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Optimization of mushroom preservation parameters using box-behnken design (BBD): A Response Surface Methodology Approach

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Abstract

The rapid perishability of *Agaricus bisporus* mushrooms presents a major challenge in post-harvest management due to microbial contamination, enzymatic browning, and lack of protective outer skin. This study aimed to optimize preservation parameters for microbial load reduction using Response Surface Methodology (RSM) with a Box-Behnken Design (BBD). Initially, a One-Factor-At-a-Time (OFAT) approach evaluated the effects of UV-C dose, gamma irradiation, storage time, temperature, and packaging thickness on microbial quality. Subsequently, BBD was applied to assess both individual and interactive effects of these variables. The results revealed that UV-C exposure at 254 nm and gamma irradiation at 2 kGy significantly reduced microbial load, particularly when combined with storage at 4 °C and packaging material of 40 µm thickness. The model yielded a significant F-value of 2.04 ($p = 0.0468$) with a non-significant lack-of-fit ($p = 0.7906$), indicating strong model adequacy. Validation trials under optimized conditions predicted microbial reduction of 1.2 log CFU/g, closely aligning with the experimental result of 1.3 log CFU/g (8.33% error). This confirms the robustness and predictive accuracy of the model. The findings are in agreement with earlier reports highlighting the efficacy of UV-C and gamma irradiation in microbial reduction without compromising mushroom quality. The developed RSM model provides a statistically sound and practical framework for enhancing mushroom shelf life and offers a scalable preservation strategy for fresh produce industries.

Keywords: *Agaricus bisporus*, Box-Behnken Design, OFAT, Microbial Content, Gamma irradiation

1. Introduction

The button mushroom (*Agaricus bisporus*) is the most commonly consumed edible mushroom, accounting for 30% of global mushroom consumption (Julian Selemin, 2023). Rich in vitamin B complex, ergosterols, selenium, and bioactive compounds like triterpenoids and glycoproteins, mushrooms are nutritionally valuable. They are low in fat and cholesterol-free, offering protein of high biological value (Somenath Das & Bhanu Prakash, 2022).

In recent years, mushrooms have gained interest in the meat industry due to their flavor and fibrous, meat-like texture. Their inclusion in muscle foods not only enhances nutritional value but also supports cleaner-label products (Stavropoulou NA et al., 2022; Singh, Awanish & Sit, Nandan, 2022). Despite these advantages, mushrooms are highly perishable, lacking a protective skin layer. This results in a shelf life of only 1–3 days at room temperature and 5–8 days under refrigeration (Devi S et al., 2020). High respiration and enzymatic activity, along with microbial spoilage, are the primary reasons for rapid degradation (Martine B, 2000).

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To address this, storage techniques such as modified atmosphere packaging (MAP) and cold storage have been developed (Rahman, M.S). In countries like Lebanon, where mushrooms are expensive [Abou Fayssal, S., et al., 2023], extending shelf life is essential to prevent economic loss and ensure affordability. Despite promising approaches like freezing, canning, and irradiation, many consumers prefer fresh mushrooms—making short-term preservation strategies critical [Kibar B, 2021; Galani et al., 2017].

Box-Behnken Design (BBD) is a statistical method within Response Surface Methodology (RSM) used to screen and evaluate multiple variables efficiently. BBD is highly effective in identifying the key parameters influencing microbial reduction and shelf life extension in perishable products like mushrooms. It serves as an initial step in RSM by assessing individual and interactive effects of variables without requiring a full factorial design. The advantage of BBD lies in reducing the number of experimental runs and allowing better estimation of quadratic relationships in systems with three or more variables [Reji, Meega & Kumar, Rupak, 2023; Sz. Gulyás et al., 2023, Wadikar et al., 2007].

This study applies BBD to identify the most influential parameters among gamma irradiation dose, substrate composition, and storage conditions on microbial contamination and physicochemical quality of *Agaricus bisporus*. The optimized results from BBD will guide the subsequent use of CCD for deeper modelling and process refinement.

2. Materials and Methods

2.1. Sample Collection and Preparation

Fresh samples of *Agaricus bisporus* mushrooms were purchased from a local market, taking care to avoid choosing any of those specimens showing visible damage, bruising, or microbial spoilage, thus maintaining sample integrity. The mushrooms were first cleaned to rid them of debris and surface contaminations and subsequently washed extensively with distilled water to remove soil particles and contaminants. Following washing, the mushrooms were evenly distributed onto a sterile tray and left to air-dry at room temperature (~25 °C) until completely dry.

2.2. Microbial Content Reduction by One-Factor-at-a-Time (OFAT)

An initial One-Factor-At-a-Time (OFAT) optimization approach was employed to evaluate the individual effects of key treatment and storage parameters on the microbial quality and overall physicochemical stability of *Agaricus bisporus.* The selected independent variables included: UV-C dose (A): 0 to 254 nm, Gamma irradiation dose (B): 1 to 2 kGy, Storage duration (C): 0 to 28 days, Storage temperature (D): 4°C to 8°C, Packaging thickness (E): 20 μm to 40 μm each factor was systematically varied while keeping other variables constant.

