STUDIES ON THE PRODUCTION AND CHARACTERIZATION OF PHYTATE DEGRADING ENZYMES IN *ASPERGILLUS NIGER*

THESIS

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By

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Dedicated

To

My Parents and my teachers

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"Thanks is such a little word
No bigger than a minute
But there is a world of meaning
And appreciation in it"

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B.S.GUNASHREE

Dr. G. Venkateswaran
Scientist
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CERTIFICATE

This is to certify that the Ph.D thesis entitled "**Studies on the production and characterization of phytate degrading enzymes in *Aspergillus niger*"** submitted to the University of Mysore, Mysore for the degree of Doctor of Philosophy is the result of work carried out by **Ms. B. S. Gunashree** in the Food Microbiology Department of Central Food Technological Research Institute, Mysore under my guidance during the period 2002- 2006.

Dr. G. Venkateswaran
Research Supervisor

DECLARATION

I hereby declare that the Ph.D thesis entitled **"STUDIES ON THE PRODUCTION AND CHARACTERIZATION OF PHYTATE DEGRADING ENZYMES IN *ASPERGILLUS NIGER*"** submitted to the University of Mysore, Mysore for the degree of Doctor of Philosophy is the result of work carried out by me under the guidance of Dr. G. Venkateswaran, Scientist, Food Microbiology Department, Central Food Technological Research Institute, Mysore during 2002- 2006.

I further declare that the results contained in this thesis have not been previously submitted for any other degree or fellowship.

(B. S. GUNASHREE)
Candidate

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LIST OF ABBREVIATIONS

%	Percent
AAM	Acetone, Acid, Molybdate
AAS	Atomic Absorption Spectrometry
AOAC	Association of Analytical Chemists
CDM	Czapek Dox Medium
CM	Complete Medium
EDTA	Ethylene diamine tetra acetic acid
EMS	Ethyl methane sulphonate
g	Gram
hrs	Hours
IMTECH	Institute of Microbial Technology
L	Litre
LAB	Lactic Acid Bacteria
lbs	Pressure in Pounds
M	Molar
mg	Milligram
ml	Millilitre
mm	Millimeter
mM	Millimolar
MM	Minimal Medium
MRSM	Mans Rogosa Sharpe Medium
MSM	Mineral Salt Medium
MTCC	Microbial Type Culture Collection
N	Normality
nm	Nanometer
npP	Non- phytate Phosphorus

NTG	N'- methyl, N- nitro, N- nitrosoguanidine
ºC	Degree celcius
PDA	Potato Dextrose Agar
PEG	Polyethylene glycol
Phy	Phytase
pP	Phytate- Phosphorus
PSM	Phytase Screening Medium
RM	Regeneration Medium
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SM	Synthetic Medium
SmF	Submerged Fermentation
sp/sps	Species
SSF	Solid state Fermentation
TEMED	N, N, N', N'- tetra methyl ethylene diamine
TP	Total Phosphorus
U/gds	Units per gram dry substrate
U/kg	Units per kilogram
U/mg	Units per milligram
U/ml	Units per millilitre
UV	Ultraviolet radiation
v	Volts
w/v	Weight per volume
WBEM	Wheat Bran Extract Medium
µM	Micromolar

SYNOPSIS

SYNOPSIS

STUDIES ON THE PRODUCTION AND CHARACTERIZATION OF PHYTATE DEGRADING ENZYMES IN *ASPERGILLUS NIGER*

Preamble:

Animal feed manufacturing is one of the important agro-industrial practices in most of the countries worldwide. Livestock practitioners are facing many problems associated with nutritional aspects due to anti-nutritional components such as phytic acid and other phenolic compounds that are predominant in the complex feed materials. Excretion of excess of minerals and other by-products poses a pollution threat to the surrounding environment. Phytic acid (myo- inositol hexakisphosphate), is a storage form of phosphorus in plant materials such as cereals, legumes and oil seeds that constitutes 1-2% by weight. Phytate-phosphorus accounts for 60 - 90% of the total phosphorus. Phosphorus bound to phytate is either non available or is available only in reduced quantities to monogastric animals such as poultry, swine and also to humans. This has forced the livestock rearers to supplement the feed with inorganic phosphates such as dicalcium or tricalcium phosphates, which are highly expensive. Further, undigested phytate- phosphorus is excreted by the monogastric birds and animals. Thus, high titres of phosphates in the environment leads to pollution of livestock areas, eutrophication of nearby water bodies that depletes oxygen content and thereby death of the aquatic animals.

The negatively charged phytic acid binds to positively charged cations such as calcium, magnesium, manganese, zinc, iron and proteins and these metal salts are made unavailable to monogastrics. Hence phytic acid is considered to be an anti- nutritional factor in feed. The phytate- protein insoluble complexes reduce the bioavailability of basic amino acids in proteins. Phytic acid is also known to inhibit certain enzymes such as α-amylase, trypsin, tyrosinase and pepsin. For the

last two decades, researchers all over the world have shown considerable interest in developing an economically viable process for the removal of phytic acid or its degradation either chemically or enzymatically.

Phytase enzyme hydrolyzes phytate and makes the bound phosphorus and other minerals available to the monogastric animals. Despite the voluminous literature on the sources of phytase, microbial source from *Aspergillus* is the most reliable one and highly characterized for application as a feed supplement.

Based on the above-mentioned facts, the present study is mainly focused **on the following objectives:**

1. **Isolation and Screening of phytase producing fungal strains**
2. **Effect of certain physico- chemical parameters for phytase production.**
3. **Nutritional studies using phytase on layer hens.**
4. **Strain improvement studies for hyper phytase production.**
5. **Purification and characterization of *Aspergillus niger* CFR 335 phytase.**

EXPERIMENTAL PLAN:

The work was carried out as follows:

1. Isolation and Screening of phytase producing fungal strains:

Microorganisms, especially fungal strains were isolated from natural habitats such as soil and poultry waste. Soil samples and poultry wastes were serially diluted and appropriate dilutions were plated on Czapek Dox Medium. About 1,500 different fungal strains were isolated by the above procedure.

The selected fungal strains and some standard *Aspergillus* strains procured from Microbial Type Culture Collection Centre (MTCC), Chandigarh were screened for phytase production by point inoculating on phytase screening

medium (PSM) containing 0.5% calcium phytate. The strains were qualitatively selected based on the clearing zones formed due to hydrolysis of calcium phytate by the phytase enzyme. In addition to fungal strains, some of the Lactic acid bacteria (LAB) cultures were also screened for phytase production. Lactic acid bacteria, being one of the interesting groups of bacteria that balance the intestinal microflora are known to produce phytase enzyme during natural fermentation process. Hence, these organisms could be directly used to feed the monogastrics that can aid in the bioavailability of essential minerals.

The phytase enzyme production was quantified by cultivating the selected fungal strains under submerged and solid- substrate fermentation conditions. All the isolates were found to produce fairly good amount of phytase enzyme in solid-state fermentation than in submerged fermentation. Finally, one of the isolate *Aspergillus niger* CFR 335 was selected for further studies. The fungus was also tested for acid phosphatase production both under SmF and SSF. It was found that the fungus only produces phytase and a very negligible amount of acid phosphatases.

2. Effect of certain physico- chemical parameters on phytase production:

In the present investigation, effect of certain physico- chemical parameters on phytase production by *Aspergillus niger* CFR 335 both in submerged and solid-state fermentation were studied. Effect of physical parameters such as temperature and pH were studied by incubating the fungus at different temperatures ranging from 10- 55°C and pH between 3.0- 8.5 respectively. The optimum temperature and pH for maximum phytase enzyme by *Aspergillus niger* CFR 335 were found to be 30°C and 4.5 respectively. The temperature and pH stability of phytase enzyme were also studied by varying the assay temperature and buffer pH. The enzyme was found to be stable upto 45°C of temperature and pH 6.5.

Optimization of chemical parameters on phytase production was also carried out. Studies on the effect of sugars on phytase production both in submerged as well as in solid- state fermentation condition were carried out by incorporating different sugars such as glucose, fructose, lactose, maltose, sucrose, xylose, sorbitol, mannose, xylitol, arabinose, cellobiose, galactose, raffinose, rhamnose and trehalose at 1% level in the cultivation media. The results showed that the media supplemented with sucrose had maximum phytase activity than with any other sugars used both in SmF and SSF.

Effect of nitrogen was studied by supplementing the cultivation media with different nitrogen compounds such as yeast extract, peptone, ammonium sulphate, ammonium nitrate, potassium nitrate and urea at 0.5% level. It was found that, the medium supplemented with peptone had a slight increase in the enzyme production while there was some declination with urea. There was no significant variation with other nitrogenous compounds.

Studies on the effect of various phosphates on phytase production was evaluated by incorporating different phosphate compounds such as sodium dihydrogen phosphate, potassium dihydrogen phosphate, p- nitrophenyl phosphate, ammonium phosphate and phosphoric acid at 0.25% level in the cultivation media. It was indicated from our results that concentration and type of phosphate play an important role in the growth and enzyme production by *A. niger* CFR 335. It was found that p- nitrophenyl phosphate did not favour growth of the fungus and all other phosphates supported the growth. However, the enzyme activity was drastically repressed when compared with the control. Thus, 0.25% concentration is considered to be a high dose for phytase production.

Surfactants are the surface-active molecules that make the cell permeable and hence increase the yield of intracellular metabolites. In liquid medium supplemented with surfactants, the fungus grows as smaller pellets that have more

of growing tips and hence the phytase enzyme production could be enhanced. The effect of different surfactants on the phytase enzyme production was carried out by incorporating 0.25% of surfactants in the cultivation media. The following surfactants were used in this study, Tween- 20, Triton- X-100, Sodium dodesyl sulphate (SDS) and Ethylene di- amine tetra acetic acid (EDTA). Total growth suppression was observed in presence of SDS in SmF, while, in SSF there was no growth suppression. Though all other surfactants supported growth of the fungus both in SmF and SSF, no enhancement in the enzyme production was observed except for Tween-20.

Effect of different metal ions on the growth and phytase enzyme production was studied by incorporating the culture media with various metal salts that includes sodium chloride, magnesium sulphate, ferrous sulphate, manganese sulphate, copper sulphate, zinc sulphate and calcium chloride. All the metal salts were used at 0.1% level. The result indicated a two- fold enhancement in the enzyme production with the liquid medium supplemented with $CaCl_2$, while all other salts reduced the enzyme level. In solid- state cultivation, there was no significant effect on the enzyme yield with any of these metal salts.

3. Nutritional studies using phytase on layer hens:

Nutritional studies on layer hens (*Gallus domesticus*) were carried out using *Aspergillus niger* CFR 335 phytase enzyme in the animal house at CFTRI, Mysore. The overall metabolism of the layer hens fed with the phytase enzyme was evaluated. In this study, three weeks old layer hens were fed with *Aspergillus niger* CFR 335 phytase along with the commercial feed devoid of phosphorus and calcium ingredients. A control group fed with only commercial feed was maintained for comparative studies. Effect of different concentration of phytase enzyme on various parameters such as body weight, egg weight, eggshell strength, leg thickness, phosphorus excretion & utilization and calcium deposition in the

bone were observed. The results indicated that the supplementation of phytase enzyme at a concentration of 400U and 600U were effective in releasing the bound phosphorus and calcium in the poultry feed.

4. Strain improvement studies for hyper phytase production:

The phytase producing fungus, *Aspergillus niger* CFR 335 was subjected to mutation through physical and chemical methods using ultraviolet radiation and ethyl methane sulphonate (EMS) & N'-methyl N'-nitro N' nitrosoguanidine (NTG). Young spores of *A. niger* CFR 335 were subjected to UV irradiation for different time duration (10, 20 & 30 minutes). In chemical mutagenesis, the spores were treated with 2, 4, 6, 8 and 10 mM concentrations of EMS solution and 5, 10, 15, 20 and 25μgml^{-1} of NTG respectively. Killing effect of all the three mutagens was plotted against the duration of UV exposure and concentration of EMS and NTG used.

Aspergillus ficuum, another phytase producer was also subjected to mutagenesis by the above procedure. *A. niger* CFR 335 and *Aspergillus ficuum* were screened to obtain auxotrophic mutants (amino acid auxotrophic marker). *Aspergillus niger* CFR 335 ala^- and *Aspergillus ficuum* val^-, $isoleu^-$ mutants were obtained and used for protoplast fusion experiment. *Aspergillus niger* CFR 335 ala^- was found to have considerable reduction in its sporulation, at the same time enzyme yield was not affected.

Interspecific protoplast fusion between *Aspergillus niger* CFR 335 ala^- and *Aspergillus ficuum* val^-, $isoleu^-$ was carried out. Young mycelial pellets of both the parents were treated with cell wall lysing enzyme obtained from *Trichoderma harzianum* and protoplasts were obtained osmotically. The protoplasts of the two strains were fused in the presence of poly ethylene glycol (PEG) 3500 containing 1M sorbitol as an osmotic stabilizer. Putative hybrids were qualitatively characterized for phytase production on phytase screening medium (PSM). In this

experiment, sixteen hybrids were selected and quantitative characterization was carried out for phytase production, biomass content, total genomic DNA content and proximate analysis (fat, protein, carbohydrate and ash content) of the biomass.

5. Purification and characterization of *Aspergillus niger* CFR 335 phytase enzyme:

Crude phytase enzyme obtained through solid-state fermentation of *Aspergillus niger* CFR 335 was subjected to three- step purification process. All the steps were carried out at 4ºC.

The enzyme purification was initiated by saturating the crude enzyme with solid ammonium sulphate upto 80% level. The precipitate was dialysed for overnight against 0.2M acetate buffer at pH 4.5. The dialysed enzyme sample was eluted through ion- exchange chromatography with DEAE- Sephadex G-50 column equilibrated with 0.2M acetate buffer pH 4.5 with a linear gradient of 0.5M NaCl. The active fractions of the protein were pooled and subjected to Sodium dodesyl sulphate – Poly acrylamide gel electrophoresis (SDS- PAGE) to determine the molecular mass of the enzyme. Finally, the purity of the enzyme was checked by HPLC. The purified enzyme was characterized for pH and temperature optima.

The results revealed that the purified enzyme had an optimum temperature and pH of 30ºC and 4.5 respectively with a molecular mass of 66 kDa.

Conclusion

Phytase enzyme has a wide range of sources that include plants, animals and microorganisms. Plant and animal sources have their own drawbacks for the application in feed industries. Microorganisms especially fungi, are highly exploited for their convenience in maintenance, production and scale- up studies. The phytase enzymes produced by the wild microbial strains are inactive at high

temperatures. Research on phytase enzyme is progressing well for the manufacture of a thermostable enzyme that can withstand high temperatures during pelletization of the feed. Associated with the expanding growth in poultry and swine industries, there is also an increased market demand for the most reliable microbial strain that produces an enzyme having a wide temperature and pH range for its activity. Thus, our study concludes that, the selected fungal strain *Aspergillus niger* CFR 335, a locally isolated fungus was a potent phytase producer capable of producing nearly 2000 U/gds in solid- state fermentation. This enzyme has been found to be stable up to a temperature of 45°C at pH 4.5. The enzyme was found to be stable for more than three months at room temperature. Due to the Generally Regarded As Safe (GRAS) status of the organism, phytase enzyme produced has a promising role to play as a nutritionally important factor in the feed industries. Phytase enzyme also combats the environmental stress due to phosphorus load by increasing the bioavailability of phosphorus and other essential minerals.

INTRODUCTION

INTRODUCTION

Enzymes are often referred to as 'biological catalysts', which are found in or closely associated with living cells. These enzymes have found application in various industrial processes that include, alcohol fermentation, baking, brewing, in effluent treatment as diagnostics and also as biosensors. They are also used in the manufacture of cheese, beverages, textiles, detergents, paper and in animal feed additive. Enzymes supplied to the food processing industries are offered in liquid, powdered, or granulated forms. They are commonly derived from controlled fermentation by microorganisms or by extraction from plant and animal tissues.

The animal feed industry is an extremely important part of the world's agro industrial activities. It is an industry that has gone through many vicissitudes in the past few years. Today, consumers and the industrialists are looking more closely than ever before into how compound animal feeds are produced, how the animals are reared and what is the end result of the systems of animal husbandry for the environment (Godfrey and West, 1996). Of the various additives used by the feed industry, enzymes are a relatively new development but one which is anticipated to grow rapidly. Certain enzymes are involved in the enhancement of general nutrient availability and phytase, mainly improves the availability of the organic phosphorus (phytic acid or phytate) found in cereals and vegetable proteins.

Phosphorus in unrefined cereals, legumes, nuts, seeds and tubers is mostly present as phytic acid. Phytic acid (myo-inositol hexakisphosphate) is a major storage form of phosphorus in cereals and legumes that constitutes approximately 1-2% by weight of cereals and oilseeds and phytate- phosphorus accounts between 60 and 90% of the total phosphorus present in these seeds (Erdman, 1979; Cheryan, 1980; Reddy *et al*, 1982). Phytate is considered to act either as a reserve of phosphorus or myo-inositol or in maintaining a phosphorus balance (Cosgrove, 1966; Asada *et al*, 1968). The interactions of phytic acid with protein and several

minerals are considered to be one of the primary factors limiting the nutritive value of cereal grains and legume seeds. Phytate-phosphorus in the meals is unavailable to monogastric animals because they lack phytase. Supplementing soybean and other meals with relatively inexpensive rock phosphate, which provide the animals with this necessary nutrient, rectifies the lack of adequate phosphorus. The excess phytate- phosphorus was disposed off in the animal manure. However, this practice added even more phosphate to the animal's feed ration and resulting ultimately in phosphorus levels in the manure that far exceeded the land's capability. Phytases catalyze the hydrolysis of phytic acid to various lower myo-inositol derivatives and inorganic phosphate. These enzymes are either absent or present in very low levels in the gastrointestinal tract of monogastric animals and nearly all the phytate phosphorus ingested by these animals is excreted into the environment resulting in phosphorus pollution in areas of intensive livestock production (Cromwell and Coffey, 1991).

Phytic acid also acts as an antinutrient because of its ability to chelate covalent metal ions such as calcium, magnesium, manganese, zinc, iron and proteins, thus rendering them insoluble and unavailable for absorption by monogastric animals. (Erdman 1979; and Erdman and Forbes, 1977). By virtue of its chemical nature the negatively charged phytic acid readily binds to positively charged molecules such as cations and also proteins. The salts of phytic acid i.e., the phytates are permanent ingredients of plant tissues and are the major storage form of phosphorus in them (Lott *et al*, 2000). Phytic acid or phytate is the primary storage form of phosphorus in plant seeds and is associated with fibre in many foods, such as soya and cereal – based products. It consists of an inositol, which is a hexahydroxycyclohexane in chair conformation, with six phosphate ester bonds. The phosphate groups confer on it a high negative charge and therefore a strong chelating ability, which reduce the dietary bioavailability of amino acids and minerals such as Ca^{2+}, Zn^{2+}, Fe^{2+} (Kennefick and Cashman,

2000). Phytic acid is known to inhibit certain enzymes such as α- amylase (Sharma, *et al,* 1978), trypsin, tyrosinase and pepsin (Graf, 1986). The insoluble phytates are closely associated with proteins in oilseeds (Fontaine *et al*, 1946) and reduce the bioavailability of basic amino acid groups in these proteins (Cheryan, 1980; Barre and van Huot, 1965). To enhance plant phosphorus utilization and to circumvent the deleterious effect of phytic acid in animal nutrition, phytases from *Aspergillus* sp. have been fed to monogastric animals (Mroz *et al*, 1990).

Phytate hydrolysis can occur during food preparation and production in the intestine, either by phytase from plants, yeasts or other microorganisms. This degradation is of nutritional importance, because removal of phosphate groups from the inositol ring results in an increased bioavailability of essential dietary minerals. Food processing techniques increasing the activity of the naturally occurring plant phytases are soaking, malting, hydrothermal treatment and fermentation. An alternative is addition of phytases or microorganisms producing phytase. Phytate degradation in the stomach and small intestine occurs as a result of activity of dietary phytase of plant or microbial origin (Ann and Thomas, 2002).

The nutritional consequences of phytic acid interactions with protein and minerals are well documented (Erdman 1979; Cheryan *et al*, 1980; Erdman and Forbes, 1977) and methods for its removal from foods of plant origin are desirable. Most research has concentrated on the removal of phytate from soybean and related products, because of their importance as sources of edible oil and as a protein source for human and animal consumption. Physical and chemical methods, such as, extraction, precipitation, roasting and autoclaving for reduction of phytic acid content in some commodities, have also been reported (Hartman Jr, 1979; Alli and Houde, 1987; Hussain *et al*, 1989; Rackis, 1974). However, application of these methods causes a partial loss of nutrients such as proteins and minerals (Gillberg and Tornell, 1976; Ford., *et al,* 1978; Ebune *et al,* 1995). Other methods like hydrolysis would be more desirable. Hence, phytate hydrolysis is

achieved enzymatically in most of the cereals and legumes that contain endogenous phytase (Chang, *et al,* 1977; Han, 1988 and Peers, 1953). Phytase enzyme is widely distributed in plants (Peers, 1953), animal tissues (Cooper and Gowing, 1983; Iqbal, *et al*, 1994) and it is also produced by many species of fungi and bacteria (Cosgrove, 1966). Strains of *Aspergillus niger* have shown to produce large amounts of extracellular phytase (Chelius and Wodzinski, 1994). By utilizing phytase in a digestive preparation, it eliminates the possibility of insoluble complexes being formed and thus increases the absorption of the cations.

Phytase, being a high- molecular weight protein, is sensitive to the presence of moisture and high temperature. Therefore, shelf life of the product must be considered and proper storage of the product needs to be maintained. Because of the heat and moisture associated with pelleting, enzymes are destroyed, therefore, stability of the enzyme during pelleting should be considered. Consequently, when phytase is added to a pelleted diet, spraying a liquid phytase product onto the cooled pellet will allow stability of the phytase (Tri- state Swine Nutrition Guide Bulletin 869-898). Hydrolysis of phytic acid to myo-inositol and phosphoric acid is considered as an important metabolic process in several bio-systems. Society's awareness and increased need for controlling agricultural pollution, particularly on phosphorus that limit the phosphorus content in manure have intensified the phytase research. The focus has mainly been on its production and use as a means of reducing inorganic-phosphorus supplementation in feed and consequent reduction in fecal phosphorus excretion. Environmental pollution due to the high-phosphate manure has resulted in the accumulation of phosphorus at various locations, especially in water bodies. This depletes the oxygen in water, leading to death of fish and other aquatic animals (Mallin, 2000). Therefore, decreasing the phytate content in plant materials to increase their nutritional values is preferable. This helps explain the increased interest in phytate- degrading enzymes, which is

evident from the published reviews (Zyla 1992; Liu *et al*, 1998; Wyss *et al*, 1999; Wyss *et al*, 1999a; Frossard *et al*, 2000).

Two important groups of animals, pigs and poultry lack the enzyme needed to efficiently digest phytate in their feed. As a result, they excrete large amounts of phosphorus into the environment that results in pollution. However, for their proper skeletal growth, these animals need phosphorus at suitable concentration. Supplementation of phytase to the feed provides an alternative to tackle both these conditions effectively. Conversion of phytin into an assimilatable form of phosphate has been an object of biotechnological interest for animal feeding. Furthermore, the generation of these intermediate products (Inositol mono, di, tri or tetra phosphates) and inositol could also have relevant health implications, as they are involved in the regulation of vital cellular functions (Shamsuddin, 2002). Phytases are particularly important in human nutrition for their possible role in the degradation of phytate during both food processing and gastrointestinal transit (Ann and Thomas, 2002). Phytase supplementation can reduce the amount of phosphorus in manure up to approximately 30% in monogastric animals (Ashok Pandey *et al*, 2001).

Phytase production in microorganisms is influenced by media components, especially carbon and nitrogen sources (Sreeramulu *et al* 1996). The effect of metal ions and physical factors such as pH, temperature, inoculum density and incubation time on phytase production have also been shown (Ashima Vohra and Satyanarayana, 2001). Lactic fermentation of maize or sorghum can shift a “low iron bioavailability” diet into an “intermediate to high bioavailability” diet. The fermentation process is considered to have a promotive effect on iron absorption, probably through the formation of organic acids. This acidification is accompanied by improvement of solubility of minerals. Since, lactic acid bacteria from bakery-leavened products degrade phytic acid and improve calcium and magnesium solubility, mineral bioavailability in fermented cereal foods will be higher than

that of unleavened products. The consumption of fermented cereal products such as bread could be recommended to provide bioavailable minerals in case of human. In whole-wheat flour medium, in which phytic acid is naturally present, fermentation leads to better mineral solubility by improving phytic acid hydrolysis through acidification (Svanberg *et al,* 1993).

Phytases (myo-inositol hexakisphosphate phosphohydrolases: EC 3.1.3.8 and EC 3.1.3.26) are enzymes hydrolyzing only phytic acid (myo-inositol hexakisphosphate) unlike acid phosphatases that can hydrolyze other organophosphate substrates (Ullah and Gibson, 1987). There was no sequence homology between the fungal phosphatases with other phosphatases except for the conserved RHGXRXP motif. This motif is characteristic of the active site of histidine acid phosphatases. Therefore fungal phytases are said to form the phytase subfamily of histidine acid phosphatases (Mitchell *et al*, 1997). The reaction mechanism of histidine acid phosphatases has been elucidated by studies on *Escherichia coli* acid phosphatase (Ostanin *et al*, 1992; Ostanin and van Etten, 1993). Greiner *et al,* (1993) have shown that the substrate specificity and the kinetic parameters of the enzyme designated as a phytase. Recently, two phytases from *Bacillus subtilis* have been isolated and the corresponding genes have been cloned (Kerovuo *et al*, 1998; Kim *et al,* 1998; Kim *et al,* 1998a). These two enzymes have shown to have approximately 90% identity on amino acid level. They are shown to have similar biochemical and biophysical properties when compared with other *Bacillus* phytases that have been characterized so far (Powar and Jagannathan, 1982; Shimizu, 1992). The ability of phytic acid to bind metal ions is lost when the phosphate groups are hydrolyzed through the action of the phytase enzyme.

The use of microbial phytase as a feed additive has been examined several times over the last 20 years, resulting in improved phosphorus availability from poultry and swine feed. However, the high cost of the enzyme, compared to the

cost of inorganic phosphate, has prevented its universal use. Recently, there has been renewed interest in phytase due to the low- cost production of this enzyme by recombinant DNA technology. Phytase has the potential to reduce the amount of phosphate in poultry and swine excretes by enhancing phosphorus retention by the animal. With the increased concern for the environment and the nutritional bioavailability for the monogastric animals, there is an increased demand for an ideal and thermostable phytase that can withstand high temperature of feed processing.

SCOPE OF THE INVESTIGATION

The antinutritional effect of phytic acid is attributed to its strong chelating effect with nutritionally essential minerals and proteins. Phytase supplementation has a promising role to play in the bioavailability of essential nutrients in monogastric feed. This enzyme catalyses the hydrolysis of phytic acid, an antinutrient in the cereal and legume based feed to release chelated phosphorus, other divalent cations and proteins. Phytase has a wide range of sources, of which microbes form the most extensive group for the production of phytases. The enzyme has a potential to reduce phosphorus load in the manure and livestock areas, thus reducing the pollution of aquatic bodies and agricultural lands. The scope of the present investigation is the production and characterization of phytate degrading enzyme in *Aspergillus niger* CFR 335. Optimization of various physical and chemical cultural conditions for phytase production, improving the fungal strain for increased production of phytase enzyme with reduction in sporulation level for the industrial application and nutritional studies on the efficacy of *Aspergillus niger* CFR 335 phytase enzyme on the overall metabolism of the layer hens. The investigation also aimed at purification of phytase which is the major phytate-degrading enzyme produced by the fungus.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The field of enzymology has expanded rapidly, particularly in the last three decades, because of intensive efforts carried out by numerous laboratories to elucidate the structures and mechanisms of a large number of enzymes. Enzymes have a rich history of use in the food and feed industries. There is not a single system that does not involve enzyme reactions. They are used in the production or processing of starch, cheese, fruit beverages, artificial sweeteners, meat and are also frequently used in brewing and wine making. Enzymes are also employed in many agricultural practices. In many processes, a cascade of complex enzyme-mediated reactions is in operation. The majority of the food and feed enzymes are hydrolases and oxidoreductases. The conventional distribution of the current world sales of enzymes is assessed by their application sectors such as dairy, detergent, starch, textiles and others which include animal feed enzymes. The use of feed enzyme is highly predominating in the animal feed industries. Feed enzyme production level is growing globally with the increase in rearing of poultry, swine and other monogastrics. The feed enzymes play a vital role in suppressing the antinutritive activity of phytic acid and other phenolic compounds. Phytase is one of the enzymes frequently incorporated with the poultry feed since it is capable of hydrolyzing phytic acid.

For most of the enzymatic processes, microbial enzymes are preferred, primarily because they are easier and cheaper to obtain. One reason for this is that many bacteria and fungi have the natural ability to secrete enzymes into their environment. This is an important trait for microbes that rely on the digestion of organic matter as a source of energy, carbon and nutrients. When microbes secrete valuable enzymes, it is relatively easy to separate the enzymes from the cells through simple centrifugation. Another reason for relying on microbes is that several microbes, including the bacteria *Bacillus subtilis* and *Bacillus licheniformis* and the fungus *Aspergillus niger,* have a long history of safe use in

the food industry. The worldwide market for industrial enzymes is around 1 billion tonnes at least 400 companies manufacture enzymes, of which 12 companies dominate the industry.

Phytic acid (PA):

Phytic acid (*myo*-inositol hexakisphosphate, IP_6), forms 60-80% of the total phosphorus found in the plant commodities. Complementary foods based on cereals are often one of the first semisolid foods introduced into the diet of infants. To improve protein quality, cereals are commonly combined with milk or legumes. However, both cereals and legumes contain relatively high amounts of phytic acid, a compound that binds strongly to nutritionally essential minerals such as Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, and trace elements that can impair their bioavailability (O' Dell and Savage, 1960; Oberleas, 1973; Lei, *et al.,* 1993). Hence, phytic acid is considered to be anti nutritional. Since the monogastrics or simple- stomached animals like swine, poultry and humans have little or no phytase activity in their gastrointestinal tract (Bitar and Reinhold, 1972), these animals are unable to utilize phytate- phosphorus (Nelson *et al.,* 1971) and thus require dietary supplementation of inorganic phosphorus, an expensive and non-renewable mineral. The unutilized dietary phytate- phosphorus is excreted by the animals, causing phosphorus pollution in areas of intensive animal production (Sweeten, 1992). The antinutritional effect of phytic acid causes deficiency related disease in monogastrics which may lead to poor quality egg and meat in case of poultry. It is estimated that 10 Kg of DCP (Dicalcium phosphate) can be replaced by 250 g of phytase enzyme. The potential demand for phytase enzyme in the swine and poultry feed is around 8000 tonnes/ annum, but the present production level is 250 tonnes/ annum only. Hence, with the growing poultry sector in the country and increased market demand for phytase enzyme, there is a need for more production of phytases and their use in food and feed formulations.

Phytic acid is the major inhibitor of iron and zinc absorption from human diets. Since it is present in cereal grains and legume seeds, it is present in most manufactured foods based on these ingredients (Nelson, *et al* 1968). In human studies with radioactive or stable isotopes, phytic acid has been reported to inhibit the absorption of iron (Hallberg, *et al* 1989), zinc (Navert, *et al* 1985), calcium (Weaver, *et al* 1991), magnesium (Bohn, *et al* 2004) and manganese (Davidsson, *et al* 1995). Influence of phytic acid on iron and zinc absorption is of great public health importance. Low absorption of these nutrients from plant- based diets in developing countries is considered to be a major factor in the etiology of iron deficiency after 4- 6 months of age (Taylor, *et al* 1995).

Phytic acid is utilized during seed germination. It supports seedling growth by supplying biosynthetic needs of the growing tissues. Young seedlings use the end products of PA hydrolysis, particularly *myo*-inositol for cell wall formation, Tsao, *et al* (1997). PA content and phosphorus availability in cereals depend on several factors like genetics, environmental conditions, locations, irrigation conditions, type of soil, year and fertilizer application (Reddy *et al,* 1989). The proportion of total P found as phytate-P increases with the dose of P_2O_5 supplied to the plant. When a plant receives a higher dose of P than it requires, surplus P seems to be stored in the form of phytic acid. PA is deposited in seeds during the seed development and its concentration changes as the kernels mature, and reaches a maximum when kernels are ripe (Barrier *et al,* 1996).

Nutritionally, inorganic- phosphorus supplementation in the diets of swine and poultry can be obviated by including adequate amounts of phytase, along with appropriate manipulation of other dietary factors (Han, *et al,* 1997). Increasing public concern regarding the environmental impact of high phosphorus levels in animal excreta has driven the biotechnological development of phytase and its application in animal nutrition.

Phytic acid structure and chemistry:

The organic phosphate first obtained as a calcium or magnesium derivatives from the aleurone grains of wheat endosperm by Pfeffer (1872) was later shown to be of widespread occurrence in seeds (Cosgrove, 1966). Winterstein (1897) on hydrolysis found that it released what was subsequently designated as inositol (*myo*- inositol) and inorganic orthophosphate. The modern terminology given was hexakisphosphate of *myo*- inositol. The term 'phytic acid' was given for the free acid, 'phytate' for a salt and 'phytin' for the calcium/ magnesium salt.

Research has traditionally focused on the unique structure that gives it the ability to bind minerals, proteins and starch and the resulting detrimental effects. Phytic acid first identified in 1885 is a natural plant compound with a unique structure that is responsible for its characteristic properties. Phytic acid has twelve replaceable protons allowing it to complex with multivalent cations and positively charged proteins and thus can be found in many forms. Phytic acid exists as free acid, phytate or phytin according to physiological pH and the metal ions present. The structure of phytic acid has been clearly demonstrated by Neuberg (1908) who has proposed a structure $C_6H_{24}O_{27}P_6$ with 18 acid hydrogens. Anderson (1914) proposed a structure containing 12 acid hydrogens $C_6H_{18}O_{24}P_6$. Until recently only *myo*- inositol was thought to exist in nature as a phosphorylated derivative but 'soil phytate' is now known to be a mixture of several inositol hexakisphosphate, has been identified as a component of *Amoeba* cell surfaces (Posternak, 1903; Courtois and Masson 1951). All the naturally- occurring inositol hexakisphosphate have been synthesized. The first successful attempt to synthesize *myo*- inositol hexakisphosphate was reported by Posternak (1919) who phosphorylated *myo*- inositol with polyphosphoric acid. The Anderson structure, a hexaorthophosphate, is now generally accepted as correct. More recently a single crystal X- ray analysis by Blank *et al* (1975) has shown that in the dodecasodium salt hydrate containing 38. H_2O (C; 4 -2.C), the conformation is inverted with the phosphate groups on C-

1, C-3, C-4, C-5 and C-6 becoming axial and the phosphate on C-2 becoming equatorial.

The evidence so far presented seems to indicate that the *myo*- inositol hexakisphosphate molecule adopts the 5e/la conformation (I) in solution, particularly if the cation is one, whose behavior closely approximates that of an ideal cation, eg. Tetrabutyl ammonia (Costello, *et al*, 1976). At higher pH values, particularly in the presence of co- ordinating cations such as Na^+ and K^+, conformational inversion takes place to give the (5a/ le) VII form found in the crystalline dodecasodium salt. The pictorial representation of phytic acid structure is given in Fig 1.

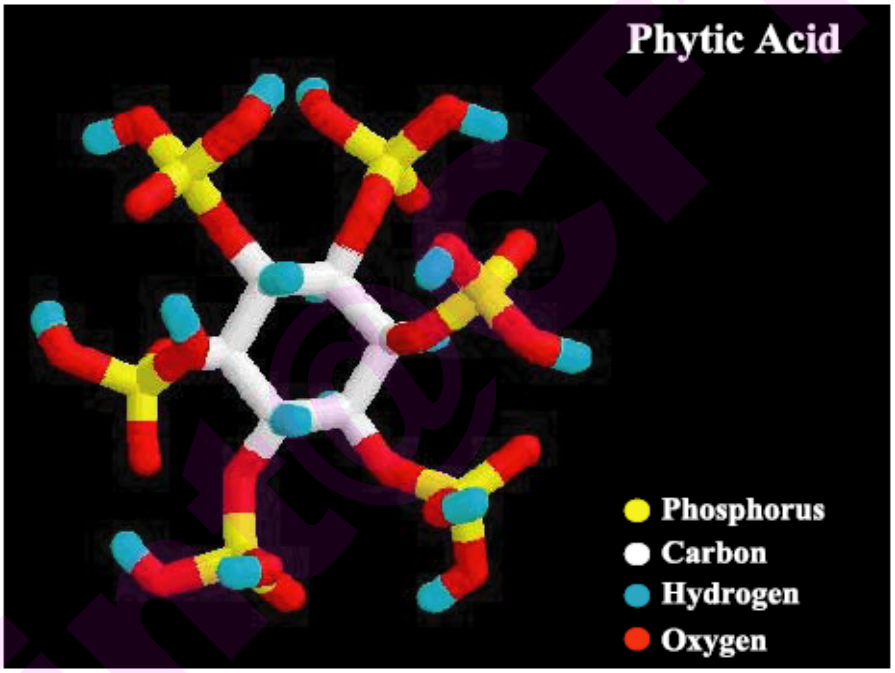
Interaction of phytic acid with different compounds:

(a) Metallic complexes:

Inorganic polymers of orthophosphoric acid (pyrophosphates, linear polyphosphates, etc.,) are able to form soluble, stable chelate complexes with Ca^{2+} and *myo*- inositol hexakisphosphate behaves in a similar way. By comparing the solubility of calcium phosphate with that of calcium *myo*- inositol hexakisphosphate as a function of pH, Bjerrum, 1941 has concluded that in the case of the complexed form, ferric iron forms acid- insoluble complexes with *myo*- inositol hexakisphosphate, but in the presence of excess iron acid- soluble complexes can be formed (Anderson, 1963). The ability of the hexakisphosphate to complex with trivalent cations is probably responsible for its stability in soil organic matter. A later study by Anderson, *et al* (1974) showed that *myo*- inositol hexakisphosphate was more strongly adsorbed by acid soils than was inorganic orthophosphate.

The ability of *myo*- inositol hexakisphosphate to complex with multivalent cations is important from the nutritional point of view and some studies of relative

Fig 1: Phytic acid structure



(Figure courtesy, W. Schmidt- USDA/ ARS)

stabilities have been made using titration methods (Vohra *et al* (1965) have listed the order of stability as $Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$ at pH 7.4. Maddaiah *et al* (1964) have found the order of stability to be $Zn^{2+} > Cu^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+}$. Metallic ions such as Fe^{3+} and Cu^{2+} are known to be effective catalysts for reactions leading to oxidative spoilage in foodstuffs. For this reason the use of *myo*- inositol hexakisphosphate as a sequestering agent has been suggested as a means of reducing spoilage in soya bean oil, ascorbic acid component of soft drinks (Niwa, *et al*, 1967) and in wines (Posternak, 1965).

(i) Phosphorus availability:

The fact that the phosphorus of P_6 *myo*- inositol is almost unavailable to young chickens was first demonstrated by Common (1939). His data suggested that phosphorus is absorbed as the orthophosphate ion. The ability of various species of poultry to utilize phosphorus from P_6 *myo*-inositol will largely depend on their ability to hydrolyze the phosphoric ester. Young birds have a very limited ability to hydrolyse P_6 *myo*-inositol.

Nelson, *et al* (1971) have studied the effect of supplemental phytase on the utilization of phosphorus from P_6 *myo*-inositol by chickens. The enzyme prepared as an acetone- dried powder from culture fluid of the fungus *Aspergillus ficuum* NRRL 3135 was added to the diet at levels up to 3g/ Kg. At this level chickens utilized phosphorus from P_6 *myo*-inositol as efficiently as supplemental inorganic phosphate. The added phytase was active in the alimentary tract of the chicken and not in the feed prior to ingestion. It was suggested by Jordan, *et al* (1906) that, ruminants are able to utilize phosphorus from P_6 *myo*-inositol which was later confirmed by Mathur (1953); Tillman and Brethour (1958); Ellis and Tillman, (1961). Rapid hydrolysis of P_6 *myo*-inositol takes place in the rumen (Reid, *et al*, 1947) and the pronounced phytase activity of rumen organisms (Raun, *et al*, 1956) suggests that this hydrolysis is not dependent on phytases present in the feed.

There is some suggestion that vitamin D enhances the utilization of phytate phosphorus (Nelson, 1967).

(ii) Calcium availability:

The anticalcifying properties of certain cereals were first noticed by Mellanby (1925), the responsible agent was later identified as P_6 *myo*-inositol (Bruce and Callow, 1934). The interrelation of dietary calcium with P_6 *myo*-inositol has been reviewed by Widdowson (1970). Evidence show that the human intestine can absorb calcium from a low- calcium "high phytate" diet as in such a situation hydrolysis of P_6 *myo*-inositol takes place in the intestine. Presumably, it is postulated that in a low- calcium situation, the P_6 *myo*-inositol is more soluble and thus is hydrolyzed more readily by intestinal phytases. The tendency to regard the role of P_6 *myo*-inositol as an important factor in calcium nutrition in humans has been shown in the works of Walker, *et al* (1948).

(iii) Zinc availability:

The first direct evidence that zinc deficiency may develop in animals fed a diet composed of natural materials was obtained by Tucker and Salmon (1955). Zinc deficiency in humans was first recognized by Prasad, *et al* (1963). Phytic acid is also shown to inhibit zinc absorption, Turnlund, *et al* (1984), Fairweather, *et al* (1992), Davidsson, *et al* (1996), Manary, *et al* (2000). In 1957 it was reported that zinc in soybean protein was less available to chickens than that in casein and eventually it became accepted that the presence of P_6 *myo*-inositol in plant products was an important factor in the reduction of zinc absorption from food stuffs (Oberleas, 1973 and O' Dell, 1969). Zinc complexes strongly with P_6 *myo*-inositol particularly at pH 6.0 and furthermore, in the presence of calcium a synergistic effect has been demonstrated (Oberleas, 1973).

