BIOPROCESS OPTIMIZATION AND CHARACTERIZATION OF PHYTASE FROM AN ENVIRONMENTAL ISOLATE *BACILLUS* MCCB 242

Thesis Submitted to the
Cochin University of Science and Technology
In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Environmental Microbiology and Biotechnology
Under the Faculty of Environmental Studies

by

Sareen Sarah John

(Reg. No.3473)

SCHOOL OF ENVIRONMENTAL STUDIES

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Ph.D. Thesis under the Faculty of Environmental Studies

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Certificate

This is to certify that research work presented in the thesis entitled "**Bioprocess optimization and characterization of phytase from an environmental isolate *Bacillus* MCCB 242**" is based on the original work done by Ms. Sareen Sarah John (Reg.No.3473) under the guidance of **Dr. Robert H. Reed, Central Queensland University, Rockhampton, Queensland 4702, AUSTRALIA** and co-guidance of **Dr. I.S Bright Singh, Professor, School of Environmental Studies, Cochin University of Science and Technology, Kochi - 682022**, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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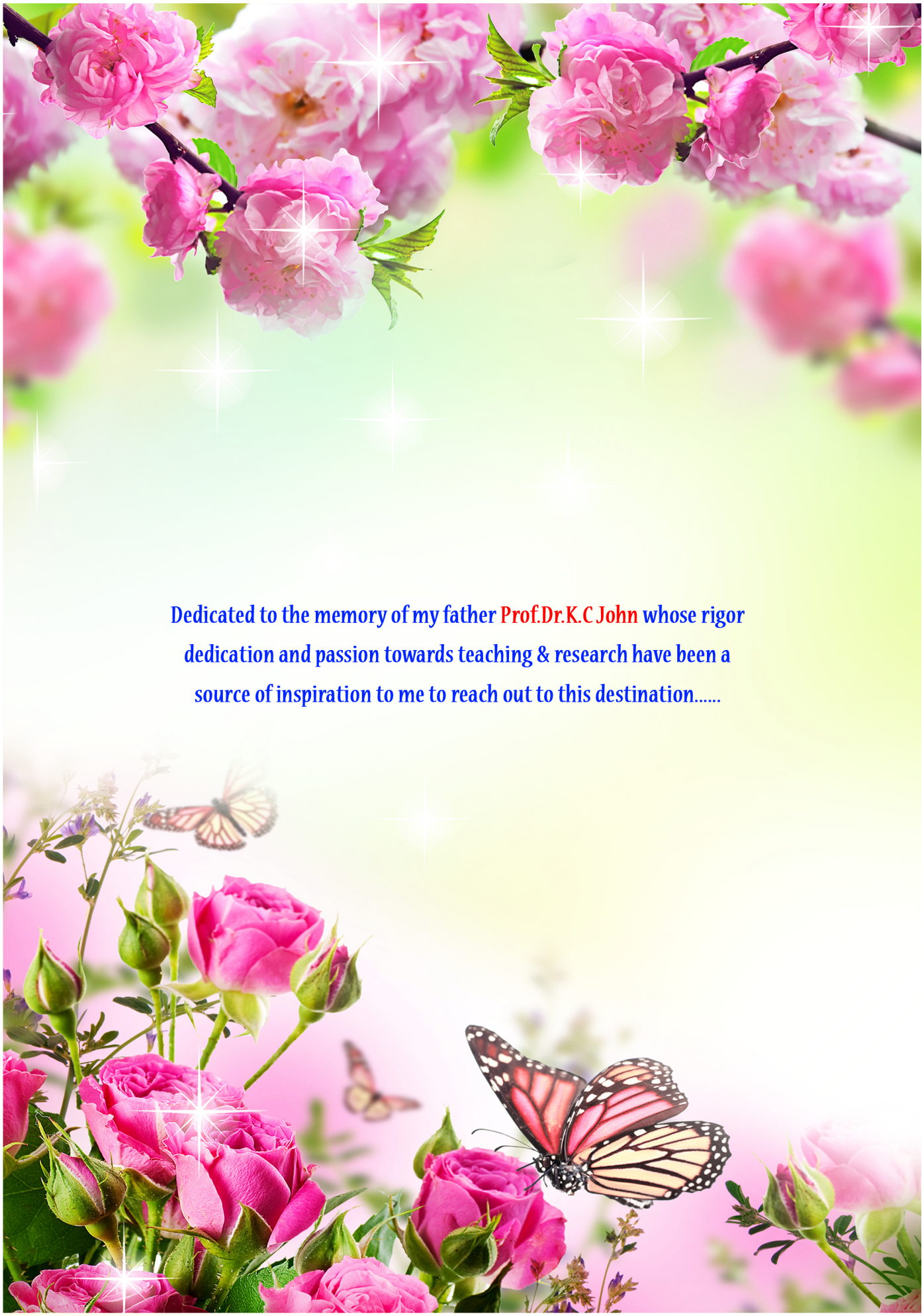

