 2019; Francisquini et al., 2020; Skryplonek et al., 2019). Lactose enzymatic hydrolysis fosters physical and chemical modifications, kept or improving dairy products' technological and sensory characteristics as viscosity, body, texture and taste of ice creams (Medeiros et al., 2019).

Transglutaminase enzyme (EC 2.3.2.13) is a transferase that catalyzes the acyl group transfer reaction between protein amino acid residues. It forms covalent interactions, cross-link between Glutamine and Lysine amino acid residues (G-L interactions), and reacts quite well with milk casein that has an open conformation, while for serum proteins, which present globular conformation, transglutaminase only reacts under conditions favorable to globular proteins structure unfolding, facilitating enzymatic action (Aloglu et al., 2018; Matsumura et

al., 2000). Additionally, the use of transglutaminase in dairy products manufacturing allows the production of smoother, low-calorie, sugar-free ice creams, providing easier spoon handling in order to obtain and high quality ice cream (Al et al., 2020; Kuraishi et al., 2001). In this context, the addition of lactase and transglutaminase enzymes, it sought to achieve an innovative ice cream product, with added value and desirable functional properties.

Crystals quantification is required to determine whether the ice cream presents the adequate amounts of crystals in terms of distribution and size resulting in a smooth and refined texture, besides providing a sense of freshness perceived by the consumer. Therefore, electron microscopy technique have been used as it is vitally important for qualitative and quantitative analyses on ice cream samples, since it allows viewing and size analyzing, quantity, and distribution of crystals and other ice cream particles (Cavender & Kerr, 2020; Hartel, 1996).

Considering the study of lactase and transglutaminase in ice cream preparation, the response surface methodology (RSM) represents an excellent tool. RSM is a mathematical and statistical method effectively used to develop, improve and optimize processes seeking the formulation of new products. The most popular optimization has been used for the development of new products and processes; existing products or processes improvements; product quality and performance optimization; manufacturing process optimization, and production costs minimization (Rodrigues & Iemma, 2014). This study used RSM as a quite useful tool to optimize ice cream production with a strictly controlled quality using lactase and transglutaminase enzyme concentrations as parameters at high temperatures, in order to establish the relation between the responses and independent variables.

The aim of this study was to apply the response surface methodology in lactase and transglutaminase enzymes activity evaluation on milk proteins combined with different temperatures, and evaluate the crystal content of strawberry ice cream formulation.

2. Methodology

Materials

The ice cream ingredients were: pasteurized milk, milk powder, and pasteurized milk cream (Frimesa, Brazil); whey protein concentrate (WPC) (Sooro, Brazil); super neutral stabilizer from League[®] (Duas Rodas, Brazil), Emustab[®] emulsifier (Duas Rodas), strawberry flavor Algemix[®] (Duas Rodas); sucralose/acesulfame-k, carmine dye from colchonilha (Gemacom,

Brazil); strawberry aroma (Givaudan, Brazil); Prozin lactase[®] enzyme (Candon Additives, Brazil); and YG-transglutaminase enzyme from Activia[®] (Ajinomoto, Brazil). The milk used in ice-cream formulations was standardized at 8% fat with pasteurized milk cream and added to WPC at 10%.

Experimental Design

A Central Composite Rotatable Design 2³ (3 factors, 5 levels, 4 center point, total of 18 treatments) was performed to evaluate the effects of lactase (0.3% to 0.9%) and transglutaminase (0.6% to 7.4%) enzyme concentrations, and incubation temperature (13 to 47 °C) on crystal content in 22 μm² (Table 1). The CCRD treatments were carried out in a random order, in triplicate (total of 54 experiments) (Table 1, Figure 1a) and microscopic analyses were performed in triplicate for treatments T1-T18 and standard treatments (ST1-ST18; without enzyme addition) (Table 2, Figure 1a).

Table 1. Matrix with coded and real independent variables and response of crystal size.

Run	Lactase (g L ⁻¹)	Transglutaminase (U g ⁻¹ protein)	Temperature (°C)	Crystal content in 22 μm ²
T1	-1 (0.4)	-1 (2.0)	-1 (20)	10.00 ^a ± 0.00
T2	-1 (0.4)	-1 (2.0)	1 (40)	3.11 ^{de} ± 3.43
T3	-1 (0.4)	1 (6.0)	-1 (20)	3.61 ^{cd} ± 3.08
T4	-1 (0.4)	1 (6.0)	1 (40)	10.00 ^a ± 0.00
T5	1 (0.8)	-1 (2.0)	-1 (20)	0.22 ^f ± 0.48
T6	1 (0.8)	-1 (2.0)	1 (40)	0.75 ^{ef} ± 1.13
T7	1 (0.8)	1 (6.0)	-1 (20)	0.94 ^{ef} ± 1.37
T8	1 (0.8)	1 (6.0)	1 (40)	5.28 ^{bcd} ± 3.50
T9	-1.68 (0.3)	0 (4.0)	0 (30)	6.89 ^{abc} ± 3.92
T10	1.68 (0.9)	0 (4.0)	0 (30)	0.25 ^f ± 0.55
T11	0 (0.6)	-1.68 (0.6)	0 (30)	1.19 ^{ef} ± 1.88
T12	0 (0.6)	1.68 (7.4)	0 (30)	1.80 ^{ef} ± 2.85
T13	0 (0.6)	0 (4.0)	-1.68 (13)	0.83 ^{ef} ± 2.00
T14	0 (0.6)	0 (4.0)	1.68 (47)	0.83 ^{ef} ± 2.00
T15	0 (0.6)	0 (4.0)	0 (30)	9.86 ^{ab} ± 0.49
T16	0 (0.6)	0 (4.0)	0 (30)	0.31 ^f ± 0.62
T17	0 (0.6)	0 (4.0)	0 (30)	2.75 ^{de} ± 3.80
T18	0 (0.6)	0 (4.0)	0 (30)	0.61 ^{ef} ± 0.84

Source: Authors.

