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Preservation and Quality Control analysis in fermented beans biscuit treated with Zinc Oxide nanoparticles

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Abstract

This study investigated the fortification of biscuits with fermented soybean flour pretreated with 40 ppm zinc oxide nanoparticles (ZnO-NPs) aiming to develop a functional bakery product with enhanced nutritional and sensory attributes. Biscuits were formulated with varying inclusion levels of the treated flour: a control (B) with 0%, and experimental samples B1 (5%), B2 (10%), and B3 (15%). A comprehensive analysis of proximate composition, physical properties, bioactivity, cytotoxicity, and sensory shelf-life was conducted. The results demonstrated a clear, dose-dependent improvement in nutritional profile upon nanoparticle incorporation. Sample B3, with the highest fortification level, exhibited the most favorable composition, characterized by significantly elevated levels of protein (8.76%), ash (3.10%), and crude fiber (7.45%), and coupled with reduced moisture (4.9%) and fat (2.56%) content compared to the control. While the physical characteristics (diameter, thickness, weight) of the biscuits were not significantly altered by the treatment, the bioactive potential was substantially increased. Furthermore, cytotoxicity assays using human fibroblast (BJ) cells confirmed the low toxicity of all treated samples (B1-B3), supporting their safety for nutraceutical application. Sensory evaluation over a 120-day storage period revealed that biscuits B2 and B3 consistently received superior scores for texture, aroma, appearance, and overall acceptability, with only a slight decline in ratings by the study's end. The integration of ZnO-NPs treatment prior to fermentation proved to be a synergistic strategy, effectively enhancing the nutritional density and bioactive compound retention without compromising safety or consumer appeal. Consequently, the B3 formulation is posited as a promising, shelf-stable functional food ingredient, offering a viable approach to enrich bakery products with health-promoting properties.

Keywords: Zinc Oxide Nanoparticles, Soybean Fermentation, Biscuits, Peroxide Value, Toxicity, Sensory Evaluation

1.INTRODUCTION

The demand for functional and nutrient-rich food products is growing rapidly due to a better understanding from consumers regarding food choices and health. Bakery products containing bioactive compounds and functional ingredients like legumes and nanoparticles are some of the most exciting developments in food products [1]. Legumes, especially soy bean, are a rich source of protein, dietary fiber, minerals, and phytochemicals, making them well suited for creating high nutritional value products [2]. However, creating baked products like biscuits from legumes can be a challenge due to flavor profiles, textural changes, and shelf life. Fermentation and nanotechnology are a viable options that provide improved nutritional quality and better food products over extended periods [3]. Through fermentation, the microorganism *Lactobacillus plantarum* can reduce complex macromolecules and anti-nutritional factors (e.g. tannins, saponins) while also producing bioactive compounds that have antioxidant potential [4].

While zinc oxide nanoparticle (ZnO-NPs) have very interesting potential applications as a food additive, as a result of their multifunctionality, antimicrobial, oxidative stability, and biocompatibility, fermentation and nanoparticles may provide a novel way to increase quality, safety, and functionality in food products [5]. However, application of nanoparticles in food requires careful consideration of evolving regulatory guidelines and safety assessments, which mandate case-by-case evaluation due to the distinct physicochemical behavior of nanomaterials. ZnO-NPs generally recognized as safe by the US Food and Drug Administration and used as a food additive (6). This study focused on a novel fermented soy bean biscuit treated with ZnO-NPs as a possible innovation for the functional bakery sector. The biscuits combined plant-based protein, dietary fibre, and essential micronutrients in a convenient form with health benefits, in addition to sensory bells and whistles. However, for any such novel product to be feasible it is necessary to conduct an appropriate range of preservation and quality control evaluation to measure nutritional stability, sensory characteristics, safety, and storage characteristics. Preservation, in food science, is the application of a variety of techniques to limit spoilage and physical and chemical deterioration while extending shelf life, nutrient quality, and maintaining safety [7]. For biscuits, the factors affecting overall quality are moisture uptake, lipid oxidation, microbial contamination, and textural changes during storage. The ZnO-NPs reduce spoilage to some extent because of their antimicrobial and antioxidant functionality, but functionality depend in part on formulation and use level and possible interactions with the food matrix. Quality control incorporated value added approaches to monitor physical, chemical, microbiological, and sensory aspects of the product evaluation from development to storage [8]. Quality control would ensure that the product is safe and/or consumer expectations. Therefore, the aim of this study were to evaluate the proximately composition, oxidative stability using peroxide value and moisture uptake trends, textural, colour, and sensory changes during storage of the biscuit, the cytotoxic potential of the ZnO-NPs in the biscuit matrix on normal human cells, and determine whether ZnO-NPs can serve as effective preservatives to prolong shelf life without compromising safety or consumer acceptability.

2. MATERIAL AND METHODS

2.1. Raw material procurement

This research was carried out in Food Analysis Laboratory in the Department of Food Science and Technology, BZU,Multan. Soy bean (Glycine max) seeds at the stage of maturity was procured from the local market of Pakistan.

2.2. Preparation of Zinc oxide suspensions

Zinc oxide nanoparticles (powder form) were purchased from Shanghai Macklin Technology Co., Ltd. These nanoparticles were dispersed in distilled water and sonicated to prevent aggregation. Seeds were soaked in 40ppm of ZnO-NPs for two hours followed by fermentation process [9].

