ISSN:2230-7346
Available online http://WWW.JGTPS.COM
Research Article
Journal of Global Trends in Pharmaceutical Sciences

Vol.2, Issue 2, pp 161-176, April–June 2011

ISOLATION OF PHYTASE PRODUCING FUNGI AND OPTIMIZATION OF PRODUCTION PARAMETERS

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ABSTRACT

Seventeen cultures of phytase producing fungi were isolated from different leguminous soil samples. Among these PF-3 exhibited maximum phytase activity. Studies were carried out to optimize the physical and chemical parameters. The optimum productivity of phytase was achieved with optimized process parameter such as Calcium phytate (0.5%) as carbon source, $(NH_4)_2$ SO_4 (0.1%) as nitrogen source, agitation speeds at 160 rpm, Tween-80 at (0.5%v/v) as a surfactant, incubation temperature of 30^0C, initial pH of 6, incubation period of 4 days, 5% inoculum level. The maximum production of phytase was under the optimized conditions an increase the yield of 1.63 times.

Keywords: Isolation, phytase, optimization, fungi, enzyme activity

Introduction:

Phytase is an enzyme that hydrolyses phytic acid to myo-inositol and phosphoric acid in a stepwise manner forming myo-inositol phosphate intermediates. Phytases (EC 3.1.3.8) are classified as histidine acid phosphatases (1). Phytic acid is chemically myo – inositol 1, 2, 3,4,5,6 – hexakisdihydrogen phosphate -

IP6 (2) is the major storage form of phosphate in plants, comprising 1-5% by weight of edible legumes, cereals, pollens and nuts. Salts of phytic are called phytates. The presence of phytases in plant foodstuffs is well documented. It is the primary source of inositol and storage form of phosphorous in plant seeds that are used as animal feed ingredient (3). Most foods of plant origin contain 50-80% of their total phosphorous as phytate. Recent studies have established the role of inositol phosphate intermediates in the transport of materials into the cell. Their role in transport as secondary messengers and in signal transduction has been confirmed (4).

The availability of phosphorous can be improved by adding microbial phytase to the feed or by using phytase rich cereal diet. The enzyme minimizes the need for supplementation with inorganic phosphorous due to improvement in the utilization of organic phosphorous in poultry (5). Phytase is used for elimination of phytate in the feed and food industries, in the preparation of myo-inositol phosphates (6), semi synthesis of peroxidase (7), as a soil emendment (8) and in aqua culture industries. (9)

Phytase hydrolyzes phytic acid and releases inorganic phosphate which could resolve some of the problems caused by phytate in animal feed. Some of the commercially important phytase producing fungi are: Aspergillus, Penicillium, Mucor and Rhizopus (10). All fungal isolates produces active extra cellular phytase among these Aspergillus species (11) produces most active phytases and they have enormous applications (12).

In the present investigation to isolate a potent fungal strain that produces a large amount of phytase in submerged culture and optimization of physical and cultural characteristics such as pH, temperature,

inoculum level, age of inoculum, agitation ,the effect of carbon, nitrogen, phosphorous sources, surfactants and their optimum concentrations.

Materials and Methods:

Screening of Fungal Isolates for Phytase production:

All the soil samples were collected near the root of leguminous plants at 4-6 inches depth in sterile screw capped test tubes. The points of collection had as wide varying characteristics as possible with regard to the organic matter, particle size, colour of soil, geographical distribution.

An enrichment technique involving Czepak's medium without phosphate was used as phosphate – deficient medium(13) and calcium phytate as sole carbon source containing $NaNO_2$, 2 g ; $MgSo_4.7H_2O$,0.5 g ; KCl, 0.5 g ; $FeSO_4$, 0.5 g ; calcium Phytate 0.5 g; distilled water up to 1000 mL, pH 5-5.6. Antibacterial agent (Penicillin –5 units/ml) was incorporated to control the bacterial contamination plated into 6 inches sterile petri plates and incubated at 28^0C for 3days. Each colony producing a translucent zone was considered a potential phytase producer. The diameter of the translucent hydrolyzed zone is an indication the amount of phytase produced. Around 17 isolates were screened and selected according to their best-hydrolyzed zone diameters. The isolates were inoculated on Potato Dextrose Agar (PDA) medium slats and maintained as master cultures.

