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*Research Article*

# Culture Condition Improvement for Phytase Production in Solid State Fermentation by *Aspergillus ficuum* Using Statistical Method

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The effective factors on phytase production by *Aspergillus ficuum* PTCC 5288 were studied using solid-state fermentation method in 250 ml shake flask. The effective process parameters on phytase production were identified using Plackett-Burman design. Four factors were identified of different variables, including glucose, moisture, $MgSO_4$, and fermentation time, which were the most significant. The optimum levels of these significant parameters were determined through response surface methodological approach as follows: 10.14% glucose, 62.69% moisture, 0.46% $MgSO_4$ and 119.23 h. The maximum predicted amount of phytase was 24.33 U/gds and the produced amount of phytase under these conditions was 25.6 U/gds, which indicates the efficacy of the model for prediction of phytase production content under different medium conditions.

## 1. Introduction

The main storage form of phosphorus in many seeds is phytate (myoinositol hexakisphosphate). In cereals, legumes and Brassicae, phytate was collected in the seeds during maturation and accounts for 50–80% of total phosphorus [1]. One of the main factors limiting the nutritional values of cereals and legumes in humans and animals is the interaction of phytic acid with essential dietary minerals (Mg, Ca, Zn, etc.), protein, or vitamins, which makes it an antinutritional factor. As a result, reducing the phytate content in plant materials to increase their nutritional values is advisable. This can help explain the increased interest in phytate-degrading enzymes [2, 3]. In order to increase the bioavailability of minerals in phytate containing seeds there is one alternative, which is phytic acid removal from the food products. Many processes have been displayed to remove phytic acid from seeds, but the best results are achieved by the use of enzymatic hydrolysis [4].

Phytases (EC 3.1.3.8 and EC 3.1.3.26) belong to the family of histidine acid phosphatases, which catalyses the hydrolytic degradation of phytic acid and its salts (phytates) also, generally yielding inositol, inositol monophosphate, and inorganic phosphate [5]. These enzymes may be either absent or present in very low levels in gastrointestinal tract of monogastric animals and almost all the phytate phosphorus ingested by these animals is excreted into the environment, leading to phosphorus pollution in areas of intensive livestock production [6].

Solid state fermentation (SSF) systems have generated much interest lately, because they offer some economical and practical advantages including higher product concentration, improved product recovery, very simple cultivation facilities, reduced wastewater output, lower capital investment, and lower plant operation cost [7, 8]. Despite several strains of bacteria, yeasts, and fungi having been used for production of phytase, two strains of *Aspergillus* sp., *A. niger* and *A. ficuum,* have most frequently been employed for commercial production [9].

The objective of this investigation was to identify the significant variables and further optimize their levels for phytase production by applying *Aspergillus ficuum* PTCC 5288 in solid state fermentation.

## 2. Materials and Methods

*2.1. Microorganism and Inoculum Preparation.* A spore suspension of *Aspergillus ficuum PTCC5288* was achieved by growing of organism on PDA at 24°C for two weeks and harvesting the spores with a known quantities of water containing 0.1% Tween-80 and then it was adjusted to $5 \times 10^6$ cfu/mL for inoculation, Therefore, the obtained spore suspension was frozen (−80°C) after addition of 23% glycerol as cryoprotectant.

*2.2. Substrate and Solid State Fermentation.* For the phytase production, the wheat bran (WB) was used as a substrate. Five grams of the dried substrate were taken into a 250 mL Erlenmeyer flask. By adding tap water, the substrate moisture was adjusted to the required level. The substrate was sterilized at 121°C and 15 psi for 15 min, cooled, and inoculated with various amount of spore suspension of *A. ficuum* strain. The contents were mixed completely and incubated at the suitable temperature. The experiments were directed on the basis of the statistical design. Variations in the process parameters were maintained according to the statistical design.

*2.3. Enzyme Extraction.* Crude enzyme (phytase) was extracted by mixing the fermented substrate with a known amount of distilled water including 0.1% Tween-80 on a rotary shaker (180 rpm) for 1 h. The suspension was then centrifuged at 7000 rpm at 4°C for 20 min and the supernatant applied for enzyme assay [10].

*2.4. Phytase Assay.* Phytase activity was assayed by measuring the amount of inorganic phosphorus which was released from sodium phytate solution by using the method of B. F. Harland and J. Harland (1980). One unit of enzyme activity was described as the amount of phytase needed to release one micromole of inorganic phosphorus per minute under the assay conditions [11].

