

Probiotic-Assisted Semi-Solid-State Fermentation of Amaranth Flour Using Response Surface Methodology for Enriched Bioactives

Mamadou Lamarana Souare^{1,*}, William Tchabo^{1,*},
Spéro Ulrich Koba Edikou¹, Gabriela Elena Bahrim², Mihaela Cotârleț²

¹Department of Technology and Control of Food Products, Higher Institute of Sciences
and Veterinary Medicine of Dalaba, PO Box .09 Dalaba, Guinea

²Faculty of Food Science and Engineering, “Dunărea de Jos” University
of Galați, 111 Domnească Street, 800201 Galați, Romania

*Corresponding author: williamtchabo@hotmail.com

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Abstract Semi-solid-state fermentation (S-SSF) of black amaranth flour, supplemented with okara (a by-product from soy beverage production), offers a sustainable route to generate value-added bioactives from underutilized crops. A three-factor, three-level central composite design was employed in this study for predicting the effects of fermentation time (40 - 80 h), okara concentration (3.30 - 11.70 %) and temperature (17 - 34°C) on total titratable acidity, soluble protein index, total polyphenols content, flavonoids content, antioxidant activity (ABTS), and antifungal activities against *Penicillium expansum* MIUG M11 strain and *Aspergillus niger* MIUG M5 strains, of the fermented products. Twenty experimental runs, including six center-point replicates, were performed with *Lactiplantibacillus pentosus* MIUG BL24 probiotic strain as the starter culture. For instance, titratable acidity, soluble protein index and antifungal activity increased with longer fermentation time and higher okara supplementation, whereas flavonoid and antioxidant activities showed antagonistic interactions between time and temperature due to phenolics biotransformation. The desirability function approach yielded an overall desirability of 0.785 at 48 h, 29.54 °C and 9.63 % (w/v) okara. Under these conditions, the model predicted titratable acidity of 9.90 mL NaOH 0.1N, soluble protein index of 0.74 mg/g DW, TPC of 3.52 mg GAE/g DW, TFC of 1.91 mg CE/g DW, antioxidant activity (ABTS scavenging assay) of 40.93 %, and antifungal activities of 70.20 % (against *P. expansum*) and 16.66% (against *A. niger*). Validation experiments at 48 h, 30 °C and 9.60 % okara confirmed the model's accuracy, with all measured responses falling within the 95 % confidence limits. These results demonstrate that combining nutrient enrichment from okara with controlled S-SSF conditions enhanced the functional properties of amaranth-based fermented products.

Keywords: *Lactiplantibacillus pentosus*, Probiotics, Black Amaranth, Semi-Solid-State Fermentation, Bioprocess Optimization, Promising Bioactivities

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1. Introduction

Grain amaranth (*Amaranthus* spp.), a member of the *Amaranthaceae* family, is an annual pseudocereal valued both as a cereal and forage crop [1]. Recognized as one of the ancient grains, it combines high yielding potential with superior nutritional quality. Amaranth seeds contain approximately 17 % protein with a balanced amino acid profile that surpasses many conventional cereals, making them a suitable complement to traditional grains [2]. Beyond proteins, its lipid content is around 7 %, with essential fatty acids accounting for more than 80 % [3].

Starch is the dominant component, while the seeds are also rich in dietary fiber, minerals such as calcium, iron, and zinc, and are naturally gluten free, an attribute of growing importance in the food industry.

Amaranth consumption has been linked to numerous health benefits, notably the reduction of cardiovascular risks through cholesterol regulation [4]. Its resilience and adaptability make it a promising crop for food security and climate change mitigation, particularly in arid and semi-arid regions of Africa [5]. Beyond enhancing regional diets, its increasing worldwide acceptance and substantial market value provide smallholder farmers with financial prospects via external trade [6]. Collectively, these nutritional, agronomic, and socio-

economic advantages underscore the importance of further valorizing amaranth in both local and international contexts.

Beyond its role as a staple food, amaranth has gained attention as a source of bio active compounds, including protein hydrolysates and peptides with antioxidant and health promoting properties [7,8]. Traditionally, enzymatic hydrolysis has been the main technique for releasing these bioactives from amaranth proteins. However, fermentation is emerging as a cost-effective, eco-friendly alternative. Microbial fermentation offers several advantages: the simultaneous action of diverse proteases, reduced antinutrient content, enhanced nutritional and sensory properties, and improved bioactivity [9,10]. Importantly, fermentation also enables the production of value-added metabolites such as organic acids, polyphenols, and flavonoids with applications in food preservation, pharmaceuticals, and biodegradable plastics.

Semi-solid-state fermentation (S-SSF) has been identified as a suitable strategy for valorizing underutilized crops like amaranth. The efficiency of S-SSF largely depends on the microbial strain and operating conditions, including temperature, pH, substrate composition, and fermentation time [11]. Statistical tools such as Response Surface Methodology (RSM) provide a powerful framework for modelling and optimizing these parameters by evaluating both individual and interactive effects on responses such as proteolysis, bioactive compound release, and acidification potential.

Semi solid state or dough like lactic fermentations have already been used by several groups to improve the functional profile of cereal and pseudocereal based foods. LAB fermentation of quinoa, buckwheat and other gluten free grains has been shown to enhance antioxidant activity, protein digestibility and shelf life through acidification, proteolysis and the release of bound bioactives [7,11,13]. Similar LAB driven strategies have been explored for the valorization of cereal and legume by products and composite flours, yielding ingredients with improved nutritional and technological properties [9,15]. More recently, our group reported that fermentation of black amaranth flour with selected probiotic strains can modulate its functional and bioactive characteristics [12]. These findings support the use of controlled lactic fermentation and response surface-based optimization as tools to tailor the properties of amaranth based ingredients.

In this context, the research aimed to optimize the production of bioactive compounds from Guinea black amaranth seeds using the selected probiotic strain *L. pentosus* MIUG BL24 strain, under semi-solid-state fermentation conditions. The fermentation process parameters (fermentation time, temperature, and okara supplementation) were investigated by using Response Surface Methodology (RSM), and optimized values were established. The present study leads to the sustainable valorization of amaranth and okara for biotransformation in high value bioproducts, improving functionality, safety, and food security through the integration of experimental design, mathematical modelling, and statistical analysis.

2. Materials and Methods

2.1. Raw Materials, Microbial Strains and Reagent

Black amaranth (*Amaranthus hypochondriacus*) seeds harvested in 2024 from the rural commune of Ditinn (Dalaba prefecture, Guinea) were portioned in batches of approximately 500g, vacuum packed and stored at room temperature in the dark until processing [12,13]. Before fermentation, the seeds were cleaned, dried and milled to obtain black amaranth flour, following Souare et al. [12]. Freeze dried okara was prepared according to Cotârleş et al. [14].

The probiotic strain *L. pentosus* MIUG BL24 belongs to the Microorganisms Collection of Dunărea de Jos University of Galați, Romania (acronym MIUG, www.mirri.org). It was selected based on previous screening in cereal and pseudo cereal fermentations [12]. The strain was maintained at -80°C in De Man, Rogosa, and Sharpe (MRS) broth (Merck Millipore Darmstadt, Germany) containing 20% (v/v) glycerol and reactivated twice on MRS agar before fermentation.