The diet strawberry ice cream samples that showed best palatability and creaminess, the most relevant aspects in sensory acceptability terms, at 40 °C, were subjected to microscopy analysis (Figure 1b). Thus, the 40 °C temperature was used to perform the treatments named TA2, TA4, TA6, TA8, TALC, TAT, and ST, using a single lactase enzyme concentration (0.4 g L⁻¹), different transglutaminase enzyme concentrations (2.0 to 8.0 U g⁻¹ protein), and no enzyme addition as seen in Table 2. The aim of this step was to evaluate a DCCR similar conditions at 40 °C and with a fixed lactase concentration, in order to evaluate only the effect of transglutaminase. Manufacturing stages for all ice cream formulations are specified in Figure 1c.

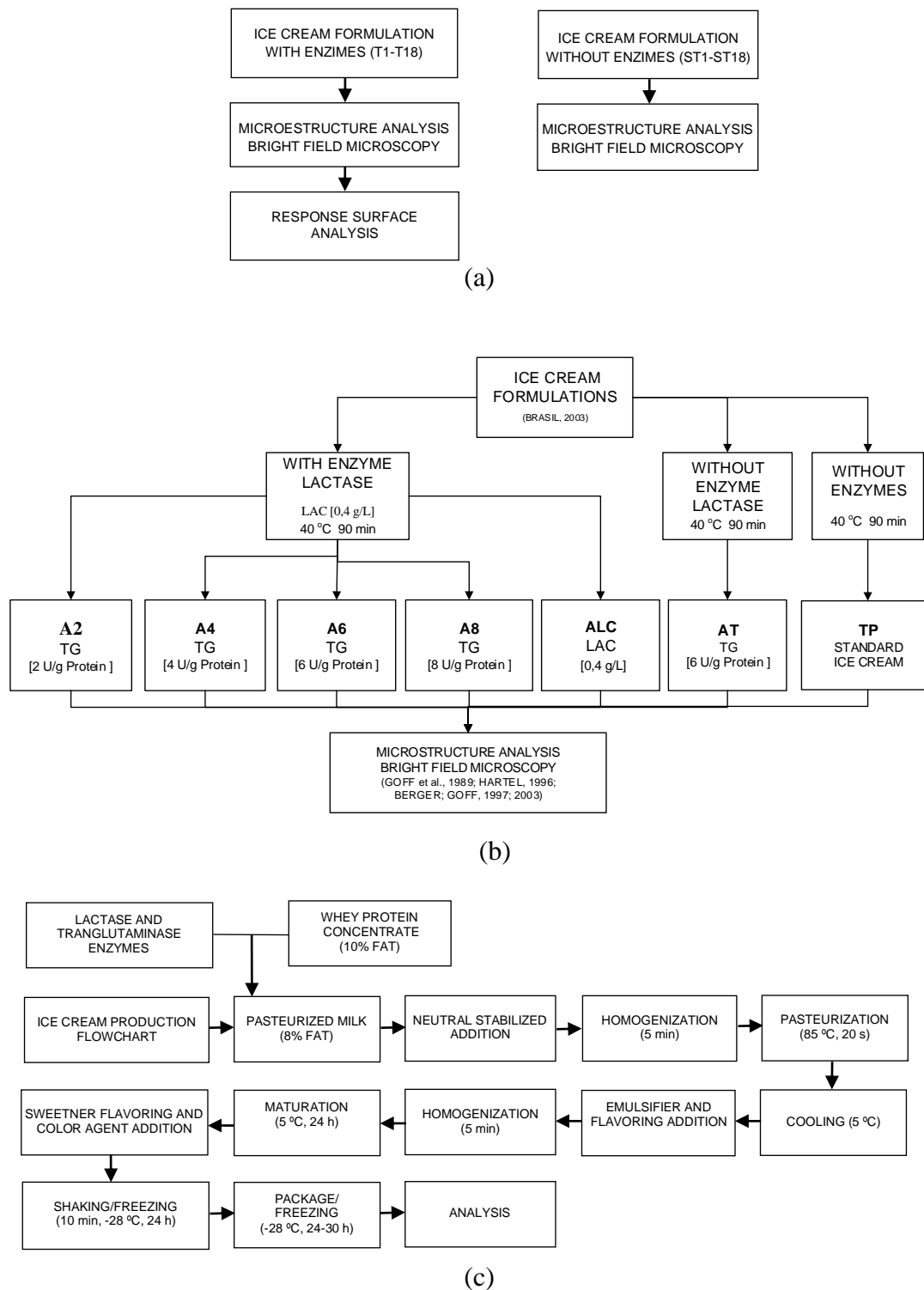
Table 2. Ice cream standard treatments (without enzyme) and treatments using a single lactase enzyme concentration, different transglutaminase enzyme concentrations, and no enzyme addition and their crystal content in 22 μm².

Run	Lactase (g L ⁻¹)	Transglutaminase (U g ⁻¹ protein)	Temperature (°C)	Crystal content in 22 μm ²
ST1	-	-	-1 (20)	10.00 ^a ± 0.00
ST2	-	-	1 (40)	10.00 ^a ± 0.00
ST3	-	-	-1 (20)	10.00 ^a ± 0.00
ST4	-	-	1 (40)	0.17 ^d ± 0.51
ST5	-	-	-1 (20)	10.00 ^a ± 0.00
ST6	-	-	1 (40)	10.00 ^a ± 0.00
ST7	-	-	-1 (20)	10.00 ^a ± 0.00
ST8	-	-	1 (40)	6.61 ^{bc} ± 3.06
ST9	-	-	0 (30)	10.00 ^a ± 0.00
ST10	-	-	0 (30)	9.67 ^a ± 0.99
ST11	-	-	0 (30)	0.47 ^{cd} ± 1.84
ST12	-	-	0 (30)	10.00 ^a ± 0.00
ST13	-	-	-1.68 (13)	10.00 ^a ± 0.00
ST14	-	-	1.68 (47)	10.00 ^a ± 0.00
ST15	-	-	0 (30)	8.53 ^{ab} ± 2.26
ST16	-	-	0 (30)	10.00 ^a ± 0.00
ST17	-	-	0 (30)	10.00 ^a ± 0.00
ST18	-	-	0 (30)	9.94 ^a ± 0.23
TA2	(0.4)	(2.0)	(40)	0.72 ^b ± 1.08
TA4	(0.4)	(4.0)	(40)	10.00 ^a ± 0.00
TA6	(0.4)	(6.0)	(40)	1.33 ^b ± 1.45
TA8	(0.4)	(8.0)	(40)	1.92 ^b ± 2.21
TALC	(0.4)	(0.4)	(40)	0.22 ^b ± 0.54

