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APPLICATION OF STATISTICAL DESIGN FOR THE ECONOMICAL PRODUCTION OF PHYTASE BY *ASPERGILLUS NIGER* USING SOLID STATE FERMENTATION

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ABSTRACT

The present study was aimed at high level and cost-effective production of phytase by *Aspergillus niger* under solid state fermentation adopting the response surface optimization method. Plackett-Burman design (PBD) and Central composite design (CCD) of Response surface methodology (RSM) were employed for screening and optimization of cultural conditions for maximum phytase production. Among 8 factors, incubation temperature, initial pH, incubation period, NH_4NO_3 and tween-40 were identified as significant factors by Plackett-Burman design (PBD) due to their positive effect on phytase production. Then, the optimization of these significant factors was conducted by Central Composite design (CCD), and incubation period (6 days), incubation temperature (35°C), initial pH (6), NH_4NO_3 (0.75%) and tween-40 (0.6%) were found to be optimum levels for best enzyme production. After statistical optimization, maximum amount of phytase production was obtained i.e. 406.45 IU/g, as it was 297.25 IU/g using conventional one factor at a time (OFAT) optimization approach. There was 1.37-fold increase in phytase yield using response surface methodology (RSM). These results indicated the efficacy of the response surface methodology to enhance phytase production by *Aspergillus niger* using solid state fermentation process.

Keywords: *Aspergillus niger*, Phytase enzyme, Response surface methodology (RSM), Solid state fermentation (SSF) and Agro-industrial waste

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INTRODUCTION

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) is the organic form of phosphorous that accounts for 60-80% of the total phosphorus present in cereal grains, oilseeds, legume seeds, pollen and nuts, and these grains and seeds are the main constituents of commercial animal feeds (Lott *et al*., 2000; Kumar and sushma, 2012; Sandhya *et al*., 2015). The non-ruminant animals such as poultry and fish cannot consume phytate bound phosphorus present in their diet due to the absence or insufficiency of phytate hydrolyzing enzyme in their digestive tract (Singh and Satyanaryana, 2010; Sandhya *et al*., 2015). Thus, most of the phytate present in their feed remains non-digested and excreted by these animals in the areas of abundant livestock and causes environmental pollution. To solve this problem, animal feed may be supplemented with phytic acid degrading enzyme i.e. phytase (Maguire *et al*., 2005; Yao *et al*., 2011).

Phytases (*myo*-inositol hexakis-phosphate phosphohydrolase) (EC 3.1.3.8) are the phosphohydrolytic enzymes that carry out the breakdown of phytic acid. Phytases usually produce lower *myo*-inositol phosphates, as well as release inorganic phosphate and important complexed minerals (Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and proteins), and in a few cases free *myo*-inositol during different stepwise reactions (Bohn *et al*., 2008; Azeke *et al*., 2011). By adding phytase enzyme in poultry diet, the availability of inorganic phosphorus and other minerals can be increased and thus the quantity of phosphorus that is excreted in their manure can also be reduced (Shah *et al*., 2009; Rasul *et al*., 2019; Zaheer *et al*., 2019).

Phytases are widespread enzymes and can be found in animals, plants and microorganisms. Microbial sources consist of many fungi, bacteria and yeast which can produce phytase using a great variety of substrates through fermentation process (Ahmad *et al*., 2017). Filamentous fungi have been investigated as a good source of phytases and have a higher production potential compared to bacteria. These fungi can lead to economic production of enzyme due to their ability to grow on various agro-residues in solid state fermentation (Salmon *et al*., 2012; Singh *et al*., 2015; Bakri *et al*., 2018). In fact, fungi are well-known for their abilities i.e., ease of cultivation, to produce large amounts of phytase with significant stability at low pH (Tahir *et al*., 2010).

