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ORIGINAL PAPER

Effect of different cultural conditions for phytase production by *Aspergillus niger* CFR 335 in submerged and solid-state fermentations

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Abstract The present article deals with the studies on the effect of media ingredients, such as carbon, nitrogen, inorganic phosphates, surfactants, and metal salts, on phytase enzyme production by *Aspergillus niger* CFR 335 in submerged (SmF) and solid-state fermentations (SSF). The results obtained showed a 1.5-fold higher enzyme yield in the presence of sucrose in both SmF and SSF, while peptone was found to be a favorable nitrogen source for SmF. Sodium dihydrogen phosphate (NaH_2PO_4) favored 34% higher enzyme yield than the control, which was followed by 19% higher activity in potassium dihydrogen phosphate (KH_2PO_4) in SSF at 0.015% w/v. The addition of Tween-20 in SmF showed a maximum yield of 12.6 U/mL while, SDS suppressed the growth of the fungus. None of the surfactants favored the enzyme yield in SSF. Calcium chloride ($CaCl_2$) was extensively efficient in stimulating more than 55% higher phytase production in SmF at 0.01% v/v. In SSF, none of the metal salts stimulated phytase production.

Keywords Extracellular phytase · Phytic acid · Monogastrics · *Aspergillus niger* CFR 335 · Media ingredients

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Introduction

Phytic acid is an antinutritional factor that forms 1–2% of most of the seeds and their co-products representing >60% of their total phosphorus [27]. Monogastric animals (chickens, swine and humans) are unable to utilize phytate phosphorus either due to lack of or insufficient amount of phytate degrading enzymes. Since phosphorus is an essential nutrient for bone formation, deficiency of this mineral would lead to osteoporosis [19, 41]. The most striking chemical impact of phytic acid is its strong chelating ability with multivalent cations to form cation–phytic acid complexes [36]. The negatively charged phytic acid chelates with positively charged divalent cations rendering a poor absorption of the bound metals in small intestine [27]. Minerals of concern in this regard include phosphorus, calcium, zinc, iron, copper, magnesium, etc. In the context of animal nutrition, the following two aspects of phytic acid are critically important [55]: (1) monogastric animals have low levels of phytate-degrading enzymes in their digestive tracts and (2) phytic acid, an antinutrient, forms complexes with proteins and a variety of metal ions, thereby decreasing the dietary availability of these nutrients. Thus, inorganic phosphorus, a non-renewable and expensive mineral is supplemented in the diets of swine, poultry, and fish to meet their nutrient requirement of phosphorus [43].

Phytic acid is also partially attributed to the wide-spreading human nutritional deficiencies of calcium, iron, and zinc in developing countries where the staple foods are of plant origin [29]. Supplementation of diets with inorganic phosphorus along with the excreted phytate phosphorus, however, imposes global ecological problems when enters into rivers resulting in cyanobacterial blooms, hypoxia, and death of marine animals [28, 34]. The supplementation of phytase in fodder improves the phosphorus bioavailability

and also reduces its excretion in the areas of intensive livestock [60]. Thus, for both environmental and economic reasons phytases and phytase producing microbes are attracting significant industrial interest. At the end of twentieth century, annual sales of phytase as animal feed additive were estimated to be US $500 million, rising further [1].

Phytases (*myo*-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8 and EC 3.1.3.26) are a special class of phosphatases that catalyze the hydrolysis of phytic acid in a stepwise manner to lower inositol phosphates, *myo*-inositol and inorganic phosphate, all utilizing a phosphohistidine intermediate in their phosphoryl transfer reaction. Phytases are found naturally in plants and microorganisms and a sizeable number of phytases have been purified and characterized from various molds [37, 38, 49, 50, 54], bacteria [11, 21, 35, 47] and yeasts [25, 26, 53]. Various researchers have shown the effect of range of parameters including the fermentation technique adopted, culture conditions, inoculum age and size, and media nutrients on the production of phytase [9, 12, 25, 52]. The fermentation conditions (pH 5.3, 30 °C, and 54.5% moisture content) for phytase production in solid-state fermentation (SSF) using canola oil cake with no additional nutrients by *Rhizopus oligosporus* are reported [42]. The culture conditions, type of strain, nature of substrate and availability of nutrients have to be taken into consideration for selecting a particular production technique, as they are critical factors affecting the yield. The effect of culture conditions, particularly inoculum age, media composition (wheat bran and full-fat soybean flour) and duration of SSF on the phytase production by *Aspergillus niger* was studied [23]. Phytase production by *Aspergillus ficuum* NRRL 3135, *Mucor racemosus* NRRL 1994 and *Rhizopus oligosporus* NRRL 5905 on canola meal, cracked corn, soybean meal, and wheat bran in SSF have been reported [7, 24]. The present investigation involves studies on the effect of different nutritional ingredients such as carbon, nitrogen, inorganic phosphates, surfactants, and metal salts on the production of phytase enzyme by *Aspergillus niger* CFR 335 in submerged and solid-state fermentations.