The fungal strains, *Penicillium expansum* MIUG M11 and *Aspergillus niger* MIUG M5, were provided by the MIUG Collection, were used as indicators for antifungal activity assay. The fungal strains were grown on Yeast Glucose Chloramphenicol Agar medium (YGC) (Merck, Darmstadt, Germany) slants at 25°C , for 5 days [15].

All analytical grade chemicals and solvents were purchased from Sigma Aldrich (USA), and distilled water was used throughout the experiments.

2.2. Semi-Solid-State Fermentation (S-SSF)

Fermentation was carried out under S-SSF conditions using amaranth flour as the main substrate. The amaranth flour supplemented with okara was moistened with distilled water, and the pH was adjusted according to the Plackett-Burman and Response Surface Methodology experimental design matrix.

The stock probiotic strain was stored in 40% (w/w) glycerol solution at -80°C . To reactivate the culture, 2 mL of stock culture was mixed with 9 mL of MRS broth (Merck, Darmstadt, Germany) and incubated for 48 h at 37°C in an incubator (Binder BF4000, Tuttlingen, Germany). A 10 μL aliquot of the reactivated culture was pipetted onto MRS agar medium (Merck, Darmstadt, Germany) enriched with 30 g/L CaCO_3 and streaked with a sterile loop to obtain single colonies [16]. To obtain the LAB inoculum, a single colony was transferred into 50 mL of MRS broth and incubated stationary for 48 h at 37°C . Then, using a spectrophotometer (Biochrom, Libra 22, Holliston, MA, USA), the optical density at 600 nm (OD600) was measured and adjusted to roughly 2.0, suggesting a cell concentration of 1×10^8 CFU/mL solution [17]. The fermentation media, consisting of the amaranth flour and okara mixture, were sterilized by autoclaving at 121°C for 15 min (Panasonic MLS-3871L, Bucharest, Romania), cooled and inoculated under aseptic conditions with *L. pentosus* MIUG BL24 in accordance with the design matrix, in an orbital shaker (Lab Companion SI-300, GMI, Minneapolis, MN, USA). Then, the fermented products (FPs) were freeze-dried at -42°C

and 0.10 mbar using a freeze-drier (Christ Alpha 1–4 LD plus, Germany).

2.3. Fermentation Design and Optimization

2.3.1. Screening of Significant Variables by Plackett–Burman (PB) Design

In order to enhance the usefulness of the amaranth seeds and okara, the independent variables for the PB experiment were selected based on the probiotic strains' growing conditions and nutritional requirements. Seven factors were analyzed: temperature and fermentation time, okara and amaranth flour concentrations, probiotic inoculum concentration, and agitation rate (Table 1).

Table 1. Coded and actual values of the independent variables in Box–Behnken Design

Variables	Levels	
	-1	+1
Temperature, °C (X1)	25.00	37.00
Time, h (X2)	48.00	72.00
Agitation, rpm (X3)	150.00	250.00
Black amaranth flour, g % (w/v) (X4)	5.00	10.00
Okara, g % (w/v) (X5)	5.00	15.00
pH (X6)	5.00	6.50
LAB inoculum, % (v/v) (X7)	2.00	5.00

Total titratable acidity (TTA), mL 0.1 N NaOH; soluble protein index (SPI), mg/g DW; total flavonoid content (TFC), mg catechin equivalents/g DW; antioxidant activity, ABTS (AA), %; antifungal activity against *P. expansum* and *A. niger*, %; and total polyphenols content (TPC), mg GAE/g DW, were the responses analyzed in the 12 runs of the Plackett–Burman experimental design.

2.3.2. Fermentation Parameters Optimization by Response Surface Methodology

A three-factor, three-level Response Surface Methodology (RSM) based on a Central Composite design was applied to investigate the influence of independent variables: time of fermentation (X_1), okara supplementation (X_2), and temperature (X_3) on bioactive characteristics of FPs [18]. The variables, their symbols, units, and coded levels are presented in Table 2.

Table 2. Codification and levels of variation of the independent variables

Independent variables, unit	Symbols	Coded levels					
		X	-α	-1	0	1	+α
Time (min)	X1	39.82	48.00	60.00	72.00	80.18	
Okara (% w/v)	X2	3.30	5.00	7.50	10.00	11.70	
Temperature (°C)	X3	16.59	33.41	25.00	30.00	33.41	

The second order polynomial regression model was used to express the relationship between the responses (Y, bioactive characteristics of FPs) and the independent variables:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i < j}^3 \beta_{ij} X_i X_j + \varepsilon$$

where β_0 is the intercept, β_i , β_{ii} , and β_{ij} are the linear, quadratic, and interaction coefficients, respectively, and ε

is the random error.

2.4. pH and Total Titratable Acidity (TTA)

The pH of fermented samples was measured as described by Nionelli et al. [19] using a calibrated pH meter (FiveEasy Plus FP20, Mettler Toledo, Greifensee, Switzerland). Total titratable acidity (TTA) was determined with an automatic titrator (TitroLine Easy, Schott Instruments, Mainz, Germany). Briefly, 10 g of the sample was homogenised with 90 mL of distilled water and titrated to a pH of 8.50. The TTA was expressed according to Nionelli et al. [19] as the volume of 0.1 N NaOH consumed in millilitres.

2.5. Antioxidant Activity (AA)

The antioxidant capacity was assessed by ABTS radical scavenging assays following the method of Souare et al. [12]. Firstly, the freeze-dried samples were solubilized in ultrapure water in a ratio of 1:10 (w/v). The mixture was vortexed for 5 sec and placed in an ultrasonic water bath (DU-32; ARGOLAB, Capri, Italy) for 30 min at 40°C. After centrifugation (Hettich Universal 320R, Tuttlingen, Germany) at 7000 rpm for 10 min at 4°C, the supernatant was collected and used for further analysis. In brief, 20 μ L of the sample was mixed with 1980 μ L of ABTS solution, vortexed for 1 min, and the absorbance was measured at 734 nm. The antioxidant activity (%) was calculated relative to the control using the following formula:

$$AA(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

2.6. Total Flavonoid Content (TFC)

TFC was determined by the aluminum chloride colourimetric method [12,20]. In brief, 250 μ L of the sample was mixed with 1.25 mL of distilled water and 75 μ L of 5% (w/v) sodium nitrite. After 5 min, 150 μ L of 10% (w/v) aluminum chloride was added. Following a 6 min incubation, 500 μ L of 1 M NaOH was added, and the final volume was adjusted to 3 mL with distilled water. Absorbance was read at 510 nm using a spectrophotometer (Libra S22 UV-VIS, Biochrom, Cambridge, UK). Results were expressed as mg catechin equivalents per g dry weight (mg CE/g DW) based on a standard curve.

2.7. Total Polyphenolic Content (TPC)

TPC was quantified by the Folin–Ciocalteu method [12,21]. A 200 μ L aliquot of sample was diluted with 7.9 mL of distilled water and reacted with 500 μ L of Folin–Ciocalteu reagent. After 5 min, 500 μ L of 20 % (w/v) sodium carbonate solution was added. The mixture was incubated in the dark for 60 min, and the absorbance was measured at 765 nm. TPC values were expressed as mg gallic acid equivalents per g dry weight (mg GAE/g DW).