TAT	-	(6.0)	(40)	10.00 ^a ± 0.00
ST	-	-	(40)	10.00 ^a ± 0.00

Note: Crystal content mean in 22 $\mu\text{m}^2 \pm$ standard deviation; lowercase letters differ vertically compared indicate statistically significant differences between the 5% probability level by the Dunn test (nonparametric). Source: Authors

Figure 1. (a) Flowchart for diet strawberry ice cream formulations analyses with lactase and transglutaminase enzymes (T1-T18) and without adding enzymes (ST1-ST18); (b) Flowchart for diet strawberry ice cream formulations analyses with lactase and transglutaminase enzymes (TA2, TA4, TA6, TA8), and with enzyme lactase addition (TALC), with enzyme transglutaminase addition (TAT), and without adding enzymes (ST); (c) Flowchart for diet strawberry ice cream manufacturing with whey protein concentrate, sucralose/acesulfame-k and lactase and transglutaminase enzymes.



Ice cream preparation. Source: Authors.

Ice creams syrups in different treatments were initially warmed at 85 °C for 20 s to achieve protein denaturing in the serum and facilitate their interaction with the casein micelles, which increases the susceptibility of protein to transglutaminase reaction (Sharma et al., 2001). The syrups were cooled down at an incubation temperature according to Table 1, added of lactase and transglutaminase enzymes, and incubated for 90 min in the defined temperature (B.O.D. model NT705). After, the treatments were inactivated following method by Rodrigues-Nogales (2006), with adaptations. For all treatments, standardized samples (ST1-ST18) were carried without enzyme addition at the same temperatures, starting then ice cream manufacturing as detailed in Figure 1c.

Microscopy analysis

Cubes of approximately 0.3 cm³ were used to evaluate the crystals size formed in ice cream samples stored at 28°C to 30°C. They were spread on slides for microscopy by adding a crystal dispenser agent (isoamyl alcohol). Isopropyl alcohol on the blades covered with glass slides was used for best sample viewing, and then cooled down for 3 min at -28 °C in cryostat (Leica Cryostats Microtomes model CM 1850, Germany) according to Regand & Goff (2003) and Costa et al. (2008), with adaptations.

All instruments and reagents used in samples preparation were pre-chilled at -28°C. The crystals images were obtained using an optical microscope (model Bx41, Olympus, Japan) with a Q-imaging 3.3 MP digital color camera and a Q-capture Pro 5.1 image capture program from Q-imaging. Different fields in each plate were photomicrographed with a 400 x magnitude to obtain the crystals measurements (300 crystals) per sample. Crystal diameter measurement was determined using the ImageJ software. The diameter values of crystals were distributed in a frequency curve to obtain the median (X 50), according to the model presented by Flores & Goff (1999).

Statistical Analysis

The statistical analysis for numeric variables comparison between treatments was carried out using analysis of variance ANOVA, adopting the Kruskal-Wallis analysis (non-parametric) followed by Dunn, at a 5% significance level assisted by BioEstat 5.0 program. All analyses were carried out in triplicates. Analysis of variance (ANOVA) and a multi-

comparison Tukey-Kramer post-test at $p < 0.05$ significance level were carried out for response surface using the software Statistica 8.0 (Statsoft, Tulsa, USA).