2.3. Fermentation of treated Soybeans with ZnO-NPs

The submerged fermentation was carried out by using *Lactobacillus Plantarum* strain. The culture was prepared in MRS broth incubated at 30^0C for 48 hrs. The biomass was centrifuged and were dried at 50^0C in oven for 3 days.

2.4. Preparation of Powder of fermented Soybeans

Dried fermented soybeans were ground to form powder using a grinder (HEAVY-DUTY-GRINDER, Pamico Tech, Pakistan) and stored in airtight bags at 4^0C for further analysis.

2.5. Product Development

Zinc oxide nanoparticles treated fermented soyabean flour was undergo the product (biscuit) development. For the biscuits, fermented soybeans (treated with zinc oxide nanoparticles) flour of 40 ppm was used at different concentrations of 5%, 15%, 20%. For the dough formation, above flour was used with plain flour, butter, baking powder, salt and icing sugar. Then

dough was molded into shapes (5 cm) after stay time of 15 mins. Biscuits were placed in preheated baking oven for 15 mins at 220 C.

2.6. Determination the effect Zinc oxide Nanoparticles on proximate composition of Biscuits

Moisture content, crude protein, crude fat, crude fiber and ash of biscuits were determined using methods of analysis in Association of Official Analytical Chemists [10]. Carbohydrates was obtained by taking out the difference. The Kjeldahl factor of 6.25 was used for crude protein, which is used for all cereals and cereal based foods [11].

2.7. Determination the effect of Zinc oxide nanoparticles on color profile of biscuits

Utilizing the methodology developed by Lohita [12], the surface color of flours was measured. After calibrating the apparatus using conventional black and white tiles, the "L" (lightness), "a" (redness–greenness), and "b" (yellowness–blueness) values were measured in a glass cell containing flour of equal sizes when it was put against the light source.

2.8. Determination the effect of Zinc oxide nanoparticles on Physical Parameters of biscuits

Biscuit width (W) and thickness (T) were measured with a Vernier caliper. Weights were determined using a Mettler digital top loading balance (Mettler, PC 400, Switzerland). Spread ratio (SR) was calculated by the method of Ordorica- Falomir and Paredes – Lopez [23] as SR = W/T [13].

2.9. Preparation of Extracts for Determination of Bioactive Compounds

Accurately measured 10g of sample flour was added in 100ml 100% (v/v) ethanol and homogenized by using orbital shaker for 8 hours. To separate the extract, the mixture was filtered by using filter paper (Whatman No.1). Rotary evaporator (Rotary Vacuum Evaporator, EYELA N.N. Series) was used to remove the residual solvent of ethanolic extract at 40 0C under reduced pressure. The obtained extract was then used to determine the radical scavenging activity and Total Phenolic Content (TPC) [14].

2.9.1. Antioxidant Activity by DPPH method

Accurately 2.9ml sample extract was dissolved with 0.1ml of 0.0mM DPPH and placed in the dark at room temperature for 30 minutes at 23 °C. The sample was filtered after 30 minutes to measure the absorbance at 517nm by using a spectrophotometer (UV-Vis 3000, ORI, Germany). A control solution was prepared by mixing 0.1ml of methanol in 2,9ml DPPH solution [14].

DPPH activity was measured by using the following equation:

DPPH %=(Absorbance of control- Absorbance of sample)/(Absorbance of control)*100

2.9.2. Total Phenolic Content

Accurately measured sample extract of 1ml was dissolved in 1ml of Folin-Ciocalteu reagent and 1ml of sodium carbonate solution (7.5%). The solution was allowed to stand for 30 minutes and then absorbance was measured at 765nm by using a UV-VIS spectrophotometer (UV-Vis 3000, ORI, Germany). The total phenolic content was expressed as mg GAE/g [15].

2.10. Cytotoxicity study

Cytotoxity of biscuit powder extract against the BJ cells in human fibroblast was assessed by H.E.J RESEARCH INSTITUTE OF CHEMISTRY from the International Center for Chemical and Biological Sciences [16].

2.11. Determination the effect of Zinc oxide Nanoparticles on Storage Stability on the basis of Moisture Content, Peroxide Value and Sensory Analysis of biscuits

Moisture Content

Moisture content was determined gravimetrically according to AOAC method 925.10. Samples were dried in a hot-air oven at 105°C to constant weight. Results were expressed as grams of water per 100 grams of sample (g/100 g, % w/w) **[17].**

Peroxide Value

AOAC (2005) was used to calculate the Peroxide Value (PV) of the lipid fraction extracted of the biscuits. In brief, lipids were extracted with chloroform and dissolved in 15 mL of glacial acetic acid and 1 mL of saturated KI aqueous solution. The sample was stirred and left in the dark for 5 minutes prior to adding 75 mL of water and 1 mL of a starch solution. Titration of formed iodine with 0.01 N sodium thiosulfate was performed. The PV was calculated as the number of milli equivalents of active oxygen per kilogram of lipids (meq O2/kg of lipids). Every sample was examined in triplicate [18].

Sensory Analysis

The tests were conducted in a sensory laboratory. A laboratory with necessary facilities, viz., separate booths, provisions for adequate diffused light and air-conditioned odor-free environment, was employed for product evaluation. Sensory evaluation of biscuits was performed by twenty trained panelists using nine-point hedonic scale (1=extremely dislike and 9= extremely like). Sensory assessment was made in terms of color, appearance, texture, flavor, and overall acceptability [19].