2.8. Soluble Protein Index (SPI)

Soluble protein index was determined using the

Bradford method [12,22]. Fermented powders were extracted in water and the absorbance of the supernatant was measured at 595 nm. The soluble protein index was calculated from a bovine serum albumin (BSA) standard curve and expressed as mg BSA equivalents per g dry weight. Under these conditions, the assay reflects the soluble, dye binding fraction of proteins and peptides rather than the total protein content of the fermented matrix.

2.9. Antifungal Activity

The fungal spores produced on Potato Dextrose Agar (PDA) slants at 25°C for 5 days were harvested in sterile distilled water containing 0.01% Tween 80 and adjusted to 105 spores/mL using a haemocytometer. Additionally, 0.5 g of the FPs was added to 45 mL of Potato Dextrose Agar (PDA) medium (Oxoid, England) and poured into Petri dishes. After that, 10 µL of the spore suspension of indicator mould strains was added to the center of the solidified medium, and it was incubated for 96 hours at 25°C. The same conditions were used for the control sample, but FPs were not added. The percentage inhibition of mycelial growth was calculated as:

$$I(\%) = \frac{C - S}{C} \times 100$$

where C is the radial growth of the control (mm) and S is the radial growth of the fungus in the presence of FPs [14].

2.10. Statistical Analysis

Design-Expert software version 8.0.2.0 (Stat-Ease, USA) was used for the design of experiments, and one-way analysis of variance (ANOVA) was assessed considering a confidence interval of 95 %. The fitting of the models was evaluated based on the regression coefficient (R²), lack of fit, and p-value < 0.05. Two parallel experimental runs were performed, and the reported results are the average values of triplicate

measurements for each analysis.

3. Results and Discussion

3.1. Screening of the Significant Fermentation Parameters by PB Design

The independent variables for the PB experiment were chosen based on the growing conditions and nutritional needs of the probiotic strain in order to increase the value of the okara, a fiber rich residue left from the soybean products [23] and gluten-free amaranth flour [24]. Temperature and fermentation time, okara and amaranth flour concentrations, probiotic inoculum concentration, and agitation rate (Table 3) were analyzed.

Seven responses were taken into account such as total titratable acidity (TTA), mL 0.1 N NaOH, soluble protein index (SPI), mg/g DW, total flavonoid content (TFC), mg catechin equivalents/g DW, antioxidant activity, ABTS (AA), %, antifungal activity *P. expansum* and *A. niger*, %, and total polyphenols content (TPC), mg GAE/g DW. The concentration of the probiotic inoculum, the addition of okara to the fermentation medium, and the temperature and time of the fermentation process all had different effects on the assessed responses, according to the ANOVA results shown in Table 4. In summary, the parameters X₅ (okara concentration, %), X₂ (fermentation time, h), X₁ (fermentation temperature, °C), and C (agitation, rpm) significantly influenced the analyzed responses (p < 0.05). In particular, the total polyphenol content was positively influenced by six of the seven parameters that were examined. Additionally, the TPC generated mathematical model had a regression coefficient R² of 0.973 and was significant (p = 0.0051) (Table 4). Our results are in accordance with Păcularu et al. [25] who reported that temperature and fermentation time, okara concentration, LAB strains inoculum, and dough yield influenced the sourdough based on chickpea, quinoa, and buckwheat flours under SSF.

Table 3. PB experimental design matrix and the responses

Runs	Independent variables							Responses						
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	A	B	C	D	E	F	G
1	37.00	48.00	250.00	10.00	15.00	5.00	2.00	10.93	0.68	0.99	62.89	55.56	62.50	3.10
2	37.00	72.00	150.00	5.00	5.00	6.50	2.00	6.20	0.73	0.96	62.53	36.11	50.00	2.71
3	37.00	48.00	250.00	10.00	5.00	6.50	5.00	7.92	0.70	1.10	57.26	52.78	56.25	2.96
4	25.00	72.00	250.00	5.00	15.00	6.50	5.00	10.23	0.68	0.90	68.74	0.00	56.25	3.05
5	37.00	72.00	250.00	5.00	5.00	5.00	5.00	6.60	0.72	0.96	65.27	41.67	50.00	2.51
6	25.00	72.00	150.00	10.00	15.00	5.00	5.00	11.52	0.64	1.02	63.56	55.56	62.50	3.84
7	25.00	72.00	250.00	10.00	5.00	5.00	2.00	7.54	0.64	1.02	73.19	55.56	68.75	2.76
8	25.00	48.00	250.00	5.00	15.00	6.50	2.00	10.85	0.58	1.06	71.21	52.78	62.50	2.72
9	37.00	48.00	150.00	5.00	15.00	5.00	5.00	10.10	0.64	1.05	64.55	55.56	100.00	3.70
10	37.00	72.00	150.00	10.00	15.00	6.50	2.00	10.01	0.66	0.97	63.74	61.11	56.25	3.33
11	25.00	48.00	150.00	5.00	5.00	5.00	2.00	3.97	0.86	1.08	68.20	52.78	50.00	3.38
12	25.00	48.00	150.00	10.00	5.00	6.50	5.00	5.90	0.80	1.17	73.14	52.78	50.00	3.71

X₁ - temperature of fermentation, °C, X₂ - time of fermentation, h, X₃ - agitation, rpm, X₄ - amaranth flour concentration, % (w/v), X₅ - okara concentration, % (w/v), X₆ - pH, X₇ - probiotic inoculum, % (v/v); A - total titratable acidity (TTA), mL 0.1 N NaOH, B - soluble protein index (SPI), mg/g DW, C - total flavonoid content (TFC), mg catechin equivalents/g DW, D - antioxidant activity, ABTS (AA), %, E/F - antifungal activity *P. expansum* and *A. niger*, %, G - total polyphenols content (TPC), mg GAE/g DW.

Table 4. ANOVA analysis of variance for the significant responses

Source	SS	df	Mean Square	F - value	p-value	R2	
Total titratable acidity, mL NaOH 0.1N							
Model	65.335	9.000	7.259	30.234	0.0324	0.993	significant
X3 - Agitation, rpm	4.723	1.000	4.723	19.671	0.047		
X5 - Okara concentration, %	45.943	1.000	45.943	191.344	0.0052		
Soluble protein index, mg/g DW							
Model	0.063	8.000	0.008	10.650	0.039	0.966	significant
X5 - Okara concentration, %	0.024	1.000	0.024	32.120	0.011		
Total flavonoid content, mg CE/g DW							
Model	0.056	6.000	0.009	20.330	0.002	0.961	significant
X2 - Time of fermentation, h	0.031	1.000	0.031	68.180	0.001		
X3 - Agitation, rpm	0.010	1.000	0.010	21.330	0.006		
Antioxidant activity (ABTS), %							
Model	237.170	7.000	33.880	8.320	0.029	0.935	significant
X1 - Temperature, °C	145.540	1.000	145.540	35.760	0.004		
X3 - Agitation, rpm	33.880	1.000	33.880	8.320	0.045		
Total polyphenols content, mg GAE/g DW							
Model	2.11	7	0.3	21.29	0.0051	0.973	significant
X1 - Temperature, °C	0.11	1	0.11	7.81	0.0491		
X2 - Time of fermentation, h	0.15	1	0.15	10.82	0.0302		
X3 - Agitation, rpm	1.07	1	1.07	75.49	0.001		
X4 - Amaranth flour, %	0.22	1	0.22	15.39	0.0172		
X5 - Okara concentration, %	0.24	1	0.24	17.13	0.0144		
X7 - Probiotic inoculum, %	0.26	1	0.26	18.41	0.0127		