The effect of dietary P_6 *myo*-inositol on zinc availability has been studied in many animals, particularly pigs, chickens and rats (Oberleas, 1973 and O' Dell, 1969). Results of a recent investigation by Davies and Nightingale (1975) showed that there was a net loss of zinc from rats maintained on a zinc- deficient diet. It would appear that dietary P_6 *myo*-inositol forms complexes in the intestine with endogenously secreted zinc and thus prevents its reabsorption. Klevay (1975) has put forward the hypothesis that coronary heart disease is predominantly a disease of imbalance in regard to zinc and copper metabolism. Zinc deficiency brings about a marked reduction in intestinal mucosal alkaline phosphatase activity (Leucke, *et al* 1968; Williams, 1972; Davies and Flett, 1978) and as suggested, intestinal phytase activity is merely a manifestation of alkaline phosphatase activity. The ability of dietary P_6 *myo*-inositol to affect zinc availability may itself be influenced by the zinc status of the animal.

(iv) Iron availability:

The negative influence of phytic acid on iron absorption was clearly demonstrated in both adults and in infants (Hallberg, *et al* 1989; Sandberg, 1999; Hurrell, *et al,* 2002). Although there is little doubt that the consumption of a diet containing added P_6 *myo*-inositol lowers iron balances in human subjects (Mc Cance, *et al*, 1943; Sharpe, *et al*, 1950; Hussain and Patwardhan, 1959; Turnbull, *et al*, 1962), the effect of the endogenous P_6 *myo*-inositol contained in brown bread or wholemeal bread is less certain. Added Na- P_6 *myo*-inositol has been reported to have no effect or only a slightly depressing effect on utilization of iron by rats (Fuhr and Steenbock, 1943; Cowan, *et al*, 1966; Ranhotra, *et al*, 1974). The ability of the rat to utilize the iron naturally present in cereals has been attributed to secretion of intestinal phytase. Morris and Ellis (1976) have reported that the major portion of iron in wheat is present as a salt extractable monoferric salt of P_6 *myo*-inositol that has a high biological availability to rats.

Ethylene diamine tetraacetic acid (EDTA) compounds have been shown to increase iron absorption from high phytate foods (INACG, 1993) and ascorbic acid increases iron absorption from soy formula (Davidsson, *et al* 1994). In addition to adding ascorbic acid or EDTA, there are two major processes to decrease the inhibitory effect of phytic acid on iron absorption. The first is phytate removal and the second is enzymatic degradation of phytate. It has been stated that an adequate intake of calcium play an important role in iron absorption than the content of dietary phytate that have no direct adverse effect on iron absorption. Calcium, iron, phosphate and P_6 *myo*-inositol appear to be mutually interacting, changes in the level of one, have an effect on the absorption of the others.

(v) Copper, manganese and magnesium availability:

Definite evidence that the presence of P_6 *myo*-inositol in diets can cause a reduction in Cu or Mn availability has only recently been obtained by Davies and Nightingale (1975) in studies with rats. Davis *et al* (1962) had reported earlier that diets containing an isolated soybean protein reduced the availability of these two trace metals for chickens, however, in view of the high P_6 *myo*-inositol content of soybean meal and the ability of soybean protein to complex with P_6 *myo*-inositol, it would seem likely that P_6 *myo*-inositol was the agent responsible.

During physiological studies on rats, Roberts and Yudkin (1960) noticed symptoms of magnesium deficiency; the symptoms were aggravated by the addition of P_6 *myo*-inositol (Na) to the diet. Reduced absorption of magnesium by rats from diets containing added P_6 *myo*-inositol has also been noticed by Scelig (1964) and Likuski & Forbes (1965). The addition of phytic acid to the diets resulted in a dose- dependent decrease of apparent Mg absorption and magnesium concentration in the plasma and femur (Rimbach and Pallauf, 1999).

(b) Protein complexes:

Formation of insoluble complex between proteins and polyphosphates is a well-known phenomenon (Posternak, 1965; Courtois and Lino, 1961). When polyphosphates such as *myo*-inositol hexakisphosphates are added to protein solutions at a pH below the isoelectric point of the protein, precipitation takes place and the complex does not redissolve until the pH is lowered to less than 2.0. This observation has been made for P_6 *myo*-inositol on a large number of proteins, and it appears that the property is common to most globular proteins. The precipitation presumably results from an aggregation, by formation of salt- like linkages, of several amino groups in a protein molecule around a molecule of P_6 *myo*-inositol. This leads to folding and a closer packing of the peptide chains and hence to the formation of an insoluble coacervate. The results of Barre and van Huot (1965) on human serum albumin showed that haxakisphosphate ion combined first with the terminal α-amino group and ε- amino group of lysine, then with histidine residues and finally with arginine guanido groups. Results from a similar study with hen ovalbumin were different; the hexakisphosphate ion combined with all arginyl residues first, then with those of lysine and finally with the histidine residues. The binding of *myo*-inositol hexakisphosphate to glycinin, a major globulin of soybean, has been investigated over the range of pH 2 - 10. The properties of protein- hexakisphosphate complexes are markedly affected by the amount of polyvalent cations present (Okubu *et al* 1976). Saio *et al* (1967, 1968, 1969) have studied extensively the effect of calcium levels on the properties and stability of tofu - gel (soybean curd), an important foodstuff in Japan.

(c) Lipid complexes:

During investigations on the inositol containing lipids of corn, Carter *et al* (1958) consistently isolated a fraction high in phosphorus and ash content. On acid hydrolysis, it yielded inositol phosphates, fatty acids, glycerol and several

ninhydrin positive substances; it was named 'lipophytin'. Further investigations showed that the metals present were mainly magnesium and calcium. The properties of lipophytin are similar in some respects to those of 1- (3-phosphatidyl)- L- myo-inositol, 3. 4- bisphosphate (triphosphoinositide) as its calcium or magnesium salt.

Phytate accumulation in plants:

A number of studies have been made on the rate of accumulation of P_6 *myo*-inositol in grains, particularly wheat. The accumulation of P_6 *myo*-inositol in broad beans (*Vicia faba*), sunflower (*Helianthus annus*) and corn (*Zea mays*) has been studied by Sobolev (1964). Walker (1974) studied the accumulation of P_6 *myo*-inositol in *Phaseolus vulgaris* and found that approximately 90% of the *myo*-inositol in the mature seed was accumulated between 24 and 30 days of embryogeny. Although most P_6 *myo*-inositol is located in the seeds or tubers there are plants in which it appears to be distributed throughout the vegetative tissue. A large proportion (68%) of the zinc in corn, a cation strongly chelated by P_6 *myo*-inositol, was located in the germ. Morton and Raison (1963) had stated previously that appreciable amounts of P_6 *myo*-inositol did occur in wheat endosperm. More recently, P_6 *myo*-inositol containing particles have been isolated from rice embryos (Ogawa *et al*, 1977). Phytic acid content of different plant material is summarized in Table 1.

Phytate accumulation in animals:

Presence of phytates in animal tissue are found in a lesser extent. Benesch and Benesch (1967, 1967a), Chanutin and Curnish (1967) and Benesch *et al* (1968) have discovered that phytates can combine with human haemoglobin to greatly reduce its affinity for oxygen. Rapoport and Guest (1941) have reported the presence of a high concentration of P_6 *myo*-inositol in avian red cells.

Table 1: Phytate-phosphorus content & phytase activities of plant feed ingredients

Ingredients	Phytate P (as % of total P)	Phytase activity (units/Kg)
Cereals		
Maize	72	15
Barley	64	582
Wheat	69	1193
Oats	67	42
Cereal by-products		
Rice bran	80	122
Wheat bran	71	2957
Rice polishing	89	-
Oil Seed Meal		
Soybean meal	60	8
Cottonseed meal	70	-
Peanut meal	80	3
Rapeseed meal	59	16
Sunflower meal	77	62
Coconut meal	49	24
Sesame meal	81	-

Source: Integrated crop Management, Purdue University, Department of Animal sciences

Physiological function of phytate:

Five physiological roles have been suggested for P_6 *myo*-inositol; these are,

i. As a phosphorus reserve store
ii. As an energy store
iii. As a competitor for ATP during its rapid biosynthesis near maturity, whereby metabolism is inhibited and dormancy is induced
iv. As an immobilizer of multivalent cations needed for control of cellular processes, such ions are later released after germination
v. As a regulator of the level of inorganic phosphate.

Toxicological studies of phytic acid:

Toxicological studies of phytic acid were extensively studied on some of the animal systems that includes human. Impaired Mg bioavailability due to 15g PA/kg diet was accompanied by an increase of hepatic thiobarbituric acid reactive substances and protein carbonyls as well as by a moderate decline in liver reduced glutathione (GSH) levels. The liver homogenates of rats receiving the diets with 7.5 and 15g PA/kg, respectively, were much more susceptible to iron- induced lipid peroxidation than those of the controls (Rimbach and Pallauf, 1999). However, PA had no scavenging effect on superoxide radicals generated in the xanthine/ xanthine oxidase reaction. At marginal dietary iron supply, phytic acid supplementation reduced apparent Fe absorption, thereby decreasing liver Fe concentration. The results obtained provide evidence for antioxidant properties of phytic acid under in vitro conditions. However, neither phytic acid nor iron had any significant effect on liver oxidant or antioxidant status in vivo in growing rats (Rimbach and Pallauf, 1998).

In a nutritional study conducted by Rimbach and Pallauf, (1997), it was shown that under conditions of high dietary Zn, phytic acid had only little effect

on the carryover of Cadmium (Cd) in growing rats. It was also shown that phytic acid decreased apparent Mg absorption significantly and apparent absorption of Ca in tendency. Cd accumulation in the liver was not significantly altered and kidney Cd accumulation slightly increased owing to phytic acid. Dietary phytate caused significant reductions in growth rates, plasma Zn concentrations and hair Zn concentrations (Davies and Olpin, 1979). Dietary phytate reduced apparent iron absorption in the groups supplemented with 7.5 and 15g/kg diet phytic acid respectively to the individually housed albino rats, whereas, when another group supplemented with *Aspergillus niger* phytase enzyme along with phytic acid, the supplemented phytase counteracted the antinutritive effects of phytic acid on iron availability (Pallauf *et al,* 1999).

Lees and Leong (1996) have shown PA toxicity to neuronal perikornia when injected into the rat hippocampus. Its potency as a toxin was approximately equal to that of the excitotoxin quinolinate. It was concluded by their study that abnormal metabolism of phytic acid might possibly contribute to neuronal death in neurodegenerative diseases. Carcinogenecity of phytic acid has been studied and Hiasa *et al,* (1992) have shown necrosis and calcification of renal papillae in Fischer 344 rats when treated with PA. A dose- dependent reduction in the mean final body weights of rats was also observed. Grases *et al* (2006) have shown in their study that phytic acid acts as an inhibitor of calcification of cardiovascular system. Thus it could be concluded that phytic acid has both beneficial and toxicological effects on animal system.

Animal feed industry:

The animal feed industry is an extremely important part of the world's agroindustrial activities. Compound animal feed production follows the general trend of global agriculture towards consolidation and concentration. Most feed production takes place in industrialized countries and in these areas the annual

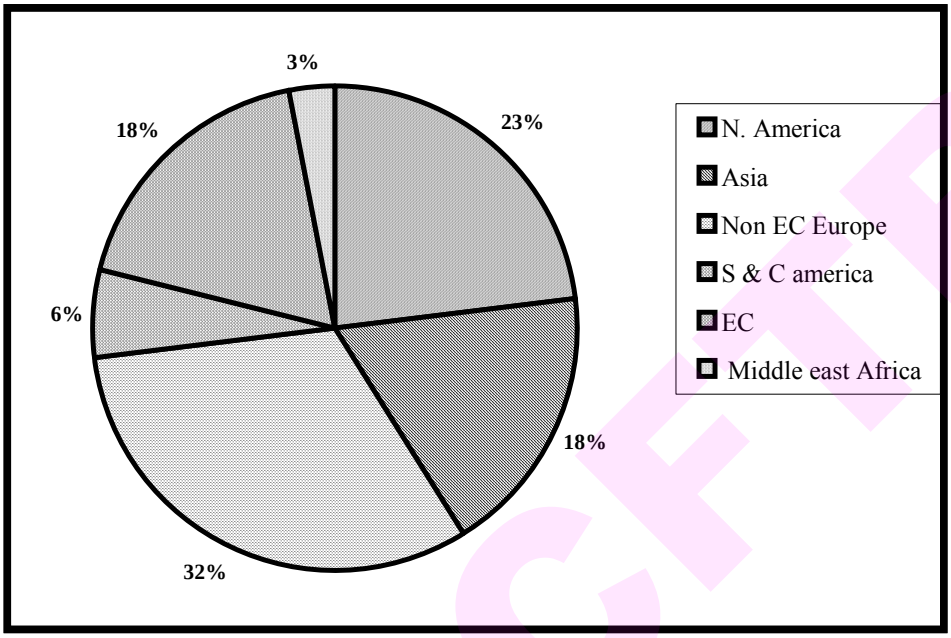
increase in tonnage is rather small. In the developing countries, however, as income and expectations rise, so does the requirement for animal production and hence these areas continue to have rapid growth in their animal feed industries. The world feed production by area and by type is shown in Figs 2 & 3. Of the various additives used in the feed industry, enzymes are relatively a new development but one which is anticipated to grow rapidly. The enzymes used as feed additive are divided into two sections; those dealing with enhancement of general nutrient availability and phytase, which improves the availability of the organic phosphorus (phytic acid) found in cereals and vegetable proteins.

Enzymes and Environmental pollution:

The intensive rearing of animals produces a considerable amount of manure that can place extra stresses on the environment. In Europe for example, the concentration of animal husbandry in parts of Holland, Northern Germany, France and Denmark has focused attention on nitrogen and phosphorus excretion and its effects on water supplies. Distribution of enzyme sales and global marketing of enzymes are shown in Figs 4 & 5.

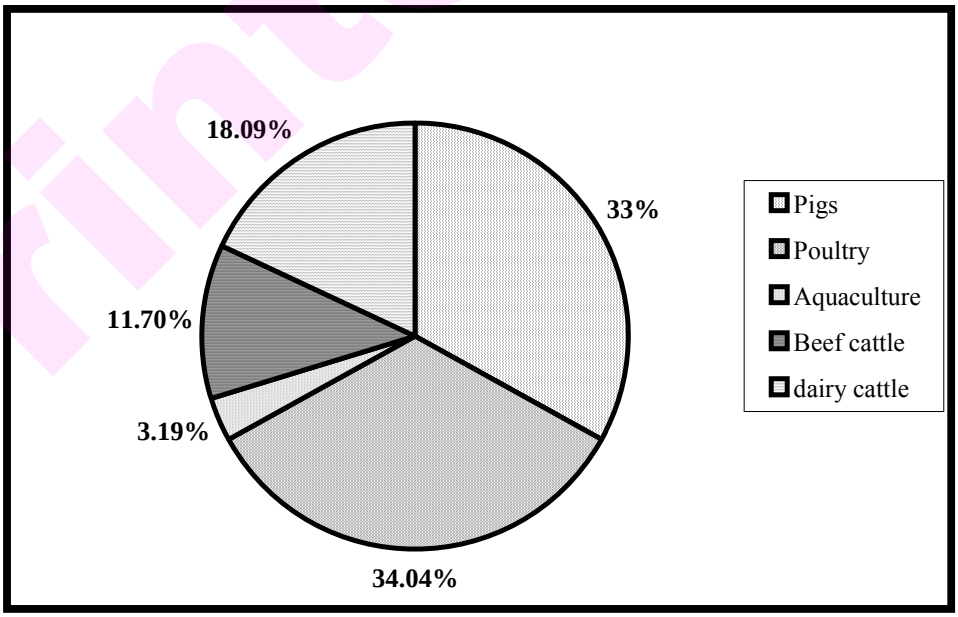
Two enzyme applications can reduce this load of pollutants on the local environment. Firstly, by improving feed digestibility, greater amounts of the feed dry matter, particularly nitrogen and soluble carbohydrates, are retained by the animals resulting in decreased excretion. For example, in the trials reported by Wiseman (1993) in which Bio- feed Plus was used on grower pig, the improvement in digestibility can be equated across the complete growing cycle to a reduced demand for feed and protein of 19 and 3Kg, respectively. Nitrogen and phosphorus excretion was found to reduce by 0.4 and 0.13Kg, respectively. This effect is significant when the total pig and poultry population of these areas is considered. Even in countries of lower animal density, local densities can be as

Fig 2: World feed production by area



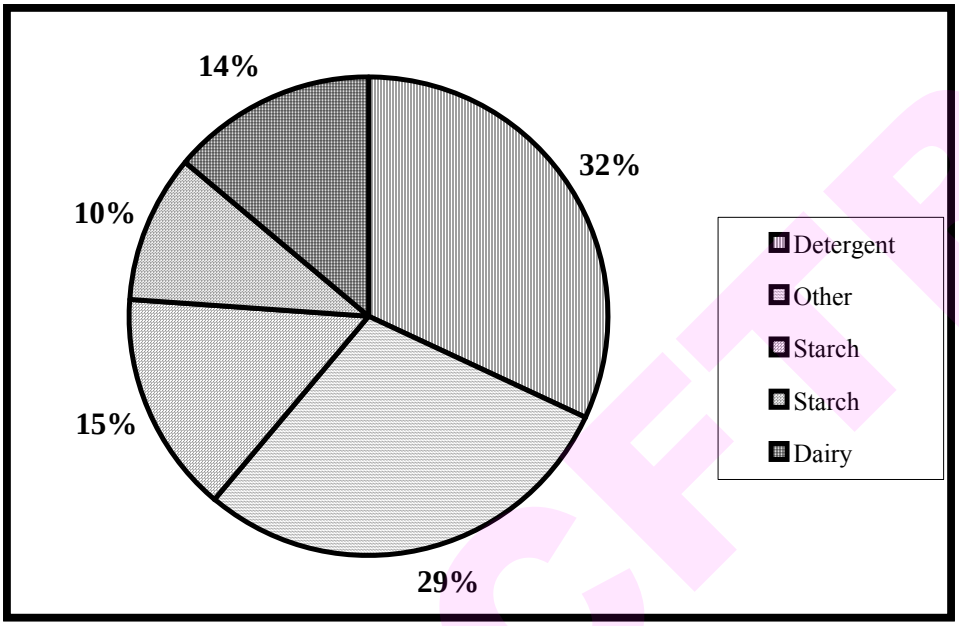
Source: W.D. Cowan, Animal Feed. In Industrial Enzymology 2nd Edition Edited by Tony Godfrey and Stuart West

Fig 3: World feed production by type



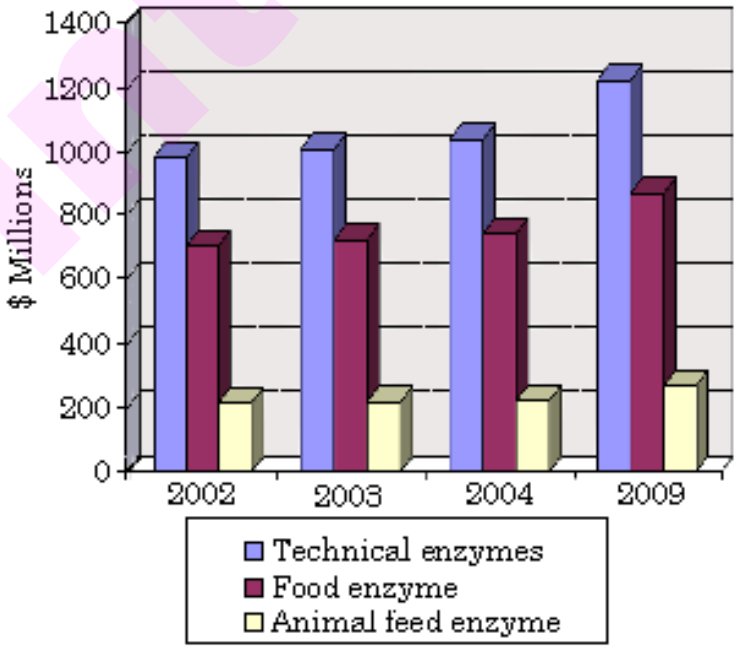
Source: W.D. Cowan, Animal Feed. In Industrial Enzymology 2nd Edition Edited by Tony Godfrey and Stuart West

Fig 4: Distribution of enzyme sales



Source: T. Godfrey and S.I. West Introduction to industrial enzymology In Industrial Enzymology 2nd Edition, Edited by Tony Godfrey and Stuart West

Fig 5: Global Marketing of enzymes



Source: BCC, Inc.

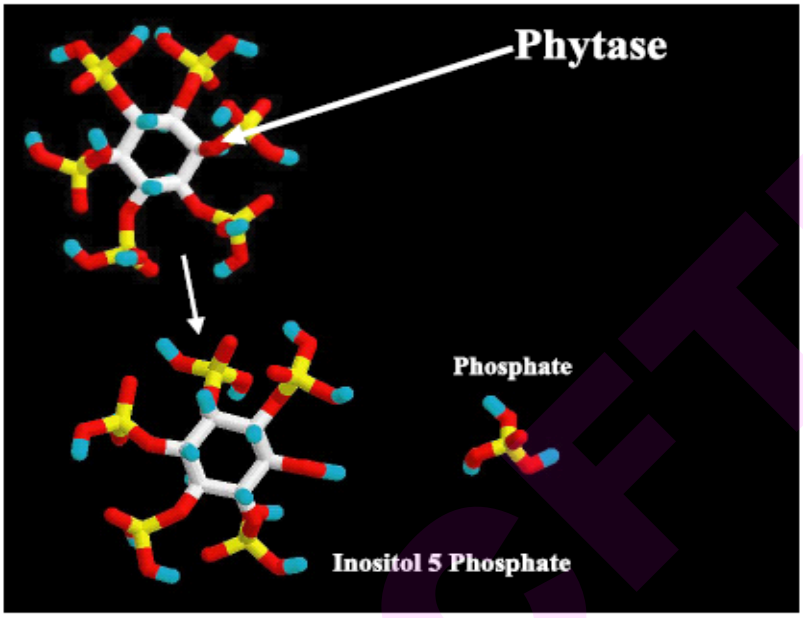
high as in Europe and enzymes can also contribute here to reduction of the local pollution load.

The second enzyme effect is that of phytase, which can liberate organic phosphorus from feed raw materials, which complexes with inositol to form phytic acid. An average figure for phytase content in feed raw materials is 1.0- 1.2 percent, although there are considerable variations both within and from raw material to raw material. Wheat and rye contain relatively high levels of endogenous phytase activity, whereas oats and soybean meal have low levels of this enzyme activity. A number of experiments have been conducted to assess phosphorus availability in feed raw materials in pigs and a set of composite values was produced by Jongbloed and Kemme, (1990). This includes the influence of the natural phytase activity and also the fact that some organic phosphorus is not found as phytate and hence is not susceptible in phytase hydrolysis.

Phytase:

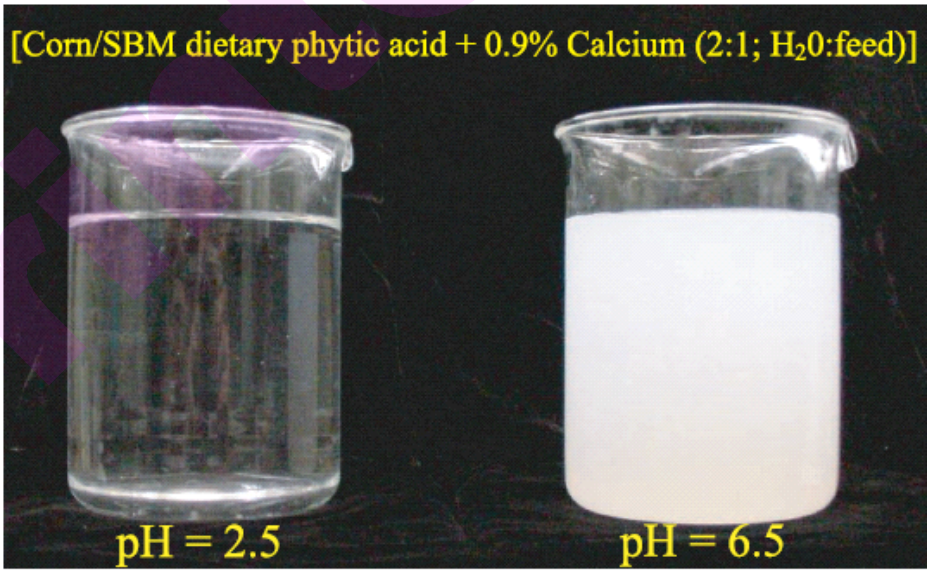
In biological system, hydrolysis of phytic acid to *myo*-inositol and inorganic phosphate is an important reaction for energy metabolism, metabolic regulation and signal transduction pathways (Vats and Uttam, 2004). The reaction is primarily catalysed by phytases (*myo*- inositol hexakisphosphate phosphohydrolase). The complete hydrolysis of phytate by the enzyme, which we propose on the basis of its capability to cleave phosphate group of phytate, is one of the highly desired properties for the biotechnological application of enzyme. During the last 15 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection, and biotechnology. Phytases are histidine acid phosphatases (HAPs), a subclass of phosphatases, which catalyze the hydrolysis of phosphate moieties from phytic acid, thereby, resulting in the loss of ability of phytic acid to chelate metal ions. The histidine residue has been proposed to serve as a nucleophile in the formation

Fig 6: Action of phytase on phytic acid



(Figure courtesy, W. Schmidt- USDA/ ARS)

Fig 7: Demonstration of what occurs to phytin- Ca complex in the stomach (pH 2.5) and small intestine (pH 6.5)



Source: Purdue University Department of Animal Sciences

of covalent phosphoenzyme intermediates (Mc Tigue and van Etten 1978; van Etten *et al,* 1982).

The physicochemical characteristics and catalytic properties of phytases from various sources indicates it to be ester- hydrolyzing enzyme with an estimated molecular weight of 35- 700 kDa depending upon the source of origin and are usually active within a pH range of 4.5- 6.0 and temperature range at 45-60°C. The catalytic action of phytase on phytic acid is shown in Fig 6. Generally, phytases from bacteria have optimum pH in neutral to alkaline range while in fungi optimum pH range is 2.5- 6.0. It is demonstrated in vitro that, in the stomach where the pH is 2.5, phytase acts on phytin- Ca complex while in small intestine (pH 6.5), phytase does not act on phytin- Ca complex and thus forms a precipitate. This is clearly shown in Fig 7. Phytases are fairly specific for phytic acid under the assay condition and it is possible to distinguish phytase from acid phosphatase that is incapable of degrading phytase.

Until now approximately a dozen fungal phytases have been characterized which includes the commercialization of *Aspergillus niger* Phy A and *Peniophora lycii* Phy A. Meanwhile, several new phytases that vary widely in their biochemical properties have been identified from bacteria. Most microbial phytases are produced extracellularly and have molecular weight ranging from 36-140kDa. The catalytic activity of the enzyme is dependent on the metal ion and is maximized by > 2mM calcium ion. The fully active form of the enzyme contains six calcium ions, while the inactive form contains only 3 calcium ions due to the loss of other 3 located at the top of the molecule (Ha and Oh, 2000).

The specific activity of the native *A. fumigatus Phy*A is found to be too low to be used in animal feed industry. Based on amino acid sequence alignments among several *Phy* A enzymes, Tomschy *et al,* (2000) hypothesized that amino acid residue 27 might be highly correlated with the difference in specific activity

between the native *A. fumigatus Phy*A and *A. terreus* enzymes. *A. fumigatus Phy*A has a glutamine instead of a leucine as in *A. terreus* at 27th position. When leucine was substituted with glutamine at that residue in *A. terreus*, specific activity was reduced from 196 to 35 U/mg. When the opposite switch in the *A. fumigatus Phy*A was made, the specific activity was increased from 26.5 – 92.1 U/mg. Several approaches have been taken to develop effective phytases that includes identifying new native phytase protein from microorganisms or plants and genetically modifying these phytases.

An ideal phytase:

Animal industries, although accept any new phytase, they look for at least three biological characteristics that seems to be necessary for an ideal phytase "effective in releasing phytate- phosphorus in the digestive tract, stable to resist inactivation by heat from feed processing and should be cheap to produce. Likewise, an enzyme that can tolerate long- term storage or transport at ambient temperature is undisputedly attractive. It is important to realize that any single phytase may never be **'ideal'** for all species or in all cases. For poultry, an enzyme would be beneficial if it is active at the near neutral pH of the crop (pH 6.5) (Riley and Austic, 1984) or at the acidic pH of the stomach. Phytases used for aqua cultural animals (Ramseyer *et al*, 1999), due to their low body temperature, require a lower temperature optimum than that for swine or poultry. Therefore, an array of phytases will be needed for different applications.

Action of phytase on phytic acid:

The enzyme reaction is likely to proceed through a direct attack of the metal- bridging water molecule on the phosphorus atom of a substrate and the subsequent stabilization of the pentavalent transition state by the bound calcium ions. The enzyme has two phosphate binding sites, the "cleavage site", which is responsible for the hydrolysis of a substrate and the "affinity site, which increases

the binding affinity for substrates containing adjacent phosphate groups. The existence of the two nonequivalent phosphate binding sites explains the formation of alternately dephosphorylated *myo*- inositol triphosphates from phytate and the hydrolysis of *myo*- inositol monophosphates.

The possible modes of binding of phytate and less-phosphorylated *myo*-inositols to the active site of phytase are investigated based on the superposition of phosphate groups of phytate on the two bound phosphate ions. It is noted that any two phosphate groups that are not adjacent to each other cannot be superposed simultaneously. In addition, because phytate is a large molecule and the active site cleft of the enzyme is narrow, successful superposition on (Pho 1 and Pho 2) without a stearic clash was limited to the pairs of 3-P & 4-P and 6-P & 1-P. The observation suggests that, initially, only two pairs of the phosphate groups are accessible for cleavage by the enzyme. Removal of 3-P or 6-P from the phytate molecule allowed for superposition of 1-P & 2-P and 4-P & 5-P, respectively, but not others. Further removal of phosphate groups alleviated stearic restriction in the superposition. The preferential binding of phytate and the selective cleavage requires two phosphate binding sites to which two adjacent phosphate groups can bind, one of which serves as a cleavage site. A phosphate group bound to the Pho 1 site would be cleaved ("cleavage Site"), while the other one bound to the Pho 2 site increases the binding affinity ("affinity site") for the substrates containing adjacent phosphate groups.

Since, the enzyme is able to cleave any of the phosphate groups of phytate, it is highly likely to hydrolyze Ins (1, 3, 5) P_3 and Ins (2, 4, 6) P_3 further at a rate comparable to that of hydrolyzing Ins P_1s. There is no stearic limitation in the simulated binding of each of the Ins (1, 3, 5) P_3 and Ins (2, 4, 6) P_3 molecules to the active site. However, under *in vitro* condition, in which produced phosphate is not removed, further degradation of the Ins P_1s should be very slow, not only due to the reduced turn- over rate for the hydrolysis of non-adjacent phosphate groups,

but also due to the increased susceptibility of the enzyme to the product inhibition. In a physiological situation, the less- phosphorylated *myo*- inositols could be further degraded by the enzyme, owing to the utilization of the produced phosphate ions.

Alkaline phosphatases and acid phosphatases, particularly purple acid phosphatases are metallo- enzymes. Purple acid phosphatases employ a nuclear Fe^{3+}/ Fe^{2+} or Fe^{3+}/ Zn^{2+} center to catalyze the hydrolysis of phosphate monoesters (Pinkse *et al*, 1999). In alkaline phosphatases, two Zn^{2+} and one Mg^{2+} are closely bound in the active center (Coleman, 1992). Mg^{2+} ion in the enzyme probably acts only to orient the phosphate containing substrate (De Silva and Williams, 1991), whereas two Zn^{2+} ions together with an arginine and a reactive serine residue are involved in the actual catalysis.

Classification of phytase:

The International Union of Biochemistry and Molecular Biology (IUBMB) in consultation with the IUPAC- IUB, Joint Commission on Biochemical Nomenclature (JCBN) have listed two phytases: 3- phytase, EC 3.1.3.8 (*myo*-inositol hexakis phosphate – 3- phosphohydrolase) and 6- phytase, EC 3.1.3.26, (*myo*- inositol hexakis phosphate – 6- phosphohydrolase). The two enzymes differ only in the position from which they remove phosphate from the substrate i.e, 3-phytase, EC 3.1.3.8, that hydrolyzes the ester bond at the 3 position of *myo*-inositol hexakis phosphate to D- *myo*- inositol 1, 2, 4, 5, 6 pentakisphosphate & orthophosphate and 6- phytase, EC 3.1.3.26 which first hydrolyzes the 6 position of *myo*- inositol hexakis phosphate to D- *myo*- inositol 1, 2, 3, 4, 5 pentakisphosphate and orthophosphate. Subsequent ester bonds in the substrate are hydrolyzed at different rates. Both the phytases are members of the hydrolase class of enzymes. In the presence of water they tend to hydrolyze the substrate phytic acid resulting in the release of inorganic phosphate (Wodzinski and Ullah, 1996;

Vats and Uttam, 2004). 3- phytases appear to be characteristic of microorganisms and 6- phytases of the seeds of higher plants (Cosgrove, 1970). Phosphatases are a group of enzymes that catalyze the hydrolysis of phosphomonoester bonds in biological systems. They differ in many ways: Eg; in optimum pH, in molecular weight and perhaps most significantly in which metal ion cofactor is required for catalysis. Phosphatases have been traditionally divided into alkaline, acid and protein phosphatases (Vincent *et al,* 1992). Acid phosphatases exhibit an optimum pH of below 7 and can be further divided into three different subclasses: low molecular weight acid phosphatases (18,000), high molecular weight acid phosphatases (50,000) and purple acid phosphatases.

Of various HAPs reported, *phy* A and *phy* B are the most extensively characterized representatives. They are shown to possess conserved active site sequence, RHGXRXP, which is unique to high molecular weight acid phosphatase (Ullah *et al,* 1991). *Phy* A is characterized by two pH optima (2.5 and 5.0), whereas, *phy* B is referred to as pH 2.5 optimum acid phosphatase. This is attributed to differences in the charge distribution at the substrate specificity sites of *phy* A and *phy* B.

Sources of phytase:

Phytase activity occurs in a wide spectrum of organisms including plants, animals and microorganisms.

Plant phytases:

Suzuki *et al,* (1907) detected phytase activity in rice and wheat bran. Reddy *et al*, (1982) and Gibson & Ullah (1990) have exhaustively reviewed the occurrence of phytase in germinating plants. They have isolated and characterized phytase in cereals, such as triticale, wheat, corn (Chong, 1967), barley (Pollard, 1969; Jronier, *et al,* 1971; Obata & Suzuki, 1976), rice and beans (Gibbins and

Norris, 1963; Chang and Schwimmer, 1977; Mandal, *et al,* 1972) such as navy beans, mung beans, dwarf beans and California small white beans. Eeckhout and De Paepe (1994) have measured phytase activity, phytin phosphorus and total phosphorus in 39 different samples of cereals, cereal by- products, oil seeds, legume seeds, roots and tubers. Phytase activity has also been shown in cotton seed (Ergle and Guinn, 1959; Wozenski and Woodburn, 1975), grass seeds (Perles, 1955), lettuce seeds (Mayer, 1958; Mayer *et al*, 1971), white mustard seed (Courtois, 1947), pea (Guardiola and Sulcliffe, 1971), potato (Pfankuch, 1936), radish (Courtois and Perez, 1949), rye (Perez, 1949; Courtois and Masson, 1951), sorghum (Adams and Novellie, 1975), soybean (Mayer, *et al*, 1961; Sudarmadti and Markakis, 1977) and wheat (Anderson, 1915; Peers, 1953; Nagai and Funahashi, 1962; Lim and Tate, 1973).

Houde *et al,* (1990) purified phytase from canola seed. This purified phytase was shown to have 232 times more activity with pyrophosphate than with sodium phytate. pH optimum of the plant phytases range from 4.0- 7.5. The seeds of higher plants are shown to contain 6- phytase. Hayakawa *et al,* (1990) have shown in their data that rice bran phytase enzyme is able to dephosphorylate at the 2 position of *myo*-inositol. The levels of phytase in plants have shown to increase by several orders of magnitude during germination. Courtois and Perez (1948) examined the seeds from a number of different species of plants and found more or less phytase activity in *Phoenix dactylifera* (date), hard wheat, soft wheat, oats, barley, *Bromus pratensis, Dactylis glomerata, Ricinus communis*, radish white mustard, *Citrus nobilis, C. vulgaris, C. aurantium, Pistacia atlantica, Faba vulgaris, Phaseolus lunatis, Lens esculenta* and *Cicer arietinum.* Wheat and barley were highest in activity, with hard wheat more active than soft wheat. Dates had least activity and the activity in all seeds increased on germination.

Phytase has also been detected in spinach leaf (Wildman and Bonner, 1947), tobacco leaf (Shaw, 1966) and orange juice (Axelrod, 1947). While the

level of activity in spinach was reasonable, that in tobacco and in orange juice was so slight as to cast doubt on the claims. Phytase activity has also been detected in the roots of higher plants (Rogers *et al*, 1940; Hayashi and Takijima, 1951; Saxena, 1964; Szember, 1960; Wild and Oke, 1966).

Animal Phytases:

Phytases in animal tissue was first reported in calf liver and in calf blood (Mc Collum and Hart, 1908). Later research (Rapoport *et al*, 1941) failed to detect phytase in the blood of mammals, including man, but detected it in the blood plasma of various lower vertebrates (birds, reptiles, fishes, batrachians) and in the erythrocytes of birds and the sea turtle. Rat intestine has been shown to posses a phytase with an alkaline pH optimum (Patwardhan, 1937; Firenzuoli & Zanobini, 1974; Bitar & Reinhold, 1972). This has also been reported in the intestinal mucosa of man and of the calf (Bitar & Reinhold, 1972). Pileggi (1959) and Maddaiah *et al,* (1969) have suggested that intestinal phytase activity may be a manifestation of alkaline phosphatase activity. This hypothesis has been supported by the work of Davies and Flett (1978) on rat intestine and that of Davies *et al* (1970), Davies and Motzok (1972), Davies and Motzok (1972a) and Mc Cuaig *et al* (1972) on chicken intestine.

Microbial Phytases:

Phytase activity in microorganisms has been detected most frequently in the fungi, in particular the *Aspergilli* (Dox and Golden, 1911; Casida, 1959; Yamada *et al*, 1968; Irving and Cosgrove, 1972; Irving and Cosgrove, 1974). Shieh and Ware (1968) have shown that of all the fungi surveyed for phytase, *Aspergillus niger* NRRL 3135 produces the most active extracellular phytase in corn starch and semi synthetic medium. *Aspergillus amstelodami, A. chevalieri, A. candidus, A. niger, A. flavus, A. repens, A. sydowi, A. versicolor, A. wentii, Botrytis cinerea, Geotrichum candidum, Mucor priformis, M. racemosus, Rhizopus oryzae, R.*

oligosporus, Saccharomyces cerevisiae have all found to be positive strains for phytase production (Howson and Davis, 1983). Shieh and Ware (1968) have shown that *A. flavus, A. niger, A. terreus, A. versicolor, Mucor* spp, *Penicillium* spp. are capable of producing phytase enzyme extracellularly. *A. awamori* ATTC 11382, *A. awamori* ATTC 11358, *A. carbonarius* NRRL 368, *A. carbonarius* PCC 1040, *A. niger* syn *A. ficuum* WB 320/ WB 364/ WB 4016/ WB 4541/ WB 4781, *A. niger japonicus saito* ATCC 1034, *A. niger* ATCC 9142/ 10864, *A. niger* var. *cinnamomeum* NRRL 348, *A. niger* NRRL 326/ 320/337/ 372/ 4361, *A. niger* van Tieghem 1, *A. niger* K/x, *A. satoi, A. tubingensis* NRRL 4875 have all shown positive for phytase enzyme production.

Phytases have been detected in some of the bacteria that include *Aerobacter aerogenes* (Greaves *et al*, 1967), *Bacillus subtilis* (Powar and Jagannathan, 1982), *B. subtilis* N77 (natto) (Shimizu, 1992), *Escherichia coli* (Greiner *et al,* 1993), *Klebsiella aerogenes* (Tambe *et al*, 1994) and *Pseudomonas* sp. (Irving and Cosgrove, 1971). Phytase activity has also been shown in yeasts (Greenwood and Lewis, 1977) and in rumen microorganisms (Raun *et al,* 1956). Soil microorganisms (Greaves and Webley, 1969) and mycorrhizal microorganisms (Greaves and Webley, 1965; Theordoron, 1968; Barlett and Lewis, 1973) have also been studied with respect to their phytase activity.

About *Aspergillus niger*:

Aspergillus species are a ubiquitous group of filamentous fungi that are commonly isolated from soil, plant debris and indoor air environments. Many species of *Aspergillus* are xerophilic, thermotolerant and show a remarkable tolerance to freezing. Several species are capable of producing secondary metabolites that are harmful to humans. *A. flavus* and *A. parasiticus* are particularly notable for producing aflatoxins, which are among the most carcinogenic substances known to man (Kozakiewicz and Smith, 1994). A few

species, including *A. fumigatus, A. flavus, A. niger, A. nidulans* and *A. terreus,* have been identified as important pathogens for immuno- compromised humans (Lewis, 1994). The industrial fermentation of *Aspergillus* cultures produces numerous commercially important enzymes and metabolites including: citric acid, amylase, glucoamylase, glucanases, phytases, lipases, hemicellulases, proteases and xylanases. *A. niger* and *A. oryzae* fermentations are "Generally Regarded As Safe" (GRAS) by the FDA (Harvey and Mc Neil, 1994).

In the present study, *Aspergillus niger* CFR 335, a local poultry soil isolate is used in all the experiments. *Aspergillus niger* belongs to family Ascomycetes (Kirk, 2001). The fungus grows rapidly on a variety of artificial substrates producing colonies that consist of a compact white or yellow basal mycelium covered by a dense layer of dark brown to black conidial heads. Reverse is pale yellow and growth may produce radial fissures on the agar. Mycelial hyphae are septate and hyaline. The species is biseriate ie, the vesicles produces sterile cells known as metulae that support the conidiogenous phialides. Conidiophores are typically 400- 3000μm, long, smooth and hyaline, becoming darker at the apex and terminate in pale- brown colored globose vesicles 40 - 60μm in diameter (Nakagawa *et al*, 1999). Metulae and phialides cover the entire vesicle. Conidia are brown to black, very rough, globose and measure 4- 5 μm in diameter (Sutton *et al*, 1998; de Hoog *et al*, 2000).

Advantages of microbial phytases over plant Phytases

(i) Easy maintenance of phytase producing microorganisms.

(ii) Microbial phytases are easy to process and scale up and are also active at wide ranges of temperature and pH.

(iii) Easy extraction since most microbial phytases are extracellular.

