Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 03:2024
DOI: 10.5281/zenodo.10792168

EFFECT OF LYSOZYME AND BROMELAIN ON PHYSICOCHEMICAL, TEXTURAL AND SENSORIAL PROPERTIES OF MOZZARELLA CHEESE

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Abstract

This study aimed to enhance the shelf life of cheese with the incorporation of various concentrations of bromelain and lysozyme. Results revealed that the inclusion of both enzymes significantly ($p<0.05$) affected the proximate composition, physicochemical and organoleptic properties of cheese. During storage, all the treatments of bromelain and lysozyme showed a non-significant effect on the fat, moisture, acidity, pH and total solid content. However, they showed a significant effect on the protein and ash contents. Likewise, textural characteristics and sensorial attributes also varied significantly among treatments and during storage days ($p<0.05$). Results concluded that lysozyme has extended the shelf life of mozzarella cheese up to 14 days with the maximum effect observed with T_5 (4% lysozyme) and in terms of bromelain T_1 (5% bromelain) showed excellent results regarding physicochemical, textural and sensorial attributes.

Keywords: Cheese, Milk, Bromelain, Lysozyme, Sensorial Attributes.

1. INTRODUCTION

Global milk production increased by more than 20% in the past ten years, from 694 million tons in 2008 to 843 million tons in 2018. After China, India, and the United States, Pakistan is the country that produces the most milk globally [1]. The most abundantly utilized milk-based product is cheese worldwide. Its consumer acceptability is highly associated with sensory attributes [2]. Humans have been manufacturing cheese for a long period to condense and conserve milk. One of the known categories of manufactured foods is cheese [3]. It is a healthful fermented milk item with a large variety of flavor and texture [4]. It is estimated that consumption of mozzarella cheese is about 70% in pizza making and its requirement is enlarging tremendously in the global market [5]. It is currently the second most popular cheese in the United States, consuming more than 3 billion pounds annually. Mozzarella cheese is very perishable due to its greater moisture content and has a shelf life of about 30 days when stored at a temperature of 2°C [6]. In this regard, different preservatives are used to enhance the shelf life of cheese.

A popular natural preservative is a soluble protein called lysozyme. It naturally presents in milk and hen's egg white. Lysozyme degrades the 1,4-beta linkages between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan. Gram-positive bacteria have peptidoglycan in their cell walls, which makes them very acceptable to the lysozyme. Gram-negative bacteria cannot be eliminated by lysozyme. It is employed as an antimicrobial agent in the food and pharmaceutical industries. It may extend the shelf life of food products by reducing the pathogenic germs [7]. Enzymes like lysozyme apply antimicrobial actions by directly criticizing the growth and development of microorganisms [8].

Bromelain is present in the pineapple stem. It has a higher capability as an anticancer and antimicrobial agent [9]. Fresh bromelain contains more proteases acquired from the pineapple fruit juice as well as useless parts. It is recommended to use it in small percentages to sustain the organoleptic properties and degree of acceptance of cheese. Utilization of pasteurized milk for coagulation with the help of bromelain improved the organoleptic properties [10]. It also performs various functions such as platelet aggregation, anti-thrombotic, anti-edematous, anti-inflammatory, immunity, regulation of cytokines, fibrinolytic activity, and skin debridement. Additionally, it facilitates digestion after swallowing, has possible post-operative effects to promote wound healing, and improves the absorption of other medications [11]. The current study was designed to enhance the shelf life of cheese with the incorporation of various concentrations of lysozyme and bromelain enzymes. Moreover, check the effect of these enzymes on the chemical composition, physiochemical, microbial and sensorial attributes.

2. MATERIALS AND METHODS

2.1 Procurement of Raw Materials

Buffalo milk was purchased from an animal farm house University of Agriculture, Faisalabad. Thermophilic culture (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*), rennet, Lysozyme, and Bromelain were procured from Sigma Aldrich, Germany. All chemicals and reagents were used of analytical grade.

2.2 Preparation of Cheese

Mozzarella cheese was prepared from buffalo milk with the incorporation of different concentrations of enzyme (Table 1) according to the prescribed method [10].

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Table 1: Development of Mozzarella cheese using various concentrations of different enzyme

Treatments	*Enzyme Concentration (%)*
T_0	Control
T_1 (Bromelain)	5
T_2 (Bromelain)	10
T_3 (Bromelain)	15
T_4 (Lysozyme)	2
T_5 (Lysozyme)	4
T_6 (Lysozyme)	8

