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ISOLATION AND IMMOBILIZATION OF ASPERGILLUS PHYTASE FOR DEPHYTINIZATION

A PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

Certified that this project report **"ISOLATION AND IMMOBILIZATION OF ASPERGILLUS PHYTASE FOR DEPHYTINIZATION"** is the bonafide work of **GAUTHAM.P(0710204012),LINCYBABU(0710204021),MOHANVARMA.R.G(0710204025), YAGNESSH.N.K (0710204056)** who carried out the project work under my supervision.

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TABLE OF CONTENTS

TABLE OF CONTENTS

ABSTRACT

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Phytases catalyse the hydrolytic degradation of phytic acid and its salts and are added to monogastric animal feed to ameliorate the negative environmental and nutritional consequences of dietary phytate. *Aspergillus niger* cultivated in wheat bran produced a phytase that displayed physicochemical characteristics likely to render it of potential industrial interest. The enzyme was partially purified by ammonium sulphate precipitation. The enzyme was immobilized using sodium alginate beads. The free enzyme displayed maximum activity at 40 ◦C and pH 4.5.Whereas immobilized enzyme displayed maximum activity at 30◦C and pH 5.0. It displayed a *K*m of 1.0mM, a *V*max of 5.5 U/mL/min. The enzyme was used for dephytinization of soy milk. Immobilized enzyme dephytinized soy milk upto 45% compared to free enzyme.

Keywords: Phytase, *Aspergillus niger*, immobilization, dephytinization, soy milk

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

EP	-	Epoxy
HPLC	-	High Performance Liquid Chromatography
Ins	-	Inositol
IP	-	Inositol Phosphate
P	-	Phosphorus
PA	-	Phytic acid
TEMED	-	Tetramethylethylenediamine

INTRODUCTION

CHAPTER 1

INTRODUCTION

Phytic acid (known as inositol hexakisphosphate (IP6) or phytate when in salt form) is the principal storage form of phosphorous in many plant tissues, especially in bran and seeds of cereals, legumes, vegetables and fruits. Phytate is not digestible to humans or non-ruminant animals, however, so it is not a source of either inositol or phosphate if eaten directly. Moreover, it chelates and thus makes unabsorbable certain important micronutrients such as zinc and iron, and to a lesser extent, also other minerals such as calcium and magnesium. The salts thus formed being sparingly soluble, are very poorly absorbed from the gastrointestinal tract. Hence, there is a strong concern that consumption of food products rich in phytates may lead to malnutrition due to reduced mineral absorption (Harland and Narula, 1999).

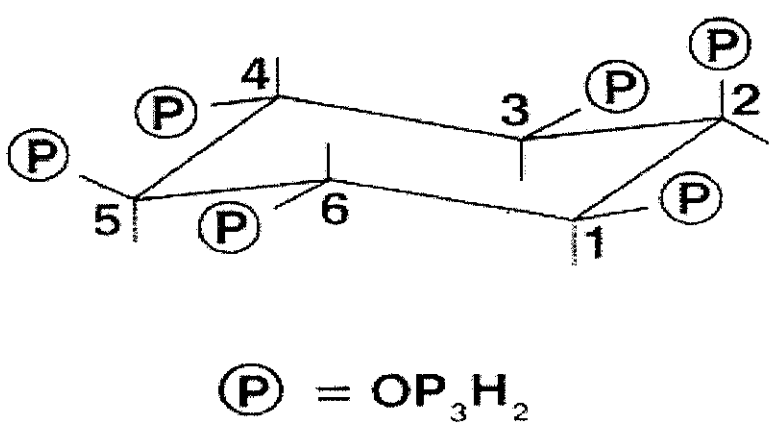

Fig 1.1 Structure of phytic acid

Phytase catalyse hydrolytic degradation of Ins P_6 to myo-inositol phosphates. Phytase is produced by a wide range of plant, bacterial, fungal and yeast sources (Liu *et al.*, 1998; Pandey *et al.*, 2001). Commercial phytases are largely sourced from *Aspergilli*,

as they are the most prolific extracellular producers of this enzyme (Howson and Davis, 1983).Various phytases have been isolated from plants and microbes, and can be grouped based on their pH optima (alkaline or acid phytases), catalytic mechanisms (histidine acid phosphatases, ß-propeller phytase, cysteine phosphatases or purple acid phosphatases), or stereospecificity of phytate hydrolysis (3- or 6-phytases) (Lei *et al.*, 2007) .The first commercial product containing phytase as animal feed additive was released under the trade name Natuphos. Natuphos is a recombinant phytase produced by expressing the phy A gene from *Aspergillus niger* NRRL 3135 in *A. niger* (Kornegay 1999).

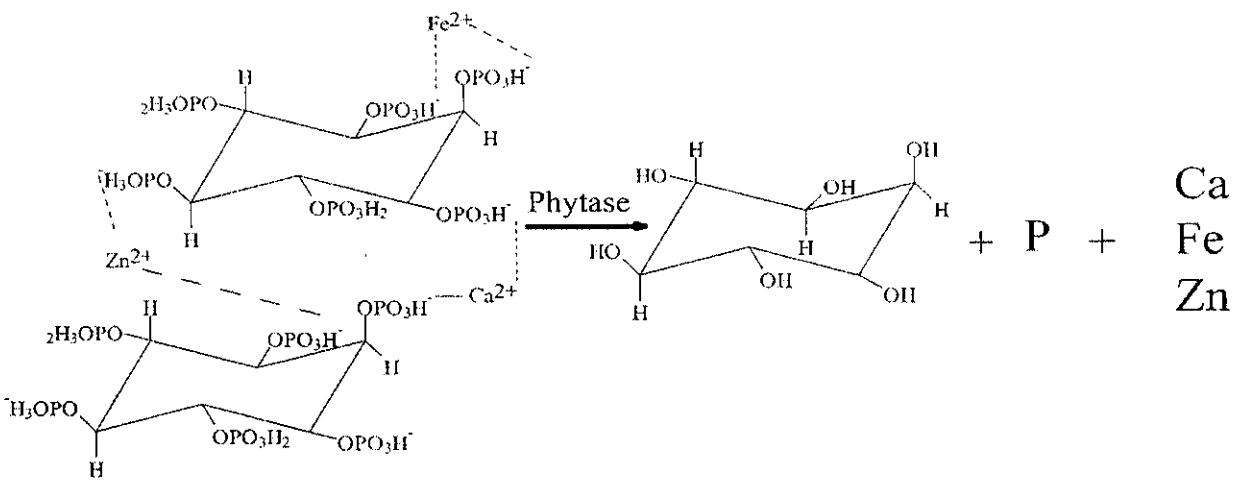

Fig 1.2 Phytate Hydrolysis by Phytase

Phytate hydrolysis by phytase into inositol, phosphate, and other divalent elements. Phytate is *myo*-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate that contains 14 to 28% phosphorus and 12–20% calcium. Phytate also chelates trace elements of iron and zinc (1 to 2%) between phosphate groups within a single phytate molecule or between two phytate molecules. Phytase is the only known enzyme that can initiate the phosphate hydrolysis at carbon 1, 3 or 6 in the inositol ring of phytate. The removal of phosphate group by phytase results in releasing of calcium, iron, zinc, and other metals.

Phytases are not synthesized in the monogastric animals such as fish, pigs and poultry birds (Rao *et al.*, 2009). Hence, phytates cannot be metabolized by the animals. In order to enhance mineral bioavailability in these monogastric animals phytases should be taken along with diet. Phytases are also environment-friendly products, which can reduce the level of phosphate pollution in intensive livestock management areas by avoiding the addition of exogenous phosphate. Disposing of manure containing excess of phytate-phosphorous can damage fresh water and other ecosystem by causing eutrophication.

Application of commercial products containing microbial phytase activity to feed ingredients has proved to be an effective method for improving phytate-P utilization by monogastric animals and thereby reducing P output in manure. Phytate behaves in a broad pH range as a highly negatively charged ion and has therefore a tremendous affinity for food components with positive charge(s), such as minerals like Zn^{2+}, Mg^{2+}, Fe^{2+}, and so on. Besides improving mineral availability, microbial phytases were also shown to ameliorate protein digestibility often reduced by phytate. Supplementation of poultry diets with phytase showed positive effects on growth performance, bone ash, toe ash, egg production, and egg quality. Phytases from different sources have been evaluated individually and in combination for their efficacy as feed additives in poultry. Bacterial phytases with neutral pH optima are being developed for use as feed additives.