Dedicated to the memory of my father Prof.Dr.K.C John whose rigor dedication and passion towards teaching & research have been a source of inspiration to me to reach out to this destination.....

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"Sometimes our light goes out but is blown into flame by another human being. Each of us owes deepest thanks to those who have rekindled this light."

- Albert Schweitzer

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The dream begins with a teacher who believes in you, who tugs and pushes and leads you to the next plateau, sometimes poking you with a sharp stick called "truth." -Dan Rather.

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Sareen Sarah John

Contents

Chapter 1

GENERAL INTRODUCTION

Contents

Over the years phytase has received tremendous importance, making it the most preferred feed additive mainly for the reason that it is eco-friendly. Microbial phytase has become an universally accepted and remarkably effective enzyme in animal feed & feed industry, as it improves the nutritional status by degrading phytate (IP6) and reducing its content. In monogastric animals and agastric fishes supplementation of phytase in feed maximize uptake of nutrients minimizing their rejection into environment through undigested feed and faecal matter (Rodehutscord *et al,.* 1995; Li and Robinson 1997; van Weerd *et al.,* 1998; Forster *et al.,* 1999; Jalal and Scheideler 2001; Robinson *et al.,* 2002; Lan *et al.,* 2002; Singh 2008; Wang *et al.,* 2013). Also, phytase increases the availability of cations (phosphorus, calcium, zinc and copper) bound to phytic acid (Odetallah, 2000)

Around 70 percent of Indian population depends on agriculture and animal farming (NCW report, 2005). Paddy cultivation is one of the major cereal crops that always require wet farming system. Cow dung is applied as the

major organic manure. Phosphorous in the manure reaches water bodies (lotic and lentic) leading to high eutrophication supporting luxuriant growth of aquatic weeds such as Salvinia and Eichornia. The high phytic acid content in plant-based feed enhances both nitrogen and phosphorus discharge and increases the level of pollution (Debnath *et al.,* 2005). Utilization of phytate from feed to the maximum extent is the only option to save the situation. Unfortunately the monogastrics are able to utilize only 20-30% of phytate because they lack phytase. In addition, leaching from animal farms and sewage effluents also enhances eutrophication. Continuous decaying of aquatic weeds and hay, release methane, a green house gas, which leads to oxygen depletion in water bodies affecting inland water fish production. Phytate phosphorus utilization and digestibility of plant protein sources could be improved by incorporation of microbial phytase in the diet of monogastric animals including fish (Vielma *et al.,* 1998; Schaefer *et al.,* 1995; Li and Robinson, 1997; Furuya *et al.,* 2001; Phromkunthong and Gabaudan, 2006).

1.1 Phytate

Phytate (myo-inositol 1, 2, 3, 4, 5, 6 hexakis dihydrogen phosphate INP6) consists of myo inositol esterified with six phosphoric acid groups, an abundant plant constituent comprising 1-5% (w/w) of legumes, cereals, oil seeds and nuts (Cheryan, 1980). It is also the principal storage form of phosphorous (Reddy *et al.,* 1989), inositol and a variety of minerals in plants, accounting for 75-80% of the total phosphorous in plant seeds. Phytate is normally found in the outer (aleuron) layers of cereal grains and in the endosperm of legumes and oil seeds, constituting about 1-3% of their dry weight (Graf, 1983; Bohn *et al.,* 2004). The terms phytic acid, phytate, and phytin denotes the free acid, salt and calcium/magnesium salt subsequently. In this chapter the word phytic acid is used interchangeably with the term phytate, which is a salt.

