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PHYTASE PRODUCTION BY FERMENTATION

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To maximize the yield of enzymes by *Aspergillus niger* nutritional and culture conditions were manipulated. The results of the study indicated that 30 –35 percent moist heat sterilized and fortified with 2-5 mg ammonium phosphate per 100 g rice bran in static cultivation, produced maximum amount of phytase in 10-17 days period.

Key words: Phytase, *Aspergillus niger*, Nutritional and culture conditions.

Introduction

After calcium (Ca), phosphorus (P) is the second most important, vital mineral for body structure and functioning of all living cells. It is 25 percent by weight of the total body minerals contents. Eighty percent of the P is bound to the bones of the body and 20 percent in the soft tissues and blood. There it is bound to proteins, lipids, carbohydrates, nucleic acids and vitamins, playing various vital functions such as energy production etc., for all living processes, as a part of many enzymes and co-enzymes.

Deficiency of P shows deprived appetite, stiff joints, muscular weakness, poor fertility and depression of oestrus. For livestock, (milk, meat, eggs and wool production) P deficiency leads to serious problems of low feed intake, stunted growth and poor production in terms of meat, milk and eggs. (Mc Donald *et al* 1981).

Animal sources of P are bone meal, fishmeal and milk etc. These are considered to be good quality sources of P, whereas the plant sources vary from good to poor quality sources for P on the basis of bio-availability of P. The principal storage form of phosphorus in plants, particularly in cereal grains, legumes and plants by-products is phytic acid (Gill 1999) (Fig 1).

Phytic acid shows strong chelating properties as a result of its structure. It forms a variety of complexes with cations, such as calcium, magnesium, copper, zinc, iron and proteins, rendering these nutrients biologically unavailable. The occurrence of protein-phytate complexes reduces the availability of proteins to the poultry because phytate-protein complex, is more resistant to proteolytic digestion than the protein alone (Zyla *et al* 1989).

The interaction of phytic acid with protein, vitamins and several minerals is one of the primary factors limiting the nutritive values of cereal grains and legume seeds (Han and Wilfred 1988). Insoluble Ca^{++} and magnesium salts of phytates occur in cereals and other plant by-products (Bird 1998). Experiments with chicks have shown that P of Ca-phytate is utilized only ten percent as compared to disodium-phosphate. Only ruminants can effectively utilize phytate-P, due to rumen microbial enzymes namely phytases (Mc Donald *et al* 1981) which hydrolyse the plant phytates to inositol and inorganic P which are in turn, hundred percent utilized (Fig 1). Thus hydrolyzing the plant phytates prior to monogastric consumption would increase the availability of inositol and inorganic phosphorus in their diet. Halander *et al* (1996) demonstrated that addition of phytase to poultry feed not only increased the bio-availability and absorption of P but digestibility of dry matter, protein, fiber was also enhanced. Thus many attempts have been made to produce microbial phytase (EC3 1.3.8) by fermentation for hydrolysis of dietary phytates to improve the feed quality (Chang *et al* 1977).

The present studies were initiated for the production of phytases, by *Aspergillus niger* and improving the yield of enzymes by manipulating and optimizing the nutritional and cultural conditions. Various plants and plant by-products were used as fermentation substrates to improve the yield of enzymes. The activity of enzymes so produced was studied on various chemicals and plant phosphorous sources at various pH.

Materials and Methods

Materials. Rice bran, wheat bran, soybean meal, mung beans, and corn meal were purchased locally and used as substrates for fermentation purpose.

Organism and growth conditions for fermentation. Locally isolated strain of *Aspergillus niger* capable of producing phytase enzyme was used for fermentation. Culture was maintained on potato dextrose agar at 30°C. One to two

