Introduction

Phytic acid

Phytic acid is the 6 phosphates ester of inositol. It is found in almost grains and fibers of plants in the form of insoluble calcium and magnesium salts [called Phytin] which are considered as the storage of organic phosphorus in plants [Ullah and Sethumadhavan, 1998]. The most outstanding feature of phytic acid is its powerful chelating function that can be adapted in biological, in foods as well as in industrial fields. Many species of plants contain appreciable amounts of phytate phosphorus [P]. In cereal grains, oilseeds and grain legumes, high levels of phytic acid were obtained, and phytate P constituted the major portion [60–82%] of total P [Dvorakova, 1998]. The various roots and tubers contained moderate amounts of phytic acid and phytate P accounted for 21–25% of the total P in this food group. Leafy greens contained negligible amounts of phytate P [Jongbloed et al, 1992]. In rice bran and the various oilseed meals, phytate P constituted 56–77% of the total P [Al Ashesh and Duvnjak, 1994]. This organically bound form of phosphorus is poorly utilized by monogastric animals, such as poultry and swine, because their simple digestive tract cannot hydrolyze substantial amounts of phytate and in order to supply enough of this nutrient, additional phosphate was required in the feed ration [Conrad et.al, 1996]. Rock phosphate soon proved to be a cost-effective means of supplying this additional phosphorus, and the excess phytin phosphorus could be disposed of easily with the animals' manure. However, this additional phosphorus creates a massive environmental problem when the land's ability to bind it is exceeded. Selective enzymatic hydrolysis of phytate phosphorus should release the phosphorus in a form available to the animal [Cosgrove, 1980].

1.1 Phytase

Phytases [myo-inositolhexaphosphate phosphohydrolase] hydrolyze phytic acid to myo-inositol and inorganic phosphates through a series of myo-inositol phosphate intermediates, and eliminate its anti-nutritional characteristics. Phytase is widespread in nature, and it occurs in microorganisms, plants and some animals. A large number of bacteria, filamentous fungi and yeasts have been reported to produce phytase extra- and intra-cellularly as well as in the cell-bound form. There are two types of phytases as classified by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology [NC-IUBMB] in consultation with the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature [JCBN]: 3-phytase [EC 3.1.3.8] that first hydrolyses the ester bond at the 3 position of myo-inositol hexakisphosphate, and is mainly reported in microorganisms; and the 6-phytase [EC 3.1.3.26] that first hydrolyses the ester bond at the 6 position of myo-inositol hexakisphosphate, and is mostly reported in plants. This had also been reported in some basidiomycetous fungi [Dvorakova,1998]. An alkaline 5-phytase from lily pollen was found to start phytate hydrolysis at the
D-5 position. Phytases can be broadly categorized into two major classes based on the pH for activity: acid phytases and alkaline phytase. More focus has been on acidic phytases because of their applicability in animal feeds and broader substrate specificity than those of alkaline phytases. Recently, phytases have also been classified as HAP [Histidine acid phosphatase], BPP [β Propeller phytase], CP [cysteine phosphatase] and PAP [purple acid phosphatase] based on their catalytic properties [Cooper and Gowing, 1983].

Although, the centaury old research on phytase, the basic problems are still unattended, like thermostability and acid tolerance, susceptibility against stomach proteases is a big problem for commercial animal farming practice, by aiming this problem firstly, we have tried to screen a thermostolerant and or acidtolerant microorganism, which produces extracellular phytase.

**Phytase as feed additive**

As discussed above phytate is considered, to be an anti-nutritional factor and its hydrolysis by phytase increases the bioavailability of various nutrients. This leads to a reduction of the costs of adding dicalcium phosphate to the feeds, as well as phosphate run off from livestock manure to water stream, which creates eutrophication [Ha et al, 2000].

