Effect of Nutritional Supplementation of Solid State Fermentation Medium on Biosynthesis of Phytase from *Aspergillus niger* NCIM 612

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Received 21 January 2014; revised 24 June 2014; accepted 15 July 2014

Extracellular phytase production by *Aspergillus niger* NCIM 612 was studied under solid state fermentation (SSF). Effect of supplementation of nutrients, such as, carbon, nitrogen, inorganic phosphate, surfactant and metal salt, on phytase production was investigated. Results showed that glucose (2.5 % w/w) as carbon source, ammonium nitrate (0.5 % w/w) as nitrogen source, potassium dihydrogen phosphate (0.2 % w/w) as phosphate source and ferrous sulphate (0.01 % w/w) as metal source were found to enhance phytase production by 1.13 fold, 1.16 fold, 1.07 fold and 1.17 fold, respectively. None of the surfactants favored phytase yield.

**Keywords**: fungal phytase, SSF, nutritional parameters, inorganic phosphate

**Introduction**
Phytic acid serves as the storage form of phosphorus in plant seeds as well as in feed ingredients like cereals and legumes. But fishes and monogastric animals such as poultry and humans are unable to utilize phosphorus from phytic acids due to lack or low phytase activities in their digestive system. The undigested phytate is passed into faecal waste and causes water and environmental pollution. Due to the antinutritive property of phytic acid, it forms insoluble complexes with calcium, zinc, magnesium, iron, decreasing their bioavailability. Phytases (EC 3.1.3.26) catalyze the stepwise dephosphorylation of phosphomonoester bonds of phytic acid to produce myo-inositol phosphate derivatives and inorganic phosphate. Therefore, supplementation of animal feed with phytases will improve phytate digestibility and nutritive value of feeds as well as reduces environmental phosphorus pollution. Production of enzymes in submerged fermentation (SmF) has long been established. Solid state fermentation (SSF) has recently gained importance due to several advantages over SmF like, simple process control, less expensive method, utilization of agricultural residues or wastes as substrate, less effluent generation and higher productivity. Effective utilization of agro-industrial residues in SSF will not only reduce disposal and environmental pollution problem but also causes value addition to these wastes. Since, some of the nutrients may be available in sub-optimal concentrations or even absent in the substrate; their supplementation is required with substrate. Secretion of enzyme is dependent on the growth rate and availability of carbon and nitrogen sources in the medium. Thus, the correct nutrient conditions should be optimized for maximum enzyme production. Therefore, in this study, the effect of nutritional conditions on extracellular phytase production by *Aspergillus niger* NCIM 612 was investigated under SSF conditions using different carbon, nitrogen, inorganic phosphate and metal sources. The compound giving highest phytase activity was further studied by varying its concentration to determine the optimum concentration in respective cases.

**Materials & Methods**

**Microorganism**
*Aspergillus niger* NCIM 612 was maintained on Potato Dextrose Agar slants by monthly subculturing and stored at 4°C. Spore suspension was prepared using 7 day old spores in sterile normal saline containing 0.01% (v/v) Tween 80.

**Chemicals**
Phytic acid sodium salt was purchased from Sigma-Aldrich Chemicals Private limited, USA. All other chemicals used were of analytical grade.
Solid state fermentation

5 gm rice straw was taken in Erlenmeyer flask (250 ml) and supplemented with distilled water (1:5 w/v) to obtain required moisture prior sterilization. The medium was inoculated with fungal spores (10^6 spores/g substrate) and incubated for 7 days at 30°C under static condition. Crude enzyme was extracted with buffer followed by centrifugation.

Phytase assay

Enzyme-substrate reaction was carried out following Sigma protocol with little modifications. The reaction was carried out for 30 minutes at 55°C and was stopped by adding 10% TCA and the liberated inorganic phosphate was measured according to King’s method. 1 Unit of phytase activity (IU) was expressed as the amount of enzyme liberating 1 micromole phosphorus per minute under the assay condition. Enzyme activity was expressed as U/g dry substrate (U/gds).

Biomass estimation

Glucosamine content of mycelia was taken as an equivalent value for biomass amount and growth. It was measured according to the method of Sakurai and expressed as mg glucosamine/gds.

Optimization of nutritional parameters

Effect of carbon sources

Fermentation was carried out with various carbon compounds, such as, dextrose, fructose, mannose, xylose, arabinose, maltose, sucrose, cellobiose, raffinose, starch, xylitol, mannitol and rhamnose at 1% w/w and enzyme assay was done.

Effect of nitrogen sources

Various nitrogenous compounds, such as, ammonium nitrate, sodium nitrate, potassium nitrate, ammonium sulphate, ammonium chloride, beef extract, peptone, yeast extract, tryptone, urea were supplemented with the substrate at 0.5% w/w and enzyme activity was measured after fermentation.

Effect of inorganic phosphate

The effects of sodium dihydrogen phosphate, ammonium dihydrogen phosphate, potassium dihydrogen phosphate, diammonium hydrogen phosphate, dipotassium hydrogen phosphate, were studied by adding them in medium at a concentration of 0.2% (w/w).

Effect of Surfactants

Triton X-100, Tween-20, Tween-80, EDTA and SDS were added to SSF medium at 0.5% (w/w) concentration for fermentation.

Effect of Metal salt

Various metal salts, such as, copper (II) sulphate, ferrous sulphate, magnesium sulphate, nickel sulphate, zinc sulphate, potassium chloride, sodium chloride, ammonium chloride, cobalt chloride, calcium chloride, ferric chloride at 0.01% w/w concentration were supplemented with the substrate and enzyme yield was determined after fermentation.

Statistical Analysis

Each experiment was carried out in triplicate. Data are presented as mean ± SD. Graphs were prepared using Origin 6.0. Statistical significance analysis was done using InStat 3.06. Values were considered significant at p<0.05 and are indicated as asterisk in figures and tables.