2.12. Statistical Analysis

The statistics presented in all tables are the mean values obtained from three separate analyses. Significant distinctions for multiple comparisons were established using one way analysis of variance (ANOVA) following the Duncan test by use of SPSS 27.0 statistics software. The result was reported as mean ±SD. The result was considered statistically significant with a $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Proximate Composition

Moisture Content (%)

As shown in Table 1, Moisture content ranged from 4.9% (B3) to 6.8% (B). The control sample (B) had the highest moisture content, while B3 showed the lowest, with significant differences ($p < 0.05$). The decreasing trend from B to B1 can be primarily attributed to fermentation. Microbial metabolism and the breakdown of hydrophilic components during fermentation could alter the water-binding capacity of the matrix, leading to a lower final moisture content after drying. Moreover, nanoparticles altered the microstructure and porosity of the biscuit matrix, potentially enhancing water removal during drying or reducing hygroscopicity [20].

Ash Content (%)

Ash content, indicative of total mineral presence, increased significantly across the treated samples. B3 recorded the highest ash value (3.10%), while the control had the lowest (1.24%) as shown in Table 1. This suggested that treatments applied in B1 to B3 may have enriched the mineral content, potentially due to nutrient release during fermentation or inclusion of mineral-rich components such as nanoparticles or additives [21].

Fat Content (%)

Fat content decreased progressively from the control (4.23%) to B3 (2.56%), with significant differences ($p < 0.05$) as observed in Table 1.This reduction may be attributed to lipid degradation or redistribution during processing. Lower fat content, especially in B3, may be beneficial from a health perspective, depending on the target application (e.g., reduced-calorie products) [22] whereas the consistent downward value with increasing NP concentration (B2, B3) suggested nanoparticles might interact with lipid components, potentially through surface adsorption or by influencing the matrix structure to alter extractable fat. This reduction could be beneficial for developing reduced-fat products [23].

Protein Content (%)

Protein content increased significantly in the treated samples. B3 and B2 exhibited the highest protein levels (8.76% and 8.79%, respectively) compared to the control (6.63%). This enhancement could be due to proteolytic activity during fermentation, improved protein extractability, or concentration effects from moisture reduction (Table 1) [24]. The increased protein content enhances the nutritional quality of the samples. Further, the effect of nanoparticle incorporation might result from a secondary concentrating effect due to moisture reduction or from NP-matrix interactions that influenced protein solubility during analysis [25].

Crude Fiber (%)

A marked increase in crude fiber was observed from B (2.24%) to B3 (7.45%), with all treated samples showing significant improvements as shown in Table 1. Sharma [26] suggested that processing may have exposed or preserved more fibrous components, enhancing the dietary fiber content. High fiber content, especially in B3, is favorable for digestive health and could improve the product's functional value.

Samples	Moisture Content (%)	Ash (%)	Fat (%)	Crude Protein (%)	Crude Fiber (%)	Carbohydrate (%)
B	6.8±0.57^{c}	1.24±0.05^{a}	4.23±0.11^{c}	6.63±0.30^{a}	2.24±0.12^{a}	78.81±0.35^{c}
B1	5.7±0.31^{b}	2.46±0.11^{b}	3.85±0.07^{c}	7.70±0.17^{b}	5.31±0.11^{b}	74.88±0.62^{b}
B2	5.3±0.45^{ab}	2.30±0.51^{b}	3.26±0.02^{b}	8.79±0.08^{c}	6.43±0.51^{c}	73.85±0.65^{ab}
B3	4.9±0.01^{a}	3.10±0.05^{c}	2.56±0.49^{a}	8.76±0.11^{c}	7.45±0.05^{d}	73.08±0.55^{a}

Table 1. Effect of zinc oxide nanoparticles on Proximate Composition of fermented soybeans Biscuits[a]

[a]All values are mean of triplicate determinations. Means within a column with different superscripts are significantly different at $P < 0.05$. Abbreviations: B: 100% Plain Flour Biscuit, B1: 5% of 40ppm ZnO-NPs fermented soybean flour biscuit, B2: 10% of 40ppm ZnO-NPs fermented soybean biscuit and B3: 15% of 40ppm ZnO-NPs fermented soybean flour biscuit.

Carbohydrate (%)

Carbohydrate content decreased from 78.81% (B) to 73.08% (B3), inversely reflecting the increases in protein, ash, and fiber (Table 1). The reduction in carbohydrate content could be attributed to fermentation-associated sugar utilization or a dilution effect due to increases in other components. Lower carbohydrate levels may support the development of lower glycemic index foods [27].

3.2. Color Profile

The color parameters (L*, a*, and b*) provide insight into the visual quality and appearance of the samples, which are critical factors influencing consumer perception and acceptance as shown in Table 2. L* values (lightness) ranged from 66.4 to 71.1 across the samples. The control (B) had the lowest L* value (66.4), indicating a slightly darker appearance. Samples B1, B2, and B3 showed progressively higher lightness values, with B2 having the highest L* (71.1), suggesting a brighter or lighter color. However, the differences were not statistically significant ($p > 0.05$), as indicated by the shared superscript "a." a* values (red-green axis) ranged from 13.4 to 15.9. The control sample had the lowest redness (13.4), while B3 had the highest (15.9). Statistically, B3 was significantly different from B ($p < 0.05$), as indicated by different superscripts ("a" vs. "b"), suggesting that B3 had a more intense red hue. B1 and B2 had intermediate a* values (15.0 and 15.2) and did not differ significantly from either B or B3, falling into the "ab" grouping. b* values (yellow-blue axis) remained relatively stable across all samples, ranging from 47.4 to 48.6, with no significant differences observed ($p > 0.05$). This indicated consistent yellowness among all samples, regardless of treatment. The increase in L* and a* values in B1–B3 compared to the control may be attributed to modifications in formulation or treatment processes that influenced the surface color characteristics, such as Maillard reactions, pigment formation, or structural changes affecting light reflectance [28]. Results suggested that even in the absence of significant differences, consistent color parameters across treatments indicated that

fermentation and nanoparticle incorporation did not negatively alter the product's visual appeal which is a critical factor for consumer acceptance.