**Application in food**

Besides a major application in the feed industry, phytase has also been found to be increasingly interesting for use in processing and manufacturing of foods for human consumption [Hoiberg-Nielsen, 2009]. Adults consume about 1 g of inositol per day from both animal and plant sources. This value could be as high as 4.5 g/day depending on the amount of plant-derived foods; an average intake of phytate is 2-2.6 g/day for vegetarian diets and inhabitants of rural areas in developing countries [Greiner and Konietzny, 2006]. An increased dietary consumption of cereal fibers, legumes and soy protein isolates leads to a higher intake of phytate. Vegetarians eating mostly wholegrain products and processed cereals, elderly people consuming unbalanced foods with large amounts of cereals, people in developing countries who eat unleavened bread, and babies eating soy-based infant formulas take in large amounts of phytate. Although phytate has been reported to be an antioxidant with anticancer activity, its negative effect related to mineral deficiency is much more severe in undeveloped and developing countries. Different strategies have been developed to reduce the risk of mineral deficiency in vulnerable groups such as child-bearing women, strict vegetarians, inhabitants of developing countries, particularly fast-growing children, but none has been very successful [Gibson and Ullah, 1988]. Since only a very low phytate-degrading activity occurs in the human intestines, supplemental
phytases to degrade phytate during food processing and in the gastrointestinal tracts has been suggested.

**Other applications of phytase**

Some lower myo-inositol phosphate derivatives are of pharmaceutical interest. Graf et al. (1987) reported antioxidant properties of myo-inositol 1,2,3,tris phosphate and myo-inositoltetraakis phosphate.

In our study, we have developed novel application of phytase; phytase producing *Klebsiella pneumonia*, which was successfully, applied as plant growth promoting strain which liberate phosphate from organic plant waste and make it available to growing wheat plant.

**Research problem:**

By surveying the literature and available information, we designed definite goal for research, as from literature review it is clearly evident that large number of publications are available on structural property, classifications, purification methods and, modification of phytase by recombinant DNA technology, but none of the research directly targeting the major issues of phytase inactivation in gut of mono-gastric animals, and inactivation during feed pelleting and processing, in our research work, we would like to address this grassroots issues pertaining the commercial application of phytase in field, therefore we have designed our goals of research by keeping these facts in mind.

**Aims and objective of the study**

The aim of present study is to characterize phytate degrading enzyme phytase from microbes. Present study is also focused on various aspects like screening, increasing the stability of phytase at high temperature and low pH, development of efficient coating strategies and its application in various fields.

- Development of in situ assay for screening of phytase producer.
- Two assays for in-situ screening of phytase producer has been reported in literature both the assays are based on zone of clearance of calcium phytate which is poorly visible in acidified medium.
- Screening of thermo-tolerant and/or acid-tolerant extracellular phytase producer on the basis of their P- utilization mechanisms.
- Our first target is to isolate microorganism, which naturally produce acid-tolerant and/or thermo-tolerant extracellular phytase.
- Medium and process parameter optimization for maximum phytase and biomass production.
- Purification and partial characterization of phytase.
Development of nanotechnology based antifouling coating technology

If naturally occurring acid-tolerant and/or thermo-tolerant phytase could not be purified than the phytase enzyme can be coated by multistep approach to tailor the phytase as per commercial need.

Application of partially purified phytase in various fields.

Results

Isolation and screening of phytase producing microorganisms

10 isolates were obtained from various screening and sampling sites like mangrove vegetation, biogas plant and highly eutrophied wetland. A novel screening strategy for phytase producing microorganism was developed to study the detail mechanism of P utilization, isolates were discriminated on the basis of their P-utilization mechanism, 2 different microbial strains were selected for different purpose by considering the objectives of targeted screening, 1 *Saccharomyces boulardii* [*a*$_w$ $\approx$ 0.7] was screened for SSF and bulk production and purification of phytase. 2 *Klebsiella pneumoniae*, non-pathogenic strain, was screened for plant growth promotion activity as it is positive for, organic acid production [73.23 mM], acid phosphatase [16.2 U/ml] production and, phytase production [0.6 U/ml], *Klebsiella* was screened for its application as phosphate solubilizer; i.e. plant growth promoting bacteria.

A novel in situ assay for detection of phytase was developed. The assay was performed by agar ditch method; a small ditch was prepared around colony and 500µl of ammonium molybdate [0.1 gm %] was poured in narrow ditch. The plates were incubated for 30 minutes on 55°C. 250 microliters 1,2,4-aminonaptholsulfonic acid [25% v/v] was added in same ditch and plates were incubated at 55°C for two minutes. Plates were observed for blue color formation around colonies. The isolates were characterized by 16s rRNA sequencing and biochemical characterization.