2.3. Statistical Optimization Using Box-Behnken Design (BBD)

A five-variable Box-Behnken design (BBD) was employed to assess the combined effect of UV-C dose, gamma irradiation, storage time, temperature, and packaging material on microbial contamination. This design allows quadratic surface modelling and generates a second-degree polynomial model with a limited number of runs. Range of Independent Variables Used in BBD represented in table -1

Number of experimental runs:

$$N = k^2 + k + Cp = 25 + 5 + 3 = 33 \text{ (As described, 46 total including replications).}$$

The experimental design and analysis were conducted using Design Expert 13.0.3.1 software.

The second-order polynomial equation used is:

$$Y = \beta0 + \sum \beta n\, Xn + \sum \beta nn\, X^2n + \sum \beta nm\, Xn\, Xm \text{ ………..Eq (1)}$$

The five factors design was analysed by following model equation:

$$Y = \beta0 + \beta1\, A + \beta2\, B + \beta3\, C + \beta4\, D + \beta5\, E + \beta11\, A2 + \beta22\, B2 + \beta33\, C2 + \beta44\, D2 + \beta55\, E2 + \beta12\, AB + \beta13\, AC + \beta14\, AD + \beta15\, AE + \beta23\, BC + \beta24\, BD + \beta25\, BE + \beta34\, CD + \beta35\, CE + \beta46\, DE + \varepsilon \text{ ………..Eq (2)}$$

Table 1 Range of Independent Variables Used in BBD

Independent Variables	Factors	Units	Low (−1)	High (+1)
UV-C dose	A	nm	0	254
Gamma irradiation	B	kGy	1	2
Storage time	C	days	0	28
Temperature	D	°C	4	8
Packaging material	E	µm	20	40

Regression analysis and analysis of variance (ANOVA) are used to fit the model according to the equation1. Adequacy of models is checked by model analysis and R_2 analysis; F value is checked to find out the significance of all the fitted equation at 5% level of significance. To visualize relationship between response and experimental levels of each factor and to find out microbial contamination, the fitted equations are expressed as contour plots, which is explained by statistical software, Design Expert 13.0.3.1 (Stavropoulou NA et al., 2022).

2.4. Validation of the experimental model

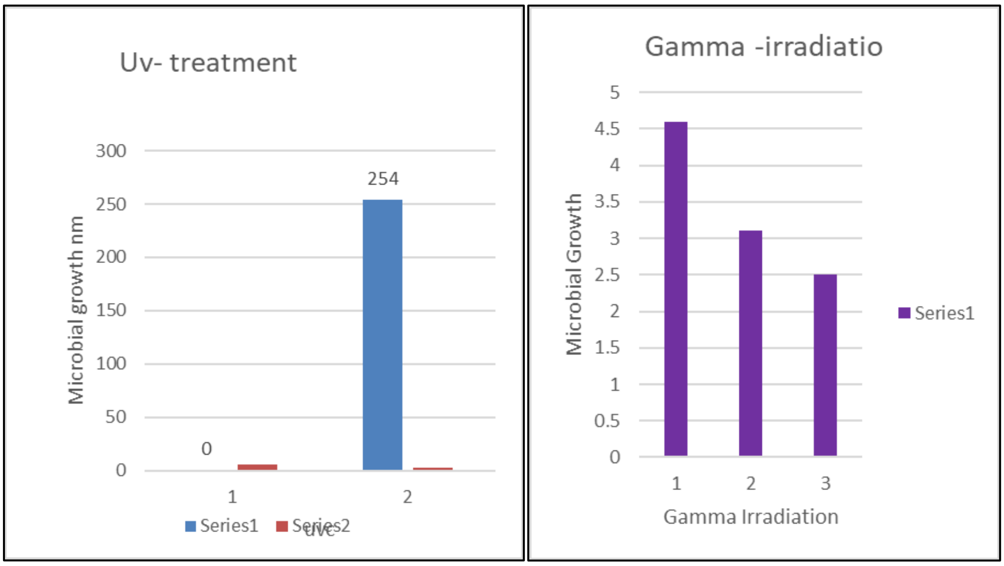
To validate the model equation. Experiments were conducted in triplicates to determine the optimum fibrinolytic enzyme production. To fit the polynomial equation was expressed as 3D dimensional (3D) surface plots to visualize the relation between responses and the experimental factors of each factor used in the design.

3. Results

3.1. Microbial Content Reduction in *Agaricus bisporus* by OFAT (one factor at a time approach):

The results of the One-Factor-At-a-Time (OFAT) investigation on microbial growth influencing mushroom spoilage are presented in this section. This analysis highlights the impact of individual parameters- UV-C dose, gamma irradiation, storage time, temperature, and packaging thickness the microbial load of *Agaricus bisporus*. By varying each factor independently while maintaining the others constant, the study identified the conditions that most significantly contributed to microbial reduction and consequently, to the extension of mushroom shelf life represented in Fig. 1

Based on the data obtained and represented, the microbial growth showed UV-C treatment at 254nm (Jing Lei et al., 2018), the gamma irradiation at 2kGy (Khan, A.A., Gani et al., 2015), the packaging with materials with a thickness of 40µm (Ban Z et al., 2014), and temperature at 4°C (Silva, M et al., 2025) showed a decrease in microbial growth compared to that of other variables tested. The period for the preservation of the mushrooms showed a gradual increase in their microbial growth on the last day, i.e, day 28; however, the results at the above-mentioned variables showed better significant values compared to those of other batches.



