Phytase production through response surface methodology and molecular characterization of *Aspergillus fumigatus* NF191

Shivraj Singh Gangoliya, Raj Kishor Gupta & Nand Kumar Singh*

Department of Biotechnology, Motilal Nehru National Institute of Technology (MNNIT), Allahabad 211 004, Uttar Pradesh, India

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Phytase play an important role in phytic acid catalysis that act as a food inhibitor in cereals. Here, we isolated high phytase producing isolates NF191 closely related to *Aspergillus fumigatus* sp. from piggery soil. DNA was isolated from the fungal culture and amplified the ITS region using ITS1 and ITS4 primer using PCR. The 400-900 bp amplicon was gel eluted and subjected to sequencing. The sequencing results were assembled and compared with NCBI data base which showed the 99% identity of *Aspergillus fumigatus*. Different carbon sources viz., fructose, galactose, lactose, dextrose, sucrose, maltose and different nitrogen sources (organic & inorganic) NH$_4$Cl, NH$_4$NO$_3$, (NH$_4$)$_2$SO$_4$, KNO$_3$, NaNO$_3$, urea, yeast extract, peptone, beef extract were tested for optimal production. The 0.3% dextrose, 0.5% NH$_4$NO$_3$ and 96 h incubation time showed the best production and enzyme activity at 45 ºC incubation temperature. The selected parameters, dextrose, ammonium sulphate and incubation time, when employed with statistical optimization approach involving response surface optimization using Box Behnken Design, gave a 1.3 fold increase in phytase production compared to unoptimized condition.

**Keywords:** Box Behnken Design (BBD), Molecular characterization, Phytic acid, RSM

Phytase (myo-inositol hexakisphosphate phosphohydrolase) catalyzes the release of phosphate from phytate (myo-inositol hexakiphosphate), the prime source of phosphorus in cereal grains, legumes, and oilseeds$^{1,2}$. As a result, phytase can be included into swine, commercial poultry, and fish diets and has a broad range of applications in animal and human nutrition as it can reduce phosphorus excretion of monogastric animals by replacing inorganic phosphates in the animal diet; contribute significantly towards environmental protection$^3$ and lead to improved availability of minerals and trace elements$^{4,5}$.

Microorganisms are the best source for commercial production of phytase because of their easy cultivation and high yields of the enzyme, compared to plants$^{6,7}$. The feeding of microbial phytase to monogastric animals alter the phytic acid complexes and increase the bioavailability of phosphorus, calcium and probably proteins to monogastric animals. Currently, phytase-producing microorganisms include bacteria, such as *Bacillus subtilis* and *Escherichia coli*$^{8,9}$; yeasts, such as *Saccharomyces cerevisiae*; and fungi, such as *Aspergillus niger*, *A. oryzae*, *A. ficuum*. Yeasts produce phytase intracellularly and the yield is comparatively less. *A. niger* produces phytase extracellularly, known to be most active and is commercialized$^{3,10}$. The microorganism is subjected to submerged fermentation together with cereal grains, legume beans or foodstuffs so as to substantially remove or reduce phytic acid$^{11,12}$. However, the increased temperature within the fermenter during fermentation significantly decrease their efficiency of removing phytic acid$^{13,14}$. In addition, fungal cells grow slowly and take longer time to produce phytase having high enzymatic activity. Hence, there is a need for thermophilic phytase-producing microorganism which can remove phytic acid during submerged fermentation.

The industrial application of such a phytase-producing microorganism is promising as the phytase produced thereby is thermostable and fermentation using the same is efficient and energy saving$^{15,16}$. In the present study, we attempted to characterize and optimize the extracellular phytase production by *Aspergillus fumigatus* NF191 strain.

**Materials and Methods**

*Sample collection*—High organic matter soil samples were collected from poultry, piggery, wheat field and compost rich soil from Allahabad, U.P., India, placed in sterilized polythene bag, and stored at 4 ºC.
Initial screening of phytate degrading fungi—The samples were dissolved with sterilized water and processed using standard serial dilution technique, plated onto PSM plate (0.1% phytic acid calcium salt, 0.3% glucose, 0.5% NH₄NO₃, 0.05% KCl, 0.05% MgSO₄·7H₂O, 0.03% MnSO₄·4H₂O, 0.03% FeSO₄·7H₂O, 1.5% Agar, pH 5.5), and were incubated for 3 day at 30 °C. Those fungal isolates showing clear zone were selected for further quantitative screening. All positive isolates were maintained on potato dextrose agar and stored at 4 °C.

Quantitative screening and phytase production using submerged fermentation—The isolates were inoculated in a 50 ml PSM broth (0.1% phytic acid Ca salt, 0.3% glucose, 0.5% NH₄NO₃, 0.05% KCl, 0.05% MgSO₄·7H₂O, 0.03% MnSO₄·4H₂O, 0.03% FeSO₄·7H₂O & pH 5.5), at 45 °C for 4 days, and centrifuged at 3000 rpm for 5 min, and the supernatant collected as enzyme solution.