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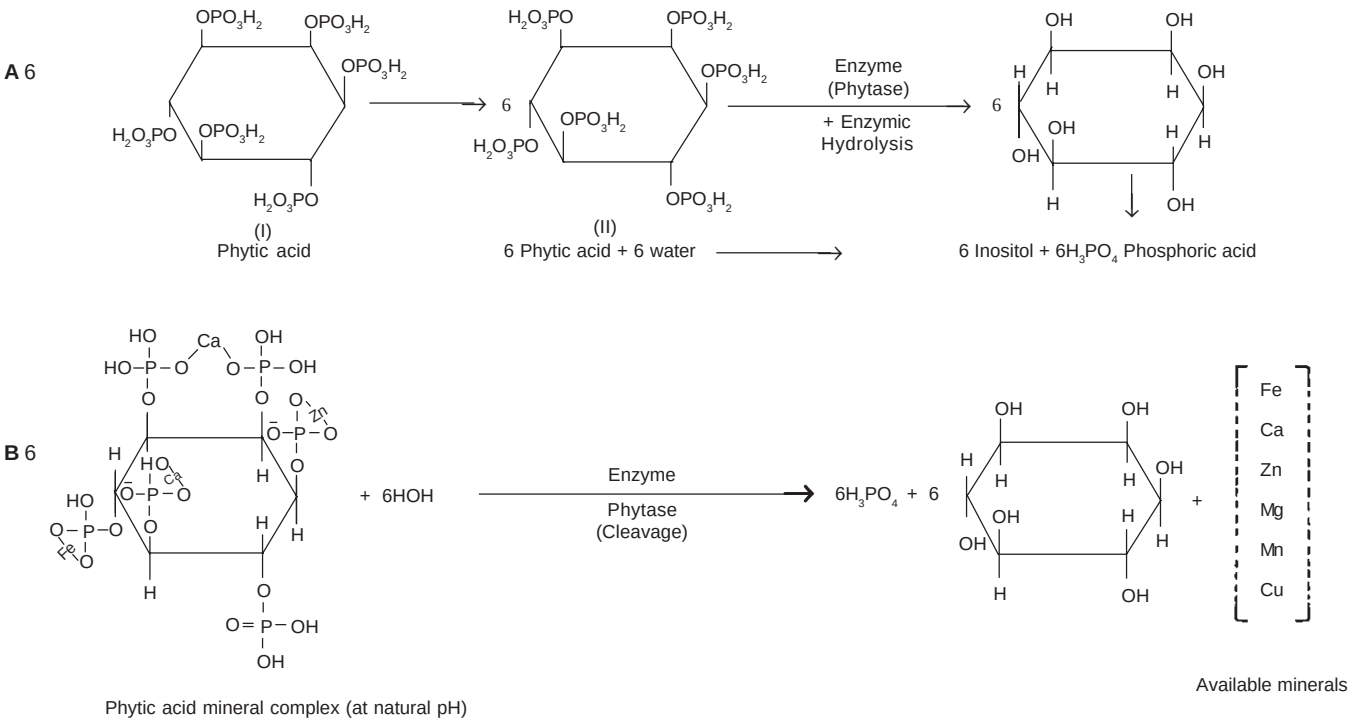

Fig 1. Phytic acid and its products.

week old culture was used for fermentation purpose. Conidia formed on the agar surface were scraped off and collected in normal saline solution. About 10^2 spores/ml were used as an inoculum. The mixtures were steam sterilized before subjecting to fungal growth. In a fermentation run 100g of substrate in 1 litre(L) flasks were inoculated with 1 ml of spore suspension and fermented at 28 to 30˚C for 1-2 weeks as semi-solid stationary culture fermentation.

The following experiments were conducted to standardize the conditions for maximum growth of *A. niger* and enzyme production.

(i) Moisture contents of substrate.

Different levels of moisture from 10 to 90 percent were tested at 28 ± 2˚C. Triplicate flasks containing rice bran as a substrate were inoculated with *A. niger.* The flasks were kept at (28 ± 2˚C) without shaking. Biomass and enzymes production after 10 days were noted.

(ii) Agitated versus static.

The triplicate flasks containing rice bran at 35 percent moisture were inoculated with *A. niger.* The flasks were incubated at 28 ± 2˚C. One set was placed on table and other on shaking machines. After 10 days the quantity of enzymes produced was estimated.

(iii) Effect of added inorganic phosphorous sources.

To the 14 flasks containing 100 g rice bran with (35 percent moisture content) various amounts of inorganic-P (Pi) from 1-1000 mg/flask, were added using ammonium phosphate. Seven flasks were used for static and other seven for agitated growth. *A. niger* was grown for 10 days and the amount of phytase produced was quantified.

(iv) Effect of growth period and enzyme quantity.

The inoculated flasks kept in static fermentation mode were opened after 5 day interval up to 30 days to ascertain the effect of time of fermentation on productivity of enzymes.

(v) Enzyme activity on various purified chemicals of phosphate sources.

Substrate specificity of *A. niger* phosphatases was ascertained using various phosphate sources at two pH values i.e 2.0 and 5.5.

(vi) Effect of various substrate sources.

Defatted rice bran, wheat bran, soybean meal, mung bean and corn meal were inoculated with *A. niger.* The growth of fungus and enzyme activity was studied according to methods of Halander *et al* (1996).