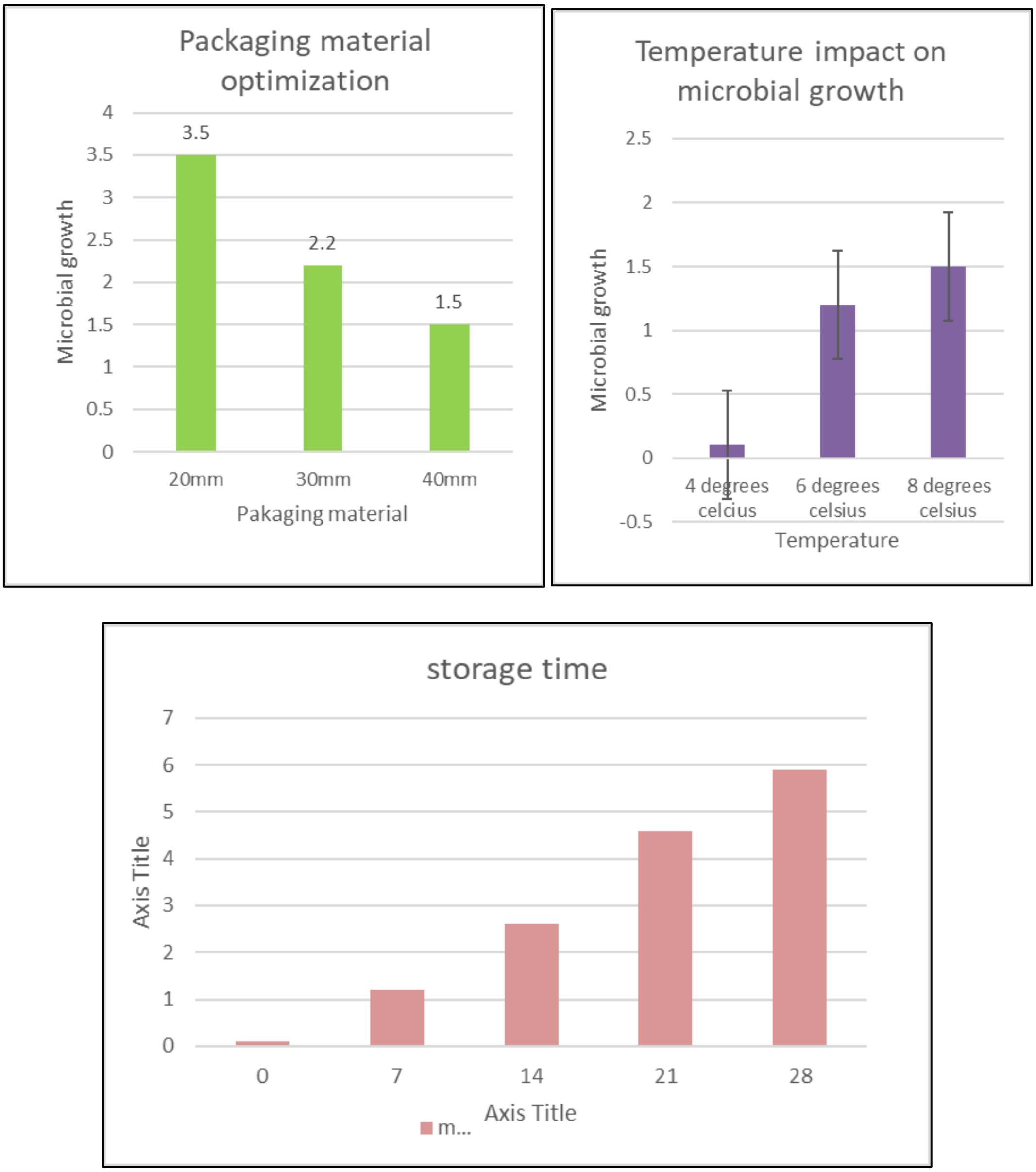


Figure 1 Microbial Content Reduction in *Agaricus bisporus* by OFAT

3.2. Optimization microbial content reduction in *Agaricus bisporus*, a Box-Behnken design (BBD):

To optimize microbial content reduction in *Agaricus bisporus*, a Box-Behnken design (BBD) was employed following preliminary screening using the one-factor-at-a-time (OFAT) approach. The OFAT method helped identify the most significant factors affecting microbial reduction, which included UV-C dose, gamma irradiation, storage time, temperature, and packaging material. These factors were then considered as independent variables in the BBD, each tested at three levels, to systematically investigate their individual and interactive effects on microbial contamination. This approach enabled the determination of optimal conditions for effective preservation of the mushrooms (Polo-Castellano et al., 2022; George & Norman 1987 ;).

Table 2 represents the Box-Behnken Design (BBD) experimental plan along with the observed microbial contamination reduction responses obtained in the laboratory. The effects of the variables-UV-C dose, gamma irradiation, storage time, temperature, and packaging material on microbial reduction were modelled using a second-order polynomial regression equation.

