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ENHANCEMENT IN IRON AND FOLATE BY OPTIMIZING FERMENTATION OF BARNYARD MILLET BY *LACTOBACILLUS PLANTARUM* USING RESPONSE SURFACE METHODOLOGY (RSM)

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ABSTRACT

The present study was conducted to optimize the fermentation process of barnyard millet by probiotic strain (*Lactobacillus plantarum)* to enhance the iron and folate content using Response Surface Methodology (RSM). *Lactobacillus plantarum* can produce folate; utilization of such kind of folate-producing bacteria is a novel strategy to amplify natural folate levels in foods. The purpose of the study was to examine the proper utilization of underutilized cereal by enhancement of its nutrients using an optimized fermentation technique. Barnyard millet was treated with probiotic fermentation at temperature (30^0C-50^0C), time (4-30 hours.) and pH (3-7). The effect of these fermentation treatments was studied with regards to iron and folate contents, antioxidant activity [Total phenolic content (TPC), DPPH(2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity and Ferric reducing antioxidant power (FRAP)], calcium, zinc, and anti-nutritional factor (tannin) content of barnyard millet with the central composite design. The optimized combination established for fermenting the barnyard millet was at the temperature of 40^0C, pH 5 and time duration of 38.86 hours. The statistical analysis showed that pH was the most important factor, had a highly significant influence ($p < 0.01$) on all the dependent variables. Mineral content and antioxidant activity significantly ($p < 0.05$) correlated with pH and temperature. The experimental results showed that at optimum condition, TPC content increased up to 76.64%, DPPH free radical scavenging activity was 72.57%, FRAP was 45.83% ,iron content was reached up to 51.7%, calcium 79.51%, zinc increased up to 56.52% and folate content was 79% respectively.

Keywords: Barnyard millet, folate, iron, Lactobacillus, fermentation Response Surface Methodology

INTRODUCTION

Lactic acid bacteria (LAB) play an immense role in food fermentation. They have been used for hundreds of years in food fermentation (Axelsson and Ahrné,, 2000). LAB are often used for the fermentation of various food groups i.e., cereals, pulses, fruits, vegetables, milk and milk products, fish and meat (Park *et al.,* 2016). They not only enhance the nutritional quality, texture, and flavor of foods but also prevent the spoilage of food caused by other microorganisms and simultaneously increasing the shelf-life of food (Velioglu *et al.,* 1998). Lactic acid bacteria refer to a heterogeneous group of Gram-positive bacteria that are naturally present in many foods and have therapeutic properties. They are anaerobic-aerotolerant homofermentative and able to produce lactic acid (Liu *et al.,* 2010). Lactobacillus is possibly the most preponderant genus (Elagoz *et al.,* 1996). *L. plantarum* is the most adaptable species for fermentation of food., as it is a non-gas producing lactic acid microorganism and has "generally recognized as safe" (GRAS) status. *L. plantarum* is widely used in the processing of raw foods and has been entitled to qualified presumption of safety (QPS) status, too (Muhammad *et al.,* 2019). *L. plantarum* has gained importance as a probiotic as it significantly reduces the symptoms of gut disorders such as inflammatory bowel syndrome and metabolic disorders. It also plays a beneficial role in dyslipidemia, hypercholesterolemia, NIDDM (non-insulin dependent diabetes mellitus) and brain health.

Millets are small-seeded grains belonging to the family of Poaceae. They are widely accepted as the sixth most important grain. Millets and cereals are included in the staple diet for people belonging to lower socioeconomic status. Barnyard millet (*Echinochloa esculenta*) is a minor type of millet cultivated in India, Japan and China in the world and used as a food and animal fodder. The barnyard millet belongs to the crops of the fastest-growing group as it can be produced within 45 days from the sowing time and grows in a semi-arid region. Barnyard millet is nutritionally adequate, with high iron content and low glycemic index (Amadou *et al.,* 2013). Fermentation of barnyard millet by *L. plantarum* has a positive influence on nutritional content as well as antioxidant activity of millet and also increases the shelf life of millet-based probiotic products. Many researchers showed that fermentation by probiotic bacteria can be significantly used to enhance the nutritional quality of millet grain increasing protein content and its digestibility. Millets are also a rich source of essential nutrients and minerals like fiber, phosphorous, magnesium, and iron. Fermentation of millet also improves the availability of nutrients by decreasing antinutritional factors like tannin and phytate

content (Dayakar *et al.,* 2017).

Barnyard millet contains up to 4.8% fat, which surpasses that in wheat, rice, kodo, sorghum and proso (Verma *et al.,* 2015). It contains 13.6% fiber, which is higher than that in rice (1%), wheat (2%), quinoa (7%), kodo (5.2%) sorghum (2%) and pearl millet (2.3%). It also has more minerals (3.7%) than rice, wheat, finger millet, pearl millet, Kodo, little millet and sorghum (Verma *et al.,* 2015). There is a requirement to develop more value-added products and processes to enable the optimal use of barnyard millet as a source of nutrients and as a replacement of rice and wheat (Kumar *et al.,* 2016). In this regard, the conversion of barnyard millet into convenience fermented food products with probiotic bacterial species could add value to non-dairy food and help product diversification for both agricultural and non-dairy foods. In view of above, the study was planned to encourage utilization of coarse cereals in our day-to-day foods through exploitation of cost-effective fermentation processing technique. The objective of this study was to optimize the fermentation process of barnyard millet by the lactic acid-producing probiotic bacteria i.e *L. plantarum* using a statistical software tool, namely response surface methodology (RSM). Validation of actual value was performed to obtain the best combination of pH, temperature, and time of fermentation in terms of antioxidant activity, mineral content and especially for enhancement of iron and folate content. RSM was used earlier to optimize the various parameters in the production of food products with the desired quality (Castro *et al.,* 2000; Inyang and Zakari 2008).

MATERIALS AND METHODS

Procurement of sample

Barnyard millet was procured from Beej bhandaar kendra, Alopibaag market, Prayagraj India. The millet was washed, dried under fans, ground to powder form and stored in a refrigerator. All chemicals used for chemical analysis and extraction were of AR (Analytical Reagent) grade.

Estimation of proximate composition and mineral content

The barnyard millet samples were analyzed for moisture, protein, fat, carbohydrate, ash and crude fiber by methods specified in AOAC (1995). Estimation of mineral content i.e calcium, zinc, iron were done by atomic absorption spectrophotometer (Ranganna, 2005) and folate was estimated by microbiological assay (Trienzyme method, Rahman *et al.,* 2015; Singh and Yadav, 2020).