Phytase producing microorganisms include filamentous fungi of the genus *Aspergillus*. Phytase from

different *Aspergillus* species such as *A. niger*, *A. oryzae* and *A. melleus* were reported (Wöstene*t al*., 2007) to have an important role in the breakdown of phytic acid into phosphate, mono-inositol, and minerals. The most remarkable and a commercial source for phytase production is *Aspergillus niger* (Liu *et al*., 2010). Hence, *Aspergillus niger* is most commonly used fungus for the production of phytase on a commercial scale in solid state fermentation (Pandey *et al*., 2000; Bhavsar *et al*., 2012; Gaind and Singh, 2015).

There is need to enhance the phytase production that can be achieved by applying statistical optimization technique rather than using only conservative, one parameter at a time (OPAT) approach. This is because, optimization of growth conditions using response surface methodology has many benefits i.e. multiple number of variables and their interactions can be studied simultaneously, optimum media formulation can be done with minimum number of experiments in short duration of time and maximum yield of the product can be obtained (Singh and Satyanarayan, 2006; Bhavsar *et al*., 2011).

Phytase enzyme has taken a very important position in biotechnological applications as it is used as an additive in the diets of non-ruminants e.g. poultry and fish to reduce the phytate content of fodder and commercial foods (Omogbenigun *et al*., 2003). Phyatse has many benefits such as, increase bio-availability of phosphorus and other important minerals in livestock feed, preserve non-renewable phosphorus sources by reducing its need of supplementation in diets and reduce environmental pollution (Yao *et al.*, 2011). Bhavsar *et al*. (2011) reported phytase production and response surface optimization by *A. niger* NCIM 563 using solid state fermentation (SSF). They obtained a 3.08-fold increase in phytase production after statistical optimization.

Keeping in view the increasing demand of phytase for food and feed industries, the aim of current study was to enhance the production of phytase employing statistical optimization approach and make the fermentation process cost-effective.

MATERIALS AND METHODS

Collection and maintenance of fungal cultures: *Aspergillus niger* was collected from the Microbiology Laboratory, PCSIR Laboratories complex, Lahore, cultured on freshly prepared Potato Dextrose Agar (PDA) slants at 37°C and stored in a cold cabinet at 4°C.

For inoculum preparation, *Aspergillus niger*'s colonies were scrapped after addition of sterilized distilled water in 5 days old fungal slants under aseptic conditions with an inoculation loop. The spore suspensions were homogenized and the number of spores (10^7 spores/ml) was adjusted with the help of a hemocytometer.

Solid state fermentation: The fermentation medium, containing rice polish, 0.1% KCl, 0.5% NH_4NO_3, 0.1% $MgSO_4.7H_2O$ and 0.1% $FeSO_4.7H_2O$, was placed in an Erlenmeyer flask (250 ml) and moistened with distilled water. After sterilization in an autoclave, the inoculation of growth medium was carried out with 10% (v/w) of inoculum under aseptic conditions and incubated at 35°C for 5 days (Mahmood *et al*., 2021).

Recovery of phytase: For extraction of crude enzyme, citrate buffer (0.2 M, pH 5.5) was added to each flask containing the fermented culture and agitated in a water bath shaker at 200 rpm for 90 min at 37°C. Muslin cloth was used to filter the suspension and the filtrate was centrifuged at 10,000 rpm for 15 min at 4°C, for removal of solid particulate matter. The clear supernatant was then used as crude enzyme extract for the estimation of phytase activity (Mahmood *et al*., 2021).

Phytase assay: Phytase activity was determined by estimating the quantity of inorganic phosphorus which was liberated from phytic acid (substrate) solution according to slightly modified method of (McKie and McCleary, 2016). One-unit of enzyme activity can be defined as the amount of enzyme that is needed to liberate 1 µ mole of inorganic phosphorus in one minute using the standard assay procedure.

Response Surface Methodology (RSM): Two statistical designs i.e. Plackett-Burman Design (PBD) and Central Composite Design (CCD) were used for the screening and optimization of culture conditions for maximum phytase production. This statistical procedure was done according to Nelofar *et al*. (2011), Suresh and Radha (2016) and Bhagat *et al*. (2019).

Identification of significant variables using Plackett-Burman Design (PBD): Plackett-Burman Design (PBD) was used for the identification and screening of culture conditions for phytase production. The independent variables, selected for the present research work through preliminary studies, and their experimental levels i.e., -1 (low level) and +1 (high level) with assigned values using PBD are presented in Table 1.

