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Enhanced Phytase Production from Indigenous *Bacillus subtilis KT004404* by Response Surface Methodology to be Used as Poultry Feed Supplement

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MY supervised the work and provided major guidance during drafting of the paper. AJ conducted all the experimental work including isolation, optimization and manuscript write up. KSK contributed during the lab experimentation and performed all the spectroscopy in the lab.

Key words
***Bacillus subtilis* KT004404, Phytase, Poultry, Centrally composite design (CCD), Response surface methodology (RSM)**

ABSTRACT

The aim of this experimental study was the optimization of phytase production from *Bacillus subtilis* KT004404 through statistical model (Response Surface Methodology) by utilizing centrally composite design (CCD). The optimal phytase activity obtained after CCD design was 132.32 U/mL at 35 °C, pH 6.5, inoculum density of 2.5mL after incubating for 24h at 75 rpm. Extracellular enzymes were then partially purified in 2 steps (ammonium sulfate precipitation and gel filtration chromatography using Sephadex G-50). The molecular weight of extracellular phytase from *B. subtilis* KT004404 estimated by SDS-PAGE was 30 kDa. The K_m and V_{max} of *B. subtilis* KT004404 phytase were 0.175 mM and 250 U/mL (phytase activity) respectively. The optimum temperature for partially purified extracellular phytase was 40°C, and the optimum pH range for extracellular phytase activity was pH 6.5-7.0. The extracellular enzyme activity was not significantly affected by Cu^{2+} and Mn^{2+} metal ions while the inhibitory effect of Ca^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+} and Fe^{2+} was recorded with 27.12%, 32%, 57.31%, 14.76% and 13.54% residual activity.

INTRODUCTION

Phosphorus is stored in plants in the form of phytic acid particularly in grains, legumes, and cereals which represents about 75%-80% of the stored phosphorous present in nature (Al-Kandari *et al*., 2021). Phytic acid has significant chelating potential under normal physiological conditions due to which it limits the bioavailability of essential mineral complexes such as zinc, calcium, iron, and magnesium (Singh, 2008). Moreover, it also inhibits the activity of digestive enzymes by binding with amino acids and proteins (Cowieson *et al*., 2006). Phytic acid due to its anti-nutrient potential also causes phosphorus pollution via manure (Toor *et al*., 2005). Phosphorous pollution in the soil can be reduced between 30% to 50% by adding the phytase supplementation in feed (Alias *et al*., 2017).

The bioavailability of phosphorous along with other minerals can be enhanced by supplementing the feed with phytic acid-degrading enzyme phytase (EC 3.1.3.8, myo-inositol hexakisphosphate phosphohydrolase). Phytase have significant application in agriculture, transgenic plant technology, and horticulture (Laboure *et al*., 1993). Phytase enzyme catalyzes stepwise hydrolysis of myo-inositol hexakisphosphate and release phosphorous to be utilized in various biochemical reactions (Vats and Banerjee, 2004).

Bacteria, fungi, and yeast are reported to produce various classes of phytase. Different phytases differ in their structure, catalytic activity, and optimal conditions (Kumar *et al*., 2017). There is no single phytase which can meet the diverse environmental and commercial need therefore, there is an increased interest in screening the microorganism with ability to produce novel phytase to improve the nutritive value of feed containing phytic acid (Alias *et al*., 2017). Due to broad industrial application, the isolation, production and optimization of phytase has attracted significant attention.

The use of enzymes in poultry feed has posed many challenges because of thermal treatment during the

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pelleting process, which causes decline or complete loss of enzyme activity due to denaturation (Kumar *et al*., 2016). Enzymes exhibiting optimal activity at high temperatures and acidic pH are significantly important for diverse applications in the poultry feed industry (Naves *et al*., 2012; Singh and Satyanarayana, 2015).

Traditional method for the optimization of enzymes known as one factor at a time (OFAT) have numerous downsides such as requirement of running large number of experimental runs and lack of information about the interaction of different parameters. However, statistical, or mathematical modeling for optimization of parameters for enzyme production offers multiple advantages over traditional OFAT method (Kumari and Bansal, 2021). Response surface methodology (RSM) is widely utilized statistical method for optimizing and analyzing the interactions between different experimental variables during optimization reaction (Jain and Singh, 2017; Shahryari *et al*., 2018; Pable *et al*., 2019; Jatuwong *et al*., 2020; Kumari and Bansal, 2021)

The main objective of the current study was directed towards the selection of media components for optimal phytase production from *B. subtilis KT004404* by using RSM and characterization of purified phytase enzyme to analyze the potential use as poultry feed supplement.

MATERIALS AND METHODS

Culture conditions and maintenance

The already identified and isolated *B. subtilis KT004404* strain from hot water spring from Kashmir (Pakistan) was obtained from International Islamic University, Islamabad, Pakistan, and cultures were maintained at 4 °C in form of glycerol stocks.