Optimization of medium components for submerged fermentation and process parameters for SSF

An effort was made to optimize the important physical, chemical and nutritional parameters that influence phytase production. Medium for submerged fermentation was optimized for optimum biomass production of *Klebsiella pneumoniae* as screened for plant growth promoting activity. The presence of organic [Phytate 5 mg/l] and inorganic phosphate [3.8 mg/l] was found significant for growth and phytase production, the analysis of results shows that phytase was produced after 48-hour incubation. In another study, the process parameters for SSF were optimized. Maximum phytase activity was observed with 48-h old inoculum, in 80-mesh size complex medium and after 96-hr of incubation was 800 U/g of
substrate. The study has demonstrated the impact of inoculum culturing conditions on fermentation yield and overall performance of the process. The study extends the understanding of SSF process parameters like requirement of optimum moisture content for optimum production of yield and biomass. The optimum moisture content for both the *Saccharomyces boulardii* is around 60% while the optimum inoculum age of the culture is 4 days. These results slightly differ from our knowledge that the inoculum should be young. In present study, the good result is obtained with aged [72 hours] microbial culture.

**Partial purification and characterization of phytase**

Enzyme was purified with combination of precipitation and chromatographic techniques [Ferrari et al, 1993]. Phytase from an overnight grown yeast culture was precipitated at 80% saturation with ammonium sulphate, subsequently the pellets are dissolved in sodium acetate buffer pH 4.5 and chromatographed in CM-Sepharose cation exchanger, which retained most of the acid phosphatase activity and phytase can easily be pooled out from the fractions under the chosen conditions [Ullah et al, 1991]. In second step fraction containing highest phytase activity [0.92 U/ml] is subjected to gel filtration, and phytase activity was measured for each fractions. A280 was measured to check the presence of other proteins. Amino acid analysis indicates that yeast phytase is rich in aspartate glutamate. HPLC analysis of amino acid composition is used to determine the molecular weight of purified phytase that is around 72KD. Native and SDS PAGE profile of purified phytase revealed the presence of 3 subunits of phytase. Optimization and substrate specificity analysis was carried out to study the physico-chemical characteristics of phytase. The optimum pH of purified yeast enzyme is 4.5, while optimum temperature is 50 °C. SDS-IEF PAGE measured the PI as 5.5.

**Antifouling coating of phytase**

The addition of phytase granules to the animal diet assists in the liberation of inorganic phosphate adsorbed by monogastric animals. Phytase granules are complex systems composed of protein incorporated in a core of various excipients and a coating to support stability. Enzymatic inactivation arises during the feed processing where steam is applied and high temperatures [T, 70-100°C] and moisture [rH, up to 100% relative humidity] are obtained.

After testing a series of experiments in the set up [total flow] and sample related parameters [such as sample size and particle size], experimental conditions were determined. Phytase granules were investigated for the effect of T, rH and time on stability; it was revealed that the combination of high temperature and high moisture dramatically decreased the stability of enzyme.
In our research we have developed the mesoporous structure \( \text{mesocellular pores} \approx 40 \text{nm} \) from carboxy-methylcellulose and silica, which is harmless for animals. Coated enzyme can withstand high temperature up to 100°C for 4 hrs, while free enzyme cannot withstand 100°C. The coated enzyme can be separated from the reaction mixture after the completion of reaction with the help of external magnetic field; this property can be utilized in aquaculture and many other fields. Moreover, the developed coating technology stabilizes the enzyme activity at low pH as low as pH 2.5 for more than 2 hours. Most of the enzymes are inactivated in the presence of protease; this developed technology gives the protease \([10 \text{ U/ml}]\) resistance. The main advantage of this antifouling coating technology is to increase the shelf life of enzyme; we found our enzyme active after 2.5 years of incubation at room temperature, with negligible loss \([0.002 \text{ U/ml}]\) of enzyme activity. The stability of enzyme was evaluated under shaking condition; it does not show impactful enzyme activity of free and coated enzymes. There is no much difference was observed in enzyme activity of free \([K_{\text{cat}}= 3.271]\) and coated \([K_{\text{cat}}= 2.897]\), the difference of 0.374 in catalytic efficiency is negligible by considering the other advantages of coating technology. The one more advantage of this technology is, the magnetic separation of enzyme from reaction mixture after its use, the reusability study shows that the same enzyme preparation can be used more than two times after that its activity decrease twofold. In summary, the developed coating technology can efficiently work under various parameters with retaining the enzyme activity for very long period at room temperature.