Antinutritional and antioxidant activity

Estimation of Total Phenolic Content (TPC)

Total phenolic content was determined with the folin ciocalteu method (Velioglu *et al.,* 1998). About 0.2 ml of extract aliquot was added to 1.5 ml of folin-ciocalteu reagent (Sigma-Aldrich) and allowed to stand at room temperature for 5 minutes. Further, 1.5 ml of sodium carbonate solution (6%) was added into the mixture. Absorbance was measured using a spectrophotometer at 725 nm after incubating the sample to stand for 1½ hours at room temperature. Results were expressed as gallic acid equivalent in mg/100 g Dry Weight (DW).

DPPH Free Radical Scavenging Assay

The free radical scavenging activity of the extracts was estimated by measuring the decrease in absorbance of ethanolic DPPH (2,2-diphenyl-1-picrylhydrazyl) solution at 517 nm in the presence of the extract (Krings, 2001). The initial concentration of DPPH was 0.1 mm and the reading was taken after allowing the solution to stand for 30 min. The antioxidant activity was expressed as follows:

$$\text{DPPH \%} = \frac{\text{control absorbance - sample absorbance}}{\text{control absorbance}} \text{x100}$$

Determination of Reducing Power

Ferric reducing antioxidant power (FRAP) of the millet extract was determined as described by Sreeramulu *et al.* ,(2009). Samples which have reduction potential react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form a ferric ferrous complex that has an absorption maximum at 700 nm.

Estimation of Tannin content

The tannin content was determined by the method of Ranganna (2005). Powdered sample (0.5 g) was boiled in 75 ml of water for 30 minutes. The sample was centrifuged at 2000 rpm for 20 minutes and the supernatant was collected. Folin Denis reagent (Sigma-Aldrich) and sodium carbonate were added to the sample extract, the solution was diluted to 100ml with water and absorbance was measured at 700 nm after 30 minutes.

Fermentation Treatment

Preparation of Inoculum

Probiotic microorganism strain *Lactobacillus plantarum* MTCC 1407 was obtained from MTCC Chandigarh. For inoculum, the glycerol stock culture tube of *Lactobacillus plantarum* was transferred in a 250 ml Erlenmeyer flask having 100 ml MRS broth. The broth was incubated for cell growth at 37 ^{0}C. Growth was observed using a spectrophotometer (590 nm). Incubation was carried out till the cell density reached 0.600 value corresponding to 9.00 log CFU/ml (colony forming unit per millilitre), a

Table.1 Experimental variables for fermentation, their coded and uncoded (actual) values

Variables	**Code**	**Coded level**				
		-1.414	-1	0	+1	+1.414
Ph	X_1	1.64	3	5	7	8.36
Temperature (°C)	X_2	23.18	30	40	50	56.82
Time (hrs.)	X_3	-4.86	4	8.17	30	38.86

Table.2 Proximate composition of Barnyard millet Echinochloa esculanta

Parameters(%)	**Echinochloaesculanta**
Moisture	8.9±0.20
Fat	1.92±0.06
Protein	6.06±0.15
Fiber	2.25±0.21
Ash	4.0±0.10
Carbohydrate	76.86±0.60

The values are mean±SD (n=3)

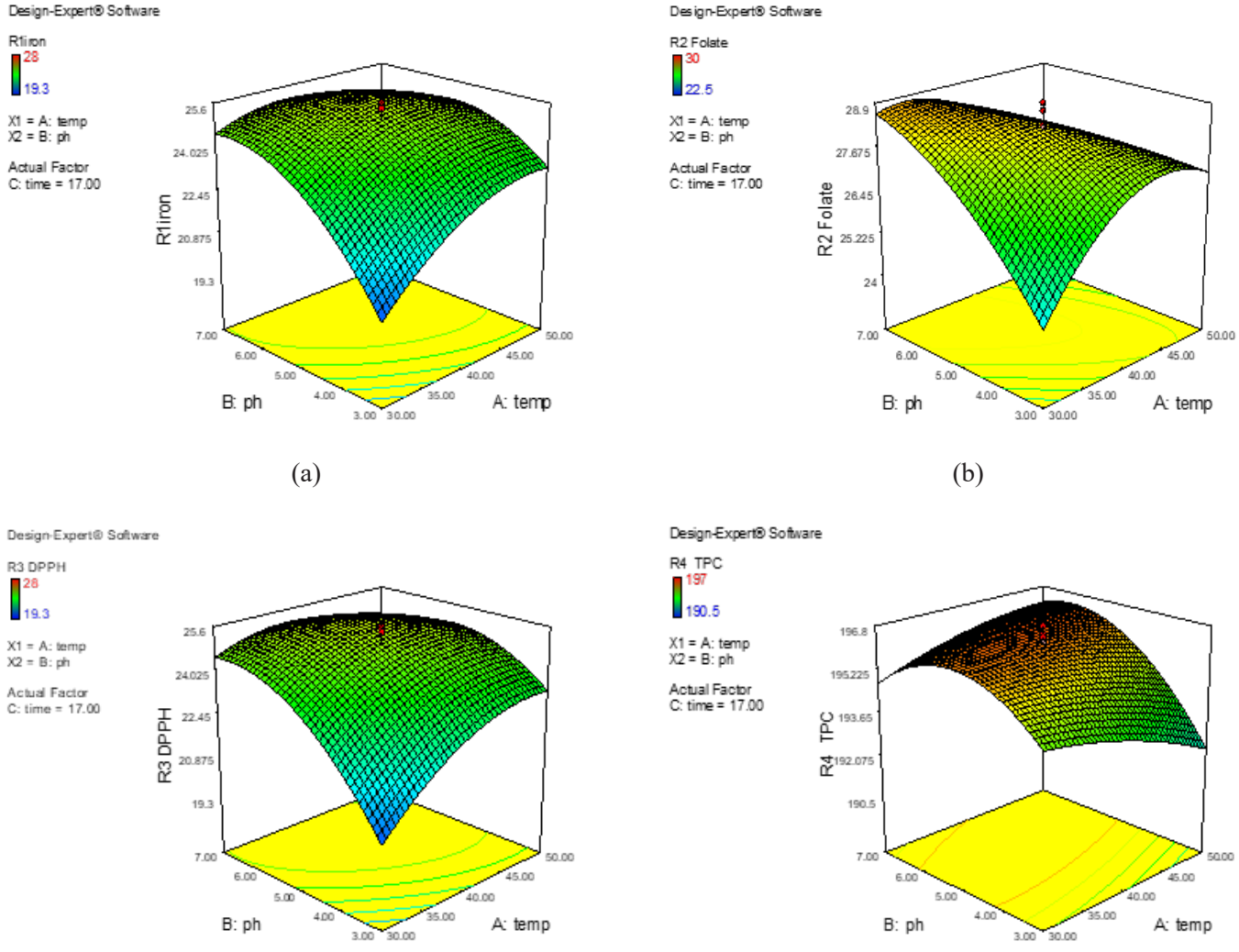


Fig.(1.) Interactive effect of temperature and pH on (a) iron content (b) folate content (c) DPPH % (d) TPC content