Bacterial inoculum preparation

B. subtilis was cultivated in 50 mL of nutrient broth (Sigma-Aldrich Co., St Louis, USA) in Erlenmeyer flasks (250 mL), 0.2 % dextrose at 37 °C and 150 rpm. The culture fluid was used as seed inoculum (CFU 1.39 × 10^6 mL^{-1}) after 24 h of incubation in the nutrient broth.

Phytase plate assay

The ability of *B. subtilis KT004404* to exogenously produce phytase enzyme was identified by confirmatory tests proposed by Yanke *et al*. (1998). Phytase screening media composition contained glucose 1% (w/v), ammonium sulfate 0.2% (w/v), urea 2% (w/v), citric acid 0.3% (w/v), sodium citrate 0.2% (w/v), $MgSO_4 \cdot 7H_2O$, 0.1% (w/v), sodium phytase 0.5% (w/v), iron sulfate 0.02% (w/v). The phytate-containing media was autoclaved at 121 °C for about 15 min at 15 psi pressure conditions to ensure sterilization. Flooding plates with 2% cobalt chloride solution with 6.25 % ammonium molybdate and 0.42% ammonium vendate (1:1). Diameters of zones of clearance, an indicator of the phytase production were measured through vernier caliper scale.

Quantitative assay for phytase activity

Phytase activity was determined by colorimetric method using ferrous sulfate-molybdenum blue method (Murphy and Riley, 1962). 100 µL phytase solution and 900 µL (1 mM) substrate solution were incubated for 30 min. After 30-min incubation, the stop solution 1 mL of TCA (5%) was added followed by addition of 1mL ferrous sulfate. 4mM solution of potassium dihydrogen phosphate was used to plot standard curve. One phytase unit is the activity of phytase that generates 1 µmol of inorganic phosphorus per min from an excess of the substrate at 37 °C. Protein concentration in filtrate was determined by Lowry method (Waterborg *et al*., 1996).

Selecting source of nitrogen and carbon via one variable at a time

Nitrogen and carbon sources of energy were screened in a preliminary study, by the one-factor optimization method (OFT). Agro-industrial substrates such as sorghum, linseed, cornmeal, soybean, millet, sesame, wheat bran, rice husk, canola meal, sorghum were selected for analyzing the best carbon source in production media for enhanced phytase production (Sadh *et al*., 2018). Similarly, during submerged fermentation, various nitrogen substrates like, peptone, casein, yeast extract, malt and beef extract, and ammonium nitrate were added in the medium (1% w/w in basal media) as nitrogen sources. Initially, production media was inoculated with 1 mL of inoculum (OD_{600} = 1.5, CFU= 1.39 × 10^6 mL^{-1}) in order to maintain a constant metabolic activity in all the experiments.

Submerged fermentation

50 ml erlenmeyer shake flasks were used to produce phytase containing different nitrogen and carbon sources of energy for bacteria. The basal medium contained wheat bran (5g/L), calcium chloride (0.2%), magnesium sulfate heptahydrate (0.2g/L), and distilled H_20 to make 1 L production media. Sterilization of the flask was done at 121 °C for 15 min at 15 lbs pressure. 1 mL (v/v) inoculum was added in the basal media and incubated at 37 °C for 24 h.

Experimental design and data analysis

Fermentation parameters optimization via RSM using centrally composite design

Response surface methodology using CCD was

used for optimization of phytase production. Varying concentrations of carbon and nitrogen substrates were used in the production medium for phytase production. Phytase activity in the medium (U/mL) was the dependent response while five independent parameters were selected to determine optimal variable combination namely temperature (X_1), medium pH (X_2), Inoculum size (X_3), the incubation period (X_4), and shaking intensity (X_5). Three different levels of all selected independent variables were studied and total of 50 experimental runs were analyzed as shown in Figure 1. The flasks containing basal medium were sterilized at 121 °C for 15 min, then inoculated with bacterial (w/v) inoculum followed by 24h incubation at varying temperature range. Combined effects of five variables on phytase production by *B. subtilis* was studied within a specific range as shown in Table I.

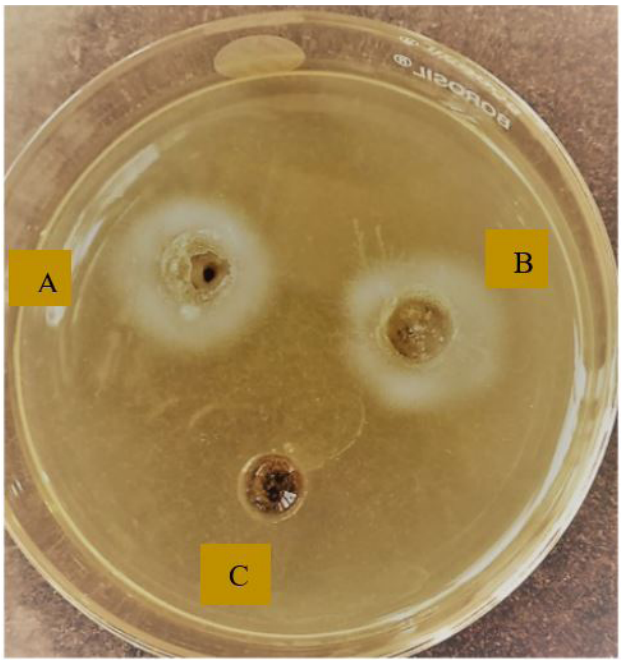

Fig. 1. Qualitative assay for detecting extracellular phytase production by *Bacillus subtilis* KT004404 on phytase-screening medium (PSM) with Na-phytate. Zones of clearance formed around the wells (A) and (B) loaded with bacterial filtrate while well (C) was loaded with control and showed no clearance zone.