Table 2 shows the experimental design of PBD for the screening of independent variables for phytase production in 12 experimental runs. Every experiment was carried out in triplicate and the average of phytase production was considered as a response/yield (Y).

The effect of individual parameters on the phytase production can be calculated using following equation:

$$Y = \beta_0 + \Sigma \beta xi$$

Where Y is the phytase yield, β_0 is the model intercept, β xi is the linear coefficient and level of the independent variable.

Table 1. Experimental levels of independent variables for phytase production using Plackett-Burman design (PBD).

Independent Variables	Units	Low Level (-1)	High Level (+1)
Incubation period (X_1)	Days	2	8
Incubation temperature (X_2)	ºC	30	40
pH (X_3)		4.5	7.5
Inoculums size (X_4)	V/W %	5	25
Moisture content (X_5)	V/W %	20	100
Substrate amount (X_6)	g	5	25
NH_4NO_3 (X_7)	W/W %	0.25	1
Tween-40 (X_8)	W/W %	0.2	0.8

Table 2. Plackett-Burman experimental design showing the observed and predicted values for phytase production.

Run No.	Variables (X)								Phytase activity (IU/g)	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Observed	Predicted
1	+1	+1	+1	+1	+1	+1	+1	+1	350.65	358.75
2	-1	+1	-1	+1	+1	+1	-1	-1	167.85	162.87
3	-1	-1	+1	-1	+1	+1	+1	-1	189.00	187.54
4	+1	-1	-1	+1	-1	+1	+1	+1	290.60	282.49
5	-1	+1	-1	-1	+1	-1	+1	+1	214.89	208.75
6	-1	-1	+1	-1	-1	+1	-1	+1	178.25	179.70
7	-1	-1	-1	+1	-1	-1	+1	-1	134.52	144.85
8	+1	-1	-1	-1	+1	-1	-1	+1	265.70	269.60
9	+1	+1	-1	-1	-1	+1	-1	-1	272.50	277.47
10	+1	+1	+1	-1	-1	-1	+1	-1	338.45	335.70
11	-1	+1	+1	+1	-1	-1	-1	+1	212.48	213.25
12	+1	-1	+1	+1	+1	-1	-1	-1	281.55	275.41

X_1 = Incubation period (Days), X_2 = Incubation temperature (ºC), X_3 = Initial pH, X_4 = Inoculum size (v/w %), X_5 = Moisture content (v/w %), X_6 = Substrate amount (g), X_7 = NH_4NO_3 (w/w %), X_8 = Tween-40 (w/w %)

Optimization of selected variables using Central Composite Design (CCD): Five significant variables (incubation period, incubation temperature, initial pH, tween-40, NH_4NO_3) selected by PBD were studied for optimization of phytase production at five different levels i.e. -2, -1, 0, +1, +2 (Table 3). Central Composite Design (CCD) used for this study consisted of 32 runs as shown in Table 4. All the experiments were conducted in triplicate and the average of phytase production was taken as the response or yield (Y).

A second order polynomial model was used to calculate the predicted response:

$$Y = \beta_0 + \sum\beta_i Xi + \sum\beta_{ii} X_i^2 + \sum\beta_{ij} X_iX_j$$

Where Y is the predicted response (yield), β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, X_i and X_j are the coded independent variables which affect the response variable.

Statistical analysis: Statistical software package STATISTICA version 7 (Stat-Ease Inc., Minneapolis, USA) and MINITAB 17 were used to construct experimental design matrix, data analysis, quadratic model building and draw three-dimensional (3-D) graphs.

Table 3. Selected variables and their optimization levels for phytase production using Central composite design (CCD).