Materials and methods

All the media ingredients and reagent chemicals were of analytical grade procured from E-Merck, Hi-Media and Qualigen Chemicals, India, Ltd. Fresh wheat bran used for solid-state fermentation was obtained from the department of Flour Milling Baking and Confectionary Technology, CFTRI, Mysore, India.

Strain

Aspergillus niger CFR 335 [13] was isolated from poultry soil using the routine mycological procedures and screened for phytase enzyme production using phytase screening medium [17] containing 0.5% calcium phytate as the substrate. The fungus was cultivated in Czapek Dox agar slants for 3–4 days at 30 °C and stored at 4 °C for further studies.

Media

Czapek Dox agar (g/L): glucose 30, sodium nitrate 3, dipotassium hydrogen phosphate 1, magnesium sulfate 0.5, potassium chloride 0.5, ferrous sulfate 0.001, pH 5.5 ± 0.2. Potato Dextrose broth: infusion was obtained by boiling 200 g of fresh potato in 800 mL distilled water to which 10 g dextrose was added and the volume was made upto 1,000 mL, pH 5.5 ± 0.2.

Culture techniques

Phytase production by Aspergillus niger CFR 335 in submerged fermentation

Submerged fermentation was carried out to study the effect of various cultural parameters on phytase production by *Aspergillus niger* CFR 335 using potato dextrose broth as the cultivation medium. Aliquots of 100 mL medium were dispensed into 500 mL Erlenmeyer flasks and autoclaved at 121 °C for 20 min. After cooling, the various components were added separately, inoculated with 0.5 mL *A. niger* CFR 335 (2×10^6 spores/mL) and incubated in an orbital shaker at 200 rpm and 30 °C for 5 days. The experiment was carried out in triplicates for each of the components.

Phytase production by Aspergillus niger CFR 335 in solid-state fermentation

Solid-state fermentation was carried out using wheat bran substrate medium. Fresh wheat bran weighing 50 g was taken in 1-L Erlenmeyer flask and autoclaved for 40 min at 121 °C. The total moisture content was maintained at 60% level with sterile distilled water. The media were supplemented with various components and inoculated with 1 mL *A. niger* CFR 335 (2×10^6 spores/mL). The flasks were incubated at 30 °C in a static horizontal position.

Effect of carbon sources

Studies on the effect of various carbon compounds in SmF was studied by replacing dextrose (glucose) in the potato dextrose broth with other carbon compounds such as monosaccharides (fructose, galactose, mannose, xylose and

arabinose), disaccharides (lactose, maltose, sucrose, cellobiose and trehalose), trisaccharide (raffinose), sugar alcohols (sorbitol and xylitol) and deoxy sugar (rhamnose) at 1% v/v providing the carbon at a range of 3.96–4.4 moles/L. In the SSF, wheat bran medium was supplemented with the carbon sources at 1% w/v. The medium with glucose served as control in both the fermentation techniques.

Effect of nitrogen sources

Various nitrogenous compounds, such as yeast extract, peptone, ammonium sulfate, ammonium nitrate, potassium nitrate, and urea were incorporated at 0.5% v/v and w/v in SmF and SSF medium, respectively. These compounds provide nitrogen in the range of 0.532–1.16 moles/L. The medium supplemented with yeast extract served as control.

Effect of inorganic phosphates

The effect of various inorganic phosphates such as sodium dihydrogen phosphate, potassium dihydrogen phosphate, *p*-nitrophenyl phosphate, ammonium phosphate, and phosphoric acid on phytase production by *A. niger* CFR 335 in both SmF and SSF was studied. The compounds were added to SmF and SSF cultivation media at 0.015 (0.019–0.047 moles of P/L) and 0.025% (0.034–0.076 moles of P/L) v/v, w/v, respectively (*p*NPP < $(NH_4)_3PO_4$ < KH_2PO_4 < NaH_2PO_4 < H_3PO_4). Medium without any additional phosphates was kept as control.

Effect of surfactants

A level of 0.25% v/v and w/v of Tween- 20, Triton X-100, SDS, and EDTA were separately added to the SmF and SSF media, respectively. The medium without any surfactant served as control.

Effect of metal salts

The effect of various metal salts on the activation or inactivation of *Aspergillus niger* CFR 335 phytase was studied by supplementing sodium chloride, magnesium sulfate, ferrous sulfate, manganese sulfate, copper sulfate, zinc sulfate, and calcium chloride at 0.005 (0.012–0.11 moles of respective metal salts/L) and 0.01% (0.024–0.22 moles of respective metal salts/L) v/v and w/v concentrations, in both SmF and SSF medium, respectively. The medium without any metal salt served as control.

Extraction of crude phytase in SmF

Extracellular crude phytase in submerged medium entailed initial filtration through whatman no. 1 filter paper. The filtrate was stored at 4 °C and used as crude phytase enzyme.

Extraction of crude phytase from SSF

Crude phytase was extracted by mixing the moldy bran with 1:5 w/v of acetate buffer (0.2 M) at pH 4.5 in a rotary shaker for 20 min at 200 rpm. The solids were separated through clean muslin cloth (0.2-mm pore size). The aqueous solution was centrifuged at 10,000 rpm for 20 min at 4 °C and the supernatant was used as crude enzyme preparation for further investigation.