Statistical software 'Design Expert 6.0' Stat-Ease, Inc., Minneapolis, USA, was used for analyzing the experimental design and to generate data (Table II).

The production of phytase in the medium was analyzed by a second-order polynomial equation for a 5-factor system fit to experimental values (phytase activity) which were obtained as following regression Equation 1.

$$Y = \beta_0 + \sum \beta i\, xi + \sum \beta ii\, xi^2 + \sum\sum \beta ij\, xi\, xj \quad \ldots(1)$$

Y (predicted response variable), β_0 represents intercepts, βi linear coefficients, β_{ii} is the squared effect; β_{ij} is the interaction coefficient. Multiple regression equation for five factors system in the full quadratic model to describe response surface is as follow (Equation 2):

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_5X_5+\beta_{11}X_{21}+\beta_{22}X_{22}+\beta_{33}X_{23}+\beta_{44}X_{24}+\beta_{55}X_{25}+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{14}X_1X_4+\beta_{15}X_1X_5+\beta_{23}X_2X_3+\beta_{24}X_2X_4+\beta_{25}X_2X_5+\beta_{34}X_3X_4+\beta_{35}X_3X_5+\beta_{45}X_4X_5 \quad \ldots(2)$$

Where *Y* represents Phytase activity, β_0 for offset term, β_1, β_2, β_3 show linear coefficients, β_{11}, β_{22}, β_{33} show squared coefficients, β_{12}, β_{23}, β_{13} are interaction coefficients which represent the following levels of Independent variable:

X_1, X_2, X_3, X_4, X_5, X_{21}, X_{22}, X_{23}, X_{24}, X_{25}, X_1X_2, X_1X_3, X_1X_4, X_1X_5, X_2X_3, X_2X_4, X_2X_5, X_3X_4, X_3X_5, X_4X_5

To validate the model, the estimated phytase activity was compared with predicted software values. Statistical analysis including standard deviation, ANOVA and Duncan multiple range test on the obtained results were applied using IBM SPSS Statistics Ver.17 (IBM corporation, USA).

Table I. Coded levels of process variables and experimental range for phytase production by *Bacillus subtilis.*

Process variables	Units	Range		
		-1	0	+1
X_1 -Temperature	°C	20	35	50
X_2 -Medium pH	pH	4	6.5	9
X_3 -Inoculum size	%	0.1	2.5	5
X_4 -Incubation time	h	24	60	96
X_5 -Shaking intensity	rpm	50	75	100

Partial purification of phytase

The crude filtrate was subjected to ammonium sulfate precipitation at different percentage saturation (20%, 30%, 40%, 50%, 60%, 70%, 80% and 90%) with constant stirring. The precipitates were collected by centrifugation (15,000×g, 20 min) and then suspended in 0.2M acetate buffer (pH 6.0). Desalting of the enzyme was done by dialyzing the enzyme overnight in 50 mM acetate buffer (pH 6.0) followed by Sephadex G-50 gel filtration chromatography column (12/60, Pharmacia), equilibrated with 0.2M acetate buffer with pH 6.0. Enzyme fractions were eluting with 0.2M Na-acetate buffer at 0.2 cm^3/min flow rate. The gel electrophoresis was performed on fractions collected by passing through 15% SDS-PAGE to determine the size of the enzyme. The gel slabs were stained with Coomassie stain (Neira-Vielma *et al.*, 2018).

Table II. Centrally composite design (CCD) of production parameters along with experimental and predicted phytase activity.