Fermentation processes:

SSF is defined as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can, in addition, be used as carbon and energy source. Solid- state fermentation (SSF) holds tremendous potential for the production of enzyme. It can be of special interest in those processes where the crude fermented products may be used directly as enzyme sources. Semisolid fermentation has been used for the production of enzymes, mycotoxins, mushrooms, fermented foods and feed (Chahal, 1985; Han and Anderson, 1975; Hang *et al,* 1982; Hesseltine, 1972; Linderfelser and Ciegler, 1975; Toyama, 1976; Zadrazil and Brunnert, 1981). In general, semisolid fermentation is simple, less expensive and yields higher amounts of product compared to liquid fermentation. The main difference between submerged liquid fermentation and semisolid cultivation is that the substrate in the former is completely dissolved and homogeneous whereas the latter employs insoluble substrate with relatively little liquid in the growth environment. In spite of its simplicity and ease of operation, due to heterogeneity of the fermentation mixture, the controls for semisolid fermentation are not as simple as those for homogeneous submerged cultures, especially in large- scale operation (Cannel & Moo- Young, 1980; Hasseltine, 1972; Knapp & Howell, 1980; Lindenfelser & Ciegler, 1975). The fermentation takes place in the absence or near absence of free water, thus being close to the natural environment to which microorganisms are adapted (Pandey *et* al, 2000). More generally, SSF can be understood as any process in which substrates in a solid particulate state are utilized (Mitchell *et al,* 2000 a).

The aim of SSF is to bring the cultivated fungi or bacteria into tight contact with the insoluble substrate and thus to achieve the highest substrate concentrations for fermentation. However, there are also several disadvantages of SSF, which have discouraged use of this technique for industrial production. The main obstructions are to the build- up of gradients of temperature, pH, moisture,

and substrate concentration during cultivation, which are difficult to control under limited water availability. This system offers numerous advantages over submerged fermentation (SmF) system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, and requirement of simple fermentation equipment (Hesseltine, 1977; Pandey, 1992; Pandey, 1994; Chahal *et al,* 1981; Nigam and Singh, 1994; Aidoo *et al,* 1982; Pandey, 1991; Doelle *et al,* 1992). It has been reported that while a strain of *A. niger* produced 19 types of enzymes, α- amylase was being produced by as many as 28 microbial cultures by SSF method (Pandey, 1992).

Wheat bran holds the key role and has most commonly been used in various processes. In SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. The substrate that provides all the needed nutrients to the microorganisms growing in it is considered as the ideal substrate. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement them externally with these. It has also been a practice to pre-treat (chemically or mechanically) some of the substrates before using in SSF process (Eg. lignocellulose), thereby making them more easily accessible for microbial growth.

It has become clear that the cost- factor for the production of "bulk- ware" enzymes in most cases favours SSF over SmF. The low estimated costs of SSF are due to the traditional preferential claim. SSF utilizes complex, heterogeneous agricultural wastes as substrates and uses low- cost technology regarding sterility and regulation demands.

Generally, smaller substrate particles provide larger surface area for microbial attack and thus, are a desirable factor. However, too small a substrate particle may result in substrate accumulation, which may interfere with microbial

respiration/ aeration and therefore result in poor growth. In contrast, larger particles provide better respiration/ aeration efficiency (due to increased inter-particle space), but provide limited surface for microbial attack. This necessitates a compromised particle size for a particular process.

Solid- state fermentation processes are distinct from submerged fermentation culturing, since microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents. Thus, it is crucial to provide optimized water content and control the water activity (a_w) of the fermenting substrate. Moreover, water has profound impact on the physico-chemical properties of the solids and this, in turn, affects the overall process productivity (Ashok Pandey *et al,* 1999).

Ideally, almost all the known microbial enzymes can be produced under SSF systems. Literature survey reveals that much work has been carried out on the production of enzymes of industrial importance, like proteases, cellulases, ligninases, xylanases, pectinases, amylases, glucoamylases, etc., and attempts are also being made to study SSF processes for the production of inulinases, phytases, tannases, phenolic acid esterases, microbial rennets, aryl- alcohol oxidases, oligosaccharide oxidases, tannin acyl hydrolase, α- L- arabinofuranosidase etc., (Bisaria *et al,* 1997; Kimura *et al,* 1995; Martinez *et al,* 1994; Germano *et al,* 1998; Ikasari & Mitchell, 1994; Ebune *et al*, 1993; Alasheh and Duvnjak, 1994).

Phytase production by fermentation:

Cultivation of *Aspergillus niger* on solid substrate is of interest as an economical means of production (Han and Gallagher 1987). The use of filamentous fungi for the production of commercially important products has increased rapidly over the past half century and the production of enzymes in submerged fermentation (SmF) has long been established. In recent years research interest in solid- state fermentations (SSF) has increased (Mitchell & Lonsane,

1992). Depending on the culture conditions and the genotype of the strain, the growth form of filamentous fungi in submerged culture varies between two extremes, the pelleting and filamentous. Each form having its own characteristics that can affect the process productivities by influencing the mass transfer rates (Thomas, 1992). In submerged condition, the fungus is exposed to hydrodynamic forces whereas in SSF, growth is restricted to the surface of the solid matrix. The characteristics of growth in such systems depend on the availability of nutrients and the geometric configuration of the matrix (Moo- Young *et al*, 1978).

Although many attempts have been made, no one has discovered a naturally occurring organism that produces more phytase in liquid culture. The characteristics of the submerged fermentation have been evaluated independently and also in terms of its suitability for use as inocula for a solid- state phytase fermentation (Papagianni, *et al* 1999). The morphology of the inoculum has been studied and correlated with process yields (Tucker and Thomas, 1992). However, published research concerning the morphological aspects of inoculum for solid-state fermentation is lacking. The state and condition of the mycelium, the number of active tips and morphology that ensure a good distribution of the inoculum to the solid- substrate are of greater importance for a successful solid- state phytase fermentation (Papagianni, *et al* 1999). Fungal strains have also been propagated and phytase enzyme is produced in submerged liquid fermentation and semi solid fermentation with cottonseed meal and soybean meal (Han, 1989; Han and Wilfred, 1988). It was suggested that phytic acid was co-precipitated with protein since the pH value was decreased during the fermentation and phytic acid tends to be soluble at acid pH values.

Fermentation activates native phytases by reducing the pH and natural fermentations can also provide microbial phytases from yeasts and moulds. The easiest way of degrading phytic acid is to add exogenous phytases to the cereal and legume mixtures or to adjust conditions for the maximum activity of native

phytases. Phytase action during soaking the whole grains causes only modest phytate losses in legume seed. Studies by Gustafsson and Sandberg (1995) demonstrate that soaking finely ground flours under optimal conditions almost completely degrades the phytate. For instance, wheat bran containing 55 μmol/g phytic acid when held at 55°C in a pH 4.5- 5.0 buffer lost about 90 % phytate within 60 minutes and virtually all within 120 mins.

Global Marketing of phytase enzyme:

Growth of the animal feed enzyme sector is somewhat higher at nearly a 4% average annual growth rate (AAGR), which is due to the increased use of phytase enzyme to fight phosphate pollution. Presently, in India, about 12 million tones of compounded poultry feed are being manufactured annually. Considering the minimum level of 1% addition in diets, about 0.12 tones of di- calcium phosphate of worth USD 400,000 is required.

The practical poultry rations are largely composed of vegetable oil cakes, meals, grains and their byproducts. Phosphorus bioavailability from such feedstuffs is only about 30 %. The hydrolysis and absorption of phytate-phosphorus by monogastrics are complex processes that are affected by certain factors namely, dietary calcium and inorganic phosphorus levels, vitamins D, age and type of birds, types of dietary ingredients, sources of fibre in diet and feed processing. Worldwide manufacturers of phytase enzyme are given in Table 2.

Phytase research in India:

In view of the above described undesirable effects of phytic acid, it is preferred to either remove it altogether or reduce its amount in poultry feed or ingredients. Though, no substantial amount of work is being carried out in India on the production and utilization of phytase, efforts have been made in different laboratories to either eliminate or reduce phytic acid content in plant feedstuffs

through chemical methods, solid- state fermentation technology, autolysis or by the use of phytase enzyme in diet. Of these, supplementation of diet with microbial phytase appears to be more promising. Considerable work has been done in India in recent years on the effectiveness of supplemental microbial phytase in improving the P bioavailability in poultry. Microbial phytase supplementation at 250- 500 Units/ Kg diet increased the body weight gain and feed intake in broiler chicken. In 1982, Powar and Jagannathan at National Chemical Laboratory, Pune, purified a phytate specific phosphatase from *Bacillus subtilis*. In 1994, Khare, *et al*., of Indian Institute of Technology, Delhi published studies on entrapment of wheat phytase in polyacrylamide gel and its application in soy milk phytate hydrolysis. In the same year another report was published (Tambe *et al,* 1994) from Baba Atomic Research Centre on the isolation of two distinct forms of phytase from *Klebsiella aerogenes.* However, there is no report on the production of recombinant phytase from any other laboratories in India.

International status of phytase research

In 1962, International Minerals and Chemicals in Skokie, Illinois have made a concentrated effort to make phytase a commercial product. Tsuong rung Shieh of same lab have screened over 2000 organisms for phytase activity and isolated an organism from the soil in a flowerpot that produced the highest yield of phytase. This was later identified as *Aspergillus ficuum* by Kenneth Raper at the University of Wisconsin based on its poor conidiogenesis. This strain was of special interest because one of the phytases termed as Phy A that it produced had 2 pH optima, one at pH 2.5 and the other at pH 5.5. The second enzyme, Phy B was acid phosphatase with an optimum pH of 2.0. The pH optima of the enzyme approximated the pH of the chick digestion tract. It showed that the enzyme would be at least partially active in the intestine. Nelson *et al*, (1968) and Nelson *et al*, (1971) have established that, inclusion of fungal phytase from *Aspergillus ficuum*

Table 2: Manufacturers of phytase enzyme world- wide

Company	Country	Trade name
Novo Nordisk	Denmark	Novozyme
Ultra Bio- Logics	Canada	Phytase
Biocon India Pvt. Ltd.	India	Phytase
Kaypeeyes	India	Phytase-2000
Ekta Biotech Pvt. Ltd	India	Ekta phytase 2500
Textan Chemicals Pvt. Ltd	India	Phytase
Alko Biotech	Finland	Finase
Altech	USA	Allzyme phytase
BASF	USA	Natuphos
Royal Gist- Brocades	The Netherlands	Phytase
Zeius Biotech Pvt. Ltd.	India	Phytase

in diet of broilers resulted in a marked improvement in the utilization of phytate phosphorus and also in bone mineralization.

In 1984, the technology that was developed at IMC was transferred to the Southern Regional Research Center (SRRC), Agricultural Research Service (ARS) and The United States Department of Agriculture (USDA). A research team at SRRC has isolated, characterized and sequenced the phytases (Phy A and Phy B) and acid phosphatase produced by *A. niger* NRRL 3135. This fundamental research provided a technical basis for cloning and gene sequencing studies at Gist- brocades, Pan Labs (Alko) and USDA.

Alko Biotechnology has supplied phytase, as Finase F, produced by their cloned version of *A. niger* NRRL 3135 to various researchers who tested its efficacy in swine (Young *et al*, 1993; Lei *et al*, 1993, 1994; Cromwell *et al* 1993). Lei *et al* (1993) have demonstrated that the addition of phytase to the diets of weanling pigs improved the bioavailability of zinc and phytate- phosphorus.

The efficacy of feeding phytase to monogastric animals has been established. The units of enzyme required are related to the phytin- P content in the diet. The use of the enzyme as a feed additive has been cleared in 22 countries. The FDA has approved the enzyme preparation as GRAS.

Thermostability studies:

The thermostability of phytase is a highly desirable property considering the high temperature employed in processing animal feed. Enzyme thermal stability is relevant in animal feed applications, where the enzyme is normally incorporated into the grains prior to pelletization and the feed briefly reaches processing temperature of 85- 90°C. In this circumstance a commercial phytase product must be able to withstand brief heating prior to encountering and animal's digestive tract at 37°C. *Thermomyces lanuginosus* phytase is found to be an

alternative enzyme with performance advantages over the commercial *A. niger* enzyme in the form of stable enzyme activity at elevated temperature and superior substrate saturation kinetics at physiological pH. Enzyme activity at elevated temperatures may be relvant in applications such as saccharification (a high-temperature industrial process to generate high- fructose corn syrup), which others have reported that the addition of phytase improves carbohydrate yields (Antrim *et al,* 1996). Phytase being a high molecular weight protein is sensitive to the presence of moisture and high temperature. Therefore, shelf- life of the product must be considered and proper storage of the product needs to be maintained.

A. niger NRRL 3135 produces an highest amount of extracellular phytase when grown on corn- starch based medium and 3 extracellular acid phosphatases when grown under phosphate limiting conditions. One of the 3 acid phosphatases were characterized as phytase (phyA) with two pH optima (2.5 and 5.0) and was heavily glycosylated with a molecular weight of 85 kDa (Ullah, 1988) while monomer protein was unglycosylated with a molecular weight of 48.5 kDa (Ullah and Dischinger, 1993). The enzyme was found to remain stable for months in crude culture filtrate and showed inherent thermostability possessing optimum temperature of 58°C with ten cysteine residues involved in forming five disulfide bridges (Ullah and Mullaney, 1996). Recently, the structure of a thermostable phytase from a strain of *Bacillus amyloliquefacience* has been determined.

Immobilization studies:

Enzymes display great specificity and are not permanently modified by their participation in reactions. Since they are not changed during the reactions, it is cost-effective to use them more than once. However, if the enzymes are in solution with the reactants and/or products it is difficult to separate them. Therefore, if they can be attached to the reactor in some way, they can be used again after the products have been removed. The term "immobilized" means

unable to move or stationary. And that is exactly what an immobilized enzyme is: an enzyme that is physically attached to a solid support over which a substrate is passed and converted to product. The stability of the enzymes can be expected to change upon immobilization. It has been found that enzymes coupled with inorganic carriers are generally more stable than those attached to organic polymers.

Fungal phytase and acid phosphatases are hydrolytic enzymes with high catalytic turnover number- typically the range being from 220- 1000 per second (Ullah, 1988; Ullah and Cummins, 1987, 1988). Thus these categories of enzymes are ideal for immobilization and construction of packed- bed bioreactors. Phytases act on *myo*- inositol hexaphosphate to degrade sequentially to liberate orthophosphates, the byproduct being *myo*-inositol penta, tetra, tri, di and monophosphate. It has been shown that an efficient bioreactor of the immobilized phytase could be used to produce various species of *myo*- inositol polyphosphates and treat soybean milk to lower the content of *myo*- inositol hexa and pentaphosphate. The higher form of *myo*inositol phosphate i.e, hexa and pentaphosphate may act as metal chelators and thus interfere with the mineral nutrition in monogastric animals. The enzymatic action of immobilized phytase on phytate may render the molecule to be a non chelator by conversion to a lower form of *myo*- inositol tetra and triphosphates.

Glycosylation of phytase enzyme:

Glycosylation is the process or result of addition of saccharides to proteins and lipids. Glycosylation is one of the more common and complex post translation modifications. This modification results in thousands of unique, bioactive glycoproteins that can be circulating, membrane bound, or confined to the cytoplasm. Two types of glycosylation exist: *N*- linked glycosylation to the amide nitrogen of asparagines side chains and *O*- linked glycosylation to the hydroxyl

oxygen of serine and threonine side chains. The role of glycosylation in the functional expression of phyA was studied by several authors and found it to be vital for enzyme's thermostability. It is hypothesized that enhancing *N*-glycosylation would improve the thermostability of the enzyme, as in the case of *A. niger* PhyA expressed in *Pichia pastoris* (Han and Lei, 1999).

Phytase inhibition:

Phytases are in general inhibited by the addition of certain divalent cations such as Zn^{2+}. *Pseudomonas* phytase (Irving and Cosgrove, 1971) and wheat bran phytase (Nagai and Funahashi, 1962) are not inhibited by cation complexing agents such as EDTA, oxalate, D – (+) tartrate or citrate which suggests that cations may not be essential for the activity of these phytases. The absence of inhibition by D – (+) tartrate distinguishes these enzymes from prostatic acid phosphomonoesterase (Kilsheimer and Axelrod, 1957). Phytase is inhibited by excess substrate (Gibbins and Norris, 1963; Irving and Cosgrove, 1971) and in some instance by the product, inorganic orthophosphate (Chang and Schwimmer, 1977; Mayer, 1958; Lim and Tate, 1973). It has been shown that fungal phytase activity is inhibited by phytate concentration higher than 750μM (Ullah, 1988). In addition both phytase and acid phosphatase are found to be inhibited by higher concentrations of phytate.

Substrate specificity of phytase:

Phytases from plant and microbiological sources have in general been described as non- specific acid phosphomonoesterases (Sloane- Stanley, 1961). Substrate selectivity studies showed that while phytase was able to degrade phytate, both pH 2.5 optimum and pH 6.0 optimum acid phosphatases were unable to hydrolyze phytate at pH 5.0 (Ullah and Cummins, 1988). Courtois (1947a); Fleury and Courtois (1946) have concluded that the seeds of higher plants contain two acid phosphomonoesterases, namely, a β- glycerophosphatase which cannot

hydrolyze phytate and a second acid phosphomonoesterase can hydrolyze both β-glycerophosphate and phytate. Courtois (1947a) named the second acid phosphomonoesterase as phytophosphatase. Ullah and Phillippy (1994) have reported that both phytase and pH 2.5 optimum acid phosphatase can efficiently hydrolyze the tested forms of *myo*- inositol phosphates. The difference in pH profiles for these enzymes indicated that the catalytic domains are not identical.

Feed studies with phytases:

Increasing public concern regarding the environmental impact of high P levels in animal excreta has driven the biotechnological development of phytase and its application in animal nutrition. Studies on enzymatic treatment of feed using microbial phytase sources have demonstrated that this method increases the bioavailability of proteins and essential minerals and provides levels of growth performance as good as or better than those with phosphate supplementation. Nelson *et al* (1968) were the first to pretreat a corn- soy diet with culture filtrate containing *A. niger* NRRL 3135 phytase and fed to one day old chicks. The chicks showed increase in bone ash due to the phytin- P released from the dietary substances by the enzyme. Natuphos, a commercial phytase enzyme produced by cloning *Phy* A from *A. niger* NRRL 3134 into *A. niger* CBS 513.88 has been used by various researchers in feeds of broilers, layers, ducks, ducklings and swine (Wodzinski and Ullah 1996; Lei, *et al* 1993).

Nelson *et al* (1971) added graded levels of solvent- precipitated *A. niger* NRRL 3135 phytase to a commercial corn soybean meal diet and fed to chicks. They measured bone ash and feed- to- gain ratios. They have reported that the enzyme was active in the animals and they were able to incorporate the released phytin- P into bone. They concluded that chicks could utilize phosphorus from phytin- phosphorus as well as supplemental phosphorus. The maximum amount of phytin- phosphorus i.e, 2.1- 3.0 g Kg^{-1} of diet was released when 1500- 2000 μM

P/hr/ml of phytase was added Kg^{-1} of diet. Nelson *et al*, (1967) have determined the effect of feeding phytase on calcium requirements. The superior activity of *A. niger* NRRL 3135 phytase and its practical application to animal feed for the removal of phytic acid has been demonstrated in many experiments (Han, 1989; Han and Wilfred, 1988; Howson and Davis, 1983; Nelson *et al*, 1968, 1971; Rojas and Scott, 1968). Recent studies show that the use of phytase- NSP blend could have a significant effect on the absorption of divalent cations such as calcium, magnesium and iron.

Feeding of phytase will increase trace mineral absorption and it also has the potential to increase amino acid digestibility. Phytase addition to swine diets will reduce the amount of phosphorus in the manure. Table-3 demonstrates the digestibility of phosphorus in selected feedstuffs in pigs. The effect of feeding phytase to animals on pollution has been quantitatively determined. The amount of reduction will depend on the diet type, inclusion rate of phytase, degree of replacement of inorganic phosphorus and the dietary phosphorus relative to the animal needs.

The inclusion of the phytase enzyme in the diet will reduce the amount of phosphorus excreted in the feces. The 30- 35 % reduction in excreted phosphorus is important on environmental point of view. In various European countries especially in the Netherlands and Germany, governments have mandated that the amount of manure that may be land- spread be governed by the actual needs of soil and the crop not by some arbitrary stocking rate. This necessitates either a more efficient method to utilize the phosphorus supplied to the animal or the necessity to remove the phosphorus from the excrement before discharge to the environment (Dunn, 1994). In order to overcome the phosphorus deficiency in the animal feed, inorganic phosphorus sources such as di or tri calcium phosphate are to be used. According to a data source of 1996, India was using about 5.3 million tones of compounded poultry feed manufactured annually. Considering the

minimal level of 1 % addition in diet, about 53,000 tones of dicalcium phosphate worth Rs. 71.5 crores is needed (Tyagi and Verma, 1996).

Table 3: Digestibility of phosphorus in selected feedstuffs determined in pigs

Feedstuff	Apparent digestibility (%) of total phosphorus
Wheat	47 ± 3
Barley	39 ± 4
Oats	27
Maize	20 ±6
Soybean meal	42 ± 5
Sunflower meal	24
Monocalcium phosphate	83 ± 4
Bone meal	68

Source: Integrated crop Management, Purdeu University, Department of Animal sciences

Mutation studies:

Increased phytase activity for *Aspergillus niger* NRRL phyA at intermediate pH levels (3.0- 5.0) has been achieved by site-directed mutagenesis of its gene at amino acid residue 300. Substitution of this residue by either basic, acidic or an uncharged amino acid residue did not yield a recombinant enzyme with the same favorable properties. Therefore, it is concluded that this residue is not only important for the catalytic function of phy A, but also essential for imparting a favorable pH environment for catalysis (Mullaney *et al,* 2002). It is shown that site- directed mutagenesis can also be used to obtain phytases with greater thermostability (Lehmann, *et al* 2000) and with greater resistance to proteolytic attack (Wyss, *et al* 1999).

The wild-type phytases from the *Aspergillus niger* strains NRRL 3135 display a three- fold difference in specific activity (103 and 32 U/mg protein), despite only twelve amino acid differences that are distributed all over the sequence of the protein. Of the twelve divergent positions, three are located in or close to the substrate-binding site. Site- directed mutagenesis of these residues in *A. niger* T213 phytase showed that the R297Q mutation fully accounts for the differences in catalytic properties observed. Molecular modeling revealed that R297 may directly interact with a phosphate group of phytic acid. The fact that this presumed ionic interaction- causing stronger binding of substrates and products- correlates with a lower specific activity indicates that product (*myo*-inositol pentaphosphate) release is the rate-limiting step of the reaction (Tomschy *et al*, 2000).

Biotechnology of phytase:

Several phytases, including fungal phytases from *Aspergillus ficuum*, (Ullah and Dischinger 1993); Ullah and Sethumadhavan 1998), and bacterial phytase from *E.coli* (Lim, *et al* 2000) have been cloned and characterized. These phytases exhibit no apparent sequence similarity to each other and to other known phosphatases, except for the RHGXRXP motif conserved in the high molecular weight acid phosphatases. The *Saccharomyces cerevisiae* pho3 gene has been cloned and transformed into *a. oryzae* expression system, which resulted in 4 –6, fold increase in phytase activity (Elliet *et al*, 1986; Yang and Schweingruber, 1990). The *Escherichia coli* appA gene, encoding a 6- phytase with pH 2.5 optimum was cloned and expressed (app A2) in *Pichia pastoris* (Greiner *et al,* 1993; Rodriguez *et al,* 1999). Both r- app A and r- app A2 proteins were markedly different in their pH profile and other catalytic properties, despite having identical sequences in the regions of the N- terminal motif (HD, 325- 326). The role of C-terminal HD motif in the catalysis of HAPs by site-directed mutagenesis has been demonstrated by Ostanin and van Etten (1993).

Two phytase genes, PHYT1 and PHYT2 in maize were identified and found to express in monocotyledons. The transcribed sequences of both genes shared little homology with *A. niger* phytase except around HAP consensus motif, RHGXRXP (Maugenest *et al,* 1997; Maugenest *et al,* 1999). Researchers at the Department of Animal Science, 252 Morrison Hall, Cornell University, USA have characterized a pH 2.5 acid phosphatase (Dassa *et al.,* 1980). This was suggested to be the phytase subsequently isolated from *Escherichia coli* by Greiner *et al.,* 1993. It was found to be a non- glycosylated, periplasmic protein with a molecular mass of approximately 42 kDa, *app A* gene from ATCC has been successfully expressed in two yeast hosts (Han and Lei, 1997; Rodriguez *et al,* 1999). The recombinant enzyme was found to be phytase and effective in releasing phytate-phosphorus and phytate- bound iron in corn- soy diets for young pigs (Stahl *et al.,* 1999, 2000). Meanwhile, same group of researchers have also cloned and expressed another *Escherichia coli* phytase gene, *app*A2 from a pig colon isolate. They have found that despite 95 % sequence homology with *app*A and *app*A2, there is a distinct biochemical difference between the two recombinant proteins (Rodriguez *et al.,* 1999; Stahl *et al*, 1999, 2000). It was found that, the recombinant phytase protein displayed several favorable characteristics: an acidic pH optimum close to the physiological pH of the stomach of pigs and chickens, higher affinity to sodium phytate than p-Nitrophenyl phosphate, greater resistance to pepsin than the commercially available *A. niger Phy*A and stronger catalytic efficiency for phytic acid than that of other known phytases.

The desire of the animal industry for a heat tolerant phytase with less activity loss during feed pelleting than the current commercial phytases has generated some interest in *A. fumigatus Phy*A (Pasamontes et al, 1997). When the purified enzyme was heated at 100°C for 20 minutes, it retained 90 % of its initial activity whereas, the *A. niger Phy*A retained only approximately 30% of its initial activity when heated at 70°C (Wyss *et al*, 1998). The gene shares 66% sequence

similarity with *A. niger Phy*A (Pasamontes et al, 1997a). Besides its thermo-tolerance, another favorable triat of the *A. fumigatus Phy*A as reported by Pasamontes *et al*, 1997a and Wyss *et al*, 1999, is its broad effective pH range, with a pH optimum at 5 and > 80% of its optimal activity between pH 4 and 7.5. They expressed the *A. fumigatus Phy*A gene in *Pichia pastoris* and demonstrated that; thermo-tolerance of the expressed phytase was modulated by the specificity of the buffers used. They also observed extreme instability of the enzyme at 0 or 4°C.

The *A. niger Phy*A gene has been successfully expressed in tobacco seeds (Pen *et al,* 1993) and soybean cells (Li *et al,* 1997). It was reported that approximately 14 % of phytase accumulated extracellularly out of the total soluble plant protein in the transformed tobacco leaves during plant maturation (Verwoerd *et al,* 1995). Apparently, plants can be used as phytase producers and carriers for animal feeding. Feasibility of this approach for phytase production depends on public acceptance for growing genetically modified organisms in the field and the effectiveness and stability of the heterologous phytases in plants used as animal feeds.

Van Hartingsveldt *et al,* (1993) and Wyss, *et al* (1999) have expressed phytase genes in *Aspergillus niger, A. terreus, A. fumigatus, Emericella nidulans* and *Mucor thermophila* and showed the secretion of active enzymes by *Aspergillus niger*. Berka *et al* (1998) have examined the use of another fungi, *Fusarium venenatum* as an expression system for the *Phy* A gene from thermophilic fungus, *Thermomyces lanuginosus.* In addition to active secretion of phytase, fungal systems also produce other proteins, including proteases at relatively high levels. Thus, there is a need for further purification or inhibition of proteases. Han and Lei (1999) have expressed a hyper- glycosylated *A.niger Phy* A (120 kDa) in *Saccharomyces cerevisiae* and found the extracellular secretion of phytase into the medium.

Pichia pastoris, methylotrophic yeast shares many beneficial characteristics with *S. cerevisiae*, such as, ease in cultivation and genetic manipulation, as well as being generally regarded as safe, therefore, both phy A and phy B phytases have been expressed to enhance the yield (Han and Lei, 1999; Rodriguez *et al,* 1999; Rodriguez *et al,* 2000; Rodriguez *et al,* 2000 a). Bacterial system has got an obstacle in producing fungal phytases i.e, their inability to sufficiently glycosylate the expressed proteins to the extent necessary for activity. Thus Phillippy and Mullaney, (1997) have expressed an inactive *A. niger Phy* A protein intracellularly in *E.coli* and Han and Lei (1999) have extracellularly expressed in *Streptomyces lividans*. In contrast, other group of researchers, Kim, *et al* 1998 and Golovan, *et al* 2000 were able to express phytases of *E. coli* and *Bacillus* sp. in *E. coli* pET expression system. Kerovuo and Tynkkyen (2000) have shown *Lactobacillus plantarum* as an extracellular system for the *Phy* C gene from *Bacillus subtilis* but the expression or secretion level was found to be too low to be considered for industrial use.

Purification studies:

Microbial phytases have been investigated but most of the data are obtained with partially purified enzyme preparations. A few reports of homogeneous enzyme preparations have been made on *A. terreus* (Yamada *et al*, 1968), *A. ficuum* (Ullah and Gibson, 1987) and *Bacillus subtilis* (Powar and Jagannathan, 1982). Phytases of *Aspergillus* sps have been reported to have a large molecular weight, 214 kDa (homohexamer) in *A. terreus* (Yamamoto *et al,* 1972), 85-100kDa (monomer) in *A. ficuum* (Ullah and Gibson, 1987). 44kDa in *A. niger* (Skowronski, 1978). An acid phosphatase was purified from cell membrane fraction of *Lactococcus lactis* sps *lactis* 303 by anion exchange chromatography on DEAE- Cellulose, hydrophobic interaction chromatography on Phenyl Sepharose and gel filtration on Sephacryl S- 200 HR and Sepharose 4B- 200. The molecular mass of the enzyme was estimated to be 430kDa by gel filtration

equilibrated with 20mM Tris- HCl buffer, pH 7.2, containing SDS and β-mercaptoethanol. Activity was optimal at pH 5.2 and 50ºC (Akuzawa and Fox, 1998).

Kerovuo *et al* (1998) have isolated, purified, characterized and cloned extracellular phytase from *Bacillus subtilis* VTTE- 68013. The enzyme had maximal activity at 55ºC, pH 7.0, required Ca^{2+} for enzyme activity and stability and showed no homology with the active site sequence of HAPs thus, designated as Phy C having phytase activity. Greiner *et al* (1997) reported a monomeric 40 kDa, a 3- phytase from *Klebsiella terrigena,* which like phyA had optimum temperature of 58ºC and is not metalloenzyme. Segueilha *et al* (1992) have purified and characterized *Schwanniomyces castelli* phytase having molecular weight of 490 kDa (tetramer) with one large subunit (125 kDa) and three identical subunits (70 kDa). The phytase from soybean did not show sequence homology with any of the known HAPs and was found to be homologous to the N- terminus of purple acid phosphatase (PAP) of *Arabidopsis thalliana* (Morgan *et al*, 1998).

A thermostable extracellular phytase from *Aspergillus niger* ATCC 9142 was purified and characterized by Casey and Walsh (2003). They employed an initial ultrafiltration step followed by chromatography using ion- exchange, gel filtration and chromatofocusing. The purified enzyme was an 84kDa monomeric protein. The enzyme possessed a temperature and pH optima of 65ºC and 5.0. Substrate specificity studies indicated that, while the enzyme could hydrolyze a range of non- phytate based phosphorylated substrates, its preferred substrate was phytate. Phytase activity was moderately stimulated in the presence of Mg^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, Zn^{2+} and F^{-} ions and the enzyme displayed higher thermostability at 80ºC. Segueilha *et al* (1992) have purified phytase from *Schwanniomyces castellii* using column chromatography and found that the molecular weight of the native phytase of *S. castellii* is 490kDa, a high value in comparison to molecular weights reported for other phytases, commonly under

100kDa (Powar and Jagannathan, 1982; Gibson and Ullah, 1990). It is a glycoprotein with an estimated glycosylation rate of 31% which is similar to the value reported for the phytase from *Aspergillus ficuum* (27.3%).

Koichi, et *al* (1968) have purified phytase from *Aspergillus terreus* by ammonium sulphate precipitation, acetone precipitation and chromatography on SE- Sephadex C-50 and Sephadex G- 200 column. The enzyme was purified about 520- folds. The purified enzyme had an optimum pH and temperature of 4.5 and 70ºC respectively. The enzyme was shown to be stable in the pH range from 1.2 to 9.0. An extracellular phytase from *Bacillus subtilis* was purified to homogeneity by ultracentrifugation. It was found to have 2 isozymes on SDS- PAGE. This enzyme was shown to differ in its metal requirement and phytate specificity. This *B. subtilis* phytase is the only known phytate- specific phosphatase (Powar and Jagannathan, 1982).

Nagashima *et al* (1999) have purified a phytase from *A. niger* SK- 57 to homogeneity in four steps by using ion- exchange chromatography, gel filtration and chromatofocussing. SDS of the purified enzyme gave a single stained band at a molecular mass of approximately 60kDa. However, the extracellular phytase from another strain of *A. niger* had a molecular weight approximately 100kDa. The pH and temperature optima were found to be 5.0 and 50ºC respectively. This enzyme was inhibited by Cu^{2+}, Zn^{2+}, Hg^{2+}, Sn^{2+} and Cd^{2+} ions and activated by Ca^{2+}, Mg^{2+} and Mn^{2+} ions (Dvorakova *et al*, 1997).

A cytoplasmic phytase from *K. terrigena* PG-2 was purified about 410- fold to apparent homogeneity with 28% recovery (Greiner *et al*, 1997). This phytase enzyme was found to behave as a monomeric protein of a molecular mass of about 40kDa. The purified *K. terrigena* phytase shares many enzymatic properties in common with other phytases (Irving, 1980; Nayini and Markakis, 1984), but the enzyme shows some differences with other *Klebsiella* sp. (Shah and Parekh, 1990;

Tambe *et al,* 1994). Phytases often have acidic pH optima (pH 4.5- 6.0) with a rapid drop in activity at pH values above 6. *Klebsiella* phytase exhibits optimal conditions for phytate degradation at pH 5.0 and 58ºC. This is a characteristic of microbial phytases, since such enzymes exhibit stability at higher temperature (55-60ºC).

MATERIALS & METHODS

MATERIALS AND METHODS

I. MATERIALS

The following chemicals and reagents were procured and used in this study.

Potato Dextrose agar, Czapek Dox agar, Mans Rogosa Sharpe medium, yeast extract, malt extract, beef extract, bacteriological peptone, calcium pantothenate, thiamine, yeast nitrogen base without ammonium sulphate and amino acid, succinic acid, glycine used in the present study were obtained from Hi- Media Chemicals. Acrylamide, bisacrylamide, ammonium persulphate, Tris (hydroxymethyl aminomethane), Ethylene diamine tetra acetic acid (EDTA), Sodium Dodecyl sulphate (SDS), N, N, N', N'- tetramethyl ethylene diamine (TEMED), bovine serum albumin, agarose, β- mercaptoethanol, Tween- 80, Coomassie Brilliant blue G 250, bromophenol blue, xylene cyanol, bromocresol green, methyl red, ethidium bromide, protein molecular mass kit, dialysis tubing, fungal cell lysing enzyme from *Trichoderma harzianum,* chloramphenicol, sodium phytate, phytase standard, p- nitrophenol, p- nitrophenyl phosphate, ammonium metavanadate, polyethylene glycol (PEG) 3500 were from Sigma Chemicals, USA.

Calcium phytate, ammonium nitrate, magnesium sulphate, ferrous sulphate, manganese sulphate, potassium dihydrogen phosphate, potassium nitrate, potassium sulphate, boric acid, calcium chloride, zinc chloride, copper sulphate, disodium hydrogen phosphate, ammonium sulphate, sodium hydroxide, ammonium molybdate, citric acid, trichloroacetic acid, sorbitol, sodium thiosulphate, sodium carbonate, silver nitrate, methanol, formaldehyde, acetone, phosphoric acid, glacial acetic acid, hydrochloric acid were of analytical grade and HPLC grade solvents like acetonotrile and trifluroacetic acid were obtained from Qualigens India Pvt. Ltd., E- Merck Chemicals, Glaxo India Pvt. Ltd., BDH Chemicals and Sisco Research Laboratory Chemicals. Ultra filter and Milli Q

water used in all the experiments were collected from Millipore Water Purification Unit, USA.

All the glasswares used in this study were obtained from Borosil Glass, India Ltd.

Healthy layer hens (*Gallus domesticus*) of 3 week old were procured from a local poultry farm and housed in well-aerated cages provided in the animal house at CFTRI, Mysore.

CULTURE MEDIA USED:

i. Potato Dextrose Agar (PDA): g/L

Potato infusion	200
Dextrose	20
Agar	20
pH	5.5 ± 0.2

ii. Phytase Screening Medium (PSM): g/L

Glucose	15
Calcium phytate	5
Ammonium nitrate	5
Magnesium sulphate	0.5
Ferrous sulphate	0.01
Manganese sulphate	0.01
Agar	20
pH	5.5 ± 0.2

iii. Czapek Dox Medium (CDM): g/L

Sucrose	30
Sodium nitrate	3
Dipotassium hydrogen phosphate	1
Magnesium sulphate	0.5
Potassium chloride	0.5
Ferrous sulphate	0.01
Agar	20
pH	7.3 ± 0.2

iv. Complete Medium (CM): g/L

Glucose	10
Yeast extract	2.5
Malt extract	5
Agar	20
pH	5.5 ± 0.2

v. Synthetic Medium (SM): g/L

Glucose	30
Yeast extract	5
Potassium dihydrogen phosphate	2.5
Potassium nitrate	1
Magnesium sulphate	0.5
Calcium chloride	0.1
Ferrous sulphate	0.02
Zinc sulphate	0.01
Manganese sulphate	0.01
Copper sulphate	0.002
pH	5.5 ± 0.2

vi. Wheat Bran Extract Medium (WBEM): g/L

Wheat bran extract	100
Glucose	10
Yeast extract	2.5
pH	5.5 ± 0.2

vii. Mineral Salt Medium (MSM): g/L

Glucose	20
Potassium dihydrogen phosphate	6
Disodium hydrogen phosphate	3
Calcium chloride	0.04
Magnesium sulphate	0.02
Ammonium sulphate	3
Calcium panthothenate	50 µg
Thiamine	20 µg
pH	6.8 ± 0.2

viii. Minimal Medium (MM): g/L

Yeast Nitrogen base without amino acid and ammonium sulphate	1.7
Ammonium sulphate	1
Succinic acid	10
Sodium hydroxide	6
Glucose	2
Agar	20
pH	5.8 ± 0.2

ix. Regeneration Medium (RM): g/L

Yeast Nitrogen base without amino acid and ammonium sulphate	1.7
Ammonium sulphate	1
Succinic acid	10
Sodium hydroxide	6
Glucose	20

Sorbitol	183
Agar	20
pH	5.8 ± 0.2

x. Mans Rogosa and Sharpe Medium (MRSM): g/L

Dextrose	20
Peptone	10
Beef extract	10
Yeast extract	5
Polysorbate 80	1
Ammonium citrate	2
Sodium acetate	5
Magnesium sulphate	0.1
Manganese sulphate	0.05
Potassium phosphate	2
pH	6.5 ± 0.2

Buffers:

1. Phosphate saline buffer 10 mM, pH 5.5
2. Acetate buffer 2 mM, pH 4.5
3. Citrate buffer 50 mM, pH 5.2
4. Glycine- HCl 200 mM, pH 2- 3
5. Sodium phosphate buffer 100 mM, pH 6-7
6. Tris- HCl buffer 100 mM, pH 7.5- 8.5

II. METHODOLOGY

SECTION 1

ISOLATION AND SCREENING OF PHYTASE PRODUCING FUNGAL STRAINS:

1.1. Isolation of phytase producing fungal strains from natural habitats:

Soil samples from different livestock areas of Bannur (Mysore District) and nearby places were collected for screening phytase producing fungi. Although microflora in the gastrointestinal tract of monogastric animals such as poultry and swine do produce some amount of phytase enzyme, the fecal matter of such monogastrics is usually loaded with complex phytic acid that may be hydrolyzed to its lower intermediates by the microbes present in the soil. With the above view, an experiment to isolate fungal strains for phytase production from the poultry wastes was also made by sampling the poultry fecal matter.

The isolation of fungal strains was carried out by the following procedure. 1 g of the soil/ poultry waste sample was dispensed in 10 ml of sterile phosphate saline buffer (10 mM, pH 5.5), mixed thoroughly and kept undisturbed for the solid particles to settle down. Then 0.1 ml of the clarified supernatant was serially diluted. Appropriate dilutions of the sample were plated on Czapek Dox medium supplemented with 0.1g/L Chloramphenicol to suppress bacterial growth due to heterogeneity of the soil sample to obtain fungal isolates. The plates were incubated at 30°C for 5 days.

1.2. Screening of phytase positive fungal cultures:

1.2.1. Qualitative Screening of fungal strains isolated from soil and poultry waste:

The fungal isolates numbering around 1,500 were qualitatively screened for phytase production by cultivating on phytase screening medium containing 0.5% Calcium phytate. All the plates were incubated at 30°C for 5 days and visualized for zone of hydrolysis around the colony.

1.2.2. Screening of MTCC cultures for phytase production:

Some of the fungal strains *viz*, *Aspergillus niger* MTCC 281, and *Aspergillus terreus* MTCC 279 were procured from Microbial Culture Collection Centre (IMTECH), Chandigarh for a comparative purpose. These fungal strains were maintained by cultivating on Czapek Dox Medium (CDM) as per the procedure specified by IMTECH Chandigarh. Qualitative screening was also made by point inoculating the strains on PSM as mentioned above (2.1).

1.2.3. Quantitative screening of selected fungal strains:

Based on the above qualitative screening of the fungal strain, the following cultures were used for enzyme quantification, *Aspergillus niger* CFR 11, *A. niger* CFR 19, *A. niger* CFR 28, *A. niger* CFR 39, *A. niger* CFR 40, *A. niger* CFR 42, *A. niger* CFR 256, *A. niger* CFR 335, *A. ficuum* SGA 01, *A. niger* SGA 03, *A. niger* MTCC 281, *A. terreus* MTCC 279, *A. fumigatus* MTCC 179 *A. fumigatus* SMU. All the fungal strains were cultivated by solid-state fermentation using wheat bran as the substrate. The flasks were incubated at ambient temperature in a well-aerated condition for 10 days. The optimum incubation time for maximum production of phytase enzyme was determined by assaying the samples periodically for 10 days.