Table 2 Represents the Box-Behnken Design (BBD) experimental

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Response 1
Run	**A: UV- C dose**	**B: Gamma irradiation**	**C: Storage time**	**D:Temperature**	**E: packaging material**	**Microbial content**
	nm	kGy	days	oc		
1	127	1.5	28	4	30	1.6
2	0	1	14	6	30	3.4
3	127	1.5	28	6	20	2.9
4	127	2	14	6	20	1.2
5	127	2	28	6	30	1.5
6	127	1	14	8	30	2.8
7	127	1.5	0	6	20	1.1
8	254	1.5	14	6	40	1.4
9	127	1.5	0	6	40	1.3
10	127	1.5	0	8	30	1.6
11	0	1.5	0	6	30	2.4
12	254	1.5	14	4	30	1.8
13	127	1.5	28	6	40	1.4
14	127	1.5	14	6	30	2.5
15	127	1	14	6	20	3.5
16	127	1.5	14	4	40	2.8
17	127	1.5	14	8	40	1.7
18	127	1.5	14	6	30	1.4
19	254	1.5	14	6	20	2.3
20	127	1.5	0	4	30	1.5
21	0	1.5	14	8	30	1.8
22	254	1.5	0	6	30	1.7
23	0	1.5	14	6	20	2.6
24	127	1	28	6	30	3.5
25	0	1.5	14	4	30	3.7
26	254	2	14	6	30	2.1
27	127	2	14	6	40	1.9
28	127	1	14	6	40	2.3
29	127	1.5	14	6	30	1.3
30	127	1	0	6	30	2.8
31	127	1.5	14	8	20	1.5
32	127	1	14	4	30	2.3
33	127	2	14	8	30	2.4

34	0	2	14	6	30	2.7
35	127	1.5	14	4	20	1.5
36	127	1.5	28	8	30	1.8
37	127	1.5	14	6	30	1.2
38	254	1.5	14	8	30	2.6
39	254	1.5	28	6	30	1.9
40	127	2	14	4	30	2.8
41	254	1	14	6	30	1.4
42	0	1.5	14	6	40	1.5
43	127	1.5	14	6	30	1.2
44	127	1.5	14	6	30	2.8
45	0	1.5	28	6	30	3.4
46	127	2	0	6	30	1.8

The ANOVA results in Table 3 shows that the overall model was significant, with a model F-value of 2.04 and a p-value of 0.0468, indicating that the model suitably fits the experimental data.

Table 3 ANOVA results

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	14.87	20	0.7434	2.04	0.0468	significant
A-UV- C dose	2.48	1	2.48	6.79	0.0152	
B-Gamma irradiation	1.96	1	1.96	5.37	0.0290	
C-Storage time	0.9025	1	0.9025	2.47	0.1286	
D-Temperature	0.2025	1	0.2025	0.5544	0.4635	
E-packaging material	0.3306	1	0.3306	0.9051	0.3505	
AB	0.4900	1	0.4900	1.34	0.2577	
AC	0.1600	1	0.1600	0.4380	0.5141	
AD	1.82	1	1.82	4.99	0.0347	
AE	0.0100	1	0.0100	0.0274	0.8699	
BC	0.2500	1	0.2500	0.6844	0.4159	
BD	0.2025	1	0.2025	0.5544	0.4635	
BE	0.9025	1	0.9025	2.47	0.1286	
CD	0.0025	1	0.0025	0.0068	0.9347	
CE	0.7225	1	0.7225	1.98	0.1719	
DE	0.3025	1	0.3025	0.8281	0.3715	
A^2	1.37	1	1.37	3.74	0.0644	
B^2	2.52	1	2.52	6.90	0.0145	
C^2	0.0038	1	0.0038	0.0104	0.9197	
D^2	0.3068	1	0.3068	0.8399	0.3682	

E^2	0.0668	1	0.0668	0.1829	0.6725	
Residual	9.13	25	0.3653			
Lack of Fit	6.54	20	0.3269	0.6303	0.7906	not significant
Pure Error	2.59	5	0.5187			
Cor Total	24.00	45				

Among the independent variables, UV-C dose (A) and gamma irradiation (B) showed significant linear effects with p-values of 0.0152 and 0.0290, respectively, while storage time (C), temperature (D), and packaging material (E) were not statistically significant at the 0.05 level. The interaction between UV-C dose and temperature (AD) was also significant ($p = 0.0347$), highlighting the importance of combined effects between these factors. Additionally, the quadratic term of gamma irradiation (B^2) showed a significant effect ($p = 0.0145$), suggesting a nonlinear influence on microbial reduction.

The lack of fit test was not significant ($p = 0.7906$), confirming that the model adequately describes the system without overfitting. Residual analysis further supported the model's validity, indicating that the experimental design and model could effectively be used to optimize microbial content reduction in *Agaricus bisporus*.