Run	X_1 Temperature °C	X_2 pH	X_3 Inoculum size %	X_4 Incubation time h	X_5 [b] Rpm	Phytase production[a] U/mL	
						[c]Actual	[d]Predicted
1	35	6.5	2.55	60	75	120	113
2	20	9	0.1	96	50	98	61.4
3	20	4	5	24	50	132	97.42
4	35	6.5	2.55	60	75	165	113.10
5	20	4	0.1	96	50	99	81.82
6	50	4	0.1	96	50	76	69.01
7	50	9	0.1	24	100	46	37.46
8	20	4	0.1	96	100	59	59.41
9	35	6.5	2.55	60	75	110	113.10
10	20	4	5	24	100	89	76.26
11	35	0.55	2.55	60	75	89	88.11
12	35	6.5	2.55	24	75	132	113.10
13	50	9	5	96	50	79	49.30
14	50	4	0.1	96	100	69	71.87
15	20	4	5	96	50	85	91.83
16	50	4	5	96	100	75	67.88
17	50	9	0.1	24	50	65	26.50
18	20	9	5	24	50	63	55.32
19	50	9	0.1	96	100	52	67.88
20	35	6.5	2.55	60	15.56	82	102.24
21	35	12.4	2.55	60	75	28	33.19
22	70.6	6.5	2.55	60	75	38	18.70
23	35	6.5	2.55	60	75	99	102.21
24	50	4	5	24	50	62	64.25
25	50	9	5	96	100	42	46.27
26	50	9	5	24	50	32	38.64
27	20	9	0.1	24	100	43	21.40
28	35	6.5	2.55	-25.6229	75	16	26.88
29	20	4	0.1	24	100	43	45.24
30	50	4	5	96	50	46	71.65
31	20	9	5	24	100	41	34.54
32	50	4	0.1	24	100	42	45.43
33	35	6.5	2.55	60	75	79	121.23
34	20	9	5	96	50	38	53.62
35	35	6.5	2.55	60	134.46	98	81.55
36	35	6.5	2.55	145.623	75	79	56.41
37	50	4	5	24	100	61	69.70
38	20	9	0.1	96	100	32	39.44

Table continued on next page.....

Run	X_1 Temperature °C	X_2 pH	X_3 Inoculum size %	X_4 Incubation time h	X_5 [b] Rpm	Phytase production[a] U/mL	
						[c]Actual	[d]Predicted
41	35	6.5	-3.277	60	75	0	13.76
42	20	4	0.1	24	50	55	60.02
43	20	9	0.1	24	50	21	35.81
44	35	6.5	2.55	60	75	96	111.21
45	-0.676	6.5	2.55	60	75	0	23.60
46	20	4	5	96	100	41	63.05
47	50	4	0.1	24	50	41	34.84
48	50	9	0.1	96	50	21	64.54
49	50	9	5	24	100	21	43.23
50	35	6.5	8.37711	60	75	42	32.53

[a] Mean of three values, SD within 10%; [b] rpm, revolution per minute; [c] Actual response: Mean phytase activity (U/mL/min) obtained as triplicate; [d] Predicted response: Response predicted by the model.

Characterization of purified enzyme

The temperature effect on phytase activity was measured at 20°C, 30°C, 40°C, 50°C, 60 °C, and 70°C by incubating enzyme fractions with sodium phytate for 20 min. The optimum pH for phytase activity was determined at different pH range of 4,5,6,7,8,9,10. Various buffers were used to determine the optimum pH such as 0.06 M glycine-HCl (for pH range 2.0-3.6), 0.2M Na-acetate (pH range 3.6-5.8), phosphate-borate (pH range 6.0-8.0). The effect of the presence of metal ions in media were investigated to determine the activation or inhibitory effect of metallic ions on the phytase activity. The effect of different metal ions (Na+, Mn^{2+}, Hg^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+}) on the phytase activity was determined by incubating the enzyme fractions with 10 mM salt solutions of NaCl, $MnCl_2$, $HgCl_2$, $FeSO_4$, $ZnCl_2$, $MgSO_4$, $CoCl_2$, $CaCl_2$. The optimized enzyme was further analyzed for immobilization and stability studies on different inert medias with the prospect of being used as poultry feed supplement.

Enzyme kinetics and substrate specificity

The kinetic parameters of phytase hydrolysis of Na-phytate (at pH 5, 0.2 M Na-acetate buffer, and 40 °C) were studied to determine enzyme-substrate affinity. Various substrates used to determine the enzyme substrate specificity included sodium phytate, D-glucose-1-phosphate, glycerophosphate, fructose-6-phosphate, and p-nitrophenyl phosphate. The rate of Na-phytate hydrolysis was studies at different substrate concentrations (0.05 to 3 mM). K_m and V_{max} were found by Line weaver–Burk plot (Dokuzparmak *et al*., 2017). All experimental data were means of triplicate determinations.

RESULTS

Phytase activity of B. subtilis

Figure 1 show zone of clearance (49 ± 1.414 mm) on phytate screening plates indicating and confirming the production of phytase enzyme by *B. subtilis.*

Our experiment has shown that wheat bran has the highest phytase production as compared to other carbon sources (71.21 U/ml). Peptone as nitrogen source gave the highest activity of phytase (80.45 U/mL) when compared with ammonium nitrate (62.3 U/mL) during fermentation.

Optimization of phytase production

Five important parameters (temperature, production media, pH, inoculation density, incubation period, and shaking flask intensity) were selected through preliminary studies were considered for centrally composite design analysis by RSM. These parameters were analyzed for the best suitable combination for optimal enzyme production. The highest phytase activity in the experimental design was 132.32 U/mL at 35 °C, pH 6.5, inoculum density of 2.5mL after incubating for 24 h at 75 rpm.