Table 3. Experimental design matrix for fermentation with responses Tannin, TPC, DPPH%, FRAP, calcium, zinc, folate, iron content and their analysis of variance

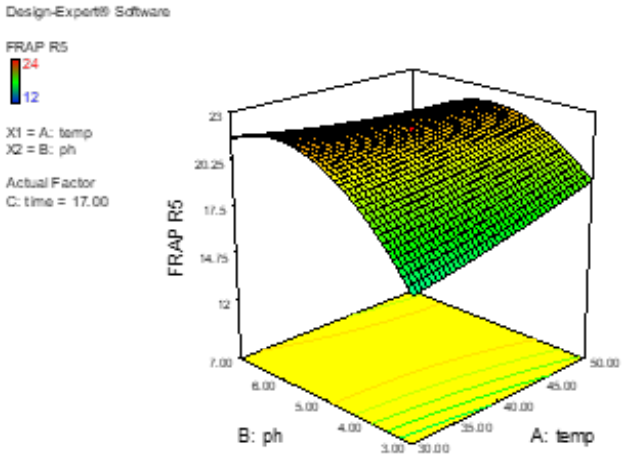
Exp. No	Coded real values*	Tannin content (mg TAE/g)	TPC (mg GAE/100g)	DPPH%	FRAP (mmol Fe(II)Eq/g)	Iron content (mg/ 100g)	Ca	Zinc	Folate(µg/ 100g)		
	X1 Ph	X2 Temp	X3 Time						(mg/100g)		
1	-1.414 (1.64)	0(40)	0(17)	0.029	190.5	23	12	19.3	25	8.7	26
2	-1(3)	-1(30)	-1(4)	0.02	192.5	25	15	19.4	22.5	4.5	22.5
3	-1(3)	-1(30)	+1(30)	0.02	195.9	26.9	17	21.9	27	11	26
4	-1(3)	+1(50)	-1(4)	0.04	191	28.1	18	22.4	27	9.5	26
5	-1(3)	+1(50)	+1(30)	0.03	193	29.5	19	25.5	26	8.3	27.5
6	0(5)	-1.414 (23.18)	0(17)	0.006	195.9	32	22	22	18.5	7.5	24.9
7	0(5)	0(40)	-1.414 (-4.86)	0.036	195	30	18.5	26	26.9	8.8	26.1
8	0(5)	0(40)	+1.414 (38.86)	0.038	197	35	24	28	29	12	30
9	0(5)	+1.414 (56.82)	0(17)	0.06	195.5	33	23	23.7	24.9	10.5	24
10	+1(7)	-1(30)	+1(30)	0.03	194.2	33	22	24.9	24	10.2	28.9
11	+1(7)	-1(30)	-1(4)	0.03	194.5	31	20	24.3	22	7.9	28.6
12	+1(7)	+1(50)	+1(30)	0.07	194	30.1	20	23.5	25.5	7.9	25.5
13	+1(7)	+1(50)	-1(4)	0.049	194.5	29.2	19	24.8	25	5.2	26
14	+1.414 (8.36)	0(40)	0(17)	0.06	194.2	27	17	24.3	28	9.7	26.9
15	0(5)	0(40)	0(17)	0.04	196.4	32	21	25.4	26.8	10.5	27.7
16	0(5)	0(40)	0(17)	0.04	196.5	32	22	25.5	27.5	10.5	28
17	0(5)	0(40)	0(17)	0.05	196.8	32	22	25.6	27.9	11	28.9
18	0(5)	0(40)	0(17)	0.04	195.7	32	22	25.5	27	11.6	28.7
19	0(5)	0 (40)	0(17)	0.05	196.3	33	21	24.9	27.5	10.9	28.3
20	0(5)	0(40)	0(17)	0.05	196.2	33	22	24	28	10.7	28.2
Mean±SD#	0.041±4.97	194.78±0.55	30.34±0.84	19.82±0.77	24.04±0.65	25.80±0.77	9.35±0.61	26.93±0.05			
Model	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic	Quadratic			
F value	19.11	22.84	26.61	30.30	22.21	21.47	21.93	22.52			
p-value; prob>F	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05			
Lack of fit	1.23	2.36	5.15	4.53	2.31	4.81	2.85	2.18			
R^2	0.94	0. 95	0.95	0.96	0.95	0.95	0.95	0.95			
Adj R^2	0.89	0.91	0.92	0. 93	0.90	0.90	0.90	0.91			
CV%	12.54	0.28	2.77	3.86	2.99	2.99	6.51	2.09			

*coded and real values (in brackets) of variables; ($p<0.05$):,
The values are mean±SD (n=3) p value: R2

Table 4: Effect of fermentation on quality parameters of Barnyard millet

Sample	DPPH (%)	FRAP Content(mmol Fe(II)Eq/g)	TPC Content (mgGAE/ 100g)	Tannin content (mgTAE/g)	Iron (mg/100gm)	Calcium Content (mg/100gm)	Zinc Content (mg/100gm)	Folate Content (mg/100gm)
Barnyard millet	25.4±0.51	11.0±0.52	151.43±1.1	2.07±0.90	14.5±0.01	23.14±0.21	6.8±0.21	2.37±0.21
Fermented Barnyard millet	35.0±0.11	24.0±0.1	197±0.9	0.006±1.41	28±0.81	29.1±0.02	12.03±0.13	3.00±0.15

The values are mean±SD (n=3)