In nature, phytate exists as metal phytate complexes with nutritional cations such as $Ca^{2+,}$ $Zn^{2+,}$ and Fe^{2+}. Phosphorus is tied up in phytins. It has been reported that phytate reduces the availability of essential minerals such as calcium, zinc, iron, magnesium and also proteins, causing wide spread deficiencies of these nutrients in populations whose staple foods are legumes (Reddy, 1982; Wu *et al.,* 2009). Meanwhile, Shamsuddin and Vucenik (1999) reported the beneficial effects phytate as well, which includes anticancer properties.

Phytate was isolated for the first time by Hartig in 1855 and 1856 as small, non-starch particles from seeds of various plants (Hartig 1855, 1856). In most cereals and legumes, phytate/ phytin/ phytic acid phosphorus constitutes the major part of the total phosphorus. They can exist in a metal free form or in metal phytate complex, based on the hydrogen ion concentration of the solution and the concentration of metal cations (Fig 1.1a). The extent of binding is dependent upon both pH and divalent metal cations to phytate ratios (Cheryan, 1980; Maenz *et al.,* 1999).

Phytate accumulates in seeds during the ripening period (Greiner *et al.,* 1998). During the germination of seed, phytate is degraded by phytases, providing phosphate and minerals for the growth of seedlings (Scott and Loewus, 1986). In addition to its role in phosphate storage, phytate acts as a strong chelator for divalent metal cations (mostly K^{+}, Mg^{2+}, Ca^{2+}, or Zn^{2+}) and exists as a stable metal-phytate complex in plants (Asada *et al.,* 1969; Reddy *et al.,* 1982).

Divalent metal cations with small radii, such as Mg^{2+} (0.65 Å), Fe^{2+} (0.74 Å), and Zn^{2+} (0.71 Å) bind in a monodentate fashion within two oxygen atoms from the phosphate groups of phytate (Fig.1.1b). Therefore, bi-dentate

metal-complex formation prefers divalent metal cations with large ionic radii (Jonson and Tate, 1969; Cheryan, 1980).