Table 1
Substrate specificity of *A. niger* phosphatase at pH 2.0 & 5.5

Substrate	pH2.0		pH 5.5	
	Pi (μM)[a]	Relative activity[b]*	Pi (μM)[a]	Relative activity*
Na-PNPP	1.058	100[a]	0.792	75 ± 3[a]
Na-Phytate	0.381	36 ± 5[b]	0.514	48 ± 3[b]
Ca Phytate	0.508	48 ± 4[c]	0.485	45 ± 6[b]
$Ca_2P_2O_7$	0.675	63 ± 3[d]	0.162	15 ± 2[c]
$Na_3P_3O_{10}.6H_2O$	0.921	87 ± 8[a]	0.338	44 ± 1[b]
$Na_3P_3O_9$	0.523	49 ± 7[c]	0.092	8 ± 8[c]
$(NH_4)_6\ P_4O_{13}.6H_2O$	0.971	92 ± 4[a]	0.162	15 ± 4[c]
Myoinositol 2-monophosphate	0.276	26 ± 5[b]	0.051	4 ± 2[d]

a. Pi liberated from reaction mixture containing 0.5 ml of substrate (10 μM Pi), 4ml of buffer, and 0.5 ml enzyme during 30 min reaction time.

b. (i) Decimal rounded to whole figure.

(ii) Relative activity on P-nitrophenyl phosphate (PNPP) at pH 2.0 is defined as 100%.

* Same superscript on means in columns show non-significant difference.

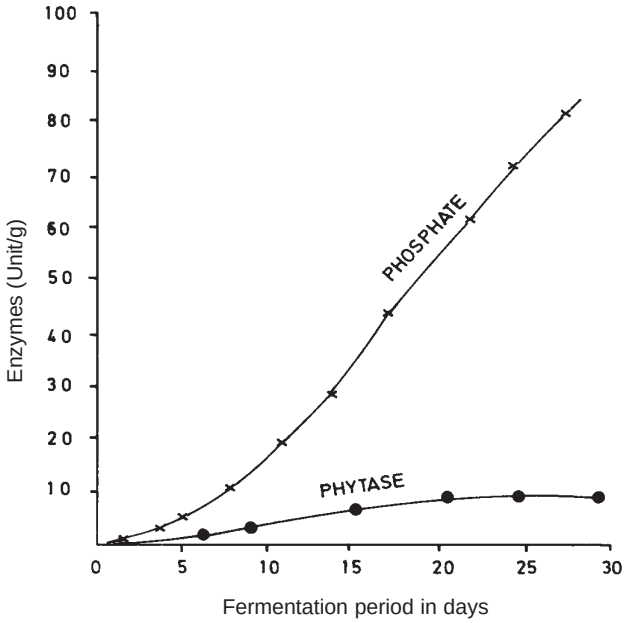

Fig 2. Effect of fermentation period on enzymes production by *A.niger*

A. ANALYTICAL METHODS

i. Determination of phytic acid. Phytic acid in the substrates and samples were determined by a rapid method of Haug and Lantzesch (1983) and inorganic (Pi) as orthophosphorous was determined by the method of Heinonen and Lahti (1981).

Table 2
Effect of inorganic phosphate (Pi) on the production of phytase by *A. niger*

Pi (mg/100 g substrate) (a)	Phytase (Units/g substrate) Static growth (b)*	Agitative growth (c)*
0	8.0 ± 2[a]	4 ± 1[a]
1	8.0 ± 1[a]	4 ± 3[a]
10	82 ± 5[c]	4 ± 1[a]
50	56 ± 3[b]	12 ± 3[b]
100	20 ± 1[a]	10 ± 3[b]
500	12 ± 1[a]	5 ± 2[a]
1000	14 ± 3[a]	4 ± 1[a]

a) Various amounts of ammonium phosphate was added.

b) *A. niger* was grown on semi-solid rice bran for 10 days at 28°C ± 2°C without agitating the substrate on rotating "Wheel shaker".

c) *A. niger* was grown on semi-solid rice bran for 10 days at room temperature with agitation on rotating "Wheel shaker".

*Same superscripts on means in column show non significant differences.