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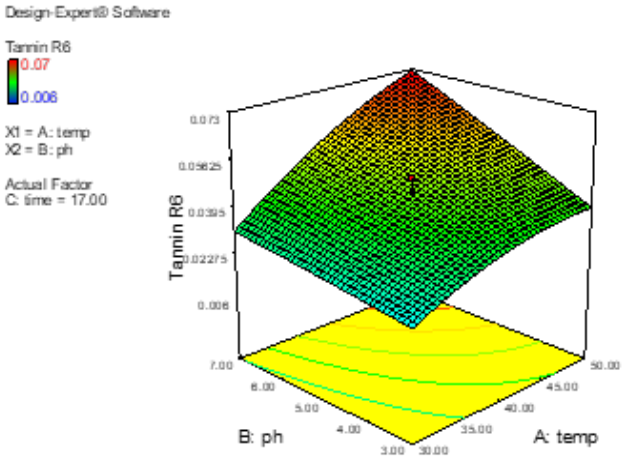

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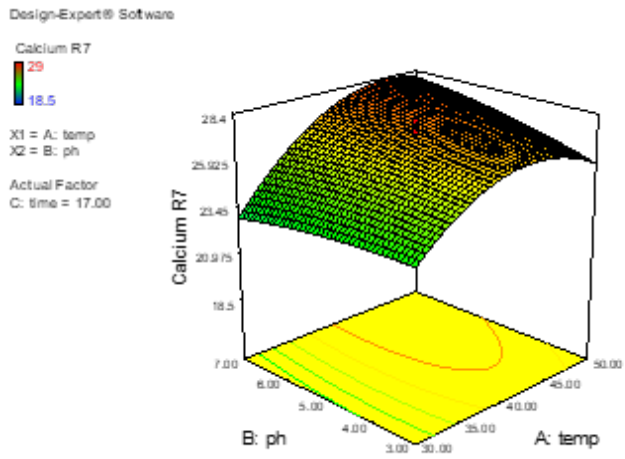

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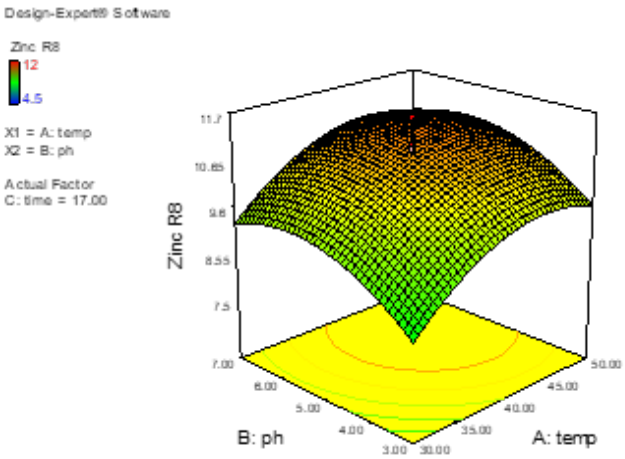
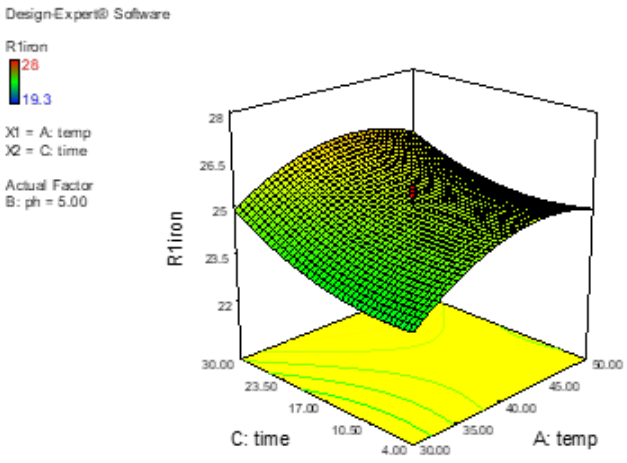
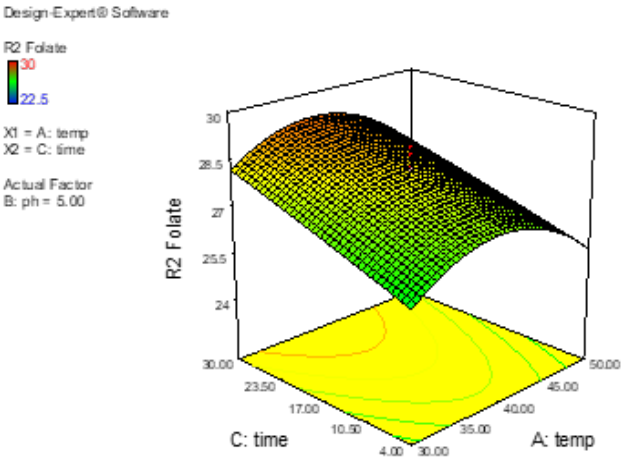


(d)

Fig.(2) Interactive effect of temperature and pH on (a) FRAP content (b) tannin content (c) calcium (d) zinc content



(a)



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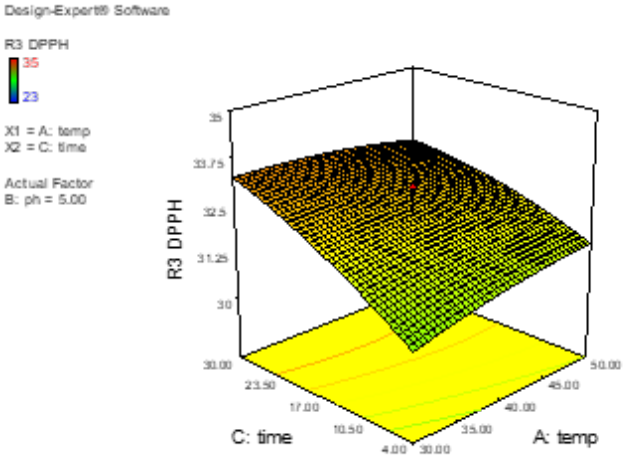

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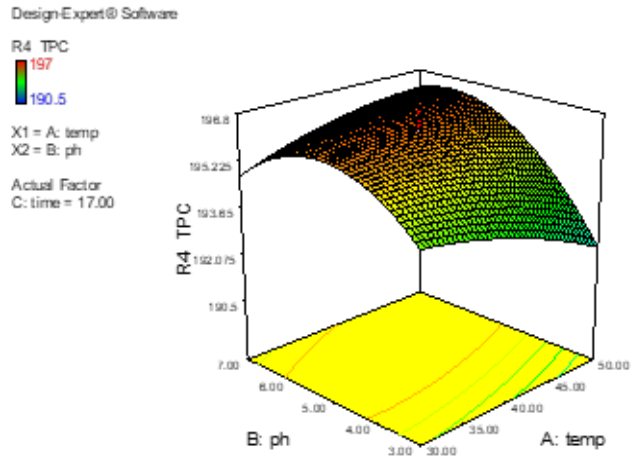