1.2.4. Screening of Lactic Acid Bacterial (LAB) cultures:

The Lactic Acid Bacterial strains obtained from Culture Collection Centre at Food Microbiology Department of CFTRI, Mysore were also screened for phytase production by inoculating them on to MRS medium supplemented with 0.5 % calcium phytate and the plates were incubated at 37°C for 20 hrs. The cultures used in the above experiment were *Lactobacillus delbrukii*, *Pediococcus acidilactici* K_7, *P. acidilactici* C_{20}, *Lactococcus* sp BA 242, *Lactococcus* sp BA 242D, *Lactococcus* sp C 2361, *Lactococcus* sp SC 516, *Lactococcus* sp B_1, *Lactococcus* sp B_4, *Lactococcus* sp C_5, *Lactococcus* sp C_6.

1.2.5. Quantitative screening of LAB cultures:

Positive lactic cultures for phytase production were selected and cultivated in MRS broth medium for 20 hrs at 37°C. After the completion of incubation time, crude extract of the enzyme was obtained by centrifuging each of the culture broth at 5,000 rpm for 10 minutes. The extracts were quantitatively assayed by the method of Heinonen and Lahti (1981).

1.2.6. Phytase Assay:

(a) Reagents:

i. 0.2M Sodium acetate buffer (pH 4.5)

(i) 0.2M Acetic acid 1.15 ml/100 ml distilled water

(ii) 0.2 M Sodium acetate 2.22 g/100 ml distilled water

pH adjusted to 4.5 by mixing the two solutions

***ii. 15mM Sodium phytate*:** 1.3g in 100 ml distilled water

***iii. 10mM Ammonium molybdate*:** 1.236 g in 100 ml distilled water

***iv. 5N Sulphuric acid*:** 13.85 ml in 100 ml Dis. Water

***v. 1M Citric acid*:** 2.1 g in 100 ml Dis. water

***vi. 15 % Trichloroacetic acid*:** 15 g in 100 ml Dis. water

vii. Pure Acetone

(b) Procedure:

Phytase enzyme assay was carried out by the method of Heinonen and Lahti (1981). 1 ml suitably diluted crude enzyme sample was mixed with 0.5 ml of 0.2 M acetate buffer and 0.5 ml of 15 mM Sodium phytate. The mixture was incubated for 40 minutes at 45°C. Reaction was terminated by adding 2 ml of 15% Trichloroacetic acid. Aliquot of 0.5 ml was taken from the above reaction mixture and mixed with 4 ml of AAM solution (2: 1: 1, Acetone; Ammonium molybdate; Sulphuric acid). To the same mixture 0.4 ml of 1 M Citric acid was added, mixed well and the optical density was read at 355 nm. A standard graph was plotted using Potassium dihydrogen phosphate with working concentration ranging from 30- 360 µM. The graph is shown in Fig 8. Phytase activity was calculated by the following formula:

Phytase activity = Concentration of phytase/ Aliquot of reaction mixture (0.5 ml) x Total reaction mixture (4 ml) x Dilution factor/ Incubation time

= U/gds in case of solid- state cultivation and

U/ml in submerged cultivation

1.2.7. Acid Phosphatase assay: The crude enzyme obtained from *Aspergillus niger* CFR 335 was also assayed for acid phosphatase by the method of Zyla *et al,* (1989).

(a) Reagents:

(i) 10 mM Disodium p- Nitrophenyl phosphate: 0.37 g/ 100 ml Dis. water

(ii) 40 mM Sodium hydroxide: 0.16 g/ 100 ml Dis. water

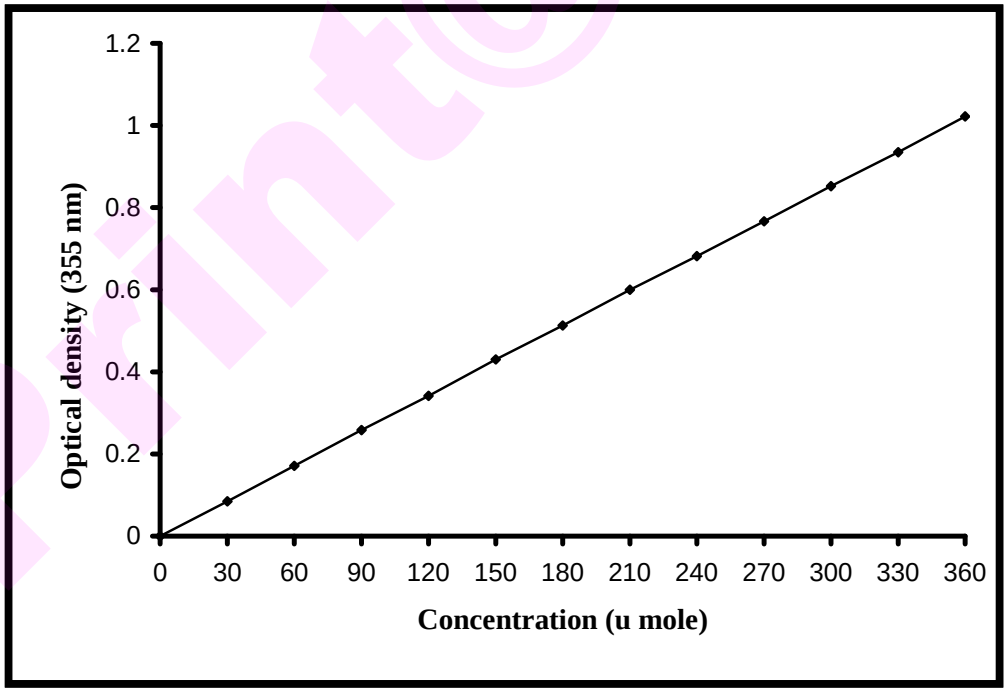
(b) Procedure:

0.2 ml of the crude enzyme extract was mixed with 1 ml of 10 mM disodium p- nitrophenyl phosphate that was used as the substrate for acid phosphatase enzyme. The mixture was incubated for 30 minutes at 37°C. After cooling the mixture to room temperature, 5 ml of 40 mM sodium hydroxide was added, mixed thoroughly and read the absorbance at 405 nm. A standard graph was prepared using p- nitrophenol with a working concentration ranging from 10-100 µM. The graph is given in Fig 9. The acid phosphatase activity was calculated by the following formula:

Acid phosphatase activity = Concentration/ Incubation time x Dilution factor x 1/ 1000 = **U/gds** in case of solid- state cultivation and

U/ml in submerged cultivation

Fig 8: Standard graph for Phytase



1.2.8. Soluble Protein estimation:

(a) Preparation of Bradford's reagent:

100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol. To this solution, 100 ml of 85% (w/v) phosphoric acid was added. The resulting solution is diluted to a final volume of 1 L. The reagent is filtered through Whatmann No.1 filter paper and stored in a clean brown bottle.

(b) Procedure:

Soluble protein in the phytase enzyme sample was estimated by the method of Bradford, (1976). 0.1ml of the protein sample is mixed with 5 ml of protein reagent and the absorbance was read at 595 nm after 2 minutes against reagent blank prepared with 0.1 ml of distilled water. A standard curve was plotted using Bovine serum albumin with the working concentration ranging from 10- 100 μg/ ml. The graph is given in Fig 10.

Specific activity = Enzyme activity (U/mgds or U/ml)/ Total protein (mg) = **U/mg**

Fig 9: Standard graph for Acid Phosphatase

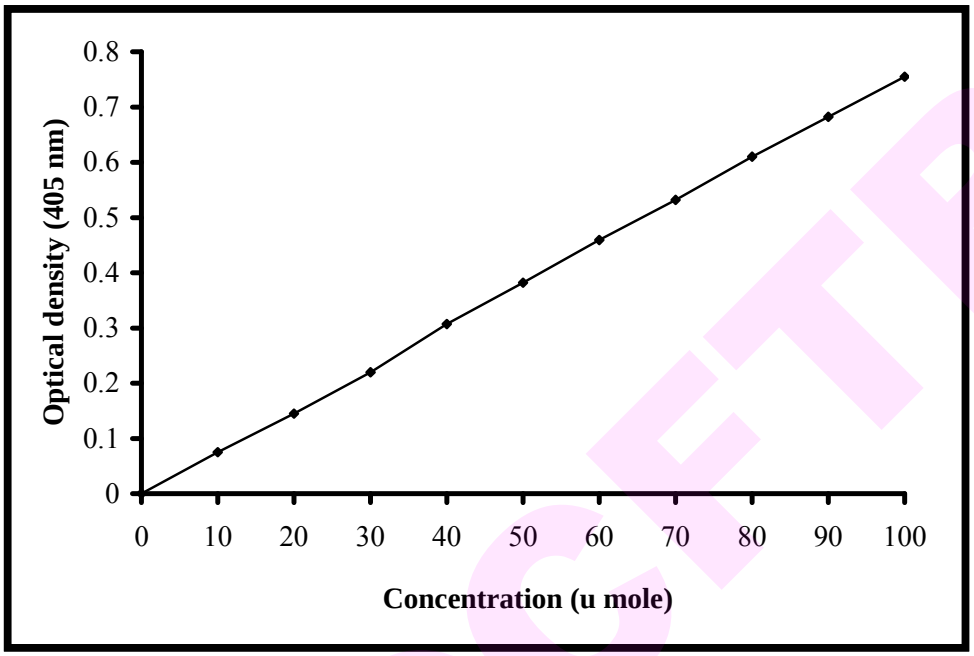
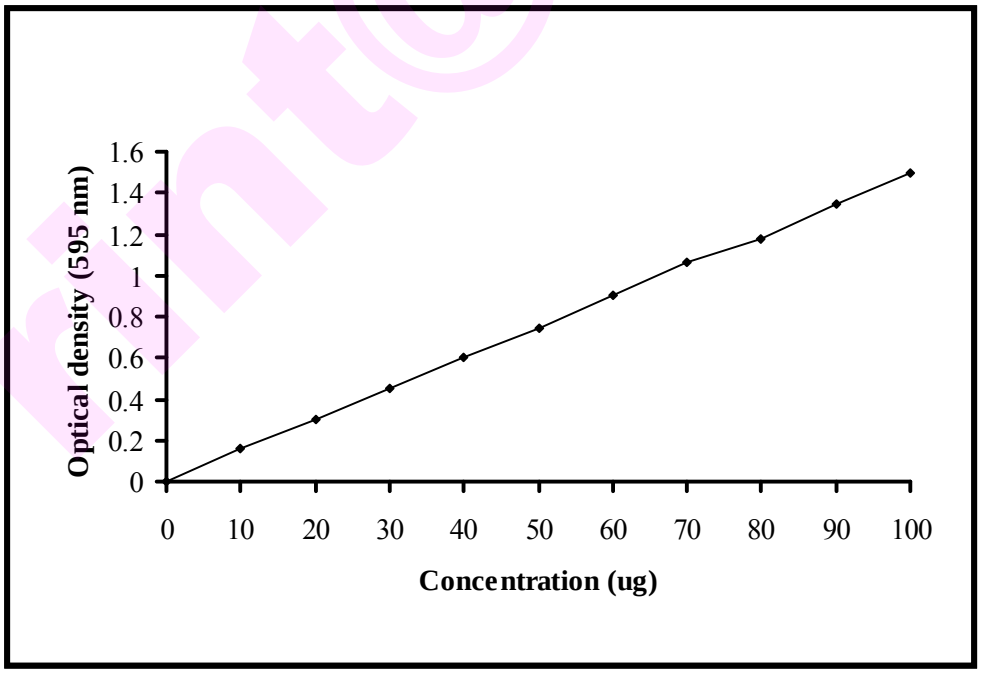


Fig 10: Standard graph for Soluble Protein



1.2.9. Solid- State Fermentation (SSF)

1.2.9.1. Media preparation:

Solid- state fermentation medium was prepared as follows: 50 g fresh wheat bran was taken in clean acid- washed Erlenmeyer flask and autoclaved for 40 minutes at 121°C. After cooling, wheat bran was moistened uniformly with sterile distilled water in an aseptic condition at 60% level. In one of the experiments, solid medium comprising 50% wheat bran, 25% rice bran and 25% groundnut cake with 60% moisture was also used. This medium was inoculated with appropriate fungal strain containing a known amount of spore count. The flasks were incubated in an inclined position to facilitate proper aeration at ambient temperature for an optimum growth period.

1.2.9.2 Inoculum preparation:

Inoculum preparation and the nature of inoculum play a vital role in deciding the quality and quantity of the ultimate product in solid- state fermentation. In the present study, inoculum for solid-state fermentation was prepared by cultivating the fungi in Czapek Dox Medium slants for 5 days at 30°C. To the fully sporulated fungal slant, 2-3 ml of sterile Tween- 80 was added and the spores were dislodged from the conidiophore by gently scraping with a sterile needle. Before inoculation spore count was taken using a Haemocytometer, Feinoptik, Bad Blankenburg, Germany. An optimized level of inoculum was used for inoculating the medium for maximum production rate.

1.2.9.3. Crude Enzyme extraction:

Moldy bran obtained after 5 days cultivation was mixed thoroughly using a clean glass rod. Crude phytase enzyme was extracted from the moldy bran with 1:5 w/v 0.2 M acetate buffer pH 4.5. The bran was mixed with buffer and kept in a rotary shaker for 30-45 minutes for proper extraction of the enzyme. The infusion

was strained through a clean muslin cloth to remove bran. The extract was centrifuged at 5000 rpm for 10-15 minutes to obtain a clarified enzyme used as crude phytase extract.

1.2.9.4. Determination of moisture content in the fermented bran:

For all the samples, 1 g of fermented bran (moldy bran) was used to measure moisture content. 1g of moldy bran was weighed exactly and dried in an oven at 55-60°C for 24 hrs until getting a constant weight. Final weight was then measured and difference between the initial weight and the final weight was found as the moisture content. This was converted into percentage moisture.

1.2.9.5. Lyophilization of the partially purified phytase enzyme:

The crude enzyme extract was partially purified by 80 % ammonium sulphate fractionation. The precipitate obtained was lyophilized for 6- 8 hrs. in an Edwards Modulyo Lyophilizer. The partially purified sample was used in the nutritional trials given in section 3 of this thesis.

1.2.10. Submerged Fermentation (SmF):

Nutritive medium with carbon and nitrogen source were used for submerged fermentation studies. In the present investigation, *Aspergillus niger* CFR 335 was cultivated in CM and the enzyme production was observed for 10 days. Similarly, the effect of different nutrient media on the growth and production of phytase enzyme was also studied by using different broth media such as PDB, CDB, CMB, SM and WBEM in a separate experiment.

1.2.10.1. Crude Enzyme Extraction:

Culture broth is filtered through a clean muslin cloth. The filtrate is centrifuged at 3000 rpm for 10 minutes. The clear filtrate obtained is used as the crude enzyme for the estimation of liberated phosphate.

1.2.10.2. Biomass estimation:

Biomass estimation in submerged fermentation was carried out directly unlike in solid- state fermentation method. Submerged culture broth of each sample, after appropriate cultivation period was filtered through a clean muslin cloth for obtaining wet biomass. Then it was dried in an oven at 55 ± 5°C to get a constant weight of the dry biomass.

1.2.13. Constitutiveness of phytase production using inorganic phosphate (Calcium phytate):

Aspergillus niger CFR 335 was cultivated in two different media, one with Complete Medium (CM) and the other Complete Medium supplemented with 0.5% calcium phytate. Both the media were inoculated with the spore suspension containing 2.2 x 10^6 spores/ ml. The flasks were incubated at 30°C in the rotary shaker at 200 rpm for 5 days. The culture filtrate was quantitatively checked for phytase enzyme by the assay method described above (2.6).

1.2.12. Effect of inorganic phosphate (Calcium phytate) on biomass content:

The effect of supplemental calcium- phytate on the biomass production of *Aspergillus niger* CFR 335 was also checked by measuring the biomass content by the method previously described (1.2.10.2).

SECTION 2

PHYSICO- CHEMICAL PARAMETERS FOR PHYTASE PRODUCTION IN SSF AND SMF:

2.1. Optimization of physical parameters:

2.1.1. Determination of optimum temperature for phytase production:

To determine the optimum temperature for maximum phytase production by *Aspergillus niger* CFR 335, the fungus was cultivated at different temperature that includes lower to higher range. The temperature ranged from 10- 55°C at an interval of 5°C. This was carried out both in submerged as well as in solid- state fermentation. The crude enzyme was checked for its inorganic phosphate release by the standard method described above (1.2.6).

2.1.2. Determination of temperature stability:

Stability of the enzyme was checked by keeping the enzyme sample obtained through the method as described earlier (2.1.1) at different temperatures of incubation ranging between 5ºC and 60ºC for 24 hr and the residual activity was determined as per the method of Heinonen and Lahti (1981).

2.1.3. Determination of optimum pH for phytase production:

The source organism, *Aspergillus niger* CFR 335 was cultivated both in submerged and solid- state fermentation media with different pH. 50 ml aliquots of complete medium was taken in 250 ml Erlenmeyer flask with initial pH ranging from 3.0- 8.5 with an interval of 0.5 were used for submerged fermentation. In case of solid-state fermentation, 50 g each of the wheat bran media with 60% moisture were also adjusted to different pH using 0.1 N HCl and 0.1N NaOH accordingly. The media were autoclaved for 20 minutes at 121°C and 15 lbs in

case of liquid broth and 40 minutes at 121°C for solid- state medium. After cooling, the flasks were inoculated with a known amount of inoculum and incubated at 30°C for 5 days.

2.1.4. Determination of pH stability:

The stability of the enzyme was tested by employing Glycine- HCl buffer for pH 3-4, sodium acetate buffer for 4-5, sodium phosphate buffer for 6-7 and Tris- HCl buffer for pH 7-8. The enzyme was kept in these buffers for 24 hrs at 4ºC and the residual activity was determined as per the method of Heinonen and Lahti (1981).

2.2. Optimization of chemical parameters:

An attempt was made to determine the effect of different carbon, nitrogen, phosphorus compounds, surfactants and metal salts on the phytase enzyme yield by *Aspergillus niger* CFR 335.

2.2.1. Effect of sugars:

The effect of different mono and disaccharides on the growth and enzyme production by *Aspergillus niger* CFR 335 was carried out by incorporating these sugars at 1% level in the complete medium broth instead of glucose. The medium with glucose served as control. The sugars used were fructose, lactose, maltose, sucrose, xylose, sorbitol, mannose, xylitol, arabinose, cellobiose, galactose, raffinose, rhamnose and trehalose. All the sugars were dissolved in distilled water and autoclaved separately for 20 minutes and were added to the medium at the time of inoculation. All the flasks were inoculated with *A. niger* CFR 335 spores and were incubated at 30°C for 5 days. Crude enzyme was extracted from both the media and was quantitatively checked for phytase enzyme (1.2.6).

2.2.2. Effect of nitrogen:

Different nitrogen sources used here were yeast extract, peptone, ammonium sulphate, ammonium nitrate, potassium nitrate and urea. Nitrogen compounds were added to nutritive media at 0.5% level. Medium with yeast extract served as control. The flasks were inoculated with *A. niger* CFR 335 and incubated at 30°C for 5 days. Crude enzyme quantifications were made by the above-mentioned assay method (1.2.6).

2.2.3. Effect of phosphate:

Various phosphates such as sodium dihydrogen phosphate, potassium dihydrogen phosphate, p-nitrophenyl phosphate, ammonium phosphate, phosphoric acid at a concentration of 0.25% were supplemented to the culture medium. Phosphate compounds were separately dissolved in distilled water, autoclaved and then aseptically added to complete medium broth at the time of inoculation. All the flasks were inoculated with appropriate amount of inoculum and incubated at 30°C for 5 days. Crude enzyme quantifications were made as per the method mentioned above (1.2.6).

2.2.4. Effect of surfactants:

In this study, different surfactants were incorporated in both SmF and SSF media. The surfactants used were Tween- 20, Triton X-100, SDS and EDTA at a concentration of 0.25% level. After incorporating the surfactants, the flasks were incubated at 30°C for 5 days. The enzyme assay was carried out by the above-mentioned method (1.2.6).

2.2.5. Effect of metal salts:

Effect of some of the metal ions on growth and phytase enzyme production by *Aspergillus niger* CFR 335 was determined by incorporating the complete

medium with different metal salts such as sodium chloride, magnesium sulphate, ferrous sulphate, manganese sulphate, copper sulphate, zinc sulphate, and calcium chloride at a concentration of 0.1% were incorporated in the culture media. All the salts were separately dissolved in distilled water and added to complete medium broth aseptically at the time of inoculation in case of Smf whereas, in SSF, the media were moistened with the salt solutions at the time of inoculation. The inoculated flasks were incubated at 30°C for 5 days. The assay method was followed as mentioned above (1.2.6).

SECTION 3

NUTRITIONAL STUDIES:

Nutritional studies on layer hens (*Gallus domesticus)* using *Aspergillus niger* CFR 335 phytase enzyme was carried out to check the efficacy of the enzyme on the overall metabolism of the layers. Young birds of three weeks old, procured from a local poultry farm were used in this study. The birds were grouped into four, based on the enzyme dosage administered. A control group without enzyme dose was also maintained. All the birds were fed with commercial feed along with appropriate levels of the enzyme in case of experimental birds.

The normal commercial feed ingredients containing mainly maize, sorghum, groundnut, fishmeal and other minor ingredients is given in Table22. The experimental diet was the commercial feed lacking calcium and phosphorus ingredients with different doses of *A. niger* CFR 335 phytase enzyme.

3.1. Estimation of non- phytate Phosphorus (npP) in feed:

The free Phosphorus or npP present in the commercial feed was estimated by AOAC (1990) method described below.

3.1.1. Phosphorus estimation:

(a) Molybdovanadate reagent:

(i) 10 g ammonium molybdate in 100 ml hot distilled water and cooled.

(ii) 1 g Ammonium metavanadate in 125 ml hot distilled water and cooled and then mixed with 125 ml of 70 % Perchloric acid.

(b) Sample preparation for phosphorus estimation:

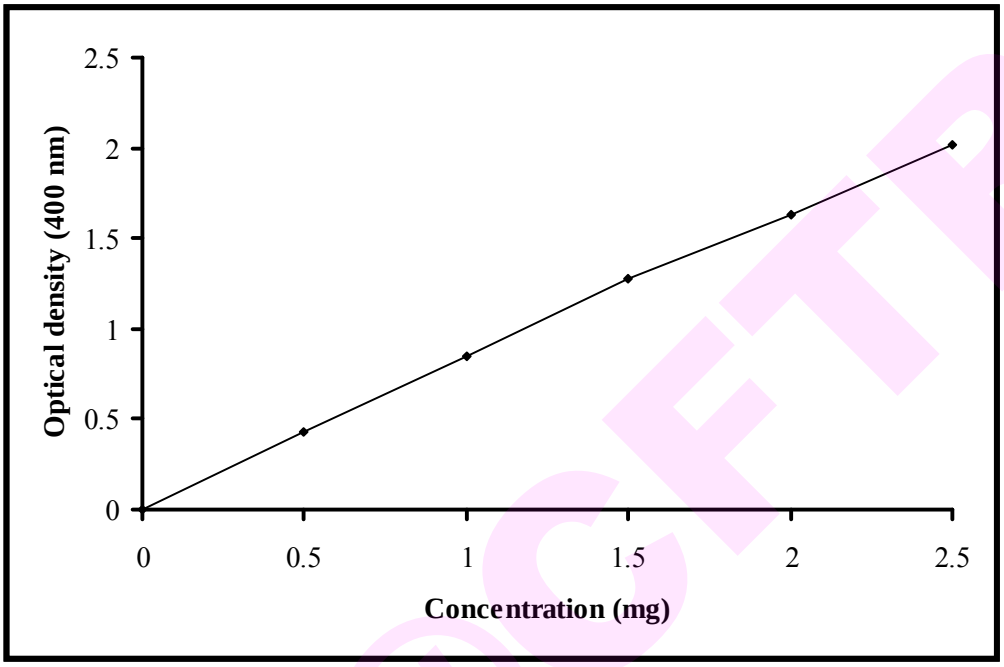
The sample for phosphorus estimation was prepared as per the method given below 2 g of the sample was dried at 60°C for 4 hrs. To the dried sample 40 ml concentrated Hydrochloric acid and few drops of Nitric acid were added, and the mixture was boiled and cooled. This mixture was transferred to 250 ml volumetric flask and the volume was made up to 250 ml. It was then filtered and the aliquots containing 0.5- 2.0 mg Pml^{-1} was taken in 100 ml volumetric flask. To the filtered sample, 20 ml molybdovanadate reagent was added and the volume made up to 100 ml with distilled water and mixed well. This mixture was kept for 10 minutes and the absorbance was read at 400 nm. A standard curve was plotted using Potassium dihydrogen phosphate. The working concentration ranged between 0.5 and 2.5 mg/ml from a stock of 10 mg/ml. The graph is given in Fig 11.

3.2. Estimation of phytate- Phosphorus (pP) in feed:

Estimation of phytate- Phosphorus in the feed was carried out by AOAC (1990) method. 2 g of the commercial feed was treated with different levels (200U, 400U, 600U and 800U and 1000U) of *Aspergillus niger* CFR 335 phytase enzyme. The feed with the enzyme was thoroughly mixed and incubated for 4-5 hrs at ambient temperature.

Phytate- phosphorus = Total phosphorus - Non- phytate phosphorus

Fig 11: Standard graph for Phosphorus



3.3. Estimation of Total- Phosphorus (TP) in feed:

Total- Phosphorus (TP) in the feed is the sum of non-phytate Phosphorus (npP) and the phytate- Phosphorus (pP).

3.4. Experimental design:

Healthy, three weeks old layer hens (*Gallus domesticus)* were procured from a local poultry farm. The birds were housed in separate cages with good ventilation. Each group containing 8 birds was taken for the experiment and one group was kept as control (Group 1) and fed with normal diet without administering the enzyme. Normally, each bird was found to take 125 g feed/day. The experimental birds, Group 2, 3 and 4 were fed with three appropriate levels of *A. niger* CFR 335 phytase enzyme, 200U, 400U, and 600U/ Kg of diet respectively, along with the experimental diet. The experiment was carried out for a period of 4 weeks. The following metabolic parameters were studied to

determine the effect of supplemental phytase on the growth and overall metabolism of the birds.

3.5. Effect of phytase on body weight of the layers:

Body weight of each bird was recorded before the feeding trial using digital electronic balance, Shimadzu UX 420 H, Japan. The subsequent body weight of the hens was recorded on weekly basis once the enzyme treatment was over. Body weight of the experimental birds was compared with the control group birds.

3.6. Effect of phytase on egg weight and eggshell breaking strength:

Eggs of each bird (group wise) were sorted out daily and their weights were determined. Eggshell strength was also determined using INSTRON Universal Texture measuring System wherein the eggs were made to break by applying a load shed of 100 Kg with a speed of 500 mm/min. The strength required for the breakage was expressed in Newton. This was compared with the control group of birds.

3.7. Effect of phytase on leg thickness:

Leg thickness of each bird (group wise) was recorded by measuring the circumference of the legs in the middle portion using Vernier Caliper scale. Measurements were taken on weekly basis after the feeding trial was over. Leg thickness of the experimental birds was compared with their control group,

3.8. Estimation of phosphorus in fecal matter:

Placing aluminium trays below each of the cages, fecal matter was collected and samples were pooled for one week. After collecting, the excreta was mixed thoroughly and dried in an oven at 60°C. The dried samples were ground to fine powder and 2 g of each sample was taken for phosphorus estimation by

AOAC (1990) method. From the above data, the amount of phosphorus excreted and the percentage of phosphorus that is utilized were determined by the following formula and compared with the control group.

% Phosphorus utilized = Total P in the feed (mg/g) - Total P in the feces (mg/g) x 100

3.9. Bone ash determination

Cleaned bones of each bird (group wise) were obtained from the experimental birds and the bone ashing was carried out by standard method. About 5-10g of the samples was dried separately at 100°C until moisture was expelled. After drying, the bone samples were ashed in the muffle furnace at 525°C to obtain white ash. Each ash sample was used for the determination of both phosphorus (AOAC, 1990) and calcium (AOAC, 2000) content. Bone ash from control birds was also estimated and compared.

3.10. Calcium estimation:

The ash samples were warmed with 5 ml of concentrated Hydrochloric acid and the volume was made up to 500 ml with distilled water in a standard volumetric flask. The sample was again diluted 2000 times prior to injecting. The diluted samples were subjected to AAS and the calcium content was calculated by the following formula:

Calcium = 500 x 2000 x Reading/ sample weight

= ------- mg /Kg = X

= X x 100 = g %

SECTION 4

STRAIN IMPROVEMENT STUDIES:

4.1. Mutation Techniques:

Aspergillus niger CFR 335 was subjected to mutation studies both by physical and chemical mutagenesis using Ultraviolet (UV) radiation and Ethyl Methane Sulphonate (EMS) & N'- methyl, N'- nitro, N'- nitrosoguanidine (NTG) respectively.

4.1.1. Mutagenesis by Ultraviolet radiation (UV):

Young spores of 18 – 20 hrs old was harvested using 0.1 % Tween-80. 1ml each of the spore suspension containing 2.4 x 10^6 cells ml^{-1} was subjected to UV irradiation for different time exposure in a sterile petridish using CAMAG UV chamber Betrachter, Switzerland make that emits radiations of 254 nm at a distance of 25 cm. The spore suspensions were intermittently agitated during the course of irradiation. After the treatment they were then incubated at 4°C in dark for overnight. The irradiated spore suspensions were suitably diluted with sterile saline and appropriate dilutions were plated on complete medium (CM). Survival percentage was plotted against the UV exposure.

4.1.2. Mutagenesis by Ethyl Methane Sulphonate (EMS):

Young spores of 18-20 hrs old were harvested from complete medium slants using 0.1 % sterile Tween- 80. Tween-80 was removed by centrifugation and the spores were resuspended in 1 ml sterile distilled water for EMS treatment. Aliquots of 1 ml of the spore suspension containing 2.4 x 10^6 spore ml^{-1} was treated with EMS at different concentrations (2, 4, 6, 8, 10 mM) in sterile distilled water. The suspensions were incubated for 1 hr at 30°C with a brief agitation. After incubation, the mutagen was completely removed by centrifugation at 2000

rpm for 10 minutes and the spore pellet was resuspended in 0.01M sterile buffer saline (pH 7.2) and then refrigerated for overnight in dark prior to plating. Appropriately diluted spore suspension was plated on CM. The plates were incubated for 4-5 days at 30°C. The survival effect of EMS was plotted against the concentration of EMS.

4.1.3. Mutagenesis by N'-methyl N'-nitro N'-nitrosoguanidine (NTG):

NTG mutagenesis was also carried out similar to EMS method (7.1.2) with different concentrations of 5, 10, 15, 20, 25 μg ml^{-1}. The survival effect of NTG was plotted against its concentration.

4.1.4. Mutagenesis of *Aspergillus ficuum*:

Aspergillus ficuum was also subjected to the above-mentioned mutagenesis to obtain an auxotrophic mutant for protoplast fusion experiment.

4.1.5. Characterization of putative mutants for phytase production:

About 2,500 putative mutants with morphological and colony variations were screened for phytase enzyme production by plating them on PSM containing 0.5% calcium phytate as substrate. Later, the positive putative mutants were selected for quantitative phytase production by cultivating them in SSF method. The enzyme activity was estimated by the method given in section 1.2.6.

4.1.6. Screening of auxotrophic mutants:

The UV, NTG and EMS mutagenised colonies grown on CM agar plates were replica plated using sterile velvet cloth on Minimal medium (MM) plates and incubated for 24-36 hrs (Venkateswaran, 1997). Colonies that did not show growth on MM plates were picked up from the master plate (CM) and individually tested for their growth factor requirement by inoculating into medium containing

appropriate concentration of individual amino acid (Holliday, 1956). About 500 colonies were screened for amino acid auxotrophic marker strain. The growth of the culture in tubes supplemented with amino acid indicated its amino acid requirement and was marked as respective auxotroph. The stability of the auxotrophic mutants was confirmed by repeated experiments and they were periodically subcultured and maintained in PDA slants for further studies.

4.2. Protoplast fusion techniques:

Protoplast fusion was carried out between the spores of *Aspergillus niger* CFR 335 ala^- and *Aspergillus ficuum* val^-, $isoleu^-$ according to the method of Peberdy (1980) with slight modification. Schematic representation of protoplast fusion technique is given in the Fig 12.

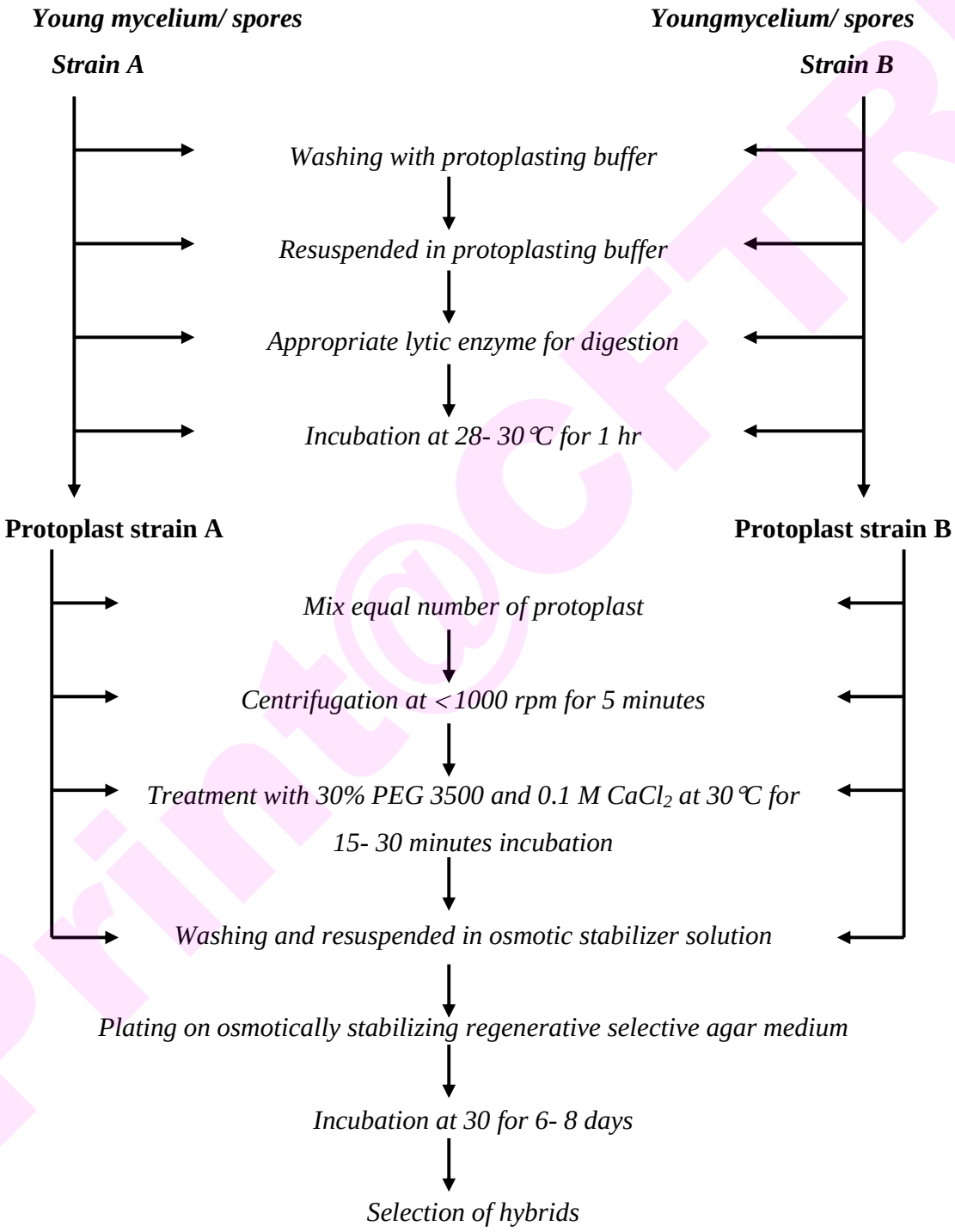
(a) Reagents

(i) ***Citrate Phosphate buffer (50 mM, pH 5.2) g/100ml*****:** 9.5 g citric acid and 4.72 g Na_2HPO_4 were dissolved and the volume made up to 100 ml with distilled water.

(ii) ***Protoplasting buffer (100 ml):*** 20 ml of 50 mM Citrate buffer, 10 ml of 60 % magnesium sulphate and 70 µl β - mercaptoethanol were mixed and the volume made up to 100 ml with distilled water.

(iii) ***Fusogen (100 ml):*** 30 g polyethylene glycol (PEG) 3500, 10mM calcium chloride (0.144 g), 10 mM, pH 5.2 Tris base (0.12 g) and 1 M sorbitol (18.22 g) were dissolved and the volume made up to 100 ml with distilled water.

Fig 12: Schematic representation of protoplast formation, fusion and regeneration



Source: Venkateswaran (1997)

4.2.1. Harvesting the fungal mycelium for protoplasting:

Aspergillus niger CFR 335 ala$^-$ and *Aspergillus ficuum* val$^-$, isoleu$^-$ were grown in complete medium broth for 18-20 hrs. The fungi were found to grow as small mycelial pellets of 1-2 mm diameter. Such mycelial pellets were harvested by filtering the culture broth through whatman No 1 filter paper. The pellets were washed thoroughly with sterile distilled water and then with sterile protoplasting buffer 2- 3 times.

4.2.2. Protoplast formation:

The fungal mycelium was treated with different concentrations (100 μg - 500 μg/ ml) of filter sterilized lysing enzyme obtained from *Trichoderma harzianum*. The mixture was incubated for 1 hr at room temperature with mild agitation. The enzyme was removed by centrifugation at 1,000 rpm for 5 minutes. The digest was re-suspended in protoplasting buffer and the protoplast devoid of cell debris was collected by filtration using specially designed equipment (Fig 13). The protoplasts were micro photographed and the percentage of protoplasting was determined by counting them using haemocytometer (Feinoptic Bad Blankenburg, Germany). Percentage of protoplast was calculated by the following formula.

% Protoplasting = Number of protoplasts obtained/ No. of spores used for protoplasting x 100

Fig 13: Apparatus used for protoplast harvesting

Courtesy: Venkateswaran (1997)

4.2.3. Regeneration of protoplasts:

Protoplasts obtained from both the fungi were tested for their regeneration by cultivating them on regeneration medium containing 1 M sorbitol as an osmotic stabilizer. The regeneration frequency was calculated using the following formula.

Regeneration Frequency = Number of colonies on Regeneration medium/ Number of protoplasts inoculated x 100

4.2.4. Protoplast fusion:

Equal number of protoplasts of both the fungi were mixed with fusogen containing polyethylene glycol 3500, 10 mM Calcium chloride, 10 mM Tris- HCl

and 1 M Sorbitol. The tubes were incubated for 1 hr to facilitate fusion between the 2 strains. Fusogen was removed by centrifugation at 1,000 rpm for 10 minutes. Fusion frequency was calculated using the formula given below after growing the fusion products on MM and CM plates.

Fusion Frequency = Number of colonies on MM/
Number of colonies on CM x 100

4.2.5. Characterization of hybrids for phytase production:

About 65 hybrids were qualitatively characterized for phytase enzyme production by inoculating the hybrid strains on PSM containing 0.5% calcium phytate. The plates were incubated for 2- 3 days at ambient temperature. The hybrids were selected based on colony size as well as the size of the zone of hydrolysis were measured and compared with their parent strains.

The negative hybrids attributed to either delayed growth or lesser yield of phytase enzyme were eliminated. Finally, sixteen hybrids were selected for enzyme quantification by the method mentioned earlier (1.2.6).

4.2.5.1. Characterization of hybrids for biomass production:

The selected sixteen hybrids were cultivated in complete medium broth for 5 days at 30°C in an orbital shaker. After incubation, the wet biomass estimation was made by the method mentioned above (1.2.10.2).

4.2.5.2. Genomic DNA extraction of the selected hybrids:

Genomic DNA is extracted from all the sixteen hybrids by the method of Maniatis *et al,* (1982).

(a) Reagents

***(i) 0.8 % Agarose*:** 0.8 g agarose is melted in 100 ml 1X TAE buffer.

(ii) ***50X TAE buffer (g/ 100 ml)*****:** 24.2 g Tris base (pH 8.0), 5. 7 ml of glacial acetic acid and 10 ml of 0.5 M, pH 8.0 EDTA were mixed and the volume made up to 100 ml with distilled water.

(iii) ***Extraction buffer (100 ml):*** 100mM Tris- HCL (pH 8.0), 0.02 M EDTA and 1% SDS.

(iv) ***DNA Loading dye (6X) (%):*** 0.25 g Xylene Cyanol, 0.25 g Bromophenol blue and 30 ml glycerol in 100 ml distilled water.

(v) ***Ethidium bromide (stock):*** 10mg ethidium bromide in 1 ml distilled water. 0.07 ml of the stock is diluted with 500 ml distilled water for working concentration.

(b) Extraction method:

A schematic chart of the protocol for genomic DNA extraction is given in Fig 14. The extracted samples were read for their absorbance at 260 nm and the quantification was made as per the method mentioned above. The samples were then electrophoresed with 0.8 % agarose gel at 60 V for 2-3 hrs.

Fig 14: Schematic chart for fungal genomic DNA extraction

1 g wet biomass ground to paste with quartz sand (3g/ g biomass)
+ 0.4 ml extraction buffer/ g sand

↓

0.5 g of the paste + 2 ml extraction buffer + 1 ml buffered phenol/ chloroform/ isoamyl alcohol (25: 24: 1)

↓

Mixture transferred to several eppendoroff's tubes and centrifuged at 16,000 rpm for 5 minutes at room temperature

↓

Aqueous phase + 0.6 ml Isopropanol

↓

Samples incubated at room temperature for 10 minutes and centrifuged at 4 ℃ for 15 minutes at 16,000 rpm

↓

Pellets rinsed with 95 % Ethanol and briefly air-dried

↓

Pellets resuspended in 340 µl TE buffer containing Ribonucleases A (20 µg/ml)

↓

Samples incubated at 37 ℃ for 30 minutes and extracted with 0.3 ml Phenol/ Chloroform/ Isoamyl alcohol

↓

Aqueous phase + ½ volume 7.5 M Ammonium acetate + 2.5 ml ethanol and incubated for 30 minutes at - 20 ℃

↓

Centrifuged at 4 ℃ for 15 minutes at 16,000 rpm

↓

Pellets rinsed with 95 % Ethanol and resuspended in 100µl TE buffer

4.2.5.3. Proximate analysis of hybrids:

4.2.5.3.1. Total sugar estimation by phenol- sulphuric acid:

Total soluble sugars in the biomass of the hybrids were estimated by phenol- sulphuric acid method (Dubois *et al*, 1951). The hybrids were grown on complete medium broth and their biomass was obtained through filtration. Wet biomass was homogenized using a Teflon tissue homogenizer. Cell debris was removed by centrifugation at 5,000 rpm for 10 minutes. Supernatant containing soluble sugars was collected for total sugar estimation. 1 ml aliquot of the sample was dispensed into clean test tube.1 ml of 5 % distilled phenol was added and mixed thoroughly. To the mixture, 5 ml of concentrated sulphuric acid was added and the tubes were gently agitated during the addition of acid. The tubes were then allowed to stay for 10 minutes. Optical density was read at 480 nm. A standard graph shown in Fig 15 was prepared using glucose with a working concentration of 1 mg/ 10 ml.