Independent Variables	Units	Level (-2)	Level (-1)	Level (0)	Level (+1)	Level (+2)
Incubation period (X_1)	Days	2	4	6	8	10
Incubation temperature (X_2)	ºC	25	30	35	40	45
pH (X_3)		4	5	6	7	8
NH_4NO_3(X_4)	W/W %	0.25	0.5	0.75	1.0	1.25
Tween-40 (X_5)	W/W %	0.2	0.4	0.6	0.8	1.0

Table 4. Central composite experimental design along with observed and predicted values for phytase production.

Run No.	Variables (X)					Phytase activity (IU/g)	
	X1	X2	X3	X4	X5	Observed	Predicted
1	0	0	0	0	0	386.80	396.61
2	0	-2	0	0	0	384.85	331.13
3	-1	1	-1	1	1	229.10	229.57
4	-1	-1	1	-1	-1	346.55	351.15
5	0	0	0	0	0	394.00	396.61
6	0	0	0	0	0	395.10	396.61
7	1	1	1	1	1	285.45	291.80
8	0	0	0	0	0	406.45	396.61
9	1	1	-1	1	-1	317.00	329.23
10	-1	-1	-1	-1	1	352.95	362.07
11	1	-1	-1	-1	-1	269.25	290.13
12	0	0	-2	0	0	377.65	351.09
13	-1	1	1	-1	1	241.50	226.64
14	0	0	0	0	-2	402.90	385.37
15	0	0	0	0	0	388.90	396.61
16	1	-1	-1	1	1	280.42	310.74
17	0	0	0	-2	0	304.95	309.04
18	1	1	-1	-1	1	303.90	305.32
19	-1	-1	-1	1	-1	357.30	377.22
20	0	0	0	0	2	366.85	352.05
21	-2	0	0	0	0	306.90	312.90
22	1	1	1	-1	-1	327.95	324.86
23	0	2	0	0	0	198.80	220.19
24	0	0	2	0	0	348.40	342.63
25	0	0	0	0	0	376.10	396.61
26	1	-1	1	-1	1	294.70	309.70
27	0	0	0	2	0	354.20	317.78
28	1	-1	1	1	-1	297.55	323.36
29	-1	1	1	1	-1	262.10	258.05
30	-1	1	-1	-1	-1	277.90	268.92
31	-1	-1	1	1	1	339.77	353.81
32	2	0	0	0	0	365.65	327.32

X_1 = Incubation period (Days), X_2 = Incubation temperature (ºC), X_3 = Initial pH, X_4 = NH_4NO_3 (w/w %), X_5 = Tween-40 (w/w %)

RESULTS

Statistical optimization of culture conditions was conducted to increase the phytase production and make the fermentation process economical.

Plackett-Burman design (PBD) for the identification of the significant variables: In the first step of response surface methodology (RSM), PBD model was used for screening of individual variables (incubation time, incubation temperature, initial pH, inoculum size, moisture content, substrate amount, NH_4NO_3 and tween-40) affecting the phytase production. Highest phytase production (350.65 IU/g) was seen with run No.1, while lowest phytase yield (134.52 IU/g) was observed with run No.7 (Table 2).

The analysis of variance (ANOVA) for screening of selected variable for phytase production is presented in Table 5 and those variables with a *p*-value of <0.05 such as, incubation time, incubation temperature, initial pH, tween-40 and NH_4NO_3 were considered as significant variables affecting the phytase production. PBD model has 0.9924 value for the coefficient of determination (R^2) and it can explain 99.24% of data variability in the response.

Pareto chart: Pareto chart was constructed to determine the significant factors for phytase production (Fig. 1). It is indicated that incubation time, incubation temperature, initial pH, tween-40 and NH_4NO_3 played significant role for phytase production and these variables were selected for further optimization studies.