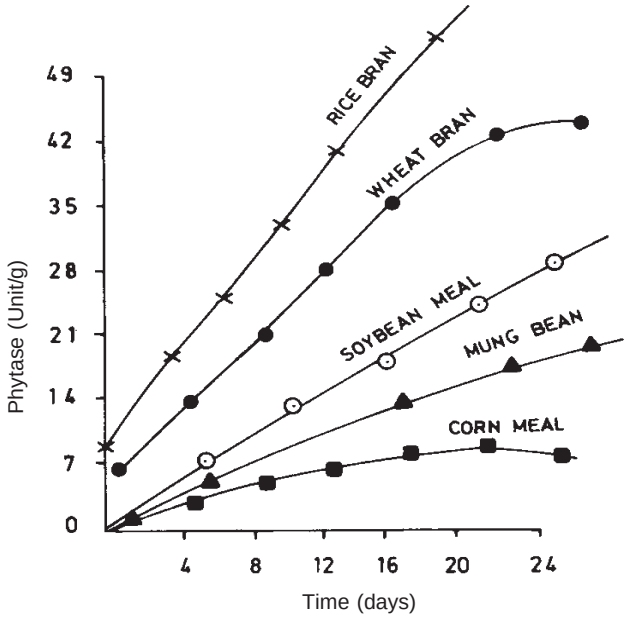

Fig 3. Production of phytase from various substrate sources

ii. Extraction of enzymes and measurement of enzyme activity. Enzymes were extracted by adding ice cold water to the fermented mixture while the medium along with biomass was blended in high speed blender and filtered through a Whatman-4 filter paper. The crude culture filtrate was used as an enzyme source. Phytase activity was assayed by following the release of phosphorous in the form of orthophosphate. The liberated inorganic phosphate (Pi) was determined by the methods of Heinonen and Lahti (1981). In the study where

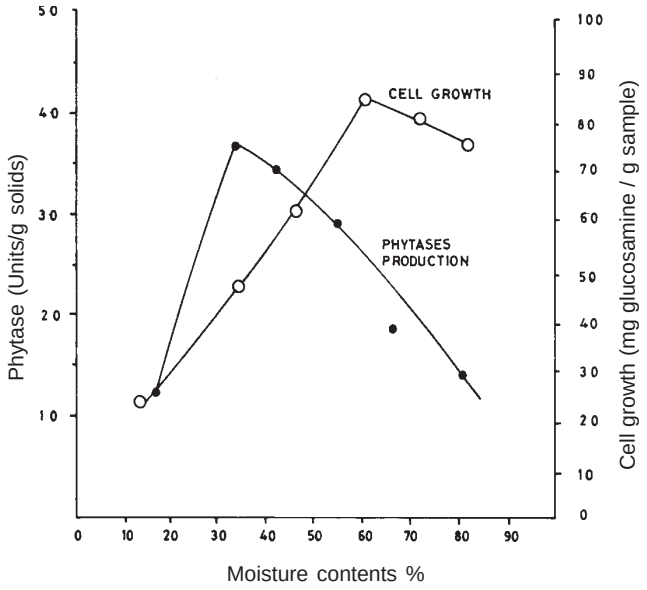

Fig 4. Effect of moisture content on phytase and cell biomass production.

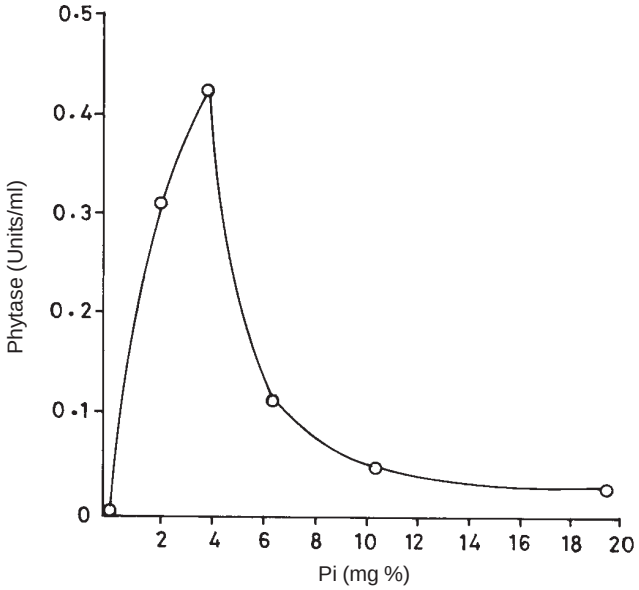

Fig 5. Effect of different levels of Pi addition on phytase production by *A. niger*.

high initial Pi interfered with the determination of Pi liberated by the enzyme, p-nitrophenyl phosphate (PNPP) was used as a substrate and the enzymic activity was reported as phosphatase activity (AOAC 1984). The enzyme reaction mixture contained 0.1 ml of suitably diluted culture filtrate, 3.0 ml of 0.1 M acetate buffer (pH 5.4), and 0.5 ml of 15 µM of PNPP or Na-phytate. The reaction mixture was incubated for 30 min at 37˚C. At the end of the reaction, the colour developed was measured by reading the optical density at 420 nm. Optical density was correlated to the units of enzyme. One unit of enzyme was defined as the amount of enzyme required to liberate 1 mM of Pi per minute under the assay conditions.