**Phytase as feed Additives**

The addition of phytase granules to the animal diet assists in the liberation of inorganic phosphate adsorbed by monogastric animals. The addition of microbial phytase has been shown to increase in overall growth performance of chicks and Rainbow trout, the weight gain and feed consumption rate were positively affected by supplemented phytase in presence of available phosphate, the increase in tibia and organ weight was observed with phytase supplementation. The modified design of aquaculture tank was proven useful for easy recovery of unconsumed phytase from effluent tank.

**Growth performance**

I. The main effects data indicated that the decrease in available phosphate \([\text{AP}]\) content from 3.5 to 2.5 g/kg depressed weight gain \([6\%; P < 0.001]\), feed consumption \([3\%; P = 0.002]\), and feed to gain ratio \([3\%; P < 0.001]\). Weight gain and feed consumption were increased quadratically \([P < 0.001 \text{ and } P = 0.002, \text{ respectively}]\) by phytase addition. This response was statistically maximized by 200 U/kg phytase \([7 \text{ and } 5\%],\)
respectively]. Feed to gain ratio was not affected by the addition of phytase.

II. Bone mineral analysis
The effects of available phosphate [AP] level, phytase supplementation on tibia ash, bone mineral and organ weights are summarized here. The main effects data indicated that the decrease in AP content from 3 to 2 g/kg caused a significant decrease [P = 0.029] in tibia ash by 1%. Mineral content in tibia ash showed a significant increase in Ca [P < 0.001] and P [P < 0.001] concentrations by 1 and 2%, respectively. However, a decrease in Zn [P < 0.001] was observed [1%] in response to decreasing AP levels in the diet. Likewise, Mg concentration in tibia ash was not affected by AP level in the diet.

Phytase supplementation to low-AP diets linearly increased tibia ash [up to 4%; P < 0.001], and Ca [upto2%;P<0.001], P[upto1%;P =0.002] and Zn[upto4%;P< 0.001] contents in tibia ash. In the case of Zn, a significant interaction [P < 0.001] was observed between AP level and phytase concentration. Mg concentration was not affected by enzyme addition.

III. The mean value of fishes growth were approximately 75 to 112 g during the 30 days of experimental feeding in tank fed with diet A0, while the mean fish growth rate of the tanks that fed with diet A1 was 75 to 124g.

Phytase as Food additive
The use of yeast phytase in bread making, and effect on bread characteristics has been studied by FT-IR spectroscopy. Phytate content during the steps of bread-making process was studied and effect of cooking and effect of phytase on phytate content was separately analyzed. From the results, it can be concluded that phytase supplementation as a bread making additive in bread formulation containing fiber leads to an acceleration of the proofing, in improvement of bread shape, a slight increase in the specific volume and also confers softness to crumbs.

Phytase for bioremediation of eutrophic water
Present study focuses on multifaceted approach for removal of phosphate from eutrophic lake; Fe₃O₄ nanoparticles [NP] are used as phosphate [P] chelators, which absorb phosphate liberated by phytase from phytate, the liberated phosphate is adsorbed on Fe₃O₄ nanoparticles without affecting its magnetic property, and nanoparticles-phosphate complex can be easily separated by applying the magnetic field. The phytase is conjugated in phytase-NP-HHMS complex; the major drawback of current method is it
removes phytase along with NP-P chelated complex. Therefore every-time reapplication of phytase is inevitable.