*2.5. Identifying of the Significant Variables by Plackett-Burman Design.* To identify the critical parameters required for increasing enzyme production Plackett-Burman, a two factorial design, was used by screening N variables in N+ 1 experiment [12]. The statistical software package "MINITAB-15" was applied for generating the experimental design and for data analyzing. The experimental design with the name, symbol code, and actual level of the variables is illustrated in Tables 1 and 2.

*2.6. Optimization by Response Surface Methodology.* Central composite design (CCD) of response surface methodology (RSM) was applied to optimize the four most significant factors (glucose concentration, moisture, $MgSO_4$ concentration, and time) identified by the Plackett-Burman design to increase phytase production. The statistical software "Design Expert 7.1" was used to produce and analyze the experimental design. The influence of each independent variable were studied at five different levels (−2, −1, 0, +1, and +2; Table 3) and a set of 28 runs were conducted. All the variables were taken at a central coded value of zero. The minimum and maximum ranges of the variables were used and the full experimental plan by considering their values in actual and coded forms is given in Table 4. The response value ($Y$) in each experiment was the average of the duplicates. The experimental results of the CCD were suited with a second-order polynomial equation by the multiple regression procedure

$$Y = B_0 + \sum_{i=1}^{4} B_i X_i + \sum_{i=1}^{4} B_{ii} {X_i}^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} B_{ij} X_i X_j, \quad (1)$$

where $Y$ is the predicted response; $B_0$ is the intercept; $B_i$ is the linear coefficient; $B_{ii}$ is the quadratic coefficient; $B_{ij}$ is the interaction coefficient; $X_i$, $X_j$ are the coded independent variables, which affect the response variables. The quality of fit of the second-order model equation was described by the coefficient of determination ($R^2$), and its statistical significance was identified by an $F$-test. The significance of each regression coefficient was tested by a $t$-test.

Table 1: Experimental variables at different levels used for the production of phytase by *Aspergillus ficuum* using Plackett-Burman design.

| Variables | Unit | Low level (−) | High level (+) |
|---|---|---|---|
| Moisture (A) | % | 50 | 70 |
| Temperature (B) | °C | 25 | 35 |
| Particle size (C) | Mm | 0.2–0.6 | 1.0–1.4 |
| Time (D) | Hour | 48 | 144 |
| $MgSO_4$ (E) | g/g dry substrate % | 0 | 0.3 |
| $KH_2PO_4$ (F) | g/g dry substrate % | 0 | 0.01 |
| $(NH_4)_2SO_4$ (G) | g/g dry substrate % | 2 | 6 |
| Glucose (H) | g/g dry substrate % | 5 | 15 |
| Tween-80 (I) | g/g dry substrate % | 0 | 0.5 |
| NaCl (J) | g/g dry substrate % | 0 | 0.3 |
| Inoculum size (K) | mL | 0.5 | 2.5 |

*2.7. Validation of the Experimental Model.* The statistical model was validated considering phytase production under the optimum conditions predicted by the model in shake-flasks level and phytase activity was determined as expressed above.

## 3. Results and Discussion

*3.1. Screening the Significance of Cultural Conditions.* Table 2 illustrates the Plackett-Burman design for 11 selected variables and the corresponding response (i.e., phytase production) and Table 5 shows the influence of each variable along with the related coefficient, $P$ value, and $t$-value. A $P$-value less than 0.05 indicates that the model terms are significant. When the value of concentration effect of the tested variable is positive, the phytase production is greater at the high concentration tested, and when it is negative, the phytase

Table 2: Plackett-Burman experimental design for screening of significant process variables affecting phytase production.

| Run order | Variables | | | | | | | | | | | Phytase activity (U/gds) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | Replication 1 | Replication 2 |
| 1 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 15.0 | 13.3 |
| 2 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | 13.6 | 11.5 |
| 3 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | 0.2 | 0.0 |
| 4 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | 1.2 | 1.0 |
| 5 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 6.0 | 3.8 |
| 6 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 9.1 | 9.5 |
| 7 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 3.3 | 2.7 |
| 8 | 1 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 1.2 | 1.1 |
| 9 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | 8.9 | 9.4 |
| 10 | −1 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | 0.9 | 1.3 |
| 11 | 1 | 1 | 1 | −1 | 1 | 1 | −1 | 1 | −1 | −1 | −1 | 5.8 | 5.3 |
| 12 | −1 | 1 | −1 | −1 | −1 | 1 | 1 | 1 | −1 | −1 | −1 | 0.0 | 0.0 |

Table 3: Experimental variables at different levels used for RSM approach.