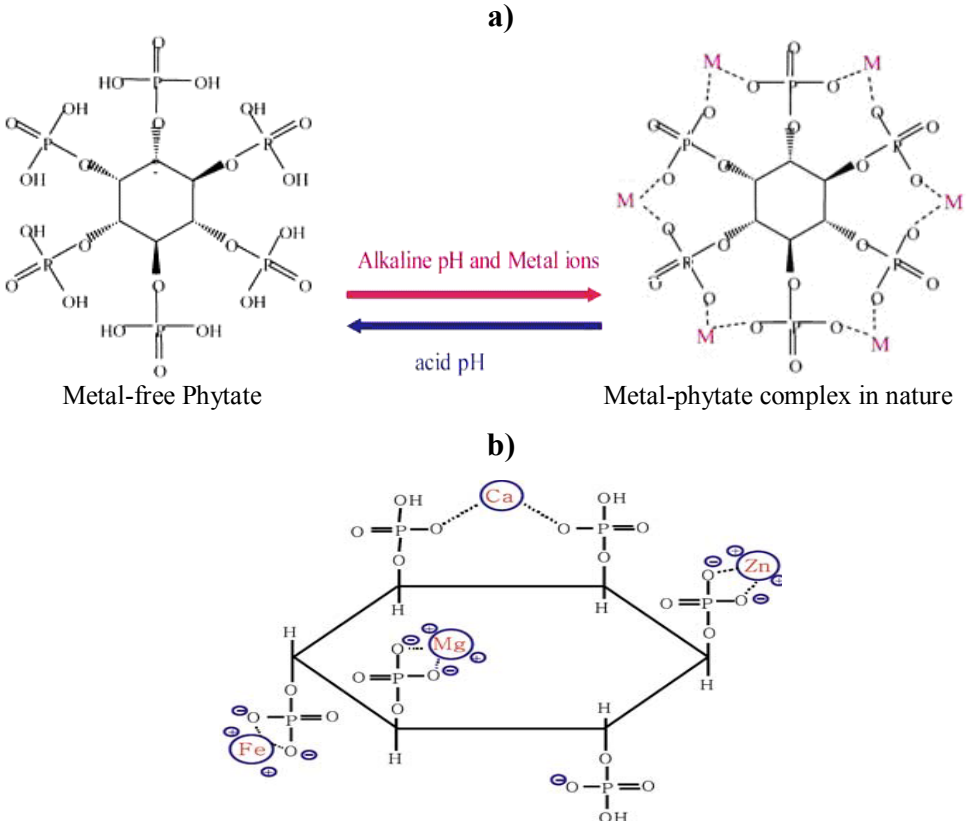

Fig.1.1 a, b: Effects of pH and divalent metal cations on physiological nature of phytate.

a) Phytate exists as a metal-free phytate or a metal–phytate complex, depending on the pH and divalent metal cations.
b) Divalent metal cations specifically bind to the phosphate groups of phytate, depending on the ionic radii of the metal cations.

1.2 Phytase

Phytase, which consists of myo-inositol hexaphosphate 3-phosphorylase (EC 3.1.3.8) myo-inositol hexakisphosphate-5-phosphorylase (EC 3.1.3.72) and myo-inositol hexakisphosphate 6-phosphorylase (EC 3.1.3.26), belongs to the family of histidine acid phosphatases that catalyze the hydrolytic degradation of phytic acid and its salts (phytates), generally yielding inositol, inositol monophosphate, and inorganic phosphate (Fig.1.2) (Badamchi *et al.,* 2013; Mittal *et al.,* 2011; Angel *et al.,* 2002; Frossard *et al.,* 2000).

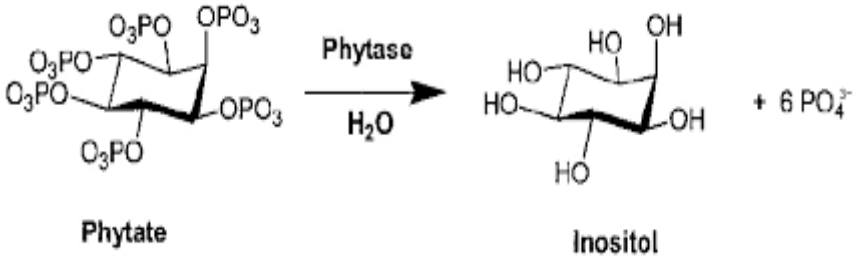

Fig. 1.2: Phytate hydrolysis by phytase

Phytase can be found in plants, microorganisms, and animals (Ocampo *et al.,* 2012). Microorganisms are the frequently used source of phytase. Till date only a few commercial phytase products are available in the market, which are very expensive.

1.2.1 Historical Overview of Phytase

The research on phytase spans more than 100 years from its discovery to its commercialization in Europe in 1993-1994. Since the discovery of phytase in 1907 (Suzuki *et al.,* 1907), a complex of technological developments has created a potential $500 million market for phytase as an animal feed additive (Mullaney *et al.,* 2000). Research has led to the increased use of cereals, grains and other plant material as protein sources in animal feed. During the 1st International Phytase Summit at Washington DC, Greiner and Bedford, (2010) reported that in the last 25 years, phytases have attracted great interest from both scientists and entrepreneurs in areas of nutrition, environmental protection and biotechnology.

In the year 1907 Suzuki, Yoshimura and Takaishi were the first investigators to detect its activity in rice and wheat bran. Dox and Golden (1911) demonstrated the production of phytase by *Aspergillus* sp. Phytic acid/ phytate/ phytin were then recognized as effective chelating agents and anti-

nutrients for monogastric animals and attempts were made to hydrolyze phytin in animal feed using live yeast. In 1962 the International Minerals and Chemicals, Skokie produced the first commercial phytase. Microbial phytase became commercially available in 1990s as a result of biotechnological intervention (Wodzinski and Ullah, 1996).