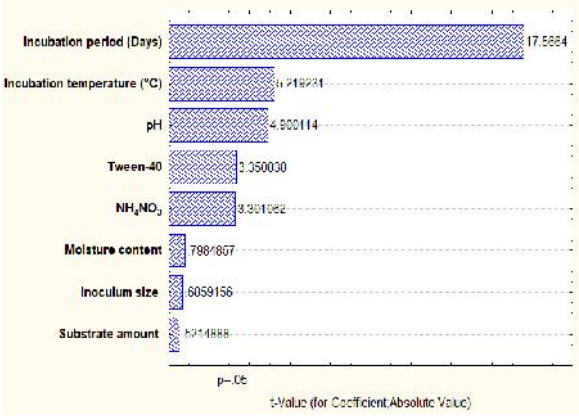


Fig. 1. Pareto chart explaining the influence of various independent variables on phytase production

Optimization of significant variables by Central composite design (CCD) for phytase production: Central composite design (CCD) was applied to optimize the levels of five selected variables and their interaction for phytase production. It is indicated from the results that there is a wide range among phytase yield (198.80 IU/g to 406.45 IU/g) in 32 trials. Maximum phytase production (406.45 IU/g) was found with run No. 8, whereas, lowest phytase production (198.80 IU/g) was found with run No. 23, as shown in Table 4.

The competency of the model was assessed using analysis of variance (ANOVA) and it was tested through Fisher's analysis. It was observed that individually, incubation temperature (X_2) and among interactions, incubation period and incubation temperature (X_1X_2) played very significant role for the production of phytase, as represented in Table 6. The coefficient of determination (R^2) of the model is 0.8762, which indicates that 87.62% of data variability in the response could be expressed by the model.

Table 5. Analysis of variance (ANOVA) for phytase production using Placket-Burman design (PBD).

SOV	SS	DF	MS	*F*-value	*P*-value
Intercept	913.21	1	913.21	7.016	0.077
Incubation period (X_1)	40163.17	1	40163.17	308.578	0.0004
Incubation temperature (X_2)	3545.48	1	3545.48	27.240	0.013
Initial pH (X_3)	3125.18	1	3125.18	24.011	0.016
Inoculum size (X_4)	61.24	1	61.24	0.470	0.542
Moisture content (X_5)	82.98	1	82.98	0.637	0.482
Substrate amount (X_6)	35.40	1	35.40	0.272	0.638
NH_4NO_3 (X_7)	1418.31	1	1418.31	10.897	0.045
Tween-40 (X_8)	1461.40	1	1461.40	11.228	0.044
Error	390.47	3	130.16		

(R^2 = 0.9924, R^2 (Adjusted) = 0.9724, R^2 = Coefficient of determination)
SOV = Source of variation, SS = Sums of squares, DF = Degree of freedom, MS = Mean sums of squares

Table 6. Analysis of variance (ANOVA) for phytase production using Central composite design (CCD).

SOV	SS	DF	MS	*F*-value	*P*-value
Intercept	4677.57	1	4677.57	4.434	0.058
X_1	1976.77	1	1976.77	1.874	0.198
X_1^2	10727.98	1	10727.98	10.171	0.008
X_2	11678.25	1	11678.25	11.072	0.006
X_2^2	26817.84	1	26817.84	25.426	0.0003
X_3	2404.23	1	2404.23	2.279	0.159
X_3^2	4536.87	1	4536.87	4.301	0.062
X_4	3739.10	1	3739.10	3.545	0.086
X_4^2	12689.53	1	12689.53	12.031	0.005
X_5	1313.13	1	1313.13	1.245	0.288
X_5^2	1426.67	1	1426.67	1.352	0.269
X_1 X_2	14301.17	1	14301.17	13.559	0.003
X_1 X_3	243.44	1	243.44	0.230	0.640
X_2 X_3	54.58	1	54.58	0.051	0.824
X_1 X_4	14.54	1	14.54	0.013	0.908

X_2 X_4	299.20	1	299.20	0.2836	0.604
X_3 X_4	2.00	1	2.00	0.002	0.966
X_1 X_5	69.10	1	69.10	0.065	0.802
X_2 X_5	933.15	1	933.15	0.884	0.367
X_3 X_5	19.47	1	19.47	0.018	0.894
X_3 X_5	311.61	1	311.61	0.295	0.597
Error	11601.96	11	1054.72		

(R = 0.936098, R^2 = 0.876280, R^2 (Adjusted) = 0.651336), R^2 = Coefficient of determination)
X_1 = Incubation period (Days), X_2 = Incubation temperature (ºC), X_3 = Initial pH, X_4 = NH_4NO_3 (w/w %), X_5 = Tween-40 (w/w %), SOV = Source of variation, SS = Sums of squares, DF = Degree of freedom, MS = Mean sums of squares

Pareto chart: Pareto chart was used to describe the influence of significant variables and their interactions on the phytase production. Pareto chart identified incubation temperature and interaction between incubation period and incubation temperature as important factors for phytase production (Fig. 2).