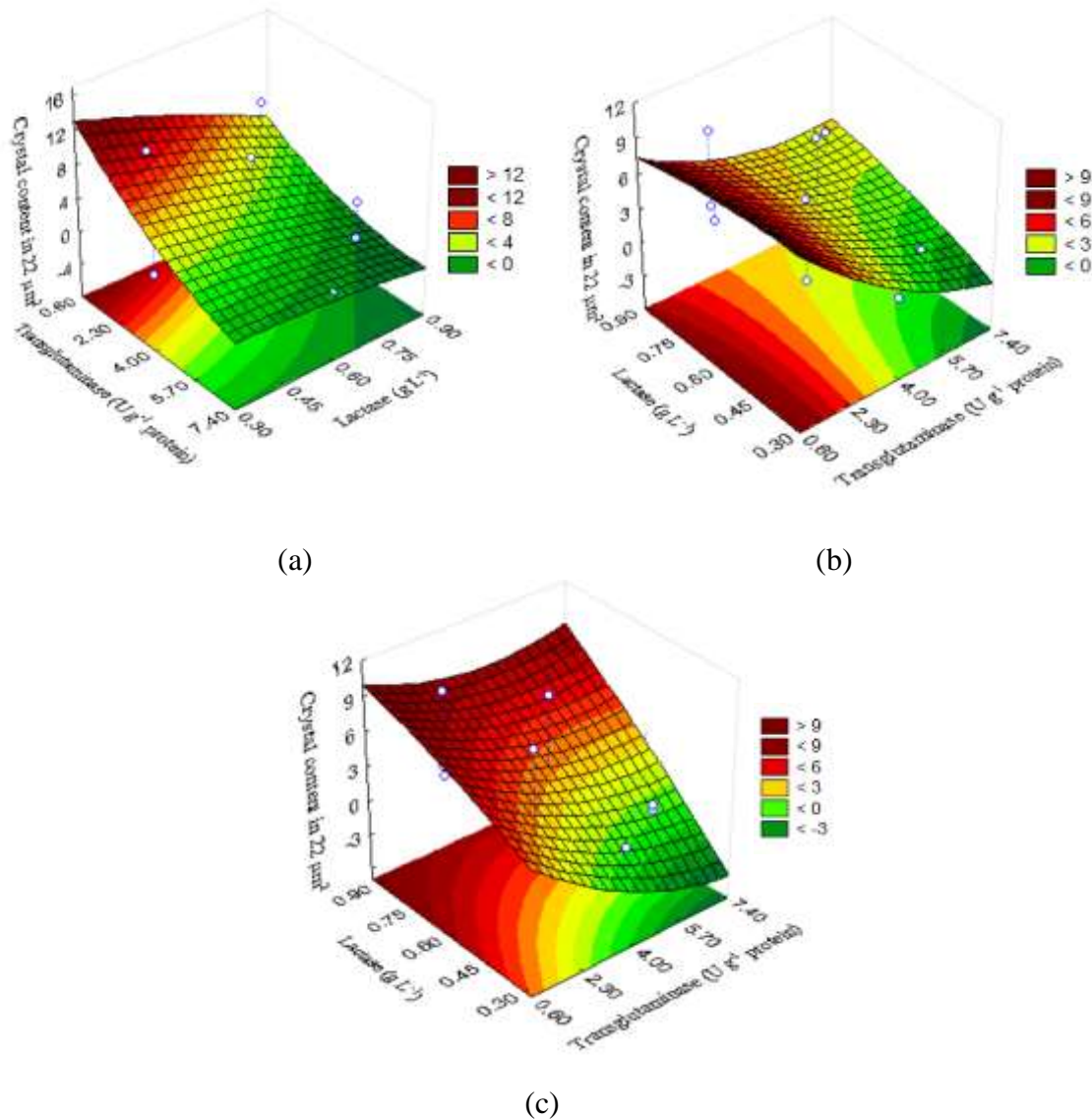
3. Results and Discussions

Crystals content at 20 °C at the presence of enzymes

Figure 2 presented the surface response of crystal content considering the influence of lactase and transglutaminase concentration at 20, 30 and 40 °C (Figure 2a, 2b and 2c, respectively). The crystal content for treatments T1, T3, T5, and T7 incubated at 20 °C suggest that transglutaminase enzyme concentrations were lower than 1.5 U/g protein, while lactase enzyme concentrations were lower than 5.5 g/L obtaining greater crystals content (> 60 crystals/mm²) (Figure 2a). The crystals content formed in mentioned treatments were still lower, probably due to lactase enzyme interactions strengthening that, which even at low temperatures was effective in lactose hydrolysis, reducing free water availability to form crystals (Horner et al., 2011). On the other hand, since the amounts of crystals formed in these treatments were lower, it infers that the synergistic effect associated with the combined use of enzymes increased cross-links formation to form large polymers, contributing to strengthening the three-dimensional network that prevented large crystals formation.

In addition, it is assumed that the performance of transglutaminase formed links between milk proteins, creating a fine network that immobilized the water in large proportions and probably the same occurred with fat to form a firm gel. This causing protein hardening, covering fat globules and taking part in the protein network, creating air bubbles, which occupy spaces in the middle by avoiding water movement as a consequence, hindering large crystals formation observed in ice cream during storage (Metwally, 2007). The crystal formation occurs during the initial freezing process, and its size is critical to determine the ice cream quality. Generally, smaller crystals are preferred, which are imperceptible to the palate, since large crystals result in coarser and sandy texture (Dekker et al., 2019; Hartel, 1996; Kaleda et al., 2018).

Figure 2. (a) Response surface analysis chart for crystals content (crystals mm^{-2}), related to changes in lactase and transglutaminase enzymes concentrations added to diet strawberry ice cream formulations under 20 °C. Figure 2 (b) Response surface analysis chart for crystals amounts (crystals mm^{-2}) relating to changes in lactase and transglutaminase enzymes concentrations added to diet strawberry ice cream formulations, at 30 °C. Figure 2 (c). Response surface analysis chart for crystals amounts (crystals mm^{-2}) in terms of regarding the variations of lactase and transglutaminase enzymes concentrations added to diet strawberry ice cream formulations at 40 °C.



Source: Authors

According to Figure 3 that shows the photomicrographs of ice cream smears in T1 to T18 treatments, the crystals content in higher transglutaminase enzyme concentrations was always above 2 crystals/ mm^2 irrespective of the lactase enzyme concentration, matching the crystals counting obtained in treatments T1 and T3 (Figures 3a and 3c), which showed amounts of crystals above this range. Thus, the response surface (Figure 2a) suggest that proteins polymerization may occurred forming a strong and cohesive protein network with the

presence of protein pellets, air bubbles, and fat, a combination that might have prevented crystals formation in larger amounts.

Figure 3. Photomicrographs of ice cream smears in T1 to T18 treatments with increasing transglutaminase and lactase enzymes concentrations, at different temperatures.