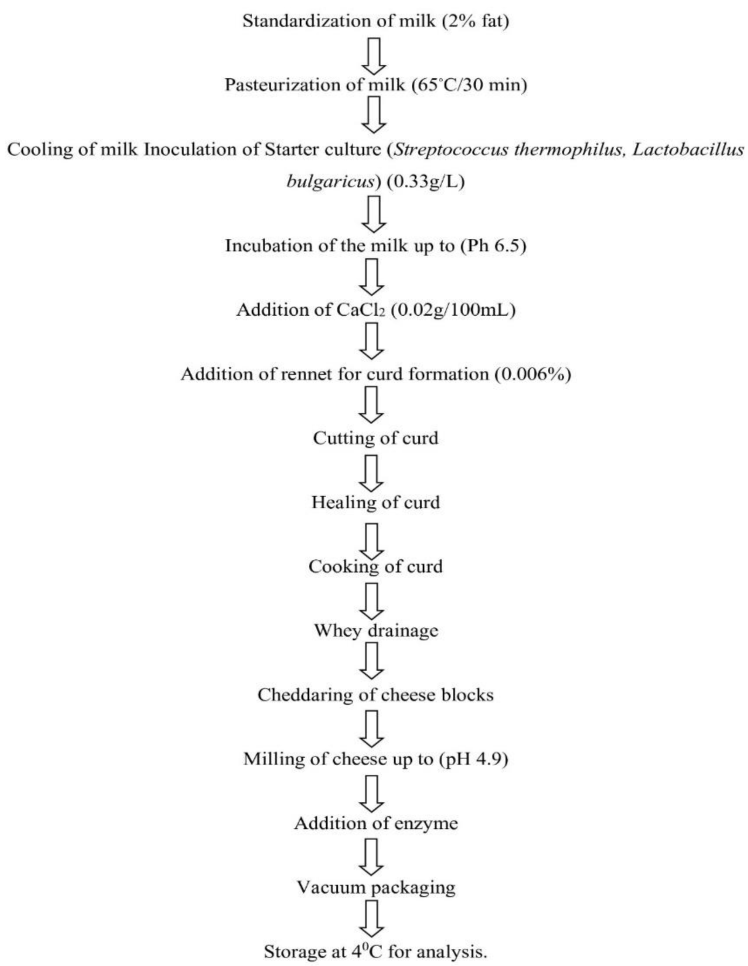

Figure 1: Flow diagram of preparation of mozzarella cheese with the incorporation of enzymes

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2.3 Chemical composition of cheese

2.3.1 Moisture

Moisture contents of cheese were determined according to the method of Komansilan *et al*., [12]. A 10g sample was put in a China dish and placed in a hot air oven (105°C) for 24 hours. After that, remove the China dish from the hot air oven kept in the desiccator for 15 mins. After that, weigh the sample and calculate the moisture content using the given equation (1)

$$\text{Moisture}(\%) = \frac{\text{Initial sample weight-Final sample weight}}{\text{Initial sample weight}} \times 100 \quad (1)$$

2.3.2 Fat

Fat contents in the cheese were measured according to the suggested method [12]. Firstly, 10g cheese and 10mL sulfuric acid were added to the butyrometer followed by the addition of 1mL iso-amyl alcohol and closed the butyrometer with a stopper. Gently mixed and centrifuged at 1300 rpm for 5 minutes. Fat contents were directly measured from the butyrometer scale.

2.3.3 Protein

Protein contents in cheese samples were measured by kjeldhal method mentioned in Komansilan *et al*., [12]. Use the 5g cheese sample, 5g digestion mixture, and 25 mL concentrated sulphuric acid in the digestion unit. Heat it at 400°C for 70 minutes until a light green color appears. Dilute the digested sample up to 250mL with distilled water. Then distillation was performed by taking a 10 mL diluted sample, 10 mL 40% NaOH, and 10 mL distilled water in a distillation tube. The distillation was performed for 3 minutes until all the nitrogen was entrapped into the boric acid. When the pink color of boric acid was changed into straw color, distillation was stopped. Then, boric acid was titrated with 0.1N sulphuric acid until light pink color appeared.

Calculate Nitrogen (%) using the given formula.

$$\text{Nitrogen}(\%) = \frac{\text{Vol.of}H_2SO_4 \times 0.0014 \times 250}{\text{Volume of sample} \times \text{Sample used for distillation}} \times 100 \quad (2)$$

$$\text{Protein } (\%) = \text{Nitrogen } (\%) \times 6.38 \quad (3)$$

2.3.4 Ash

Ash contents were find out according to the suggested method by Yadav *et al.*, [13]. 10g of moisture-free cheese sample was taken in crucible and placed in muffle furnace at 550^{o}C for 6 hours. Removed the sample from the muffle furnace and placed it in desiccators for half an hour. Calculate the ash content using the given equation,

$$\text{Ash}(\%) = \frac{\text{Weight of ashed sample}}{\text{Initial sample weight}} \quad (4)$$

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2.4 Physicochemical analysis

2.4.1 pH

The pH of cheese samples was determined according to the standard method mentioned by Yadav *et al.*, [13]. pH meter calibrated with buffers. Then, 20mL cheese was poured into the graduated beaker. Inserted the pH meter rod and recorded the pH readings.

2.4.2 Acidity

The acidity of cheese samples was determined by using the prescribed method [10]. Took 10mL grated cheese in a titration flask. Titrate with 0.1N NaOH using phenolphthalein until a light pink color appeared. Noted NaOH volume used. Calculated acidity with the given equation,

$$\text{Acidity (\%)} = \frac{\text{Volume of NaOH used} \times 0.009}{\text{Sample weight}} \times 100 \quad (5)$$

2.4.3 Total Solids

The total solids contents of cheese were measured according to the prescribed method [13]. 10g of sample was taken in dried china dish and heated in a water bath for 15 minutes.