Phytase assay—Enzyme substrate was prepared with 1 ml crude enzyme mixed with 0.5 ml sodium acetate buffer (0.2 M, pH-4.5) and 0.5 ml sodium phytate (15 mM). To 1 ml enzyme solution, 1 ml enzyme substrate was added and allowed to react at 40 °C for 45 min, and at the end 2 ml trichloroacetic acid (TCA) (15%) was added to stop the reaction. Assay mixture of 0.5 ml was then mixed with 4 ml of 2:1:1 v/v of acetone, 10 mM ammonium molybdate and 5N H₂SO₄, and 0.4 ml of citric acid (1 M) and measured for absorbance at 400 nm. The standard curve was made using KH₂PO₄. A unit of phytase activity was defined as 1 μmol phosphate produced by 1 ml enzyme solution per minute. All the experiments were carried out in triplicate. The values reported are mean of three such experiments.

Molecular characterization of selected high phytase producing microorganism—DNA was isolated from the fungal culture following Liu et al., and was used in PCR to amplify the ITS region using ITS1 (5′TCCTCCGCTTATGATATGC3′) and ITS4 (5′TCCTCGATTATTAGATATGC3′) primers for identification and characterization of fungi through PCR amplification and sequencing. The 400-900 bp amplicon was gel eluted and subjected to sequencing. The sequencing results were assembled and compared with NCBI data base.

Optimization of process parameter for phytase production—Effects of various process parameters on phytase production by Aspergillus fumigatus NF191 that exhibited maximum enzyme activity after screening was estimated under submerged fermentation. The parameters optimized for enhanced phytase production were incubation time (72-120 h), carbon sources (maltose, dextrose, fructose, sucrose, galactose and lactose at 0.3%), and organic and inorganic nitrogen sources (ammonium nitrate, ammonium sulphate, ammonium chloride, sodium nitrate, potassium nitrate, urea, yeast extract, peptone and beef extract at 0.5%). We optimized the physical and chemical parameters through ‘one-at-a time’ approach (keeping the rest constant) to search the optimum level and further through statistical method using RSM approach for maximum enzyme production.

Optimization of phytase production by response surface methodology—By above means and based on preliminary studies, independent variables (dextrose, ammonium sulphate, incubation time, temperature and pH) were identified as most significant factors affecting phytase production and chosen for optimization by response surface methodology using Box–Behnken design (BBD) experiments. BBD is useful for optimization of a small number of variables at few levels. It is used to estimate the best fit parameters of the quadratic model, detection of lack of fit of the model, construction of sequential designs, and hence good design for RSM studies. Each variable was studied at three different levels (-1, 0, +1). A mathematical correlation between the three independent variables on phytase production was developed. The experimental data obtained from the design were fitted to a second-order polynomial equation. The dependent variable (phytase activity) can be described as a function of the values of independent variables X₁, X₂, X₃. The polynomial equation was as follows:

\[ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \]

where Y is the predicted production of phytase (U/ml) and X₁, X₂ and X₃ are independent variables. \( \beta_0 \) is the intercept, \( \beta_1, \beta_2, \beta_3 \) are linear coefficient, \( \beta_{11}, \beta_{22}, \beta_{33} \) are square coefficients and \( \beta_{12}, \beta_{13}, \beta_{23} \) are cross product coefficients.

The statistical software package MINTAB 16 was applied for constructing the design matrix data analysis and generating response surface plots.

Results and Discussion

Isolation and screening of phytate degrading fungi—Fifty fungal isolates were screened out through halo zone formation in 0.1% calcium phytate containing phytase specific media (PSM). These fungal isolates have shown clear halo zone formation.
in PSM that presented the particular microorganism having ability to hydrolyse phytic acid into myo-inositol mono, di, tri, tetra or penta phosphate. The fungal isolates that had clear halo zone in PSM were put to secondary screening using submerged fermentation. By quantitative determination of phytase activity, among 50 isolates, 8 isolates from different sources confirmed better activity in which only NF191 produced maximum phytase (Table 1). As a result, this isolate was chosen for further investigations.

**Molecular characterization of NF191 fungal isolate**—The DNA of this selected strain was isolated by rapid mini preparation method. Internal transcribed spacer (ITS) sequencing was selected for identification and characterization of fungi. ITS region is widely used in taxonomy and molecular phylogeny as it has a high degree of variation even between closely related species. Based on conserved regions of 18S rDNA, established universal fungal primers (ITS1: 5’TCCGTAGGGTAACTGCGG3’ ITS4: 5’TTCCTCCGTTATTGATATGC3’) are available and large numbers of 18S rDNA sequence are available in NCBI GenBank that makes similarity search easy. Characterizations of fungi on the basis of 18S rRNA gene sequence analysis have been reported by several researchers. The DNA used in PCR to amplify the ITS region using ITS1 and ITS4 primers and the ITS sequencing for NF191 isolate (Fig. 1) showed 99% identity at 100% coverage with the sequence from *Aspergillus fumigatus* strain P2BL1 (Fig. 2), the sample isolate NF191 is most probably *Aspergillus fumigatus*.