Phytase assay

Crude enzyme extracted from both the media was quantitatively assayed for phytase enzyme [15]. The assay was initiated by mixing 1 mL of diluted (1:10) crude enzyme with 0.5 mL of sodium acetate (0.2 M) buffer of pH 4.5 and 0.5 mL of sodium phytate (15 mM) (Sigma Chemicals Co, USA). The reaction mixture was incubated at 40 °C in a water bath for 45 min. The reaction was terminated by adding 2 mL of 15% trichloroacetic acid. 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 5 N sulfuric acid (AAM solution) and 0.4 mL of citric acid (1 M). The amount of free phosphate released was determined spectrophotometrically at 355 nm. A standard graph was plotted using potassium dihydrogen phosphate with working concentration ranging from 30 to 360 μM. Protein quantifications were made by the method of Bradford [6] and compared with the standard prepared using bovine serum albumin.

Statistical analysis

Data are presented as the standard error means (±SEM). Comparisons between different C, N, P, surfactants and metal salts used in the production of phytase enzyme by *Aspergillus niger* CFR 335 through submerged and solid-state fermentations were made with the analysis of variance [46]. Values were considered significant at $P < 0.05$ and are indicated as asterisk in tables and figures.

Results and discussion

Effect of carbon compounds on phytase activity by *Aspergillus niger* CFR 335

The results indicated that sucrose is a suitable carbon source for maximum phytase activity of 14.2 U/mL, which was 1.5-fold higher than the control in SmF (Fig. 1). The medium with glucose serving as control showed phytase

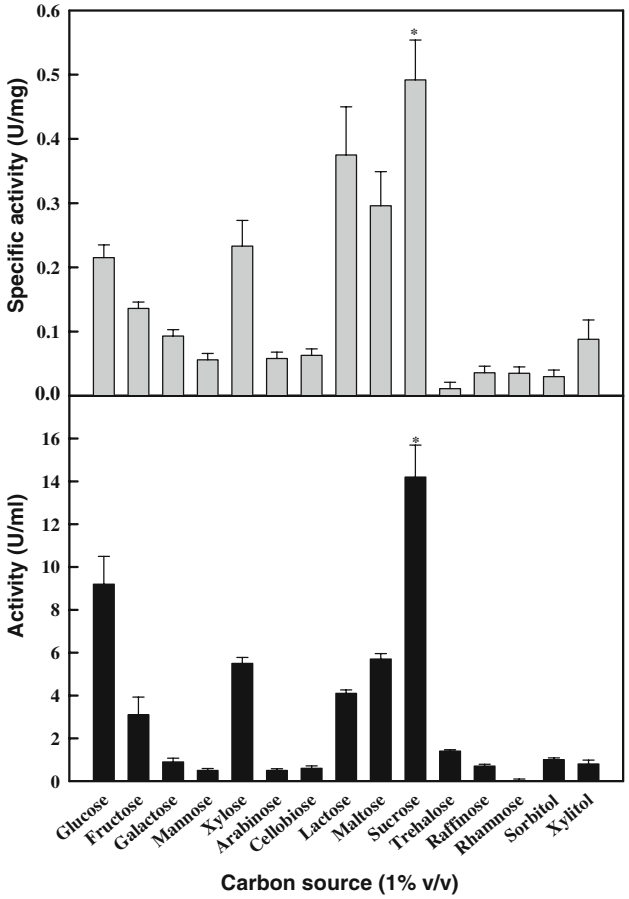

Fig. 1 Effect of different carbon sources on phytase production by *Aspergillus niger* CFR 335 in submerged fermentation. *Asterisks* indicate significant difference ($P < 0.05$) in phytase production as compared to control