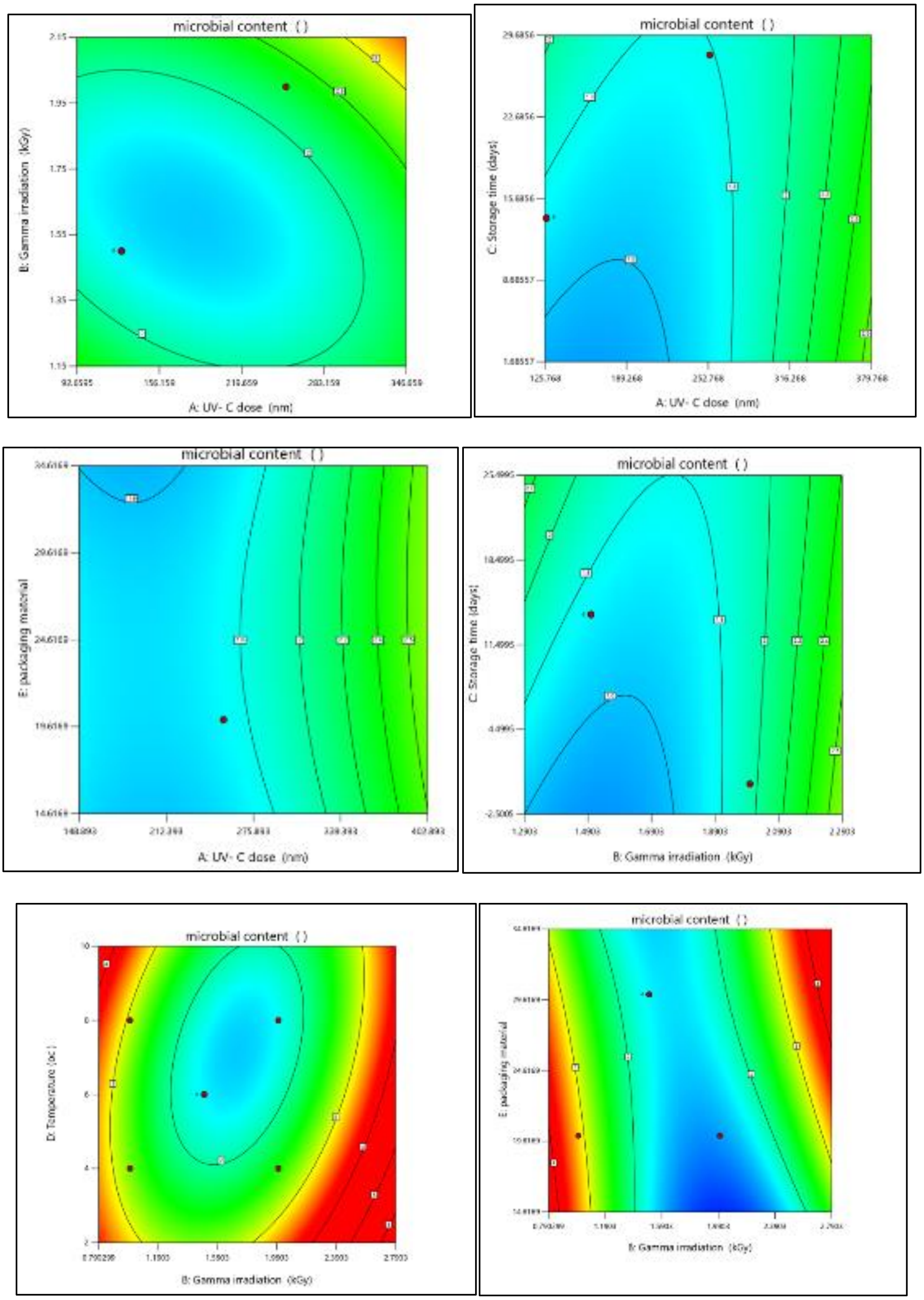
3.3. Model Fitting and Statistical Analysis