Results & Discussion

Effect of carbon sources on phytase production

Maximum phytase activity 120.88 U/gds was obtained with glucose compared to control of activity 110 U/gds (Graph 1). Similar result was reported by Ramachandran et al. Fructose, xylose, mannose, mannitol, raffinose and rhamnose did not favor enzyme production. Better phytase production was obtained with cellobiose (115.15 U/gds) and Xylitol (115.15 U/gds) compared to control. Phytase production was inhibited by sucrose (87.65 U/gds) and starch (65.88 U/gds) and that did not correlate with the reports by others.

2.5% (w/w) Concentration of glucose showed maximum enzyme production (125.2 U/gds) and fungal growth (11.2 mg glucosamine/gds) compared to control (Table 3).

Graph 1—Effect of supplementation of different carbon sources on production of enzyme and cell growth. Results are presented as mean ± SD (n = 3). Asterisks indicate significant increase (p<0.05) in phytase production as compared to control (110 U/gds).
Effect of nitrogen sources on phytase production

Among all the nitrogen sources added, the highest enzyme production (128.1 U/gds) was observed with 0.5 % (w/w) ammonium nitrate compared to control (109.44 U/gds) and biomass (12.4 mg glucosamine/gds) over control (8.8 mg glucosamine/gds) (Graph 2). Similar result was reported by others\textsuperscript{24,31}. However, higher concentrations of ammonium nitrate were ineffective (Table 3). Other effective nitrogen sources were ammonium sulfate (126.24 U/gds), beef extract (124.35 U/gds), peptone (122.65 U/gds) and yeast extract (117.69 U/gds).

Effect of inorganic phosphate on phytase production

Table 1 illustrates the production of phytase using different inorganic phosphate sources, such as dipotassium hydrogen phosphate, potassium dihydrogen phosphate, diammonium hydrogen phosphate, sodium dihydrogen phosphate and ammonium dihydrogen phosphate. These compounds provide phosphorus at the level of 0.027\% (w/w), 0.045\% (w/w), 0.046\% (w/w), 0.051\% (w/w) and 0.053\% (w/w), respectively. It was observed that potassium dihydrogen phosphate gave highest phytase production (115 U/gds) compared to control (109.2 U/gds). Also, phytase production decreases with increasing phosphorus concentration. This observation agrees with the report that inorganic phosphate acts as a regulator of phytase synthesis\textsuperscript{32-36}. However, inspite of having lowest phosphorus concentration (0.027\% w/w), dipotassium hydrogen phosphate did not favor phytase production.

On further studies with varying concentrations of KH$_2$PO$_4$ (0.02 % - 0.4 \% w/w), no increase in production of phytase was observed at lower or higher concentration than 0.02\% (w/w) (Table 3). Similar effect was observed with \textit{Aspergillus ficuum}\textsuperscript{37,38}. However, cellular growth was not inhibited at high concentrations of potassium dihydrogen phosphate.

Effect of surfactants on phytase production

Supplementation of surfactants showed inhibition of enzyme production (data not shown) which correlates with the report of Gunashree \textit{et al.}\textsuperscript{29}.

Effect of Metal salts on phytase production

All the supplemented metal salts (0.01\% w/w), enhanced phytase production (114.23 U/gds – 128.79 U/gds) compared to control (110 U/gds) (Table 2). Ferrous sulphate enhanced biomass (12.5 mg glucosamine/gds) as well as phytase production (128.79 U/gds) maximally (Table 2 & 3). There is a report that metal salt supplementation did not

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
Inorganic phosphate salts & Phytase activity (U/gds) & Biomass (mg glucosamine / gds) \\
\hline
Control & 109.2±0.4 & 8.8±0.66 \\
K$_2$HPO$_4$ & 109.6±0.5 & 9.8±0.33 \\
KH$_2$PO$_4$ & 115±0.3 & 11.8±0.13 \\
(NH$_4$)$_2$PO$_4$ & 113.6±0.67 & 9.8±0.22 \\
NaH$_2$PO$_4$ & 111.6±0.47 & 9.8±0.32 \\
NH$_4$H$_2$PO$_4$ & 110±0.8 & 10.5±0.03 \\
\hline
\end{tabular}
\caption{Effect of different inorganic phosphate salts in phytase production and biomass. [●] indicates significant increase (p<0.05) in phytase production as compared to control (109.2 U/gds).}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
Metal salts & Phytase activity (U/gds) & Biomass (mg glucosamine / gds) \\
\hline
Control & 110±2 & 9±0.66 \\
CuSO$_4$ & 127.49±0.49 & 12±0.16 \\
FeSO$_4$ & 128.79±0.29 & 12.5±0.36 \\
MgSO$_4$ & 125.05±0.05 & 11.8±0.63 \\
NiSO$_4$ & 122.69±0.39 & 10.1±0.11 \\
ZnSO$_4$ & 126.92±0.42 & 10.2±0.24 \\
KCl & 126.92±0.92 & 12±0.43 \\
NaCl & 125.05±0.05 & 11±0.32 \\
NH$_4$Cl & 122.69±0.15 & 10±0.23 \\
CaCl$_2$ & 126.92±0.57 & 10±0.61 \\
CoCl$_2$ & 114.23±0.23 & 9.6±0.42 \\
FeCl$_3$ & 122.69±0.71 & 10±0.36 \\
\hline
\end{tabular}
\caption{Effect of different metal salts on phytase production and biomass. [●] indicates significant increase (p<0.05) in phytase production as compared to control (110 U/gds).}
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New Delhi, for this research work.

Acknowledgements

The authors gratefully acknowledge the financial assistance of University Grant Commission, New Delhi, for this research work.

References