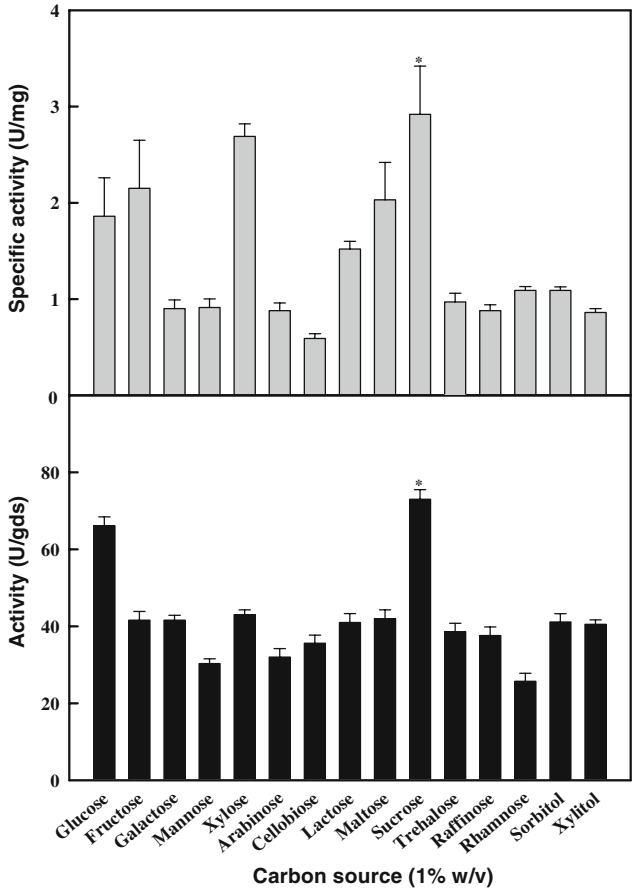

Fig. 2 Effect of different carbon sources on phytase production by *Aspergillus niger* CFR 335 in solid-state fermentation. Abbreviation is as shown in Fig. 1

activity of 9.2 U/mL and 0.215 U/mg specific activity. The solid-state fermentation showed activity of 66 U/g dry solids (gds) with specific activity of 1.86 U/mg in control while an enhanced activity of 73 U/gds and specific activity of 2.92 U/mg was found in medium with sucrose (Fig. 2). Phytase activity was enhanced by none other carbon sources, tested in both SmF and SSF.

Trehalose, a non-reducing sugar formed of two glucose units linked through a glycosidic bond (1–1α) makes it highly resistant to acid hydrolysis and therefore stable in solutions at high temperatures even under acidic conditions [48]. Thus, trehalose may not be a suitable carbon source for normal growth of the fungus and phytase accumulation. Cellobiose, maltose and lactose provide same number of carbons (4.21 moles/L) as that of sucrose, however they did not support phytase production by *A. niger* CFR 335. It may be assumed that the fungus lack mechanisms for converting these disaccharides into simpler glucose molecules. Raffinose, a trisaccharide provides higher moles of carbon (4.28 moles/L) than any other compounds used, but the phytase activity was not improved than the control. This may be due to repression of enzyme activity at high carbon concentrations [45, 54]. Fructose and galactose did not support the enzyme production. Xylose and arabinose being pentose sugars were also found to be less suitable for phytase production, although they provide same amount of carbons (3.96 moles/L) as that of other monosaccharides. Other carbon compounds such as sorbitol, xylitol, mannose (3.96 moles/L) and rhamnose (4.4 moles/L) grouped under sugar alcohols, sugar monomer and deoxy sugar were also proved to be inefficient carbon sources for phytase production.

The effect of various sugars and non-sugars as carbon sources on the profile of cell mass, enzyme activity and extracellular protein production by *A. niger* van teigham have been checked, where a maximum enzyme production was obtained when starch was used as carbon source, followed by a combination of glucose and starch [51]. Fructose did not activate phytase production, which may be due to the formation of larger pellets and in some cases a single large clump that reduced the number of growing tips responsible for the accumulation and release of extracellular enzyme [49]. It is known that disaccharides provide

higher dose of carbons (4.21 moles/L) than the monosaccharides when used at same concentrations, but none other than sucrose enhanced the phytase activity. Glucose and fructose were not effective in enhancing the phytase activity when used individually, while in combined form as sucrose, the activity was considerably higher. This may be due to the fact that α-D-glucopyranosyl- (1↔2)-β-D-fructofuranoside linkage in sucrose makes the carbon atoms available to the fungus.

Effect of nitrogen compounds on *Aspergillus niger* CFR 335 phytase activity

The submerged fermentation medium with yeast extract serving as control showed activity of 8.2 U/mL and 0.187 U/mg specific activity (Fig. 3). In SSF, the phytase activity of 67.3 U/gds and specific activity of 2.79 U/mg was observed in control (Fig. 4). The SmF medium with peptone showed increased activity of 8.5 U/mL. This is similar to previous report where the significant role of nitrogen in growth and phytase production in *Aspergillus niger* van teigham was studied and a maximum phytase production was obtained with bio-peptone followed by ammonium nitrate and ammonium sulfate [52]. Organic forms of nitrogen such as 1% peptone along with 1% yeast extract have been used extensively for the production of phytase in *Aerobacter aerogenes* and *Klebsiella aerogenes* [18]. There was no enhancement in the phytase activity with peptone in SSF in the present study. About 50–70% reduction in the enzyme activities was found in both SmF and SSF in the presence of potassium nitrate and urea that provide 0.686 and 1.16 moles of N/L, respectively. These concentrations may be too high for the fungus to produce phytase enzyme. Similar results of inhibition were also reported when different organic and inorganic nitrogen sources were tested for phytase yield by *Aspergillus ficuum* and *Rhizopus oligosporus* [39]. It could be thus stated that an appropriate concentration of peptone can be used as a suitable nitrogen source for improved phytase activity by *Aspergillus niger* CFR 335 in submerged fermentation.