Phytase was shown to be an excellent bread making improver (Haros *et al., 2001*). Besides reduction in phytate content in doughs and fresh breads, fermentation time was shortened by phytase addition without affecting the dough pH. An increase in bread volume and an improvement in crumb texture were also observed. In all formulations the hardness or firmness of the bread crumbs was reduced, so softer crumbs were obtained with phytase supplementation. Other texture parameters such as gumminess and chewiness were also decreased. These improvements in bread quality were suggested to be associated with an indirect impact of phytase on a-amylase activity. Addition of phytase during breadmaking results in lower phytate levels in the final breads. Even so, a complete removal of phytate was not achievable. This, in turn, releases calcium ions, which are essential for a-amylase activity, from calcium-phytate complexes. In the final

breads no phytase activity could be detected. Thus intrinsic cereal as well as supplemented microbial phytases was inactivated during baking.

Due to their good nutritional and functional properties, the application of plant protein isolates and concentrates has been found increasingly interesting in food production. However, the relatively high content of phytate present in plant seeds and grains and its interaction with proteins under alkaline conditions, which are, in general, applied for protein extraction, negatively affects the yield and quality of the protein isolates obtained by using common production processes. By interacting with phytate, the solubility of the proteins decreases leading to a reduced protein content in the final concentrate. In addition, a considerable amount of the phytate ends up in the protein isolate affecting its nutritional as well as functional properties. Introducing an exogenous phytase into the production process, however, was reported to result in significantly higher protein yields and an almost complete removal of *myo*-inositolhexakis-, pentakis-, tetrakis-, and trisphosphates from the final plant protein isolate (*Wang* et al., 1999). Due to an improvement in mineral bioavailability, their amino acid composition as well as their *in vitro* protein digestibility, these phytate-reduced plant protein isolates were suggested as suitable protein sources for infant formulae. In addition, some phytate-reduced plant protein isolates are discussed as functional additives in food products, because of their good foaming, emulsifying and gelling properties.

Steeping is a process required in wet milling of maize to obtain the valuable corn steep liquor and to soften the maize kernel as well as to break the maize cell wall. The key issues of corn wet milling are starch yield, corn steep liquor quality and steeping time. Maize comprises phytate, which to a large extent ends up in the corn steep liquor and constitutes an undesirable component. Phytate-free corn steep liquor is easier to concentrate and this concentrate is used in the fermentation industry for the production of compounds such as enzymes, yeast, polysaccharides, antibiotics, and amino acids as well as a high-energy liquid animal feed ingredient. By adding phytases together with plant cell wall degrading enzymes to the steep liquor, corn steep liquor that was entirely free from phytate was obtained (Caransa *et al.* 1988).

OBJECTIVES

The objectives of the present study were:

- To screen and isolate phytase from *Aspergillus niger*.
- To immobilize phytase and study its reusability.
- Dephytinization of food products using partially purified enzyme.

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1 Phytic Acid:

Phytate (*myo*-inositol hexakisphosphate, InsP6) widely occurs in plant seeds and/or grains, roots and tubers, fruits and vegetables, nuts, pollen of various plant species, and organic soils. The phytate fraction of organic soil contains a mixture of phosphorylated derivatives of *myo*-, *chiro*-, *scyllo*-, and *neo*-inositol. Inositol phosphates with fewer than six phosphate groups, such as *myo*-inositol 1,3,4,5,6 pentakisphosphate, have been isolated and identified from the nucleated erythrocytes of birds, turtles, and freshwater fish (Rapaport, S. 1940).

2.1.1 Occurrence and Distribution of Phytic acid:

Phytic acid serves as the storage form of P in plant seeds. Cereals and grain legumes that are commonly used as feed ingredients all have similar phytate levels, approximately 0.25% of dry matter. In general, oilseed meals tend to have higher levels of phytate-P as indicated by the percentage of dry matter composition for soybean(0.39%), rapeseed meal(0.70%), cotton seed meal(0.84%) and sunflower meal(0.89%). On average 70% of the total P in feed ingredient is found as phytate-P.

In plants,phytic acid complexes potassium and magnesium ions, and to a lesser extent calcium ions, to form phytin. Phytin is deposited within the proteinaceous matrix of membrane-bound protein vacuoles or protein bodies. Under the electron microscope,phytin is often visible within the protein bodies as insoluble globoid crystal structurs. In rice grains, globoid crystals are composed of 67% phytic acid, 19% K, 11% Mg and 1% Ca on a dry weight basis (Ogawa *et al.*, 1975). The size of the globoid crystal is dependent upon the ratio of divalent cations/ K in protein bodies. Ratios that favour divalent cations result in large insoluble crystals .Legumes such as soybeans and peas

have higher levels of K and thus smaller globoid structures and a proportionally greater amount of phytin as predominantly K bound soluble forms of phytic acid. This soluble phytin tends to exist as phytin-protein complexes that are evenly dispersed within protein bodies.

The location of phytin-containing protein bodies in the seed varies considerably between plants. In wheat and rice phytin is found primarily in the aleurone layer and the bran, while in maize phytin is concentrated within the endosperm(O'Dell *et al.*, 1972).Phytin tends to be distributed evenly throughout the seed in oilseeds and legume grains . The form and location of phytin within the seeds are likely to be important variables that impact on the efficiency of phytate hydrolysis. One could hypothesize that phytin as insoluble globoid crystals in the fibrous layer of the seed coat would be relatively indigestible in comparison with phytin that exist predominantly as soluble forms in the germ portion of the seed.

2.1.2 Levels of phytic acid:

The amount of phytate varies from 0.06 to 2.22% in cereals, 0.08 to 6% in cereal-milled fractions and protein products, 0.03 to 2.41% in various types of breads and other products, 0.05 to 3.29% in ready-to-eat cereal products, and 0.06 to 1.38% in infant cereals. Among all cereals, polished rice contains the lowest amounts (<0.25%) of phytate. Some of the ready-to-eat cereals such as wheat cereals (100% Bran, Shredded Wheat, Wheaties, Raisin Bran), and infant cereals have the highest phytate content. Phytate phosphorus accounts for the major portion (>80%) of total phosphorus in cereals and cereal products (Reddy., 2002). Of the total phosphorus, phytate phosphorus represents 73.7–81% in brown rice, 51–61% in polished rice (Reddy and Salunkhe, 1980) , 60–80% in wheat , 55–70% in barley, 48.7–70.9% in oats, 38–66% in rye (Lolas *et al.*,1976), 18–73% in triticale (Singh and Reddy, 1977), 71–88% in corn, 87.1% in high lysine corn , 63.9–90.5% in sorghum, 88.9% in high tannin sorghum , 58.3–78% in ragi (common millets) (Rao and Deosthale, 1988), 70.4% in foxtail millets, 64–85.7% in rice bran, 49.6–93% in wheat bran, 59–76% in wheat middlings , 54% in oat bran , 80%

in oat white flour , 84% in Quaker instant oats with bran and raisins , 34% in Quaker old fashioned oats , 15–33% in white breads , 55% in brown bread , and 38–66% in whole wheat bread (Tongkongchitr *et al.*, 1981).

Phytate content ranges from 0.17 to 9.15% in whole beans, 0.58 to 4.20% in bean flours and bean protein products, 0.05 to 5.20% in bean-based foods, and 0.004 to 0.03% in crude soybean oil. Phytate phosphorus accounts for 50–70% in soybeans , 27–87% in lentils, 40–95% in chickpeas , 39.5–95% in broad beans , 36–53% in peas, 75–76% in pigeon peas , 70–87% in linseed (Ferrando., 1983), 31–60% in lima beans (Olghobo and Fetuga, 1982) , 63.2–69% in green gram (Kumar et al.,1978) , 37–54% in dolique beans , 29.8–71.8% in cowpeas, 74.4–79% in black gram (Reddy.,1978) , 57–81% in navy beans , 68-72% in red kidney beans, 55–80% in Great Northern beans, 73.2–93.3% in dolichos beans, 57–81.6% in peanuts , 44–73% in winged beans , 70% in California small white beans , 20–54.7% in lupine, 58.6% in velvet beans , 77% in tempe and 94.5% in tofu ,60–60.9% in soybean meals 87% in defatted soy flour , and 62% in soy protein isolate (Thompson and Erdman,1982). Among all leafy products and vegetables, tomatoes and okra have high amounts of phytate. Most fruits contain low amounts of phytate. However,some fruits, namely apples, apricots, bananas, pears, peaches, and grapefruit, contain no detectable phytate (Wolters *et al.*, 1993). Nuts are reported to contain high amounts of phytate compared with tubers, fruits, and leafy products and vegetables (Ravindran *et al.*, 1994).