| Variables | Units | Symbol code | Levels | | | | |
|---|---|---|---|---|---|---|---|
| | | | −2 | −2 | 0 | +1 | +2 |
| Moisture | % (w/w) | $X_1$ | 55 | 60 | 65 | 70 | 75 |
| $MgSO_4$ | g/g dry substrate % | $X_2$ | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 |
| Glucose | g/g dry substrate % | $X_3$ | 4 | 7.5 | 11 | 14.5 | 18 |
| Time | Hours | $X_4$ | 72 | 96 | 120 | 144 | 168 |

production is more at the low concentration. The analysis showed that moisture, particle size, fermentation time, $MgSO_4$, $KH_2PO_4$, glucose, and Tween-80 have significant effects on phytase production. Fermentation time, $MgSO_4$, and initial moisture content have the most significant effects, respectively. Among the significant factors, moisture, time, $MgSO_4$, and glucose, which had positive effect on phytase production, were further studied by an RSM design.

*3.2. Optimization by Response Surface Methodology.* The four significant factors influencing phytase production were further optimized using RSM design. Considering the Plackett-Burman results, particle size of the substrate was kept constant at its lower level, that is, 0.2–0.6 mm. The mean predicted and observed responses are illustrated in Table 6. The regression equation coefficients were calculated and the experimental data were fitted to a second-order polynominal equation. The response (phytase activity) can be described as a function of the values of moisture ($X_1$), $MgSO_4$ ($X_2$), glucose ($X_3$), and fermentation time ($X_4$):

$$Y = 23.875 - 3.2833X_1 - 0.4X_2 - 0.5833X_3 - 0.0733X_4 - 7.0717X_1^2 - 0.9717X_2^2 - 2.5717X_3^2 - 8.4417X_4^2 - 0.75X_1X_4 + 3.05X_3X_4. \quad (2)$$

The ANOVA and model coefficients are illustrated in Table 6. The statistical analysis for the model (Table 6) showed the "lack of fit" was not significant ($P = 0.7837 > 0.05$). $P$ value for the model was less than 0.0001, indicating that the model was significant and could be applied to

Table 4: Exprimental design and the results of central composite design for phytase production.

| Run | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Phytase activity (U/gds) | |
|---|---|---|---|---|---|---|
| | | | | | Observed | Predicted |
| 1 | −1 | −1 | −1 | −1 | 22.4 | 21.95 |
| 2 | −1 | −1 | −1 | 1 | 20.6 | 21.39 |
| 3 | −1 | −1 | 1 | −1 | 19.4 | 19.52 |
| 4 | −1 | −1 | 1 | 1 | 21.6 | 22.00 |
| 5 | −1 | 1 | −1 | −1 | 21.5 | 21.35 |
| 6 | −1 | 1 | −1 | 1 | 20.0 | 20.79 |
| 7 | −1 | 1 | 1 | −1 | 18.7 | 18.92 |
| 8 | −1 | 1 | 1 | 1 | 21.2 | 21.40 |
| 9 | 1 | −1 | −1 | −1 | 18.4 | 18.52 |
| 10 | 1 | −1 | −1 | 1 | 16.9 | 17.20 |
| 11 | 1 | −1 | 1 | −1 | 17.0 | 16.74 |
| 12 | 1 | −1 | 1 | 1 | 18.0 | 18.47 |
| 13 | 1 | 1 | −1 | −1 | 18.2 | 18.32 |
| 14 | 1 | 1 | −1 | 1 | 16.8 | 17.00 |
| 15 | 1 | 1 | 1 | −1 | 17.0 | 16.54 |
| 16 | 1 | 1 | 1 | 1 | 17.3 | 18.27 |
| 17 | −2 | 0 | 0 | 0 | 20.3 | 19.76 |
| 18 | 2 | 0 | 0 | 0 | 13.5 | 13.19 |
| 19 | 0 | −2 | 0 | 0 | 23.3 | 22.98 |
| 20 | 0 | 2 | 0 | 0 | 22.7 | 22.18 |
| 21 | 0 | 0 | −2 | 0 | 22.0 | 21.56 |
| 22 | 0 | 0 | 2 | 0 | 20.8 | 20.39 |
| 23 | 0 | 0 | 0 | −2 | 15.7 | 16.49 |
| 24 | 0 | 0 | 0 | 2 | 19.3 | 17.66 |
| 25 | 0 | 0 | 0 | 0 | 24.4 | 23.87 |
| 26 | 0 | 0 | 0 | 0 | 24.0 | 23.87 |
| 27 | 0 | 0 | 0 | 0 | 23.2 | 23.87 |
| 28 | 0 | 0 | 0 | 0 | 23.9 | 23.87 |