4.2.5.3.2. Fat Estimation by Soxhlet method:

Total fat content of the hybrids was estimated by Soxhlet method (Shaw, 1965). The hybrids were grown on complete medium broth to obtain their biomass. The dried biomass, finely powdered using acid wash sand (1:2) was wrapped in whatmann No 1 paper and macerated by boiling in 0.1N HCl for 1 hr on a boiling water bath. Hydrochloric acid was removed by washing thoroughly through running tap water. Macerated samples were dried at 55°C for overnight. The total fat was extracted by using analytical grade petroleum ether (40-60°C bp).

4.2.5.3.3. Protein estimation by Kjeldhal Method:

(a) Reagents:

(i) ***Digestion mixture*****:** Powdered potassium sulphate, copper sulphate and selenium di- oxide were mixed thoroughly in 5: 2: 1 proportion respectively.

(ii) ***40 % Sodium hydroxide*****:** 40 g Sodium hydroxide pellets were dissolved in about 50 ml distilled water and the volume was made up to 100 ml in a standard volumetric flask.

(iii) ***2% Boric acid*****:** 2 g Boric acid was dissolved in hot distilled water and the volume was made up to 100 ml in a standard volumetric flask.

(iv) ***Mixed indicator*****:** 0.1% solution each of bromocresol green and methyl red indicators were prepared in absolute alcohol and then 5 parts of bromocresol green solution was mixed with 1 part of methyl red solution.

(v) ***Standard N/70 HCl*****:** Approximately 1.3 ml of AR grade concentrated HCl was mixed with distilled water and the volume was made up to 1 L in a standard volumetric flask.

(vi) ***Standard Ammonium sulphate solution*****:** 0.942 g of Ammonium sulphate is dissolved in distilled water and the volume is 1 L in a standard volumetric flask (5 ml of this solution is equal to 1 mg of Nitrogen).

(b) Procedure**:**

Kjeldhal method for protein content determination is given in the schematic chart (Fig 16).

Percentage protein in the biomass = Titre value **-** blank x 0.21 x volume of the digest made up x 6.25 x 100/ aliquot used for distillation x weight of sample taken for digestion x 1000.

Fig 15: Standard graph for total sugars

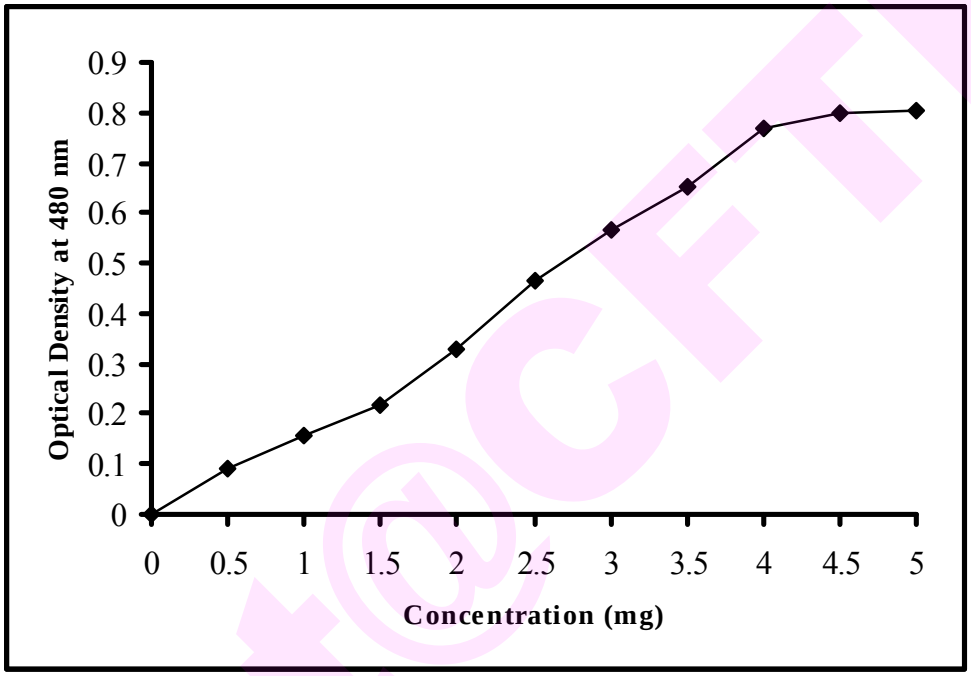


Fig 16: Schematic chart for Kjeldhal method

2 g dried fungal biomass + digestion mixture

↓ *Digested for 5- 6 hrs*

20 ml Con. Sulphuric acid

↓

Digest cooled and volume made up to 250 ml with distilled water

↓

5 ml of the above sample + 20 ml 40 % NaOH + 5 ml 2 % boric acid + 2 drops of mixed indicator

↓ ***Titrated against standard HCl***

Pale pink through pale green

SECTION 5

PURIFICATION OF *ASPERGILLUS NIGER* CFR 335 PHYTASE ENZYME:

The purification of phytase enzyme started with extraction of crude enzyme from the moldy bran of solid- state fermentation. The extraction was carried out as mentioned in section 1.2.9.3. All the steps were carried out at 4°C.

5.1. Ammonium sulphate fractionation:

To the crude enzyme preparation, solid ammonium sulphate was added slowly while stirring and brought to 80% saturation (Raghuramulu, *et al*). The standard chart followed for ammonium sulphate saturation is given in Table 4. After equilibration for overnight at 4°C, the precipitate was collected by centrifugation at 10,000 rpm for 20 minutes. The precipitate was dissolved in small volume of 0.2 M acetate buffer (pH 4.5).

5.2. Dialysis:

The enzyme sample obtained in the previous step was dialyzed extensively against 0.2 M acetate buffer, pH 4.5 for overnight. The supernatant was collected by centrifugation at 5,000 rpm for 10 minutes. This fractionation was subjected to further purification by ion- exchange chromatography.

5.2.1. Preparation of dialysis tubing:

The dialysis tubing was prepared as per the method of Maniatis, 1982. The tubing was cut into pieces of convenient length (10-20 cm), boiled for 10 minutes in a large volume of 2 % sodium bicarbonate and 1mM EDTA solution. After rinsing thoroughly in distilled water, it was again boiled for 10 minutes in distilled water, allowed to cool and stored at 4°C in 70% ethanol.

Table 4: Ammonium Sulphate Fractionation

Final concentration of Ammonium sulphate % saturation at 0°C. g solid ammonium sulp/ 100 ml solution

Initial satn.	20%	25%	30%	35%	40%	45%	50%	55%	60%	65%	70%	75%	80%	85%	90%	95%	100%
0	10.6	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	65.0	69.7
5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57.0	61.5	66.2
10	5.3	8.1	10.9	13.9	16.9	20.0	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1	62.7
15	2.6	5.4	8.2	11.1	14.1	17.2	20.4	23.7	27.1	30.6	34.3	38.1	42.0	46.0	50.3	54.7	59.2
20	0.0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	34.9	38.7	42.7	46.9	51.2	55.7
25		0.0	2.7	5.6	8.4	11.5	14.6	17.9	21.1	24.5	28.0	31.7	35.5	39.5	43.6	47.8	52.2
30			0.0	2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5	48.8
35				0.0	2.8	5.7	8.7	11.8	15.1	18.4	21.8	25.4	29.1	32.9	36.9	41.0	45.3
40					0.0	2.9	5.8	8.9	12.0	15.3	18.7	22.2	25.8	29.6	33.5	37.6	41.8
45						0.0	2.9	5.9	9.0	12.3	15.6	19.0	22.6	26.3	30.2	34.2	38.3
50							0.0	3.0	6.0	9.2	12.5	15.9	19.4	23.0	26.8	30.8	34.8
55								0.0	3.0	6.1	9.3	12.7	16.1	19.7	23.5	27.3	31.3
60									0.0	3.1	6.2	9.5	12.9	16.4	20.1	23.9	27.9
65										0.0	3.1	6.3	9.7	13.2	16.8	20.5	24.4
70											0.0	3.2	6.5	9.9	13.4	17.1	20.9
75												0.0	3.2	6.6	10.1	13.7	17.4
80													0.0	3.3	6.7	10.3	13.9
85														0.0	3.4	6.8	10.5
90															0.0	3.4	7.0
95																0.0	3.5
100																	0.0

Source: A manual of laboratory techniques Ed. N. Raghuramulu, K. Madhavan Nair and S. Kalyanasundaram, National Institute of Nutrition, Indian Council of Medical Research

5.3. Ion-Exchange Chromatography:

DEAE- Sephadex G- 50 was swollen at room temperature for overnight in 0.2 M acetate buffer pH 4.5 in a glass column (3 x 20 cm) equilibrated with the same buffer. The dialyzed fraction from ammonium sulphate fractionation was loaded on to DEAE- Sephadex column washed thoroughly with the same buffer. The adsorbed enzyme was eluted from the column with a linear gradient of 0.5 M NaCl with a flow rate of 20 ml/ hr. Active fractions were pooled, concentrated by freeze drying (lyophilization) (2.9.5) and were used for molecular mass estimation.

5.4. Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS-PAGE):

The concentrated enzyme sample obtained through ion- exchange chromatography was subjected to SDS- PAGE and the molecular mass was determined according to the method of Laemmli (1970) and Maniatis *et al*, (1982).

5.4.1. Stock solution preparation:

(a) ***30 % acrylamide:*** 30 g of acrylamide and 0.8 g bisacrylamide were dissolved in distilled water and then the volume was made up to 100 ml, filtered and stored in brown bottle at 4°C.

(b) ***Separating gel buffer (1.5 M Tris- HCl buffer, pH 8.8):*** 18.16 g Tris base was dissolved in 90 ml distilled water, pH adjusted to 8.8 with concentration HCl and volume made up to 100 ml with distilled water.

(c) ***Stacking gel buffer (0.5 M Tris- HCl, pH 6.8):*** 0.303 g Tris base was dissolved in 8 ml distilled water, pH adjusted to 6.8 with concentration HCl and volume made up to 10 ml with distilled water.

(d) ***Reservoir buffer:*** 14.4 g Glycine, 1 g of Tris and 0.5 ml of 10 % SDS were

dissolved in distilled water and the volume made up to 1 L with distilled water.

(e) ***10% SDS:*** 10 g SDS in 100 ml distilled water.

(f) **10% Ammonium persulphate*:*** 0.1 g/ ml distilled water.

(g) ***Sample buffer*:** All samples before loading onto the SDS- PAGE were denatured by boiling in the SDS- sample buffer for 5 minutes on a water bath. The protein samples were diluted with equal volume of the sample buffer. The composition of sample buffer was 0.125 M Tris- HCl buffer, pH 6.8, 0.5 ml 10 % SDS, 20 % glycerol (v/v), 10 % β- mercaptoethanol (v/v) and 0.002 % bromophenol blue (w/v).

(h) ***N, N, N', N'- tetra methyl ethylene diamine (TEMED):*** Stock available from Sigma Chemicals, USA.

5.4.2. Electrophoresis:

Electrophoresis was carried out on a slab gel obtained from Bangalore Genei, India, Pvt. Ltd with 10 % acrylamide. The separating gel (20 ml) was prepared by mixing the following reagents.

Reagent	**(ml/ 20 ml)**
30% Acrylamide	4.0
Separating gel buffer	5.0
10 % SDS	0.2
TEMED	0.016
10 % Ammonium persulphate	0.2
Distilled water	10.6

The above mixture was poured between 2 clean glass plates, sealed at three sides and separated by 1.5 mm thick spacers. Gel was allowed to polymerize for 30 minutes.

Stacking gel (5 ml) was prepared by the following mixing.

Reagent	**(ml/ 5 ml)**
30% Acrylamide	0.83
Stacking gel buffer	0.63
SDS (10 %)	0.05
TEMED	0.005
Ammonium persulphate (10 %)	0.05
Distilled water	3.4

Stacking gel buffer was overlaid the separating gel and this was also allowed to polymerize for 30 minutes by placing 1.5 mm comb over the gel. Electrophoresis was carried out at room temperature at 60 V for 6 hrs. Molecular mass of the protein was determined by loading a standard molecular mass protein mixture with molecular masses 29,000, 45,000, 66,000, 1,16,000 and 205,000 Daltons.

The gel was stained by silver nitrate method (Maniatis *et al*, 1982)

5.4.3. Silver nitrate staining:

(a) ***Reagents***

(i) ***Fixing solution*****:** 50 % Methanol, 12 % Acetic acid and 0.5 % Formaldehyde, mixed and the volume made up to 100 ml with distilled water.

(ii) ***50 % Ethanol*****:** 50 ml distilled ethanol made up to 100 ml with distilled water.

(iii) ***Sodium thiosulphate*****:** 0.02 g in 100 ml distilled water.

(iv) ***Staining solution*****:** 0.2 g Silver nitrate and 0.075 ml of 37 % Formaldehyde in 100 ml distilled water.

(v) ***Developing solution*****:** 6 g Sodium carbonate and 0. 5 ml of 37 % Formaldehyde in distilled water and the volume made up to 100 ml with distilled water.

(b) ***Procedure:***

a. The gel was fixed in fixing solution for 1 hr/ overnight.
b. The fixed gel was washed twice in 50 % Ethanol for 30 minutes.
c. It was pretreated with Sodium thiosulphate solution exactly for 1 minute and rinsed 2-3 times with distilled water for 20 seconds.
d. The gel was then kept in staining solution for 20 - 40 minutes and rinsed twice with distilled water for 20 seconds each.
e. It was then developed in a developing solution containing Sodium carbonate and formaldehyde until the bands are distinct visible.
f. The reaction was terminated by adding 3 ml of acetic acid directly at the corners of the box.

(c) ***Destaining:***

The gel was repeatedly washed with 10 % acetic acid and stored in 5 % acetic acid.

5.5. High Performance Liquid Chromatography (HPLC):

The active fractions obtained through ion- exchange chromatography were subjected to reverse phase HPLC (RT- HPLC). This was conducted on a Shimadzu LC- 10A VP system equipped with a UV. A reverse phase C-18 column

(Water Radialpak) was equilibrated with the linear gradient of mobile phase comprised with 0.1% trifluroacetic acid (solvent A) and 95% acetonitrile (solvent B). The solvent gradation started with 0% and raised to 100% acetonitrile containing 0.1% trifluoroacetic acid in 20 minutes. The elution profile was monitored at 280 nm with a flow rate of 1 ml/ minute.

5.6. Determination of optimum temperature of enzyme:

Optimum temperature for activity of the purified enzyme was determined according to the method given in section 2.1.1.

5.7. Determination of temperature stability:

The temperature stability of the purified enzyme was checked by the method given in the section 2.1.2.

5.8. Determination of optimum pH of enzyme:

Optimum pH for activity of the purified enzyme was tested by the method given in section 2.1.3.

5.9. Determination of pH stability:

Stability of the purified phytase enzyme was tested by the method given in section 2.1.4.

All the methods used in this study were carried out very carefully and wherever necessary, experiments were repeated until obtaining a clear cut/ consistent results.

RESULTS
&
DISCUSSION

RESULTS AND DISCUSSION

SECTION 1

Isolation and screening of fungal strains for phytase production:

Phytase is an enzyme that is highly recommended as a feed supplement of poultry and swine. This enzyme aids in the bioavailability of essential minerals and combat environmental pollution attributed to complex excretion of phytic acid. Microorganisms, being a diversified group of organisms, are reliable sources for a number of useful products such as enzymes, amino acids, antibiotics and other bioactive molecules. In the present study, about 1500 fungal strains were screened for phytase activity from different natural habitats such as soil and poultry wastes. Among these, 150 strains were found to possess phytase activity and 12 potent strains belong to genus *Aspergillus* were isolated for enzyme quantification. Some of the standard cultures from Microbial Type Culture Collection (MTCC) strains were also screened and quantified for phytase activity. The fungal strains screened for phytase enzyme are shown in Figs 17, 17a, 17b & 18. One of the isolates *Aspergillus niger* CFR 335 was found to have the highest specific activity of 1.864 U/mg on the fifth day of its cultivation in a solid medium containing wheat bran with 60% moisture level. Another strain, *Aspergillus terreus* MTCC 279 showed a specific activity of 1.89 U/ mg on the tenth day of cultivation. This delay in the enzyme production is an undesirable trait of this strain. Two more strains *A. fumigatus* MTCC 179 and *A. fumigatus* SMU also produce fairly good amount of phytase and are known to secrete certain toxins (fumigatin) extracellularly. The results are given in Tables 5, 5a and 5b. Since *Aspergillus niger* is placed in GRAS status by FDA (Harvey and Mc Neil, 1994), the isolate, *A. niger* CFR 335 was used for further experiments in this study.

Vats and Banerjee (2002) have screened phytase-producing organisms from soil samples collected from live stock units, poultry farms and agricultural fields

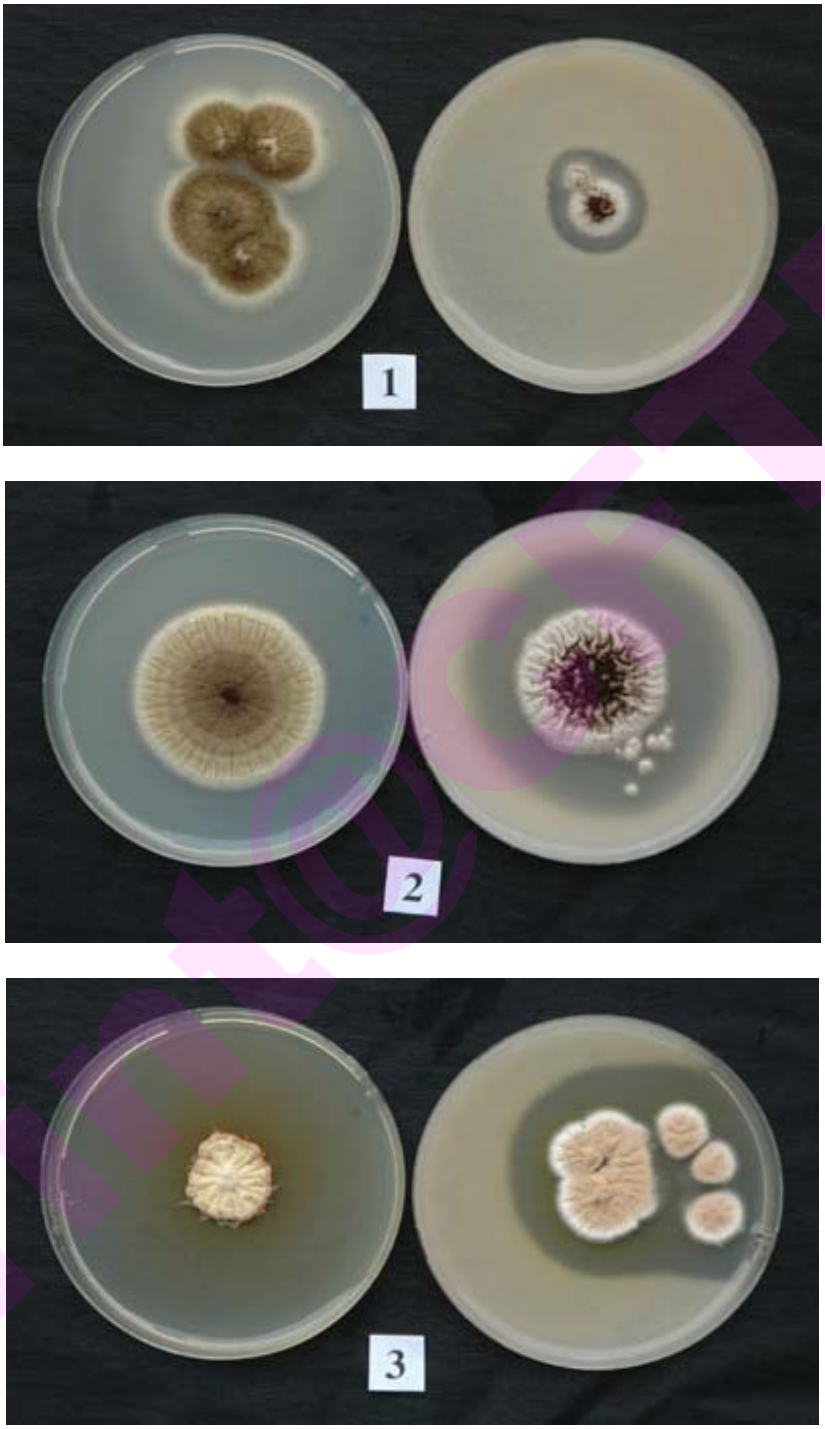


Fig 17: 1-3; Fungal cultures screened for phytase production

Complete Medium (Left side); Substrate Medium (Right side)

1. *Aspergillus niger* MTCC 281 2. *Aspergillus niger*CFR 42

3. *Aspergillus terreus* MTCC 279

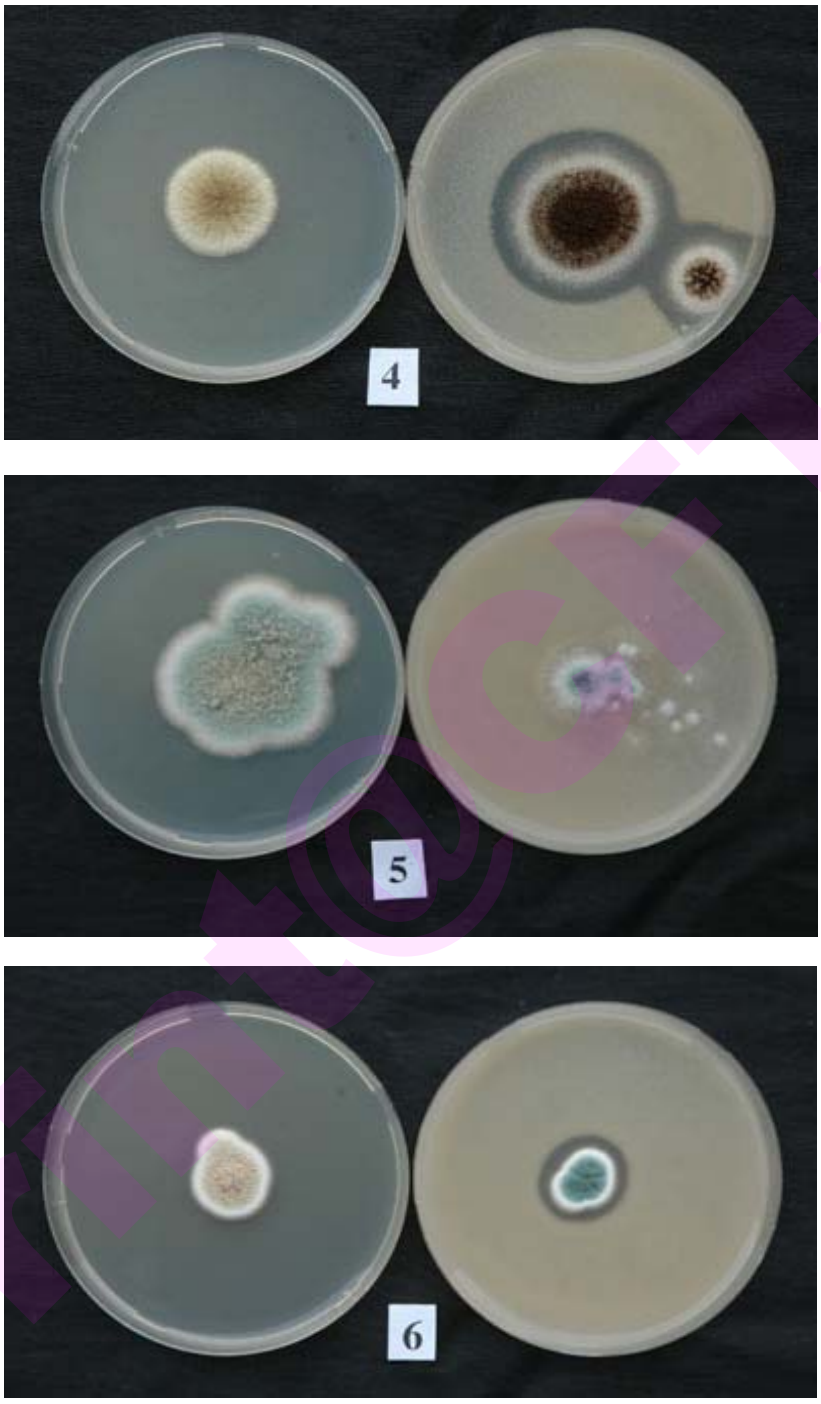


Fig 17a: 4-6; Fungal cultures screened for phytase production

Complete Medium (Left side); Substrate Medium (Right side)

4. *Aspergillus niger* CFR 335 5. *Aspergillus fumigatus* MTCC 179

6. *Aspergillus fumigatus* SMU

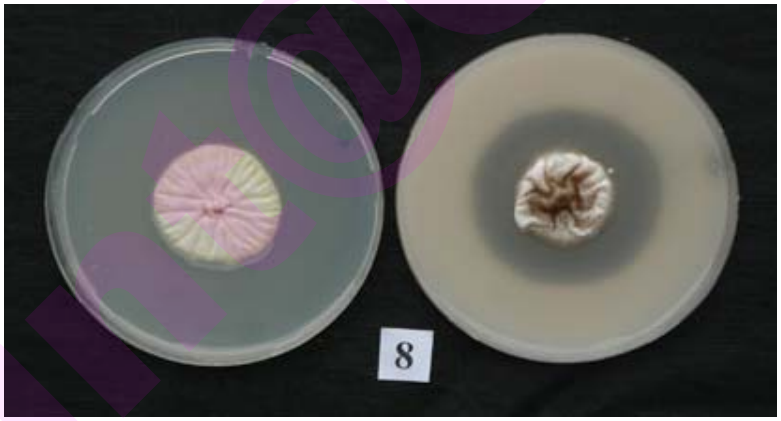


Fig 17b: 7 & 8; Fungal cultures screened for phytase production

Complete Medium (Left side); Substrate Medium (Right side)

7. *Aspergillus niger* SGA 03

8. *Aspergillus ficuum* SGA 01

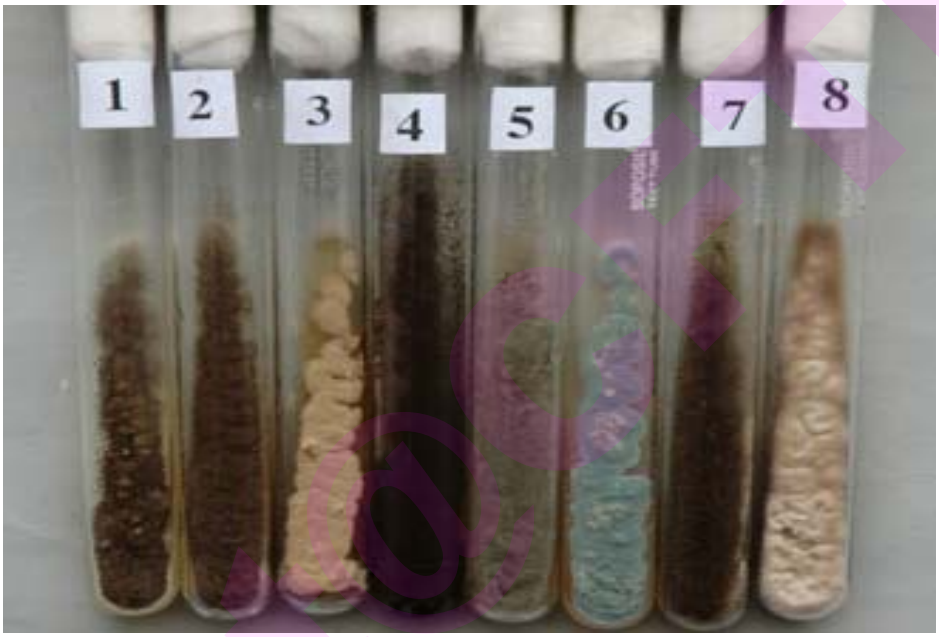


Fig 18: Phytase producing cultures on slant

1. *A. niger* MTCC 281
2. *A. niger* CFR 42
3. *A. terreus* MTCC 279
4. *A. niger* CFR 11
5. *A. fumigatus* MTCC 179
6. *A. fumigatus* SMU
7. *A. niger* SGA 03
8. *A. ficuum* SGA 01

Table 5: Screening of fungal isolates for phytase activity

Incubation time (day/s)	Fungal Isolate									
	A. niger CFR 6		*A. niger* CFR 8		*A. niger* CFR 11		*A. niger* CFR 19		*A. niger* CFR 28	
	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
1	432	0.193	469	0.236	256	0.124	293	0.144	656	0.398
2	689	0.404	770	0.483	306	0.195	370	0.226	903	0.496
3	886	0.436	1289	0.597	381	0.303	886	0.423	1253	0.774
4	2105	1.356	381	0.303	442	0.354	1160	0.613	1710	0.934
5	2230	1.383	442	0.354	516	0.373	987	0.463	1441	0.867
6	1051	0.567	2126	1.368	550	0.386	745	0.383	1042	0.759
7	983	0.523	2115	1.356	1200	0.673	701	0.339	964	0.523
8	873	0.428	2113	1.353	2908	1.507	640	0.298	921	0.503
9	637	0.396	1903	1.183	2780	1.463	433	0.226	674	0.432
10	383	0.178	1600	0.985	2173	1.236	277	0.127	388	0.243

*** 1 Unit of the enzyme is the activity of the enzyme to release one μmole of inorganic phosphorus in one minute at 45°C**

Table 5a: Screening of fungal isolates for phytase activity

Incubation time (day/s)	Fungal Isolate									
	A. niger CFR 39		*A. niger* CFR- 40		*A. niger* CFR 42		*A. niger* CFR 335		*A..niger* MTTC 281	
	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
1	440	0.196	193	0.193	404	0.172	846	0.523	836	0.286
2	753	0.419	252	0.252	634	0.388	1121	0.818	1316	0.450
3	1110	0.573	432	0.432	836	0.433	1176	0.838	1017	0.556
4	1713	0.947	697	0.697	1116	0.621	1282	0.86	1096	0.610
5	1977	1.223	573	0.573	1187	0.738	1783	1.292	1316	0.776
6	1367	0.983	432	0.432	1326	0.883	**2067**	**1.864**	1540	0.994
7	1228	0.976	483	0.483	1586	1.087	2038	1.538	1668	1.012
8	1028	0.653	577	0.577	2231	1.386	2000	1.399	1777	1.625
9	973	0.618	396	0.396	1870	1.138	1560	0.948	1666	1.463
10	556	0.423	236	0.236	1560	0.948	1280	0.864	1365	1.226

*** 1 Unit of the enzyme is the activity of the enzyme to release one μmole of inorganic phosphorus in one minute at 45°C**
Highest activity is indicated in bold (*A. niger* CFR 335)

Table 5b: Screening of fungal isolates for phytase activity

Incubation time (day/s)	Fungal Isolate							
	A. niger CFR- 256		*A. niger* SGA - 03		*A. ficuum* SGA - 01		*A. terreus* MTTC- 279	
	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
1	617	0.158	778	0.196	638	0.339	534	0.193
2	834	0.362	1192	0.378	865	0.449	884	0.447
3	936	0.444	1393	0.593	986	0.687	987	1.223
4	1116	0.603	1496	0.641	1623	0.983	1479	1.48
5	1226	0.723	1506	0.739	1113	0.756	1556	1.283
6	1326	0.884	1656	0.877	1353	0.483	1612	1.891
7	1756	1.146	1799	1.709	1584	0.896	1587	1.596
8	2231	1.595	1538	1.283	1793	1.684	1454	1.711
9	1593	1.404	1516	1.166	1734	1.336	1263	1.787
10	1356	1.394	1505	1.091	1684	0.919	1891	1.040

*** 1 Unit of the enzyme is the activity of the enzyme to release one μmole of inorganic phosphorus in one minute at 45°C**

and at random from rotten wood- logs. They have isolated thermostable and acid stable phytase producing organisms by plating the soil suspension on to phytase screening medium (PSM). The plates were incubated at various temperatures (30, 40, 50 and 60°C) and checked for the appearance of a zone of hydrolysis produced by phytic acid degrading organisms at 24 h intervals. By this screening process, they have isolated most potent phytase producer, which was later identified as *Aspergillus niger* var. teigham. This isolate was shown to have reproducible phytase activity with pH and temperature optima between 2.0 - 2.5 and 50 - 55°C respectively.

Chen (1998) has developed a bioassay method for the screening of extracellular phytase producing microorganisms. Cells of *Corynebacterium glutamicum* were used as indicator strain in his procedure. Volfova *et al,* (1994) screened 132 micro- organisms for phytase production. About 1200 yeast strains were screened from CBS Collection for the efficiency to grow on phytate as a sole source of carbon and phosphate (Sano *et al,* 1999). Yanke *et al,* (1998) have screened 334 strains, out of which 22 species of anaerobic ruminal bacteria were shown to possess phytase activity. Strains such as *Selenomonas ruminantium, Megasphaera elsdenii, Prevotella ruminicola, Mitsuokella multiacidus* and *Treponema* sp. were shown to have phytase activity.

Shieh and Ware (1968) have screened around 2000 cultures from 68 soil samples for phytase enzyme production. Howson and Davis (1983) have surveyed 84 fungi for phytase production. A bacterial strain producing extracellular phytase was isolated from soil near the roots of the leguminous plants and it was identified as *Enterobacter* sp.4. Optimum conditions for enzyme production by this organism in phytase screening medium were found to be pH 5.5 and 3 days of cultivation at 37°C (Yoon *et al*, 1996).

Our studies indicated that *A. niger* CFR 335 isolated from poultry soil produces maximum amount of phytase on the 5th day of the incubation.

Screening of Lactic cultures for phytase production:

The human gut is populated by at least 500 different species, which coexist in dynamic equilibrium with the host. The beneficial microbial groups develop an array of metabolic, trophic and protective functions, which have profound repercussions on human health (Guarner and Malagelada, 2003). Among them, nutrient metabolism represents an important biochemical activity of the human body and results in salvage of energy, generation of absorbable compounds and production of vitamins and other essential nutrients (Bomba *et al*, 2002; Reid *et al,* 2003). As a consequence, dietary strategies that favour the prevalence of health-promoting intestinal bacteria have been developed. In our experiment, some of the lactic strains were also screened for phytase enzyme. Quantitative estimation of phytase enzyme was made by cultivating the strains in submerged fermentation medium. The results indicated that one of the lactic strains, *Lactococcus* K_7 showed maximum phytase activity and specific activity of 259.6 U/ml and 0.38 U/mg respectively which was followed by *Lactococcus* C_{20}, *Lactococcus* BA 242 D, *Lactococcus* B_1, *Lactococcus* B_4, *Lactococcus* C_5. The results are given in Table- 6.

Lactic acid bacteria (LAB) are one of the interesting groups of bacteria, some of which are classified as probiotics. These probiotics form a major class due to their nutritional and pharmaceutical point of view. Probiotics are capable of producing a large number of antibiotics that may be antibacterial or antifungal. Lactic acid bacteria are in general able to survive at low pH values. However, their activity is inhibited when pH drops below 4.0. Some of the lactic strains are capable of producing phytase enzyme under optimal fermentation condition. *Lactobacillus plantarum* was found to be able to decrease phytate in a medium in

which phytic acid was the only source of phosphate (Marklinder *et al*, 1995). *Schwanniomyces castellii* CBS 2863 was shown to have highest phytase activity in the presence of phytate (Lambrechts *et al*, 1992).

Table 6: Screening of Lactic cultures for phytase activity

Strain	Activity (U*/ml)	Sp. Act. (U/mg)
Lactococcus sp. B_1	92.4	0.07
Lactococcus sp.BA 242D	195.5	0.16
Lactococcus sp.C_{20}	252.4	0.34
Lactococcus sp.C_6	72.7	0.04
Lactococcus sp.K_7	**259.6**	**0.38**
Lactococcus sp.C_5	71.1	0.03
Lactococcus sp.B_4	71.1	0.03

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**
Highest activity is indicated in bold

Solid- state fermentation for phytase production:

Solid- state fermentation (SSF) is normally handled by many industries for enzyme production through fungal sources. *Aspergillus niger* CFR 335 was found to grow luxuriantly in wheat bran substrate medium. A maximum growth was attained at an early period of 40- 48 hrs of incubation. Fermented bran is shown in Fig 19. The fungus produced the enzyme extracellularly into the substrate. The enzyme activity was observed periodically and the results are given in Table-7. It was found that, the enzyme activity increased exponentially and maximum phytase activity of 1879 U/gds and specific activity of 1.711 U/mg on 5^{th} day of incubation at room temperature. With subsequent cultivation period, a decline in the activity was observed. On 10^{th} day the enzyme activity reduced more than 40% when compared to the optimum activity on 5^{th} day.

Fig 19: 1; Moistened wheat bran, 2; Fermented bran

Table 7: Phytase activity of *Aspergillus niger* CFR 335 in SSF

Incubation time (days)	Activity U*/gds	Specific activity U/mg
1	569	0.560
2	594	0.612
3	797	0.759
4	965	0.901
5	**1879**	**1.711**
6	1052	1.012
7	875	0.994
8	839	0.922
9	775	1.123
10	730	0.973

***1 Unit of the enzyme is the activity of the enzyme to release one μmole of inorganic phosphorus in one minute at 45°C**

Highest activity is indicated in bold

A considerable amount of work has been done in recent years to understand the biochemical engineering aspects of SSF processing (Mitchell *et al*, 2000, 2000a; Pandey, 2003). About 5,000 years ago fungi were cultivated in SSF for the production of food, the oldest known fermentation of rice by *A. oryzae* used to initiate the koji process. *Penicillium roquefortii* has been used for cheese production for 4,000 years, and soy sauce produced in Asia and bread in Egypt since 3,000 years ago (Pandey *et al,* 2001). An important biological factor in favour of SSF is the low catabolite repression, which appeared to be the cause for limiting enzyme production by *A. niger* in SmF (Nandakumar *et al,* 1999). Similar observation was also made in this study.

The production of phytase from *Aspergillus ficuum* NRRL 3135 has been achieved by three different cultivation methods, namely, solid- state (Ebune *et al*, 1995), semi- solid (Han and Gallagher 1987) and submerged fermentations (Howson and Davis, 1983 and Ullah and Gibson, 1987). Ebune *et al* (1995) have used canola meal for phytase production by A*spergillus ficuum* with optimum moisture of 64%. Alasheh and Duvnjak (1995) have produced phytase using *A. carbonarius* strain on canola meal with optimum moisture ranging from 53- 60%. The reduction of phytic acid content in canola meal was found with the production of phytase enzyme by *Rhizopus oligosporus* NRRL 2990, *Aspergillus niger* NRC 5765, *A. carbonarius* NRC 401121, *A. ficuum* NRRL 3135 and a wild strain, *Saccharomyces cerevisiae* in solid- state fermentation has also been reported (Nair and Duvnjak, 1991).

The effect of culture conditions, particularly inoculum age, media composition and duration of SSF on the phytase production by *A. niger* was studied by Krishna and Nokes (2001). Bogar *et al*, (2003) have reported phytase production by *A. ficuum* NRRL 3135, *Mucor racemosus* NRRL 1994 and *Rhizopus oligosporus* NRRL 5905 on canola meal, cracked corn, soybean meal and wheat bran in SSF. The fermentation conditions (pH 5.3, 30°C, 54.5%

moisture content) for phytase production in SSF using canola oil cake with no additional nutrients by *R. oligosporus* were reported (Sabu *et al*, 2000).

The phytic acid in soybean meal and cottonseed meal was hydrolyzed by phytase produced by *A. ficuum* that was grown on semisolid soybean meal. *A. ficuum* was found growing well on steamed wheat bran, soybean meal and corn meal without any added nutrients. The highest amount of phytase was produced on wheat bran, followed by soybean meal and corn meal. The levels of phytase production on semisolid substrates were higher than in the liquid medium. It was found that *A. ficuum* produced 45 Units of phytase per gram of wheat bran, whereas less than 10 Units/ml enzyme was produced in liquid medium after 2 weeks of cultivation (Han *et al,* 1987). In our findings, when the fungus was cultivated separately in SmF as well as in SSF, a maximum phytase activity of 1879 U/gds was observed in SSF when compared with SmF cultivation (248 U/ml). A nine- fold increase in enzyme activity was observed on 5^{th} day of incubation at room temperature.

Acid phosphatase:

Acid phosphatase is another member of histidine group phosphatases that has broad substrate specificity. Acid phosphatase act on a large number of phosphate compounds and release lower intermediates. In the present study, assay for acid phosphatase was carried out in *Aspergillus niger* CFR 335 as per the method given in section 2.7 of materials and methods. The results indicated that the fungus did not produce significant amount of acid phosphatase at 30°C. It was found that a very negligible amount was produced. The production was found to increase slightly with increase in the incubation time and the maximum acid phosphatase produced was 33U/gds. The results are given in Table 8.

There are some fungi that are capable of hydrolyzing a large spectrum of phosphates due to their secretion of acid phosphatase. Acid phosphatase activity

was determined by monitoring the rate of hydrolysis of p- nitrophenyl phosphate (Palacios *et* al, 2005).

Table 8: Acid phosphatase activity of *Aspergillus niger* CFR 335 in SSF

Incubation time (days)	Activity (U*/gds)	Specific activity (U/mg)
1	3	0.01
2	6	0.009
3	8	0.038
4	12	0.030
5	14	0.064
6	17	0.085
7	21	0.113
8	23	0.126
9	27	0.176
10	**33**	**0.246**

*** 1 Unit of the enzyme is the activity of the enzyme to release one μmole of inorganic phosphorus in one minute at 45°C**
Highest activity is indicated in bold

Submerged fermentation for phytase production:

Submerged fermentation is one of the industrially important techniques where the fungal products of biotechnological interest, such as enzymes, secondary metabolites and spores are developed for use in moist solid substrates. Although submerged fermentation technology is considered to be a kind of violation of the natural habitats of wild- type microorganisms, very efficient microbial strains, well adapted to submerged fermentation by genetic engineering are available for enzyme production on an industrial scale.