Production of Enzyme:

The spore crop from master culture was subcultured on PDA and incubated for 72 hrs at 28^0C on rotary shaker (125 rev/min). The spore crop of each slant was scrapped into 5 ml sterile water and shaken well with sterile glass beats on rotary shaker for 30 min to break the spore chains and to make a uniform suspension. A 5ml of

inoculum was used to inoculate 45ml of sterile fermentation medium in 250ml Erlenmeyer flasks and incubated for 8 days on rotary shaker to allow the growth of the organism. Samples were drawn on each day and the amount of phytase activity was assayed.

Phytase Assay:

Pipette out aliquots of standard phosphorous solution into a series of tubes then add to each of the tubes (including blank) the following solutions in the order. 0.4ml of 10% of Trichloroacetic acid (TCA), 0.4 ml of molybdate solution, 0.2 ml of Amino napthosulphonic acid(ANSA) reagent and 4ml of double distilled water, mix well after each addition. Keep the tubes as such for 5 minutes. Measure the blue colour formed at 640nm. Phytase activity was assayed by estimating the amount of inorganic phosphate liberated from calcium phytate (or) phytic acid by the action of phytase. When we add Ammonium molybdate solution and 10% TCA solution to standard phosphorous solution, it forms phosphomolybdic acid by combining with Ammonium molybdate solution in the presence of TCA which is a protein precipitant. Phosphomolybdic acid reacts with reducing agent such as ANSA reagent, to form the blue colour by reducing molybdinum in phosphomolybdic acid.

A standard graph was prepared with concentration of phosphorous on X-axis and Optical density on Y-axis.

Determination of phytase activity:

Phytase activity was determined by mixing 0.1ml of suitably diluted culture filtrate and 0.9 ml of 0.2M acetate buffer, pH 4.5, and containing 0.5 mg of calcium phytate. The mixture was incubated for 20 min at 37^0C. Liberated orthophosphate was determined

by the method of (14).

Optimization of culture media for phytase production:

In submerged fermentation, the substrate is liquid broth in which various nutrients were dissolved. Successful fermentation means (high yield of enzyme) will occur only when all the essential features are brought together. The strain's production potential not only depends on the genetic nature, but also on nutrients supply and the cultural conditions. So, it is important to know about the suitable nutrients and cultural conditions required to achieve higher productivity. It was proposed to study the following parameters to optimize the production of phytase.

All experiments were conducted in triplicate. Three different production media were tried for phytase activity and their composition is presented in Table .1.

50ml of basal medium in 250ml flask was inoculated with 72 hr old inoculum and incubated on rotary shaker at 28^{o}C for 96 hr.Samples in triplicate were withdrawn from each flask and assayed for extracellular phytase content.

Effect of various Incubation Temperatures:

To study the optimal incubation temperature for maximum phytase production, the flask containing production medium was incubated at various temperatures (25^{0}C, 30^{0}C, 37^{0}C,50^{0}C) for 6 days keeping all other conditions at their optimum level. Samples were withdrawn periodically on 3rd, 4th and 5th day. The samples were assayed as described earlier. The optimum incubation temperature achieved by this step was fixed for subsequent experiments.

Effect of Initial pH:

While optimizing the initial pH of the production medium, the pH was varied from 5.0 to 9.0 with 1N HCl or 1N NaOH. The fermentation was carried out at 30^0C keeping all other conditions at their optimum level. Samples were assayed as described earlier. The optimum initial pH achieved by this step was fixed for subsequent experiments.

Effect of Various Incubation Periods:

To study the various incubation periods on phytase production, the flasks with the production medium were inoculated and incubated at 30^0C for 8 days samples were withdrawn periodically on 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th}, $7^{th}$and 8^{th} day and samples were assayed as described earlier. The optimum incubation period achieved by this step was fixed for subsequent experiments.

Effect of Level of Inoculum:

The fermentation profile of the organism is affected by the size of inoculum and its physiological conditions. Inoculum level of 2.5%, 5% 7.5%, 10% (v/v) were added to the production medium. The inoculated flasks were incubated at 30^0C for 6 days on rotary shaker keeping all other conditions at their optimum level and samples were assayed for phytase activity as described earlier. The optimum level of inoculum achieved by this step was fixed for subsequent experiments.

Effect of various Carbon, Nitrogen and Phosphorus Sources:

The production medium was supplemented with different carbon sources at 1%w/v (glucose, cornstarch, combination of glucose & cornstarch, calcium phytate, Sucrose); different both organic and inorganic nitrogen sources at 1%w/v (Yeast

extract, Casein hydrolysate, Peptone, $NaNO_3$, $(NH_4)_2$ SO_4). The samples were assayed as described earlier and the best sources and their optimized concentrations obtained were used in the subsequent experiments.