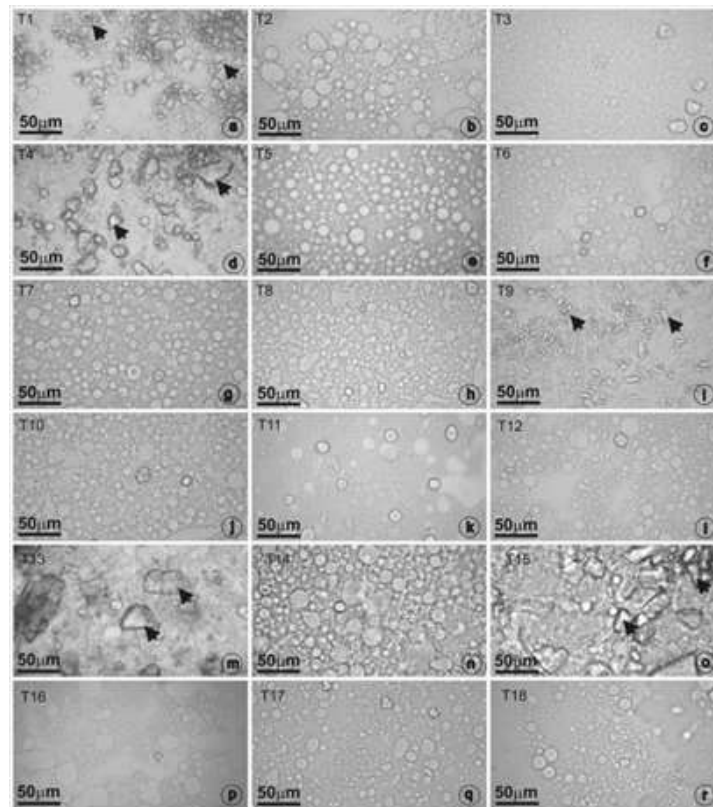


Figure 3a-3r Photomicrographs of ice cream smear in the different treatments (1-18) without staining: (a) Treatment 1 (T1); (b) Treatment 2 (T2); (c) Treatment 3 (T3); (d) Treatment 4 (T4); (e) Treatment 5 (T5); (f) Treatment 6 (T6); (g) Treatment 7 (T7); (h) Treatment 8 (T8); (i) Treatment 9 (T9); (j) Treatment 10 (T10); (k) Treatment 11 (T11); (l) Treatment 12 (T12); (m) Treatment 13 (T13); (n) treatment 14 (T14); (o) Treatment 15 (T15); (p) Treatment 16 (T16); (q) Treatment 17 (T17); and (r) Treatment 18 (T18). Arrows indicate the lactose crystals. Source: Authors

According to Figure 2a, the lowest crystals content was obtained on the transglutaminase enzyme concentrations above 6 U/g protein, associated with lactase enzyme concentrations above 0.9 g/L. The photomicrographs confirm the results obtained for T5 and T7 regarding crystal content (Table 1 and Figures 3e and 3g), which used a 0.8 g/L lactase enzyme concentration and proteins cross-link formation may have occurred, with fat globules agglomeration in casein micelles, increasing the medium viscosity, and reducing water mobility. A greater array viscosity increases flow resistance, and more time is needed to spread water on the ice cream structure; consequently, small crystals are formed (Muse &

Hartel, 2004). According to Wang et al., (2007), the crystal growth could be inhibited by proteins or polypeptides adsorption in ice-solution interface by means of hydrogen bonds.

The results obtained for crystals content in treatments T1, T3, T5, T7, and T9 (Table 1), show statistically significant differences between the 5% probability level done by the Dunn test (nonparametric). Presumably, transglutaminase performance forming links between milk proteins, creating a fine network that immobilized water in large proportion and probably fat, forming a firm gel caused a protein hardening effect, covering fat globules and taking part in the protein network, creating air bubbles which occupy spaces in the middle and avoiding water movement in the middle (Metwally, 2007).

However, in higher transglutaminase enzyme concentrations, the amounts of crystals were generally above 2 crystals/mm², regardless of the lactase enzyme concentration, corroborating with the crystals content in treatments T1 and T3 (Table 1 and Figures 3a and 3c), which showed crystals content above this range. Enzymes polymerization may have occurred, forming a strong and cohesive protein network, and the presence of protein pellets, air bubbles, and fat, have possibly prevented crystal formation in larger quantities. As previously stated, the crystal formation in ice cream determines its final quality, and their size is critical to determine the quality of the ice cream; usually, smaller crystals are preferred, which are imperceptible to the palate, as large crystals result in a coarser and sandy texture (Hartel, 1996; Kaleda et al., 2018).

Crystals content at 30 °C at the presence of enzymes

For treatments incubated at 30 °C, the crystal content for T9, T10, T11 T12, T15, T16, T17, and T18 treatments suggest that at transglutaminase enzyme concentrations smaller than 1 U g⁻¹ protein, the enzyme lactase concentration did not influence crystals formation in ice cream treatments (Figure 2b); the number of crystals formed in all lactase enzyme concentrations were greater than 24 crystals/mm². An exception was observed for treatment T11, which showed a small growth of crystals, because the content formed was smaller than 24 crystals/mm², demonstrating that there was no polymerization and lactase hydrolysis forming cross-linked proteins as seen in Figure 3k. Possibly, a large agglomeration of fat globules, casein micelles, and air bubbles occurs in ice cream studies. Metwally (2007) observed that fat is covered by a partly coalesced protein layer stabilizing the air bubbles and foam structure, which makes for better ice cream texture, avoiding large crystals formation.

Lactose hydrolysis may have favored higher protein polymerization by the transglutaminase enzyme forming a more cohesive structure, decreasing the free water in the middle and, consequently, forming less crystals, hence decreasing ice cream firmness. According to (Cruz et al., 2009), adding air during ice cream processing increases product volume in relation to the syrup. The air bubbles are surrounded by fats and milk proteins and defining the final product structure, providing both greater foam stability and texture (Tsuchiya et al., 2017). Several factors are crucial to develop air bubbles in the ice cream, such as shear force applied during freezing, nonfrozen matrix (syrup) viscosity, and the partial fat globules coalescence degree (Chang & Hartel, 2002).

Although with little variation, lactase enzyme concentrations influence on the number of crystals formed from 5 U g⁻¹ protein transglutaminase enzyme concentrations. However, in T10, T12, T16, T17, and T18 treatments (Table 1), a small crystals formation occurred, as shown in Figures 3j, 3l, 3p and 3q. The small crystals resulted from the limitation caused by the large amounts of fat and air bubbles, which possibly prevented the formation of large crystals.

For crystals growth in ice cream, free water availability is needed to start the process. This occurs when the total ice cream solids are moved to the crystal's growth region, immediately increasing the medium concentration, elevating instantly the boundaries between the crystals and the unfrozen phase. In contrast, microviscosity increases with the improvement of concentration in the middle, forming a water movement kinetic dependent gel. Therefore, this increase in microviscosity will delay water migration surrounding each crystal growing in the area, hindering growth (Regand & Goff, 2003).