Then, the sample was dried in a hot air oven at 105^{o}C until constant weight was obtained. After that, sample was placed in desiccator for half an hour and the total solid contents was calculated using given equation,

$$\text{Total solid(\%)} = \frac{\text{Weight of dried sample}}{\text{Initial sample weight}} \times 100 \quad (6)$$

2.5 Texture Analysis

Texture of mozzarella cheese was determined using the texture analyzer as per method described by Al-Baarri *et al.* [7]. The condition for texture analysis was force (10g), distance (5mm), and speed (1mm/s). Hardness, springiness, and cohesiveness were shown on the texture analyzer at the conclusion of the compression cycle.

2.6 Sensory analysis of Cheese

The samples of mozzarella cheese were assessed for sensory attributes (color, flavor, taste and overall acceptability) through semi-trained panelists at National Institute of Food Science and Technology, University of Agriculture, Faisalabad. 9-point hedonic scale was used to assess the sensorial attributes of cheese samples.

The cheese samples were labelled with codes and presented in separate booth equipped with white fluorescent light. Then, samples were offered to panelists in white polystyrene plates at room temperature (25℃). The plain water was provided to panelists before each evaluation to rinse their mouth and neutralize their taste buds [10, 14].

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2.7 Statistical Analysis

All the experiments were carried out in triplicates and analyzed statistically using two-way analysis of variance (ANOVA) techniques to check the effect of treatments on storage days and their interaction. The Duncan Multiple Range Test was used to compare the treatments.

3. RESULTS AND DISCUSSION

3.1 Chemical composition of cheese

3.1.1 Moisture contents

Moisture contents in cheese was determined and results are presented in Figure 2. Results showed that incorporation of bromelain significantly ($p<0.05$) decreased the moisture contents of cheese. It may be due to the addition of bromelain which act as a coagulating agent and produced firm curd with reduced moisture contents. In lysozyme incorporated cheese, moisture contents were 2.42 times lower than control (2% lysozyme).

Increasing the enzyme concentration (4-8%) in cheese then reduction of the moisture contents was observed from 3.54 and 3.35 times as compared to control sample. The lysozyme enzyme can bind water molecules. Water molecules can be retained more successfully when lysozyme is added to cheese because it interacts with the water that is already present in the cheese matrix [15]. At 14^{th}days of storage moisture contents were reduced in bromelain incorporated treatments and also in control treatments. The decrease in moisture contents in cheese sample is due to fluctuation of storage conditions and improper packaging [16]. These findings are in line with previous literature [17].

3.1.2 Fat contents

Fat contents in cheese was measured and results are presented in Figure 2. Results showed that addition of enzyme in cheese significant effect on the fat contents in cheese. Incorporation of bromelain slightly increased the fat contents.

These results are correlated with literature findings [18]. Similarly, increasing the concentration of lysozyme (4-8%) increased the fat contents. It may be due to incorporation of lysozyme which restrict the growth of bacteria that result in unwanted byproducts, indirectly affecting the fat level [19]. With the passage of 14 days fat contents are reduced in all these 3 bromelain treatments.

Bromelain appear to be the main contributors of lipolysis in all bromelain containing cheeses samples. Lipolysis is the catabolic process leading to the breakdown of triglycerides stored in fat cells and release of fatty acids and glycerol [20]. Fat contents in all lysozyme containing treatments including control treatment were decreased significantly ($p<0.05$) during storage of 14 days. It is mainly due to the ability of lysozyme to suppress reactive oxygen species generation that leads to the suppression of fat contents [19].

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3.1.3 Protein contents

Protein contents in cheese sample was find out and results are depicted in Figure 2. Results revealed that protein contents were increased compared to control samples with the increase in enzyme concentration (5-15%).

It may be due to the supplementation of bromelain which significantly increased the protein contents of cheese because bromelain is an enzyme, and enzymes are protein in nature. Similar results were observed for lysozyme, increasing the lysozyme concentration (4-8%) significantly ($p<0.05$) upsurge the protein contents as compared to control sample. It may be due to the incorporation of lysozyme which increased the protein contents in cheese.

At 14th days of storage, protein contents were decreased in bromelains incorporated cheese, because bromelain caused the proteolysis (break down of protein into amino acid due to enzymes action) and showed the reduction in protein contents [17].

Similarly, protein contents in lysozyme incorporated cheese sample were also decreased at 14th days. This reduction may be due to inappropriate storage conditions, which may be caused the degradation and inactivity of protein and resulted in reduced protein contents [21].

3.1.4 Ash Contents

Ash contents in cheese sample was determined and results are presented in Figure 2. Results indicated that addition of bromelain dramatically increased the ash contents of cheese.

Although bromelain does not directly enhance the mineral concentration, the proteolytic action it has on proteins may cause bound minerals that were formerly a part of the protein structure to be released. As a result, the cheese may have somewhat greater ash content [17].

Similarly, increasing the lysozyme concentration on cheese (4-8%) significantly increased the ash contents as compared to control sample. During storage, the ash contents in cheese was decreased in bromelain incorporated sample at 14th days along with control treatment.