Effect of Various Agitation Speeds:

To study the effect of various agitation speeds, flasks with production medium were agitated at 100, 150, 200, 250 &300 rpm for 6days. At the end of the fermentation, samples were assayed as described earlier. The optimum level of agitation speed achieved by this step was fixed for subsequent experiments.

Effect of Various Surfactants

To study the effect of various surfactants (Tween-80 and Triton X-100) were added to the production medium at 0.5% v/v and fermentation was carried out (15). At the end of fermentation samples were assayed as described earlier.

Results and Discussion:

In the present investigation, it was proposed to isolate phytase producing fungi from various soil samples of leguminous plants collected from different places. About 17 fungal isolates were isolated from screening procedure. Among isolates, 5 isolates, viz., PF-3, PF-2, PF-1, PF-5, and PF-4 showed significant Phytase activity (Fig.1).PF- 3 was found to be the best phytase producer according to its maximum hydrolyzed zone diameter (15.7mm). Phytase production capabilities of these 5 isolates were further confirmed by shake flask fermentation. From the results it was confirmed that PF-3 was producing maximum yield of phytase.This potent isolate was selected for the optimization studies. Suitable inoculum mediums as well as basal production medium were selected for detailed fermentations.

Of the three media studied Medium III gave maximum phytase yield - 47U/ml (Fig.2) was selected as a basal medium for subsequent studies.

The effect of incubation temperature was studied on phytase production (Fig.3) and 30^0C (48.5U/ml) was found to be optimum temperature. It was found that the maximum phytase production (50 U/ml) was obtained at pH 6 (Fig.4).As the metabolic activities of the microorganisms are very sensitive to changes pH, phytase production was affected if the pH level was higher or lower compared to the optimum value. Newly isolated fungal strain PF-3 was able to grow and produced highest phytase activity (50.5 U/ml) at 4 days incubation period (Fig.5).

Inoculum level is an important factor for production of phytase, lower inoculum density may give insufficient biomass causing reduced product formation and where as higher inoculum may produce too much biomass leading to the poor product formation. The highest phytase production (52 U/ml) was obtained at 5 % v/v (Fig.6). So the inoculum level of 5% v/v was found to be optimum for maximum phytase production.

Carbon is a major component of the cell and the rate at which a carbon source is metabolized can often influence the production of metabolites. The influence of different carbon sources on phytase production was shown in Fig.7.Among different carbon sources studied, the highest yield of phytase was obtained with Calcium phytate (64.5U/ml).

After establishing the best carbon source, it was planned to evaluate the optimum concentration of calcium phytate for better production of phytase. Calcium phytate at different concentrations like 0.5, 1, 1.5, 2% w/v were studied. The results indicated that maximum yield of phytase (70.5 U/ml) was obtained at 0.5% w/v

concentration of calcium phytate (Fig.8).Among various both organic and inorganic nitrogen sources better phytase production (73 U/ml) exhibited by $(NH_4)_2 SO_4$ (Fig.9) with optimum concentration of 0.1% (Fig.10). Among various agitation speeds better phytase production (75 U/ml) exhibited at 160 rpm (Table.2). In a medium containing surfactant, growth was, however, dispersed and phytase yields increased. High phytase production (77 U/ml) exhibited by Tween-80(Table.2) at concentration of 0.5%v/v.

Production of Phytase with all Optimized Conditions:

Based on the results obtained with all optimized parameters, an attempt was made to evaluate the extent of improvement in the modified production medium. The results were presented in Fig.11

Table.1. Composition of different media for Phytase production

	Quantities (g/L) of different media		
	Medium No. I	Medium No.II	Medium No.III
Corn starch	-	80	-
Glucose	10	30	-
Malt extract	3	-	-
Yeast extract	3	-	2
Sucrose	-	-	10
Peptone	0.5	-	-
Tryptone	-	-	3
$MgSo_4.7H_2O$	-	0.5	0.5
$MnSo_4. 7H_2O$	-	0.01	0.01
$FeSo_4. 7H_2O$	-	0.1	0.01
$(NH_4)_2So_4$	-	-	2
K_2HPO_4	-	0.2	-
KCl	-	-	0.5
pH	6.8	5.4	7.4

Table.2.Effect of various agitation speeds and concentration of surfactant on Phytase activity

Agitation speed(rpm)	Phytase activity(U/ml)	Concentration of surfactant	Phytase activity(U/ml
100	59	Tween-80	77
130	63.5	Triton X-100	63.5
160	75		
220	70.5		