Table 2. Effect of zinc oxide nanoparticles on Color Profile of fermented soybeans Biscuits[a]

Samples	L*	a*	b*
B	66.4±0.57[a]	13.4±0.05[a]	48.4±0.11[a]
B1	69.3±0.31[a]	15.0±0.11[ab]	48.6±0.07[a]
B2	71.1±0.45[a]	15.2±0.51[ab]	47.4±0.02[a]
B3	70.2±0.01[a]	15.9±0.05[b]	48.4±0.49[a]

[a]All values are mean of triplicate determinations. Means within a column with different superscripts are significantly different at $P < 0.05$. Abbreviations: B: 100% Plain Flour Biscuit, B1: 5% of 40ppm ZnO-NPs fermented soybean flour biscuit, B2: 10% of 40ppm ZnO-NPs fermented soybean biscuit and B3: 15% of 40ppm ZnO-NPs fermented soybean flour biscuit.

3.3. Diameter, Width and Weight of fermented soybeans Biscuits

The physical dimensions and weight of the samples did not show statistically significant differences ($p > 0.05$), as shown in Table 3. This suggested that the variations introduced in samples B1, B2, and B3 (likely due to treatment, formulation, or processing changes) did not significantly affect the overall size or mass compared to the control sample (B). Diameter ranged from 5.3 to 5.8 cm, with B1 and B2 showing slightly higher values than B and B3, although not significantly different. Width values were relatively similar, except B3 showed a slightly increased width (0.73 cm) compared to others (0.56–0.63 cm), but again without statistical significance. Weight varied minimally across all samples (15.4–15.8 g), indicating consistent mass distribution. These findings suggest that the modifications applied in B1, B2, and B3 did not compromise the physical integrity or uniformity of the samples. Maintaining consistent physical parameters is particularly important in product development, where uniform size and weight are critical for consumer acceptability, packaging, and process control [28].

Table 3. Effect of zinc oxide nanoparticles on Diameter, Width and Weight of fermented soybeans Biscuits[a]

Samples	Diameter (cm)	Width (cm)	Weight (g)
B	5.4±0.57[a]	0.63±0.57[a]	15.4±0.57[a]
B1	5.8±0.31[a]	0.56±0.31[a]	15.6±0.31[a]
B2	5.8±0.45[a]	0.56±0.45[a]	15.8±0.45[a]
B3	5.3±0.01[a]	0.73±0.01[a]	15.6±0.01[a]

[a]All values are mean of triplicate determinations. Means within a column with different superscripts are significantly different at P < 0.05. Abbreviations: B: 100% Plain Flour Biscuit, B1: 5% of 40ppm ZnO-NPs fermented soybean flour biscuit, B2: 10% of 40ppm ZnO-NPs fermented soybean biscuit and B3: 15% of 40ppm ZnO-NPs fermented soybean flour biscuit.

3.4. Bioactive Compounds

Table 4 observed a significant and dose-dependent enhancement in the bioactive profile of the fermented soybean biscuits was observed upon the incorporation of zinc oxide nanoparticle (ZnO-NP)-treated flour. The antioxidant capacity, as measured by DPPH radical scavenging activity, was found to increase progressively from 41.3% in the control biscuit (B) to a maximum of 74.6% in the sample containing 15% treated flour (B3). Correspondingly, the total phenolic content (TPC) was elevated from 18.0 mg GAE/g in the control to 39.0 mg GAE/g in the B3 formulation. This represented an increase of 81% in antioxidant activity and 117% in phenolic compounds for the B3 sample compared to the control, as detailed in the presented data . The clear incremental rise noted in the intermediate samples, B1 and B2, further confirmed the consistent positive influence of the treatment concentration. This pronounced improvement was likely attributable to the role of ZnO-NPs as an elicitor, which may have stimulated the biosynthesis or enhanced the retention of phenolic compounds during the fermentation and baking processes. Consequently, the potential for developing a nutritionally fortified functional food was successfully demonstrated [29]. However, these promising results regarding bioactive enhancement were necessarily considered alongside the imperative for thorough safety evaluations concerning the application of nanomaterials within the food system.

Table 4. Effect of zinc oxide nanoparticles on Bioactive Compounds of fermented soybeans Biscuits[a]

Samples	DPPH (%)	Total Phenolic Content (mg Gallic acid/g)
B	41.3±0.57[a]	18.0±0.57[a]
B1	55.6±0.31[b]	23.6±0.31[b]
B2	63.6±0.45[c]	31.6±0.45[c]
B3	74.6±0.01[d]	39.0±0.01[d]

[a]All values are mean of triplicate determinations. Means within a column with different superscripts are significantly different at p < 0.05. Abbreviations: B: 100% Plain Flour Biscuit, B1: 5% of 40ppm ZnO-NPs fermented soybean flour biscuit, B2: 10% of 40ppm ZnO-NPs fermented soybean biscuit and B3: 15% of 40ppm ZnO-NPs fermented soybean flour biscuit.