The results in Table II exhibit that predicted values for phytase production and experimental values by *B. subtilis* KT004404 are not significantly different. These results were analyzed by ANOVA. The second-order polynomial equation for phytase production as a function of temperature, pH, inoculum density, incubation time, and rpm was fitted to the model led to following regression Equation 3.

$$Y=-49.91+2.81X_1+12.06X_2+28.22X_3+1.42X_4+0.312X_5+0.105X_1X_2-0.05X_1X_3+0.005X_1X_4+0.016X_1X_5-0.72X_2X_3+0.010X_2X_4+0.001X_2X_5-0.077X_3X_4-0.021X_3X_5-0.002X_4X_5-0.072X_1^2-1.48X_2^2-2.649X_3^2-0.009X_4^2-0.005X_5^2 \quad \ldots(3)$$

Where X_1 -temperature for enzyme production, X_2

-pH of the medium, X_3 -inoculation density, X_4 -Incubation period, and X_5 -Shake flask rpm. The 3D response surface plots were generated to study the interaction of physicochemical parameters on enzyme production.

Centrally Composite Design was used to analyze the effect of five independent variables for optimizing the production of Phytase. Variability in temperature, pH, incubation substantially effect phytase production during fermentation.

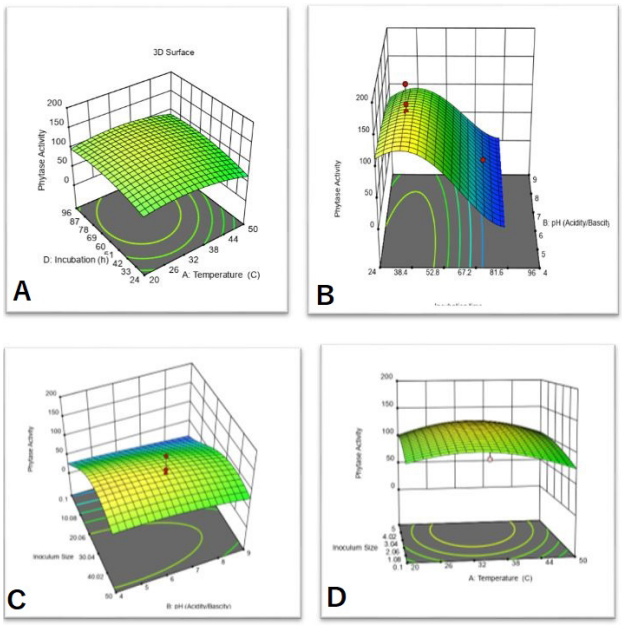

Fig. 2. Response surface curve for production of phytase as a function of temperature incubation (A), pH-incubation (B), inoculum size-pH (C), temperature-inoculum size (D).

Validation of phytase production

As predicted by the model the combination of 5 different types of variables were determined for optimized phytase production. Higher similarity index was found between the predicted and experimental values for phytase production by *B. subtilis*. Predicted values were in agreement with experimentally determined phytase activity. The coefficient of determination (R^2) for the model was 0.705 which implies that there is 70.5% compatibility of predicted and experimental data. Phytase activity was measured in triplicates in all predicted model 50 experimental runs. Signal to noise ratio is measure by model precision. A ratio above 4 is desirable for reliable results. A ratio of 6.030 shows a suitable signal hence, this model can be used to navigate the design space. The model Prob > F (0.01) and F value (3.43) indicates significance of the model and specify that only 0.1% chance that model F value equivalent to this would occur due to noise (Tables III, IV).

Table III. ANOVA for response surface quadratic model (CCD).

Source	Sum of squares	[a] df	Mean square	[b] F value	p value	
Model	44859.50	20	2242.97	3.47	0.001	significant
A-Temp	45.97	1	45.97	0.0711	0.002*	
B-pH	5773.77	1	5773.77	8.93	0.005*	
C-inoculum size	674.26	1	674.26	1.04	0.315	
D-incubation	1669.32	1	1669.32	2.58	0.01*	
E-shaking int.	859.50	1	859.50	1.33	0.25	
AB	504.03	1	504.03	0.7799	0.3844	
AC	108.78	1	108.78	0.1683	0.6846	
AD	306.28	1	306.28	0.4739	0.4967	
AE	1287.78	1	1287.78	1.99	0.1687	
BC	639.03	1	639.03	0.9888	0.3283	
BD	30.03	1	30.03	0.0465	0.8308	
BE	0.2812	1	0.2812	0.0004	0.9835	
CD	1498.78	1	1498.78	2.32	0.1386	
CE	81.28	1	81.28	0.1258	0.7254	
DE	116.28	1	116.28	0.1799	0.6746	
A^2	14684.04	1	14684.04	22.72	< 0.0001	
B^2	4778.35	1	4778.35	7.39	0.0109	
C^2	14052.24	1	14052.24	21.74	< 0.0001	
D^2	8866.73	1	8866.73	13.72	0.0009	
E^2	762.63	1	762.63	1.18	0.2863	
Residual	18741.32	29	646.25			
Lack of fit	13833.82	22	628.81	0.8969	0.6112	not significant
Pure error	4907.50	7	701.07			
Cor total	63600.82	49				

* Statistically significant at 99% probability level; [a] df, degrees of freedom; [b] F-value, F distribution showing variation between sample means.