Table 5: Results of regression analysis for Plackett-Burman design.

| Term | Coefficient | $t$ value | $P$ value |
|---|---|---|---|
| Intercept | 5.17 | 33.89 | <0.0001 |
| Moisture | 1.17 | 7.67 | <0.0001 |
| Temperature | −0.14 | −0.90 | 0.3852 |
| Particle size | −0.95 | −6.20 | <0.0001 |
| Fermentation time | 3.35 | 21.98 | <0.0001 |
| $MgSO_4$ | 2.25 | 14.72 | <0.0001 |
| $KH_2PO_4$ | −1.22 | −8.0 | <0.0001 |
| $(NH_4)_2SO_4$ | 0.15 | 1.01 | 0.3323 |
| Glucose | 1.11 | 7.29 | <0.0001 |
| Tween-80 | −1.20 | −7.89 | <0.0001 |
| NaCl | −0.20 | −1.28 | 0.2236 |
| Inoculum size | −0.26 | −1.72 | 0.1110 |

$R^2 = 0.96$.

Table 6: Analysis of variance (ANOVA) for the quadratic model.

| Source | Sum of squares | df | $F$ Value | Prob > $F$ |
|---|---|---|---|---|
| Model | 211.1123 | 10 | 127.256 | <0.0001 |
| $X_1$ | 64.68167 | 1 | 389.8933 | <0.0001 |
| $X_2$ | 0.96 | 1 | 5.786764 | 0.0286 |
| $X_3$ | 2.041667 | 1 | 12.30692 | 0.0029 |
| $X_4$ | 0.023048 | 1 | 0.138928 | 0.7142 |
| $X_1X_4$ | 0.5625 | 1 | 3.390682 | 0.0842 |
| $X_3X_4$ | 9.3025 | 1 | 56.07434 | <0.0001 |
| $X_1^2$ | 73.18313 | 1 | 441.139 | <0.0001 |
| $X_2^2$ | 1.381663 | 1 | 8.328495 | 0.0108 |
| $X_3^2$ | 9.678248 | 1 | 58.33931 | <0.0001 |
| $X_4^2$ | 65.78006 | 1 | 396.5143 | <0.0001 |
| Residual | 2.654333 | 16 | | |
| Lack of fit | 1.906833 | 13 | 0.58868 | 0.7837 |
| Pure error | 0.7475 | 3 | | |
| Total | 213.7667 | 26 | | |

$R^2 = 0.988$; Adj $R^2 = 0.98$.

monitor the optimization. The coefficient of determination ($R^2$) of the model is 0.988, which indicates that 98.8% of the variability in the response could be expressed by the model. Values of "Prob > $F$" less than 0.05 indicate that the model terms were significant. $X_1$, $X_2$, $X_3$, $X_3X_4$, $X_1^2$, $X_2^2$, $X_3^2$, and $X_4^2$ were significant model terms for phytase production.

To identify the levels of each variable for maximum phytase production, three-dimensional response surface plots were constructed by plotting the response (phytase production) on the $z$-axis against any two independent variables, while the other variables were kept constant at the level of zero (Figures 1–3). The shape of the response surface curves (Figures 1 and 2) illustrated an average interaction between these tested variables.

Figure 1 shows the interaction between moisture and fermentation time. Lower and higher levels of both moisture and fermentation time did not lead to higher enzyme activities. Primary moisture content is a critical factor for growth and production of enzyme. Moisture is an essential factor

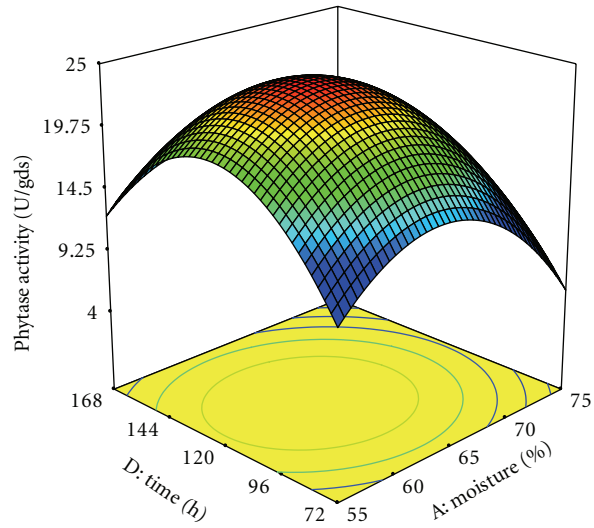


Figure 1: Contour plot to study the effect of moisture (%) and time (h) on the phytase production (U/gds) at $MgSO_4$ and glucose coded level of zero.