3.5. Cytotoxicity

The cytotoxicity assessment on BJ human fibroblast cells revealed that all tested samples (B1, B2, B3) showed mild inhibition of cell viability at 200 µg/mL, with sample B1 showing the highest inhibition (16.15%) as shown in Table 5. This indicated low cytotoxic potential of the samples toward normal human fibroblast cells, which may be advantageous if the compounds are being developed for biomedical or neutraceutical purposes. In contrast, doxorubicin—used as a standard—demonstrated very high cytotoxicity (98.3%) at just 16.3 µg/mL, with an IC_{50} of 0.08 ± 0.09 µg/mL, confirming its potent cytotoxic nature. The significant difference in cytotoxicity between the test samples and doxorubicin highlights the biocompatibility of B1–B3, especially toward non-cancerous cells like BJ fibroblasts [30]. Low cytotoxicity of B1–B3, making them potentially safe for applications where interaction with normal cells is expected. B1 has the most noticeable cytotoxic effect among the test samples, indicating it may have some bioactive compounds worth further exploration. The test concentration of 200 µg/mL was selected to represent a conservative, high-

end exposure scenario, simulating potential direct and prolonged cellular contact in a biomedical context. This concentration allows for a clear safety assessment by differentiating between low-level bioactive effects and significant toxicity, as demonstrated by the stark contrast with the potent control agent, doxorubicin. Further analyses, such as texture, moisture content, or structural properties, could offer additional insight into how these samples differ in quality or performance despite physical similarity.

Table 5. Effect of zinc oxide nanoparticles on Cytotoxicity of fermented soybeans Biscuits[a]

Samples	Conc. (µg/mL)	% Inhibition	IC_{50}±SD
B	-	-	-
B1	200	16.14±0.01[c]	-
B2	200	10.51±0.00[b]	-
B3	200	8.06±0.00[a]	-
Std. Doxorubicin	16.30	98.3	0.08±0.09

[a]All values are mean of triplicate determinations. Means within a column with different superscripts are significantly different at $P < 0.05$. Abbreviations: B: 100% Plain Flour Biscuit, B1: 5% of 40ppm ZnO-NPs fermented soybean flour biscuit, B2: 10% of 40ppm ZnO-NPs fermented soybean biscuit and B3: 15% of 40ppm ZnO-NPs fermented soybean flour biscuit.

3.6. Storage Stability

3.6.1. Peroxide Value

The results indicated a progressive increase in the measured values of all samples (B, B1, B2, B3) throughout the 120-day storage period, suggesting a gradual increase in oxidative changes or deterioration over time as shown in Figure 1. Sample B, which showed the highest initial value (0.66) at day 0, also recorded the greatest increase, reaching **1.3** by day 90, with no further rise observed up to day 120, indicating possible stabilization or early saturation of oxidative products [30]. In contrast, samples B1, B2, and B3 exhibited significantly lower initial and final values**,** suggesting better oxidative stability or improved preservation. Among these, B3 demonstrated the most stable profile, starting at 0.37 and only reaching 0.71 by day 120. B2 showed a similar trend, rising from 0.38 to 0.70**,** with a steady and moderate increase. B1 displayed slightly higher values than B2 and B3, particularly after the 60th day, possibly indicating a lesser antioxidant protection or greater susceptibility to degradation. However, its final value (0.81) still remained well below that of B, reinforcing that the treatments applied to B1, B2, and B3 were effective in slowing down the rate of oxidative change. These findings suggest that formulations B1–B3, likely enhanced with natural antioxidants, modified ingredients, or protective packaging, were more resistant to oxidative deterioration during storage compared to the control sample (B). The data support the conclusion that such treatments can play a significant role in improving product stability over extended shelf life.