In this study, *Aspergillus niger* CFR 335 was cultivated by submerged fermentation method (SmF) in complete medium broth with sufficient aeration and agitation. The fungus produced discrete pellets with the vegetative mycelium intertwined to form tough pellets within 48- 50 hrs of incubation at 30°C. Phytase activity of 248 U/ ml and 0.173 U/mg of specific activity on 5th day of the cultivation were observed. The enzyme activity was found to increase exponentially and there was a decline in the activity beyond optimum incubation period. A 40% reduction in the specific activity of the protein was observed on 10th day of the incubation period. The results are given in Table 9.

Table 9: Phytase activity of *Aspergillus niger* CFR 335 in SmF

Incubation time (day)	Activity (U*/ml)	Specific activity (U/mg)
1	19	0.126
2	46	0.128
3	72	0.128
4	106	0.244
5	**248**	**0.173**
6	116	0.163
7	78	0.153
8	36	0.147
9	19	0.123
10	12	0.100

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**
Highest activity is indicated in bold

Ahmad *et al* (2000) used maize starch- based medium for the production of phytase by *A. niger* by submerged fermentation. Activity of the enzyme was found to be 10.8 U/minute/ ml of the crude culture filtrate at pH 5.5 and 40°C. A strain of *Aspergillus niger* var. teigham has been checked in starch medium at 30°C and

the isolate produced 184 nKat/ ml phytase activity after 17 days of incubation with a parallel increase in cell mass and extracellular protein. After 18 days, there was a decline in phytase production despite continued culture growth (Vats and Banerjee 2002). *Bacillus* sp. DS11 was used for the production of a thermostable, extracellular phytase at 37°C under submerged fermentation medium consisting of wheat bran and casein hydrolysate (Kim *et al,* 1998; Kim *et al,* 1998a).

Effect of different media in SmF:

Aspergillus niger CFR 335 was cultivated in different broth media to compare the effect of media components on the production of phytase enzyme activities. Potato dextrose medium showed an activity of 256 U/ml with 0.783 U/ mg specific activity. Two- fold increase of 505 U/ml was observed in wheat bran extract medium with specific activity of 1.188 U/mg. This was followed by synthetic medium and czapek dox medium. Potato dextrose medium was found to be less suitable for phytase yield in liquid cultivation. The results are shown in Table 10.

Table 10: Effect of different culture media on phytase production in *Aspergillus niger* CFR 335 by SMF

Media	**Activity (U*/ml)**	**Specific activity (U/mg)**
Potato Dextrose broth	256	0.783
Complete Medium broth	302	0.938
Wheat bran extract broth	**505**	**1.188**
Czapek Dox Medium broth	444	1.099
Synthetic medium broth	402	1.036

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**

Highest activity is indicated in bold

Induction/ repression of phytase enzyme:

Experiment was carried out to show the induction or repression of phytase enzyme in presence of inorganic calcium phytate. Calcium phytate was found to induce phytase production when the culture medium was supplemented with 0.5 % calcium phytate. Biomass production was also enhanced by calcium phytate. The results showed more than 25% increase in phytase activity and the biomass increased up to 55% when the fungus was cultivated in the presence of phytate. Optimum enzyme activity of 682 U/ml and a specific activity of 1.829 U/mg were found on the 6^{th} day of incubation in submerged cultivation. The results are shown in Table 11. The effect of calcium phytate on biomass production in *Aspergillus niger* CFR 335 is shown in Fig 20.

Lambrechts *et al*, (1992) have shown highest phytase activity in yeast, *Schwanniomyces castellii* CBS 2863 in the presence of phytate. It has been reported that phytase synthesis is repressed by excess orthophosphate where the extent of repression depending on the strain and that repression can be reversed by providing a slow- released organic phosphate source. The phenomenon of phosphate repression on phytase production was observed not only in fungi but also in many yeast strains where, an increased level of phytase production was found in phosphate-depleted medium (Nakamura *et al*, 2000). Our studies showed an enhancement of phytase activity when the fungus was grown in the presence of calcium phytate at 0.5% level.

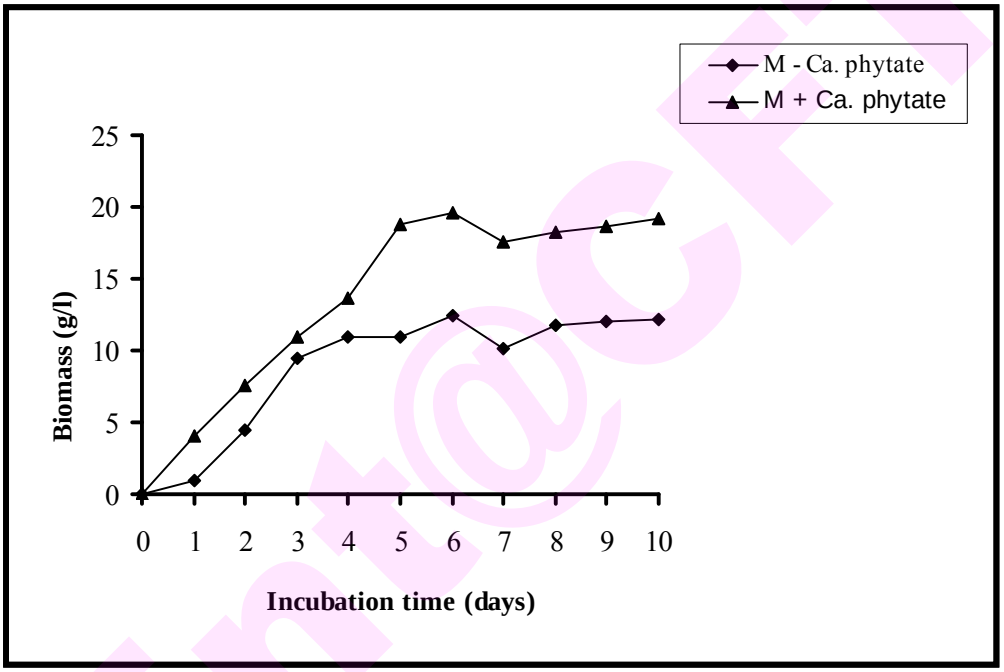
Table 11: Effect of calcium phytate on phytase production in *Aspergillus niger* CFR 335

Incubation time (day/s)	Medium without Calcium phytate		Medium with calcium phytate	
	Activity (U*/ml)	Sp. Act. (U/mg)	Activity (U*/ml)	Sp. Act. (U/mg)
1	149	0.823	422	0.976
2	165	0.894	476	1.025
3	191	1.116	498	1.106
4	200	1.178	509	1.13
5	271	1.28	571	1.279
6	**338**	**1.366**	**682**	**1.829**
7	384	0.825	558	1.507
8	344	0.748	467	1.439
9	233	0.712	453	0.988
10	136	0.735	260	0.881

*** 1 Unit of the enzyme is the activity of the enzyme to release one μmole of inorganic phosphorus in one minute at 45°C**

Highest activity is indicated in bold

Fig 20: Effect of calcium phytate on biomass content of *Aspergillus niger* CFR 335 in SmF



SECTION 2

Physico- chemical parameters for enzyme production:

Temperature studies:

Temperature is one of the vital physical factors that play a role in growth and metabolism of all the organisms. An optimum temperature exists for every organism that may enhance the growth and facilitate the production of metabolites. In the present study, *A. niger* CFR 335 was cultivated at various temperatures ranging from 10- 55°C both in SmF and SSF media. Maximum activity of 284

U/ml and specific activity of 0.234 U/mg were found at 30°C in submerged cultivation. The activity was found to decline above 35°C. About 24% decline was observed when the fungus was cultivated at 45°C. Similar result was observed in case of solid- state cultivation. A maximum phytase activity of 1925 U/gds was found at 30°C with a specific activity of 2.608 U/mg. More than 35% decline in the enzyme activity was observed at 45°C. The fungus failed to grow above 50°C. The results are given in Table 12. It was evident from the result that, in both SmF and SSF, optimum temperature for maximum phytase activity was 30°C.

Table 12: Effect of temperature on phytase production in *Aspergillus niger* CFR 335 by SmF and SSF

Temperature (°C)	SmF		SSF	
	Activity (U*/ml)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
10	NG **	-	NG	-
15	137	0.124	415	1.305
20	248	0.188	922	1.897
25	256	0.205	1233	1.964
30	**284**	**0.234**	**1925**	**2.608**
35	234	0.212	1299	2.102
40	228	0.186	1199	1.959
45	215	0.118	1176	1.903
50	NG	-	NG	-
55	NG	-	NG	-

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C ** NG = No growth**
Highest activity is indicated in bold

Temperature stability studies of phytase enzyme of *A. niger* CFR 335 obtained through SmF and SSF conditions were carried out. In SmF, our result revealed that the enzyme was stable up to 40°C and 80% of its residual activity was retained up to 3 months period at room temperature. Beyond 40°C, the enzyme lost > 55% of its activity. In SSF condition, similar observations were made when the enzyme was stored at room temperature. More than 50% loss of enzyme activity was observed after 3 months. In conclusion, enzyme production under SSF condition was found to be highly satisfactory. The results are given in Table 13.

Table 13: Effect of temperature on stability of *Aspergillus niger* CFR 335 phytase by SmF and SSF

Temperature (°C)	SmF		SSF	
	Activity (U*/ml)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
10	256	0.212	1779	1.877
15	263	0.218	1828	1.936
20	268	0.226	1856	2.216
25	277	0.231	1878	2.432
30	**286**	**0.238**	**1932**	**2.626**
35	282	0.219	1896	2.516
40	253	0.193	1734	2.114
45	182	0.181	1363	1.896
50	143	0.158	1098	1.356
55	087	0.104	894	1.115

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**

Highest activity is indicated in bold

Vats and Banerjee (2002) have shown the effect of different temperature, 25, 30, 37, 45 and 50°C on *A. niger* var teigham by cultivating the isolate in shake flasks. The strain was shown to grow best at 30°C with a sharp decline at 37°C and there was no growth at 45°C and above. So also our observation in *A. niger* CFR 335.

Nakamura *et al*, (2000) have reported that the optimal temperature for *A. ficuum* was always higher than 60°C, often around 75°C when measured at pH 4.5. This temperature resistance required the presence of the substrate. In the absence of phytate, incubation of the enzyme at their optimal reaction temperature for 30 minutes always resulted in a massive inactivation. *Pichia spartinae* enzyme showed the highest optimal reaction temperature among the species examined (75-80°C). When *Schwanniomyces castelii* CBS 2863 previously studied by Segueilha *et al*, 1992 was examined simultaneously with *Pichia spartinae* enzyme, the latter had a clearly higher optimal temperature by about 3°C. *Pichia spartinae* enzyme invariably retained a large fraction of its activity even at more than 80°C.

The temperature optimum for phytase activity of *A. ficuum* was shown to be 55°C (Howson and Davis 1983). Skowronski (1978) reported a similar optimum of 53°C for the closely related species *A. niger* while reports for *A. terreus* gave a range between 60 and 70°C (Yamada *et al,* 1968 and Yamada *et al,* 1968 a). Similarly, higher optimum temperatures for phytase activity have been reported from other microorganisms (Greaves *et al*, 1967) and from plants (Peers, 1953). *A. carbonarius* phytase produced in SSF had an optimum temperature of 55°C. Thus, we conclude in this study that *A. niger* CFR 335 had an optimum temperature for phytase production at 30°C and the stability of the enzyme was found to be maximum up to 3 months period of storage at the same temperature.

pH Studies:

pH of the cultivation medium is the most important factor for a fermentation process. The microbial growth, production of enzymes and other byproducts of microbial origin are always directly related to the pH and temperature of the growth medium and other cultivating conditions such as aeration, inoculum size and moisture. To know the optimal pH for the phytase, enzyme production was carried out at various pH in the range 3.0 - 8.5 by both SmF and SSF methods. It was evident that a maximum enzyme activity of 388 U/ml and specific activity of 0.363 U/mg was found when the fungus was grown in SmF medium at pH 4.5. With increase in the pH of the cultivation medium, the enzyme activity declined and there was more than 60% reduction at an alkaline pH of 8.5. In solid substrate medium, the enzyme activity and the specific activity were found to be 1901 U/gds and 2.61 U/mg respectively at pH 4.5. Beyond this pH, the enzyme activity declined. More than 50% of the activity was lost when the fungus was cultivated in a medium with pH 8.5. The results are given in Table 14.

Table 14: Effect of pH on phytase production in *Aspergillus niger* CFR 335 by SmF and SSF

pH	SmF		SSF	
	Activity (U*/ml)	**Specific activity (U/mg)**	**Activity (U*/gds)**	**Specific activity (U/mg)**
3.0	221	0.232	1060	1.959
3.5	256	0.290	1202	2.062
4.0	302	0.318	1422	2.279
4.5	**388**	**0.363**	**1901**	**2.61**
5.0	352	0.281	1436	2.140
5.5	342	0.257	1312	1.915
6.0	312	0.229	1189	1.815
6.5	243	0.176	986	1.533
7.0	203	0.125	865	1.400
7.5	188	0.104	800	1.176
8.0	166	0.087	465	0.998
8.5	227	0.154	119	0.553

*** 1 Unit of the enzyme is the activity of the enzyme to release one μmole of inorganic phosphorus in one minute at 45°C**

Highest activity is indicated in bold

Table 15: Effect of pH on the stability of *Aspergillus niger* CFR 335 phytase by SmF and SSF

pH	SmF		SSF	
	Activity (U*/ml)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
3.0	322	0.277	1698	2.591
3.5	328	0.283	1687	2.574
4.0	336	0.328	1899	2.581
4.5	**349**	**0.363**	**1891**	**2.73**
5.0	339	0.358	1716	2.61
5.5	341	0.332	1683	2.563
6.0	296	0.341	1451	1.867
6.5	274	0.284	1204	1.531
7.0	212	0.263	868	1.264
7.5	156	0.222	733	0.971
8.0	117	0.118	625	0.776
8.5	69	0.076	519	0.623

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**

Highest activity is indicated in bold

pH stability studies of phytase enzyme was also carried out by incubating the enzyme sample at different pH (2.1.4). The enzyme was obtained by cultivating the fungus at pH 4.5. The result indicated that the enzyme was stable up to pH 5.5 and more than 70% loss was observed at pH 8.5 both in submerged and solid- state fermentation medium. The results are summarized in Table 15.

Vats and Banerjee (2002) have studied the phytase production at various pH in the range 3.5- 7.5. Maximum growth and enzyme activity by *Aspergillus niger* var teigham were observed when the initial pH of the medium was 6.5. A gradual increase in the enzyme activity, extracellular protein content and cell mass concentration were observed when the initial pH of the medium moved from acidic towards neutral range. Above pH 7.0, a decline in enzyme activity was observed.

Optimum pH for maximum phytase production was recorded with the pH of the mineral salt solution as 6.0 for *A. ficuum* and 7.0 for *Rhizopus oligosporus.* Further increase in the pH of the mineral salt solution led to the reduction of enzyme production and poor growth of the fungi (Pinky *et al,* (2002). Nakamura *et al*, (2000) have shown that all the yeast enzymes examined were acid phytases with an optimal pH range of 4- 5 and these enzymes were measured at an optimal or suboptimal temperature of 50- 60°C. It has been observed in our experiment, that the level of enzyme synthesis was dependent on the initial pH of the substrate. The optimum pH for phytase production was between 4.0 and 5.0.

The pH profile for phytase production by *A. ficuum* on semisolid substrate was similar to that reported on liquid cultivation of the same organism (Han and Gallagher, 1987). The optimum pH of the media for phytase production from *A. niger* 307 was shown to be 5.0 (Gargova and Sariyska 2003). Tadashi *et al,* (1999) have investigated the pH optimum and pH stability by performing the phytase assay at pH range of 2.0- 9.0 with a variety of buffers by standard assay method.

Their phytase was shown to have double pH optimum of 5.5 and 2.5 and was virtually inactive above pH 7.0. The enzyme was found to be stable at the pH range of 5.0- 7.0. It was also suggested that phytic acid was co-precipitated with protein since the pH value was decreased during the fermentation and phytic acid tends to be soluble at acid pH values.

Studies on Sugars:

The growth and metabolism of fungi requires different carbon sources in an easily available form. Hence, the effect of different carbon sources in the fermentation medium on phytase enzyme production was evaluated by incorporating various sugars. The fungus was cultivated in a fermentation medium containing specific sugars at 1% level. The medium with glucose served as control and had an activity of 277 U/ml and 0.215 U/mg of specific activity. The results of SmF indicated that sucrose was more suitable for maximum phytase production. The medium supplemented with sucrose had 1.5-fold increase in the enzyme activity in shake flask cultivation.

In solid- state cultivation, the enzyme activity was found to be 1982 U/gds and the specific activity was 1.86 U/ mg in presence of glucose. Enzyme activity in sucrose medium was 2194 U/gds with specific activity of 2.92 U/ mg which is slightly higher than the control. The enzyme activity was found to decline by 80-90% in presence of other sugars such as sorbitol, mannose, xylitol, arabinose, cellobiose, galactose, raffinose, rhamnose and trehalose in both SmF and SSF cultivation. The results are given in Table 16.

The effect of various sugars and non- sugars as carbon sources on the profile of cell mass, enzyme activity and extracellular protein production was studied by Vats and Banerjee (2002). They obtained maximum enzyme production when starch was used as carbon source, which was followed, by a combination of glucose and starch. The isolate was found to produce 184 nkat/ml phytase activity.

They have shown little or no enzyme production with simple sugars. Their studies indicated a strong repression of enzyme synthesis in the presence of simple sugars. The phenomenon of glucose repression has been reported in *Arxula adeninivorans*, which was overcome by replacing glucose with galactose (Sano, *et al* 1999). Shieh and Ware (1968) have shown that when simple sugars, such as glucose or fructose are used as sole source of carbon for phytase production by *Aspergillus niger* NRRL 3135, mycelia formed pellets and the enzyme was produced in lower amounts. This phenomenon is also supported by Han and Gallagher (1987).

Table 16: Effect of sugars on phytase production in *Aspergillus niger* CFR 335 by SmF and SSF

Sugar (1%)	SmF		SSF	
	Activity (U*/ml)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
Glucose **	277	0.215	1982	1.86
Fructose	93	0.136	1248	2.15
Lactose	123	0.375	1230	1.52
Maltose	172	0.296	1261	2.03
Sucrose	**425**	**0.492**	**2194**	**2.92**
Xylose	166	0.233	1290	2.69
Sorbitol	30	0.030	1232	1.09
Mannose	14	0.056	1214	0.912
Xylitol	23	0.088	910	0.86
Arabinose	15	0.058	1067	0.97
Cellobiose	19	0.063	960	0.59
Galactose	28	0.093	772	0.9
Raffinose	20	0.036	1159	0.88
Rhamnose	8	0.035	1248	1.09
Trehalose	44	0.011	1129	0.88

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**

**** Control**

Highest activity is indicated in bold

A 2^3 central composite experimental design was used to study the combined effects of the medium components, tryptone, yeast extract and NaCl. The optimized medium with glucose showed a highest phytase activity of 2250 U/L (Sunitha *et al*, 1999). Vohra and Satyanarayana (2003) showed in their studies that *Pichia anomola* produced maximum enzyme titres of 140 U/g dry weight when

the glucose concentration in the medium was 0.5% and beef extract concentration was 0.1%. Increasing the glucose concentration to 4% led to a slight decline in phytase production, however on increasing the beef extract beyond 0.5% phytase production declined significantly. The biomass production was shown to increase with increase in the glucose and beef extract concentration up to 5.5% and 2% respectively. Phytase production was low at this concentration indicating that phytase production was not directly related to growth. Maximum phytase production by *Lactobacillus amylovorus* was observed in MRS medium at a concentration of 1% glucose. With further increase in glucose concentration the enzyme production declined significantly (Sreeramulu *et al*, 1996).

As indicated in our studies, 1% sucrose in the cultivation medium showed the maximum phytase activity of 425U/ml in SmF and 2194 U/gds in SSF. There was no significant increase in the enzyme in the above cultivation media where glucose served as C- source. Due to the cost- effectiveness of glucose than sucrose or any other sugars, it is concluded that glucose could be used in the cultivation media for the production of the enzyme by *A. niger* CFR 335.

Studies on nitrogen:

Nitrogen being one of the major components of protein is also an important part of the genetic material. Effect of different inorganic nitrogen sources on the production of phytase enzyme was also tested in both SmF and SSF medium, supplemented with 0.5% of six different nitrogenous compounds. Of the nitrogen compounds used, the medium with peptone showed maximum activity of 425 U/ml & 0.492 U/mg specific activity in SmF and 2194 U/gds & 2.92 U/mg of activity and specific activity respectively. Pinky *et al* (2002) have studied the impact of supplementation of organic and inorganic nitrogen sources to the fermentation medium. They have shown an inhibitory effect of four different organic as well as inorganic nitrogen sources on phytase yield by both *A. ficuum*

and *Rhizopus oligosporus*. This indicated that there was no need to add external nitrogenous sources in the fermentation medium. They have shown more than 15-25% inhibition in the phytase production when potassium nitrate and urea were used as inorganic nitrogen sources. Similarly, in the present study, a two- fold and four- fold reduction in enzyme activity was observed in presence of potassium nitrate and urea respectively. Slight increase in the activity in SmF was observed in the peptone medium whereas, more than 20% decline in the activity was observed with urea in SSF. The results are shown in Table 17.

Table 17: Effect of Nitrogen sources on phytase production in *Aspergillus niger* CFR 335 by SmF and SSF

Nitrogen (0.5%)	SmF		SSF	
	Activity (U*/ml)	**Specific activity (U/mg)**	**Activity (U*/gds)**	**Specific activity (U/mg)**
Yeast Extract **	245	0.187	**2020**	**2.79**
Peptone	**254**	**0.232**	1562	3.21
Ammonium sulphate	221	0.216	951	0.98
Ammonium nitrate	243	0.215	1054	1.87
Potassium nitrate	115	0.155	934	1.07
Urea	90	0.138	536	0.664

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**

**** Control**

Highest activity is indicated in bold

A maximum phytase production when biopeptone was used as nitrogen source (Vats and Banerjee 2002). This was followed by ammonium nitrate and ammonium sulphate. Organic forms of nitrogen such as 1% peptone have been

used extensively for the production of phytase in *Aerobacter aerogenes* (Greaves *et al*, 1967) and in *Klebsiella aerogenes* (Jareonkitmongkol *et al*, 1997) that was also supplemented with 1% yeast extract. From our result it is observed that nitrogen in the growth medium has no significant role in the growth and phytase production in *Aspergillus niger* CFR 335.

Studies on phosphates:

Phosphate forms back bone of nucleic acid and hence are a very essential nutrient component for any medium used for microbial cultivation. High phosphorus conditions are known to repress the synthesis of acid phosphatases and phytases, while limiting phosphate conditions result in their expression. In the present study, five different inorganic phosphates at 0.25% level were incorporated in the cultivation medium to study their effect on phytase production. The results indicated that 0.25% inorganic phosphate had a negative impact on phytase production. A maximum repression of 84% of phytase synthesis was observed when sodium dihydrogen phosphate was incorporated in the liquid culture medium at a concentration of 0.25%. Potassium dihydrogen phosphate and ammonium phosphate showed 80% reduction whereas, a reduced repression rate of 41% was seen when phosphoric acid was used. p-nitrophenyl phosphate completely suppressed the growth of the fungus in shake flask cultivation. Phytase production has shown a little higher activity when cultivated on solid substrate medium. Phytase production was repressed up to 71, 66, 68 and 48% respectively in the presence of potassium dihydrogen phosphate, sodium dihydrogen phosphate, ammonium phosphate and phosphoric acid. Even though the fungus grew well in the presence of p-nitrophenyl phosphate in SSF medium, phytase synthesis was repressed up to 82%. These results are shown in Table 18.

Table 18: Effect of inorganic phosphates on phytase production in *Aspergillus niger* CFR 335 by SmF and SSF

Phosphate (0.25%)	SmF		SSF	
	Activity (U*/ml)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
Control **	**256**	**0.176**	**179**	**1.733**
Potassium dihydrogen phosphate	52	0.078	523	0.683
Sodium dihydrogen phosphate	42	0.056	610	0.719
Ammonium phosphate	52	0.065	122	1.17
Phosphoric acid	152	0.068	93	1.08
p- nitrophenyl phosphate	NG***	-	333	0.446

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**

**** Medium without phosphate supplementation**

***** No growth**

Highest activity is indicated in bold

Effect of the above phosphates on acid phosphatase production by *Aspergillus niger* CFR 335 was also studied. All the phosphates except phosphoric acid showed higher activity than the control indicating an induction in the acid phosphatase production when supplemented at a concentration of 0.25% in the cultivation medium. Medium with p- nitrophenyl phosphate showed an higher activity of 17 U/ml and specific activity of 0.014 U/mg in SmF. Phosphoric acid showed a very negligible activity which was beyond calculation. In SSF, similar to SmF, higher activity of 49 U/gds and 0.029 U/mg of activity and specific activity

were observed in presence of p- nitrophenyl phosphate. Phosphoric acid suppressed the activity, which was below that of the control. The results are given in Table 19. Thus, it is evident from the result that eventhough acid phosphatase has a wide spectrum of substrates, p- nitrophenyl phosphate is the most effective phosphate compound. Phosphoric acid is less suitable for acid phosphatase production. It could be concluded from this study, that *Aspergillus niger* CFR 335, a phytase producing fungus used in the present study can be induced to produce acid phosphatases using an optimum level phosphates in the cultivation medium.

Chelius and Wodzinski, (1994) during the strain improvement studies of *A. niger* NRRL 3135 by UV radiation, reported that phyA production in the mutant strain was repressed (60%) when the Pi concentration was 0.006% (w/v) whereas phytase production in wild type strain was not influenced significantly by the said concentration of phosphorus. Vats and Banerjee (2002) have shown a sharp decline in phytase production by *A. niger* var teigham even at a final concentration of 0.05% and there was no enzyme production at 0.1% and above. The cell mass and protein concentrations were not affected by phosphate concentration in the medium. Phytase synthesis is repressed by excess orthophosphate. The phenomenon of phosphate repression on phytase production was observed not only in fungi but in many yeast strains where an increased level of phytase production was found in phosphate depleted medium (Nakamura, *et al* 2000). It is concluded from our observation that the extent of repression depends on the strain and that repression is reversed by providing a slow- release organic phosphate source.

Wodzinski *et al,* (1998); Kim *et al*,(1999); Gargova *et al,* (1997) and Chelius and Wodzinski (1994) have reported that the phosphorus concentration plays an important role in phytase production and by regulating the phosphate concentration in the growth medium, production of enzyme can be substantially regulated. It has also been shown that, repression of phytase synthesis by inorganic

phosphorus was less significant in a medium with complex composition. These findings appear to deviate from our results.

Table 19: Effect of inorganic phosphates on acid phosphatase production in *Aspergillus niger* CFR 335 by SmF and SSF

Phosphate (0.25%)	SmF		SSF	
	Activity (U*/ml)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
Control **	3	0.001	13	0.006
Potassium dihydrogen phosphate	8	0.004	26	0.015
Sodium dihydrogen phosphate	6	0.002	18	0.013
Ammonium phosphate	11	0.006	32	0.021
Phosphoric acid	ND***	ND	7	0.003
p- nitrophenyl phosphate	**17**	**0.014**	**49**	**0.029**

*** 1 Unit of acid phosphatase in the activity of the enzyme to release 1μmole of p-nitrophenol in 1 minute at 37°C**

**** Medium without phosphate supplementation**

***** Not determined**

Highest activity is indicated in bold

Ware and Shieh (1967) have discovered that the available inorganic phosphorus content of the medium control the synthesis of the enzyme. Shieh and Ware (1968); Shieh *et al*, (1969) have demonstrated the interaction of total phosphorus content with the carbon source in the medium. The optimum concentration of phosphorus was found to be 0.4mg/100 ml of medium. The relative amounts of pH 2.0 (phy B) and pH 5.5 (phy A) specific phytases are shown to differ depending on the amounts of inorganic phosphorus in the medium.

Howson and Davis (1983) confirmed the regulatory effect of high P on phytase production. Han and Gallagher (1987) have also confirmed that high P concentrations inhibited phytase synthesis by *A. niger* NRRL 3135. They have also noted that 1-5mg Pi/100 ml was needed for maximum phytase synthesis while 8mg Pi/100 ml medium was required for maximum cell growth. Utt (1987) tested the effect of initial P concentration of eight different sources of commercial cornstarch on yields of phytase. Gibson (1987) also confirmed their results that the levels of phosphorus in the medium control phytase synthesis. In a survey of phytase producing microorganisms, *A. ficuum* was shown to produce highest amount of phytase (113 nkat/ ml in shake flask in 5 days) when the inorganic phosphorus content was in the range of 0.0001- 0.005%, optimum being 0.4 mg/ 100 ml with 8% cornstarch (Wodzinski and Ullah 1996).

It is evident from our results that 0.25% of the inorganic phosphate is a higher dose that repressed the phytase production and there was no effect on the growth of the fungus. However, with the above concentration p- nitrophenyl phosphate did not favour the growth in liquid cultivation, while a slight activity was absorbed in solid- substrate cultivation. Although phosphates supported growth of the fungus and slightly enhanced the acid phosphatase production both in SmF and SSF, the production rate was not very much significant for the fungus to be concluded as a potent producer of acid phosphatases.

Studies on surfactants:

Several researchers have shown that incorporation of surfactants would lead to formation of small pellets in the liquid medium and hence higher yield of phytase enzyme could be obtained. Surfactants are surface active agents that have got a number of application that includes, lowering of surface and interfacial tensions, wetting and penetration actions, emulsification, detergency, gelling, flocculating actions, microbial growth enhancement etc. All the applications have tempted researchers to use surfactants to induce pellet formation in liquid cultures.

It is shown that pelletization increases the extra cellular enzyme synthesis in submerged fermentation.

Different surfactants were incorporated in the cultivation medium at 0.25% level. Our result indicated a two-fold increase in the enzyme activity, when the medium was supplemented with 0.25% Tween- 20 in the liquid medium. There was no significant difference with the addition of Triton- X- 100. A three- fold decrease in the enzyme activity was observed when the medium was incorporated with EDTA, while total growth suppression occurred in presence of sodium dodecyl sulphate (SDS). In case of SSF, Tween- 20, Triton- X-100 and EDTA showed reduced enzyme activity of 57%, 64% and 85% respectively, SDS supported the growth of the fungus but the enzyme activity was not significant than its control. The results are presented in Table 20.

Alasheh and Duvnjak (1994) studied the effect of surfactants such as Tween- 80, Triton-X- 100, Na- oleate on the phytase production and reduction of phytic acid content in canola meal by *Aspergillus carbonarius* in SSF. They have also shown that addition of glucose at lower concentrations and surfactants such as Na- oleate or Tween- 80 in the medium increased biomass growth and enzyme synthesis. The phytase production increased in the presence of 1% Na- oleate and Tween- 80 suggesting alteration of the cell permeability resulting in the higher release of enzyme. Han and Gallagher (1987) have shown that in a medium containing surfactant (0.5% Triton -X- 100, Tween- 80 and Na- oleate (v/v), growth was dispersed and phy A yields were 4.7 fold higher that in control. Similarly, it has been reported, a 30% increase in phytase activity of *A. niger* NCIM 563 when 0.5% Triton- X- 100 was added to the production medium.

EDTA, a chelating agent was found to readily inactivate *Bacillus* phytases (Kerovuo *et al*, 1998; Powar and Jaggannathan, 1982; Shimizu, 1992), whereas fungal phytase activity was stimulated by EDTA (Wyss *et al*, 1999). Contradictory

to this finding, our results indicated that fungal phytase activity was repressed in SmF while in SSF the activity was not significantly affected.

Table 20: Effect of surfactants on phytase production in *Aspergillus niger* CFR 335 by SmF and SSF

Surfactant (0.25%)	SmF		SSF	
	Activity (U*/ml)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
Control **	263	0.184	1788	1.731
Tween- 20	**377**	**0.438**	794	0.741
Triton X- 100	213	0.345	687	0.622
EDTA	51	0.062	256	0.249
SDS	NG	-	**1157**	**1.305**

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**

**** Medium without surfactant**

Studies on metal salts:

Certain biochemical reactions are catalyzed in presence of metal ions and hence fasten the reaction. In the present study, different metal salts were incorporated into the medium and their effects on the phytase enzyme activity of *Aspergillus niger* CFR 335 was evaluated. It was found in our results that all the metal salts used in the study had a negative impact on the enzyme production except for calcium chloride where there was two- fold enhancement in the enzyme activity when the medium was incorporated with 0.01% calcium chloride in shake flask cultivation. A 50% reduction in the enzyme production was observed with all other salts. In the case of SSF, there was no enhancement of enzyme activity in presence of all the metal salts used including calcium chloride. There was > 20% reduction in the activity in presence of magnesium chloride whereas, all other salts showed still lesser phytase enzyme activity. Copper sulphate reduced the activity

by more than 35%. As stated above, calcium chloride in the cultivation medium enhanced the phytase activity by two- fold in SmF and at the same time in SSF there was 30% reduction in enzyme activity indicating that calcium induced the enzyme activity in SmF. The results are given in Table 21.

Table 21: Effect of metal salts on phytase production in *Aspergillus niger* CFR 335 by SmF and SSF

Metal salts (0.01%)	SmF		SSF	
	Activity (U*/ml)	Specific activity (U/mg)	Activity (U*/gds)	Specific activity (U/mg)
Control **	256	0.203	1932	1.793
NaCl	11	0.094	1364	1.749
$MgCl_2$	153	0.125	**1435**	**1.194**
$FeSO_4$	57	0.040	1212	1.509
$MnSO_4$	6	0.080	1173	1.372
$CuSO_4$	66	0.072	959	1.116
$ZnSO_4$	73	0.073	1187	1.368
$CaCl_2$	**396**	**0.462**	1113	1.272

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**

**** Medium without metal salts**

Highest activity is indicated in bold

Nakamura *et al* (2000) have shown that yeast phytases are generally resistant to high salt concentration. Their study indicated that at 1 M NaCl, *Pichia rhodanensis* enzyme could function at more than 50% of its maximal rate. Kerovuo *et al* (2000) studied the metal ion requirement of *Bacillus subtilis* phytase and showed that the removal of metal ions from the enzyme by EDTA resulted in complete inactivation of the product. The activity of *Bacillus* phytase is shown to be a metal ion dependent. No metal ion requirement for enzymatic activity has

been reported to any other phytases. However, the activities of some plant phytases have shown to be stimulated by calcium salt (Hara *et al,* 1985). Thus, our study showed an increase enzyme activity only in presence of calcium chloride in shake flask cultivation while in SSF no metal salt enhanced the enzyme activity.

SECTION 3

Nutritional studies:

Phytase feeding experiment was carried out in our Institute's Animal House where the experimental birds and control group birds were properly maintained in well aerated, clean cages. This study was aimed to determine the efficacy of different levels of *Aspergillus niger* phytase enzyme on the utilization of phytate-phosphorus in the feed, excretion of phytate-phosphorus in the feces and other parameters.

Phytase enzyme liberates phosphorus from phytic acid, thereby making dietary phosphorus available to monogastric animals and birds. This has the dual effect of promoting more efficient growth of animals, as well as imposing less of an environmental burden in the form of excess phosphorus in water streams and surrounding area. Use of phytase as a growth feed supplement is well known. However in the past, focus has always been given on adding exogenous phytase to animal feed or to increase the level of phytase expression in seeds used in feed formulation.

In this experiment, the feed ingredients were composed of mainly cereal materials that contain phytic acid (Table 22). The diet was prepared according to the local poultry farm manufacturers and the experimental diet as mentioned earlier was made by eliminating major phosphorus and calcium supplements. The

Table 22: Commercial feed ingredients

Ingredient	Kg/ton
Maize	250
Sorghum	250
Ragi	250
Broken rice	150
Ground nut cake	20
De oiled rice bran	15
Sun Flower cake	15
Shell grit	15
Salt	8
Fish Meal	12
Mineral mixture	1
Vitamin B- complex	1 bottle
Liver tonic	1 bottle
Di calcium phosphate	10
Trace mineral solution	100 ml
Calcium powder	5
Mahua Flower powder	1

Table 23: Phosphorus content in the commercial poultry feed

Feed Phosphorus	Phosphorus (mg/g)
Total Phosphorus (TP)	0.45
Non- phytate Phosphorus (npP)	0.26
Phytate Phosphorus (pP)	0.19

effect of phytase enzyme on different growth parameters of the layer birds such as body weight, egg weight, leg thickness, eggshell strength, phosphorus excretion and phosphorus utilization is discussed below. The effect of microbial phytase intake at different levels on growth performance of layers is summarized in Tables 24-29. The enzyme was found to be effective in the 3rd week of the supplementation at 600Ukg^{-1} diet levels. The nutritional studies using *Aspergillus niger* CFR 335 phytase was initiated by estimating the total phosphorus, phytate phosphorus and non- phytate phosphorus in the commercial feed. One gram of the feed was estimated to contain 0.45 mg of total phosphorus of which phytate phosphorus content was 0.19 mg and the non- phytate phosphorus was 0.26 mg. This is shown in Table 23.

(i) Body weight:

Supplementation of feed with 200U and 600U/kg diet of phytase enzyme did not show a significant improvement in the body weight of layer birds in all the test groups. But the layers treated with 400Ukg^{-1} diets have shown an increase in body weight by 5% when compared to that of control hens, which is shown in Table 24.

(ii) Egg production, egg weight, and eggshell thickness:

The overall performance of egg production, egg weight, and eggshell thickness was found to be good when the enzyme was administered at the level of 600Ukg^{-1} diets. All the experimental hens have shown an increased weight and quality of eggs. The result showed that when the experimental hens were fed with 400Ukg^{-1} diet of phytase enzyme, an overall increase of 11.2% of weight in the eggs was observed. Quality of eggshell is also an important factor since poor shell quality leads to a loss in poultry industries due to easy breakage and brittleness of eggs during transportation. The result obtained in our study indicated an increase

of 16% strength in the eggshell, after supplementing the feed with 600Ukg^{-1} of the enzyme. The results are summarized in Tables 25 and 26.

(iii) Leg thickness:

The effect of phytase enzyme from A.niger CFR 335 on leg thickness of layers is given in Table 27. The overall leg thickness in group 4 fed with 600U Kg^{-1} diet increased to 8-10% level than the control group that proves the availability of free phosphorus, effectively.

(iv) Phosphorus excretion and utilization:

The total Phosphorus in the feces was also estimated by the AOAC (1990) method. Our results indicated that the total phosphorus excreted in the fecal matter was much higher in case of control group when the phytase enzyme was not administered into the diet. Whereas, the experimental birds fed with different doses of phytase enzyme (200 U - 600 U) showed decreased phosphorus excretion in their fecal matters. In the experimental group, after 3 weeks of feeding with enzyme, excretion of total phosphorus showed more than 40% decreased level when compared to control group of layers. The excretion level of phosphorus was highly reduced when 600Ukg^{-1} diet of enzyme was administered with the diet in 3 weeks period. The results are shown in Table 28. The result also indicated that the availability of feed phosphorus was increased by 46% with 400Ukg^{-1} of phytase enzyme. The results are given in Table 29.

(v) Phosphorus content of the bone:

It is known that Phosphorus and Calcium are the major components of bones. Usually, they get deposited in thigh and leg bones of the animals for keeping their overall strength. In this angle, our study was also focused to find out the Phosphorus content in thigh and leg bones of experimental layer hens. The study indicated that the availability of feed phosphorus was increased by 20% and

25% with 400U and 600UKg^{-1} diet of phytase enzyme when compared with control group.

Table 24: Effect of phytase enzyme on body weight (Kg) of the layers

Group	Week			
	1	2	3	4
1*	1.16	1.22	1.18	1.2
2	1.26	1.32	1.3	1.28
3	1.2	1.25	1.2	1.18
4	1.23	1.28	1.24	1.26

*** Control Group**

Table 25: Effect of phytase enzyme on egg weight (g) of the layers

Group	Week			
	1	2	3	4
1*	50.13	50.16	52.54	55.4
2	54.9	56.23	56.6	58.8
3	54.4	57.2	59.16	57.1
4	50.6	51.71	52.12	58.9

*** Control Group**

Table 26: Effect of phytase enzyme on the eggshell strength (Newton)

Group	Week			
	1	2	3	4
1*	24.5	27.0	27.25	25.25
2	27.0	27.3	29.5	26.9
3	28.2	29.0	29.5	26.95
4	23.3	28.2	32.5	28.6

*** Control Group**

Table 27: Effect of phytase enzyme on leg thickness (cm) of the layers

Group	Week			
	1	2	3	4
1*	3.5	3.6	3.6	3.6
2	3.7	3.9	3.9	3.8
3	3.9	4.0	4.0	3.9
4	3.6	4.0	3.8	3.9

*** Control Group**

Table 28: Effect of phytase enzyme on phosphorus excretion (mg/g) in the layers

Group	Week			
	1	2	3	4
1*	0.4	0.39	0.36	0.45
2	0.43	0.45	0.38	0.25
3	0.37	0.34	0.26	0.35
4	0.34	0.32	0.21	0.28

*** Control Group**

Table 29: Effect of phytase enzyme on phosphorus utilization (%) in the layers

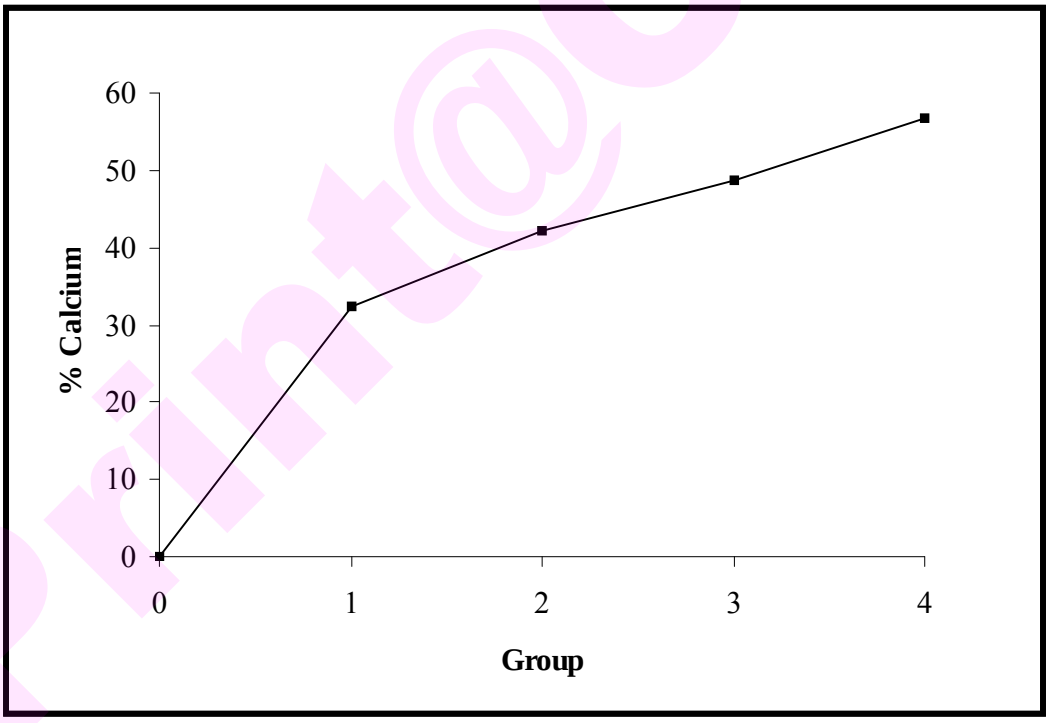
Group	Week			
	1	2	3	4
1*	46.0	44.5	37.5	39.0
2	49.0	50.0	59.0	44.0
3	52.0	53.5	63.0	54.0
4	55.0	57.5	70.0	61.5

*** Control Group**

(vi) Calcium content in bone:

Calcium content of the same bone samples was also carried out which showed an increased trend with increase in the enzyme dose. There was 50% increase in the bone calcium deposition when the birds were fed with 600U of phytase enzyme. The result is shown in the Fig 21. Thus our investigation proved that the phytase enzyme from *Aspergillus niger* CFR 335 played an important role in the diets of the experimental birds by rendering absorption of phosphorus and other essential minerals including calcium that were present in the feed materials procured from the local poultry industry.