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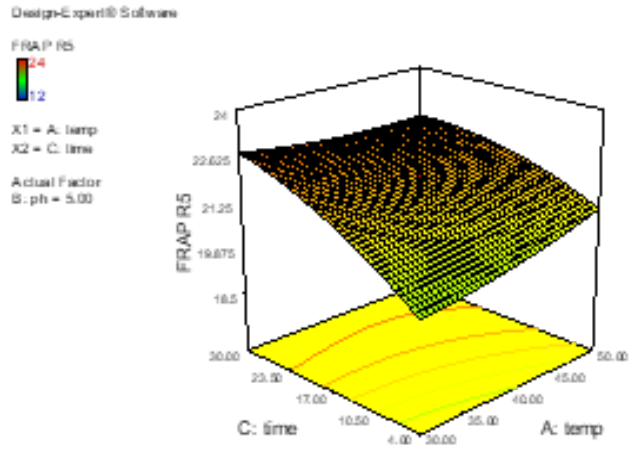


(e)

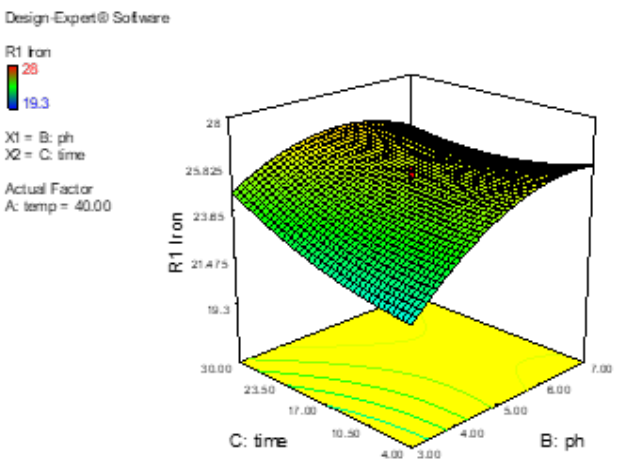
Fig. (3) Interactive effect of temperature and time on (a) iron content (b) folate content (c) DPPH% (d) TPC content and (e) FRAP content

scale designed by McFarland standard (McFarland, 2009).

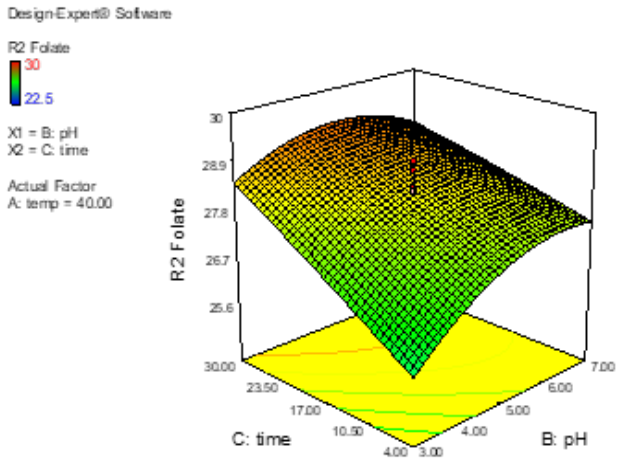
Inoculation and Fermentation

Barnyard millet flour was subjected to lactic acid fermentation by *L. plantarum* at different combinations of temperatures (30-50°C), time (4-30 hrs.) and pH (3-7). The fermentation conditions were optimized using the Response surface methodology (RSM). The pH of millet was adjusted with sodium bicarbonate and citric acid (0.1 N). Then, pre-determined concentration (7.00 log CFU/ml) inoculum was added to millet as recommended for probiotic foods (Panghal *et al.,* 2017).

Experimental design and analysis



(a)



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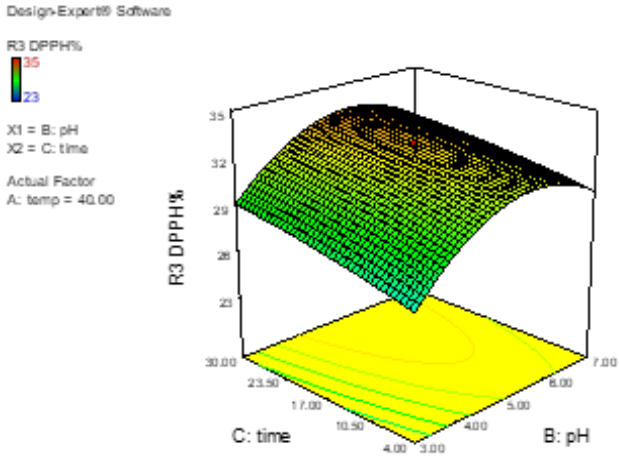

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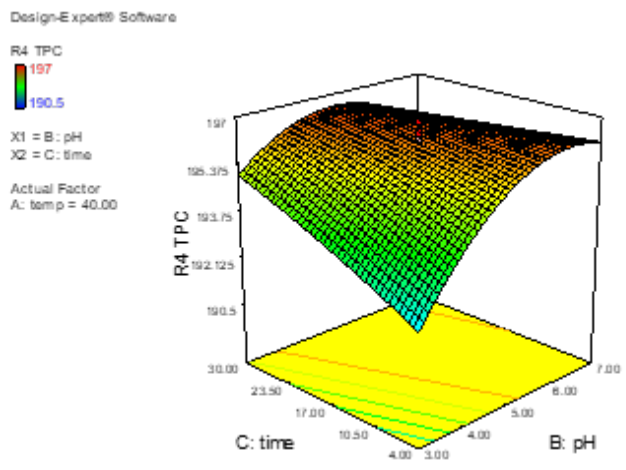