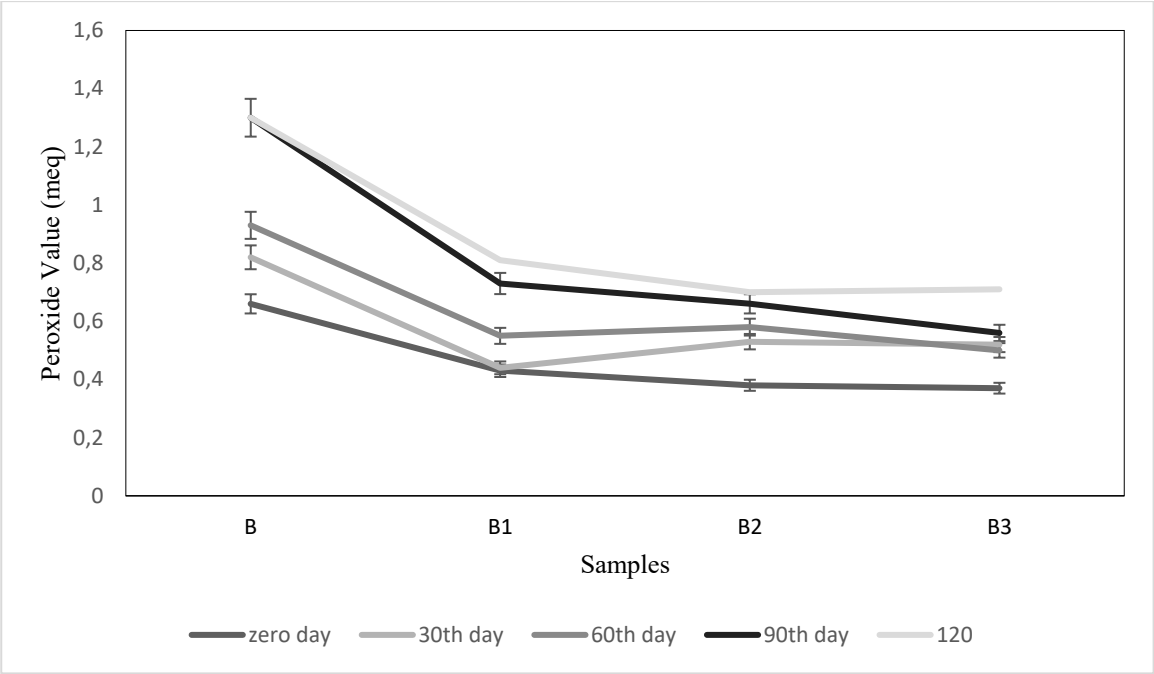


Figure 1. Effect of zinc oxide nanoparticles on Storage Stability on the basis of Peroxide Value of fermented soybeans Biscuits over 120 days[a]

All values are mean of triplicate determinations. Means within a column with different superscripts are significantly different at $P < 0.05$. Abbreviations: B Fermented soybean flour biscuit, B1: 5% of 40ppm ZnO-NPs fermented soybean flour biscuit, B2: 10% of 40ppm ZnO-NPs fermented soybean biscuit and B3: 15% of 40ppm ZnO-NPs fermented soybean flour biscuit.

3.6.2. Moisture Content

The data revealed a general upward trend in the measured parameter across all samples (B, B1, B2, B3) during the 90-day storage period indicating progressive deterioration or chemical change over time as shown in Figure 2. The moisture content data expressed as a percentage (g/100 g) revealed critical differences in hygroscopicity and shelf-stability potential among the biscuit formulations over 90 days. The control (B) began with the highest initial moisture (0.99%) and showed a progressive increased to 1.12% indicating a significant tendency to absorb environmental moisture during storage. In contrast, all treated samples (B1, B2, B3) started with substantially lower initial moisture levels. Sample B1 (fermented only) increased from 0.64% to 1.03%, demonstrating that fermentation alone improved the matrix's resistance to moisture uptake compared to the control. The most striking stability was observed in samples containing ZnO nanoparticles. B2 and B3 began with the lowest moisture (0.40% and 0.32%, respectively) and maintained significantly lower levels throughout storage. B3, with the highest nanoparticle concentration, exhibited exceptional stability, showing no increase for the first 30 days and reaching only 0.91% at day 90. This indicated that ZnO-NPs likely altered the microstructure, reducing porosity and hydrophilicity, thereby creating a more effective barrier against moisture migration. The sequential reduction in moisture uptake from B to B3 clearly demonstrated a synergistic effect where fermentation and nanoparticle incorporation combined to produce a physicochemically more stable, shelf-stable product with reduced risk of microbial spoilage and textural degradation [12]

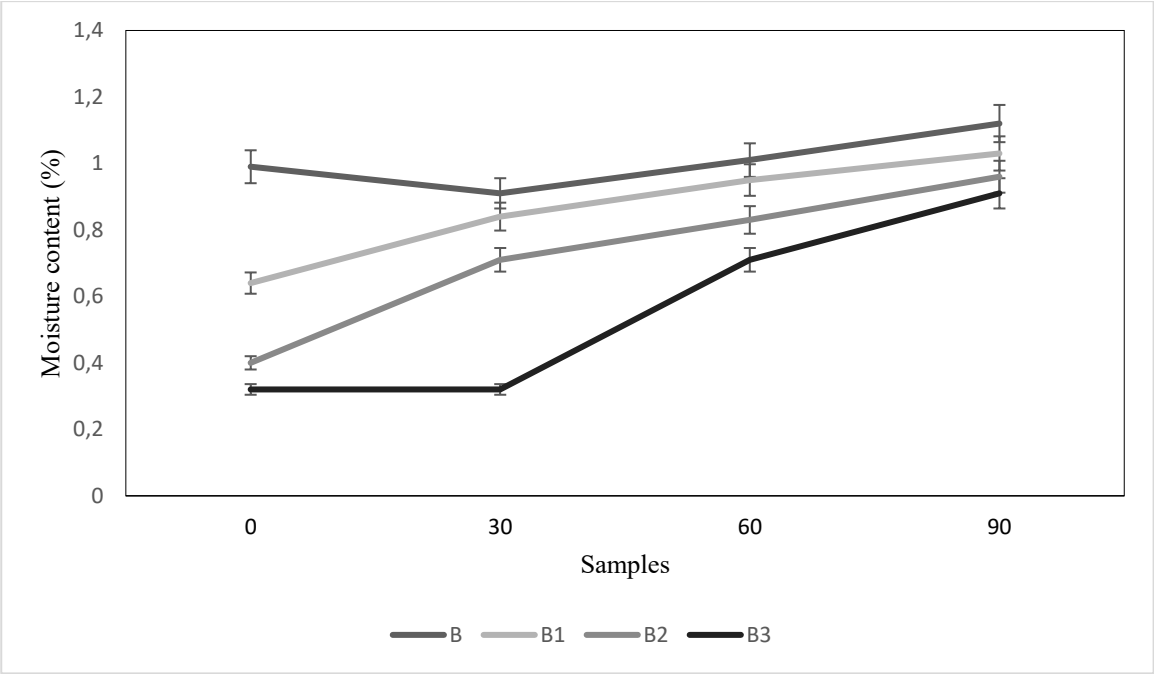


Figure 2. Effect of zinc oxide nanoparticles on Storage Stability on the basis of Moisture Content of fermented soybeans Biscuits over 120 days[a]

All values are mean of triplicate determinations. Means within a column with different superscripts are significantly different at $P < 0.05$. Abbreviations: B Fermented soybean flour biscuit, B1: 5% of 40ppm ZnO-NPs fermented soybean flour biscuit, B2: 10% of 40ppm ZnO-NPs fermented soybean biscuit and B3: 15% of 40ppm ZnO-NPs fermented soybean flour biscuit.