Fig.1-Hydrolysed zones to different fungal screened isolates

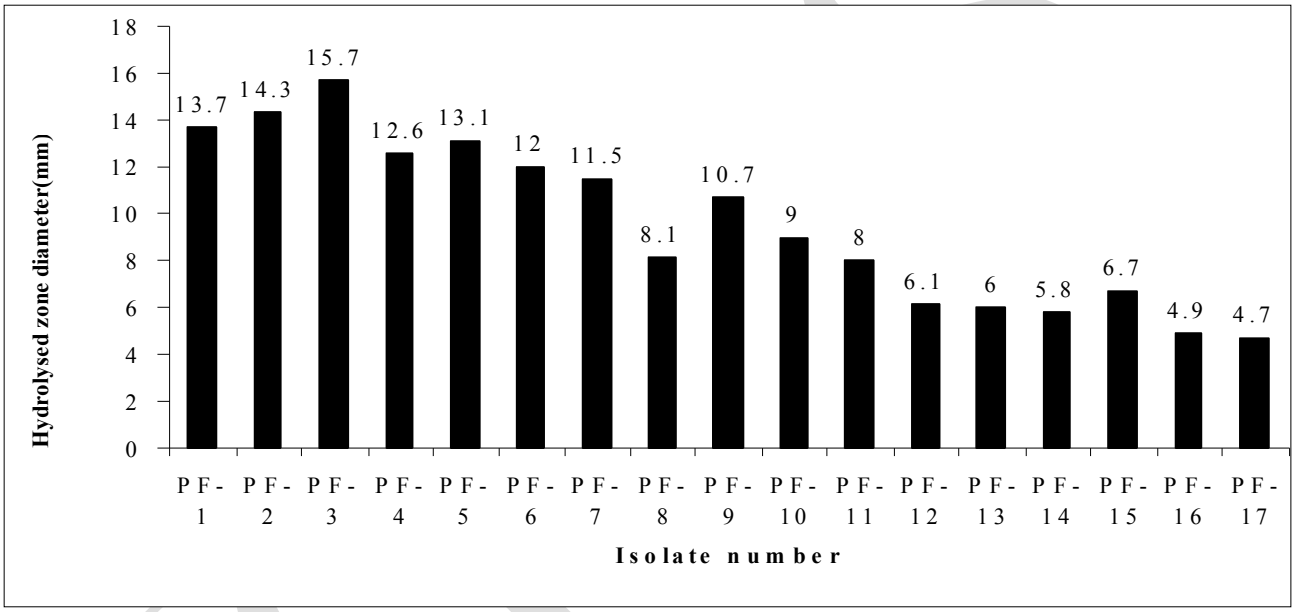


Fig.2-Effect of different media compositions on phytase activity

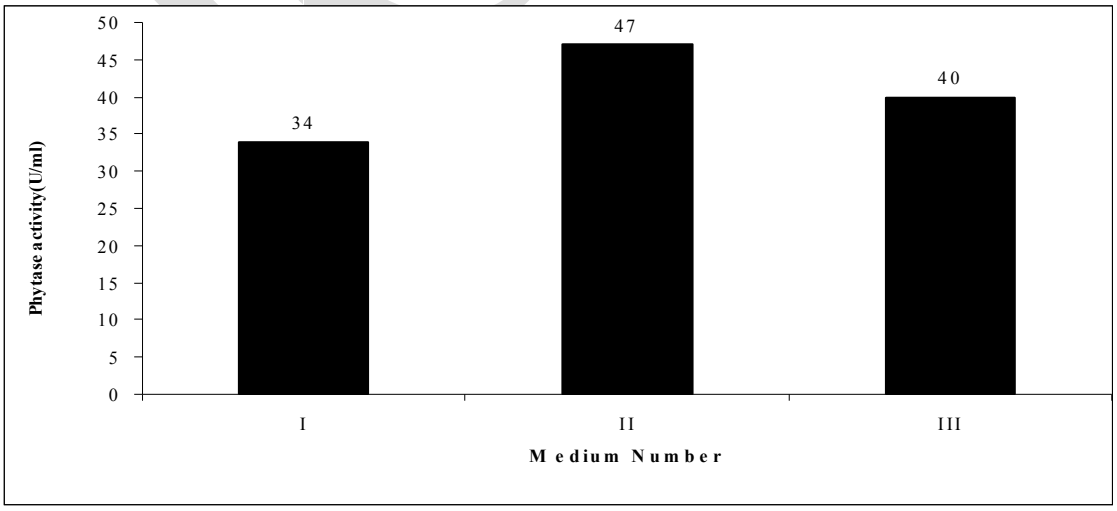


Fig.3-Effect of incubation temperature on phytase production

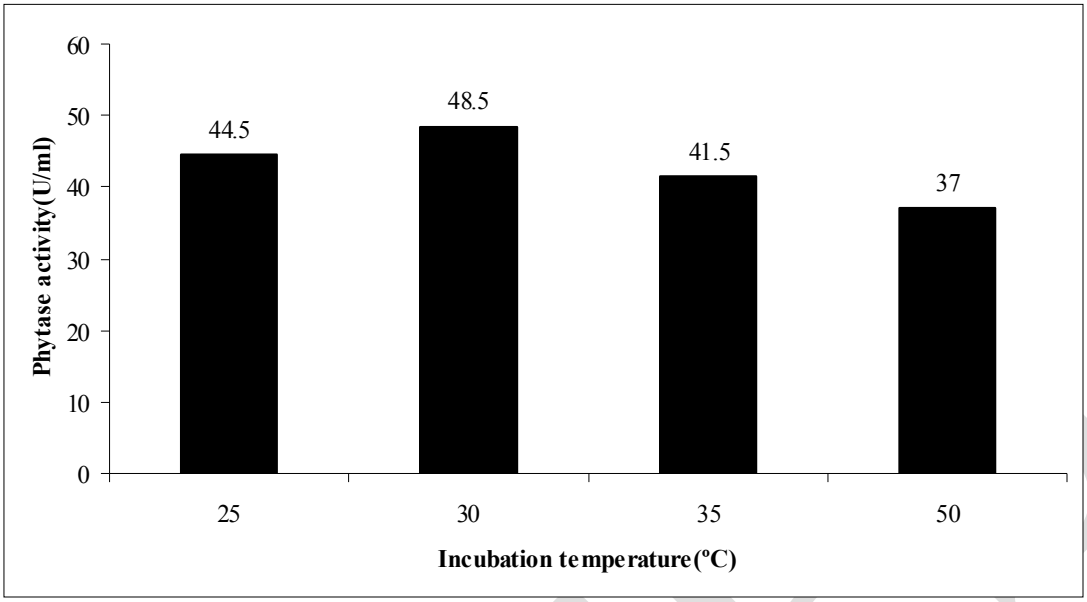


Fig.4-Effect of initial pH on phytase production

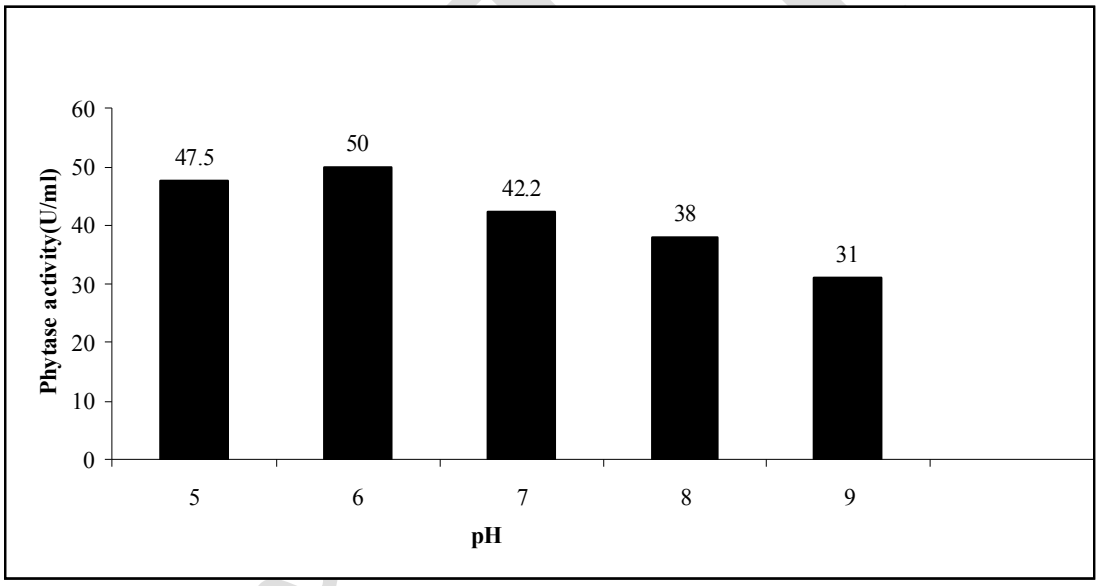