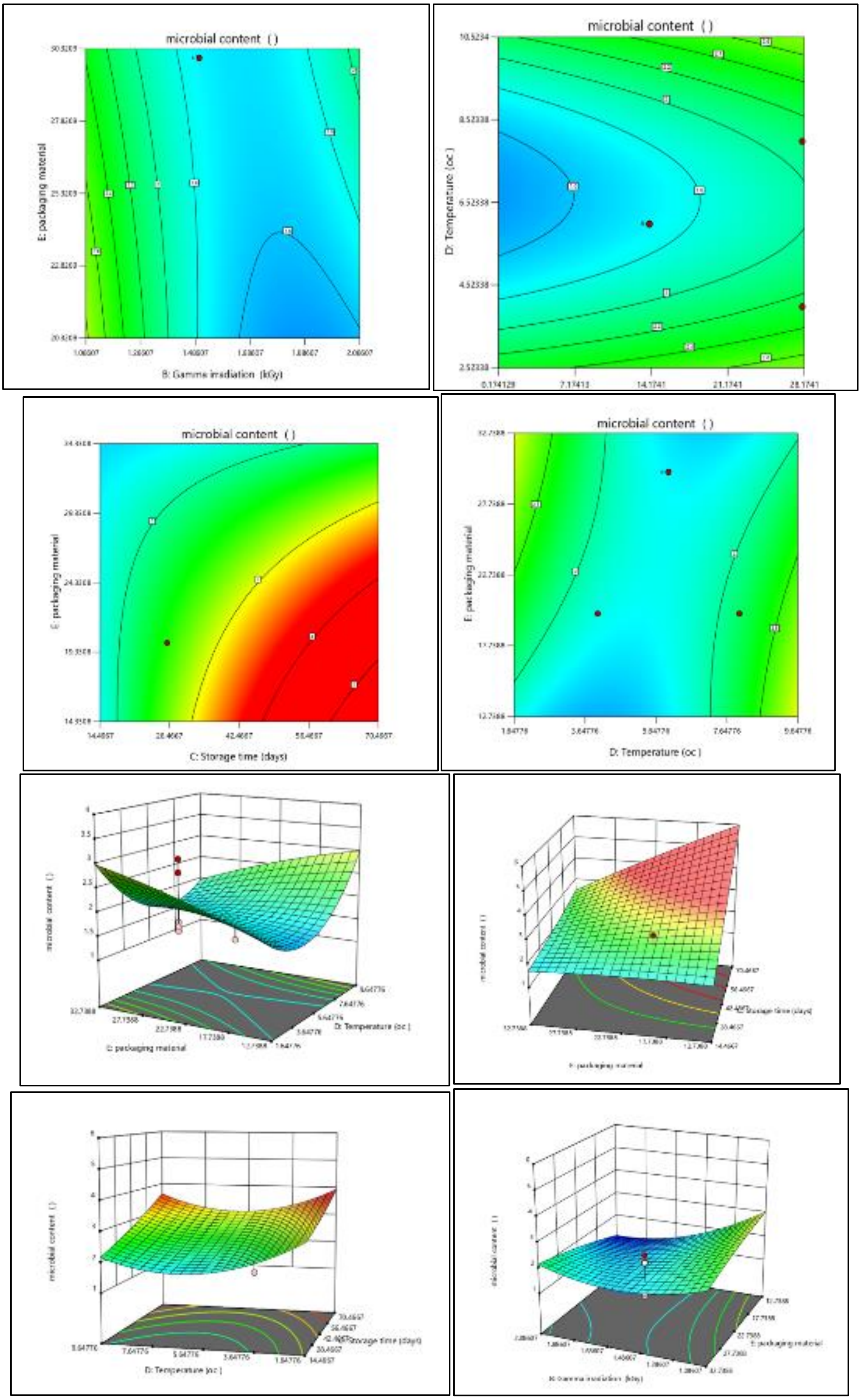
The polynomial regression equation for microbial content reduction, based on the five variables UV-C dose (A), gamma irradiation (B), storage time (C), temperature (D), and packaging material (E), is represented as follows:

$$Y=+1.73-0.3937A-0.3500B+0.2375C-0.1125D-0.1438E+0.3500AB-0.2000AC+0.6750AD+0.0500AE-0.2500BC-0.2250BD+0.4750BE+0.0250CD-0.4250CE-0.2750DE+0.3958A_2+0.5375B_2+0.0208C_2+0.1875D_2-0.0875 \quad \ldots\ldots\ldots\ldots Eq\ (2)$$

Where Y, is the microbial content (log CFU/g or % reduction), and A, B, C, D, and E represent the independent variables as mentioned above.

The statistical significance of this second-order quadratic model was evaluated using the F-value, p-value, and analysis of variance (ANOVA), as summarized in Table 5. The significance of each coefficient was determined by their p-values, where $p < 0.05$ indicates statistical significance. The analysis of variance (ANOVA) for the model showed that the Model F-value of 2.04 indicates the model is significant. There is only a 4.68% probability that such a large F-value could arise due to random noise, confirming the model's validity in explaining microbial content reduction. Model terms with p-values less than 0.05 are considered statistically significant. In this study, the significant factors affecting microbial reduction were UV-C dose (A), gamma irradiation (B), the interaction between UV-C dose and temperature (AD), and the quadratic term of gamma irradiation (B^2). Conversely, terms with p-values greater than 0.10 were considered not significant, and these could be candidates for model simplification to enhance model performance without sacrificing accuracy. The Lack of Fit test resulted in an F-value of 0.63, which is not significant relative to the pure error, with a 79.06% chance that a lack of fit this large could occur due to noise. This non-significant lack of fit confirms the model's adequacy, indicating it fits the experimental data well and is reliable for prediction within the studied range. The 2D contour plot is used to determine the interaction strength between the two variables according to the radius of the curved surface of the arc. The elliptic order of contour indicates that the interactions between corresponding variables were significant, while the circular order reveals non-significant interactions [Yan L et al., 2021].


microbial content ( )
B: Gamma irradiation (kGy)
A: UV- C dose (nm)
microbial content ( )
C: Storage time (days)
A: UV- C dose (nm)
microbial content ( )
E: packaging material
A: UV- C dose (nm)
microbial content ( )
C: Storage time (days)
B: Gamma irradiation (kGy)
microbial content ( )
D: Temperature (oc)
B: Gamma irradiation (kGy)
microbial content ( )
B: Gamma irradiation (kGy)