2.1.3 Function of phytic acid:

PA biosynthesis initiates shortly after flowering and it accumulates during development until seed maturation and desiccation. The primary functions of PA in seeds are storage of phosphates as energy source. In addition to its role in phosphate storage, phytase may function as an antioxidant in seeds (Graf and Eaton, 1987). In plant seeds, most of the iron is complexed with phytate (Morris and Ellis, 1976) thereby alleviating the potentially lethal combination of free iron and unsaturated fatty acids in close proximity. Phytate as the mineral bound salt of PA is also an important mineral reserve in

seeds and it is stored in protein storage vacuoles in the aleurone cell layer of embryo of the seed. Lower inositol phosphates are also involved in stress response, membrane biogenesis and intracellular signaling of seeds (Bohn *et al.*, 2008 and Sung *et al.*, 2005).

2.1.4 Dietary intake of phytate:

Average American (weighing 75 kg) consumes about 750 mg phytate per day.(Harland et al. 1988). Vegetarians consume a higher amount of phytate compared to non-vegetarians. Phytate intake also varies with season. For instance, phytate intake in self-selected diets of omnivorous females varied from 585 mg/day in spring to 734 mg/day in winter and from 781 mg/day in spring to 762 mg/day in winter for omnivorous males. Cereals were the major source of phytate for the omnivorous males consuming self-selected diets.

Indian and Nigerian diets also provide high phytate intake because they consist mainly of cereals and beans. The large variation reported for intake of phytate in India may be due to the differences in the socioeconomic groups (urban vs. rural) involved in these studies. The diets of urban populations were reported to be varied and contained more vegetables, fruits, milk, and leafy vegetables. This variation contributed to lower phytate intake in urban populations compared with the variation in phytate intake of rural vegetarian Indian children, adolescents, and adults. On the other hand, Indian vegetarian diets consisted mainly of cereal-based cooked foods (unleavened chapaties) and cooked beans that are known to contain high amounts of phytate .Bindra et al. (1986) indicated that lacto-ovo-vegetarian diets of Punjabi immigrants in Canada contained higher levels of phytate than the diets reported for both omnivorous and vegetarian American diets. The phytate intake for Punjabi immigrants was estimated to be 1487 mg/day. An average Nigerian may consume as much as 2000 to 2200 mg of phytate per day using a typical menu of kidney bean balls, rice, plantains, yams, gari, and pudding .This is three times the estimated intake of phytate in the North American population. Middle Eastern inhabitants also consume very high amounts of phytate in their diets.

2.1.5 Phytate mineral interactions:

Phytic acid has a potential for binding positively charged proteins, amino acids, and/or multivalent cations or minerals in foods (Harland, B.F. 1989). The resulting complexes are insoluble, difficult for humans to hydrolyze during digestion, and thus, typically are nutritionally less available for absorption. Phytate forms chelating conjugates with nutritionally important minerals such as calcium, magnesium, copper, iron (Fe2 and Fe3+), zinc, cobalt, and manganese. Solubility is a prerequisite for absorption of most minerals, although solubility at neutral pH has been shown to be less important for calcium absorption. The chemical structure of phytic acid is indicative of strong chelating potential. Phytic acid has six strongly dissociated protons (pKs 1.1 to 2.1) and six weakly dissociated protons (pKs 4.6 to 10.0). The effect on minerals is observed through the formation of phytate-mineral (M) or peptide-mineral-phytate complexes. These complexes have stoichiometries of the M+(n)-phytate type (n = 1–6). Phytate forms a wide variety of insoluble salts with divalent and trivalent cations. Usually, the divalent cations (e.g., Zn2+, Ca2+, Mg2+) form insoluble penta- and hexa-substituted salts. The insolubility of these complexes is regarded as the major reason for the reduced bioavailability of minerals due to diets high in phytic acid. When the complex includes peptides, bioavailability of proteins and enzymatic activity may be reduced .Humans lack sufficient intestinal phytase to degrade the complexes. As much as 30–97% of the intake of phytic acid (0.3–3.7 g/d) may be undigested before it reaches the colon, and this phytic acid may have protective or adverse effects on colonic health. Several factors determine the effect of phytate on mineral bioavailability: pH, size and valence of the mineral, mineral and phytate concentrations and ratios, and food matrix that includes the presence of enhancers and/or inhibitors.

2.1.6 Determination of phytic acid:

An HPLC procedure was developed for analysis of InsP6 in which error due to coelution with the solvent front was eliminated (Lee, K. and Abendroth, J.A. 1983). Precipitation

methods are based on the principle that InsP6 forms an insoluble stable complex with ferric ion in dilute acid and, presumably, are the only phosphate compound with that property.Phytic acid has been determined by ion exchange procedure (Harland and Oberleas, 1977), Rapid colorimetric method (Latta and Eskin, 1980) and by titration (Reever *et al.*, 1979). Leslie Young (1935) method of phytate determination is widely applied to plant products and food stuffs.

A disadvantage of these methods is the lack of specificity in distinguishing between $InsP_6$ and its degradation products. Because inositol phosphates with three to five phosphate groups ($InsP_3$–$InsP_5$) as well as $InsP_6$ have been shown to be nutritionally significant, it is of great importance to have a reliable method for the determination of the individual inositol phosphates. There are also difficulties in determining low $InsP_6$ levels using the precipitation and anion-exchange methods. The amount of detectable inositol phosphates should be at least in the nanomolar range, due to the low inositol phosphate concentrations in biological samples. Thus, a sensitive analytical method for the determination of $InsP_6$ is required. With the development of ion-pair HPLC procedures and capillary electromigration methods, it became possible to study $InsP_6$ and some of its hydrolysis products during food processing and digestion. These methods are relatively easy to handle with a short and simple procedure, but they do not differentiate isomeric forms of inositol phosphates (Skogland and Sandberg) applied to plant products and food stuffs.

2.1.7 Methods for decreasing the levels of phytic acid:

A number of methods have been reported for decreasing phytate such as quick cooking, dehulling, soaking, cooking and germination of seeds to increase phytase activity (Kyriakidis *et al.*, 1988).

1) Reduction by diffusion: Soaking and cooking belong to this group. Only diffusion of phytic acid into medium could explain the reduction of phytic acid since phytic acid has been shown to be heat stable (Oatway *et al,* 2001). With diffusion of phytic acid,

minerals will also be lost. The content of IP_6+IP_5 (one phosphorus in IP_6 was removed by hydrolysis) in rice flour was reduced by 60% and 65% after 1 and 6 h soaking in water, and almost completely after 12 h soaking (Perlas and Gibson, 2002). The phytic acid concentration of brown rice and white rice was decreased by 24% and 65% respectively after cooking.

2) Reduction by degradation of phytic acid: fermentation, germination and application of phytase belong to this group. By the activity of endogenous or exogenous phytases, phytic acid is hydrolysed and degraded to lower inositol phosphates. These treatments could only affect contents of IP_6, while having little effect on mineral levels. Natural fermentation can achieve a large reduction in phytic acid in rice flour by the action of bacterial as well as grain phytases. These reduce the hexa form of phytic acid into lower forms, which have a lower binding capacity for metals like iron and zinc. Results of fermentation on wheat products (bread) showed that it can improve in vivo bioavailability of minerals significantly. Studies on legumes, millets, oat, and barley indicated that germination could decrease phytic acid with 30 to 90%, depending on the type of cereal. Decreased phytic acid levels resulted in a significantly improved bioavailability of micronutrients (Mamiro *et al,* 2001).