It may be due to several changes during storage. Cheese proteins can be broken down by proteolytic enzymes. When these breakdown products interact with minerals, they can create complexes that dissolve in the aqueous phase of cheese.

These minerals are removed from the cheese matrix when dissolve in moisture, which lowers the amount of ash present in cheese sample [10].

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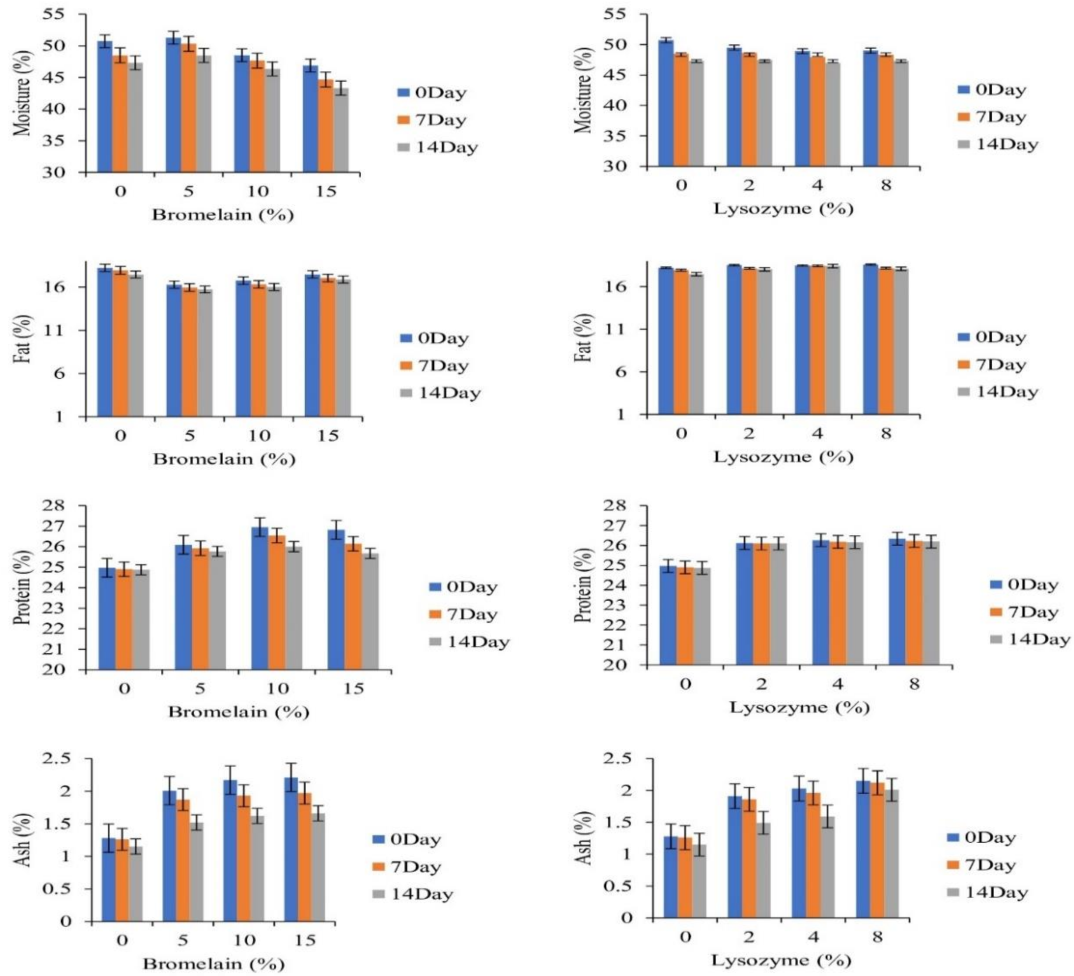

Figure 2: Chemical composition of mozzarella cheese incorporated bromelain and lysozyme enzyme

3.2 Physicochemical properties of cheese

3.2.1 pH

pH of cheese sample was measured and results are depicted in Figure 3. Results indicated that pH was significantly ($p<0.05$) decreased with the incorporation of bromelain. It may be attributed that the interaction between organic components of the cheese induced by temperature and action of bromelain enzymes [10].

Similarly, addition of lysozyme (2%), pH was decreased4.46 times lower than control. Increasing the enzyme concentration (4-8%) caused the decreases in pH of 4.66 and 3.49 times as compared to control sample.

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It may be due to the *activity of some microorganisms and conversion of sugar to acids or some electrolytes [15]*. During storage, pH was decreased in bromelains and lysozyme incorporated cheese. The gradual decrease in pH during storage can be attributed to the interaction between organic components of the cheese induced by temperature and action of bromelain enzymes [10]. Results indicated that enzymes and storage had highly significant effect on pH of cheese.

3.2.2 Acidity

Acidity of cheese sample was determined and results are shown in Figure 3. Results indicated that addition of bromelain and lysozyme in cheese significant effect ($p<0.05$) on the acidity. Increasing the concentration of bromelain significantly increased the acidity of cheese. It may be due to the oxidation of acid due to the bromelain which is a good oxidizing agent [22].