microbial content ( )
E: packaging material
B: Gamma irradiation (kGy)
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D: Temperature (oc)
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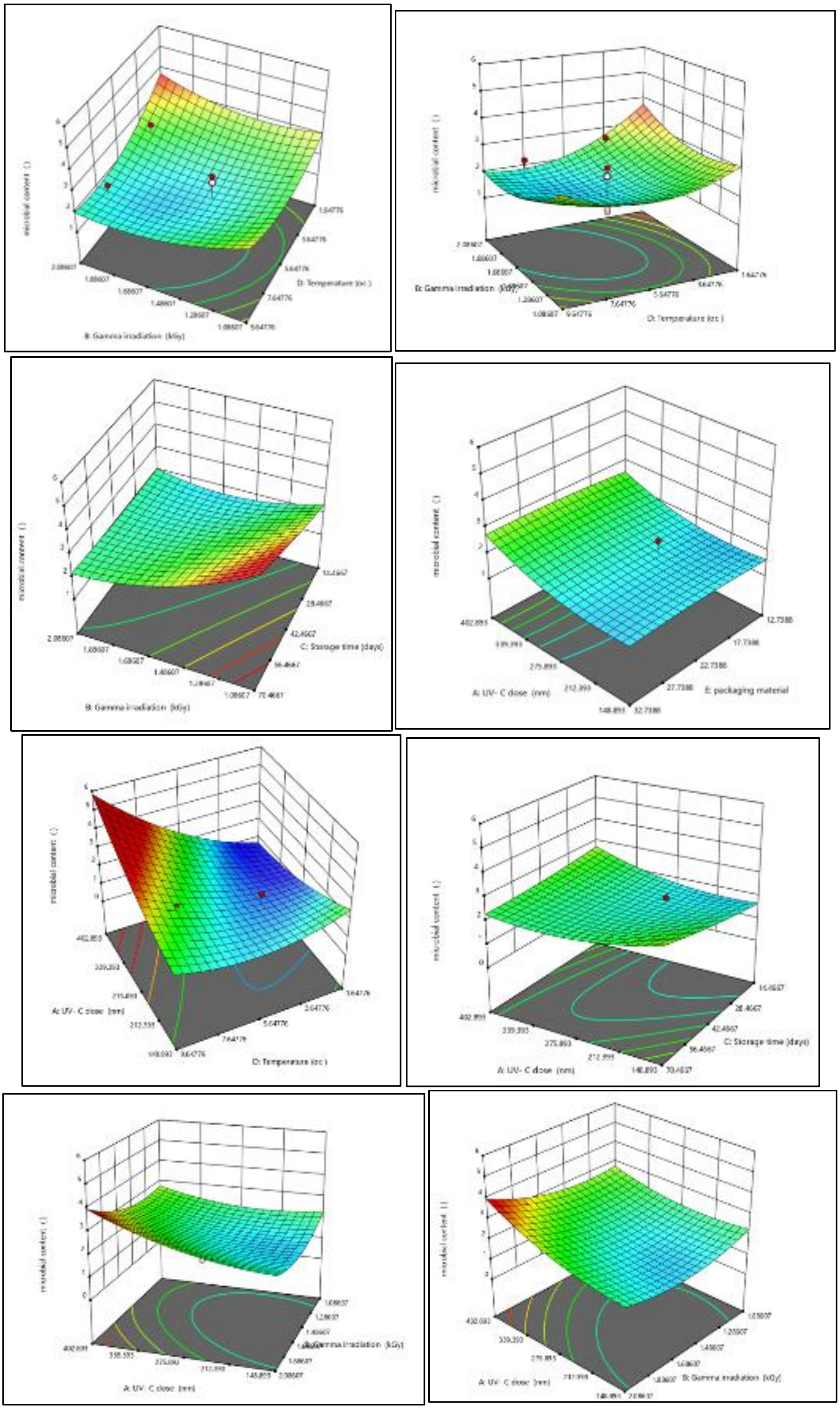
microbial content ( )
D: Temperature (oc)
C: Storage time (days)
E: packaging material


microbial content ( )
B: Gamma irradiation (kGy)
D: Temperature (oc)
C: Storage time (days)
A: UV- C dose (nm)
E: packaging material

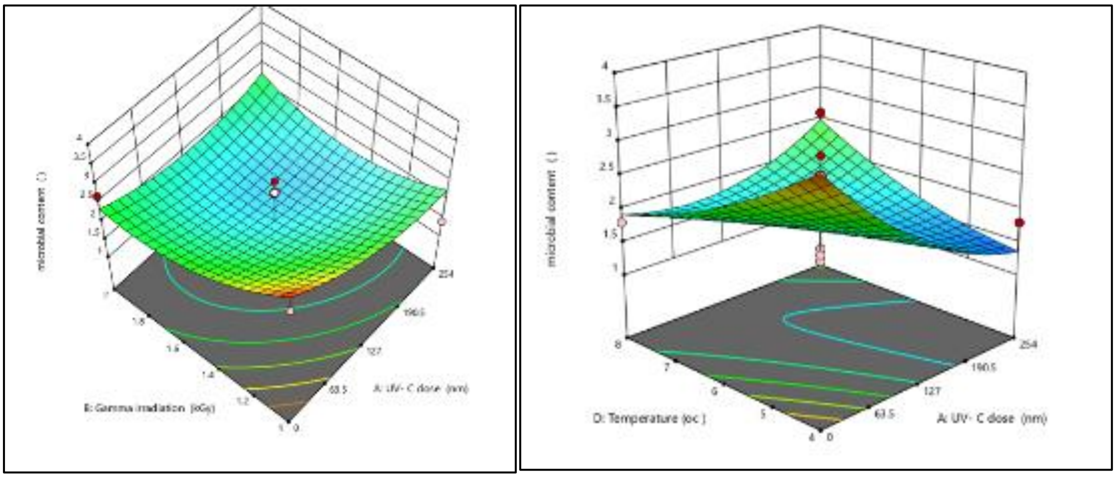


Figure 2 RSM Plots of BBD methods.