Fig 21: Effect of phytase enzyme on calcium deposition of the bone



As demonstrated by Mitchell and Edwards (1996), both ash and plasma phosphorus are the most reliable methods for determining the bird's/ animal's phosphorus need. This is evident in our experiment that phosphorus availability in

the feed as well as spent phosphorus in fecal matter showed an increase and decreased levels respectively when phytase was supplemented with feed. Supplemental microbial phytase of *Aspergillus niger* CFR 335 was very effective in increasing phytate- phosphorus retention about 46% in 3- 4 weeks of feeding. These results are consistent with other reports (Van der Klis *et al,* 1997, Carlos and Edwards, 1998, Coon and Leske, 1999). But the extent of increased availability of free phosphorus reported by many investigators is varied from 15-25%. This is probably due to the design of the experiment, levels of phytase dosage and the methods of determination of phosphorus availability in the diet i.e., ideal retention properties of phytase enzyme.

Our study concludes that the dietary supplementation of *A. niger* CFR 335 phytase at the level of 400- 600 U/ kg diet enhances the overall performance which includes, body weight, egg weight, egg quality, egg shell thickness, free phosphorus availability in feed, reduction of phosphorus level in the fecal matter and calcium deposition as shown in the eggshell strength. Supplementation of microbial phytase has a great deal of potential existence for reducing the environmental pollution attributed to phosphorus excretion in layers without reducing the production performance.

The feed treated with 600U of phytase enzyme kg^{-1} of feed was found optimum for maximum pP liberation. Higher amount of the enzyme treatment showed a constant P value. Results are not shown here. Phytase enzyme has been shown to consistently improve the utilization of phytate-phosphorus and phytate-bound Ca, Zn and Mn as well. Phytate found in plant based animal diet usually bind dietary proteins, lipids, carbohydrates and inhibit the proteolytic enzymes thus making the probiotics non-functional in the gut. Studies on enzymatic treatment of feed using microbial phytase have demonstrated that this method increases the bioavailability of proteins and essential minerals and provides levels of growth performance as good as or better than those with phosphate

supplementation. Microbial phytase obtained from *Aspergillus niger* is an accepted feed additive that is used extensively in commercial animal and poultry diets. Nelson *et al* (1968) were the first to pre treat a corn- soy diet with culture filtrate containing *Aspergillus niger* NRRL 3135 phytase and fed to one day old chicks. The chicks showed increase in bone ash due to the phytin – phosphorus released from the dietary substances by the enzyme.

Nelson *et al* (1971) added graded levels of solvent- precipitated *Aspergillus niger* NRRL 3135 phytase to an experimental and to a commercial corn soybean meal diet and fed it to chicks. They measured bone ash and feed- to- gain ratios. They have reported that the enzyme was active in the animals and they were able to incorporate the released phytin- P into bone. They concluded that chicks could utilize the phosphorus from phytin- P as well as supplemental P. The maximum amount of phytin- P i.e., 2.1- 3.0 gKg^{-1} of diet was released if 1500- 2000U of phytase was added per Kg of diet. Nelson *et al* (1968) have determined the effect of feeding phytase on calcium requirements. The superior activity of *Aspergillus niger* NRRL 3135 phytase and its practical application to animal feed for the removal of phytic acid has been demonstrated in many experiments (Han, 1989; Han and Wilfred, 1988; Howson and Davis, 1983; Nelson *et al,* 1971; Rojas and Scott, 1968). Simons *et al*, (1990); Jongbloed and Kemme (1990) in the Netherlands collaborated to reconfirm the pioneering studies of Nelson *et al,* (1971) of phytase feeding to broilers. They concluded that addition of 1000 U of phytase/ Kg of diet was sufficient to provide levels of performance equal to or better than that attained by adding supplemental inorganic phosphate to a broiler diet. The results also indicated an improved performance when compared with birds fed control diets when the enzyme dose was increased to 1500 U. It is also reported that the microbial phytase when fed to low- P diets of broiler, the availability of P increased to 60% and the amount of P in the droppings decreased by 50%. Researchers have established the potential benefits that the use of phytase

in commercial diets would have on abating phosphate pollution in soil and water pollution. Thus, our study reveals that *Aspergillus niger* CFR 335 phytase enzyme was effective in making 31.5% of the total phosphorus available to the layers when fed with 600 Ukg^{-1} of the enzyme. It is evident from our study that, the enzyme can be effectively used in reducing the phosphorus load in the livestock areas.

SECTION 4

Mutation studies:

Much of the detailed information now available about the metabolism and activities of microorganism has come from the study of mutant strains that have lost a specific cellular function. By observing the effect of genotypic changes on the cell's phenotype one can deduce the cellular function of the gene product. The effective mutagenic treatments are able to increase the frequency of mutant cells (Roger *et al*).

Mutation is hereditary changes in the information content or in the distribution of the hereditary material in an organism. A mutation may lead to morphological changes or to biochemical requirements. Mutation frequency in nature is very less. Chromosome breaks *per se* do not constitute mutations because, they usually lead to the death of the cell itself or all of its early progeny, owing to the loss of some vital information. However, in most cells, broken chromosome can heal either by restitution or by the exchange of two broken chromosome portions that happen to be close enough in space and time. As a result of this exchange, mutations can arise, indeed, all known chromosome-breaking agents also produce mutations (Ernst Freese, 1971).

The maintenance of correct genetic information in a cell is so important that many mechanisms have evolved to protect chromosomes against damage and to

repair damage once it has been inflicted. Some mutagenic agents, which are very effective on isolated DNA, may not exert much of a mutagenic effect in cells, because (i) they cannot enter the cell or the nucleus or (ii) they are inactivated by enzymes (Peroxidase) or (iii) alterations such as thymine (generally pyrimidine)-dimers induced by UV are repaired in certain organisms (Smith, 1966; Witkin, 1966). Since mutagens can be activated by some enzymes and inactivated by others, it is impossible to predict whether a compound that is strongly mutagenic in one organism is mutagenic in another.

In the present study, mutants of *Aspergillus niger* CFR 335 were developed by exposing the young spores to UV irradiation at different time intervals. Fungal cultures used for mutation studies are shown in Fig 22 and microphotographs of these parental cultures are given in Figs 23 & 24. Non-ionizing radiations like UV create highly reactive free radicals that usually cause lesions in double stranded DNA. UV in the 254 nm wavelength is absorbed by DNA and is therefore mutagenic. Thymine dimers are induced by UV and cause mispairing during DNA replication if not repaired. This leads to permanent mutation. This technique is frequently employed in the field of microbiology for improving industrially important microbial strains for product development.

Young spores of *Aspergillus niger* CFR 335 when exposed to UV for 30 minutes, about 26.3 % killing was achieved in our experiment. The results showed a minimum killing rate of 10.2% with 10 minutes of UV exposure which is 16% lower than the highest killing rate (26.3%). With *Aspergillus ficuum,* a killing rate of 15.3% with 10 minutes exposure and a maximum of 46.2% killing were observed when the spores were exposed to UV for 30 minutes. It was observed that the killing rate increased with the increase in the exposure time and an inhibitory effect on growth was observed in CM. This is given in Fig 25.

Chemical mutagens on the other hand are normally intercalating, deaminating or alkylating agents that usually cause mispairing and base

substitutions in DNA sequence. N-methyl N-nitro N-nitrosoguanidine (NTG) and Ethyl Methane Sulphonate (EMS) are the two alkylating agents used in the present study for improving *Aspergillus niger* strain for phytase production and reduced sporulation level.

Fig 22: Cultures for mutation
Pale yellow: *Aspergillus ficuum*; Black: *ASpergillus niger*

In the present study, young spores of *Aspergillus niger* CFR 335 were treated with five different concentrations of EMS ranging from 2-10mM. The results indicated that, 10mM concentration of EMS was found to be optimum in giving 95% killing rate. A lower killing rate of 32% was observed when treated with 2 mM concentration. Similarly with *Aspergillus ficuum,* 100% killing was observed with 10mM with a lower killing rate of 33% with 2 mM of EMS

concentration. The survival rate decreased with the increase in the concentration of EMS. This is given in Fig 26.

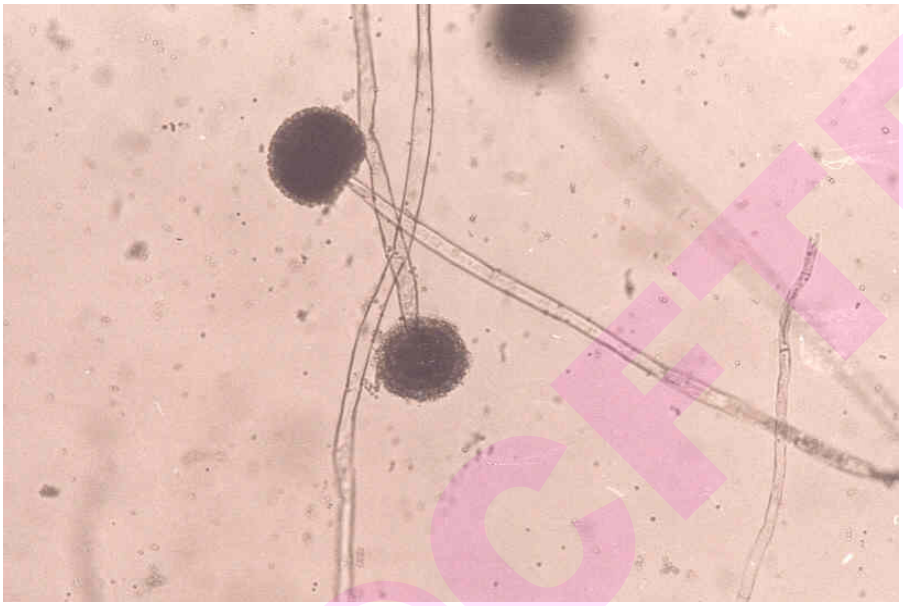


Fig 23: Microphotograph of *Aspergillus niger* CFR 335

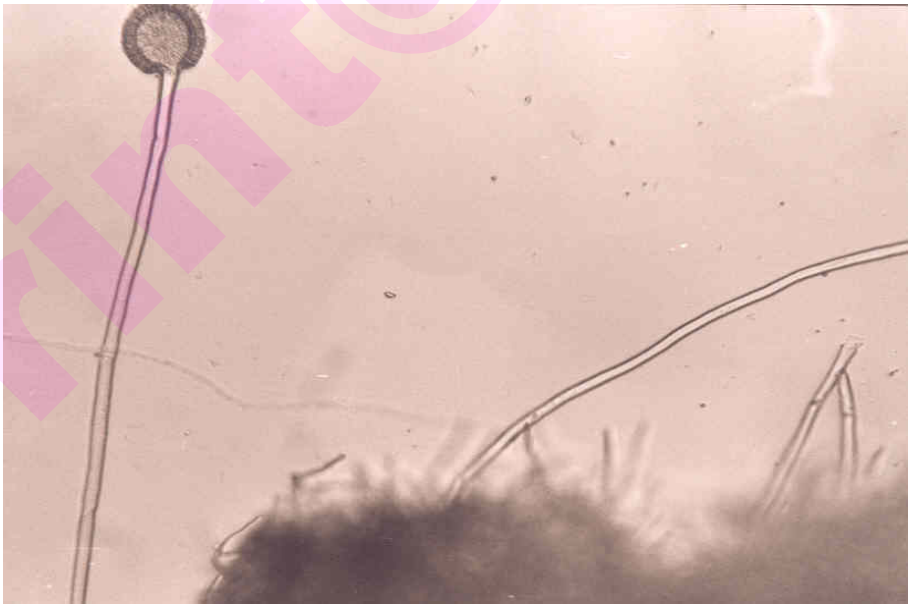


Fig 24: Microphotograph of *Aspergillus ficuum*

Fig 25: Influence of UV (minutes) on % killing of *Aspergillus niger* CFR 335 and *Aspergillus ficuum*

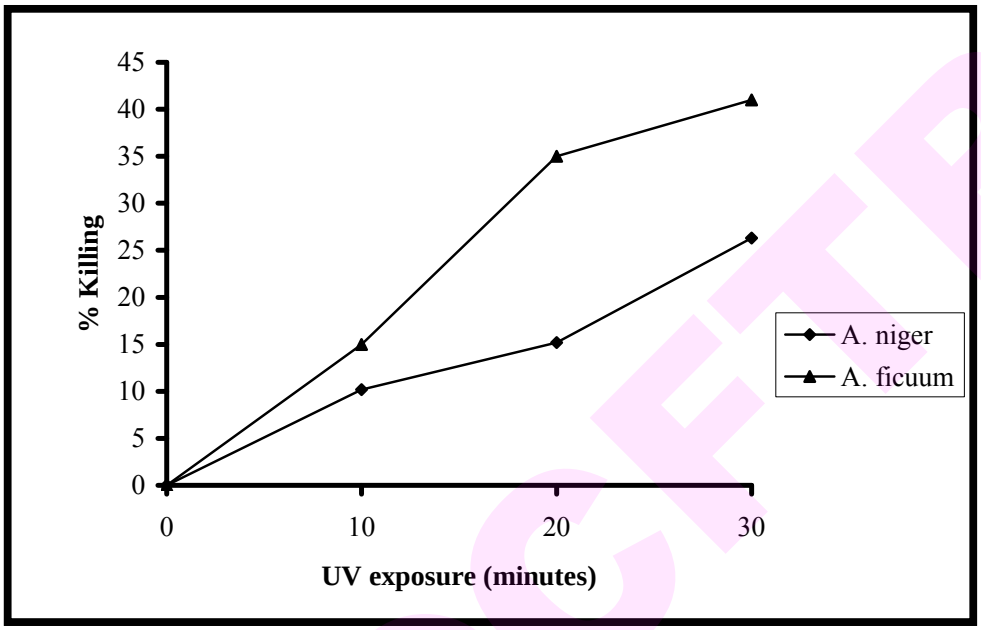
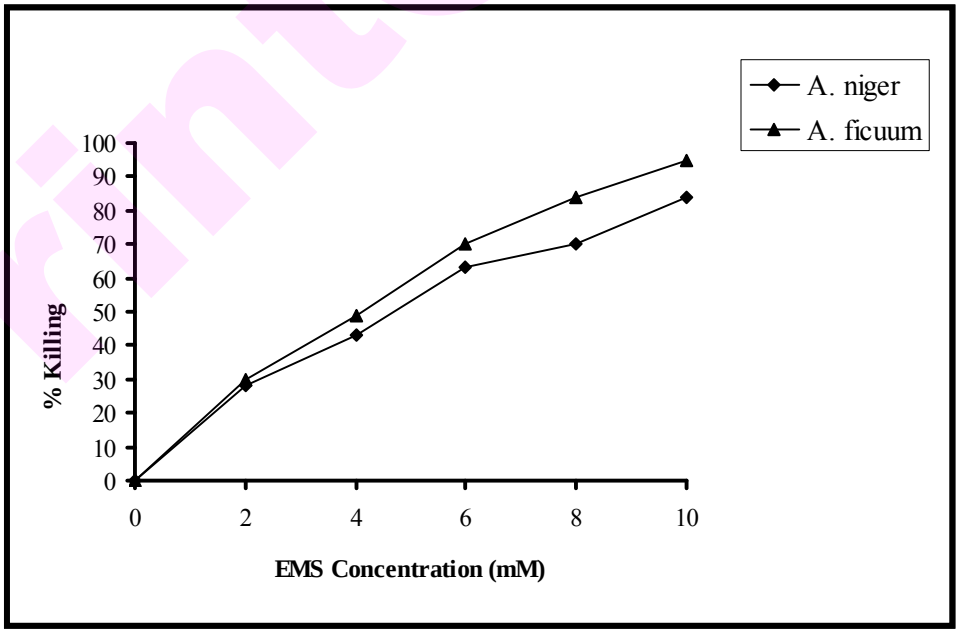


Fig 26: Influence of EMS (mM) on % killing of *Aspergillus niger* CFR 335 and *Aspergillus ficuum*



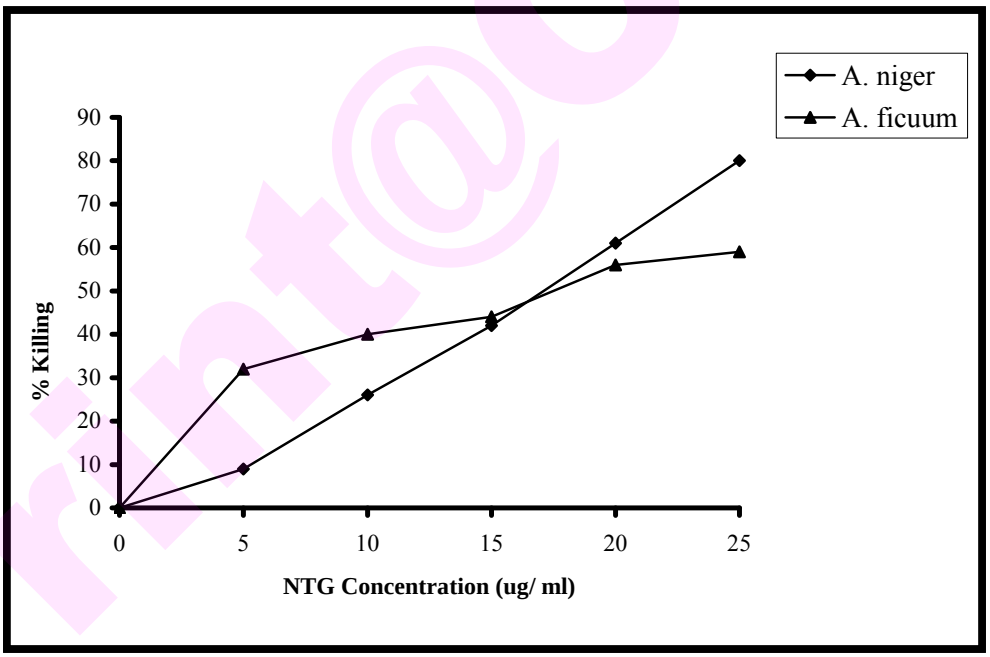
Ethyl methane sulfonate (EMS) is highly mutagenic in lower organisms but only moderately carcinogenic in mice and rats. No case reports or epidemiological studies on the hazards of EMS to humans are known. EMS produces almost all kinds of genetic effects in all the organisms tested. The relationship of mutation frequency to the dose is non- linear for EMS which is shown in case of *Salmonella typhimurium.* The main effect of EMS on DNA *in vitro* and *in vivo* without activation by cellular components is alkylation. However, the main product, 7-alkylguanine is not necessarily the major cause of mutagenesis and the major mutagenic effects of EMS must be examined by biological means.

N'- methyl- N'- nitro- N'- nitrosoguanidine (NTG) reacts with the sulfhydryl groups for conversion to very reactive carbonium ions or alkyl diazonium ions. This is a widely used laboratory mutagen and carcinogen known by a number of abbreviations that includes most commonly MNNG. It has been applied to a wide array of organisms starting from prokaryotes through mammals and has been clearly demonstrated to produce a spectrum of genetic alterations in nearly all test systems.

Studies of specific- locus MNNG mutability have been conducted at a number of loci in *Neurospora*. The vast majority of induced mutations were found to behave like point mutations of the base pair substitution type and a few base pair deletion mutations were also obtained. The lowest effective dose was $3.7\mu gml^{-1}$ for 15 minutes has been reported in *Neurospora*. MNNG was found to induce both forward and reverse mutation in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* with a linear dose response relationship and the lowest effective dose tested was $10\mu gml^{-1}$. In addition to point mutation, MNNG was found to induce both mitotic crossing over and gene conversion in *S. cerevisiae* (John and Seymour, Ed. Frederick and Michael).

In this experiment, a maximum killing rate of 80% was observed when the spores were treated with 25µgml^{-1} of NTG, which is higher than the reported value. A killing rate of 100% was observed when the concentration of NTG increased more than 25µgml^{-1}. Results obtained indicated maximum survival rate when treated with 5µgml^{-1} of NTG. Unlike in UV and EMS, results obtained through NTG treatment of *Aspergillus ficuum,* the killing rate was less than that of *Aspergillus niger* CFR 335. It was found to be only 60% killing when the spores were treated with 25µgml^{-1}. Survival rate decreased with the increase in the concentration of NTG. The result is indicated in Fig 27.

Fig 27: Influence of NTG (µgml^{-1}) on % killing of *Aspergillus niger* CFR 335 and *Aspergillus ficuum*



Comparatively few reports are published regarding the improvement of phytase production via mutagenesis (Rodriguez *et al*, 2000). The catalytic efficiency and thermo stability of *Escherichia coli* pH 2.5 acid phosphatase/ phytase expressed in *Pichia pastoris* was improved by site-directed mutagenesis (Mullaney and Ullah, 2003). An increase in the phytase activity of *Aspergillus*

niger NRRL 3135 phytase A (phy A) at intermediate pH levels (3.0- 3.5) was shown by site directed mutagenesis of its gene at amino acid residue 300 (Utt, 1987). Their results have shown that, a single mutation, K300E, improved phytic acid hydrolysis at 37°C by 56 and 19 % at pH 4.0 and 5.0 respectively. Ramakrishnan, (1994) have devised an improved medium that produced microcolonies and permitted large number of mutant colonies per plate.

A genetically modified *Bacillus subtilis* was shown to produce extracellular phytase (2.0 Units/ ml), which constituted over 90% of the total protein. The yield was 100- fold higher than the wild type *B. amyloliquefaciens* DS11 (Kim *et al,* 1996).

By the above experiments, it was able to obtain auxotrophic strains, namely *Aspergillus niger* CFR 335 ala^- and *Aspergillus ficuum* val^-; $isoleu^-$. The mutant *Aspergillus niger* CFR 335 ala^- was found to have considerable reduction in its sporulation this is shown in Fig 28. The mutant also showed morphological variations with white vegetative mycelium unlike its wild strain counterpart, which is pale yellow in colour (Fig 29). Interestingly, the quantitative production of phytase by the auxotroph did not differ much with that of the wild type strain. *Aspergillus ficuum* val^-, $isoleu^-$ mutant had a slight variation in the morphology when compared with its wild strain (Fig 30).

Even though, the relationship between amino acid auxotrophy and colony morphology variations were concisingly proved, the exact mechanism of occurrence of this phenotype is not known (Ullah and Gibson, 1987). Amino acid auxotrophy might play a vital role in suppressing the spore formation. In our experiment, physical and chemical mutations were carried out to select a marker, preferably amino acid. The mutagenized spores were plated on both complete medium as well as on a nutritionally deficient medium. The composition of the complete medium includes carbon and nitrogen source. When the growth was

observed on minimal medium plates after screening about 2000 colonies, two colonies did not show any growth. When these two colonies were checked for amino acid requirements, one of the colonies grew in alanine-supplemented medium and the other colony required the presence of valine and isoleucine. The mutant that had val^- and iso leu^- markers reverted back to its original form after repeated subculturing. Whereas, the ala^- mutant did not revert back even after several subcultures and retained its auxotrophic nature. These amino acids play vital role in the formation of spores especially in most deuteromycetous fungi.

Fig 28: 1. *Aspergillus niger* CFR 335 (Wild) 2. Asporulating strain of *Aspergillus niger* CFR 335

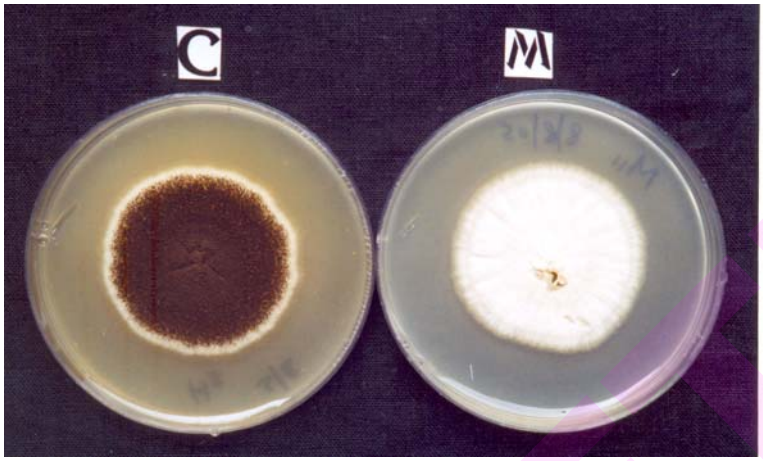


Fig 29: C. *Aspergillus niger* CFR 335 (Wild), M. *Aspergillus niger* CFR 335 (Asporulating mutant)

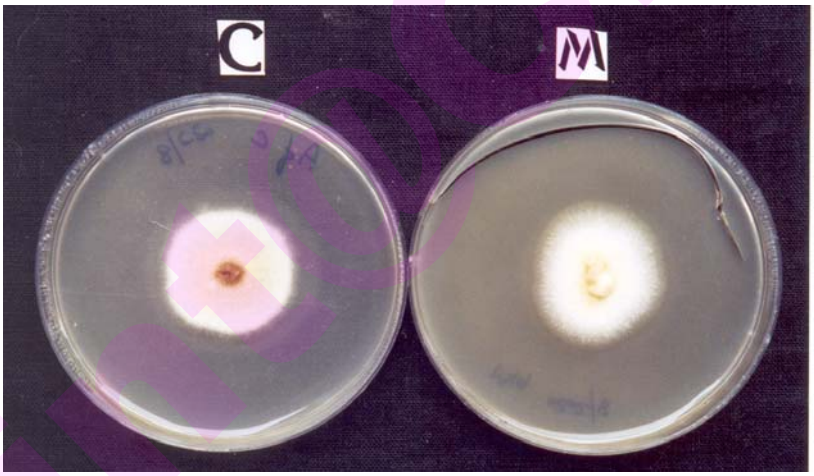


Fig 30: C. *Aspergillus ficuum* (Wild), M. *Aspergillus ficuum* (Mutant)

The above discussion on fungal asporulation process has clearly indicated that asporulation is an important genotypic change that bears relationship to utilization of nitrogen, carbon and certain vital amino acid deficiencies. Colonies with slight morphological variation were also observed during the experiment, which did not show much reduction in sporulation effect. This may be due to slight genetic defect occurring in the organism. The possibility of the involvement of membrane diffusion caused by mutagens cannot be ruled out for delayed

sporulation in *Aspergillus niger.* This needs further investigation to look into the mechanism of sporulation in these fungi. Mutants of *Aspergillus niger* CFR 335 and *Aspergillus ficuum* with morphological variation is shown in Fig 31. The selected mutants of *Aspergillus niger* CFR 335 and *Aspergillus ficuum* were characterized for phytase enzyme production and compared with their wild strains. This is shown in Tables 30, 30a &31.

Fig 31: Mutants of *Aspergillus niger* CFR 335 and *Aspergillus ficuum* on CM plates

Table 30: Characterization of *Aspergillus niger* CFR 335 mutants for phytase activity

Incubation time (Day/s)	Control		Mutants									
			M 1		M 2		M 3		M 4		M 5	
	Activity (U/gds)	Specific activity (U/mg)	Activity (U/gds)	Specific activity (U/mg)	Activity (U/gds)	Specific activity (U/mg)	Activity (U/gds)	Specific activity (U/mg)	Activity (U/gds)	Specific activity (U/mg)	Activity (U/gds)	Specific activity (U/mg)
1	340	0.46	450	0.72	700	1.13	880	1.26	850	0.87	740	0.75
2	490	0.51	420	0.53	790	0.79	1280	1.10	1030	1.03	740	0.75
3	580	0.59	860	0.58	1200	0.97	**1360**	**1.19**	**1410**	**1.25**	810	0.77
4	670	0.39	1110	0.77	1240	0.67	890	0.72	840	0.84	940	0.79
5	1490	0.85	180	1.26	**1240**	**1.35**	970	0.76	890	0.76	1000	0.81
6	**1830**	**1.77**	1400	1.87	1000	0.50	880	0.67	1390	1.00	**1540**	**1.16**
7	1290	0.79	**1600**	**1.89**	1400	0.73	970	0.75	1070	0.77	1340	1.08
8	870	0.74	1500	1.60	810	0.44	1160	1.00	990	0.73	1160	1.09
9	780	0.55	1500	1.44	920	0.56	910	0.88	1270	0.87	1140	0.84
10	660	0.57	1540	1.40	1340	0.92	1300	1.10	840	0.93	1130	0.93

*** 1 Unit of the enzyme is the activity of the enzyme to release one μmole of inorganic phosphorus in one minute at 45°C**

Highest activity is indicated in bold

Table 30a: Characterization of *Aspergillus niger* CFR 335 mutants for phytase activity

Incubation time (Day/s)	Mutants											
	M 6		M 7		M 8		M 9		M 10		M 11	
	Activity (U/gds)	Specific activity (U/mg)	Activity (U/gds)	Specific activity (U/mg)	Activity (U/gds)	Specific activity (U/mg)	Activity (U/gds)	Specific activity (U/mg)	Activity (U/gds)	Specific activity (U/mg)	Activity (U/gds)	Specific activity (U/mg)
1	650	0.83	480	0.72	180	0.34	720	0.86	380	0.49	650	0.85
2	760	0.91	420	0.42	790	0.80	720	0.73	440	0.52	650	0.76
3	1070	0.95	610	0.45	1140	0.72	1100	0.73	960	0.65	840	0.59
4	1100	0.86	1150	0.73	1270	0.91	**1270**	**0.79**	**1290**	**0.84**	940	0.83
5	1300	0.99	1100	0.75	1320	0.95	1240	0.69	1250	0.84	**1380**	**1.29**
6	**1350**	**1.10**	940	0.77	**1440**	**1.08**	1000	0.56	1240	0.84	650	0.46
7	950	0.96	1140	1.05	1170	0.97	720	0.42	1180	0.75	980	0.78
8	970	0.92	**1300**	**1.15**	800	0.80	1240	0.85	1050	0.72	790	0.81
9	860	0.86	1180	1.07	800	0.69	960	0.72	860	0.75	760	0.95
10	830	0.78	1030	0.81	860	0.86	1130	0.93	890	0.70	840	1.01

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**

Highest activity is indicated in bold

Table 31: Characterization of *Aspergillus ficuum* mutants for phytase activity

Incubation time (Day/s)	Control		Mutants									
			M1		M2		M3		M4		M5	
	Activity (U/gds)	Specific Activity (U/mg)	Activity (U/gds)	Specific Activity (U/mg)	Activity (U/gds)	Specific Activity (U/mg)	Activity (U/gds)	Specific Activity (U/mg)	Activity (U/gds)	Specific Activity (U/mg)	Activity (U/gds)	Specific Activity (U/mg)
1	860	1.06	650	1.09	730	1.79	900	1.64	1000	1.96	560	0.44
2	1190	1.57	870	1.06	600	0.99	830	1.46	1080	1.89	612	0.55
3	1510	1.72	880	1.27	990	3.00	1180	1.68	1330	2.42	840	0.671
4	**1960**	**3.04**	810	1.76	1740	3.22	1940	3.66	1600	2.82	940	1.27
5	1900	2.22	1170	2.06	1160	3.6	2230	3.97	2020	2.97	**1760**	**1.84**
6	1630	1.81	1480	2.08	**2300**	**3.63**	**2700**	**4.37**	**2730**	**3.37**	1310	1.26
7	1600	2.36	**1560**	**2.44**	2000	2.86	2000	4.63	2150	2.69	980	1.18
8	1170	1.72	1400	2.15	169	3.38	1700	4.13	1660	2.68	890	0.97
9	920	1.05	1600	1.69	1600	3.64	1700	3.42	1220	1.58	750	0.87
10	790	0.97	1500	1.69	1340	3.2	1630	3.08	1050	1.18	660	0..68

*** 1 Unit of the enzyme is the activity of the enzyme to release one µmole of inorganic phosphorus in one minute at 45°C**

Highest activity is indicated in bold

Protoplast fusion studies:

The field of induced fusion of microbial protoplasts (inter & intra generic and inter & intra specific protoplast fusion) is a rapidly expanding one, and many data have accumulated in both basic and applied areas since the first report on complementation of auxotrophic mutants by controlled protoplast fusion (Ferenczy *et al*, 1972; and Ferenczy *et al*, 1974). The discovery of the spontaneous protoplast fusion of bacteria was made as early as 1925 (Mellon, 1925). This was followed by many fungal species such as *Saccharomyces* and *Candida* sps (Muller, 1970), *Polystictus versicolor* (Strunk, 1967) and *Fusarium culmorum* (Lopez *et al,* 1966).

In the present study, interspecific protoplast fusion between *Aspergillus niger* CFR 335 ala^- and *Aspergillus ficuum* val^- & iso leu^- was carried out. The extent of protoplasting depended on the effectiveness of the cell wall lytic enzyme that makes the wall permeable to release its contents for the formation of osmotically fragile bodies called protoplasts. Protoplasting frequency was calculated using the formula given in section 7.2.2. The total protoplasting frequency of *Aspergillus niger* CFR 335 ala^- and *A. ficuum* val^- & iso leu^- was found to be 26.4% and 37.2% respectively. The protoplasts of *Aspergillus niger* CFR 335 ala^- and *Aspergillus ficuum* val^- & iso leu^- are shown in Figs 32 & 33.

The frequency of protoplast viability may be very low because at the time of segregation, some of the protoplasts may be devoid of nucleus and hence may not regenerate. Only those protoplasts with one or more nuclei will regenerate when cultivated on regeneration medium with hypertonic compound. Regeneration frequency of the protoplasts of *Aspergillus niger* and *A. ficuum* was calculated using the formula given in section 7.2.3. It was found to be 0.32% and 0.47% respectively.

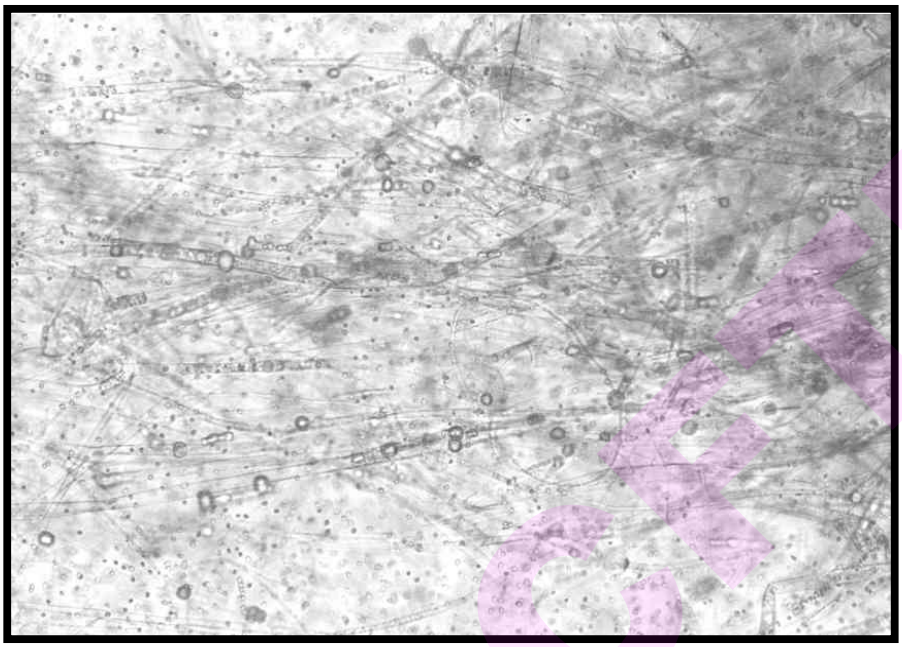


Fig 32: Protoplasting of *Aspergillus niger*

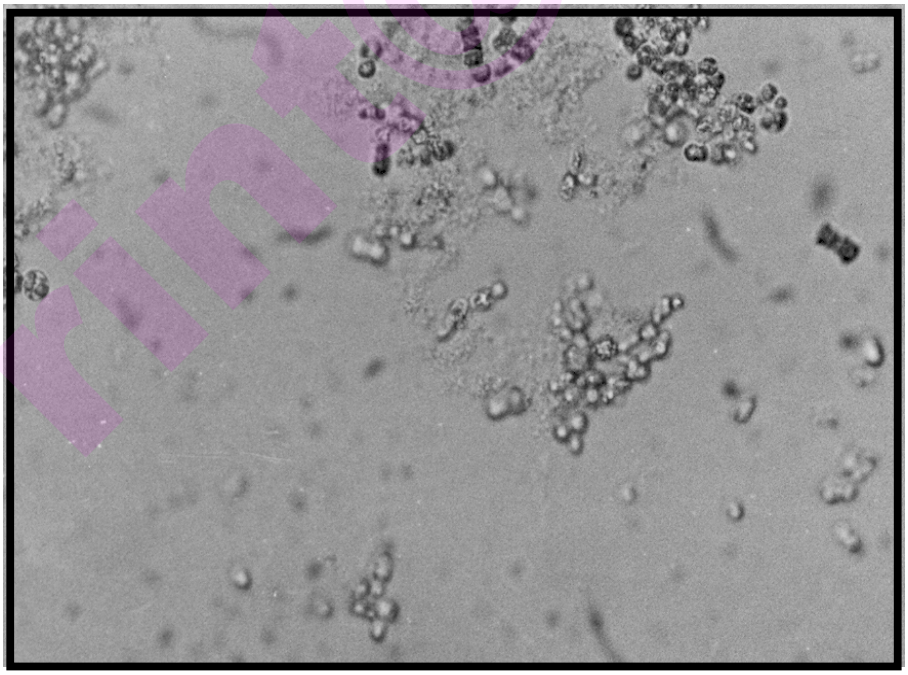
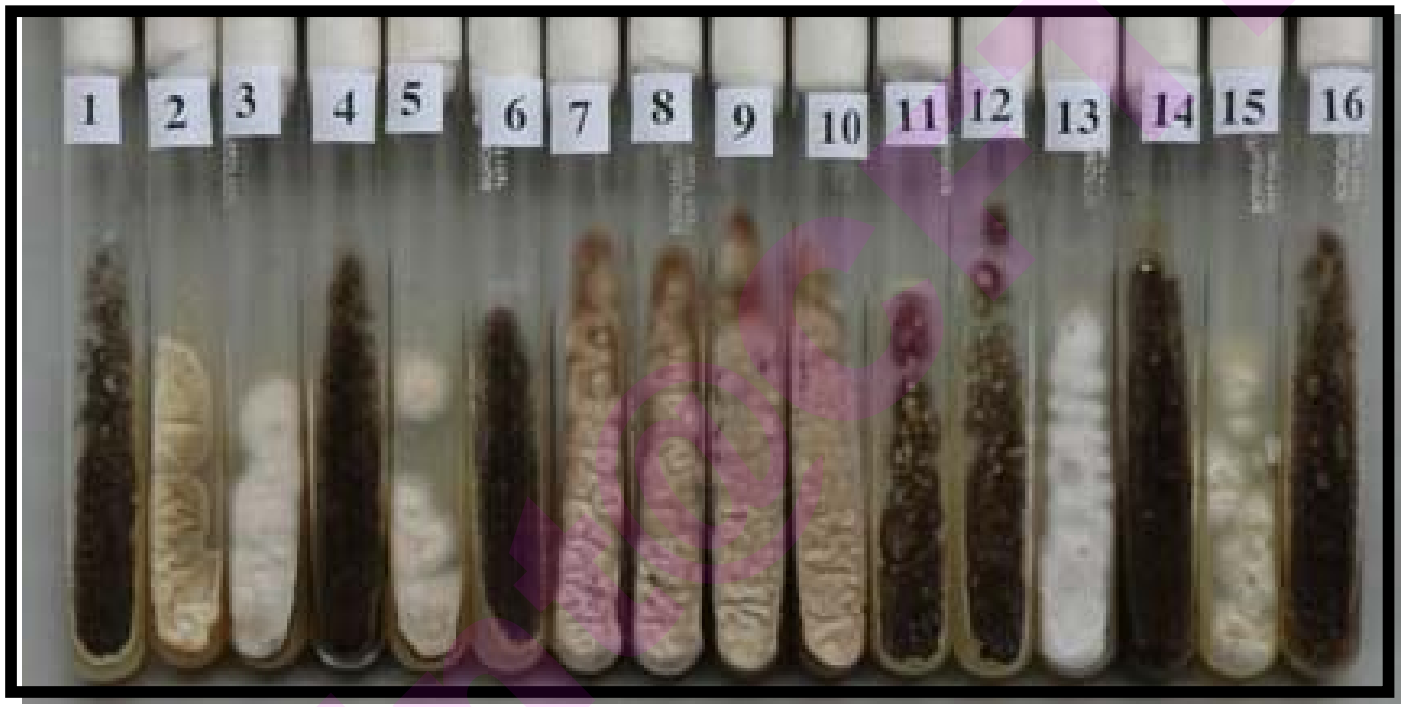


Fig 33: Protoplasting of *Aspergillus ficuum*

Fig 34: Hybrids of *Aspergillus niger and Aspergillus ficuum*



Protoplasts of the two fungi were fused in presence of a fusogen that leads to sharing of genetic material between the two parents. Fusion frequency between the protoplasts from *Aspergillus niger* and *A. ficuum* was calculated based on the ratio between the number of colonies on minimal medium to the number of colonies on complete medium. The fusion frequency was found to be 0.9%.