Transgenic mice expressing bacterial phytase has been recently developed as an experimental model to study the efficiency of indigenous phytase expression (Golovan *et al.,* 2001). During 1980's Gist-Brocades (Dutch) cloned, sequenced and over-expressed phy A from *Aspergillus niger* NRRL 3135 along with amyloglucosidase promoter having 52-fold improvement of phytase yield. This bioengineered phytase is approved by FDA as GRAS (Generally Recognized As Safe) for use as feed additive for swine and poultry and marketed in the United States from January 1996 as Natuophos®. Research is in progress on to discover new phytases, engineering better phytases based on three dimensional structures and developing more cost effective expressive systems, using microorganisms, animal tissues etc.

In India, Powar and Jagannathan (1982) reported the first work on the production and purification of phytate specific phosphatase from *Bacillus subtilis*. Di-calcium phosphate (DCP) is used in animal feeds and it has been noted that phytase supplementation can replace its 50-60%. Well documented reports showed that 10 kg DCP can be replaced by 250 gm phytase.

Phytase, a phosphoesterase can release not only phosphate from phytic acid/ phytate/ phytin, but also chelate divalent metals (Ca, Mg, Zn and Fe), from phytate (Fig1.3). Phytase also releases minerals, amino acids and proteins, which are complexed with the phytate molecule.

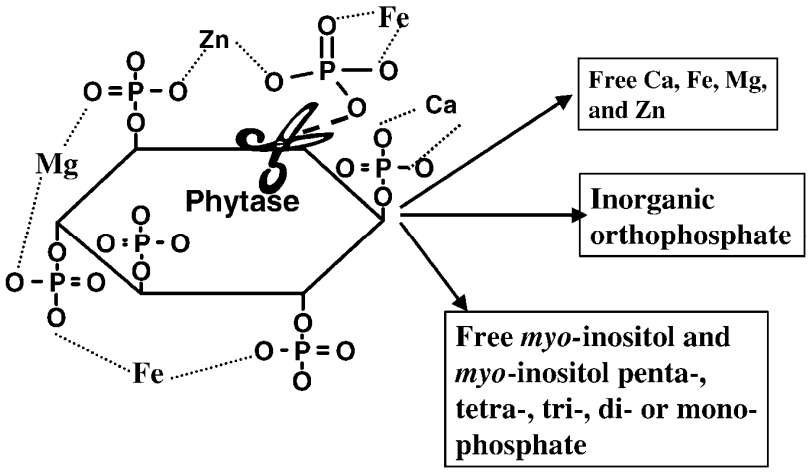

Fig.1.3: Schematic illustration of phytase function. Phytase, showing as a pair of scissors, initiates the removal of phosphate groups from phytate at the carbon ring positions 1 or 3 (3-phytase) or 6 (6-phytase), producing free inorganic phosphate, metals, and myo-inositol or its intermediate metabolites (Lei and Stahl., 2001)

1.2.2 Phytase Classification

The International Union of Biochemistry and Molecular Biology (IUBMB) in consultation with the IUPAC-IUB, Joint Commission on Biochemical Nomenclature (JCBN) have listed three phytases:

The three classes of phytase are 3-phytases, 5-phytases and 6-phytases

a) 3-phytase,EC 3.1.3.8(myo- inositol hexakisphosphate-3- phosphohydrolase)

b) 5-phytases EC 3.1.3.72 (myo- inositol hexakisphosphate-5- phosphohydrolase)

c) 6-phytase, EC3.1.3.26, (myo-inositol hexakisphosphate-6- phosphohydrolase).

3-phytase initially removes orthophosphate from the D-3 position of phytate, whereas 6-phytase preferentially initiates the phytate dephosphorylation at the L-6 (D-4) position of the myo-inositol rings. 5-phytase starts hydrolysis at the D-5 position of the inositol ring. 5-phytases (EC 3.1.3.72) from *Medicago sativa*, *Phaseolus vulgaris*, and *Pisum sativum* initiate phytate hydrolysis at the fifth phosphate group (Rao *et al.,* 2009). In general depending on the type of phytase the first hydrolysed group of myoinositol ring