Table 5. Experimental design matrix and their influence on the bioactive characteristics of the FPs

Runs	Independent variables			Responses						
	Time (min)	Okara (%)	Temperature (°C)	A	B	C	D	E	F	G
	X ₁	X ₂	X ₃							
1	72.00	10.00	30.00	12.08	0.74	1.95	39.48	72.65	91.00	3.17
2	48.00	5.00	30.00	7.98	0.76	2.36	37.40	40.00	91.13	2.80
3	60.00	7.50	33.41	10.01	0.78	2.13	35.28	60.00	91.67	3.48
4	72.00	10.00	20.00	13.08	0.76	2.31	40.64	46.7	90.3	3.09
5	39.82	7.50	25.00	9.12	0.79	2.11	41.93	66.67	90.83	2.54
6	72.00	5.00	20.00	7.78	0.67	1.99	40.25	64.08	92.08	2.71
7	48.00	5.00	20.00	6.04	0.77	1.69	37.73	66.67	91.67	2.88
8	48.00	10.00	20.00	9.49	0.81	2.21	41.70	42.96	91.25	3.14
9	60.00	3.30	25.00	6.23	0.65	1.77	38.88	60.00	91.67	2.50
10	72.00	5.00	30.00	10.10	0.70	1.89	40.08	40.04	91.67	2.53
11	60.00	7.50	25.00	10.86	0.73	2.09	37.94	66.67	91.40	3.50
12	60.00	7.50	25.00	10.37	0.77	1.77	40.82	66.67	91.67	3.68
13	60.00	7.50	16.59	6.73	0.86	1.99	35.83	53.33	91.67	3.49
14	48.00	10.00	30.00	10.46	0.74	2.19	40.68	72.25	91.67	3.2
15	60.00	7.50	25.00	11.09	0.71	1.56	38.93	66.67	91.25	3.08
16	60.00	11.70	25.00	13.00	0.76	2.15	42.81	66.67	91.10	3.30
17	60.00	7.50	25.00	10.54	0.73	1.90	38.00	53.33	91.67	3.94
18	60.00	7.50	25.00	9.90	0.73	1.59	37.25	60.00	91.25	3.24
19	60.00	7.50	25.00	9.80	0.71	1.71	38.51	66.67	91.67	3.32
20	80.18	7.50	25.00	12.14	0.64	1.90	40.98	66.67	91.03	2.42

A – TTA, mL 0.1 N NaOH, B – SPI mg/g DW, C – TFC, mg CE/g DW, D – antioxidant activity (ABTS), %, E/F – antifungal activity *A. niger* and *P. expansum*, IR %, G – TPC, mg GAE/g DW.

3.2. Design of the Experiments and Responses Evaluation

Considering the previously discussed PB analysis results, three significant parameters were chosen for the RSM based on their statistical relevance ($p < 0.05$), as follows: X₁ (fermentation time, h), X₂ (okara concentration, %) and X₃ (fermentation temperature, °C). The other parameters of the fermentation, such as probiotic inoculum concentration, pH, amaranth flour

concentration and agitation rate, were maintained constant. The experimental results obtained from the 20 runs (including 6 replicates at the centre points to evaluate pure error) were performed according to the Central Composite Design (Table 5). The results obtained ranged from: titratable acidity (6.04 – 13.08 mL NaOH), soluble protein index (0.64 – 0.86 mg/g DW), total flavonoid content (1.56 – 2.36 mg CE/g DW), ABTS (35.28 – 42.81 %), antifungal activity against *P. expansum* MIUG M11 (40.00 – 72.65 %), and *A. niger* MIUG M5 (90.30 –

92.08 %), and total polyphenolic content (2.42 – 3.94 mg GAE/g DW). These findings revealed that time of fermentation, okara concentration and temperature have a synergistic influence on the various responses. Therefore, data from Table 4 were fitted to a variety of models using regression analysis and analysis of variance (ANOVA) to determine which model fitted the responses (TTA, SPI, TFC, ABTS, antifungal activity, and TPC). To assess the model's adequacy, the coefficient of determination (R^2) was also calculated [26]. The adequacy of model summary output from the ANOVA (Tables 3 and 4) indicated that the quadratic model was highly significant and sufficient to represent the actual relationship between the responses and significant parameters with low p-value, (< 0.0001 for TTA, < 0.001 for SPI and antifungal activity against *A. niger* MIUG M5 strain, < 0.05 for TFC, ABTS, antifungal activity against *P. expansum* MIUG M11 strain, and TPC). The p-value was used as a tool to determine the significance of every coefficient (Table 3 and Table 5). The smaller the p-value, the more significant the corresponding coefficient. Values of p less than 0.05 indicate that the model terms are significant. The lack-of-fit test was not significant for any response ($p > 0.05$), indicating no evidence of systematic lack of fit and supporting the reliability of the model predictions.

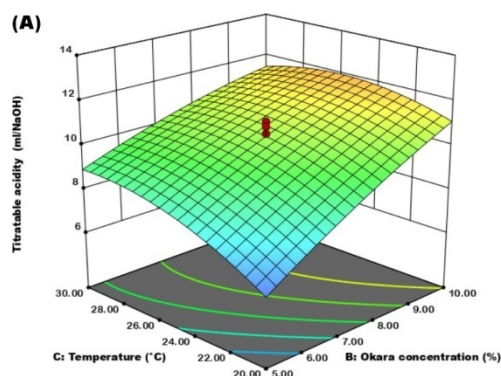
3.2.1. Titratable Acidity of Fps in Correlation with Variation of the Independent Variables

The quadratic model calculated for titratable acidity, as shown in Table 6, was found to be significant ($p < 0.0001$). The linear effect of independent variables was significant. The quadratic model calculated for titratable acidity was significant ($p < 0.0001$), and the linear effects of time of fermentation (X_1), okara concentration (X_2), and temperature (X_3) were also significant. Each of these variables independently increased titratable acidity, which can be explained by the microbial metabolic pathways and substrate utilization mechanisms involved in fermentation. Extended fermentation time allowed lactic acid bacteria (LAB) to sustain glycolytic activity for longer periods, converting available carbohydrates into pyruvate and subsequently into lactic acid through lactate dehydrogenase, which led to progressive pH reduction and increased titratable acidity [27,28]. Temperature influenced the kinetics of these pathways by activating enzymatic reactions that drive glycolysis and lactic acid fermentation, thereby accelerating the metabolic flux toward lactic acid production; within the optimal range, LAB multiplied more rapidly, exhibited higher enzymatic efficiency, and produced more acid, confirming the central role of incubation temperature in the acidification process [29]. Okara supplementation further enhanced this process by modifying substrate availability, since it is rich in proteins, fibers, and soluble carbohydrates that provided fermentable sugars and nitrogen sources supporting microbial growth and metabolism; carbohydrates were directed into glycolysis and lactic acid synthesis, while amino acids promoted biosynthetic pathways that improved bacterial viability, resulting in higher lactic acid accumulation and shorter lag phases compared with non-fortified products [30]. The interaction between okara concentration and temperature (X_2 X_3) was also

significant ($p < 0.05$, Fig. 1-A), and this synergistic effect occurred from the combination of enhanced nutrient supply and optimal enzymatic activity, which together accelerated carbohydrate metabolism and acid production, producing a rapid and pronounced increase in titratable acidity and highlighting how substrate enrichment and environmental conditions jointly regulate the metabolic dynamics amaranth flour fermentation.