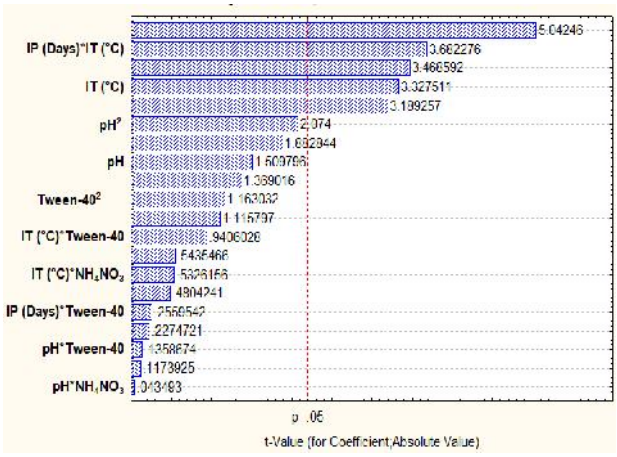
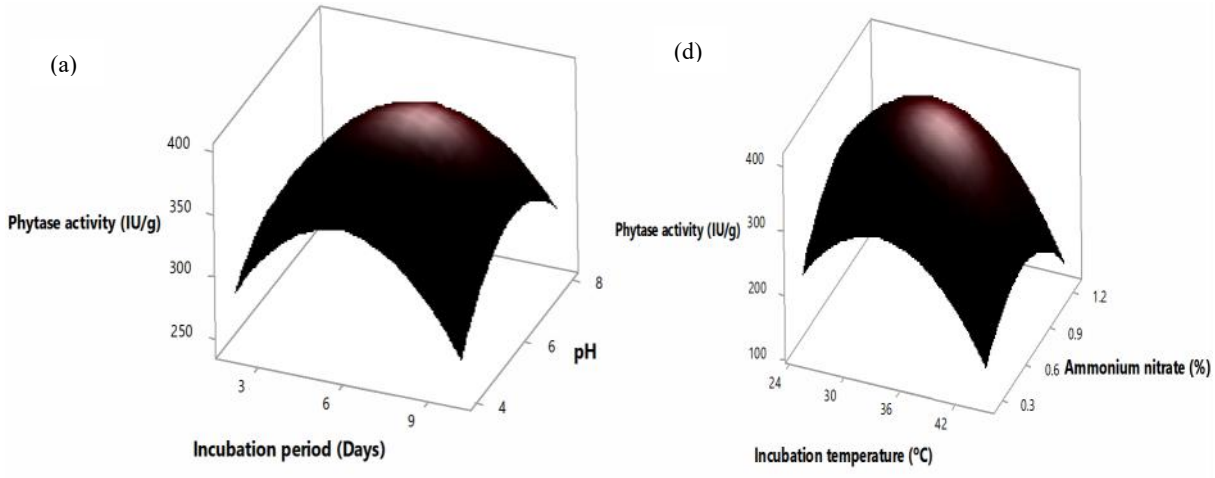


Fig. 2. Pareto chart explaining the influence of different significant variables and their interactions on the phytase production

Experiments were conducted and the response (phytase production) was calculated through second order polynomial regression equation as a function of the values of incubation period (X_1), incubation temperature (X_2), pH (X_3), NH_4NO_3 (X_4) and tween-40 (X_5). Phytase production can be estimated by the model given below:

Enzyme activity (IU/g) = -1410 - 61.7 X_1 + 70.6 X_2 + 153 X_3 + 679 X_4 + 503 X_5 - 4.78 X_1X_1 - 1.209 X_2X_2- 12.44 X_3X_3 - 332.8 X_4X_4 - 174 X_5X_5+ 2.990 X_1 X_2 + 1.95 X_1 X_3 + 1.9 X_1 X_4+ 5.2 X_1 X_5 - 0.37 X_2 X_3 - 3.46 X_2 X_4 - 7.64 X_2 X_5- 1.4 X_3X_4 - 5.5 X_3 X_5- 88 X_4 X_5

Three-dimensional (3-D) response surface curves were drawn to find out the optimum level of each variable and their interactive effects for maximum response (phytase production) by plotting the response on the z-axis against pairs of independent variables, while the other variables were kept constant at the level of zero (central values). The peaks in the 3-D plots can be used to identify the optimal values of each variable (Fig. 3a,b,c,d,e&f).These graphs showed that each parameter had significant effect on phytase production by *Aspergillus niger* in solid state fermentation.



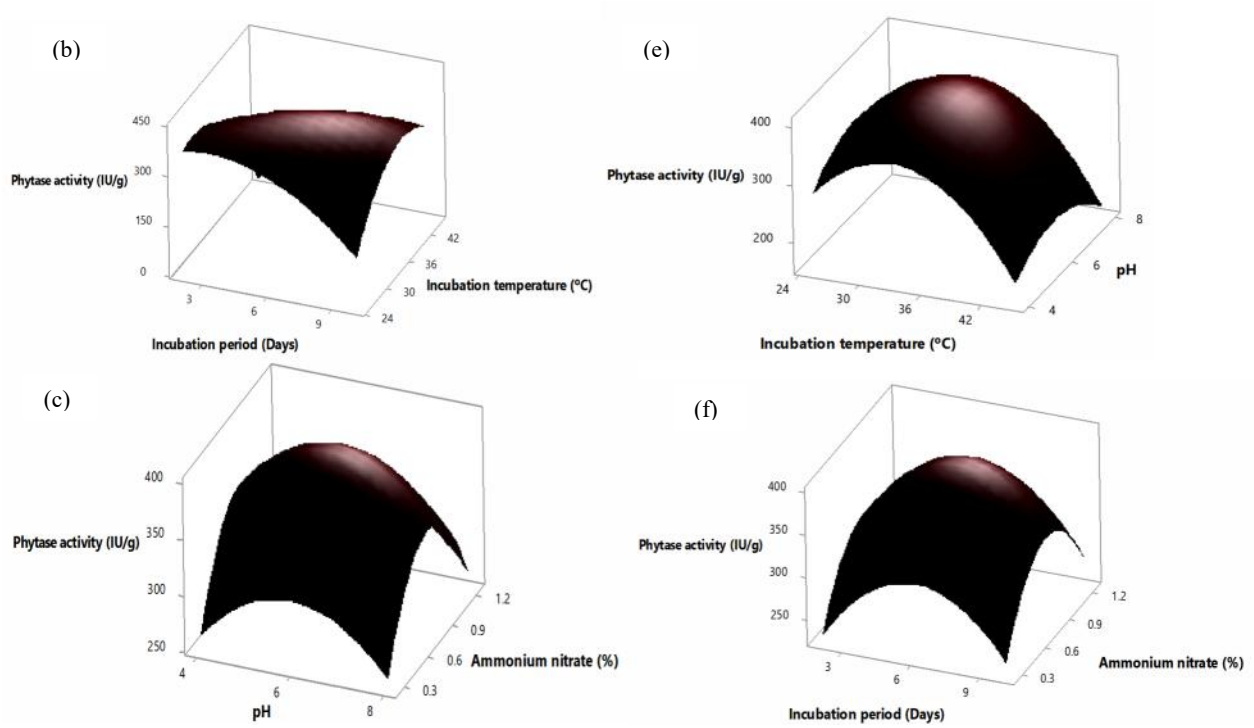


Fig. 3. Three-dimensional response surface graphs exhibiting the effect of (a) incubation period and pH, (b) incubation period and incubation temperature (c) pH and NH_4NO_3 (d) Incubation temperature and NH_4NO_3 (e) Incubation temperature and pH (f) Incubation period and NH_4NO_3 on phytase production in solid state fermentation (SSF).