varies in position and generates positional isomers (Ronald *et al.,* 2014). In the presence of water, phytase tend to hydrolyze the phytic acid and release inorganic phosphate (Wodzinski and Ullah, 1996; Vats and Banerjee, 2004). Phytases from microorganisms are considered to be 3-phytases (EC3.1.3.8), whereas seeds of higher plants are said to contain 6-phytases (EC 3.1.3.26). (Johnson and Tate, 1969; Cosgrove, 1970). The phytases from rye, barley, spelt, oat, wheat bran, rice and mung bean are included in this category (Hayakawa *et al.,* 1990; Greiner 2001). The phytases from *Sacchromyces cerevisiae* (Greiner *et al.,* 2001) *Pseudomonas* (Cosgrove, 1970) *Klebsiella terrigena* (Greiner *et al.,*1997) and *Aspergillus niger* (Irving and Cosgrove, 1972) generate D-Ins (1,2,4,5,6) B5 as the major myo-inositol pentakisphosphate. Hence, these enzymes are considered as B-phytases. In certain seeds phytases are found in multiple forms and these forms exhibit stereo specificity of myo-inositol hexakisphosphate dephosphorylation.

1.2.3 Sub Classes of Phytase

Three sub classes of phytase are described based on their structural differences and varied catalytic properties. These include members of histidine acid phosphates (HAPs), alkaline phosphatase (EC 3.1.3.1) and purple acid phosphates (PAPs). All acid phytases (EC 3.1.3.2) are in a subfamily of the high molecular weight HAPs (Van Etten *et al.,* 1991). The catalytic mechanism of phosphomonoester hydrolysis by HAPs was elucidated by site-directed mutagenesis (Ostanin *et al.,* 1992; Ostanin and Van Etten, 1993) and by the crystal structure of transition-state analogue complexes (Lim *et al.,* 2000). The catalytic mechanism of phytate hydrolysis by alkaline phytase was characterized by site-directed mutagenesis, kinetic analysis, and the crystal structure of the phytase (Oh *et al.,* 2001; Shin *et al.,* 2001).

Structurally, most of the 3-phytases show homology to betapropeller phosphatase (BPP) or histidine acid phosphatases (HAPs). Unlike HAPs, BPPs (EC 3.1.3.8) is a recently discovered class of enzyme with a novel mechanism for hydrolyzing its substrate. BPPs have been isolated and their genes cloned from *Bacillus subtilis* (phyC) (Kerovuo *et al.,* 1998) and *Bacillus amyloliquefaciens* (Kim *et al.,* 1998a). BPPs are tightly bound to three calcium ions and two phosphate groups before hydrolysis can occur (Bohn *et al.,* 2008). The end product has been suggested to be inositol triphosphate either Ins (1, 3, 5) P3 or Ins (2, 4, 6) P3 (Kerovuo *et al.,* 2000; Shin *et al.,* 2001). Substrate specificity and affinity are important properties of phytases, specifically related to the physiological nature of the substrate (Fig 1.4a, b). Purple acid phosphates found in 6-phytase group are the ADP phosphoglycerate phosphatase (related to EC 3.1.3.28) (Bohn *et al.,* 2008). PAP is found as a homodimeric glycoprotein mainly in plant species, with a Fe (III)-Zn (II) active site (Dionisio *et al.,* 2007).

HAPs exhibit a broad specificity for phytate and other phosphate esters. Compared to HAPs the substrate specificity of alkaline phytases is much narrower. The reciprocal relationship between the substrate specificity and crystal structure of histidine acid phytases or alkaline phytases provides a new approach into the nature of phytate and into molecular recognition of phytases. EDTA which has the ability to sequester metal ions strongly inhibits alkaline phytase activity (Powar and Jagannathan, 1982; Kerovuo *et al.,* 1998; Kim *et al.,* 1998b; Liu *et al.,* 1998). The reaction mechanisms of phosphate ester hydrolysis by HAPs follow the nonspecific acid phosphatases properties (Gibson and Ullah, 1988). Specifically for phytate as a substrate, HAPs can hydrolyze metal-free phytate at acidic pH, when phytate exists as a metal-free phytate (Maenz *et al.,* 1999; Wyss *et al.,* 1999a).