Similarly, the 2% addition of lysozyme, acidity was increased 2.02 times as compared to control. Increasing the enzyme concentration (4-8%) significantly increased the acidity of cheese 6.01 and 4.0 times as compared to control. It may be due to the incorporation of lysozyme which fluctuate the acidity in cheese. Compared to untreated cheese, cheese coated with an Alginate/Lysozyme Nano-Laminate showed a lower pH and this decrease of pH caused greater titratable acidity values [23]. During storage, acidity of bromelain incorporated cheese significantly increased at 14^{th} days.

This phenomenon was possible due to oxidation of acid during storage [22]. *Bromelain is a predominant oxidizing agent [24]*. Likewise, acidity of lysozyme incorporated cheese sample also increased during storage at 14^{th} days. It may be attributed that antibacterial effect of lysozyme is achieved by dissolving bacterial cell walls, specifically by concentrating on the peptidoglycan layer that is present in many bacteria. D-glutamic acid is one of the several amino acids that are released when lysozyme digests the peptidoglycan. D-glutamic acid can cause cheese to become more acidic during storage if it is present [23].

3.2.3 Total Solids

Addition of bromelain significantly ($p<0.05$) increased the total solids contents of cheese. It may be due to the coagulating properties of bromelain, and decreased in the moisture contents which result in an increase in total solid contents. Similarly, increasing the lysozyme addition in cheese (4-8%) significantly ($p<0.05$) increased the total solids contents. It does not directly enhance the solids contents in cheese, but it can indirectly through a number of processes including whey retention, curd firming, and prevention of proteolysis [25]. During storage, the total solid contents in cheese was increased in bromelain and lysozyme containing cheese sample at 14^{th} days. It may be due to inhibition of proteolytic and lipolytic activities of microorganisms by low storage temperature or by addition of preservatives including different enzymes [22]. Other reason of increasing the total solid in cheese may be due to the decrease of moisture content which significantly increased the total solid content during storage [26].

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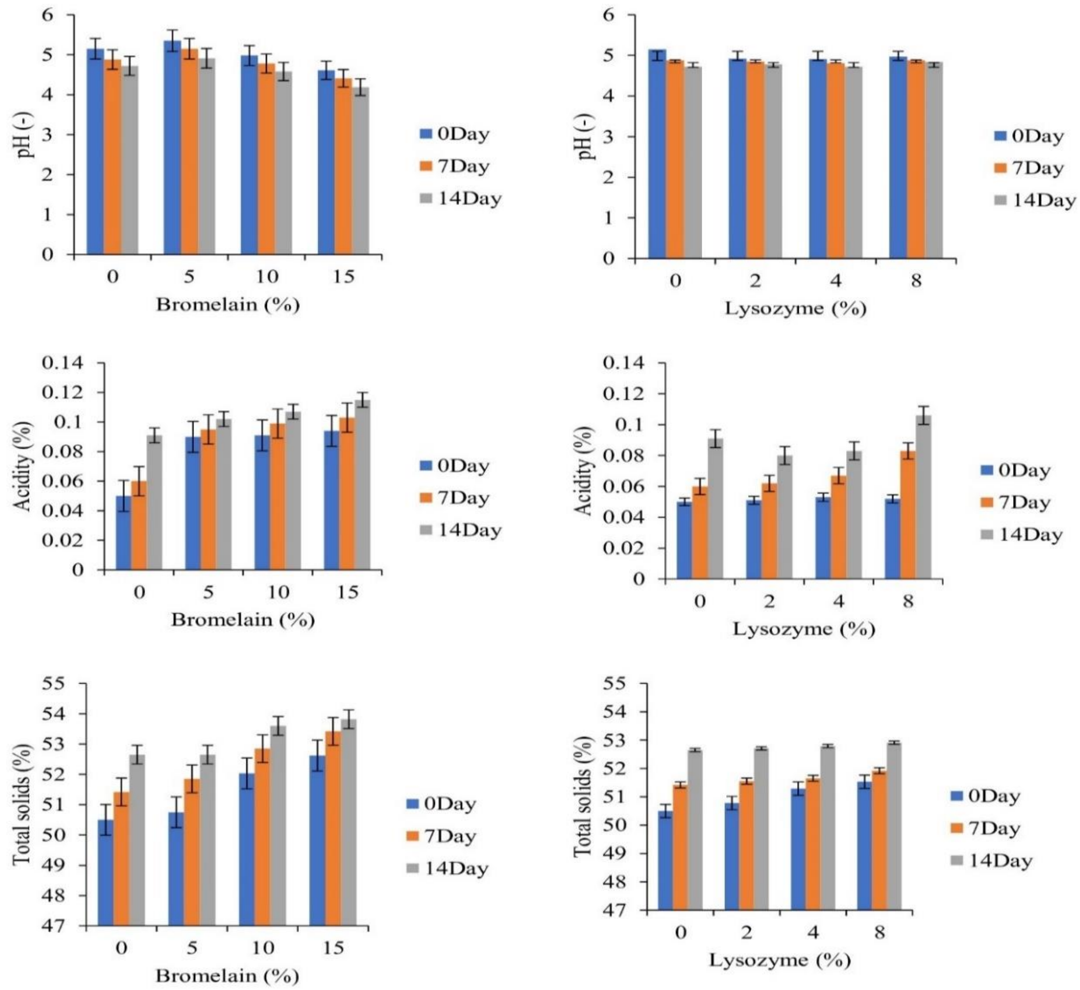