3.4. Validation of RSM Model for Microbial Content Reduction in *Agaricus bisporus*

To validate the reliability and predictive accuracy of the second-order polynomial model developed using the Box-Behnken Design (BBD), experimental trials were conducted at the optimized conditions identified through the response surface methodology (RSM). The optimal conditions determined for significant microbial content reduction were: UV-C dose at 254 nm, gamma irradiation at 2 kGy, storage time of 14 days, storage temperature at 4°C, and packaging material thickness of 40 µm. The model predicted a microbial reduction response of approximately 1.2 log CFU/g under these optimal conditions. Experimental validation was performed in triplicate, and the observed microbial count averaged 1.3 log CFU/g, closely matching the predicted value. The calculated percentage error between predicted and experimental results was 8.33%, confirming the model's high degree of accuracy. Additionally, 3D surface plots and 2D contour plots generated using Design Expert 13.0.3.1 visually confirmed the predicted optimal region and illustrated strong interactions between UV-C dose and gamma irradiation, as well as between other factors represented in the fig-2 . Residual analysis showed random distribution with no apparent trends, and the normal probability plots indicated that residuals followed a normal distribution, supporting model adequacy. The non-significant lack-of-fit result (p = 0.7906) further validated that the model did not overfit the data and was statistically sound. These outcomes collectively demonstrate that the RSM model is robust, precise, and suitable for practical application in optimizing post-harvest treatments to reduce microbial load in *Agaricus bisporus* mushrooms.

4. Discussion

Savita Kumari et al. (2020) reported that the mushrooms stored after 3 days of storage in ambient conditions, the deterioration of colour and texture was observed in mushrooms, and then began to deteriorate, accompanied by fungal growth. The magnitude of the change in colour depends on the time and temperature for storage. Fernandes et al. (2016) indicated that the application of gamma irradiation at a dose of 2 kGy appeared to have no significant impact on the chemical profiles of the tested mushrooms. Furthermore, their antioxidant properties exhibited enhanced bioactivity in the majority of instances, suggesting an additional benefit, and Pewlong et al. (2019) reported the decrease of the microbial load when mushrooms were irradiated with gamma irradiation of 2kGy. The study focused on reducing microbial content in *Agaricus bisporus* using a combination of optimization techniques. Initially, the One-Factor-At-a-Time (OFAT) approach was applied to identify significant individual parameters-UV-C dose, gamma irradiation, storage time, temperature, and packaging thickness-affecting microbial growth and mushroom spoilage. These variables were then optimized using a Box-Behnken Design (BBD), where the combined and interactive effects of the selected factors were evaluated through a second-order polynomial regression model. The model showed statistical significance ($F = 2.04$, $p = 0.0468$), with UV-C dose, gamma irradiation, their interaction with temperature, and the quadratic term of gamma irradiation having significant effects on microbial reduction. The adequacy of the model was confirmed by a non-significant lack of fit ($p = 0.7906$), indicating a reliable predictive capability.

5. Conclusion

This study effectively utilized statistical optimization through Response Surface Methodology (RSM) using Box-Behnken Design (BBD) to enhance the post-harvest preservation of Agaricus bisporus by minimizing microbial contamination. Initially, the One-Factor-At-a-Time (OFAT) approach identified UV-C dose, gamma irradiation, storage time, temperature, and packaging thickness as significant parameters influencing microbial spoilage. The BBD approach revealed that UV-C irradiation at 254 nm and gamma irradiation at 2 kGy were the most influential in reducing microbial load, aligning with findings reported by Jing Lei et al. (2018) and Khan et al. (2015). The interaction between UV-C and temperature also played a significant role ($p = 0.0347$), confirming the interactive effect on microbial reduction, which is consistent with previous optimization studies (Polo-Castellano et al., 2022). Experimental validation under optimized conditions (UV-C 254 nm, gamma irradiation 2 kGy, storage time 14 days, 4°C, and 40 µm packaging) produced microbial counts of 1.3 log CFU/g, closely matching the model's predicted value of 1.2 log CFU/g, with a minimal error of 8.33%. This outcome validates the predictive accuracy of the developed model and confirms its practical applicability for extending mushroom shelf life. The model's adequacy was supported by non-significant lack-of-fit ($p = 0.7906$) and good residual distribution. These findings align with reports that gamma irradiation not only reduces microbial content but also retains the antioxidant and nutritional quality of mushrooms (Fernandes et al., 2016; Pewlong et al., 2019). The study demonstrates that BBD-RSM modeling is a powerful tool for identifying and optimizing multiple interacting variables, offering a cost-effective and consumer-preferred alternative to conventional preservation techniques for maintaining the microbial quality of fresh mushrooms.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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