Fig.5-Effect of incubation period on phytase production

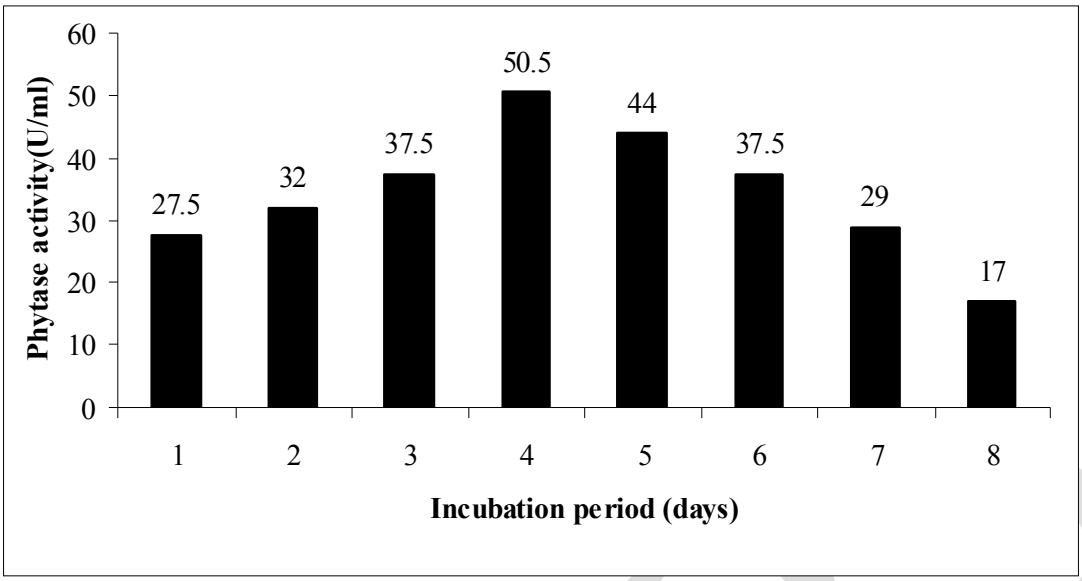


Fig.6-Effect of inoculum level on phytase production

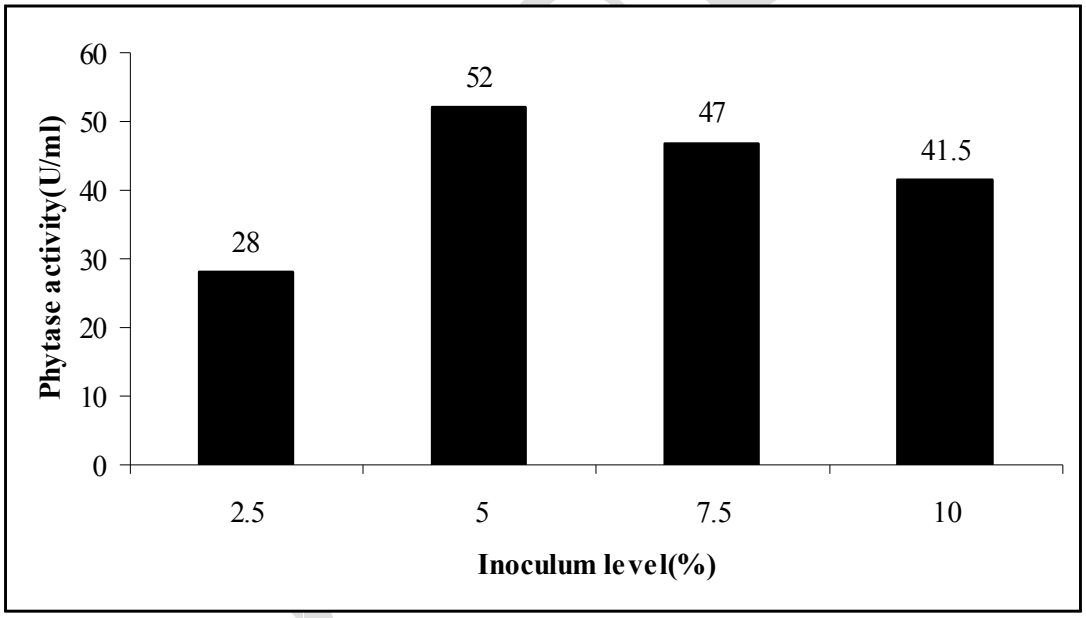


Fig.7. Effect of supplementary carbon sources on phytase production

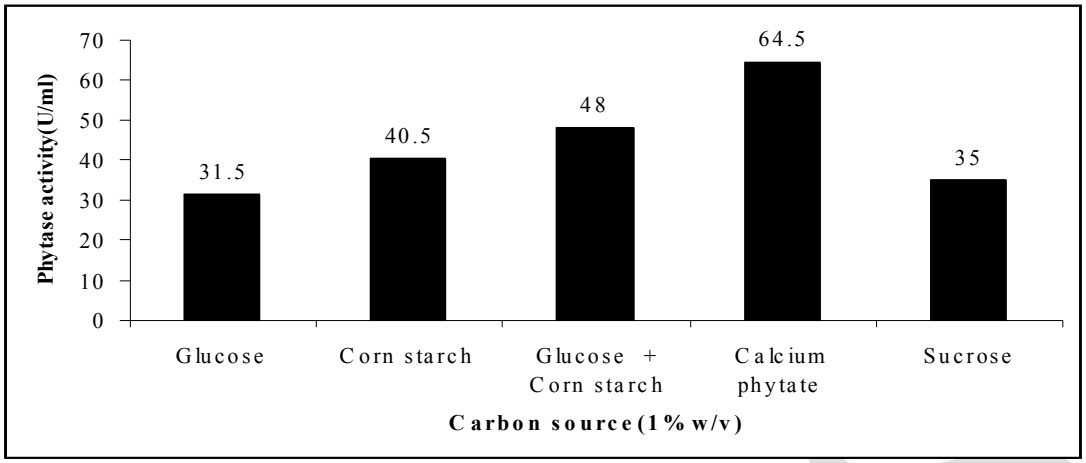


Fig.8. Effect of different concentrations of calcium phytate

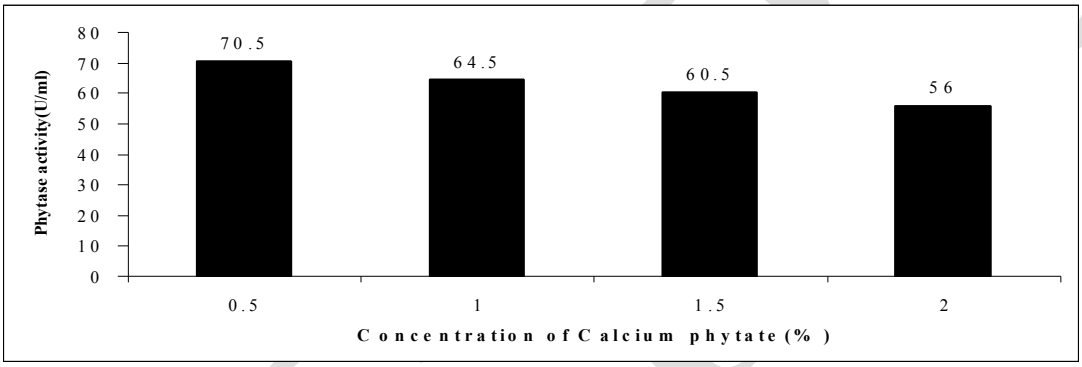