3) Reduction by removal: milling belongs to this group. Reduction of phytic acid in this group depends on the distribution of phytic acid in rice kernels. Minerals will also be lost with decrease of phytic acid. Loss of phytic acid during milling was about 70% (Tabekhia and Luh, 1979).

2.2 PHYTASE:

Phytase enzyme preparations have a wide range of applications in animal and human nutrition. Phytases decompose phytates (myo-inositol-1,2,3,4,5,6-hexakisphosphates), the salts of phytic acid Phytate is regarded as the primary storage form of both phosphate and inositol in plants (Cosgrove 1966). The phosphorus fraction stored as phytate range from 30% in roots up to 80% in seeds and cereals. Phytic acid is a

polyanionic chelating agent that forms complexes with several divalent cations of major nutritional importance, e.g., Ca2+, Mg2+, Zn2+, Cu2+, Fe2+, and Mn2+ (Harland and Oberleas 1999). The stability of the different salts mainly depends on the type and concentration of cation and pH. Phytic acid can also form complexes with proteins and amino acids at both acidic and alkaline pH. The term phytase (myo-inositol hexakisphosphate phosphohydrolase) describes a class of phosphatases with the in vitro capability to release at least one phosphate from phytate. Despite this definition, up to now, myo-inostitol pentakisphosphate (IP5) has yet to be identified as the final product. Usually, the degradation ends with the less phosphorylated myo-inostiol phosphates IP3 (Hara et al. 1999). The International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC–IUB) distinguish two classes of phytate degrading enzymes, 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.28), initiating the dephosphorylation at the 3 and 6 positions of phytate, respectively. Phytases are widespread in nature because they can be found in animals, plants, and microorganisms.

2.2.1 Microbial phytases:

Most of phytases isolated from fungi and yeast are 3-phytases, histidine acid phosphatases, glycosylated, and active for a wide variety of substrates.There have been a number of reports on purification and characterization of phytase from variety of microbial species.Best characterized is the phytase from *Aspergillusficuum* (Ullah A., Gibson D.M. (1987)). Also purified and characterized are phytases from *Aspergullus oryzae* (Shimizu M. (1993)), *Aspergillus niger* (Nagashima T., Tange T., Anazawa H. (1999)), *Aspergillus fumigatus* (Pasamontes L., Haiker M., Wyss M., Tessier M., van Loon A.P.G.M. (1997)), *Bacillus subtilis* (Shimizu M. (1992)), *Schwanniomyces castellii* (Segueilha L., Lambrechts C., Boze H., Moulin G., Galzy P. (1992)).Although a variety of phytate-degradingenzymes differing in their pH- and temperature optima, specific activity, and substrate specificity have been identified and purified to homogeneity or near-homogeneity screening for enzymes with properties useful for application is of interest. The use of filamentous fungi for the production of commercially important metabolites 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 batch solid-state fermentation (SSF) has addressed the production of many innovative and high value products, *e.g.*single-cell protein (SCP), protein enriched feed, ethanol,enzymes, mycotoxins, from starchy materials and a variety of wastes utilized by fungi (D. A. Mitchell, B. K. Lonsane,1992). In general, SSF in terms of equipment and process control, less expensive and often results in higher yields compared to submerged fermentation.

Among the factors which influence process yields, the quality of the inoculum is regarded as the parameter that determines the course of a fungal fermentation (C. Parton, P. Willis,1990).Inoculum morphology has been studied and correlated with process yields for a number of important submerged fermentation processes (R. Steel, S. M. Martin, C. P. Lentz, *Can. J.* (1954–1955). On the other hand, published research concerning morphological aspects of inoculum for solid-state fermentations is very limited. *Aspergillus niger* PhyA was the first well-characterized and commercialized phytase. Phytases isolated from bacteria are non-glycosylated histidine acid phosphatases or alkaline phytases with a ß-propeller structure. *Escherichia coli* AppA phytase is a periplasmic protein with a molecular mass of approximately 42 kDa. Because of its acidic optimal pH, it has a strong resistance to pepsin hydrolysis and high specific activity for phytic acid, *E. coli* AppA phytase is more effective than *A. niger phytase* in releasing phytate-phosphorus in diets for swine and poultry (Lei *et al.,* 2007).There is only one report on occurrence of phytase activity in *Escherichia coli* but without any characterization of the enzyme(s).Four acid phosphatases have been identified in the perisplasmatic space of *Escherichia coli.*

2.2.2 Plant phytases:

Plants may express high levels of phytases in storage compartments such as seeds for the purpose of utilizing Pi and energy required for growth of the germinating plant. Phytase enzymes have been isolated and characterized from a number of plant sources; the initial preparation of phytase made from rice bran (Suzuki et al., 1907). It is, however, only recently that some phytases have been isolated and purified to

homogenity. Considering the in vitro degradation of InsP6 by plant phytases such as wheat phytase and the phytase from rye, spelt and barley are reported to be 6(4)-phytases. The major InsP5 in raw soybean was dl- Ins (1,2,4,5,6)P5 and thus soybean phytase seems to be a 3-phytase. Another leguminous plant, pea, was also found to have a degradation pathway of InsP6 dissimilar to that of cereals. The isomeric pattern during degradation of InsP6 in oats, rye and barley were found to be similar to that formed by hydrolysis by wheat phytase. The same InsP6 degradation pathway may be assumed in these cereals.

2.2.3 Animal phytases:

Although phytase activity has been detected in tissues of several animal species (Bitar and Reinhold, 1972), there is no complete molecular characterization of any of animal-derived phytases. Many of these enzymes display an optimal pH in the neutral to alkaline range, with K_m for phytate ranging from 0.03 to 2.6 mM. However, phytase detected in brush border vesicles of poultry showed an optimal pH of 5.5–6.0 and phytase in the hybrid stripped bass showed an optimal pH of 3.5–4.5.Phytase activity found in the large intestine or rumen is mainly microbial origin.

2.2.4 Market Trends and manufacture:

'Cenzyme' is a product from Cenzone, which is a unique blend of concentrated digestive enzymes including phytase from a fungal source. It has application in animal feed. Gist Brocades manufactures feed enzyme containing phytase under the trade name of 'Natu-phos'. The largest market share (40%) in feed enzymes is held by Finnfeeds International, a unit of Finland's Cultor, followed by BASF (probably the number two feed enzyme producer). However, the latter, which has marketing agreement with Dutch enzyme producer company Gist Brocades, is the leading marketer of phytase world-wide. Finnfeeds has recently developed a phytase. Novo industry too is marketing a phytase in Europe. Alltech has established a manufacturing facility in Mexico for the production of phytase (Pandey *et al.*, 2001)

S.No	Company	Trademark	Phytase source	Production strain
1.	BASF	Natuphos	*Aspergillus niger*	*Aspergillus niger*
2.	AB Enzymes	Finase	*Aspergillus awamori*	*Trichoderma reesei*
3.	Novozymes	Bio-Feed Phytase	*Peniophora lycii*	*Aspergillus oryzae*

Source: Haefner *et al.*, 2005

2.3 APPLICATIONS OF PHYTASES:

Phytase can be used in food and feed industry to eliminate phytate. The main reason for removal of phytate is its undigestibility for monogastric animals including man. Increased dietary consumption of cereal fibers, legumes and soy protein isolates results in an increased intake of phytate (Dvarakova., 1998). Vegetarians eating mostly whole grain products and extruded cereals, elderly people consuming unbalanced food with a lot of cereals, people who eat unleavened bread and babies fed with soy based infant formulas take in large amounts of phytate. Phytase is applied in bakery technology and animal feed additive. Phytate phosphorous is largely unavailable to monogastric animals due to lack of adequate levels of phytate degrading enzymes in their gastro intestinal tract. Since phytic acid cannot be utilized as source of P, feeds for pigs and poultry are commonly supplemented with inorganic phosphate to meet their P requirement. Supplemented inorganic P and phytate P impose global ecological problems (eutrophication) when entered into rivers, lakes and other water bodies, resulting in algal blooms, hypoxia and death of marine animals (Singh and Sathyanarayana, 2010). Phytase passes undigested through the digestive tract of monogastric aanimals as they produce little or no intestinal

phytase activity. As a result monogastric feed must usually be supplemented with inorganic phosphate (Casey and Walsh, 2004)

2.3.1 Application in animal nutrition:

Phytase is incorporated into commercial poultry, swine, and fish diets to improve the availability of phosphorus, minerals, amino acids, and energy. Cereals constitute a large portion of pig and poultry diets. Adequate levels of phosphorus are critical to the growth and development of all organisms for a range of functions such as macromolecular structure, energy generation, and metabolic regulation. The demand for phosphorus increases dramatically during periods of rapid cell growth and division, such as seed germination (Hegeman *et al.*, 2001). About two-thirds of the phosphorus in cereal grains are present in the form of phytate-phosphorus. Phosphorus in this form is nearly unavailable to monogastric animals because they lack the enzyme phytase required to cleave the phosphorus from the phytate molecule.