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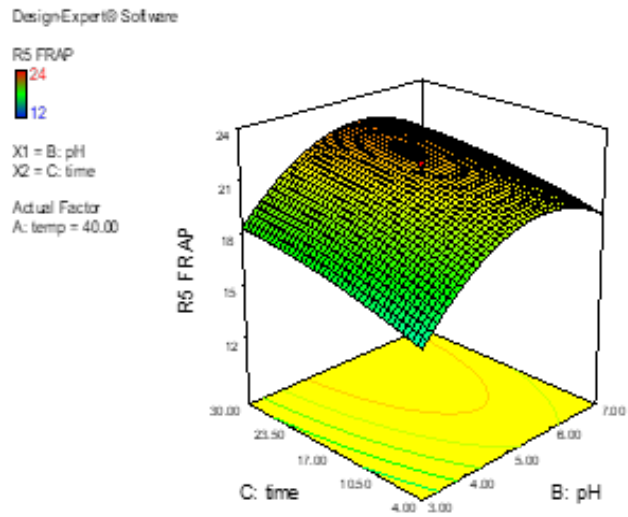

(e)

Fig.(4) Interactive effect of time and pH on (a) iron content (b) folate content (c)DPPH% (d) TPC content and (e) FRAP content

The objective was to optimize a response (output variable) which is influenced by several independent variables (input variables). RSM was used to investigate the influence of the fermentation variables namely temperature, pH, and time on the TPC (Total phenolic content), DPPH, FRAP, tannin, calcium, zinc, folate, and iron content of barnyard millet (Table 1). A Central Composite Design (CCD) with 3 factors was used to fit a second-order response surface. The responses were analyzed by multiple regressions through the least-squares method to fit the following equation

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \qquad (4.1)$$

...eq (1)

Where, Y= measured response variable; β_0,β_i, β_{ii} and β_{ij} are constant, linear, quadratic and cross-product regression coefficients of the model, respectively; and X_i and X_j represent the independent variables in coded values. The coefficients of the response function, their statistical significance and process conditions for maximum tannin, TPC, DPPH%, FRAP, calcium, zinc, folate and iron content of barnyard millet were evaluated by the statistical soft DPPH %=(control absorbance-sample absorbance)/(control absorbance)×100 ware "Design Expert, version 11"(Stat-Ease DPPH %=(control absorbance-sample absorbance)/(control absorbance)×100 Inc., Minneapolis, USA).

RESULTS AND DISCUSSION

Proximate composition of Barnyard millet

The proximate composition of barnyard millet is reported in Table 2. The moisture content of barnyard millet was found to be 8.9±0.2%. The protein content of barnyard millet was 6.06±0.15% whereas fat content was 1.92±0.06%. The fiber, ash, and carbohydrate content were 2.25±0.21%, 4.0±0.1% and 76.86±0.65%, respectively. Similar chemical analysis of barnyard millet has been shown by Verma and her co-workers (2015), results depicted that it is a good source of fiber and mineral content as it contains about 11.93% moisture, 4.27% ash, 2.98% crude fibre, 2.02% crude fat, 6.93% crude protein and 71.87% of carbohydrate (Verma *et al.,* 2014).

The present investigation was conducted to optimize the process of fermentation of barnyard millet for the enhancement of iron and folate content. Tannin, TPC, DPPH, FRAP, calcium, zinc, folate, and iron contents analyzed after fermentation under various experimental conditions are presented in Table 3. Tannin content ranged between 0.006 to 0.07 mg/100g. The maximum tannin content of 0.07 mg/100g was obtained at pH 5, temperature 17ºC and fermentation period of 4 hours, whereas the minimum concentration of 0.006 mg/100g was obtained at pH 5, temperature 23.18ºC and time of fermentation about 17hours. TPC content of barnyard millet during fermentation ranged from 190.5 to 197 mg/100g.The maximum concentration obtained was 197 mg/100g at pH 5 temperature 40ºC and fermentation period of 38.86 hours whereas the minimum concentration of 190.5mg/100g was obtained at 40ºC, pH 1.64 and fermentation time of 17 hours. DPPH of barnyard millet during fermentation ranged between 23 to 35%. The highest DPPH content (35%) was obtained at pH 5, temperature 40ºC and fermentation period of 38.86 hours. The minimum DPPH% i.e. 23%was obtained at pH 1.64, temperature 40ºC and time of 17 hours. Ferric reducing antioxidant power (FRAP) was also analyzed during fermentation which ranged between 12 to 24 mg/g. The minimum range of ferric reducing antioxidant power was obtained 12 mg/g at temperature 40ºC, pH 1.64, and fermentation time of 17 hours, whereas the maximum concentration of FRAP obtained was 24 mg/g at temperature 40ºC, pH 5 and time 38.86 hours. The iron content was also estimated during fermentation, it ranged from 19.3mg/100g to 28 mg/100 g. The minimum iron concentration was obtained at 40ºC at pH 1.64 and a time duration of 17 hours, whereas the maximum concentration of iron 28mg/100g was obtained at temperature 40ºC, pH 5 and fermentation time of 38.86 hours. The maximum concentration of calcium 29 mg/100g was obtained at temperature 40ºC, pH 5 and time 38.86 hours and minimum concentration (18.5mg/100g) was obtained at 23.18ºC, pH 5, and a period of 17 hours. The maximum concentration of zinc 12mg/100g was obtained at temperature 40ºC, pH 5, and time 38.86hours. The minimum zinc concentration 4.5mg/100g was obtained at 30ºC at pH 3 and time duration of 4 hours. The folate concentration was found to be minimum 24µg/100 g at 56.82ºC at pH 5 and time duration of 17 hours, whereas its maximum concentration 30µg/100 g was obtained at a temperature of 40ºC, pH 5 and fermentation time of 38.86 hours.

Equation 1 was fitted into the response variables i.e iron, folate content, tannin content, TPC content, DPPH, FRAP, calcium and zinc using least square regression analysis. The ANOVA and statistical analysis are given in Table 4 for the selected response variables. Regression results showed that all the variables had a significant effect on the responses viz. iron, folate, tannin, TPC, DPPH, FRAP calcium and zinc content at the quadratic level. In the following equations the independent variables i.e: temperature, pH and time are donated as A, B and C respectively. The combined effects of these three independent variables were studied by CCD. They are fitted in equation number 1.For tannin content, F value suggests that the model was significant at $p<0.0001$. The coefficient of determination, R^2 was 0.9451 suggesting 94.51% of the variability in the data was explained by the model. Therefore, model was adequate to predict the response and interpret the effect of variables on the response.