3.2.2. Soluble Protein Index Variability of FPs According to Independent Variables Effect

The quadratic model calculated for the soluble protein index (SPI) and the linear effects of time (X_1), okara concentration (X_2), and temperature (X_3) (Table 6). Okara supplementation had the most direct impact because it is naturally rich in protein, with concentrations ranging from 25 % to 29 % [14]. Increasing okara level provides more substrate for the proteolytic system of *L. pentosus* and favors the formation of soluble peptides and amino acids that react with the Bradford reagent. The higher SPI at elevated okara levels is therefore consistent with a greater release of soluble protein fragments from the matrix. These soluble peptides and free amino acids may also support functional properties such as antioxidant activity [31]. Fermentation time contributed by promoting the proteolytic activity of the starter culture. As incubation progresses, amaranth and okara proteins are progressively cleaved into lower molecular weight fragments that are more easily solubilized in water, which increases SPI. This reflects changes in protein structure and solubility rather than an increase in the total amount of protein. Temperature had a double role. Moderate temperatures support microbial growth and enzyme activity, which favors proteolysis and solubilization. Very high temperatures or very long incubations can destabilize the protein network and promote aggregation or denaturation of proteins and may also impair starter activity, which tends to reduce SPI [32]. The interaction between time and temperature highlights this balance. At long fermentation times combined with elevated temperatures, structural changes and possible loss of enzyme activity can outweigh the release of new soluble fragments. In these conditions the proportion of material that remains soluble and dye binding in the aqueous extract decreases and SPI is reduced. Overall, okara fortification, fermentation duration and incubation temperature influence SPI through interconnected mechanisms of substrate supply, proteolysis and structural modification of the protein matrix, without demonstrating a net increase in total protein content of the fermented powders.



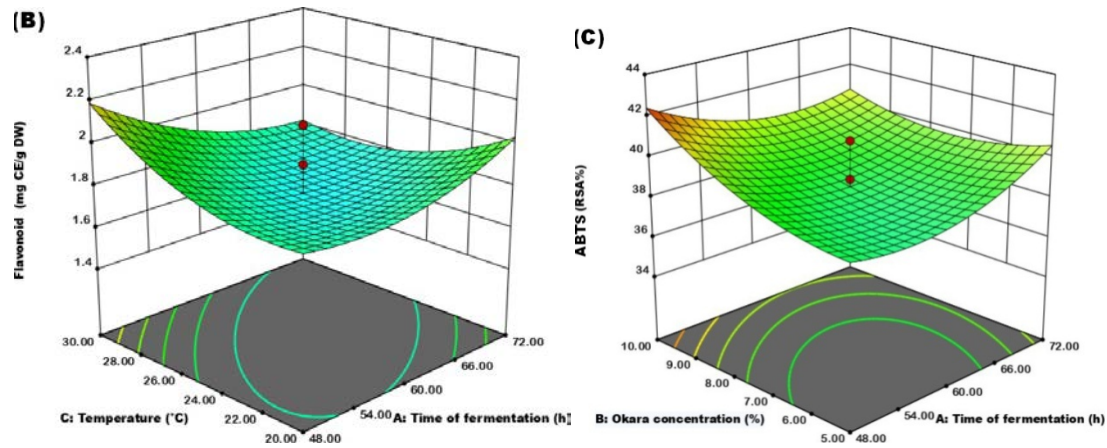


Figure 1. Response surface of the effect of temperature and okara concentration on the TTA (A), time of fermentation and temperature on the TFC (B), and okara concentration and time of fermentation on the antioxidant potential (ABTS) (C)

Table 6. Analysis of variance of the second-order polynomial model for the response variables explaining the effect of the independent variables on the TTA, SPI, TPC, and ABTS

Source	TTA			SPI			TFC			AA		
	CE	SS	p-Value	CE	SS	p-Value	CE	SS	p-Value	CE	SS	p-Value
Model	10.42	76.79	< 0.0001***	0.73	0.05	0.0008**	1.77	0.78	0.0263*	38.56	68.39	0.002**
X1	1.04	14.66	< 0.0001***	-0.03	0.02	0.0004**	-0.05	0.03	0.275	0.10	0.13	0.7232
X2	1.80	44.30	< 0.0001***	0.02	0.01	0.0035*	0.10	0.14	0.035*	1.00	13.64	0.0042*
X3	0.71	6.96	0.001**	-0.02	0.00	0.0377*	0.03	0.01	0.4629	-0.26	0.95	0.3532
X1X2	0.17	0.23	0.4286	0.01	0.00	0.1746	0.00	0.00	0.942	-0.93	6.99	0.0247*
X1X3	-0.20	0.32	0.3541	0.01	0.00	0.3022	-0.14	0.15	0.0307*	0.00	0.00	0.9964
X2X3	-0.54	2.30	0.0255*	-0.01	0.00	0.1302	-0.12	0.11	0.053	-0.21	0.35	0.5667
X12	0.10	0.15	0.5192	0.00	0.00	0.4524	0.09	0.13	0.0413*	1.11	17.67	0.0018*
X22	-0.26	0.95	0.1225	-0.01	0.00	0.1301	0.08	0.09	0.0771	0.89	11.44	0.007**
X32	-0.70	7.01	0.001**	0.03	0.01	0.0005**	0.11	0.18	0.0186*	-0.98	13.85	0.004**

X₁ – time of fermentation, h, X₂ – Okara concentration, % (w/v), X₃ – Temperature, °C; CE (coefficient estimate), SS (sum of squares), Asterisks (*, ** and ***) indicate 5 %, 0.1 % and 0.01 % significant levels, respectively.

3.2.3. Polyphenols Content Variation

The significant linear effect of okara (X₂) on total polyphenol content in the response surface model reflects its multifaceted role in enhancing the release of phenolic compounds during S-SSF. Okara is rich in bound phenolics, especially isoflavone glycosides such as daidzein and genistein. These compounds are often conjugated to sugars or structural components of the plant matrix, limiting their bioavailability [33]. During microbial fermentation, enzymes like β -glucosidase, feruloyl esterase, and cellulase are produced by fermentative microbes such as *Bacillus subtilis*, *Lactobacillus plantarum*, or *Saccharomyces cerevisiae*. These enzymes catalyze the hydrolysis of glycosidic and ester bonds, releasing phenolic compounds into more extractable and bioactive aglycone forms [34,35]. This enzymatic activity contributes to a measurable increase in total phenolic content and antioxidant activity, which has been consistently reported in fermented okara systems [33,34]. In addition to serving as a source of phenolic precursors, okara also functions as a prebiotic substrate. Its high content of dietary fiber, residual carbohydrates, and protein supports microbial colonization and growth during fermentation, which in turn promotes the biosynthesis of key hydrolytic enzymes [36]. The moisture retaining and porous structure of okara further enhances microbial activity by creating favorable conditions for oxygen transfer and metabolic flux. These structural and nutritional characteristics contribute

synergistically to the biotransformation and release of bound phenolics from the plant matrix. The observed increase in polyphenol content with increasing okara levels is therefore consistent with these mechanisms. The fermentation process not only releases native phenolic compounds present in okara but also enables the enzymatic hydrolysis of phenolics bound within the co-fermented substrate. This dual action supports the significant positive effect of okara in the predictive model and underscores its functional role in improving the nutraceutical value of fermented amaranth products.