Fig.9. Effect of supplementary nitrogen sources on phytase production

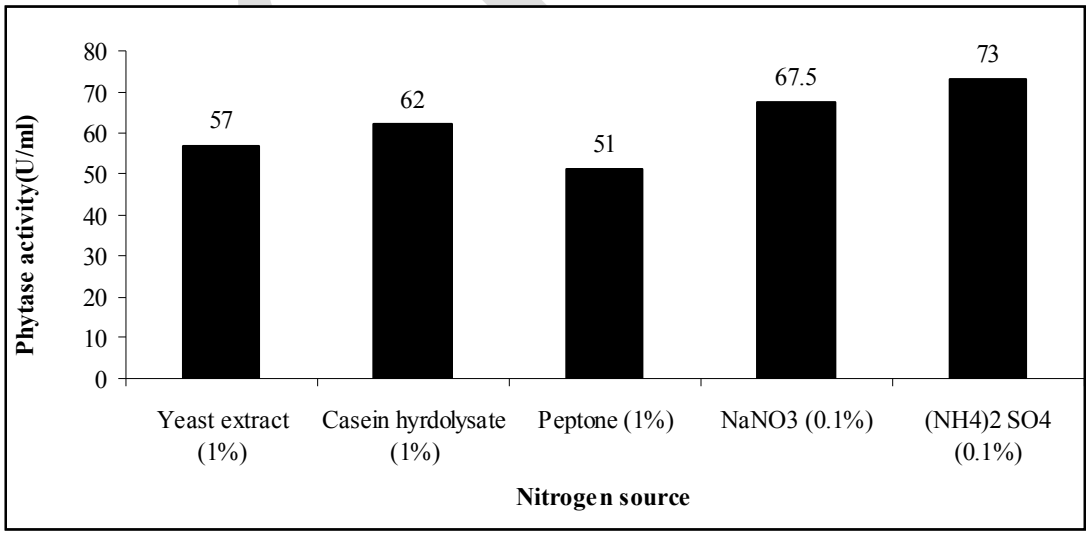


Fig.10. Effect of different concentration of Ammonium sulphate on phytase production

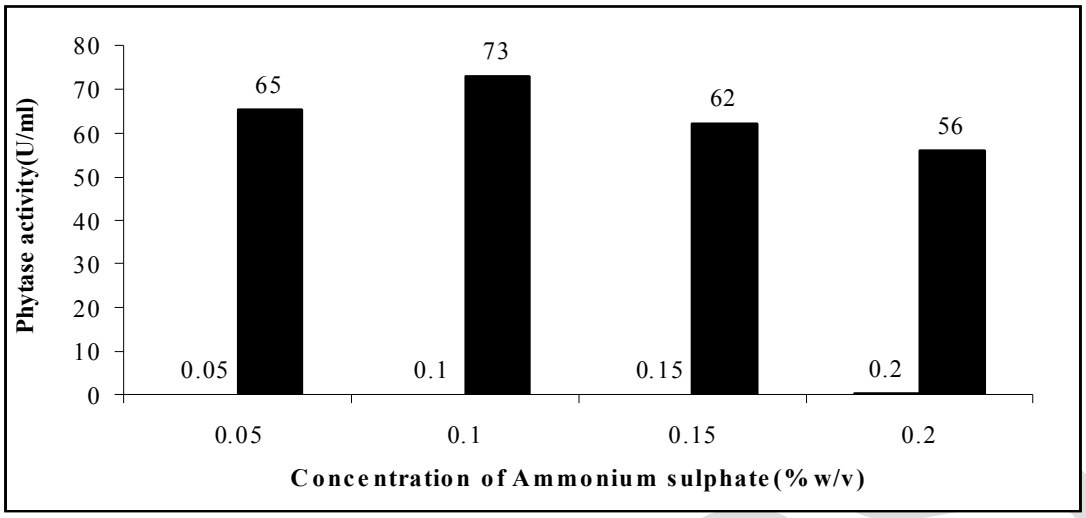
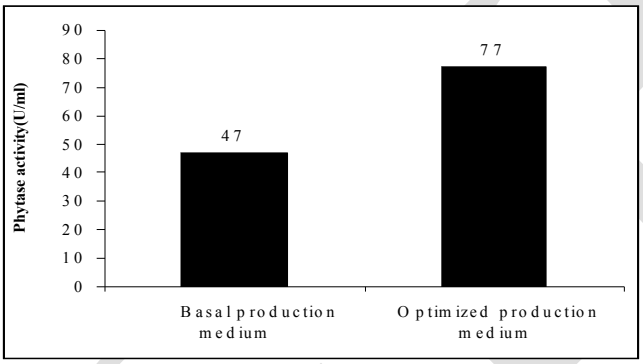


Fig.11.Phytase production in the basal production medium and optimized production medium



Conclusion:

In the present study phytase production by our fungal isolate (PF-3). Initially Phytase activity was determined in 3 different media. Maximum production was obtained in medium III. The optimum productivity of phytase was achieved with optimized process parameter such as Calcium phytate (0.5%) as carbon source, $(NH_4)_2$ SO_4 (0.1%) as nitrogen source, agitation speeds at 160 rpm, Tween-80 at 0.5%v/v as a surfactant, incubation temperature of 30^0C, initial pH of 6, incubation period of 4 days, 5% inoculum level.The maximum production of phytase was under the optimized conditions 77U/ml.This represents an increase the yield

of 1.63 times.

Acknowledgements:

The author thanks the Principal and Head of the University College of Pharmaceutical Sciences, Andhra University, and Visakhapatnam, India, for providing infrastructure for this study.

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