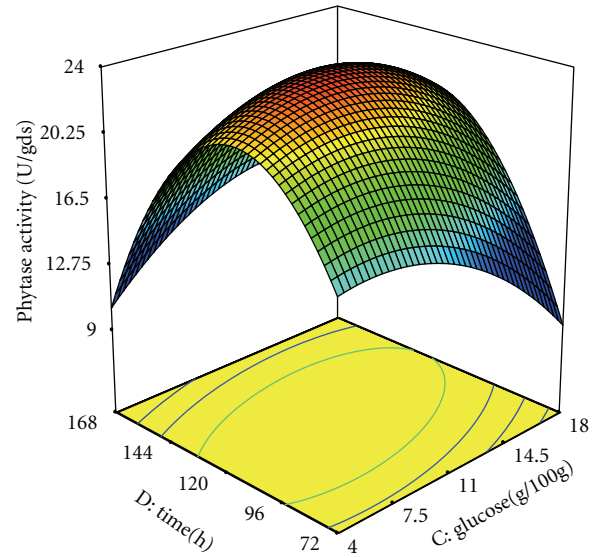


Figure 2: Contour plot to study the effect of glucose (%) and time (h) on the phytase production (U/gds) at $MgSO_4$(%) and moisture (%) coded level of zero.

for new cell synthesis; hence it is intimately related to SSF [13]. Filamentous fungi, when cultivated on agroindustrial residues during SSF, grow best when the substrate moisture content is commonly between 50 and 75% according to the type of fungi and substrate [10].

The response surface curve for the interaction of glucose and fermentation time is shown in Figure 2. The maximum production of phytase was after 120 h of incubation. A decrease in enzyme activity after this period may be the result of a reduction in nutrient availability in the medium or catabolic repression. Figure 3 illustrates the response surface plot achieved as a function of $MgSO_4$ concentration versus

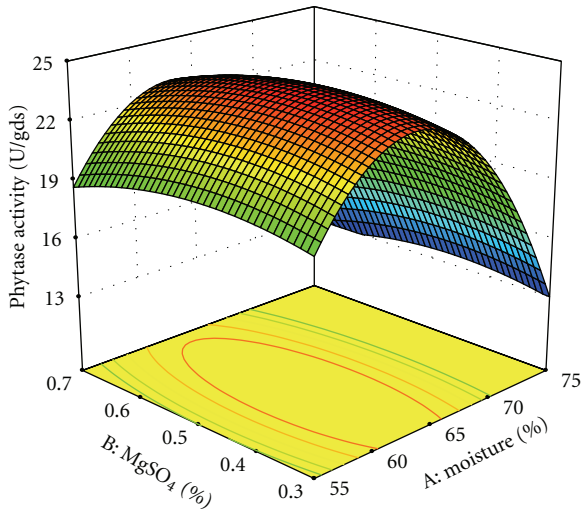


Figure 3: Contour plot to study the effect of moisture (%) and $MgSO_4$(%) on the phytase production (U/gds) at time (h) and glucose (%) coded level of zero.

moisture. It is obvious from Figure 3 that $MgSO_4$, concentration had no significant effect on phytase production in the studied range ($P < 0.01$).

According to Figures 1–3, almost the central values of moisture, fermentation time, $MgSO_4$ and glucose lead to maximum phytase activity.

*3.3. Validation of the Model.* In order to obtain the maximum activity of phytase, the optimization of the model was performed applying RSM autoanalysis software by setting the maximum phytase activity value ($Y$) as the objective. The resulting maximum phytase activity value ($Y$) of 24.33 U/gds was predicted under the following optimal conditions: moisture ($X_1$) was 62.69%, $MgSO_4$ ($X_2$) was 0.46%, glucose ($X_3$) was 10.14%, and fermentation time ($X_4$) was 119.23 h. To confirm the validity of the model, three assays were done under the stated optimal conditions. The estimated phytase value was 24.33 U/gds and the experimental phytase value was 25.6 U/gds indicating the efficacy of the model for prediction of the quantity of phytase production under different medium conditions.

## 4. Conclusion

In the present work, we demonstrated the optimization of *A. ficuum* culture conditions and media composition using Plackett-Burman and RSM designs that result in a substantial increase in phytase production activity. This study demonstrated the effect of different process parameters on the enzyme activity. Further, production of phytase was found to be significantly affected by moisture content, glucose, $MgSO_4$, and fermentation time. Using statistical approach, the phytase production could be enhanced from 13.1 U/gds in (nonoptimized media) to 25.6 U/gds in optimized media.

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