Table IV. ANOVA for regression analysis.

Std. Dev.	25.42	R^2	0.7053
Mean	63.06	Adjusted R^2	0.721
C.V. %	40.31	Predicted R^2	0.769
		Adeq precision	6.0298

Partial purification and optimization of enzyme activity

The optimum temperature of *B. subtilis* phytase activity was found to be 40°C, while the enzyme remains stable until 70°C. The partially purified phytase exhibited

stability at acidic pH, while the optimal enzymatic activity was found between pH 6.5-7.0 (enzyme activity of 267U/mg) and completely inactive above 9.0 (Figs. 3, 4).

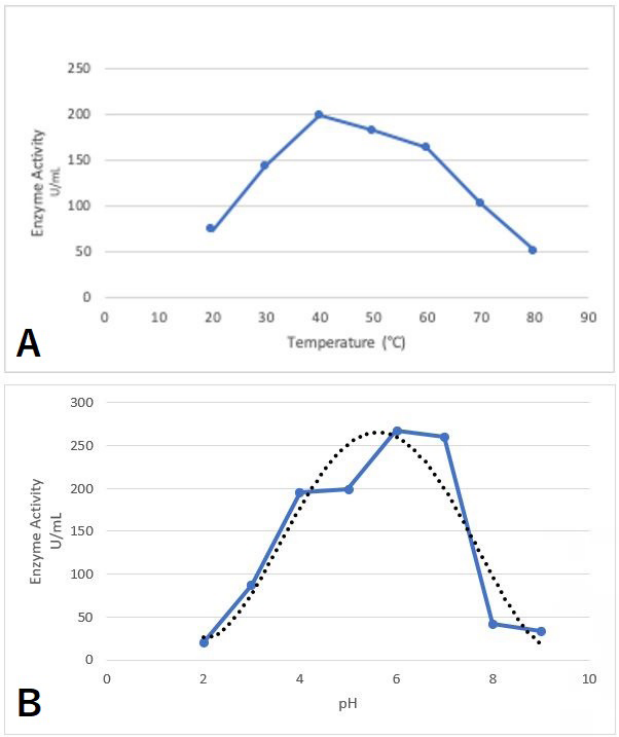

Fig. 3. One factor plots for the effect of temperature (A) and pH (B) on phytase production.

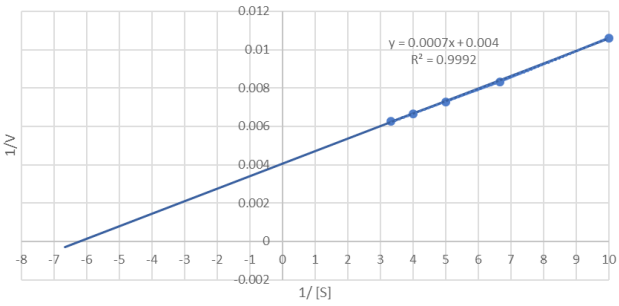

Fig. 4. Lineweaver Burk plot drawn to calculate Vmax and Km values.

The residual activity of phytase was assayed following pre-incubation with different metal ions. Few metal ions after incubation were tested to have non inhibitory effect like Mn^{2+} and Cu^{2+} with residual activities as 185.8, 215.21 U/mL, respectively, where as some had a slight inhibitory effect like Ca^{2+} (27.12% residual activity), Co^{2+} (32%), Zn^{2+} (57.31%), Hg^{2+} (14.76% residual activity) Fe^{2+} (13.54%) of its original activity, respectively.

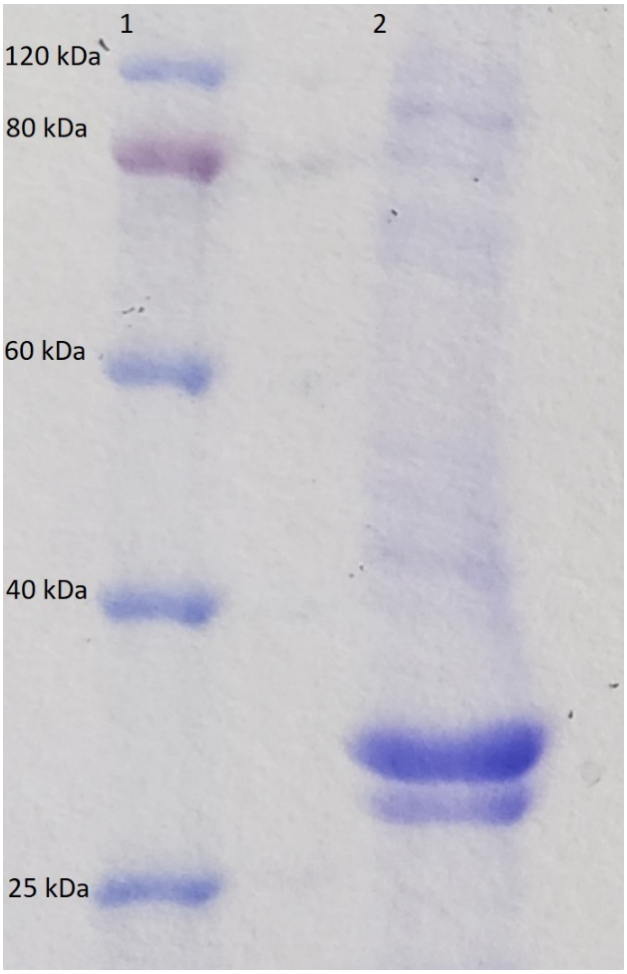

Fig. 5. SDS-PAGE Lane 1: Protein ladder (PAGE-MASTER Protein Standard) Lane 2: Partially Purified Phytase.