The present study is conducted with artificial phytate-polluted water in fixed column system. In this study we developed an enzyme phytase armoured with Fe₃O₄ nanoparticles covalently linked in hierarchically ordered, mesocellular, mesoporous silica [HHMS]. Earlier similar kind of approach was used to study the possibilities of enzyme and nanoparticles co-adsorption in HMMS with chymotrypsin as model enzymes. This approach efficiently removed phytate – P up to 2-PPM concentration.

**Klabsiella pneumoniae; A phytase producing strain as bio-fertilizer**

The use of phytase producing strain of Klebsiella pneumoniae is studied in vitro in inorganic phosphate limiting condition, and small-scale pot study has been conducted. The study includes its efficient localization to plant root, and its effect on wheat plant, when incubated with soil containing external source of phytate and phosphate. Increase in dry weight and shoot/root ratio was measured in the presence of microbial culture. Moreover the efficient technology was developed for targeted delivery of Klebsiella, same technology can be utilized for delivery of other microbes too.
Conclusions

Screened microbial cultures, *Saccharomyces boulardii* and *Klebsiella* are efficiently used for their targeted applications, both proven successful on laboratory experiments. *Saccharomyces boulardii* is generally found in gut of broiler, detection of phytase activity in this strain can stimulate its use in future as poultry probiotic strain. Phytase from yeast strain can be easily produced by low cost solid-state fermentation process on complex medium.

The developed novel in-situ assay will be useful for screening of phytase producing microorganisms. The developed assay can be optimized for other pH range too; our study was concentrated on acidic pH. The novel strategies of screening are very efficient in distinguishing the various mechanisms of phosphate solubilization, which minimizes the number of quantitative estimation at primary stage. *Saccharomyces boulardii* strain is novel in the list of phytase producing microorganisms, this strain was first isolated from poultry gut, it can be employed as poultry probiotic strain, as study of probiotic is beyond aspects of our study, this point we are keeping open for future study. The other important outcome of this study is *Klebsiella* strain that solubilize both organic and inorganic phosphate, by production of broad-spectrum acid phosphatase that utilize phytate-P as well as P from other inorganic phosphate source, and organic acid production.

The study on optimization of process parameters shows the significance of statistical evaluation of all treatment combinations for determination of optimal fermentation strategy. This study has demonstrated the impact of inoculum culturing conditions on fermentation yield and overall performance of the process. Statistically based experimental designs are valuable tools in optimizing operational conditions and inoculum performance for maximal phytase production. The strong relationship between growth and yield in the fermentation process shows promise for establishing consistency and reliability of the production process. The optimized medium for submerged fermentation contains glucose (10 g/l), calcium phytate (10 g/l), ammonium nitrate (2.55 g/l), and KCl (0.3 g/l). The optimum fermentation time for SSF process is 96-h with 1-day-old primary seed culture, and 48-h old secondary seed culture, the maximum phytase production was observed 800 U/g of substrate. The study also compares the enzyme production in two different types of medium, complex medium and standard liquid medium; the result shows that both media can be used for production of phytase by
Saccharomyces boulardii. Our study optimizes the parameters for both complex (pineapple waste) and Standard liquid medium. The significance of conducted study is, it decreases fermentation time by 24-h for maximum phytase production. The study also emphasized the role of substrate particle size for SSF; smaller the particle size higher the production of phytase was observed.

The partial characterization of yeast phytase revealed the multisubunit structure of yeast phytase with approximate molecular weight 72 KD., the results are also supported by literature available.