The consequence is that the phytate-phosphorus is excreted with the manure and contributes to phosphorus pollution especially in regions with intensive animal production. The amount of phosphorus excreted can be markedly reduced by providing the animal diets with an efficacious source of phytase which is capable of hydrolysing the dietary phytate during gastrointestinal passage. Besides additives of microbial phytases commercially available nowadays, there are some cereals like rye, triticale, wheat and to a lesser extent barley that contain high phytase activities (Zimmerman *et al.*, 2002).

2.3.1.1 Phytase additive in swine feed:

The biological availability of plant phosphorus for the young pig varies between 15 and 50% in grains and 18 and 39% in soybean meal. The addition of microbial phytase to a diet in which most of the phosphorus was from organic sources resulted in improved rate and efficiency of gain, phosphorus digestibility, serum phosphorus, serum alkaline phosphatase activity, bone ash, and weight of phosphorus in

bone, indicative of increased dietary phosphorus availability. Microbial phytase reduced the requirement for total dietary phosphorus and decreased fecal phosphorus (Young *et al.*, 1993). *E. coli* phytase had superior efficacy compared with either Natuphos or Ronozyme in the young chick at equivalent activity levels. The P-release values, while consistently high for E coli derived phytase, were unexpectedly low for both commercial phytases. The pig trial revealed a high P-release value for E.Coli derived Phytase, but the P-release values for Natuphos and Ronozyme were greater—the former much greater—than in the chick. Hence, the advantage for the *E. coli* phytase compared to the commercial phytases was much greater in the chick than in the pig (Augspurger *et al.*, 2003).

2.3.1.2 Phytase additive in poultry feed:

The dietary supplementation with a multi-enzyme complex containing nonstarch polysaccharide enzymes and phytase is efficient in reducing the P, energy, protein, and amino acid specifications of corn-soybean meal diets (Francesch and Geraert, 2009). Three experimental phytase enzyme preparations derived from the same *Escherichia coli* gene but produced in *Saccharomyces cerevisiae* (A), *Pichia pastoris* (B), and *Pseudomonas fluorescens* (C) were compared with a commercial enzyme preparation by addition to wheat-soybean meal diets fed to broiler chicks. Chick performance and nutrient digestibility showed that the pelleting process inactivated enzymes A and C and the commercial enzyme. When added to the negative control diet, enzyme B had positive effects on broiler performance and calcium and phosphorus digestibility, and increasing levels of enzyme had greater positive effects (Silversides *et al.*, 2004). Wheat bran phytase improved the production performance of laying hens and utilization of total phosphorus and crude protein. It is feasible to completely replace the addition of inorganic phosphorus by wheat bran and microbial phytase in laying hen diets (Yao *et al.*, 2007). Birds fed with phytase-supplemented diets also showed increased plasma magnesium and zinc levels when compared to the birds fed on deficient diet (Rao *et al.*, 2009).

2.3.1.3 Phytase additive in Aquafeed:

Plant ingredients as feed are rich in phytic acid, which reduces the bioavailability of nutrients like minerals and protein to the fish, thereby causing aquaculture pollution. Dietary phytase treatment reduces the aquaculture pollution by improving the bioavailability of nutrients, and reduces the feed cost (Baruah *et al.*, 2004). Supplemental phytase can enhance the digestibility and bio-availability of P, nitrogen and other minerals reduce the amount of inorganic-P supplement to maximize growth and bone mineralization, and markedly decrease P load to aquatic environment (Cao *et al.*, 2007). Atlantic salmon (*Salmo salar*) fed with phytase-supplemented meal exhibited enhanced growth and neutralized the negative effect of phytate on protein digestibility (Sajjadi and Carter, 2004).

2.3.2 Human:

Effective and complete degradation of phytate occurred in the stomach when *Aspergillus niger* phytase was given with the meal was reported by Sandberg *et al.*, 1995. Low bioavailability of Zn from soy formula is a function of its phytate concentration and can be overcome by the removal of phytate (Lonnerdal *et al.*, 1988).

2.3.3 Food processing applications:

Haros et al., 2001 investigated the possible use of phytase in the process of bread making. Different amounts of fungal phytase were added in whole wheat breads, and it was shown that phytase is an excellent bread-making improver. The main achievement of this activity was the shortened fermentation period without affecting the bread dough pH. An increase in bread volume and an improvement in crumb texture were also observed. Fermentation time was shortened by phytase addition without affecting the dough pH (Greiner and Konietzny, 2006).

2.4 Immobilization of phytase:

Celam and Onal in 2009 immobilized phytase from soyabean sprouts on Epoxy activated Sepabead EC-EP. The optimum temperature and pH for the activity of both free and immobilized enzymes were found as 60 °C and pH 5.0. It was applied to degrade soyamilk phytate. Free and immobilized enzyme hydrolysed the soymilk phytate very quickly within 5 h (free enzyme hydrolysed 90% and immobilized enzyme hydrolysed 76% of phytate). Avocado Phytase was also immobilized on epoxy activated Sepabead EC-EP.

The optimum pH values of free and immobilized enzyme were pH 4.0 and 5.5 respectively. Free enzyme showed 92% activity at 60°C whereas immobilized enzyme showed 86% activity at 60°C. Soluble phytase degraded 56% and immobilized phytase degraded 65% of phytate in soymilk.

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1. MATERIALS:

Some of the chemicals used are Phytic acid dodecasodium salt from Sigma Chemical Co (USA). Trichloro acetic acid and Magnesium sulphate from Merck.

3.2. MAINTENANCE OF *Aspergillus niger* CULTURES:

Aspergillus niger isolated from soil was sub-cultured in potato dextrose agar for a period of 5 days.Pure strain of *Aspergillus niger* thus obtained was used.

Fig 3.1 Sub-culture of *Aspergillus niger* on potato dextrose agar for the experiments.

3.3. SOLID STATE FERMENTATION:

Five growth substrates Wheat Bran, Coconut oilcake, Groundnut oilcake, Sesame oilcake and Neem oilcake were used for the growth studies of *Aspergillus niger*. 20 g of each substrate was taken in a conical flask and 30 ml of water was added to each. Inoculation of *Aspergillus niger* in these five substrates was done aseptically. From the third day of growth, enzyme was extracted using calcium chloride and assay was carried out from day 1 to day 5.

3.4. CRUDE ENZYME SEPERATION:

The enzyme was extracted from the growth substrate by adding 100 ml of 2% aqueous solution of calcium chloride for 20 g of the substrate used. Flask was kept on a rotary shaker at 200 rpm for a period of 2 hours at room temperature. After 2 hours, the suspension was squeezed through a double layer of muslin cloth and it was centrifuged at 5000 rpm for 20 minutes at 4°C to obtain an extract containing crude phytase.

3.5. ASSAY OF PHYTASE ACTIVITY:

Phytate degrading activity was determined at 50°C in 350µL of 100mM sodium acetate buffer pH5 containing 1.03mM sodium phytate. The enzymatic activity was done by adding 10µL of enzyme solution to the assay mixture. After incubating for 30 minutes at 50°C the liberated phosphate was measured accordingly to ammonium molybdate method (Jukka, Heinonen, Lahti, 1981) with some modification.