Effect of inorganic phosphates on *Aspergillus niger* CFR 335 phytase activity

The result obtained through the studies on the effect of inorganic phosphates on *Aspergillus niger* CFR 335 phytase production in SmF is shown in Table 1. The SSF studies showed that the phytase activity was 45 and 34% higher with 0.015 (0.037 moles of P/L) and 0.025% (0.056 moles of P/L) w/v of NaH_2PO_4, respectively, than the control. This was followed by 30 and 20% higher activities with 0.015 (0.034 moles/L) and 0.025% (0.056 moles/L) w/v of KH_2PO_4, respectively (Table 2). It is clear from the results

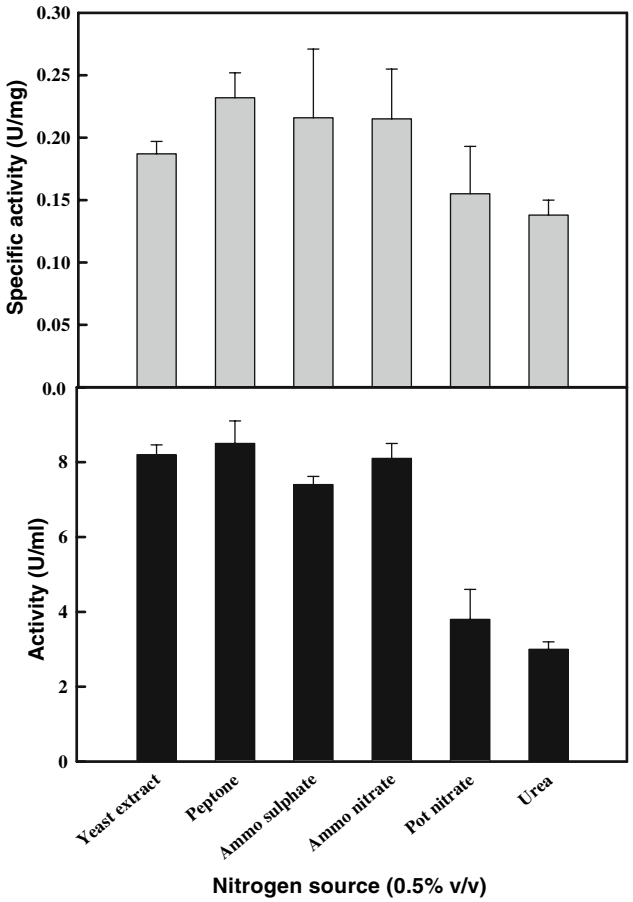

Fig. 3 Effect of different nitrogen sources on phytase production by *Aspergillus niger* CFR 335 in submerged fermentation

that the phytase activity decreased with increase in the phosphate concentration. The results showed a maximum repression of >80% with *p*NPP, $(NH_4)_3PO_4$ and H_3PO_4 in SSF. There was about 10–80% repression with all the inorganic phosphates in SmF. However the fungus failed to grow in submerged cultivation medium with *p*NPP. Even though *p*-nitrophenyl phosphate supported growth of the fungus in SSF medium, the enzyme activity was suppressed. The result suggests that the phytase activity is suppressed with higher concentration of phosphates, which is proved by H_3PO_4 that liberates 0.047 moles/L of phosphates.

It is reported that phosphate plays an important role in phytase production and by regulating the concentration in the growth medium, production rate can be substantially regulated [9, 22, 58]. Phytase repression by excess orthophosphate is reported and the extent of repression depends on the strain and is reversed by providing a slow-releasing organic phosphate source [10]. An insignificant repression of phytase synthesis has been shown by inorganic phosphorus in a complex medium [8]. A sharp decline in phytase production by *A. niger* van teigham at a final concentration

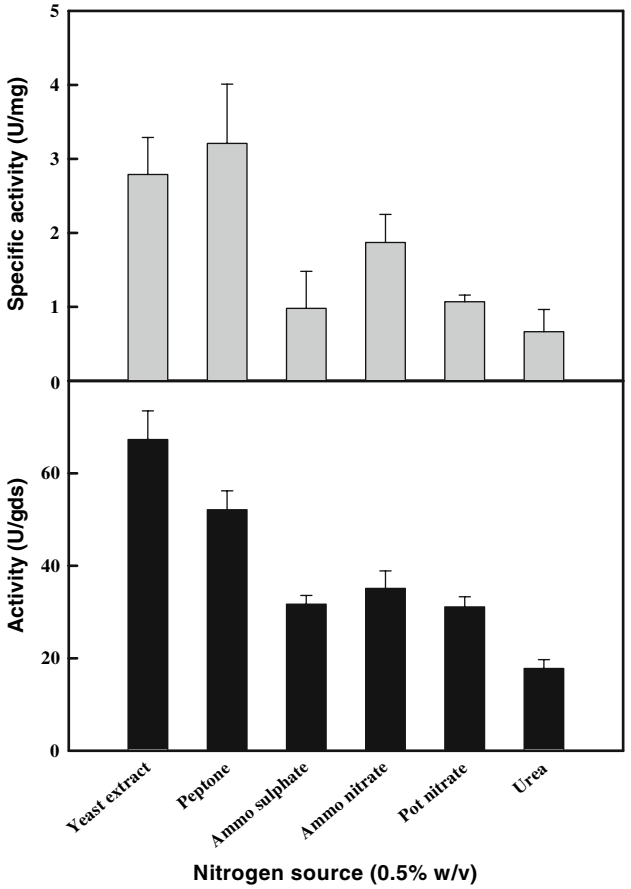

Fig. 4 Effect of different nitrogen sources on phytase production by *Aspergillus niger* CFR 335 in solid-state fermentation