The selected hybrids are shown in Fig 34. They were characterized for phytase production. All the hybrids were found to produce the enzyme in par with their parents except for one hybrid that showed slight increase in the production. The results are indicated in Table 32. Biomass estimation was made for all the selected hybrids and the results indicated that almost all the hybrids such as H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, and H14 showed more than 18% elevation in the biomass content. This is attributed to the genetic complementation of the two parents. The results are shown in Table 33.

DNA quantification of the hybrids was also made. Wild strains of *Aspergillus niger* CFR 335 and *Aspergillus ficuum* had DNA content of 42.5 and 39 $\mu g ml^{-1}$ respectively. Some of the hybrids such as H1, H2, H4, H6, H15, and H16 showed an higher DNA content of 96.5, 84, 82.5, 83.5, 83.5 and 80.0µg/ml respectively. The results are given in Table 34.

The selected hybrids were also subjected to proximate analysis such as, estimation of total protein, total fat, total sugars and ash content of the biomass were determined. The results are given in Table 35.

The total fat in the biomass was estimated by Soxhlet method given in section 4.2.5.3.1. The results obtained in our study showed that all the hybrids varied between 1.0- 8.4%. A slight difference in the total fat was observed between the parents and the hybrids.

Table 32: Characterization of hybrids for phytase production

Sample	Activity (U*/gds)	Specific activity (U/mg)
A. niger ala$^-$	1763	1.724
A. ficuum val$^-$, isoleu$^-$	1992	1.87
H1	1289	0.933
H2	1072	0.816
H3	1634	1.338
H4	1150	0.736
H5	**2186**	**1.939**
H6	2095	1.747
H7	1613	1.549
H8	1231	1.358
H9	2185	1.670
H10	1997	1.581
H11	1521	1.453
H12	1721	1.503
H13	1047	0.833
H14	2100	1.814
H15	1585	1.301
H16	1364	1.246

*** 1 U of the enzyme is the activity of the enzyme to release 1 μmole of inorganic phosphorus in one minute at 45°C**

H1, H2……..Hybrid

Highest phytase activity is indicated in bold

Table 33: Characterization of hybrids for biomass production

Sample	**Biomass (g/ l)**
A. niger ala$^-$	7.6
A. ficuum val$^-$, isoleu$^-$	8.0
H1	**9.4**
H2	**11.6**
H3	**9.4**
H4	**11.2**
H5	**10.8**
H6	**10.6**
H7	**10.0**
H8	**9.8**
H9	8.0
H10	**9.2**
H11	**11.8**
H12	**10.2**
H13	6.4
H14	**11.0**
H15	8.8
H16	6.2

Highest biomass content is indicated in bold

H1, H2……..Hybrid

Table 34: DNA Quantification of hybrids

Sample	**DNA (μg/ ml)**
A. niger ala$^-$	67.0
A. ficuum val$^-$, isoleu$^-$	75.5
H*1	**96.5**
H2	**84.0**
H3	48.0
H4	**82.5**
H5	69.5
H6	**83.5**
H7	76.5
H8	33.0
H9	43.0
H10	58.0
H11	63.0
H12	39.5
H13	68.5
H14	59.5
H15	**83.5**
H16	**80.0**

Highest DNA content is indicated in bold

H1, H2…… Hybrid

Estimation of crude protein was made by Kjeldhal method (section 4.2.5.3.2). The method involves an oxidation of organic compound by concentrated sulphuric acid to form carbon di-oxide and water and release of ammonia. Ammonia combines with sulphuric acid to form ammonium sulphate.

Ammonia is again released by the reaction of ammonium sulphate with strong alkali (40 % Sodium hydroxide) and the liberated ammonia is held in dilute boric acid (2 %) solution, which in turn is back titrated with dilute acid (Standard HCl).

Table 35: Proximate analysis of hybrids

Sample	Total lipid (%)	Protein (%)	Carbohydate %	Ash (%)
A. niger	3.1	38.8	57.9	0.2
A. ficuum	5.2	44.1	50.52	0.18
A. niger ala^{+}	5.8	26.9	63.19	0.3
A. ficuum val^{+}, isoleu^{+}	1.7	28.6	48.43	0.27
H1	7.4	56.2	61.06	0.34
H2	1.9	40.8	41.36	0.24
H3	6.6	36.3	61.84	0.26
H4	2.8	26.6	50.6	0.38
H5	3.1	30.45	58.07	0.38
H6	5.7	46.9	62.21	0.19
H7	3.4	27.6	52.83	0.17
H8	1.0	32.31	43.43	0.26
H9	4.5	42.6	62.54	0.36
H10	4.1	38.4	57.22	0.28
H11	8.4	20.24	59.17	0.19
H12	1.9	26.7	43.08	0.32
H13	2.15	18.36	49.32	0.17
H14	6.25	18.85	54.59	0.31
H15	6.5	23.6	54.76	0.14
H16	5.1	21.3	62.39	0.21

Highest values are indicated in bold

H_1, H_2……..Hybrid

Three of the selected hybrids showed an increased protein level of 56.5, 55.31 and 54.7% while the wild strains, *Aspergillus niger* CFR 335 and *A. ficuum* showed 38.8 and 44.1% respectively. The mutants had 30.71 and 49.6% protein respectively in their biomass.

The total sugars in the biomass of the hybrids were estimated by the method given in section 4.2.5.3.1. The total soluble sugars in the biomass of parent strains and hybrids were estimated by phenol- sulphuric acid method given in section 4.2.5.3.1. The results showed that the total soluble sugars in all the hybrids ranged from 41- 62%. There was no significant difference in the total sugar content between the parents and the hybrids.

The ash content of all the strains valued less than 0.5%.

A series of basic discoveries on induced and controlled microbial protoplast fusion e.g, the first successful fusion experiments and the subsequent characterization of the fusion products, the establishment of methods for obtaining high fusion frequency, the first interspecific protoplast fusion and recognition of new possibilities of microbial gene transfer in both basic and applied fields are all based upon research on protoplast fusion of filamentous fungi. Between 1972 and 1976, microbial protoplast fusion meant exclusively the protoplast fusion of filamentous fungi. Stable auxotrophic mutants of *Geotricum candidum* were selected for protoplasts formation and their fusion was induced by centrifugal force followed by long incubation (Ferenczy, 1981). The attainment of gene transfer between two different species has been reported in two major genera of filamentous fungi *Aspergillus* (Croft *et al*, 1980) and *Penicillium* (Anne *et al*, 1976). The technique of protoplast fusion seems to be the only means of obtaining interspecific heterokaryons. It is an acceptable general belief, supported by several facts, that protoplast fusion can be induced between two partners independently of their taxonomic relationship. On the other hand, very little is known about the

genetics or biochemistry of compatibility and incompatibility events in these interspecific microbial fusion products.

It has been observed that unlike sexual conjugation, transformation or transduction, where only genetic material is unidirectionally transferred into the recipient cell, in protoplast fusion, total cytoplasm is involved.

Protoplast preparations are used in the following biochemical and genetic studies (Spencer and Spencer, 1988).

a. Preparation of cell membrane and the study of cell wall structure
b. For obtaining nuclei, tonoplast, etc.,
c. Preparation of cell organelles such as mitochondria, ribosome, endoplasmic reticulum etc.,
d. Study of cell- free extract
e. Study of osmotic system and permeability
f. For growth studies
g. Study of transport mechanism of solutes
h. For testing the resistance for physical factors
i. Investigation of protein and enzyme synthesis
j. Studies of localization of enzymes in cells
k. To study the mode of action of antibodies and surface active agents
l. To study the regeneration of cell wall and its biosynthesis
m. For the studies of conjugation between protoplasts
n. To study spore formation

Studies on interspecific protoplast fusion in yeasts have also been carried out (Whittaker and Leach, 1978). Protoplasts of stable auxotrophic strains of *Kluyveromyces lactis* and *K. fragilis* were fused and stable prototrophic colonies were obtained. The fusion products were larger than cells of the parental strains with an elevated DNA content. There were indications of both the loss of

chromosomes after fusion and also the occurrence of multiple fusions. In the protoplast fusion of auxotrophic mutants of *Schizosaccharomyces pombe* and *S. octosporus*, the latter proved dominant in the fusion products, since only *S. octosporus* could be recovered from the hybrids.

The present work was carried out for the possibility of producing hybrids between *A. niger* CFR 335 ala^- and *A. ficuum* val^-, $isoleu^-$. The aim of fusing the protoplasts of these two strains was to reduce the sporulation in *A. niger* CFR 335 and at the same time to retain its phytase production which was observed in our experiment.

SECTION 5

Purification of phytase enzyme:

The various stages of purification are summarized in Table 36. The crude enzyme extract exhibited lowest specific activity of 1.68U/mg and highest protein content of 1100 mg than the purified. Different proteins can be gradually precipitated out from their aqueous solutions using highly soluble salts such as ammonium sulphate. As the concentration of the salt increases, they compete with proteins for water molecules and lead to their gradual precipitation based on their requirement for water molecules to be in the soluble form. By convention, the final concentration of ammonium sulphate required for precipitation is expressed in terms of percentage of saturation. During this step of purification, the protein precipitate obtained after 80% ammonium sulphate fractionation showed a lesser protein content and higher specific activity of 665 mg and 4.19 U/mg respectively. The sample, after dialyzing for overnight against 0.2M acetate buffer, pH 4.5, showed 432mg protein and 6.96 U/mg specific activity respectively.

In the third step of purification, phytase enzyme was eluted through NaCl gradient of DEAE- Sephadex G-50 column chromatography. Protein elution profiles from chromatographic columns were monitored by measuring fractional

absorbance at 280 nm (Layne, 1957). The protein elution pattern is given in Fig 35. Maximum activity was observed with 0.5 M NaCl concentration. Active fractions with optical density more than 0.2 were pooled and subjected to electrophoresis. This sample had a highest specific activity of 9.97 U/mg and lowest protein content of 358 mg. Electrophoresis with silver nitrate staining showed that the extract was pure with a single discrete band. The enzyme was stored for months without much activity loss.

Table 36: Purification scheme of *Aspergillus niger* CFR 335 phytase

Step	Protein (mg/ml)	Activity (U)	Specific activity (U/mg)	Fold purification
Crude extract	1100	1846	1.68	1
Ammonium sulphate fractionation	665	2789.5	4.19	2.5
Dialysis	432	3008.5	6.96	4.15
DEAE- sephadex column chromatography	358	3568.74	9.98	5.94

Molecular mass of *A. niger* CFR 335 phytase was found to be 66 kDa when the enzyme sample was loaded on to 10% SDS gel with silver nitrate staining (Fig 36). The eluted fractions were also anlysed by HPLC, which showed a single major peak indicating the purity of the enzyme (Figs 37-40). Retention time of the peak was compared with authenticated standard phytase obtained from Sigma Chemical Co. USA. It could be concluded from our study that extracellular phytase enzyme obtained from *A. niger* CFR 335 had an optimum pH and temperature of 4.5 and 30°C respectively (Figs 41 & 42) with a molecular mass of 66 kDa. This suggested that the enzyme was a monomeric protein and the enzyme showed a 6-fold increase in the activity when compared with the crude enzyme extract.

Fig 35: Elution profile of phytase in DEAE- Sephadex G-50 column

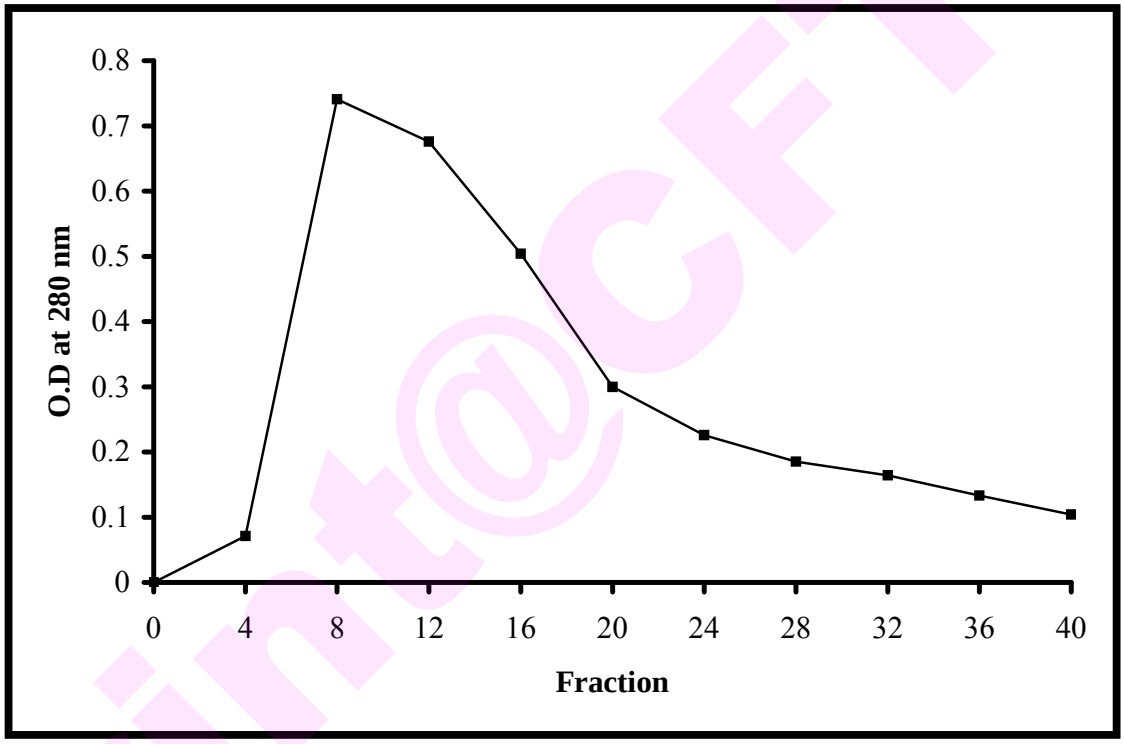
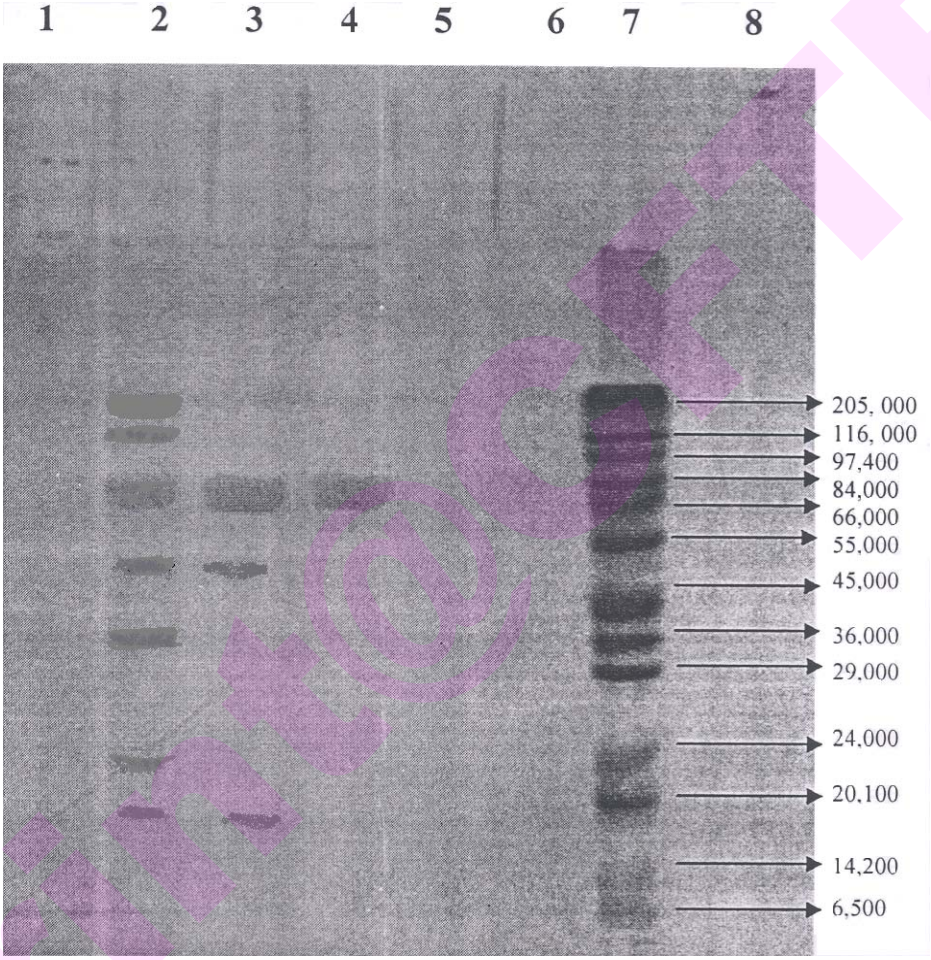


Fig 36: SDS- PAGE



Lane 2: Crude enzyme sample
Lane 3: Partially purified enzyme (dialysed sample)
Lane 4: Purified enzyme
Lane 7: Standard protein marker

Fig 37: HPLC of standard phytase enzyme

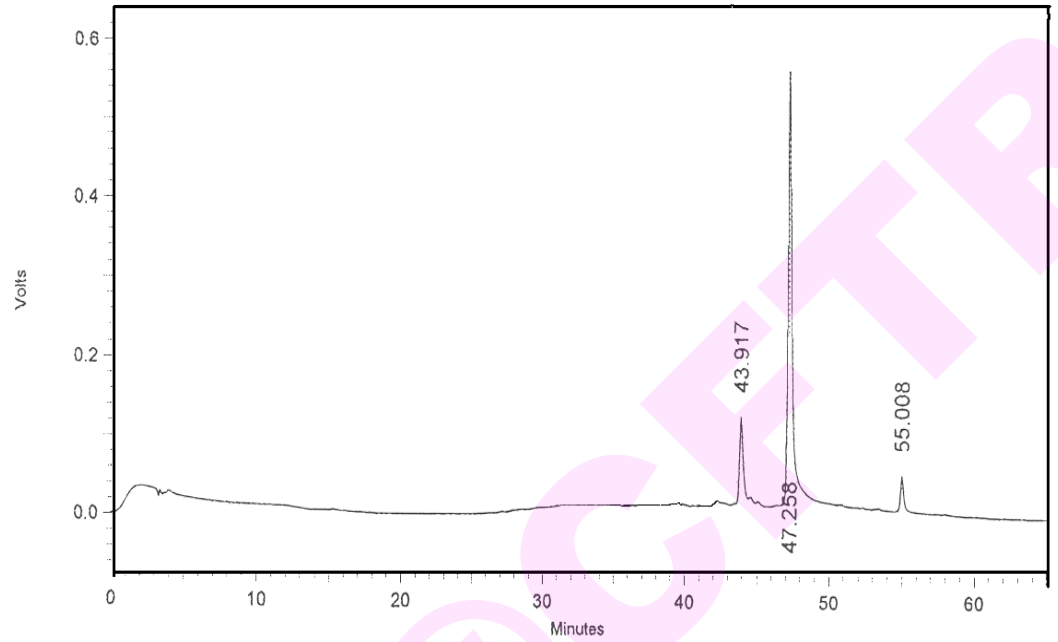


Fig 38: HPLC of crude phytase enzyme

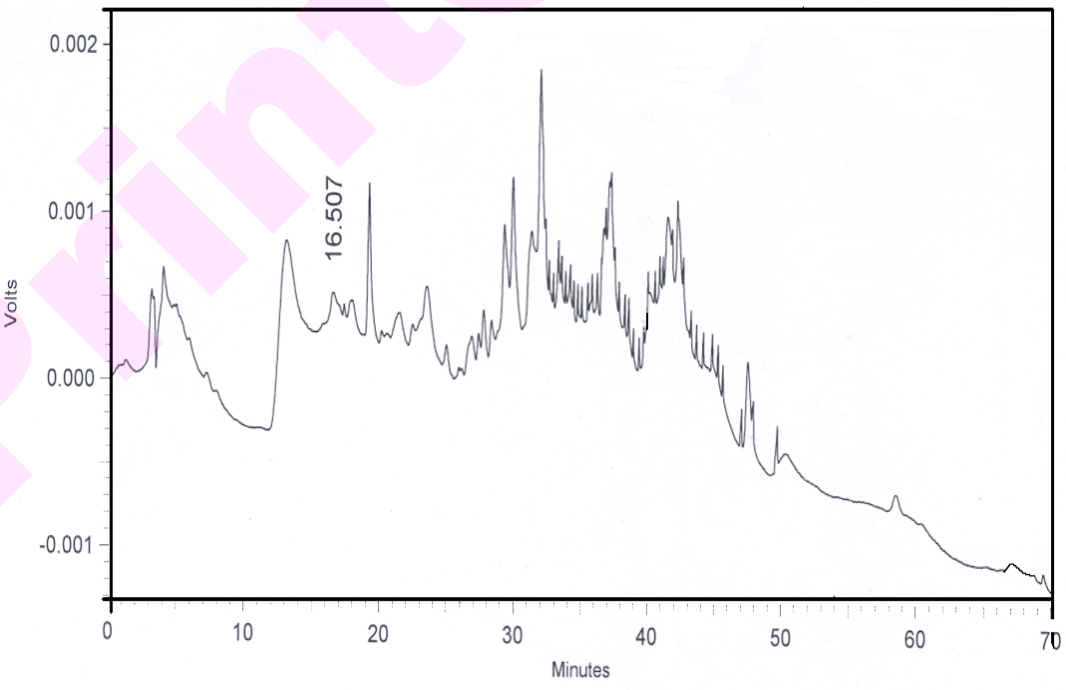


Fig 39: HPLC of dialysed phytase enzyme

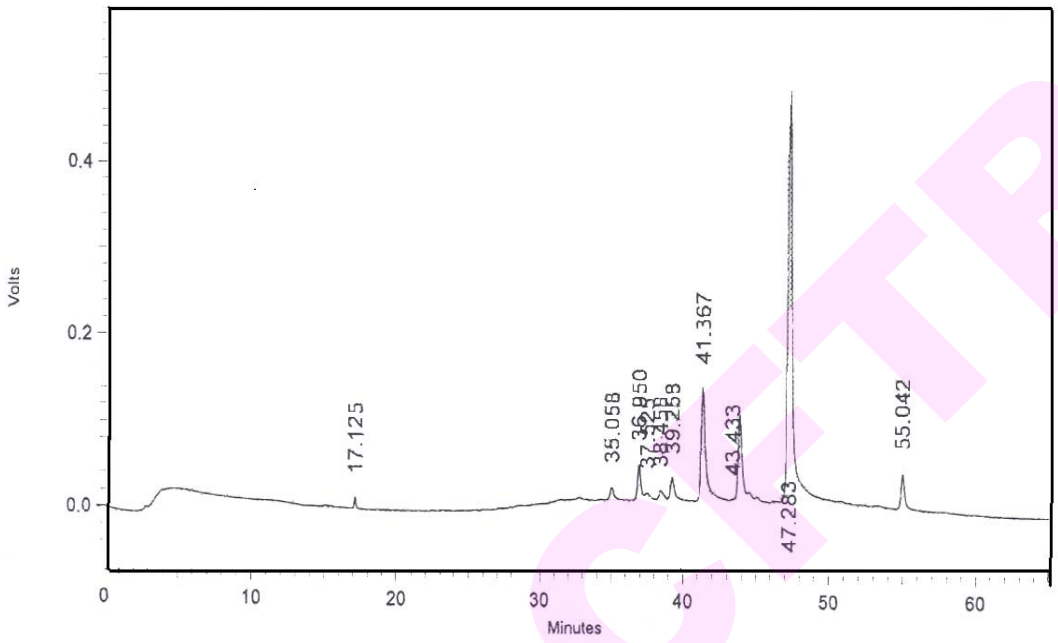


Fig 40: HPLC of column-purified phytase enzyme

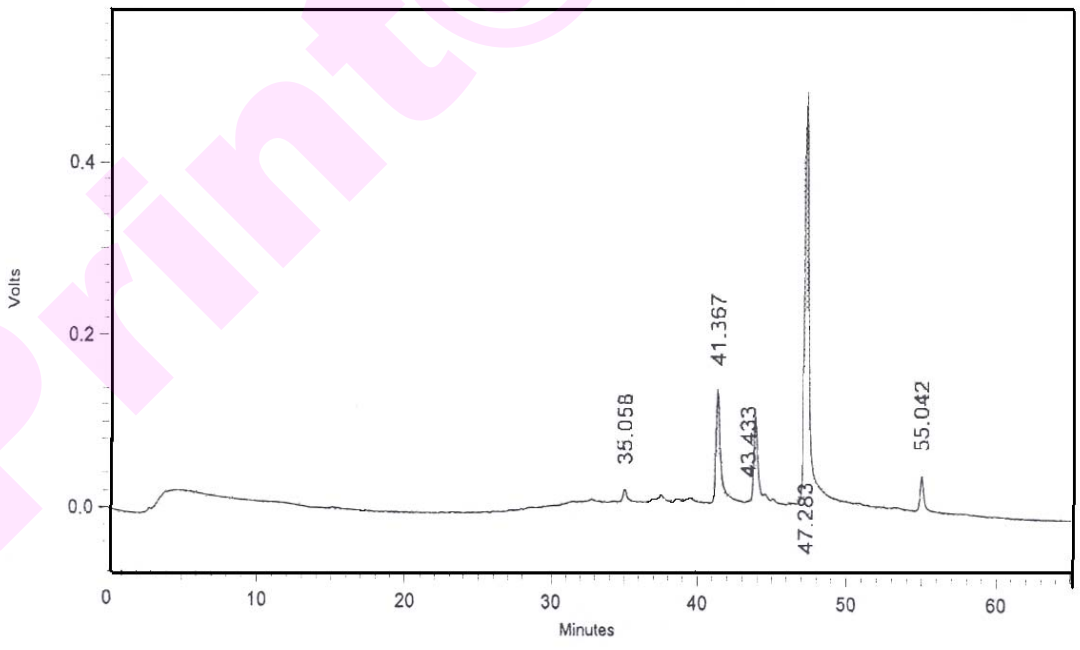


Fig 41: Effect of temperature on purified phytase activity

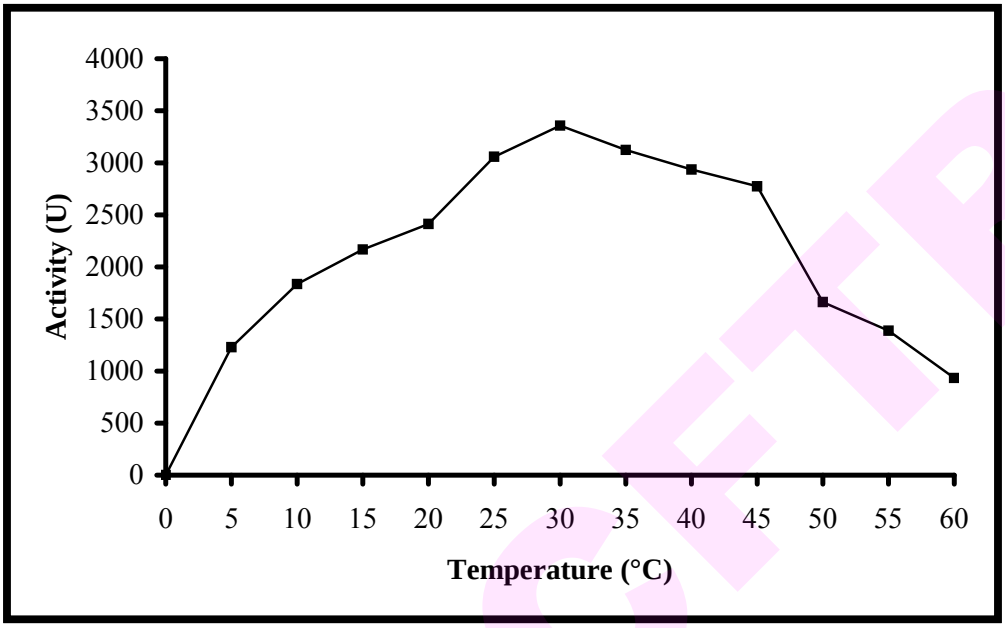
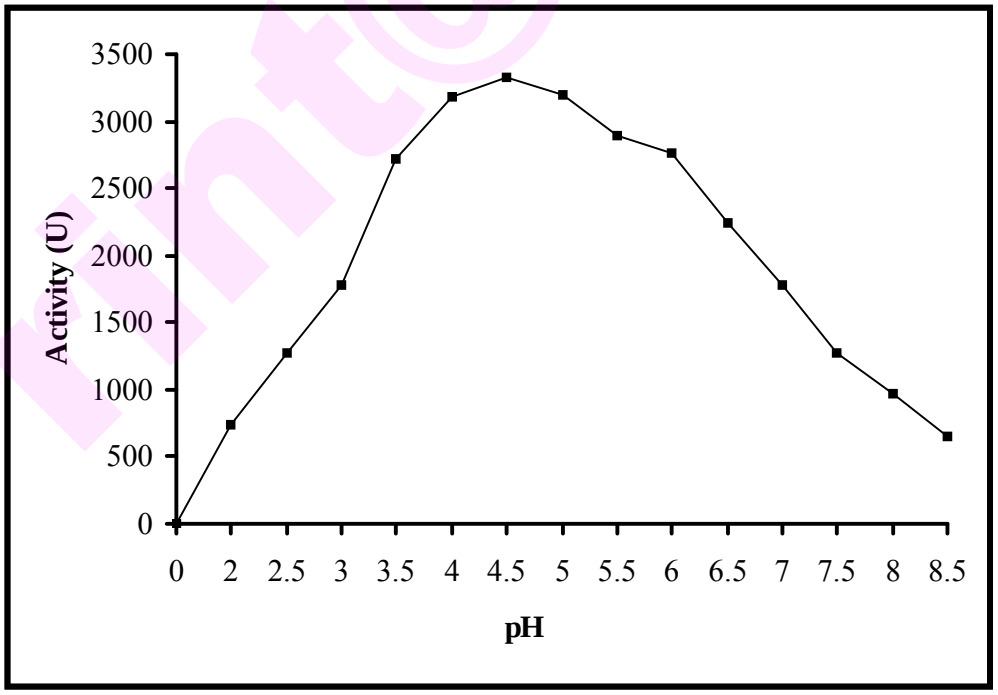


Fig 42: Effect of pH on purified phytase activity



Shimizu, (1993) have purified an extracellular phytase and an extracellular acid phosphatase from *Aspergillus oryzae* K_1 to homogeneity on gel filtration, chromatofocussing, PAGE and SDS- PAGE. Molecular masses of K_1 phytase and K_1 acid phosphatase were estimated to be 120kDa & 140kDa on PAGE and 60kDa & 70kDa on SDS- PAGE respectively, suggesting that both enzymes are composed of two subunits. This enzyme showed similarity in substrate specificity, K_m, optimum pH and temperature with *A. oryzae* NRRL 1988, but differed in molecular mass.The active enzymes of *A. oryzae* were found to be dimers unlike *A. niger* NRRL 3135 phytase and pH 6.0 optimum acid phosphatase which are monomers. The molecular masses of *A. oryzae* phytase and acid phosphatase were found to be 60 and 70 kDa respectively.

An extracellular phytase from *Bacillus subtilis* (natto) N-77 was purified 322-fold by gel filtration and DEAE chromatography and combination of Sephadex G- 100 and DEAE- Sepharose CL- 6B column chromatography. Molecular weight of this purified enzyme was estimated to be 36kDa on gel filtration and 38 kDa on SDS- PAGE suggesting that the native enzyme was a monomeric protein. Activity of this enzyme was greatly inhibited by EDTA, Zn^{2+}, Cd^{2+}, Ba^{2+}, Cu^{2+}, Fe^{2+} and Al^{3+} (Shimizu, 1992). Greiner *et al*, (1993) have purified two periplasmic phytases P_1 and P_2 from *Escherichia coli* for about 16,500- fold to an apparent homogeneity with a recovery of 7 and 18% respectively. The enzymes behave as monomeric proteins with molecular mass of about 42kDa. These two purified *E. coli* phytases share many kinetic parameters in common with other phytases (Ullah, 1988). An extracellular phytase from *Bacillus subtilis* was purified to homogeneity by ultracentrifugation. It was found to have 2 isozymes on SDS- PAGE. This enzyme was shown to differ in its metal requirement and phytate specificity. This *B. subtilis* phytase is the only known phytate- specific phosphatase (Powar and Jagannathan, 1982). They have pH optimum of 4.5 and temperature optimum at 55ºC. Both the periplasmic enzymes

were shown to be very specific for phytate. A phytase was also purified from yeast (Nayini and Markakis, 1984). The bacterial enzyme resides at the periplasm and it has high affinity for p- nitrophenyl phosphate (Dassa *et al*, 1980). The fungal pH 6.0 optimum acid phosphatase was found to be a highly glycosylated extracellular metalloenzyme (Ullah and Phillippy, 1994). Ullah & Gibson (1987 and Ullah & Cummins, (1987) have purified the secreted proteins phy A, phy B and pH 6.0 optimum acid- phosphatase from *A. ficuum* at room temperature using ion-exchange chromatography and chromatofocusing.

Golovan *et al* (2000) have purified a phytase from *E. coli* with molecular mass of 44.7kDa. The purified enzyme was further separated by chromatofocussing into two isoforms of identical size with isoelectric points of 6.5 and 6.3. The isoforms had identical pH optima of 4.5 and were found stable at pH values from 2.0- 10.0. Optimum temperature for both the isoforms was 60°C. A phytase from *Klebsiella* sp. No PG- 2 was purified 50-fold by ammonium sulphate fractionation, ion exchange chromatography and gel filtration. The pH and temperature optima of this enzyme were 6.0 and 37°C respectively (Shah and Parekh, 1990). Tadashi *et al* (1999) have purified the phytase enzyme and the temperature profile of purified phytase was determined from 4- 60°C by standard assay at the given temperature. The optimum temperature was found to be 50°C.

Aspergillus fumigatus phytase was subjected to cation- exchange chromatography at pH 5.0 and the enzyme was eluted as a single peak at 500 mM NaCl (Pasamontes *et al*, 1997). The activity of the purified *A. fumigatus* enzyme at different pH with phytic acid and 4- nitrophenyl phosphate as substrates. With phytic acid as the substrate, the enzyme showed activity between pH 2.5and 8.0 with optima at pH 4.0 and 6.5 and maximum activity at latter. With 4- nitrophenyl phosphate as the substrate, enzymatic activity occurred at pH values that were lower than those for phytic acid. The highest level of activity was measured at pH 5.0 and roughly 60% of the activity occurred between pH 3.0 and 3.5. Most

remarkable characteristic of this enzyme and not described so far for any other phytase or acid phosphatase is the resistance of the enzyme to high temperatures. Incubation at 90 or even 100ºC for 20 minutes resulted in only a minor loss of activity (10%). Even after exposure to 90ºC for 120 minutes, 70% of the initial activity remained. Two possible explanations are (i) that the *A. fumigatus* phytase displays increased resistance to heat inactivation, similar to enzymes from hyperthermophilic organisms, and (ii) that this enzyme has the ability to refold properly after denaturation.

It is concluded in our study, that unlike other fungal strains that produce histidine acid phosphatases with two or three different pH optima, our isolate *Aspergillus niger* CFR 335 produces only phytase enzyme with pH and temperature optima of 4.5 and 30°C respectively. The enzyme was purified in a three- step purification method and was found to be active at pH between 3.5 and 5.5 with an optimum of 4.5. The enzyme was also found to be active at temperature up to 45°C and the optimum being 30°C. Above this temperature, activity of the enzyme diminished and at 60°C there was about 80% loss in the enzyme activity. The purified enzyme in aqueous form was stable up to one month, while in lyophilized form, it was stable up to three months.

It is found from our study that the enzyme obtained from *Aspergillus niger* CFR 335 efficient in releasing phytate- phosphorus and has no drastic effect on health of the layers hens. The enzyme was also found to play a very important role in combating environmental pollution by reducing excessive phosphorus excretion by poultry birds. Hence use of the enzyme, a cost- effective product obtained from *Aspergillus niger* CFR 335, as a feed supplement is highly recommended in the poultry industries.

The future work can be focused on incorporation of highly purified phytase enzyme in infant and geriatric food formulations, which ultimately enhance the bioavailability of phosphorus and other minerals.

SUMMARY
&
CONCLUSION

SUMMARY & CONCLUSION

The increasing economic pressures currently being placed upon animal producers demand more efficient utilization of low- grade feedstuffs. In addition, consumer awareness and new legislation require that any increase in animal production cannot be achieved via growth- promoting drugs or other chemical substances. A scientific approach to this problem is to supplement animal diets with hydrolytic enzymes to aid the digestion and absorption of poorly available nutrients, or to remove antinutritional factors from the diet. Phytic acid, one of the antinutritional components, constitutes about 60-80% of all the plant materials that include cereals, legumes and oil seeds. The antinutritional nature of phytic acid is attributed to its chelating effect on certain nutritionally important minerals such as Ca, Mg, Mn, Zn, Fe, certain proteins and few enzymes. The bound minerals and proteins are unavailable to the monogastric animals and birds, due to lack of intrinsic phytase enzyme in their gastrointestinal tract. Supplementation of phytases in such diets has desirable results. Phytases from microbial sources offer techno-economical feasibility for their production and application.

The present study was initiated by isolation and screening of fungal strains for phytase enzyme. More than 1,500 fungal strains were isolated from soil and poultry wastes obtained from different geographical areas. 150 strains possessed phytase activity among the isolates. *Aspergillus niger* CFR 335 showed an highest phytase activity of 2067 U/gds and specific activity of 1.864 U/mg in solid- state fermentation condition. This enzyme was induced up to >25% in presence of 0.5% calcium phytate. The fungus was found to be a non- acid phosphatase producer. Some of the Lactic Acid Bacteria were also quantitatively characterized for phytase enzyme during this investigation.

The study was also focussed on certain physical parameters, such as the effect of temperature and pH on phytase production by *A. niger* CFR 335. The data obtained revealed that the enzyme production was maximum at 30ºC and 4.5 of temperature and pH respectively, when the fungus was cultivated at temperatures ranging from 10- 55ºC and pH between 3.0 and 8.5. Effect of different carbon, nitrogen and phosphate compounds, surfactants and metal salts on phytase production by the fungus was tested. Sucrose was found to be a good carbon source, which yielded 2194 U/gds in SSF at 1% level. In case of nitrogen compounds, with 0.5% peptone, the activity was found to be same as that of sucrose. Among the different inorganic phosphates tested for phytase production, there was no significant increase in the enzyme level under the culture conditions. p- nitrophenyl phosphate did not support growth of the fungus. An experiment was also carried out to test whether the fungus produces acid phosphatase in presence of different phosphates. More than five- fold increase in acid phosphatase activity was observed in presence of p- nitrophenyl phosphate. The enzyme level was not significantly higher when tested with four different surfactants at 0.25% level. Enzyme production was also checked in presence of 0.01% level of different metal salts that resulted in more than 20% reduction in the enzyme activity with all the metal salts.

Animal experiment on phytase enzyme was carried out nutritionally by feeding 3 week old layer birds and found that the dietary supplementation of *A. niger* CFR 335 phytase enhanced the overall performance of layer birds when the enzyme was used at a level of 400- 600 U/gds.

Mutation studies were carried out to improve the strain for higher yield of enzyme. Two auxotrophic mutants having *A. niger* CFR 335 ala^- and *A. ficuum* val^-, $isoleu^-$ markers were obtained that were not reported earlier. These two mutants having amino acid tags were subjected to hybridization by protoplast fusion method in the presence of PEG. The regeneration frequency of protoplasts

of both the parents was found to be 0.32 and 0.47% respectively with a fusion frequency of 0.9%. This resulted in obtaining 16 stable hybrids. All the hybrids were selected and characterized for phytase activity and their DNA content was also quantified.

Purification studies revealed six- fold increase in the enzyme activity when the crude enzyme was subjected to three- step purification procedures. Molecular weight of the enzyme was found to be 66 kDa. The enzyme was found to have an optimum temperature and pH of 30ºC and 4.5 respectively. The enzyme retained 80% of its residual activity upto a temperature of 45ºC and pH up to 6.5. The purified enzyme was stable up to three months at room temperature.

Phytase is considered to be a special type of acid phosphatase, which is capable of splitting off phosphate from phytate as well as other diversified organophosphates. Although a sizeable number of phytase producing organisms are reported, it has been observed that a thermostable and acid stable phytase with broad substrate specificity and high specific activity is still desirable for animal nutrition purposes and is of great commercial importance. Currently, phytase utilization is restricted to those locations where there is a considerable pollution load but if increase in the efficiency is achieved, then, this enzyme application would become more widespread in the future. Thus, to obtain better and alternative source of phytases, there is always an ongoing interest in screening new organisms producing novel and efficient phytases with the ultimate aim to produce this enzyme to cost effective level and establish the suitability for its industrial application.

The enzymatic degradation of phytic acid will not produce mutagenic and highly toxic by- products, thus, the exploitation of enzymes in the industrial process would be environmental friendly and assist in the development of novel cleaner technologies. Furthermore, the enzyme could be used via coupling for

diagnostic purposes in clinical medicine such as diagnosing hyperinositolemia. Phytases also have an immense importance in the food industries because they improve the nutritional status by degrading phytic acid, which acts as an antinutritional factor and the enzyme finds application in infant and geriatric food formulations. Hence, there is a need for the engineering of a thermostable and more ecofriendly phytases from microorganisms using cheap raw materials to meet the world demand of this enzyme.

It is concluded from our study that the enzyme obtained from *Aspergillus niger* CFR 335 efficient in releasing phytate- phosphorus and has no drastic effect on health of the layers hens. The enzyme was also found to play a very important role in combating environmental pollution by reducing excessive phosphorus excretion by poultry birds. Hence use of the enzyme, a cost- effective product obtained from *Aspergillus niger* CFR 335 as a feed supplement is highly recommended in the poultry industries.

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