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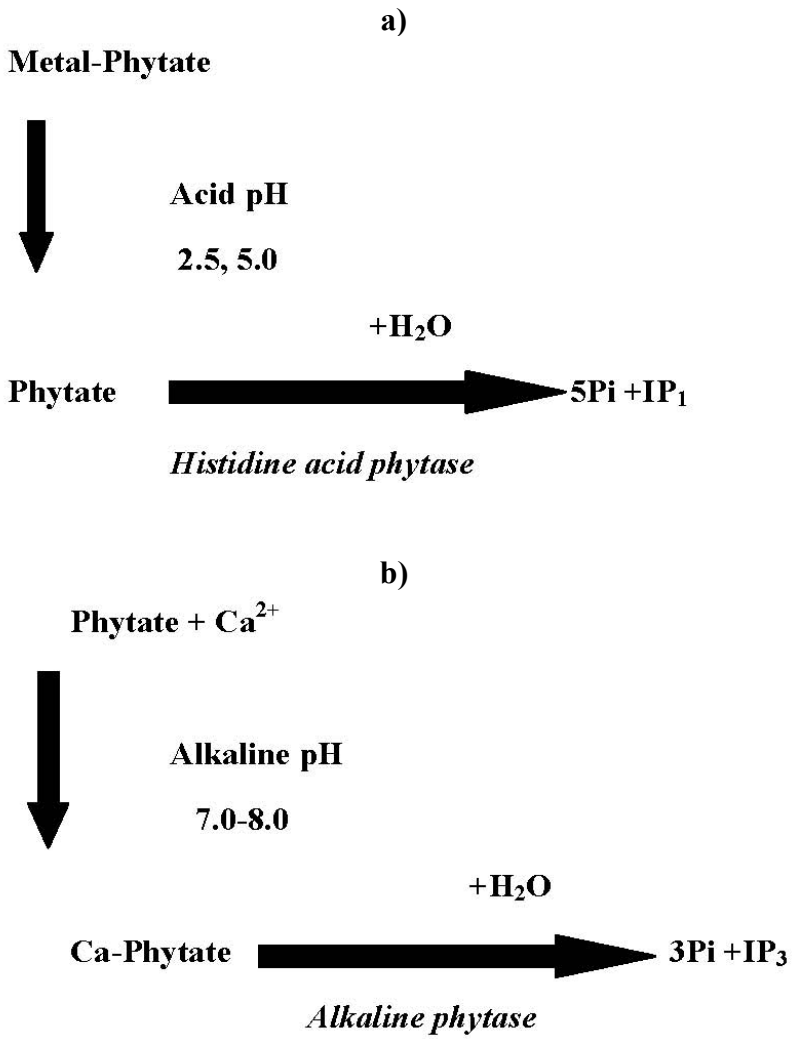

Fig1.4 a, b: Schematic illustrations of substrate hydrolysis by histidine acid phosphatases (HAPs) and alkaline phytases. a) HAPs are able to hydrolyze five phosphate groups from phytate at acidic pH, yielding myo-inositol monophosphate as the final product. b) Alkaline phytases are able to hydrolyze three phosphate groups from specific calcium–phytate complex at alkaline pH, producing myo-inositol trisphosphate as the final product (Oh *et al.,* 2004)