Table 1 Effect of inorganic phosphates on phytase production by *Aspergillus niger* CFR 335 in submerged fermentation

Inorganic Phosphate	Concentration (% v/v)	Activity (U[a]/mL)	[b]Specific activity (U/mg)
Control	–	8.5 ± 0.18	1.50 ± 0.07
KH_2PO_4	0.015	2.2 ± 0.13	0.36 ± 0.04
	0.025	1.7 ± 0.10	1.80 ± 0.06
NaH_2PO_4	0.015	2.0 ± 0.06	0.20 ± 0.03
	0.025	1.4 ± 0.13	0.70 ± 0.04
$(NH_4)_3PO_4$	0.015	2.6 ± 0.06	0.30 ± 0.04
	0.025	1.7 ± 0.09	1.10 ± 0.05
H_3PO_4	0.015	7.7 ± 0.15	1.05 ± 0.03
	0.025	5.1 ± 0.60	2.15 ± 0.06
p-NPP	0.015	NG	–
	0.025	NG	–

NG No growth of the fungus

[a] One Unit is the activity of the enzyme to release 1 μM of inorganic phosphorus from the substrate in 1 min at 40 °C

[b] Specific activity is defined as the amount of substrate the enzyme converts, per mg protein in the enzyme preparation, per unit of time

Table 2 Effect of inorganic phosphates on phytase production by *Aspergillus niger* CFR 335 in solid-state fermentation

Inorganic Phosphate	Concentration (% w/v)	Activity (U[a]/gds)	[b]Specific activity (U/mg)
Control	–	60.0 ± 2.2	1.7 ± 0.09
KH_2PO_4	0.015	78.6 ± 3.1*	0.9 ± 0.06
	0.025	71.4 ± 2.8*	2.5 ± 0.08
NaH_2PO_4	0.015	91.0 ± 2.2*	1.7 ± 0.08
	0.025	80.2 ± 2.6*	3.1 ± 0.11
$(NH_4)_3PO_4$	0.015	4.1 ± 0.9	1.2 ± 0.08
	0.025	7.3 ± 1.3	1.3 ± 0.09
H_3PO_4	0.015	3.1 ± 0.6	1.1 ± 0.07
	0.025	3.6 ± 0.9	1.2 ± 0.10
p-NPP	0.015	11.1 ± 1.6	0.5 ± 0.04
	0.025	12.2 ± 2.2	0.8 ± 0.08

[a,b] Abbreviation as shown in Table 1

* Indicates significant difference ($P < 0.05$) in phytase production as compared to control

of 0.05% with no enzyme production at 0.1% and above was observed [51]. The phenomenon of phosphate repression on phytase production was also observed in many yeast strains where an increased level of phytase production was found in phosphate depleted medium [32]. In a survey of phytase producing microorganisms, *A. ficuum* was shown to produce highest amount of phytase when the inorganic phosphorus content was in the range of 0.0001–0.005% [57]. In the present study, a maximum phytase activity of 91 U/gds was found at 0.015% (0.037 moles/L) of NaH_2PO_4. Thus the appropriate concentration of inorganic phosphates plays an important role in the phytase production.

Effect of surfactants on *Aspergillus niger* CFR 335 phytase activity

The result indicated that the liquid medium supplemented with Tween–20 increased 40% phytase activity. A threefold decrease in the activity was observed with EDTA, while there was no growth in presence of SDS (Fig. 5). In case of SSF (Fig. 6), there was >50% inhibition in the phytase activity in presence of Tween-20, Triton X-100 and EDTA. SDS supported the growth of the fungus, but not the enzyme activity. Overall, none of the surfactants activated enzyme production in solid-state cultivation.

Several researchers have shown the incorporation of surfactants inducing the formation of smaller pellets in the liquid medium and hence higher yield of phytase [3, 5, 33]. Surfactants are surface active agents having a number of applications that include, lowering of surface and interfacial tensions, wetting and penetration actions, emulsification, detergency, gelling, flocculating actions, microbial