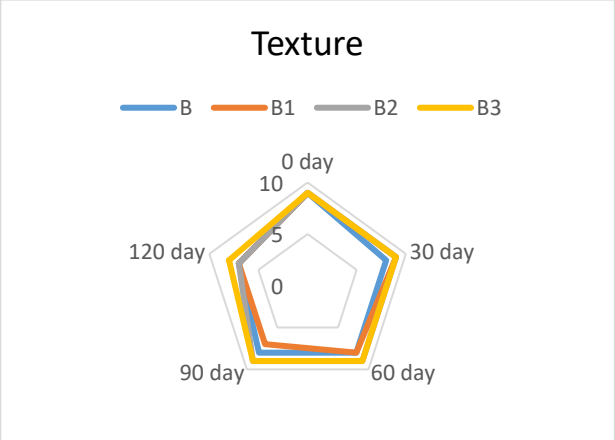
3.6.3. Sensory Characteristics

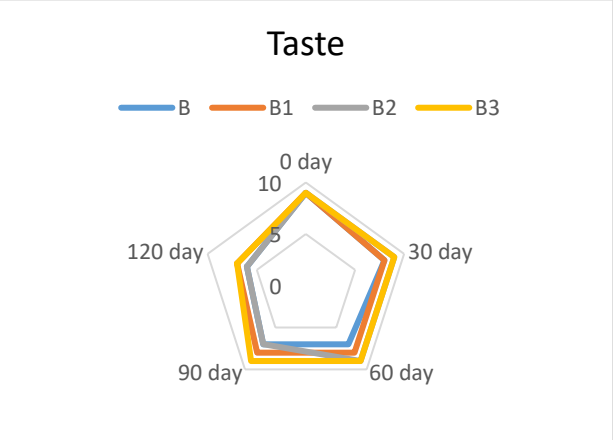
Texture

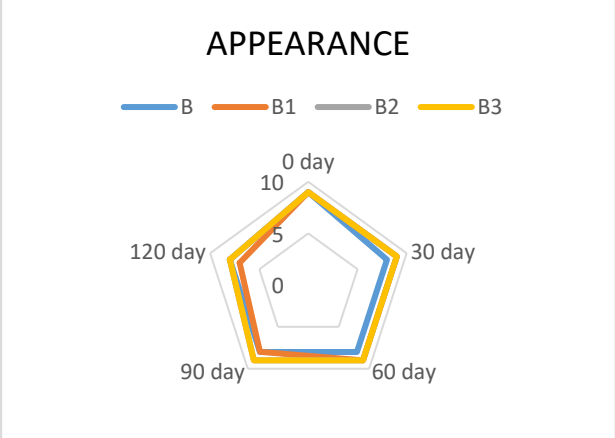
Texture is a critical quality attribute that affects consumer acceptance, particularly in food products with extended shelf lives. The texture scored for samples B, B1, B2, and B3 were evaluated at intervals of 0, 30, 60, 90, and 120 days to assess the impact of treatment and storage duration on textural stability (Figure 3). All samples began with a texture score of 9, indicating excellent texture quality at the start of storage. This consistency confirmed that the initial formulation or processing did not negatively affect texture. Control sample (B) showed a gradual decline in texture, dropping from 9 to 7 over 120 days. This indicates moderate texture deterioration over time, likely due to moisture migration, staling, or structural breakdown. B1 also declined in texture quality, decreasing to a score of 7 by day 90, similar to the control. This suggests that the treatment used in B1 provided limited benefit in preserving textural quality beyond 60 days. B2 maintained a perfect texture score of 9 through 90 days, only dropping to 7 at day 120. This extended textural stability suggested a more effective treatment, possibly due to better water-holding capacity, improved structural integrity, or reduced retrogradation. B3 showed the best overall textural stability, maintaining a score of 9 up to 90 days and only slightly declining to 8 by day 120. This indicates that B3 was the most effective at preserving texture throughout the storage period. The textural decline across all samples by day 120 is expected due to aging-related changes such as starch retrogradation, moisture redistribution, or protein network breakdown. However, the rate and extent of decline varied significantly among the samples: B2 and B3 demonstrated superior textural preservation, suggesting that their specific treatments (possibly involving fermentation, crosslinking, or nanoparticle incorporation) contributed to a more stable matrix. In contrast, B and B1 showed earlier and more pronounced degradation, indicating a need for improved stabilization strategies.