Added to the assay mixture was 1.5 mL of a freshly prepared solution of acetone:5N sulphuric acid:10mM ammonium molybdate (2:1:1 v/v) and 100 µL of acetic acid. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355nm.To calculate the enzyme activity, a calibration curve was produced over the range of 5 to 600 mM phosphate.

$$\text{Units/ml enzyme} = \frac{(\mu\text{moles of Phosphate released})*(df)}{(T)*(0.01)}$$

Where,

df = Dilution factor;
T = 30 minutes (incubation); and
0.01 = Volume (in milliliters) of enzyme used.

3.6. ESTIMATION OF PROTEIN:

Protein concentration was measured by the method of Lowry *et al.*, (1951) using Bovine Serum Albumin as a standard. Protein assay mixture consisted of 0.1 ml of sample and 0.9 ml of distilled water. To this solution 2.10 ml of alkaline copper reagent was added. This was then kept at room temperature for 10 mins. After which 0.2 ml of Folin's reagent was added and the mixture was incubated at room temperature for 30 mins. Blue colour developed was measured at 660 nm. Distilled water was used as blank.

3.7. PARTIAL PURIFICATION OF PHYTASE:

The crude extract was used for an ammonium sulfate precipitation at 4 °C and 90% saturation. The precipitate was collected by centrifugation at 10000 rpm for 10 min and suspended in 200 mM sodium acetate buffer and dialyzed against 50 mM sodium acetate buffer, pH 5.0.

3.8. DEPHYTINIZATION OF SOY MILK:

5mL of soy milk was mixed with 5 mL of distilled water and incubated at 37 °C, 100 rpm with enzyme sample (10U/ml). Control without the addition of enzyme was performed and phytic acid determination was done in both control and test samples.

3.9. DETERMINATION OF PHYTIC ACID:

Phytic acid was determined by the method of Wheeler and Ferell, 1971. In this method phytic acid is extracted with 3% TCA for 30 min with mechanical shaking. Suspension is centrifuged and 10 mL aliquot is transferred to a centrifuge tube.4 mL of $FeCl_3$ is added by rapid blowing. The tubes are heated in boiling water bath for 45 min.The precipitate is washed twice by dispersing in 20-25 mL TCA (3%), heated in boiling water bath 5-10 min and centrifuged. The precipitate is dispersed in a few mL water and 3 mL of 1.5N NaOH is added and mixed. Volume is made upto 30 mL with water and heated in boiling water bath for 30 min, filtered and precipitated with 60-70 mL hot water and filtrate is discarded. Precipitate is dissolved with hot 3.2 N HNO_3. 5 mL aliquot is diluted to 70 mL and 1.5 M KSCN and color read at 480 nm. Iron content is calculated from ferric nitrate standard. The phytate phosphorous is calculated from the iron results assuming a 4:6 iron: phosphorous molecular ratio.

3.10. IMMOBILIZATION:

3.10.1. ENZYME IMMOBILIZATION IN AGAR:

Tablet strips were used for the purpose of casting agar. It could be easily poured into the tablet strips to obtain the agar tablets of uniform shape and size. In order to optimize the percent immobilization, various concentrations of agar (0.5 to 10% w/v) and protein were used. For immobilization, the molten agar and soluble phytase were mixed and immediately poured into moulds before the time of solidification. Care was taken to prevent the formation of air bubbles.

3.10.2. ENZYME IMMOBILIZATION IN AGAROSE:

Soluble phytase and agarose were mixed and immediately casted into moulds. Immobilization of soluble phytase done at two concentration of agarose (2 & 2.5%).

3.10.3. PHYTASE IMMOBILIZATOIN IN ALGINATE GEL:

Enzyme solution of 1ml was added to 1 ml of 3% (wt) sodium alginate solution. The beads were formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100 ml) of stirred 0.2M $CaCl_2$ solution with a syringe and a needle at room temperature. The beads were left in the calcium solution to cure for 0.5-3 hours.

3.10.4. PHYTASE ENTRAPMENT IN GELATIN GEL:

Enzyme solution of 1 ml was added to 1 ml of the gelatin solution. 2 ml of the Hardening Solution was added to the above enzyme solution. The enzyme solution was frozen at -28°C for 4 hours to facilitate the gel formation. When the gel is set, warm the gel to room temperature simply by leaving it on a lab bench. The gel was cut into small cubes of approximately 3mm per side. The gel was washed liberally with deionized water.

3.10.5. PHYTASE IMMOBILIZATION IN POLYACRYLAMIDE GEL:

Resolving gel of 10%, 12% and 15% were prepared and enzyme solution of 1 ml was mixed with resolving gel. Finally it was casted into moulds. After gel gets set Enzyme assay was carried out by slicing the gel into pieces.

Composition of gel	10%	12%	15%
Stock Acrylamide (ml)	1.625	1.995	2.49
Acetate Buffer (ml)	0.830	1.000	1.25
TEMED (µl)	1.25	2.5	3.125
Ammonium per sulphate (µl)	20.83	25	31.25
Deionized water (ml)	2.26	2.715	3.393

3.10.6. ASSAY OF IMMOBILIZED PHYTASE:

The agar tablets, Agarose tablet, Calcium alginate beads, Polyacrylamide and Gelatin moulds were incubated with 20 ml of Reaction Mixture (10 ml acetate buffer pH 5,5 ml distilled water, 0.40 ml Magnesium Sulfate and 4.40 ml of phytic acid)After the desired time interval, 1.0 ml of this reaction mixture was taken for color development as described for soluble phytase and absorbance was recorded spectrophotometrically at 660nm.

3.10.7. REUSABILITY OF IMMOBILIZED PHYTASE:

Reusability experiments were performed at 37°C. After incubation, immobilized enzyme beads were removed and washed three times with acetate buffer (0.2 M, pH 5). Activity was determined in the same manner as enzyme assay.

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

4.1. SOLID STATE FERMENTATION:

Aspergillus niger was grown in different substrates such as wheat bran, coconut oilcake, groundnut oilcake, sesame oilcake and neem oilcake.

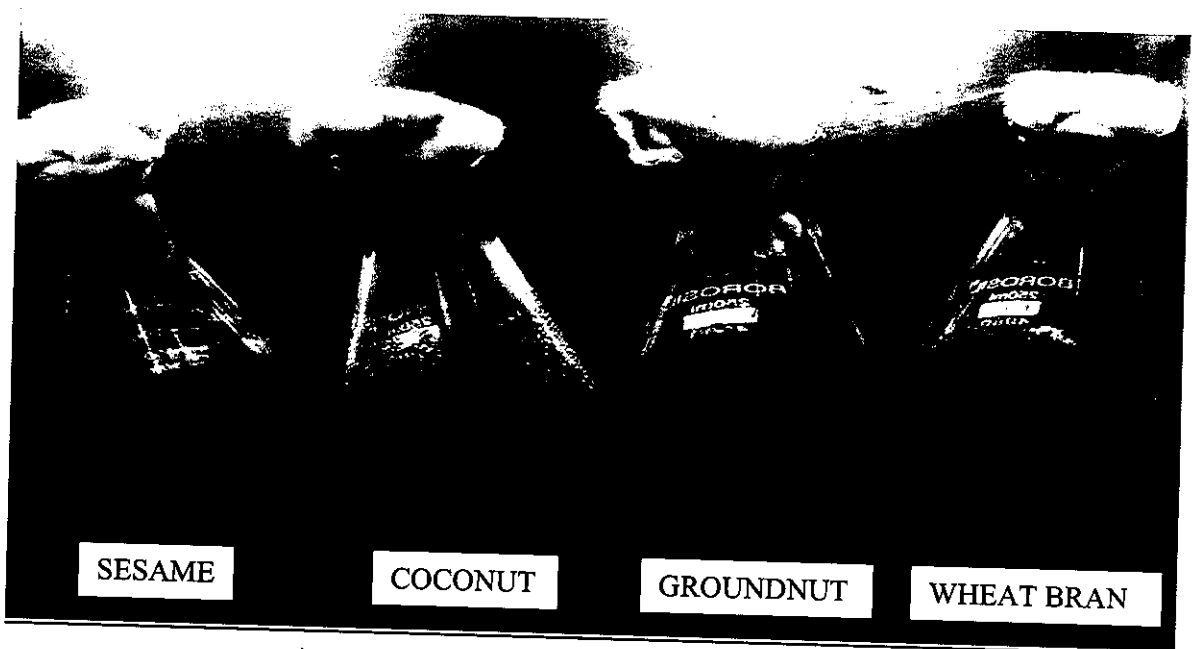

Fig 4.1: Solid state fermentation

Aspergillus niger was well grown in only four substrates such as sesame oilcake, coconut oilcake, groundnut oilcake, wheat bran. There was no growth observed in neem oilcake probably due to its antimicrobial property.