$Y=0.046+0.015A+0.010B-1.324\times10^{3}C+6.031\times10^{3}AB-2.680\times10^{3}AC+1.797\times10^{4}BC4.374\times10^{3}A^{2}-3.094\times10^{4}B^{2}-3.596\times10^{3}C^{2}$……Eq (2)

Similarly the response; for TPC, F value suggests that the model was significant at $p<0.0001$. Coefficient of determination, R^2 was 0.9536; suggesting 95.36% of the variability in the data was explained by the model:

$Y=196.28-0.17A+1.02B+0.46\ C+0.89A\ B-0.41\ A\ C-0.98\ B\ C-0.32A^{2}-1.51\ B^{2}-0.15\ C^{2}$

…….Eq(3)

For DPPH and FRAP also, the F value suggests that the model was significant at $p<0.0001$ coefficient of determination for DPPH, R^2 was 0.9601; suggesting 96.01% of the variability in the data (Eq. 4). Similarly, the coefficient of determination for FRAP, R^2 was 0.9646; suggesting 96.46% of the variability (Eq. 5). The model was found to be adequate for predicting the responses and interpret the effect of variables on the responses.

$$Y=32.44+0.13\ A+1.43B+1.00\ C-1.43A\ B-0.32\ AC-0.16BC-0.13\ A^2-2.79\ B^2-0.23C^2$$

……Eq (4)

$$Y=21.76+0.22A+1.44B+1.04\ C-1.09\ A\ B-0.37A\ C-0.12\ BC+0.16\ A^2-2.68B^2-0.37C^2$$

……Eq (5)

For iron content also, the F value suggests that the model was significant at $p<0.0001$ coefficient of determination, R^2 was 0.9524; suggesting 95.24% of the variability in the data was explained by the model. Thus, the model obtained was adequate to predict the response and interpret the effect of variables on the iron content after fermentation.

$$Y= 25.17+0.62\ A+1.22\ B+0.60\ C-0.95AB-0.18\ AC-0.80\ BC-0.91A^2-1.29B^2+0.54C^2$$

…..Eq (6)

For calcium content, F value suggests that the model was significant at $p<0.0001$. coefficient of determination, R^2 was 0.9508; suggesting 95.08% of the variability in the data was explained by the model given in Eq.7.

$$Y=27.45+1.76A+0.32B+0.29C+0.79AB-1.56AC-0.81BC-1.97A^2-0.28\ B^2+0.22C^2$$

…..Eq (7)

For Zinc content, F value suggests that the model was significant at $p<0.0001$ coefficient of determination, R^2 was 0.9518; suggesting 95.18% of the variability in the data was explained by the model. Thus, the model was obtained which adequate to predict the response and interpret the effect of variables on the zinc content after fermentation.

$$Y=10.91+0.70A+0.50\ B+0.56\ C-2.404\times10^3AB-1.92AC-1.04B\ C-0.76A^2-0.70B^2-0.31\ C^2$$

…..Eq (8)

For folate content also F value suggests that the model was significant at $p<0.0001$ coefficient of determination, R^2 was 0.9530; suggesting 95.30% of the variability in the data was explained by the model. The model obtained is given in Eq 9.

$$Y=28.27-0.28A+0.53B+0.98C-1.53AB-0.095AC-0.40BC-1.35A^2-0.65B^2-0.043C^2$$

…..Eq (9)

Optimization of the process variables

Design Expert (11.0) was employed to maximize iron, folate, antioxidant activity and mineral content during fermentation using a numerical method of optimization. The optimized conditions found were temperature 40^{O}C, fermentation time of 38.86 hours and pH 5 for which folate content was 30 µg/100 g, iron content was 28 mg/100g, TPC 197 mg GAE/100 g, DPPH % was 35%, FRAP 24 mg/100 g. Tannin content was minimized to be 0.006 mg TAE/g, at temperature 23.18ºC, pH 5 and fermentation time of 17hours.

Interactive effect of temperature and pH on selected responses

The interactive effect of temperature and pH on iron, folate, DPPH%, and TPC content of barnyard millet is illustrated in Fig.1 (a), (b), (c), and (d) respectively. Fig.2 (a), (b), (c), and (d) show the interactive effect of temperature and pH on FRAP, tannin, calcium and zinc content of barnyard millet respectively. The interactive effect of pH and temperature on the responses increased as temperature and pH increased (alkalinity increased). Similar results have been obtained earlier in two varieties of pearl millet. The correlation results suggest that DPPH radical scavenging activity and reducing power assay in the varieties was largely due to the presence of flavonoid content (Adetuyi *et al.,* 2014). Similar trends were also obtained in non-thermal processing (fermentation) of four cultivars of cowpea (EC-4216, BL-2, Kohinoor and Gomati) for DPPH%, TPC and FRAP content for 16h to 40 hours fermentation period **(**Yadav *et al.,* 2018). Hemalatha (2007) found that fermentation also increases the bioaccessibility of iron. The HCl extractability of iron increased significantly ($p\leq0.05$) with the fermentation period **(**Sokrab *et al.,* 2014). Tannin, calcium and zinc of barnyard millet increased as temperature, time and pH increased. Similar results were obtained when two corn genotypes Var-113 (high phytate) and TL-98B-6225-9×TL617 (low phytate), respectively, were processed by natural fermentation **(**Sokrab *et al.,* 2014). A similar trend was also obtained during the natural lactic acid fermentation in pearl millet, sorghum and maize (Osman, 2004; Cui *et al.,* 2012). Similar results for calcium content in sorghum have been observed by Varghese *et al.,* (2008).

Interactive effect of temperature and time on selected responses

The interactive effect of temperature and time on iron, folate, DPPH%, TPC and FRAP content of barnyard millet is illustrated in Fig.3 (a), (b), (c), (d) and (e) respectively .The interactive effect of time and temperature of fermentation on selected responses increased as

temperature and time increased. Similar results have been obtained earlier in cereals and pseudocereals on TPC, DPPH%, FRAP and antioxidant activity (Dordevic *et al.,* 2010). Time and temperature of fermentation also increased the antioxidant activity in *Arthrospira platensis* (Castro *et al.,* 2019).

Interactive effect of time and pH on selected responses

The interactive effect of time and pH on iron, folate, DPPH%, TPC content and FRAP content of barnyard millet is illustrated in Fig.4 (a), (b), (c), (d) and (e) respectively. The interactive effect of time and pH of fermentation on selected responses increased with increase in pH and time.