Figure 3: Physicochemical properties of mozzarella cheese incorporated with bromelain and lysozyme enzyme

3.3 Textural Analysis

3.1 Hardness

Hardness of cheese was determined by texture analyzer and results are shown in Figure 4. Results revealed that addition of bromelain and lysozyme had significant effect on the hardness of cheese. Increasing the concentration of both enzyme in cheese significantly ($p<0.05$) decreased the hardness of cheese. It may be due to the increased overall concentration of amino acids with the incorporation of bromelain enzyme [27]. Other reason might be the addition of lysozyme which lowered the cheese firmness. Loss of moisture and proteolysis inhibition by lysozyme may be the causes of the decrease in hardness [28]. During storage hardness of cheese was decreased in enzymes incorporated cheese, hardness of control sample was increased at 14th days. The

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decrease of hardness during storage period might be due to prevention of proteolysis by lysozyme and loss of moisture [28].

3.2 Cohesiveness

The incorporation of bromelain in cheese significantly ($p<0.05$) increased the cohesiveness of cheese. It may be attributed that incorporation of bromelain in cheese can disintegrates the protein, resulting in smaller protein pieces which have ability to interact and bind together more successfully and increased the cohesiveness [29]. Similarly, the increase of lysozyme enzyme concentration (4-8%) significantly increased the cohesiveness as compared to control samples. It may be due to the incorporation of lysozyme which increased the cohesiveness in cheese. Koca and Metin [30] investigated that Lysozyme can improve the cohesion of cheese through a number of processes, including: Reduced syneresis, protein cross-linking, and proteolysis inhibition. Results showed that cohesiveness of bromelain incorporated cheese was significantly decreased during storage at 14th days. The cohesiveness of cheese during storage may be affected by the addition of bromelain. Here's why bromelain might make things less cohesive: protein degradation, protein cross-Linking defects, and texture Softening [27].

Likewise, during storage cohesiveness significantly increased with the increasing lysozyme concentration at 14th days. It may be attributed that peptidoglycan fragments may be released when lysozyme breaks down bacterial cell walls. These pieces may serve as water molecule-binding sites, facilitating water retention in the cheese matrix. Increased water retention can change the cheese's texture by making it softer and less cohesive[15].The decrease in moisture contents and proteolysis during ripening are the causes of the loss in cohesiveness that was seen in the control and other enzyme-treated groups. It has been demonstrated that cheese cohesiveness declines as cheese moisture content does [31].

3.3 Springiness

Springiness can be defined as the degree to which sample returned to its original shape after being compressed between palate and tongue [32]. Results of springiness of enzymes incorporated cheese is depicted in Figure 4. Results showed that inclusion of bromelain significantly ($p<0.05$) decreased the springiness of cheese as compared to control. Increasing the bromelain concentration in cheese significantly increased the springiness. Cheese with a lower springiness at ripening was obtained using a *different concentration of papain (bromelain) and a longer coagulation time [27]*. Inclusion of lysozyme considerably decreased the cheese's springiness. Calcium was substituted by sodium in cross links, decreasing the value of springiness and lowering the springiness of mozzarella cheese [33]

Likewise, during storage, springiness values are lower in bromelain containing cheese and higher in the control group at 14th days. *Springiness and moisture showed a positive correlation*, bromelain treated cheese sample display reduction in springiness timely but control treatment show reduction after 3-4 months [34]. Similarly, springiness values in all lysozyme containing treatments are decreased with the passage of 14 days. Tunick,

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[33] stated that decrease in value of springiness attributed due to the fact that calcium was replaced by sodium in cross linkages, thus reducing springiness of mozzarella cheese.