However, treatments T9 and T15 presented crystals larger than in other treatments. Figure 3 shows that no fat droplets or air bubbles are noticed on those treatments, which hinder the growth of crystals, as described previously. According to the literature, crystals formation may occur at the start of the ice cream shaking process. The conditions for this to occur are close to the cylinder surface, which is cooled down by a coolant, at a very low temperature, causing crystals nucleation. These surface-formed nuclei are scraped into the mixture (area furthest away from the surface), where temperature is more similar to freezing start, favoring crystals growth (Hartel, 1996).

The largest variation in the number of crystals formed as a function of lactase enzyme concentration was the transglutaminase enzyme concentration between tracks 7 and 8 U g⁻¹ protein (Figure 2b). At this temperature, crystals formation is more affected by transglutaminase enzyme concentration than by the lactase enzyme one. It maybe observed in

T12 treatment with a transglutaminase concentration of 7.4 U g^{-1} protein (Table 1 and Figure 3l), where protein polymerization by the transglutaminase enzyme can be seen, with very few crystals, showing many round-shaped air bubbles, well-defined fat droplets and some casein micelles or denatured serum proteins, present in the ice cream emulsion, forming a firm emulsion, hence avoiding crystals formation. Studies by Flores & Goff (1999) proved that a certain content of air present in the ice cream is required to impact the microstructure. Thus, the presence of air bubbles may affect the ice cream thermal properties, acting also as a fixed barrier during freezing. Crystal collision is less probable in an air-dispersed structure, reducing there-crystallizing phenomenon.

Crystals content at 40 °C at the presence of enzymes

The ice cream crystal content for T2, T4, T6, and T8 incubated at 40°C suggest that higher transglutaminase concentrations associated with low lactase concentrations resulted in low content of formed crystals (fewer than $26 \text{ crystals mm}^{-2}$) (Figure 2c). On the other hand, low transglutaminase concentrations associated with high lactase concentrations resulted in a greater number of formed crystals (more than $30 \text{ crystals mm}^{-2}$). Lactase levels variation had lesser influence in crystals formation (ranging from 14 to over 30 crystals/mm^2) at low transglutaminase concentrations (below 1 U protein^{-1}) than in higher concentrations, which may vary from crystals absence to more than $30 \text{ crystals mm}^{-2}$.

Crystal content for T2, T4, T6, and T8 treatments (Table 1) showed significant statistical differences between the 5% probability level by the Dunn test (nonparametric). T2, T6, and T8 treatments showed small crystals content associated with a great presence of air bubbles, fat globules and probably some casein micelles (Table 1 and Figures 3b, 3f, 3h), which are related to fewer crystals formation. In these treatments, the three-dimensional network water by means of capillary forces, allowed crystals formation and growth; however, such growth is also inhibited by the presence of numerous air bubbles and fat globules, as discussed earlier.

The few crystals observed in those treatments influenced the ice cream structure, visible through the microscope. It should be stressed that small crystals are necessary as they help to keep the ice cream structure intact, but they should always be imperceptible to consumers' palate avoiding the coarse mouthfeel (Kaleda et al., 2018).

T1, T4, T13, and T15 treatments presented larger crystals formation (Table 1 and Figures 3a, 3d, 3m, 3o) predominant in microscopy image, as crystals growth is related to fat

globules and air bubbles presence. In this treatment, it may be noticed that protein polymerization by transglutaminase enzyme did not occur. Large content of free water molecules in the middle favored the growth of large single crystals. For some authors, the abrupt fluctuations in temperature during freezing may cause water movement, thus initiating crystals formation. The addition of air causes greatly impacts growth, distribution, and size of crystals, affecting the ice cream microstructure. When small amounts of air form in ice cream, the air structure is unable to prevent crystals movement, since not finding any barrier, collide against each other, forming large heaps of crystals (Flores & Goff, 1999).

Crystals content in standard ice creams treatments – without enzymes

Table 2 detailed the ice crystal content in $22 \mu\text{m}^2$ in ice cream standard treatments (without enzyme) and treatments using a single lactase enzyme concentration, different transglutaminase enzyme concentrations, and no enzyme addition. Standard treatments microscopic analysis (without enzymes), showed no statistically significant differences between the crystal content averages of all treatments at a 5% probability level by the Dunn test (nonparametric), except for ST4, ST1, and ST15 treatments, which presented differences between the averages of all treatments at a 5% probability level (Table 2). Figure 4 shows the crystal photomicrographs of ice cream ST1 to ST18.

Figure 4. Photomicrographs of ice cream smears of ST1 to ST18 standard treatments without adding lactase and transglutaminase enzymes, at different temperatures.