4.2. PHYTASE ACTIVITY ON VARIOUS SUBSTRATES:

The enzyme was extracted from these four substrates from first day of inoculation till fifth day. The enzyme was assayed from first day till fifth day. It was observed that maximal activity of phytase was produced around the second to third day of incubation in various substrates.

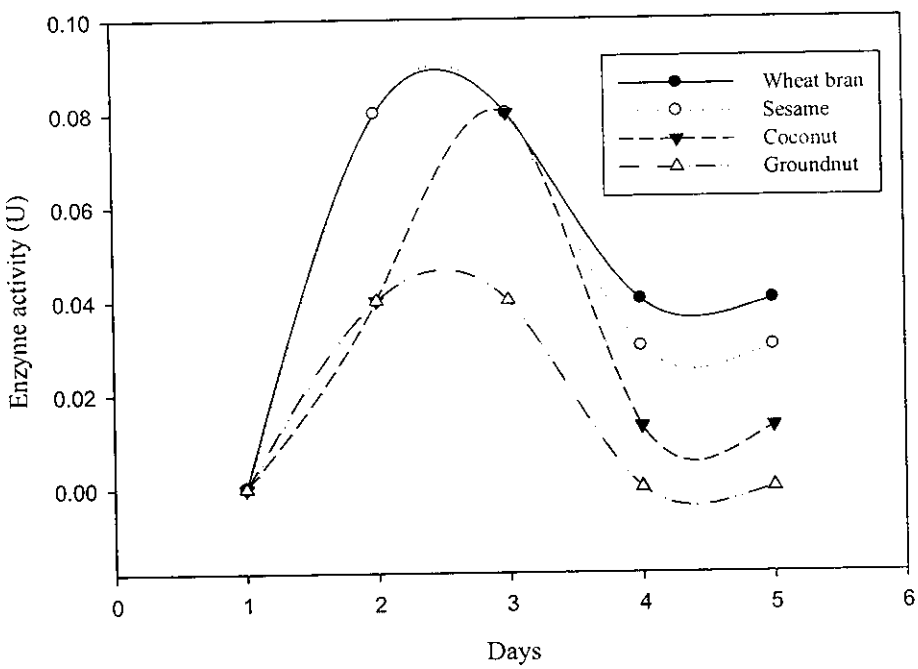

Fig 4.2. Effect of different substrate on enzyme activity

4.3. PROTEIN ESTIMATION:

Protein estimation of the biomass was done by Folin Lowry's method.Protein levels during growth on most substrates peaked and plates used at around 2^{nd} -3^{rd} day of growth.

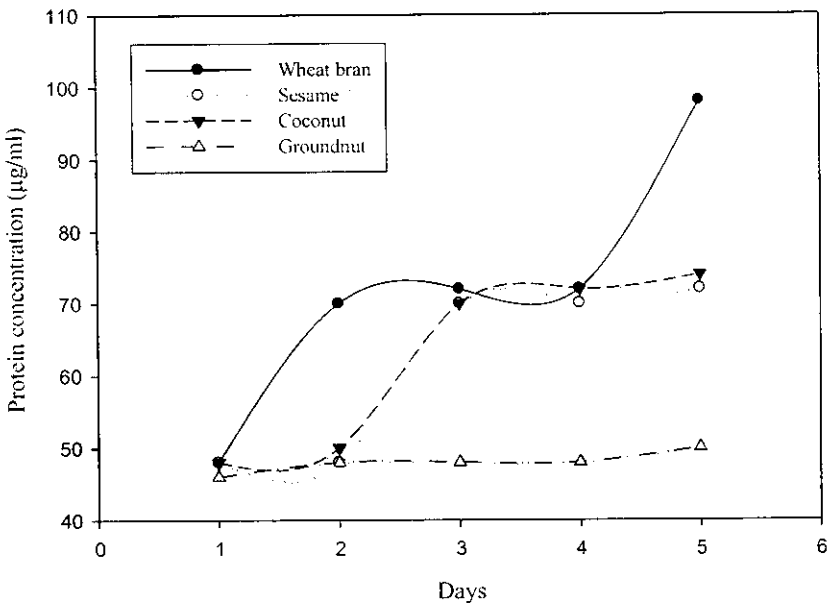

Fig 4.3. Effect of different substrate on protein levels

However maximum Protein concentration was found in enzyme extracted from **wheat bran** beyond four days.

4.4. PARTIAL PURIFICATION OF PHYTASE FROM *Aspergillus niger*

Table 4.1. Partial Purification of phytase from *Aspergillus niger*

S.No	Purification step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Fold Purification
1.	Crude Extract	294	240	0.816	1
2.	90% Ammonium sulphate	9	10	0.9	1.10

Phytase from *Aspergillus niger* was partially purified and it had a Specific activity of 0.9 (U/mg). Further purification increases the cost of enzyme, partially purified phytase is used in dephytinization of food products.

4.5 CHARACTERIZATION OF PHYTASE ENZYME

4.5.1 EFFECT OF pH ON ENZYME ACTIVITY:

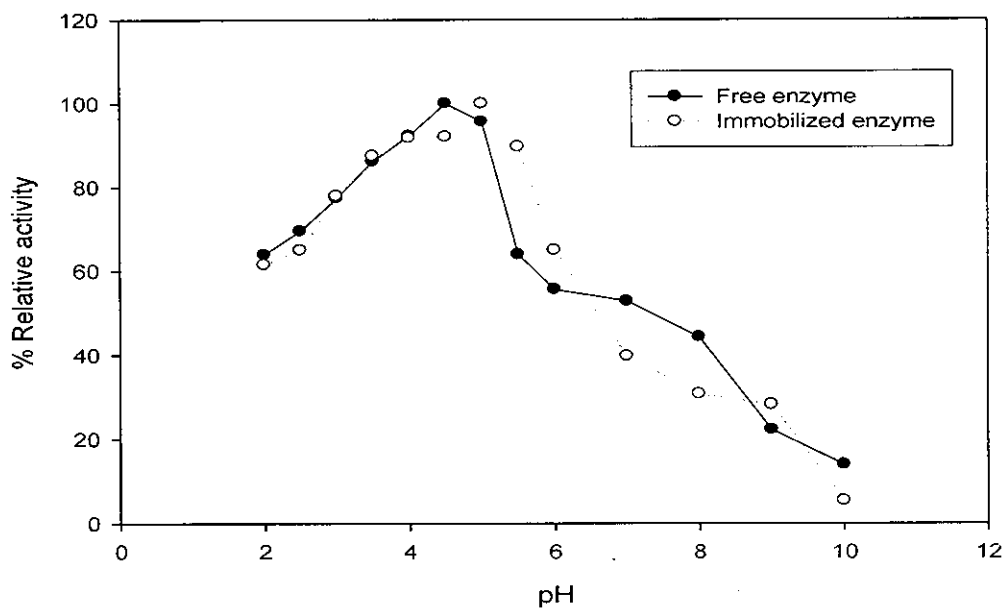

Fig 4.4. EFFECT OF pH ON ENZYME ACTIVITY

- The optimum pH of the free phytase enzyme was found to be **4.5.**
- The optimum pH of the immobilized phytase enzyme was found to be **5.0.**