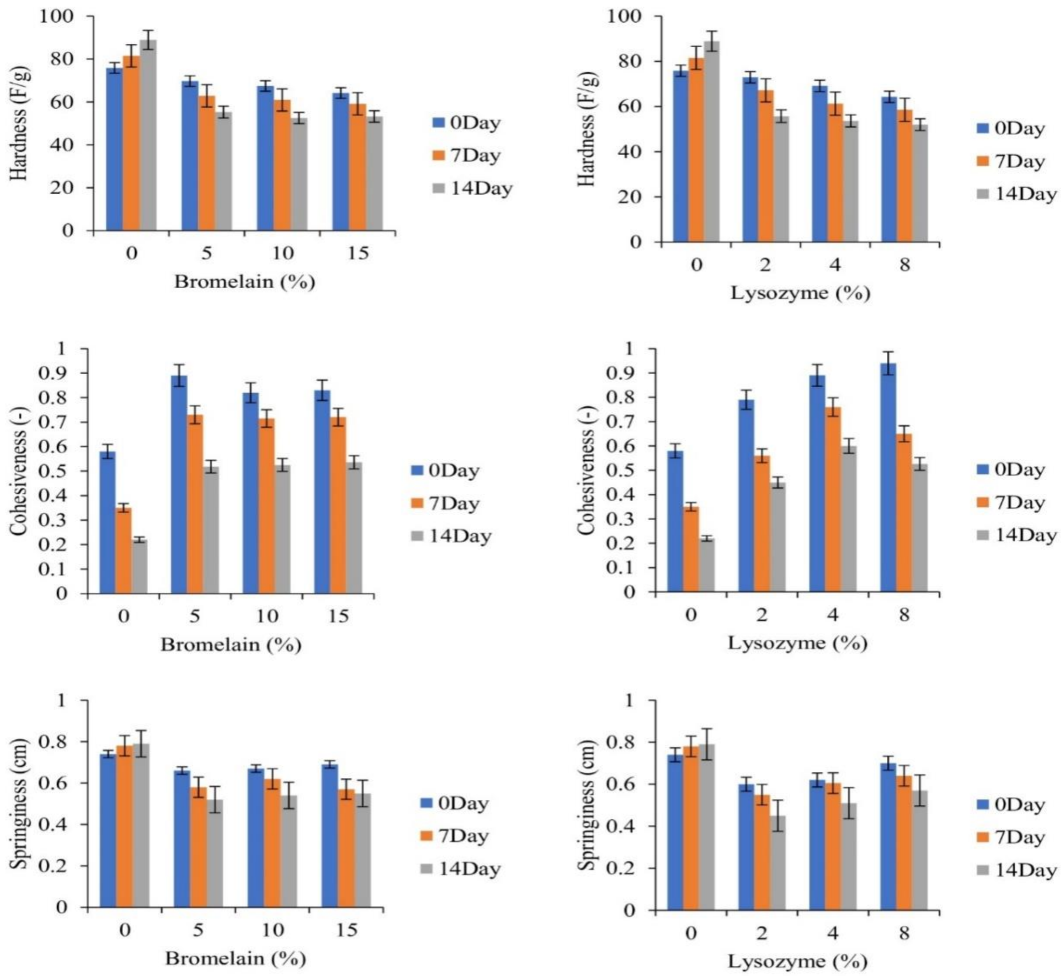

Figure 4: Textural characteristics of mozzarella cheese incorporated with bromelain and lysozyme enzyme

3.4 Sensory Analysis

3.4.1 Color

Incorporation of bromelain in cheese significantly ($p<0.05$) effect on the color score. Results indicated that addition of bromelain increased the color score as compared to control. During storage, color score was decreased. It may be due to the breakdown of bromelain to produce a lightening or discoloration effect [17]. Similarly, increasing the inclusion of lysozyme in cheese (4-8%) significantly increased the color score. It may be due to the incorporation of lysozyme which has antimicrobial activity, and inhibits the growth of bacteria, [35].

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However, during storage color score was decreased at the 14th day. It may be due to the proteolytic activity of the enzyme resulted in the discoloration and decreased the color score [17].

3.4.2 Flavor

Flavor was significantly affected with the addition of bromelain and lysozyme enzyme. Results showed that the inclusion of bromelain in cheese significantly ($p<0.05$) decreased the flavor score. It may be due to addition of *bromelain enzyme which subsequently metabolized into volatile compounds*, and contribute to the fluctuation of flavor of cheese [27]. Similarly, increasing the lysozyme concentration significantly increased the flavor score. It may be due to the inclusion of lysozyme which speed up the inhibit the growth of *Clostridium tyrobutyricum*, which causes off flavors [35].

During storage, the flavor score of bromelains incorporated cheese was significantly ($p<0.05$) decreased at 14th day. It may be due to the hydrolysis with bromelain which reduced the flavor score [36]. Likewise, in lysozyme incorporated cheese the flavor score was significantly increased during storage at 14th days. It may be due to the addition of lysozyme, *accelerates the ripening and prevents growth of Clostridium tyrobutyricum* which is responsible for late blowing and off-flavors in cheese [35].

3.4.3 Taste

Addition of bromelain significantly effect of enzyme (bromelain and lysozyme) incorporated cheese. Results showed that increasing the concentration of bromelain decrease in taste score was observed. Similarly, opposite trend was observed in lysozyme incorporated cheese. It may be due to inclusion of lysozyme which used to prevent the growth of bacteria, particularly Gram-positive bacteria [8]. During storage the score of taste was decreased at 14th days in enzyme (bromelain and lysozyme) incorporated cheese. It may be due to proteolytic activity of bromelain and inhibition of bacterial growth of lysozyme which caused the decrease in taste score of cheese [17].

3.4.3 Overall Appearance

Incorporation of enzyme (bromelain and lysozyme) in cheese significantly effected the overall acceptability of cheese. Results revealed that increasing the concentration of both enzyme the score of overall acceptability was decreased. It may be due to the enzymatic activity on proteins, bromelain used in cheese production might alter the overall appearance of the cheese [37]. Increase in lysozyme concentration (4%) led to increases in score of overall appearance when compared to control samples. Lysozyme is used to prevent the growth of bacteria, particularly Gram-positive bacteria that ruin food goods like cheese [8]. But when lysozyme concentration increased up to 8%, score of overall appearance become decreased 3.02 times when compared to control sample. Lysozyme is a protein; its inclusion considerably fluctuates the overall appearance. All three bromelain treatments, along with control, exhibited a drop in overall appearance points after 14 days. Overall appearance score in all lysozyme containing treatments are decreased with the passage of 14 days. Many microbial proteases and lipases bring

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change and cause decline in the overall appearance of cheese with the passage of time[35]. Overall appearance of control treatment affected more in contrast to all other cheese samples which are treated with bromelain and lysozyme.