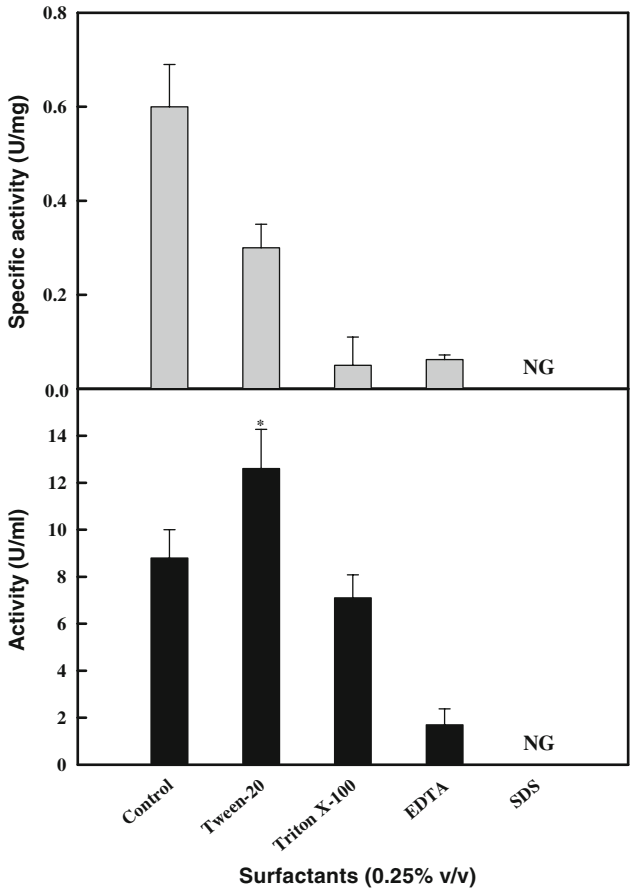

Fig. 5 Effect of different surfactants on phytase production by *Aspergillus niger* CFR 335 in submerged fermentation. Abbreviation is as shown in Fig. 1 (*NG* no growth)

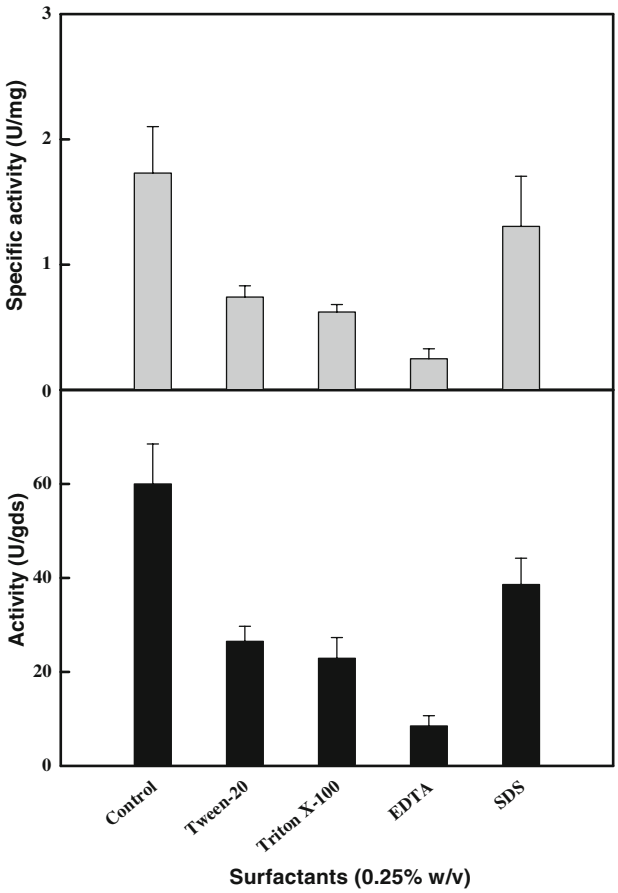

Fig. 6 Effect of different surfactants on phytase production by *Aspergillus niger* CFR 335 in solid-state fermentation

growth enhancement etc., [31]. All the applications have tempted researchers to use surfactants to induce pellet formation in liquid cultures. Increase in the pelletization increases the extra cellular enzyme synthesis in submerged fermentation [42]. In the present study, Tween-20 has proved to be an efficient surfactant, which results in flocculation and formation of smaller pellets for better yield of the enzyme in submerged cultivation medium.

During the growth of *Aspergillus carbonarius*, phytase production and reduction of phytic acid content in canola meal during SSF were higher in the presence of Na-oleate or Tween-80 than in the control medium without any surfactants [2]. In the present study, Tween-20 promoted higher phytase synthesis by *A. niger* CFR 335 suggesting increased cell permeability in SmF. EDTA, a chelating agent was found to readily inactivate *Bacillus* phytases [21, 40, 45], whereas *Aspergillus fumigatus* phytase activity was stimulated by EDTA [59]. In other studies, EDTA being a sequestering agent is shown to bind and form strong complexes with Mn^{2+}, Cu^{2+}, Fe^{3+}, Co^{2+} etc., suppressing the fungal growth thereby inactivating phytase production [16]. Similarly in the present investigation, *A. niger* CFR 335 phytase was inactivated by EDTA which may be due to its antifungal activity. Such antifungal activities of EDTA against *Candida albicans* and in human salivary mucin have been demonstrated [44, 56]. Higher yields of phytase by *A. niger* NRRL 3135 with 0.5% Triton X-100, Tween-80 and Na-oleate v/v on 17th day of incubation and by *A. niger* NCIM 563 with 0.5% Triton X-100 has been reported [14, 30]. The present investigation showed 40% enhanced phytase activity on 5th day of incubation in submerged cultivation medium supplemented with 0.25% Tween-20 than in control, which is of high industrial impact.