Although phytase is a kind of phosphatase, the two enzymes were distinguished on the basis of their activity towards substrates, P-nitro phenyl phosphate (PNPP) for phosphates and sodium phytate for phytase, respectively.

B. ESTIMATION OF GLUCOSAMINE

Glucosamine was determined on the basis of amine-N (AOAC 1984) by the method of Lowry *et al* 1951, using bovine serum albumin as standard. The following buffer systems were used throughout the studies. (pH 1.0 - 2.2) glycerin – HCl (pH 2.8 – 3.2) and citric acid - Sodium citrate (pH 3.0 – 6.2).

C. STATISTICAL METHODS

The data on various parameters were tabulated and subjected to statistical analysis according to Steel and Torrie (1966). The comparison of means was done by Duncan's multiple range test (Duncan 1955) at 5% level of significance, whenever significant difference in means was observed.

Results and Discussion

A. niger produced a non-specific acid phosphatase, rather than phytin-specific phosphatase, which hydrolysed a variety of phosphates (Table 1). The crude enzymes hydrolyzed a variety of phosphates.

The organism produced phosphatases and phytases slowly and the enzyme activity increased continually during the one month period (Fig. 2) but phytase production remained stable after 17 days of growth. However, the levels of enzyme activities towards PNPP (phosphatase) and Na-phytate (phytase) were different depending on the pH. The optimum pH for phosphatase and phytase was 2.5 and 5.5, respectively.

Aspergillus niger grew well on steamed brans i.e. defatted rice bran, wheat bran, than on soybean meal, mung bean and corn meal. The highest amount of phytase was produced on rice bran followed by wheat bran, soybean meal, mung bean and corn meal (Fig 3). The enzyme production was less on corn meal and mung beans as compared to rice bran, wheat bran and soybean. The water content of substrate plays an important role for both cell growth and enzyme production in the solid state fermentation as reported by Cannel and Young (1980) and Toyama (1976).

Addition of water causes swelling of the substrate and facilitates its utilization by the microorganisms. However, the optimal amount of water required varied (Fig 4). About 50 to 60 percent moisture showed the best results in terms of cell growth while optimal moisture content for phytase production was between 30 and 35 percent. The activity of phytase produced was drastically reduced when water contents ex-

ceeded 40 percent. The results are in line with those of Han *et al* (1987) and *Kim et al* (1985), who reported that the optimum water content for cellulase production was lower and the range was narrower than that for cell growth in case of *Tricoderma reseii* and *Sporotrichum cellulophillum* grown on semi solid wheat bran medium.

The initial phosphates in the growth medium at pH 2.0 and pH 5.5 level showed importance for the production of phosphatases (Table 1) *A. niger* required less than 4 mg/100 ml of inorganic (Pi) phosphorous. Ammonium phosphate and sodium phosphate gave the best results. In general, a high level of inorganic phosphate (Pi), inhibits the synthesis of phosphate. The type and amount of phosphatase synthesized are dependent on the concentration of Pi, present in the growth medium (Ohta *et al* 1968; Shieh *et al* 1969).

The addition of more than 5 mg Pi per 100 g substrate (Rice bran) severely depressed phytase synthesis in static growth media (Fig.5).

The mode of cultivation in semi-solid fermentation also affected the optimal concentration of Pi. The static culture produced more enzymes than the agitated culture. Shaking the medium inhibited the production of enzymes. The results are in line with Han and Gallagher (1987) and Tambiev *et al* (1997) who reported product production of extracellular enzymes by *A. niger* when grown on semi solid substrates. The enzymes phytase produced by *A.niger* were efficient to hydrolyse plant phytates. Hence, it can be concluded from the results that 30-35 percent moist, heat sterilized rice bran, fortified with 2-4 mg ammonium phosphate per 100 g rice bran, in static cultivation produced maximum amount of enzyme (phytase) by *Aspergillus niger*, in 10 to 17 days old culture.

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