Purified phytase

The phytase was partially purified using ammonium persulfate precipitation and Sephadex G-50 column via chromatography. The purification of phytase increased approximately 87-folds as compared to crude extract with a 46.27 % yield. Purified phytase showed a high specific activity of 408.2 U/mg protein. Production of extracellular phytase in *B. subtilis* was marked with approximately 87 folds purification and 407 phytase U/mg of total protein (Table V).

The molecular weight of phytase obtained from Sephadex G- 50 column determined by SDS-PAGE was 30 kDa (Fig. 5). A single band was observed during the SDS-PAGE hence the assumption that *B. subtilis* phytase consists of identical subunits.

Table V. Purification of phytase from *Bacillus subtilis* KT004404.

Steps	[a] Total activity (U/mL)	[b] Total protein (mg)	[c] Specific activity (U/mg)	[d] Yield %	[e] Purification (fold)
Enzyme production before RSM[f]	45.3	4.8	9.4	-	1
Crude extract of enzyme (after RSM)	132.32	6.3	21.00	100	2.3
Ammonium sulfate precipitation	78.34	1.7	46.08	59.20	2.19
Elusion Chromatography Sephadex G-50	61.23	0.4	153.07	46.27	7.2

[a] Total activity or phytase activity as the amount of phytase which can liberate the phosphorus from catalyzed phytate in one minute; [b] Total protein content determined by Lowry method using BSA as standard; [c] Specific activity = Total activity/ Total protein; [d] Yield: Recovered enzyme activity; [e] Purification fold: Increase in specific activity; [f] Media parameters (35 °C, pH-7.0, 100rpm, Na-phytate as substrate).

Kinetics of enzyme activity

Kinetic parameters of phytase enzyme were studied at varying Na-phytate concentrations. The optimal reaction velocity (V_{max}) and Michaelis constant (K_m) of *B. subtilis* phytase on sodium phytate was calculated via Lineweaver-Burk plot 250 U/mL of protein and were 0.175 mM of sodium phytate, respectively. K_m value is significantly higher than those reported for phytase from *A. vacuum* – 40 µmol but lower than 1.77mM in *Geobacillus* sp., and 2.19 mM *B. subtilis*. Value of Km is sensitive towards conditions such as pH, temperature, and ionic strength. The phytase activity reaches saturation in presence of a small amount of substrate concentration if the K_m value is small while a large Km demonstrates requirement of higher sodium phytate concentrations to reach V_{max}. When binding site of enzyme is saturated with substrate it demonstrates the maximum velocity of the specific reactions.

DISCUSSION

A large number of studies have reported the phytase production from different microbial species with potential use in poultry feed (Oh *et al*., 2001; Zhang *et al*., 2011; Jorquera *et al*., 2011; Singh and Satyanarayana, 2015). Kumari *et al*. (2016) reported the production of phytase from thermophilic mould *Sporotrichum thermophile* as a potential candidate to be used in poultry feed. B propeller phytase from *Bacillus* spp. isolated from high temperature habitat has also been reported to have a significant importance in the feed industry (Choi *et al*., 2001; Tye *et al*., 2002; Wulandari *et al*., 2015). These studies support the idea of optimizing the production of phytase from thermostable *B. subtilis* KT004404 for large scale enzyme production to be used as poultry feed supplement (Powar and Jagannathan, 1982; Shimizu, 1992; Brown *et al*., 2011; Singh and Satyanarayana, 2015). Bacterial production of enzyme during submerged fermentation has multiple benefits such as easy growth, fast replication, and easier genetic manipulation for production of recombinant enzymes as compared to other sources such as plants, fungi and animals (Yang *et al*., 2017). *B. subtilis* is also reported to be benign as it doesn't possess the pathogenic or toxigenic traits which makes it as a better candidate to be used as source of exogenous enzyme (Mingmongkolchai and Panbangred, 2018). Although many studies have reported the use of bacterial and fungal species for phytase production with potential industrial application, however, this is the first study which has reported the use of statistical model (RSM) for optimization of parameters for phytase production from *B. subtilis* KT004404.