Effect of metal salts on *Aspergillus niger* CFR 335 phytase activity

Result presented in Table 3 shows more than 15 and 50% increase in the phytase activities with 0.005 (0.018 moles Ca^{2+}/L) and 0.01% (0.036 moles Ca^{2+}/L) v/v of calcium chloride supplemented in liquid medium when compared to control. It was found that all other metal salts used had a negative effect with >50% suppression of the phytase

Table 3 Effect of metal salts on phytase production by *Aspergillus niger* CFR 335 in submerged fermentation

Metal salt	Concentration (% v/v)	Activity (U^a/mL)	^{b}Specific activity (U/mg)
Control	–	8.5 ± 1.20	2.42 ± 0.081
NaCl	0.005	0.3 ± 0.08	0.70 ± 0.070
	0.010	0.4 ± 0.08	0.33 ± 0.054
$MgCl_2$	0.005	3.3 ± 0.30	1.20 ± 0.053
	0.010	5.1 ± 0.73	1.10 ± 0.066
$FeSO_4$	0.005	0.6 ± 0.25	0.74 ± 0.050
	0.010	1.9 ± 0.07	0.77 ± 0.071
$MnSO_4$	0.005	0.2 ± 0.01	0.06 ± 0.005
	0.010	0.2 ± 0.02	0.08 ± 0.008
$CuSO_4$	0.005	0.9 ± 0.09	0.75 ± 0.043
	0.010	2.2 ± 0.07	1.54 ± 0.081
$ZnSO_4$	0.005	1.2 ± 0.02	0.91 ± 0.062
	0.010	2.4 ± 0.06	0.89 ± 0.060
$CaCl_2$	0.005	9.8 ± 0.09*	2.75 ± 0.093
	0.010	13.2 ± 1.1*	3.42 ± 0.091

[a,b] Abbreviation as shown in Table 1

* Indicates significant difference ($P < 0$ .05) in phytase production as compared to control

Table 4 Effect of metal salts on phytase production by *Aspergillus niger* CFR 335 in solid-state fermentation

Metal salt	Concentration (% w/v)	Activity (U^a/gds)	^{b}Specific activity (U/mg)
Control	–	64.41 ± 2.4	1.79 ± 0.12
NaCl	0.005	55.63 ± 1.8	6.61 ± 0.22
	0.010	45.52 ± 2.2	1.75 ± 0.13
$MgCl_2$	0.005	53.60 ± 1.4	5.32 ± 0.07
	0.010	47.84 ± 3.6	2.19 ± 0.09
$FeSO_4$	0.005	36.81 ± 1.1	2.75 ± 0.16
	0.010	40.42 ± 2.0	1.51 ± 0.05
$MnSO_4$	0.005	48.60 ± 1.3	3.61 ± 0.14
	0.010	39.13 ± 2.9	1.37 ± 0.11
$CuSO_4$	0.005	39.36 ± 0.9	3.34 ± 0.05
	0.010	32.02 ± 2.0	1.12 ± 0.08
$ZnSO_4$	0.005	41.62 ± 1.1	4.10 ± 0.16
	0.010	39.61 ± 2.5	1.37 ± 0.07
$CaCl_2$	0.005	58.72 ± 1.6	7.22 ± 0.09
	0.010	37.11 ± 2.1	1.27 ± 0.11

[a,b] Abbreviation as shown in Table 1

activity. Similarly in SSF, none of the metal salts used stimulated phytase production (Table 4).

Vats and Banerjee [51] have shown in their studies that certain microbial phytases are activated in the presence of metal ions. It is also shown that yeast phytases are generally resistant to high salt concentrations where, *Pichia rhodanensis* phytase was found to function >50% of its maximal rate at 1 M NaCl [32]. The metal ion requirement of *Bacillus subtilis* phytase has been studied and their removal from the medium by EDTA resulted in complete inactivation of the product while the enzymatic activities of fungal phytases are reported not to require metal ion [20]. *Rhizopus oligosporus* phytase activity was found to be unaffected or moderately stimulated by a range of metal ions [4]. Similarly, *Aspergillus niger* CFR 335 phytase was activated in presence of Ca^{2+} in SmF at 0.01% (0.036 moles/L) level indicating Ca^{2+} ion dependency of phytase, which might be acting as its cofactor.

In conclusion, sucrose was found to be a suitable carbon source instead of glucose in both the fermentation media, NaH_2PO_4 in SSF, peptone, Tween-20 and $CaCl_2$ in SmF were found to enhance the phytase activity of *Aspergillus niger* CFR 335. Despite considerable economic interest and large number of phytase producing microorganisms, low yield and high cost for production are the limiting factors in using this enzyme in animal diet. The potential demand for phytase in cattle and poultry feed is around 4,000 tons per annum. Thus, there is an ongoing interest to obtain significant titers of phytases with an ultimate aim to produce the enzyme to cost effective level and establish the suitability for its industrial application.

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