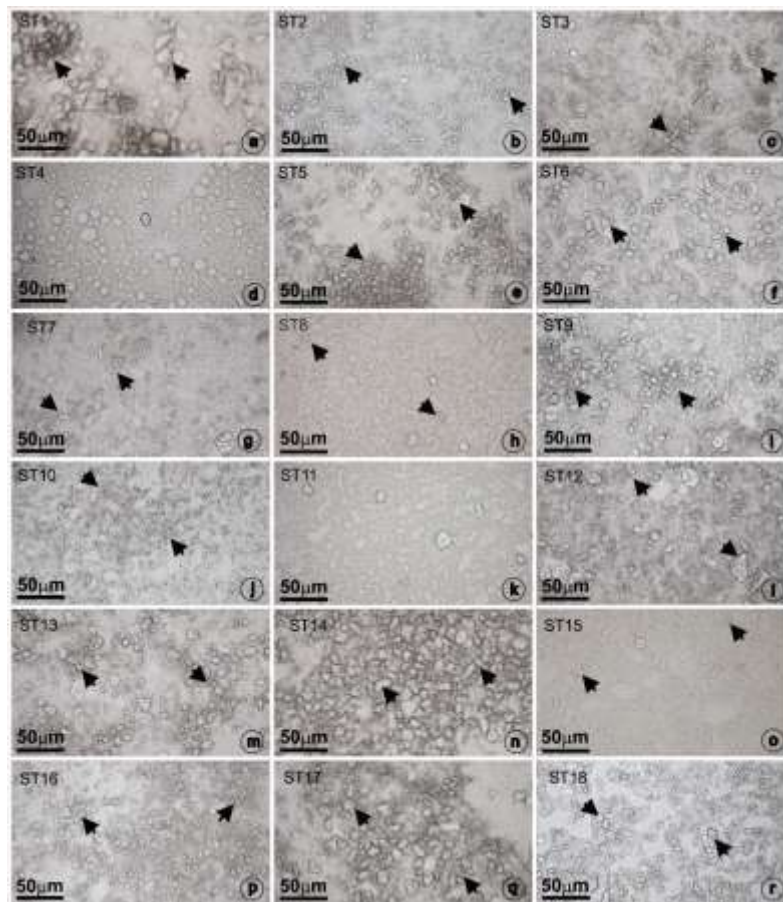


Figure 4a-r Photomicrographs of ice cream smear, without staining, in different standard treatments (without the addition of enzymes) (1-18): (a) Standard treatment 1 (ST1); (b) Standard treatment 2 (ST2); (c) Standard treatment 3 (ST3); (d) Standard treatment 4 (ST4); (e) Standard treatment 5 (ST5); (f) Standard treatment 6 (ST6); (g) Standard treatment 7 (ST7); (h) Standard treatment 8 (ST8); (i) Standard treatment 9 (ST9); (j) Standard treatment 10 (ST10); (k) Standard treatment 11 (ST11); (l) Standard Treatment 12 (ST12); (m) Standard treatment 13 (ST13); (n) Standard treatment 14 (ST14); (o) Standard treatment 15 (ST15); (p) Standard treatment 16 (ST16); (q) Standard treatment 17 (ST17); and (r) Standard treatment 18 (ST18). Arrows indicate the lactose crystals. Source: Authors

The results indicated crystal growth in all treatments, except for ST4, ST11, and ST15 standard treatments (Table 2 and Figures 4b, 4k and 4o) that showed a globule-shaped air bubble and small fat globules formation, isolated or clustered, and a piled-up concentration of small crystals, predominantly irregularly shaped, rectangular or polygonal (Figure 4). We concluded that no temperature variations occurred during these treatments manufacturing, mainly in freezing storage, which may have prevented larger crystals formation. The final quality of an ice cream, particularly its refined texture and its sense of freshness perceived by the consumer. It depends on manufacturing processing factors such as freezing temperature, the main agent for stabilizing the emulsion formed during the air addition to ice cream mass;

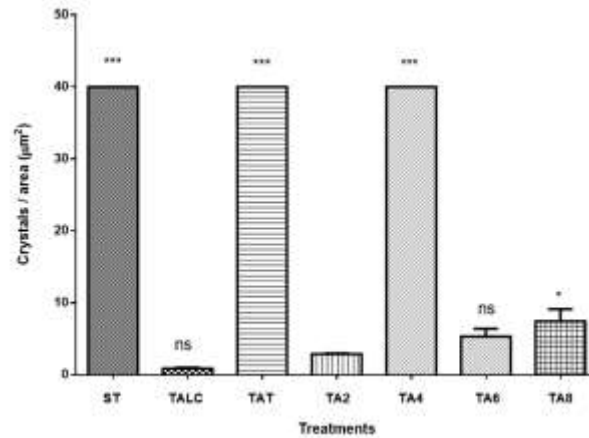
formulation; their structure, defined primarily by distribution, size, and air bubbles morphology and crystals (Hartel, 1996).

The freezing process starts in the ice cream maker, where air is added during the shaking process. When the ice cream syrup is shaken, crystals are formed in the ice cream maker drum's metallic surface, which are scraped and incorporated into the middle (Costa et al., 2008; Goff, 2008). ST4, ST11, and ST15 treatments images (Table 2 and Figures 4b, 4k and 4o) show low number of crystals, probably caused by the lack of ideal conditions for new crystal cores formation inside the ice cream maker's drum. One of the critical points also considered in this analysis refers to primary and secondary nucleation. During primary nucleation, the crystals are formed by crystal-crystal collision, generating small crystal aggregates, forming cores that tend to reach a critical radius, giving rise to the nucleus for a secondary nucleation (Hartel, 1996; Pandalaneni & Amamcharla, 2016). Another point that may affect crystals formation is related to the cream emulsion stabilization after its removal from the ice cream maker in a semi-solid consistency with more than half the amount of water frozen (Chang & Hartel, 2002); the freezing process is completed during storage at temperatures below -28 °C. However, all other treatments presented crystal growth (Table 2). Temperature oscillations may have occurred during the freezing and/or storage, resulting in partial melting followed by recrystallization. According to Ndoye & Alvarez (2014), temperature fluctuation that affect the ice cream during processing and storage stages cause crystal growth, responsible for the recrystallization process, which may give a sandy aspect, often found in low-quality ice creams.

Crystals content in ice creams with equal lactase enzyme concentrations and different transglutaminase enzyme concentrations, and without enzymes addition at 40 °C

There were no statistically significant differences among crystal content of TA4, TAT, and ST treatments. Likewise, there were no statistically significant differences among TA2, TA6, TA8, and TALC treatments (Table 2). However, significant differences were found between these two groups at a 5% probability level by the Dunn test (nonparametric). Moreover, the ANOVA analysis used to determine the amount of crystals mm⁻² in ST, TA4, TA6, TA8, TAT, TALC (Figure 5 and Figures 6a, 6c, 6d, 6e, 6f, and 6g), demonstrated that (***) indicated statistically significant differences ($p < 0.0001$) and (*) indicated no significant differences ($p > 0.001$) in all treatments.

Figure 5. Statistical analysis chart of crystals content (crystals mm^{-2}) for TA4, TA6, TA8, TALC, TAT, and ST treatments considering the same enzyme lactase concentration, different transglutaminase enzyme concentrations, and treatment without enzymes addition, all at 40 °C.