The amount of phytase production obtained through statistical model (response surface methodology) was 132.32 U/mL at 35 °C, pH 6.5, inoculum density of 2.5mL after incubating for 24h at 75 rpm. Multiple studies have utilized the centrally composite design (CCD) of response surface methodology (RSM) for optimization of production parameters for exogenous enzymes through different microbial resources to determine the interaction and impacts of various production parameters (Vohra and Satyanarayana, 2003; Pable *et al*., 2019; Kumari and Bansal, 2021). Thermostable enzymes have attracted profound interest in feed industry due to high temperature treatment during feed preparation and pelleting which affects the catalytic activity of exogenous enzymes (Vasudevan *et al*., 2019).

Enzymes that exhibit catalytic stability at acidic pH are of significant importance in poultry due to acidic pH of gizzard (pH=3.4) and intestine (pH=6.4) (Svihus, 2011). The phytase produced from *B. subtilis KT004404* reported during the current studies exhibited stable catalyzing potential between 4.0-7.0 pH due to which it is assumed that acidic pH of digestive tract of poultry will not be a limiting factor for in vivo phytase activity (Fengying *et al*., 2011).

In the current studies the optimal phytase production was found in the medium containing wheat bran as principal carbon source (Kumari and Bansal, 2021). These results are in agreement with previously conducted studies in which the wheat bran is reported to be an efficient source of carbon for bacterial metabolism for exogenous

enzyme production (Wang *et al*., 2011; Gupta *et al*., 2014; Shahryari *et al*., 2018). Bala *et al*. (2014) reported highest phytase yield when wheat bran (source of carbon), and ammonium persulfate (as a nitrogen source) were added in the fermentation medium during SSF using *H. nigrescens* BJ82. Several other studies also reported ammonium persulfate as suitable nitrogen source medium (Grothe *et al*., 1999; Bajaj and Singh, 2010). A major advantage of using the agricultural residues as compared to expensive synthetic media is a significant reduction in production cost for enzyme production at commercial scale specially in agricultural country such as Pakistan (Jabeen *et al*., 2019). Temperature is reported to be one of the most crucial factor for bacterial propagation and to maintain catalytic activity of enzymes (Vohra and Satyanarayana, 2003; Ajith *et al*., 2019). In the present study, the optimal enzymatic activity of phytase produced by *B. subtilis KT004404* was achieved at 35 °C. Our findings are in agreement with number of studies which have reported the optimal temperature for phytase activity produced from different microbial sources within temperatures range from 33-45 °C (Quan *et al*., 2001; Gunashree and Venkateswaran, 2008; Gaind and Singh, 2015; Sanni *et al*., 2019).

Demir *et al*. (2018) characterized 43.5 kDa phytase from *Lactobacillus coryniformis* MH121153 which exhibited optimal activity at 5.0 pH and 60 °C. Choi *et al*. (2001) reported the isolation of 44 kDa phytase from *Bacillus* sp. KHU-10 with optimal activity at 40 °C and optimal pH between 6.5–8.5 (Choi *et al*., 2001). Similarly, Fujiti *et al*. (2003) also reported the optimal pH for microbial phytase between 4.5-5.5 at 50 °C. The effect of different metal ions on the enzyme activity depends on the microbial species. The current study shows that phytase activity was declined in presence of metal ions. Similar results have been reported in numerous previously conducted studies (Roy *et al*., 2012; Yu and Chen, 2013). Phytase V_{max} values reported in the literature for *A. vacuum*. (55.9 nmol s^{-1}), *Rhizopus oligosporus* 32 nmol s^{-1}, *Bacillus licheniformis 4.7mM A. niger* 54.35 U/mL and *Geobacillus* sp. TF16 526.28 U/mg (Casey and Walsh, 2003; Chadha *et al*., 2004; Ekren and Metin, 2013; Fasimoye *et al*., 2014; Dokuzparmak *et al*., 2017) support values obtained during current studies (K_m= 0.175 mM and V_{max}= 250 U/mL).

Plant based feed stuff such as soybean, corn, and rapeseed contain a high proportion of phytic acid. Poultry birds are unable to indigenously catalyze the phytic acid to release phosphorous (Abbasi *et al*., 2019). Therefore, farmers have to rely on expensive inorganic phosphorous source to meet the phosphorus requirement of poultry which results in increased cost of feeding in poultry sector (Woyengo and Nyachoti, 2011). Several studies have reported positive impacts of using exogenous phytase supplements on the growth performance during poultry trials (Cowieson and Adeola, 2005; Selle *et al*., 2009; Ravindran, 2013). Due to the ability of phytase produced from *B. subtilis KT004404* to retain catalytic activity within a wide range of pH and temperature, it offers a potential use in the feed industry for improving the nutrient utilization and growth performance of poultry birds.

CONCLUSION

Bacillus subtilis KT004404 has been proven to be a potential candidate for producing exogenous phytase. As compared to OFAT, the RSM based CCD model resulted in 2.3-fold increase in phytase production in the optimized culture media. The results of the current study indicated that the temperature, pH and concentration of wheat bran are significantly important parameters for producing bacterial phytase. Due to the ability of phytase to withstand temperature and acidic pH condition, it appears to have a potential use in poultry industry for improving nutritional properties of feed.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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