Effect of fermentation on antinutritional and antioxidant activities of barnyard millet

The effect of fermentation on anti-nutritional and anti-oxidant activities, FRAP and iron, calcium, zinc, and folate content of barnyard millet is shown in Table 4. Fermentation reduced the tannin content in the raw samples from 2.07 to 0.006 mg TAE/g. Reduction in tannin content may be due to the binding of tannins with cotyledon endosperm that is usually unobserved by a regular method, due to their insolubility in the solvent or may be due to bacterial phenoloxidase action. Hassan *et al.,* (1995) reported that natural fermentation of three varieties of sorghum for 24 hrs significantly reduced its tannin content by 20.4 to 38 % (llham and Abdullahi, 1995). Fermentation processing by LAB proves to be effectual in enhancing the digestibility of protein and starch in cereals and legumes. This increase in the digestibility of protein may be accredited to the degradation of antinutritional factors (tannins and phytic acid) by bacteria as stated by Vikas (2015). Tannins are known to have a rigorous taste which affects palatability of food intake. It can also form a complex with protein and reduce their absorption and digestibility. Therefore, the reduction of an antinutritional factor of millets through fermentation would improve its nutritional value. Fermentation of millets is a major cause of the reduction in trypsin and amylase inhibitors activities and the phytic acid content. Several researchers have observed that fermentation can be successfully used to improve the nutritional superiority of cereal grains by increasing protein content and digestibility (Inyang and Zakari, 2008; Hag *et al.,* 2002*)* and available lysine content (Hamad and Field, 1979). Fermentation was also found to decrease trypsin inhibitory activity (TIA), amylase inhibitor activity, phytic acid, and tannins (Osman and Gassem, 2013; Mohamed *et al.,* 2007). Fermentation by *L.plantarum* brings about a significant ($p < 0.05$) increase in the DPPH radical-scavenging ability from 25.4 to 35%. During fermentation, several significant biochemical changes occur. The liberation of phenolic isoflavone aglycones by the activation of enzyme β-glucosidase and the formation of reductones could donate to an increase in antioxidant activities. Investigators have found that fermentation by LAB does increase the DPPH radical-scavenging ability of pulses, legumes and some species of Cruciferae family (Oboh *et al.,* 2011; Dajanta *et al.,* 2013; Foluso *et al.,* 2011). Fermentation of okra seeds for 24 h observed the highest DPPH % which was significantly ($p < 0.0001$) diverse from unfermented seeds. Time of fermentation did notably affect the DPPH activity, (Adetuyi *et al.,* 2014). It has also been observed by former investigators that the correlation was predictable among phenolic content and antioxidant activities (Dajanta *et al.,* 2013). Natural fermentation increases the reducing sugar, antioxidant activity, mineral content and nutrients of foods through the biosynthesis of amino acids, vitamins and protein; by recovering the quality of protein and digestibility of fiber and reduces tannin and phytate content in pearl millet as reported by Srivastava *et al.,* (2020).

The total phenolic content (TPC) of the barnyard millet was indicated as mg gallic acid equivalent (GAE) per 100 g sample as shown in Table 4. Phenolic content for the barnyard millet samples analyzed in the study extended from 151.43±1.1 mg GAE/100g to 197±0.9 mg GAE/100g sample. The total phenolic content (TPC) of the fermented barnyard millet was found to be significantly ($p < 0.0001$) higher than the unfermented barnyard millet. Phenolic compounds that are found in millets are bound with sugar of millet which lowers their accessibility to an organism. In fermentation, protease, proteinase and other proteolytic enzymes from the lactic acid bacteria hydrolyze complexes of insoluble phenolics into soluble phenols and more biologically active compounds that are selectively absorbed. This was constant with findings reported by Olaniyi (2013). The TPC for the okra seeds examined in the study varied from 185 mg GAE/100 g to 1460 mgGAE/100g sample. Fermentation of seeds for 24 hrs. had the maximum ($p < 0.05$) phenolic content of 1460 mgGAE/100g, while untreated okra seeds had the minimum phenolic content of 185 mgGAE/100g (Adetuyi, 2014).

The reducing power (FRAP) of the fermented barnyard millet was significantly ($p = 0.0001$) higher than that of the unfermented millet. The unfermented barnyard millet had a reducing power of 11.0±0.52 mmol Fe (II) Eq/g, while fermentation increased the reducing power 24.0±0.52 mmol Fe (II) Eq/g. This was consistent with findings reported by Ganiyu *et al.,* (2011) i.e., fermentation enhanced the reducing power of groundnut and soybean.
The mineral content of the barnyard millet was determined and reported as shown in Table 4. The fermented barnyard millet showed significantly ($p = 0.0001$) higher mineral content than the unfermented millet. The unfermented barnyard millet had 14.5±0.01mg/100gm iron content, 23.14±0.21 mg/100 gm calcium content, 6.8±0.21 mg/100gm zinc and 2.37±0.21 mg/100gm folate content. Fermentation caused an increase in minerals,

such as 28±0.81 mg/100gm iron, 29.1±0.02 mg/100gm, calcium content, 12.03±0.13mg/100gm zinc and 3±0.15 mg/100gm folate. There are three mechanisms by which fermentation enhances the mineral bioavailability and bioaccessibility. Firstly, millet minerals are present in bound form as complex with nondigestible materials like cell wall polysaccharides and some antinutritional factors such as phytate, etc. Because of this complex, they have very low bioavailability. Fermentation increases the bioavailability of minerals by reducing phytic acid, tannin and oxalate that bind minerals. The second reason for the enhancement of mineral content during fermentation is microbial enzymes. Enzymes loosen the complex matrix that immerses minerals. Both phytase and α-amylase make the matrix loose by degrading anti nutritional factors and starch, respectively. Lactic acid bacteria can degrade fiber which loosens the food matrix. Another reason for the enhancement of mineral during fermentation is the low pH of fermentation. Low pH acquired during fermentation increases iron absorption due to change from ferrous iron (less absorbable), to ferric iron i.e. readily absorbable (Nkama, 2001).

CONCLUSION

Consequently, results of the study indicate that RSM was successfully used to optimize pH, temperature and time during fermentation by *L. plantarum* of barnyard millet to enhance iron, folate; maximize TPC, DPPH%, FRAP, calcium and zinc content in barnyard millet flour. The fermentation process reduced the anti-nutritional factor and increased antioxidant activity and mineral content. The outcome of the result of this study affirmed the fact that products developed through fermentation by *L. plantarum* of millets can be used as a good alternative to chemically processed foods in various food industries. Since fermentation of foods not only minimize the health effects which occur due to additives and chemicals used during the processing of food but it also enhances the nutritive value of food and simultaneously improves the shelf life of the food product.

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