3.2.4. Flavonoid Content Variation

The quadratic model for total flavonoid content (TFC) was significant ($p < 0.05$, Table 6). Regression analysis further revealed that the linear effect of okara concentration (X₂) was a significant factor, indicating a direct and positive correlation between okara supplementation and the TFC measured in the fermented amaranth matrix. This finding is consistent with the known composition of raw okara, which retains numerous soybean isoflavones such as malonyl glucosides, isoflavone glucosides and aglycones [37]. Because LAB do not synthesize flavonoids, the observed increase in TFC reflects the physical incorporation of these plant-derived phenolics into the fermentation substrate during fortification. Furthermore, fermentation modulates both the extractability and chemical form of these flavonoids. Isoflavones in unfermented okara are predominantly

glycosylated and therefore less soluble. During fermentation, microbial β -glucosidase enzymes cleave the glycosidic bonds of isoflavone glucosides such as daidzin and genistin, yielding their more soluble aglycone counterparts daidzein and genistein [37]. This bioconversion enhances the solubility of isoflavones and improves their recovery in colourimetric assays. Studies on probiotic fermented okara beverages show that the concentration of glycoside isoflavones decreases markedly during the first day of fermentation, while aglycone content and total flavonoid levels increase; the overall phenolic content also rises compared with unfermented controls [38]. These observations indicate that fermentation not only releases flavonoids from the okara matrix but also converts them into forms that are more readily detected. In addition, the interaction between fermentation time (X_1) and temperature (X_3) was significant ($p < 0.05$, Fig. 1 B) and displayed an antagonistic pattern. Flavonoid compounds are thermolabile; during thermal processing, they can undergo heterocyclic ring cleavage, hydroxylation, dehydroxylation and deglycosidation reactions that diminish their integrity [39]. Consequently, short fermentation at moderately elevated temperatures may favor rapid enzymatic release of flavonoids before degradation becomes substantial, whereas prolonged exposure at high temperature accelerates oxidation and enzymatic breakdown, resulting in a net decrease in measurable TFC. Because raw okara retains endogenous enzymes that may persist during fermentation, extended high temperature incubations could further amplify degradation compared with heat-treated substrates.

3.2.5. Antioxidant Potential Variability

The model describing the ABTS radical scavenging activity was significant, and regression analysis showed that okara concentration exerted a particularly strong positive effect. This relationship occurs because soy residue contains various phenolic acids and isoflavones that confer inherent antioxidant potential [37]. Fermentation further amplifies this effect; during solid state fermentation of okara with *Bacillus subtilis* TISTR001 strain, the ABTS radical scavenging activities of both free and bound phenolic fractions increased, a change attributed to structural modifications mediated by microbial enzymes [33]. These enzymes release phenolic compounds from the insoluble matrix, enhancing their accessibility and reactivity in the ABTS assay. The interaction between fermentation time and okara exhibited an antagonistic pattern. High okara levels coupled with shorter fermentation times favor rapid enzymatic release of antioxidants before degradation processes become dominant, whereas prolonged incubation, especially at elevated temperatures, can lead to oxidative and enzymatic degradation of phenolic compounds. Polyhydroxy flavonols and related phenolics are known to be heat sensitive; thermal processing can cause ring cleavage, dehydroxylation and other reactions that diminish their radical scavenging capacity [39]. Consequently, while okara enrichment enhances ABTS activity through its phenolic content and enzyme mediated release, careful control of fermentation time and temperature is essential to maximize antioxidant retention. The relationship between ABTS values and the measured

TPC and TFC also suggests that non phenolic components contribute to radical scavenging in the fermented powders. During S-SSF, proteolysis of amaranth and okara proteins can release peptides with antioxidant activity, including sequences able to donate electrons or protons and to chelate transition metals. In parallel, the metabolism of *L. pentosus* can generate low molecular weight antioxidants such as glutathione and other thiol containing compounds, together with redox active intermediates formed during carbohydrate and protein transformations. Because the ABTS assay responds to a wide range of electron donating species, it reflects the combined effect of phenolic compounds, bioactive peptides and bacterial metabolites. Overall, these contributions may account for the relatively high ABTS values observed despite more modest changes in TPC and TFC and underline the need for future targeted profiling of peptide based and microbial antioxidants.

3.2.6. Antifungal Activity Against *P. expansum* Strain

The second order model for antifungal activity against *P. expansum* was statistically significant ($p < 0.05$, Table 7). Analysis of the model terms revealed a highly significant synergistic interaction ($p < 0.0001$, Table 7, Fig. 2-A) between Okara concentration (X_2) and temperature (X_3). The response surface plot showed that maximum antifungal activity occurred at higher okara levels combined with lower incubation temperatures, especially when fermentation time was extended. This interaction can be explained by the metabolic behavior of *Lactiplantibacillus pentosus* MIUG BL24 under semi solid-state fermentation. Lactic acid bacteria are known to produce a wide range of antifungal compounds, including lactic and acetic acids, phenyllactic acid, hydrogen peroxide, carbon dioxide, fatty acids, small peptides and volatile metabolites, many of which exhibit strong inhibitory activity against *Penicillium spp* [40,41,42]. At mild temperatures *L. pentosus* can maintain prolonged metabolic activity, convert available sugars into organic acids and direct part of its metabolism towards the synthesis of secondary metabolites such as phenyllactic acid and phenolic derivatives, while limiting thermal degradation of these compounds [41,42]. Reduced temperatures also slow *P. expansum* growth directly, which enhances the net antifungal effect [40]. Prolonged fermentation allows these inhibitory metabolites to accumulate in the matrix. Okara plays a central role by acting as a phenolic rich co substrate. It supplies precursors such as ferulic and p coumaric acids that can be transformed by *L. pentosus* enzymatic activity into more bioactive antifungal forms [38,43]. These hydroxycinnamic and phenyllactic derivatives can disrupt fungal membranes or interfere with key enzymatic functions. In parallel, the proteolytic system of *L. pentosus* can release small peptides from amaranth and okara proteins, and some of these peptides may further contribute to antifungal activity. Additionally, prolonged enzymatic hydrolysis releases bound antioxidants and small molecules that can synergies with LAB derived organic acids to inhibit fungal proliferation. At the microbiological level, inhibition of *P. expansum* in this system is therefore likely to result from a combined action of low pH, accumulation of organic acids and production of strain dependent antifungal metabolites by *L. pentosus* MIUG BL24. The sensitivity of *P.*

expansum to changes in time, temperature and okara level reflects not only the acidification profile but also the dynamic balance between release, biotransformation and possible degradation of these antifungal metabolites. Since individual antifungal compounds were not quantified in this study, this mechanistic interpretation remains tentative and should be confirmed in future work by targeted profiling of organic acids, phenolic derivatives and peptides under different okara–temperature combinations.