**Optimization of process parameter**—Submerged fermentation (SmF) utilizes free flowing liquid substrates, such as molasses and broths. An additional advantage of this technique is that purification of

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### Table 1—Sources and phytase activities of different fungal isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Isolated source</th>
<th>Enzyme activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF191</td>
<td>Piggery</td>
<td>80.2 ± 0.3</td>
</tr>
<tr>
<td>NF411</td>
<td>Poultry</td>
<td>79.0 ± 0.6</td>
</tr>
<tr>
<td>NF141</td>
<td>Poultry</td>
<td>76.3 ± 0.4</td>
</tr>
<tr>
<td>NF381</td>
<td>Wheat field</td>
<td>75.6 ± 0.4</td>
</tr>
<tr>
<td>NF363</td>
<td>Compost</td>
<td>75.2 ± 0.5</td>
</tr>
<tr>
<td>NF391</td>
<td>Poultry</td>
<td>72.7 ± 0.6</td>
</tr>
<tr>
<td>NF194</td>
<td>Piggary</td>
<td>68.3 ± 0.7</td>
</tr>
<tr>
<td>NF351</td>
<td>Compost</td>
<td>66.3 ± 0.3</td>
</tr>
<tr>
<td>MTCC 8652</td>
<td>MTCC</td>
<td>70.3 ± 0.4</td>
</tr>
</tbody>
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**Fig. 1**—ITS sequencing for NF191 isolate  
**Fig. 2**—Phylogenetic relationship of NF191 isolate
products is easier. SmF is primarily used in the extraction of secondary metabolites that need to be used in liquid form. Phytase was obtained using submerged fermentation from *Thermoascus auranticus* and its activity was found to be higher.

Carbon sources in easily available form are required for growth initiation and metabolism in fungi. Of the different carbon sources tested for their effect on phytase production, dextrose achieved maximum phytase productivity of 77.8 U/ml and lactose showed the lowest, 42.7 U/ml (Fig. 3a). Dextrose support phytase production by *Aspergillus adeninivorans; Candida krusei* and *Aspergillus niger* NCIM has been reported earlier. Supplementation with nitrogen source enhanced the phytase production. Among the different nitrogen sources used, the (NH₄)₂SO₄ was the most favourable nitrogen source for phytase production which exhibited maximum productivity (87.8 U/ml), while the beef extract had lowest productivity, 30.7 U/ml (Fig. 3b).

Incubation period is one of the major factors in cost effective enzyme production. To get optimum phytase activity, fermentation was carried out for 2-7 days. The highest phytase production was found after 4 days of incubation (Fig. 3c).

Response Surface Methodology (RSM) approach towards reach optimum condition—The PSM components dextrose, ammonium sulphate and incubation time (Table 2) identified as the significant variables for phytase production on the basis of preliminary study, were further optimized by RSM using Box-Behnken design (BBD). The experimental design in coded level, experimental and predicted results of phytase activity are shown in Table 3 and analysis of variance (ANOVA) for phytase activity is shown in Table 4. Highest phytase production was seen with run order number 10 using 0.3% dextrose, 0.5% ammonium sulphate and 4 day incubation time. The maximum experimental phytase production by *Aspergillus fumigatus* NF191 was 101.79 U/ml while the predicted value was 85.30 U/ml. The experimental results of phytase production were incorporated into MINITAB 16 and analysis was carried out.

Identification of the levels of each variable for maximum phytase production, 3D response surface plots were constructed by plotting the phytase activity.
production on the z-axis against any two independent variables. The 3D response surface plots showing the optimal levels and nonlinear interactions among the variables for phytase production are shown in figs (4 and 5). The shape of the response surface curves demonstrated an average interaction between the tested variables and the optimum values of each variable were identified on the basis of 3D plot. The phytase yield varied significantly upon changing the level variables and the central values of these variables lead to maximum phytase activity.

**Conclusion**

The present study established that *Aspergillus fumigatus* NF191 isolated from piggery soil produces phytase efficiently in submerged fermentation. SmF is an efficient, less costly, simple and directly applicable for Optimization of different process parameter resulted in considerable increase in phytase production. Further, phytase production was significantly influenced by dextrose, ammonium sulphate and incubation time. Response surface optimization using BBD gave a 1.3 fold increase in phytase production compared to unoptimized approach. The statistical optimization revealed an increase of phytase production by 1.3 fold for *Rhizomucor pusillus*, 1.7 fold for *Aspergillus ficuum* and 1.8 fold for *Mucor racemosus*. These observations suggest that every fungus requires different nutrient and physicochemical parameter for growth and production of enzyme and each fungal strain needs optimization. The
phytase produced by *Aspergillus fumigatus* has potential application for reduction of phytic acid in animal feed.

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**References**