(***) Indicated statistically significant differences ($p < 0.0001$) and (*) indicated no significant differences ($p > 0.001$) by Tukey test. Source: Authors

Figure 6. Photomicrographs of ice cream smears in ST, TA2, TA4, TA6, TA8, TAT, and TALC treatments with equal lactase enzyme concentrations, different transglutaminase enzyme concentrations and without adding enzymes, at room temperature 40°C.

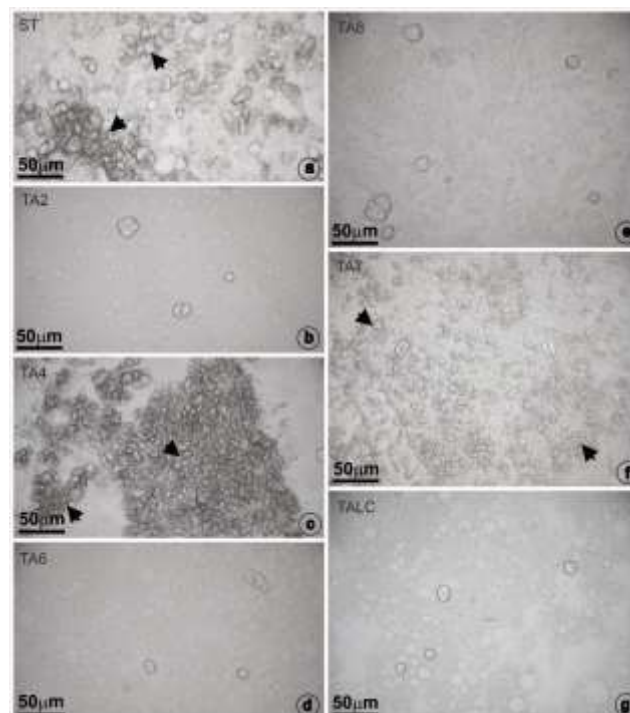


Figure 10a-g Photomicrographs of ice cream smear, without coloring in the different (ST, TA2, TA4, TA6, TA8, TAT, and TALC) treatments: (a) Treatment ST; (b) Treatment TA2; (c) Treatment TA4; (d) Treatment TA6; (e) Treatment TA8; (f) Treatment TAT; and (g) Treatment TALC. Arrows indicate lactose crystals. Source: Authors

For treatments where small crystals were predominantly found (Figure 6) at higher transglutaminase and lactase enzymes concentrations (TA4, Figure 6c), crystal clusters were formed, not seen in TAT treatment (Figure 6f), where only the same transglutaminase enzyme concentration was added. When compared with the standard enzyme-free ice cream, it became clear that the combined effect of enzymes favored such formation. Patel et al. (2006) observed that, in milk origin emulsion, the increase of proteins concentration in the middle favor crystals formation with smaller diameters. According to Schorsch et al. (2000), binding with transglutaminase affects the system microstructure, favoring small aggregates formation that remain intact under storage conditions preventing the water from leaving the gel structure, making it impossible to form large crystals, as observed in the treatments' microscopy.

Crystal formation in the medium size sample (Figure 6) can be seen in ST treatment. These crystals might have grown due to temperature fluctuations during processing and storage. Physical phenomena, such as sample's thermal exchange with external environment, temperature variation, gravity, density and/or viscosity affect the ice cream structure, forming crystals, which may have occurred in this treatment (Costa et al., 2008; Goff, 2008; Ndoye & Alvarez, 2014).

Microscopic evaluation in TA2, TA6, and TA8 treatments (Table 2 and Figure 6b, d, e) detected various air bubbles, large amounts of fat globules and casein micelles or denature proteins, indicating proteins polymerization by the transglutaminase enzyme, forming firm cross-links, preventing water movement in a three-dimensional network, hence preventing crystals formation. The addition of air during processing increases product volume in relation to syrup (Cruz et al., 2009). Air bubbles are surrounded by fats and milk proteins, establishing the structure of the final product, as well as giving a larger foam stability and greater texture to the ice cream, avoiding crystals formation (Tsuchiya et al., 2017).

It is important to mention that these treatments' incubation temperature (40 °C), near-optimum lactase and transglutaminase enzyme temperatures provided the right conditions for enzyme activity. Studies indicate that β - and κ -casein incubation with transglutaminase led to intramolecular bonds formation in casein micelles, even more so in κ -casein (Kruif et al., 2002). It should be stressed that κ -casein greater reactivity is due to its location on the micelle surface, and also due to κ -casein macropeptides susceptibility to transglutaminase attack (Rodrigues-Nogales, 2006), fostering a firmer protein network formation.

TALC treatment (Table 2 and Figure 6g) did not present any noticeable crystal formation. In this case, lactose hydrolysis by lactase enzyme increased solids concentration

(proteins), avoiding the movement and the availability of free water to form a firmer three-dimensional network, with the presence of air bubbles, large amounts of fat globules and casein micelles. Fat globules are stable under static conditions; however, shearing forces on the base syrup caused by the ice cream maker beating phase starting cause fat globules to collide, favoring the partial coalescence phenomenon occurrence. The partially coalesced fat globules build a semi-continuous network stabilizing the bubbles, strengthening the protein network producing a uniform and stable emulsion, diminishing crystals formation during storage, resulting in beneficial properties for a smoother and softer texture (Costa et al., 2012; Goff, 2002).

4. Conclusion

All treatments prepared at 40 °C (T2, T6, and T8) and TA2 treatment (T2 treatment similar formulation), showed small content of crystals associated with a large presence of air bubbles, fat globules and probably some casein micelles, making them ideal for small crystals agglomeration that form a firmer, smooth and cohesive texture. Therefore, the effects of the combined use of lactase and transglutaminase enzymes in the formulations have proven to be a viable, efficient and easy technology for ice cream production. Furthermore, the use of response surface methodology was effective in selecting the best formulation in relation to desirability features, which ensures their usage in the development of ice cream formulations. For future researches more aspects maybe explored, such as the application studies on an industrial scale, study on probiotic and prebiotic ice cream, use of fruit pulp, mineral and vitamin enrichment and the study of other technological properties and stability during the useful life.

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