Table 7. Analysis of variance of the second-order polynomial model for the response variables explaining the effect of the independent variables on the antifungal activity and TPC

Source	Antifungal activity <i>P. expansum</i>			Antifungal activity <i>A. niger</i>			TPC		
	CE	SS	p-Value	CE	SS	p-Value	CE	SS	p-Value
Model	63.501490.84	0.0033*		91.56	2.84	0.0004**	3.46	2.95	0.0067*
X1	-0.59	4.73	0.6728	-0.02	0.01	0.6732	-	0.08	0.263
X2	1.86	47.07	0.2	-0.24	0.76	0.0005**	0.25	0.85	0.0036*
X3	1.86	47.12	0.1998	0.01	0.00	0.7924	-	0.05	0.4527
X1X2	2.04	33.25	0.2756	-0.31	0.78	0.0004**	0.03	0.01	0.7263
X1X3	-1.29	13.37	0.4813	0.05	0.02	0.4293	-	0.02	0.8657
X2X3	12.041159.61	<0.0001***		0.26	0.53	0.0016*	0.10	0.07	0.2898
X12	0.06	0.06	0.9623	-0.21	0.66	0.0008**	-	0.34	1.68 0.0003**
X22	-1.11	17.90	0.4172	-0.05	0.04	0.2696	-	0.16	0.36 0.0344*
X32	-3.47	173.69	0.0249*	0.05	0.03	0.3234	-	0.05	0.04 0.4165

X₁ – time of fermentation, h, X₂ – Okara concentration, % (w/v), X₃ – Temperature, °C; CE (coefficient estimate), SS (sum of squares), Asterisks (*, ** and ***) indicate 5 %, 0.1 % and 0.01 % significant levels, respectively.

3.2.7. Antifungal Activity Against *A. niger* Strain

The second-order model describing antifungal activity against *A. niger* was statistically significant ($p < 0.001$, Table 7). Regression analysis confirmed that the linear effect of okara concentration (X₂) was significant ($p < 0.001$, Table 7). This positive correlation indicates that higher levels of okara contribute to higher antifungal efficacy. A possible explanation is that okara provides phenolic acids, isoflavones, and dietary fibers that serve as substrates for LAB. During fermentation, LAB can transform these compounds into bioactive antifungal metabolites such as phenyl lactic acid and hydroxycinnamic acid derivatives, which are known to inhibit *Aspergillus* spp. by disrupting cell membranes and interfering with mycotoxin biosynthesis [38,41].

Thus, the phenolic fraction of okara appears to be particularly important as a precursor pool for microbial bioconversion. Two significant interaction effects were also detected. The interaction between fermentation time (X₁) and okara concentration (X₂) was antagonistic ($p < 0.001$, Fig. 2-B). This suggests that while okara provides essential precursors, extended fermentation may lead to the degradation or microbial consumption of active compounds, thereby reducing antifungal potency. At

shorter fermentation times, by contrast, higher LAB activity promotes the release and conversion of phenolics, resulting in stronger inhibitory effects. Moreover, the interaction between okara concentration (X₂) and temperature (X₃) was significant and antagonistic ($p < 0.05$, Fig. 2-C). High okara levels combined with low incubation temperatures reduced antifungal activity, likely because low temperatures preserve bioactives but limit LAB enzymatic activity, slowing the conversion of okara-bound phenolics into antifungal derivatives. Conversely, higher temperatures can accelerate microbial transformation but may also risk degrading thermolabile metabolites if not carefully controlled. In addition, the response surface describing antifungal activity as a function of fermentation time and temperature (Fig. 2-C) exhibits a depressed valley, with lower inhibition at intermediate conditions and higher inhibition toward the extremes of the factor space. This pattern likely reflects several overlapping processes rather than a single pathway. At relatively short times and moderate temperatures, *L. pentosus* MI-UG BL24 produces lactic and acetic acids and lowers pH rapidly, creating conditions that are already unfavorable to *A. niger* growth. At longer fermentation times and at either lower or higher temperatures, prolonged microbial activity and matrix modification favor the buildup of a broader spectrum of antifungal metabolites. These may include phenyllactic acid, hydroxycinnamic acid derivatives and small antifungal peptides derived from amaranth and okara proteins, in addition to organic acids, as reported for related LAB systems [38,41]. These compounds can disrupt fungal membranes, interfere with energy metabolism and reduce mycelial expansion, acting together with low pH to enhance inhibition. At intermediate conditions, acidification or metabolite accumulation may be insufficient, which is consistent with the valley observed in the response surface. Since individual antifungal compounds were not quantified in this study, this interpretation remains a mechanistic hypothesis that should be tested in future work by targeted profiling of organic acids, phenolic derivatives and peptides at different time–temperature combinations. Taken together the antifungal results show a consistently high inhibitory effect on *A. niger* (above 90 %) and a lower, more variable inhibition of *P. expansum* (about 40–72 %). This difference probably reflects the combined action of organic acids, pH reduction and matrix associated phenolic compounds and peptides produced by *L. pentosus* MIUG BL24, acting on fungi with distinct stress tolerance. Differences in cell wall structure, membrane composition and weak acid resistance between *Aspergillus* and *Penicillium* species can modulate their sensitivity to lactic fermentation derived inhibitors, making *P. expansum* intrinsically more tolerant under the conditions created in the amaranth–okara matrix. These findings suggest that the optimized S-SSF process is particularly suitable to control *A. niger*, while further work aimed at identifying the key antifungal metabolites and adjusting process conditions would be needed to maximize efficacy against *P. expansum*.