A novel approach of antifouling coating of phytase is developed. We have developed a unique approach for multifunctional nanocomposites of enzyme phytase and magnetic nanoparticles in polymeric silica cage; this approach employs a simple, two-step process that involves the co-adsorption of enzyme and magnetic nanoparticles into polymeric silica followed by glutaraldehyde treatments. Cross-linking enzymes within mesocellular pores of a uniquely designed mesoporous material successfully retained the resulting enzyme aggregates, retained in the pores, providing high loading capacity. Using this ship-in-a-bottle approach, greatly improved stability was achieved with model enzymes, phytase. Since this ship-in-a-bottle approach of cross linked enzyme aggregates [CLEAs] in polymeric silica is very simple and effective for enzyme stabilization. Biological polymeric silica was synthesized using a single biological non-toxic polymeric agent under neutral conditions. Most of the porous materials reported so far are meso-macroporous or micro-macroporous materials. This biological polymeric silica is very easy to synthesize in laboratory and not very expensive. While mostly all the mesoporous polymers synthesis needs very specific chemicals and they are expensive. In addition, biological polymeric silica has two advantages over other mesoporous materials. First, the overall synthetic process is very cost-effective because inexpensive sodium silicate is employed as the silica source and the synthesis is conducted under mild, neutral conditions. Secondly, the synthetic procedure using a single biological polymer is much simpler than those employed for the synthesis of other mesoporous materials. These nanocomposites, termed Magnetic-CLEAs, are proven to be magnetically separable, highly loaded with enzymes, stable under harsh shaking conditions like high temperature, high pH, shaking condition, and recyclable for repeated use with negligible loss of enzyme activity.
It offers great potential to expand to any other enzyme and any other nanostructure in the matrix for the development of stable enzyme system in many enzyme-catalyzed reactions.

The study on phytase supplementation as food additive shows the positive effect of phytase supplementation in bread-making process, additionally, the study also provides the other efficient and rapid method of phytate measurement during bread-making process. It minimizes the major steps of traditional reviewed methods; the developed procedure is cheap, rapid and efficient.

From the results, it can also be concluded that phytase supplementation as a bread-making additive in bread formulations containing fiber leads to an acceleration of the proofing, an improvement of the bread shape, a slight increase of the specific volume, and also confers softness to the crumb. In addition, from the nutritional point of view, a further hydrolysis of the phytate [considered as anti-nutritional compounds] is reached by adding exogenous yeast phytase. By considering its anti-nutrient activity, we carried out step-by-step measurements of phytate, our data shows that direct measurement of phytate from wheat flour by the FT-IR method is simple and rapid compare with chemical method.

By using this method as prototype it can be employed for estimation of phytic acid content from other food and feed products. The successful incorporation of phytase in bread may be useful for development of other phytate free products.

The phytase have lot to offer for sustainable environment. Our study develops an operational method of phosphate removal from eutrophic lake. The experimental methods cover the P removal from viscous and non-viscous water. By using this study as a base of novel technology, an effective approach for phosphate removal can be developed for eutrophic aquifers and water treatment plants, by incorporating nanotechnological advances, this technology can be employed for removal of multiple chemical species by conjugating multiple enzymes in silica matrix. The viscosity and the sedimentation rate are two important factors which affect the dispersion of ternary complex, before establishing the actual experiment on field it is too important to optimize these two factors. This process efficiently extracts inorganic and organic phosphate from the various layers of water. Phytase-NP-HHMS complex is very stable in normal storage conditions, in our study it is found active up to one-year storage period without
any significant loss in enzyme activity. It is very easy to sprinkle over the surface water layer and easy to separate under the strong magnetic field without harming the delicate ecosystem of eutrophic aquifers. This developed technology is simple cheap and rapid. The major constraint of technology is dispersion of this ternary complex in very large aquifer. No genotoxicity was observed in primary study performed on onion root mitosis, no abnormality was observed in seed germination pattern. The present separation method also removes other iron particles, which was not intended and important trace element for the growth of aquatic animals; however, re-supplementing the required concentrations of iron in eutrophic lake can solve this problem.

Phytase can also be employed for development of sustainable agriculture practices. Phytase producing strain of *Klebsiella pneumoniae* that solubilize organic and inorganic phosphate. This strain was efficiently utilized for its field application. The bio-priming of *Klebsiella pneumoniae* to wheat plant is proved beneficial in increasing the total weight gain, and height of shoot compare to roots. Moreover, it also increased the growth rate rapidly in early stages of growth. The organisms can solubilize phosphate by all 3 mechanisms as per the conditions, the broad spectrum of choice of P-source make this strain as ideal strain as P-solubilizer for field applications. The developed technology of gellan-based coating is successful in maintaining the viability of culture as well as seeds for long period.

In summary of our research work, studies on microbial phytase offers great promises for technological advancement in Century old knowledge, the research work provides a new insight from different perspectives for novel application of phytase.