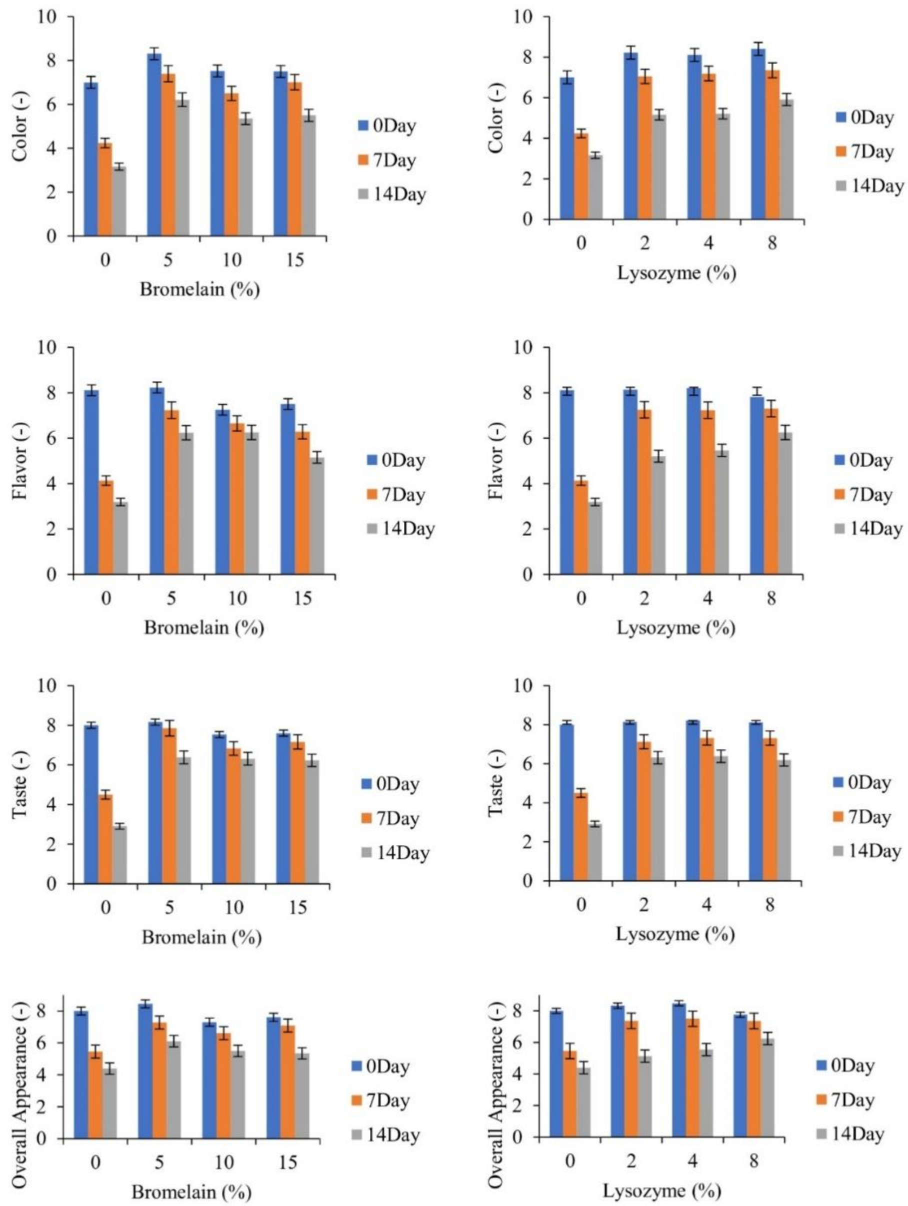

Figure 5: Sensorial attributes of mozzarella cheese incorporated with bromelain and lysozyme enzyme

ISSN (Online):0493-2137
E-Publication: Online Open Access
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4. CONCLUSION

In this study, mozzarella cheese was prepared from buffalo milk and incorporated with various concentration of bromelain and lysozyme enzyme. The inclusion of lysozyme and bromelain both increased the to extend the shelf life of mozzarella cheese up to 14 days. Higher concentration of lysozyme produced more pronounced results as compared to lower levels of lysozyme. But in case of bromelain lower concentration produced more noticeable results. Moreover, lysozyme and bromelain both improved texture and sensory properties of mozzarella cheese. But in comparison of lysozyme, bromelain show more good results in term of physicochemical, textural, and sensorial properties.

Conflicts of interest

All authors declare no conflict of interest.

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ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 03:2024
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Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 57 Issue: 03:2024
DOI: 10.5281/zenodo.10792168

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