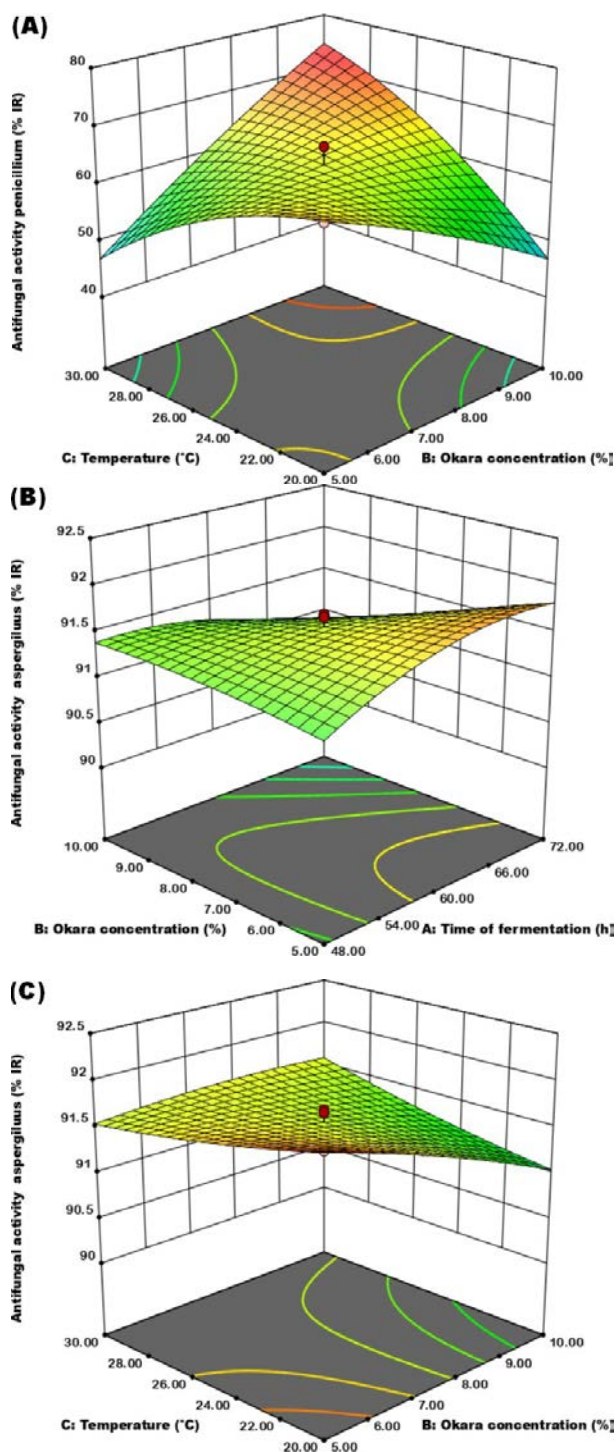


Figure 2. Response surface of the effect of temperature and okara concentration on the antifungal activity against *P. expansum* (A), okara concentration and time of fermentation on the antifungal activity against *A. niger* (B) and temperature and time of fermentation on the antifungal activity against *A. niger* (C)

3.3. Optimal Semi-Solid-State Fermentation conditions for increasing the bioactive characteristics of FPs

The optimal conditions for the S-SSF process for obtaining an FP with improved bioactive characteristics were determined by maximizing the responses, such as titratable acidity, soluble protein index, polyphenols, flavonoids, antioxidant activity, and antifungal potential.

The model predicted that the highest overall desirability of 0.785 would be achieved with a fermentation time of 48 h, a temperature of 29.54 °C, and an okara supplementation of 9.63 % (w/v). To validate the model's predictive accuracy, verification experiments were conducted under slightly modified, more practical conditions (48 h, 30 °C, 9.60% (w/v) okara). The experimental results confirmed the model's robustness, showing excellent agreement with the predictions. The titratable acidity measured at a value of 10.13 ± 0.40 mL NaOH 0.1N closely matched the predicted value of 9.90 mL NaOH 0.1N. Similarly, the soluble protein index was 0.80 ± 0.05 mg/g DW against a prediction of 0.74 mg/g DW. The bioactive content also aligned closely with the model: TPC reached 3.43 ± 0.03 mg GAE/g DW (predicted: 3.35 mg GAE/g DW), TFC were 2.02 ± 0.13 mg catechin/g DW (predicted: 1.91 mg catechin/g DW), and antioxidant activity (ABTS) was 41.81 ± 0.95 % (predicted: 40.93 %). Furthermore, the critical antifungal activities were successfully validated, with inhibition of *P. expansum* MIUG M11 at 69.44 ± 0.69 % (predicted: 70.20 %) and *A. niger* MIUG M5 at 15.94 ± 1.25 % (predicted: 16.66 %). For all responses, the experimental values fell within the model's 95 % confidence interval, conclusively demonstrating its suitability for predicting the optimized biotechnological conditions for obtaining a probiotic FP with improved bioactive characteristics, starting from amaranth flour and okara, by S-SSF bioprocessing. Taken together, the screening step, the RSM models and the multi response optimization show that S-SSF performance can be tuned in a rational way by adjusting fermentation time, temperature and the level of a phenolic and fiber rich co-substrate such as okara. In this sense, the present work goes beyond a simple optimization of a single formulation and proposes a methodological framework that links process variables to multiple technological and bioactive targets. The sequential strategy that combines screening, central composite design and desirability functions provides a structured way to balance acidification, protein enrichment, antioxidant capacity and antifungal activity. This approach can be adapted to other plant-based matrices by redefining the factor ranges and the responses of interest. Although the quantitative optimum determined here is specific to black amaranth flour supplemented with freeze dried okara and fermented with *L. pentosus* MIUG BL24, the underlying mechanisms are expected to be relevant to other pseudocereals and agro-industrial by products with comparable composition. In such substrates, a co-ingredient rich in phenolics, proteins and dietary fiber can play a role similar to okara. It can act as a precursor pool for LAB mediated release and biotransformation of bioactive compounds as well as for the formulation of antifungal metabolites. Differences in starch structure, protein and fiber profiles, buffering capacity and endogenous enzyme activity will shift the numerical values of the optimal conditions. However, similar interactions between temperature, co-substrate level and fermentation time are likely to occur. The present S-SSF system should therefore be viewed as a case study that illustrates a broader process design approach. This approach can be transposed to other cereal and by product matrices, provided that it is supported by matrix specific modelling and validation. Furthermore, it should be noted

that this study did not assess the sensory profile or consumer acceptance of the fermented amaranth okara powders. Food products formulated with these fermented powders were also not evaluated. This is a major limitation, despite the improvements observed in nutritional and bioactive properties. The higher titratable acidity and the expected buildup of lactic and other organic acids suggest that the incorporation of the optimized amaranth okara powders may lead to more pronounced sour notes and a lactic cereal type aroma. Protein hydrolysis and the contribution of okara may also modify mouthfeel and reduce some beany or astringent notes compared with raw okara. These points remain speculative, since no sensory tests and no instrumental analysis of volatile compounds or taste active peptides were carried out. Future work should therefore combine the present S-SSF approach with a systematic sensory programme. This should include descriptive profiling and consumer acceptance studies on relevant product prototypes, together with targeted analysis of organic acids and key flavor compounds, in order to link the compositional changes reported here with perceived flavor, aroma and texture.

4. Conclusions

Response surface methodology enabled the identification of S-SSF parameters that simultaneously contribute to maximizing the functional properties of fermented amaranth flour. The optimized conditions, as follows: 48 h of fermentation at 30 °C with 9.60 % (w/v) okara supplementation, produced high total titratable acidity and robust inhibition of *P. expansum* and *A. niger* while preserving soluble protein index and enhancing total polyphenols and total flavonoids content. Significant interactions between time of fermentation, okara concentration and temperature highlight the need to balance substrate availability with enzymatic activity, such as extended fermentation at moderate temperature promotes LAB metabolism and antifungal metabolite accumulation, whereas prolonged time of fermentation can negatively affect the phenolic compounds. Using okara as a co-substrate not only supplies fermentable sugars and nitrogen but also provides phenolic precursors that *L. pentosus* converts into bioactive compounds, thereby enriching antioxidant and antifungal properties. Validation of the model underscores its reliability for predicting multi-response outcomes, suggesting that the identified biotechnological conditions can be extended for scaleup applications. The results offer valuable perspectives for okara and amaranth flour valorization through S-SSF to develop nutritionally enhanced products. Future research should explore sensory attributes, shelf stability, and the synergistic use of other microbial strains or substrates to further improve the functional and commercial potential of black amaranth-based fermented foods. These perspectives are challenges in improving life quality, food safety and food security, in the global context of the healthy food resources.

Credit Author Statement

Mamadou Lamarana Souare: Conceptualization, data curation, formal analysis, investigation, methodology, writing - original draft; **William Tchabo:** Methodology, original draft, editing; **Spéro Ulrich Koba Edikou:** Visualization, writing- review and editing; **Gabriela Elena Bahrim:** project administration, funding acquisition, Conceptualization; **Mihaela Cotârleț:** Supervision, review and editing.

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Ethical Approval

The conducted research is not related to either human or animal use.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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