DISCUSSION

Productivity of any fungal fermentation is affected by process parameters and media composition (Bhavsar *et al*., 2012) and therefore the present investigation was performed to statistically optimize the process parameters and medium components for the high-level production of the phytase from *Aspergillus niger* using Plackett-Burman design(PBD) and Central composite design (CCD) methodologies.

The conventional methods of optimization studies alone are ineffective, prolonged and expensive. Therefore, statistical optimization approach has been commonly used now a days for optimization of fermentation conditions in a few experiments in short duration of time (Mao *et al*., 2005; Nelofar *et al*., 2011) and also predicts the effects of interactions between fermentation factors that affects the response (yield)(Shahid *et al*., 2017).

All significant variables involved in phytase production were evaluated by PBD because it can test a large number of variables while avoiding the loss of any important information in subsequent optimization studies (Bhavsar *et al*., 2012). The results of Plackett-Burman design in the current study showed that there was a wide range of variation among the phytase production (134.52 IU/g to 350.65 IU/g) of all 12 runs (Table 2). This variation in the response was due to application of different combinations of cultural conditions and their interactive effects on phytase production. The highest phytase production i.e. 350.65 IU/g was obtained with best combination of these parameters. Based on analysis of pareto chart (Fig. 1) and analysis of variance (Table 5) of PBD, five key variables i.e. incubation period, incubation temperature, pH, NH_4NO_3 and tween-40 were identified as significant factors for phytase production and selected for further optimization studies by Central composite design (CCD).

Badoei-Dalfard *et al*. (2019) employed Plackett-Burman design and Central composite design for medium optimization for a thermostable, acidic - phytase production from *Bacillus tequilensis* Dm018, using response surface methodology (RSM). Statistical optimization approach leads to 2.3-fold increase in phytase production by *Bacillus* sp. Dm018.

After screening of significant variables for phytase production by PBD, a multifactorial response surface approach employing Central composite design (CCD), an effective design strategy, for studying the effects of key variables and their mutual interactions, was used. The results achieved from the Central composite design during this research work exhibited that phytase yield ranged from 198.80 IU/g to 406.45 IU/g in the 32

trials. These variations in phytase yield expressed the importance of optimization of culture conditions for obtaining maximum phytase yield. This study resulted in an overall 1.37-fold (297.25-406.45 IU/g) increase in phytase production using statistical optimization technique.

Plackett-Burman design and Central composite design were used by Jafari-Tapeh *et al*. (2012) to identify the important cultural parameters for higher phytase production by *Aspergillus ficuum* using solid state fermentation. Four selected factors and their optimum levels were 0.46% $MgSO_4$, 10.14% glucose, 62.69% moisture and 119.23 h incubation period. Using statistical approach, the yield of phytase was increased from 13.1 U/gds to 25.6 U/gds.

After statistical optimization, 1.3, 3.08, 2.07, 1.74, 1.85 and 5-fold enhancement in phytase production were achieved by *Rhizomucor pusillus* (Chadha *et al*., 2004), *Aspergillus niger* NCIM 563 (Bhavsar *et al*., 2011), *Sporotrichum thermophile* (Singh and Satyanarayan, 2006), yeast *Pichia anomala* (Vohra and Satyanarayana, 2002), *Mucor racemosus* (Bogar*et al*., 2003) and *Pichia anomala* (Kaur and Satyanarayana, 2005).

Conclusion: In this research work, the screening and optimization of growth parameters were successfully carried out for maximum phytase production from *Aspergillus niger* using a combination of Plackett-Burman design (PBD) with Central composite design (CCD). This study reported that incubation period, incubation temperature, initial pH, NH_4NO_3 and tween-40 are the significant factors for high phytase production. Due to statistical optimization, 1.37-fold of increase in phytase production was achieved. Thus, the statistical optimization methodology was confirmed to be an efficient and reliable technique for high level and economical production of phytase using solid state fermentation.

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