4.5.2 EFFECT OF TEMPERATURE ON ENZYME ACTIVITY:

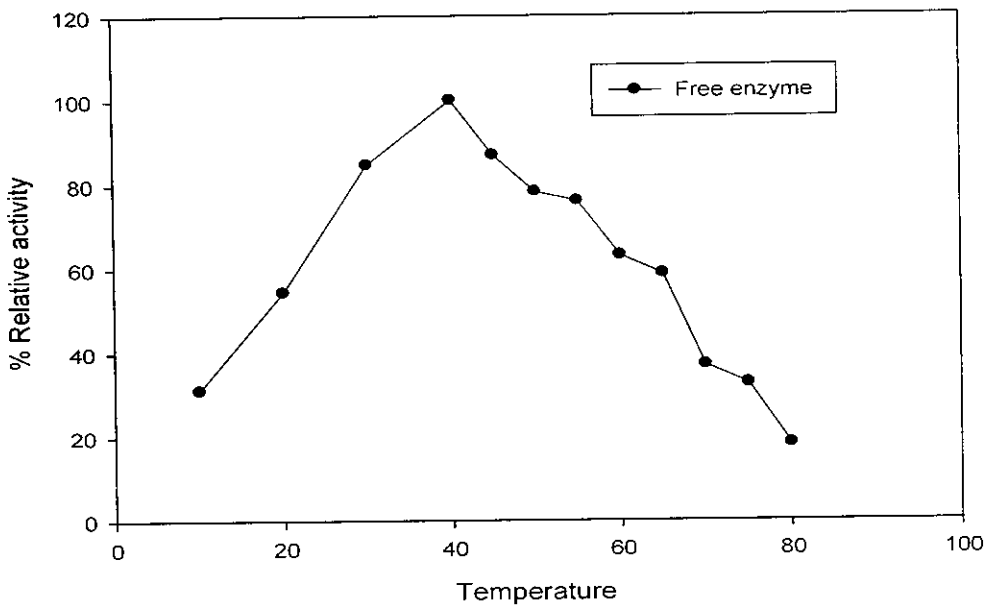

Fig 4.5. Graph showing the optimum temperature of phytase enzyme.

- The optimum temperature of the free phytase enzyme was found to be **40°C.**
- The optimum temperature of the immobilized phytase enzyme was found to be **30°C.**Beyond the optimum temperature the immobilized beads were disintegrated.

4.5.3. EFFECT OF SUBSTRATE CONCENTRATION ON ENZYME ACTIVITY:

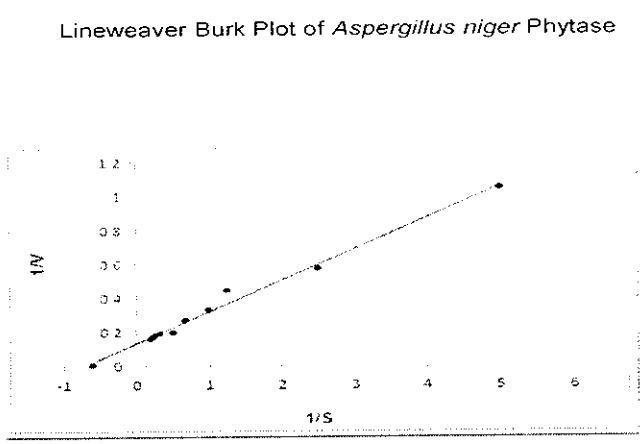

Fig 4.6 Lineweaver Burk plot of the effect of substrate concentration.

From the Lineweaver Burk plot the kinetic constants was found to be **Km=1.0mM** and the value of **Vmax=5.5U/ml/min.**

4.6: IMMOBILIATION OF THE PHYTASE ENZYME:

- The enzyme was immobilized in various matrixes like agar, gelatin, polyacrylamide, agarose and sodium alginate beads.
- The enzyme phytase obtained from *Aspergillus niger* was successfully immobilized only in sodium alginate beads and polyacrylamide.

TABLE 4.2: REUSABILITY OF IMMOBILIZED ENZYME:

No. of reactions	Relative Activity (%)
1	100
2	68
3	32
4	No activity

From the above data, we come to the conclusion that phytase under these conditions can be reused for the maximum of three reactions. Therefore further studies should be carried out to improve stability and reusability of the enzyme by addition of additives etc.

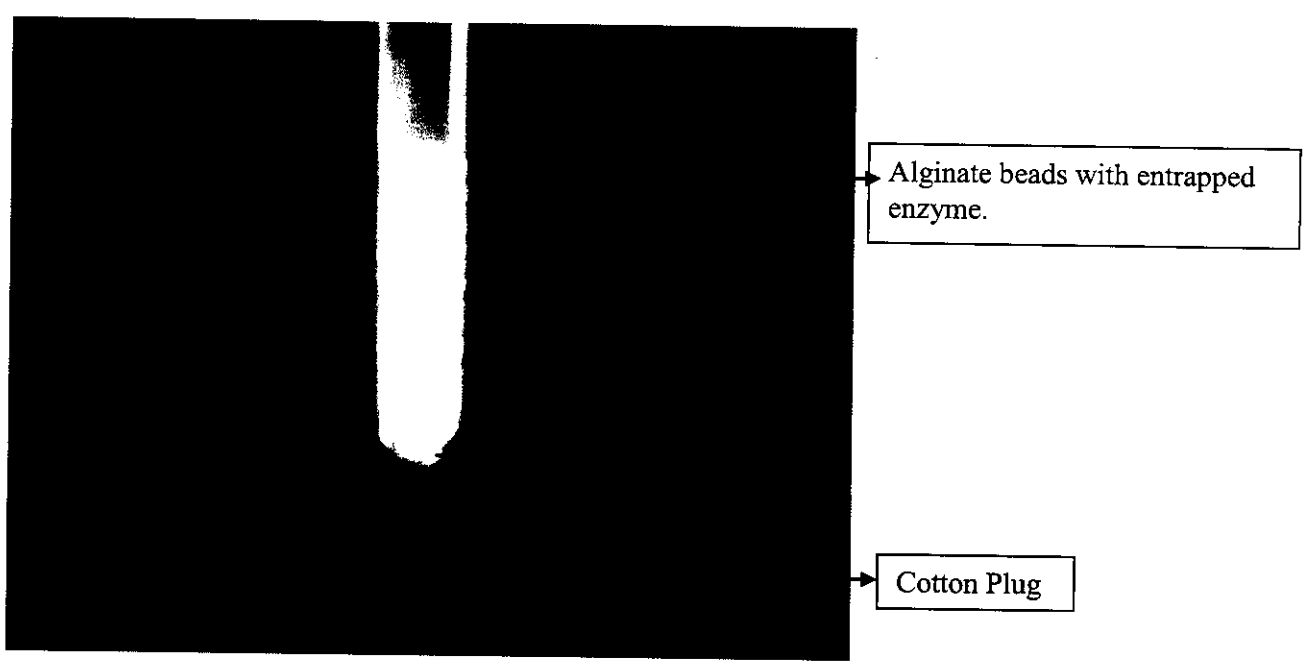

Fig 4.7 Enzyme immobilized and packed in a column.

4.7: DEPHYTINIZATION OF UNPROCESSED SOY MILK

Table 4.3: Dephytinization of Unprocessed soy milk by Phytase from *Aspergillus niger*

S.No.	Type of enzyme	Initial phytate content (mg/100g)	Final phytate content (mg/100g)	% Dephytinization
1	Free enzyme	590	286	51.52
2	Immobilized enzyme	590	453	23.22

Free enzyme dephytinized soy milk up to **51.52%** while immobilized enzyme dephytinized soy milk up to **23.22%.**

4.8: EFFECT OF PHOSPHOROUS RELEASE BY PHYTASE FROM *Aspergillus niger*

The effect of phosphorous release by phytase from *Aspergillus niger* on soy milk was performed.

Table 4.4: Effect of Phosphorous Release by Phytase from *Aspergillus niger* on soy milk.

Type of enzyme	Food Product	Free Phosphorous before Enzyme treatment (µg/ml)	Free phosphorous liberated after Enzyme treatment (µg/ml)
Free enzyme	Soy milk	223	389
Immobilized enzyme	Soy milk	223	316

CONCLUSION

CONCLUSION

Aspergillus niger was grown under solid state fermentative conditions with four different substrates like wheat bran, sesame oilcake, groundnut oilcake, coconut oilcake out of which wheat bran has proved to be the effective substrate for obtaining maximum enzyme activity. *Aspergillus niger* grown using wheat bran as substrate was used to isolate phytase followed by partial purification. The partially purified enzyme was immobilized and its reusability was studied. The enzyme was characterised with respect to pH, temperature and substrate concentration. Both free enzyme and immobilized enzyme were employed for dephytinization of soy milk. It was found that free enzyme dephytinized soy milk better than immobilized enzyme.

REFERENCES