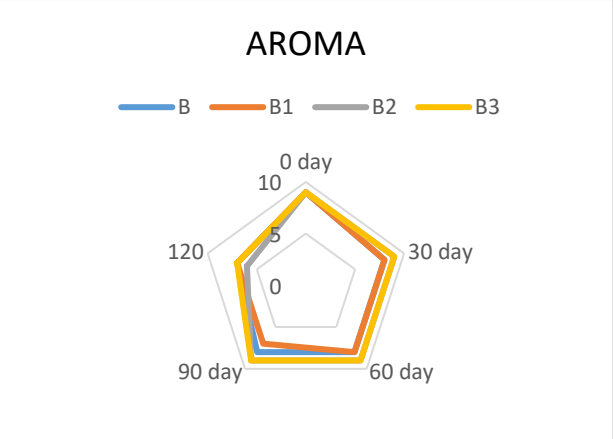
Aroma

Sample B (Control) showed a gradual decline in aroma, decreasing to a score of 7 by day 120. This reflects a moderate loss of volatile aromatic compounds, possibly due to oxidative degradation or volatilization during storage (Figure 3). Sample B1 followed a similar trend, maintaining a stable aroma until day 60, but then decreasing to 7 by day 90 and staying at that level. This suggests that B1 had limited aroma preservation, potentially due to inadequate antioxidant protection or structural stability. Sample B2 exhibited superior aroma retention through day 90 with consistent scores of 9, but dropped sharply to a score of 6 at day 120. This indicated a sudden decline in aroma stability, which may be linked to threshold degradation or the accumulation of off-flavors after extended storage. Sample B3 maintained a score of 9 through day 90 and only decreased slightly to 7 at day 120. This suggested excellent aroma preservation, with delayed onset of degradation compared to the control and other samples.









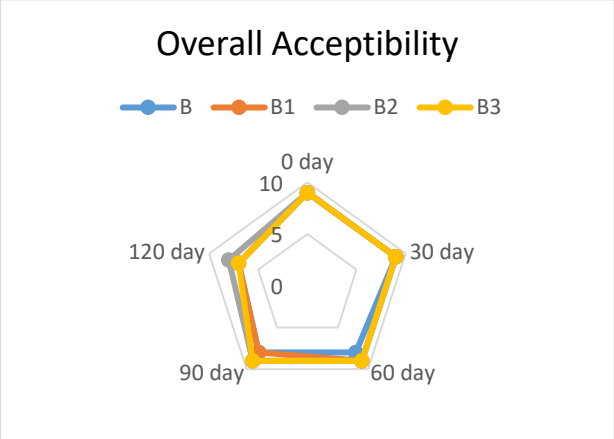


Figure 3. Effect of zinc oxide nanoparticles on Storage Stability on the basis of Sensory Characteristics of fermented soybeans Biscuits over 120 days[a]

All values are mean of triplicate determinations. Means within a column with different superscripts are significantly different at $P < 0.05$. Abbreviations: B Fermented soybean flour biscuit, B1: 5% of 40ppm ZnO-NPs fermented soybean flour biscuit, B2: 10% of 40ppm ZnO-NPs fermented soybean biscuit and B3: 15% of 40ppm ZnO-NPs fermented soybean flour biscuit.

Appearance

The control sample (B) showed a slight decrease in appearance score from 9 to 8 by day 30, which remained stable throughout to day 120 (Figure 3). This indicated a minor but consistent decline in visual quality that could be due to factors like surface drying, color fading, or texture changes affecting appearance. Sample B1 maintained a perfect score of 9 until day 60, then declined to 8 at day 90 and further to 7 at day 120. This gradual decrease suggested some visual degradation over time, possibly related to subtle color changes, surface irregularities, or loss of glossiness. Samples B2 and B3 both maintained high appearance scores of 9 through day 90, with only a slight decrease to 8 by day 120. This indicated that these treatments were more effective at preserving the visual quality during storage, potentially through protective effects against oxidation, moisture loss, or enzymatic browning.

Over all acceptability

The control sample (B) maintained a high acceptability score of 9 through 30 days but declined to 7 by day 120 (Figure 3). This gradual decreased suggested sensory deterioration likely due to cumulative changes in texture, aroma, and appearance over time. Sample B1 showed a similar trend, with consistent acceptability of 9 up to 60 days followed by a decrease to 7 at day 120, indicating moderate sensory stability but eventual decline likely from flavor or texture changes. Sample B2 maintained the highest overall acceptability, with scores of 9 up to 90 days and a slight decrease to 8 by day 120. This indicates superior sensory preservation, possibly due to better maintenance of texture, aroma, and appearance, which contributed positively to consumer acceptance over a longer period. Sample B3 also retained an acceptability score of 9 through 90 days but decreased to 7 at day 120, suggesting good but slightly less stable sensory quality compared to B2 toward the end of storage.

4. CONCLUSION

This study demonstrated that ozone treatment induces notable structural changes in both WPI and EW proteins. DSC analysis showed increased denaturation temperatures for WPI, suggesting enhanced thermal stability, while enthalpy values decreased for both protein types, indicating partial structural disruption. Optical activity measurements revealed that ozonation increased the laevorotation of WPI, reflecting alterations in its secondary and tertiary structure. HPLC analysis confirmed significant degradation of major protein components, with the appearance of new peaks suggesting the formation of oxidation products. Overall, ozone significantly affects protein conformation and stability, with a more pronounced impact observed in WPI compared to EW proteins. Collectively, the results confirm that ozone treatment induces both conformational and compositional changes in food proteins. These structural alterations may have implications for the functional and nutritional properties of proteins in food systems, highlighting the need for controlled application of ozone in food processing environments.

DECLARATION OF CONFLICTING INTERESTS

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