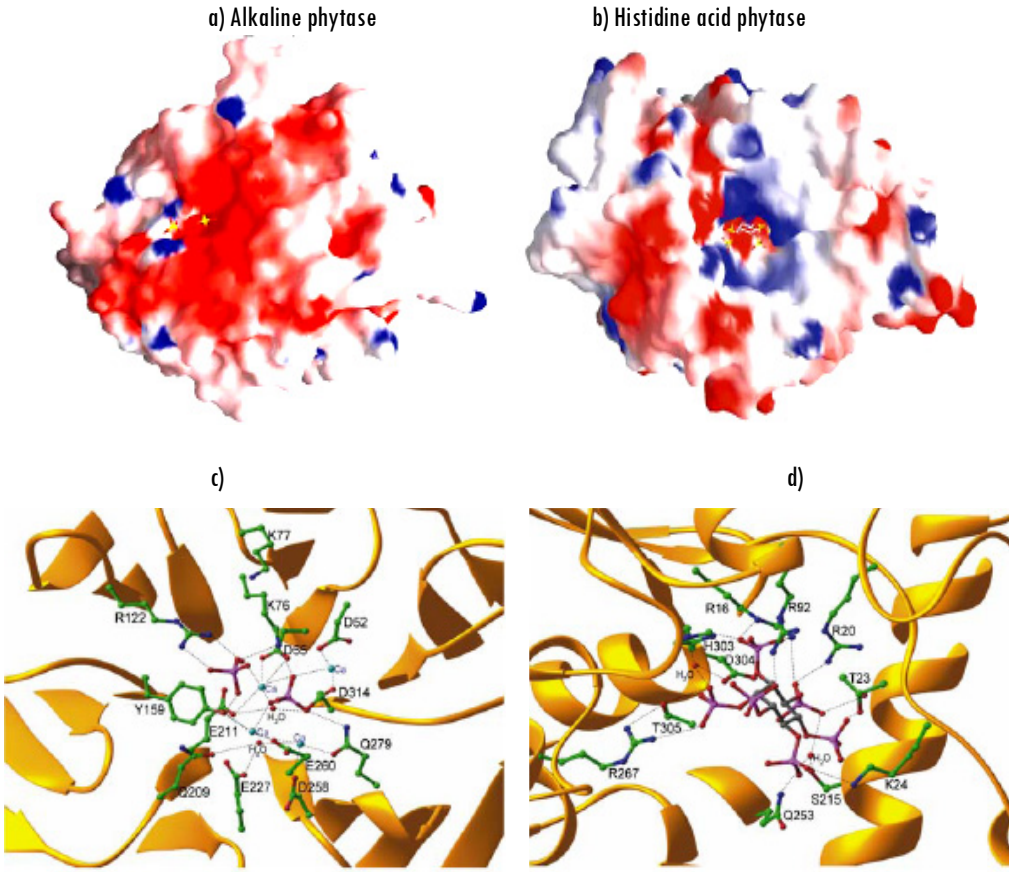

Fig.1.5: a-d Electrostatic surface potential of alkaline phytase and HAP in the active-site region. The surfaces of the substrate-binding sites of *Bacillus amyloliquefaciens* (a) and *Escherichia coli* phytase (b) are colored according to their local electrostatic potentials, ranging from −7 kt/e in red to +7kt/e in blue, using GRASPP (Honig and Nicholls 1995). Stick models of two phosphates (a, c) and phytate (b, d) are shown in the substrate-binding site. The positively charged active site of the phytase from *E. coli* prefers metal-free phytate. In contrast, a negatively charged active site of the phytase from *B. amyloliquefaciens* provides a favorable electrostatic environment for the positively charged calcium–phytate complex (Oh *et al.,* 2004).

The crystal structure of the phytase from *Bacillus amyloliquefaciens* DS11 demonstrated that a negatively charged active site provides a favorable electrostatic environment for the positively charged calcium–phytate complex (Ha *et al.,* 2000; Shin *et al.,* 2001) (Fig 1.5 a, c). Therefore, in *E.coli,* the phytase crystal structure clearly shows that most of the phosphate groups from phytate interact with the active-site pockets of the phytase (Fig. 1.5 b, d).

The large active-site region allows HAPs to accommodate various kinds of phosphate esters, such as pNPP, AMP, ATP, fructose 1,6-bisphosphate, and glucose 6-phosphate. In contrast, the active site region of the phytase from *Bacillus amyloliquefaciens* can only bind two phosphate groups of the calcium-phytate complex, which endows alkaline phytases with very strict substrate specificity for the calcium–phytate complex.

Phylogenetic analysis of the subclasses of phytase clearly shows that alkaline phytases are not a subfamily of HAPs but are indeed novel phytases (Fig.1.6). Most significantly, this group requires calcium for catalytic activity and has an optimal pH of ~7.0-8.0 (Powar and Jagannathan, 1982; Shimizu, 1992; Kerovuo *et al.,* 1998; Kim *et al.,* 1998b; Choi *et al.,* 2001; Idriss *et al.,* 2002). The optimal temperature of these phytases is ~55-70°C; and they are quite thermostable at a temperature range of 80-95°C (Kim *et al.,* 1998b; Choi *et al.,* 2001; Tye *et al.,* 2002).

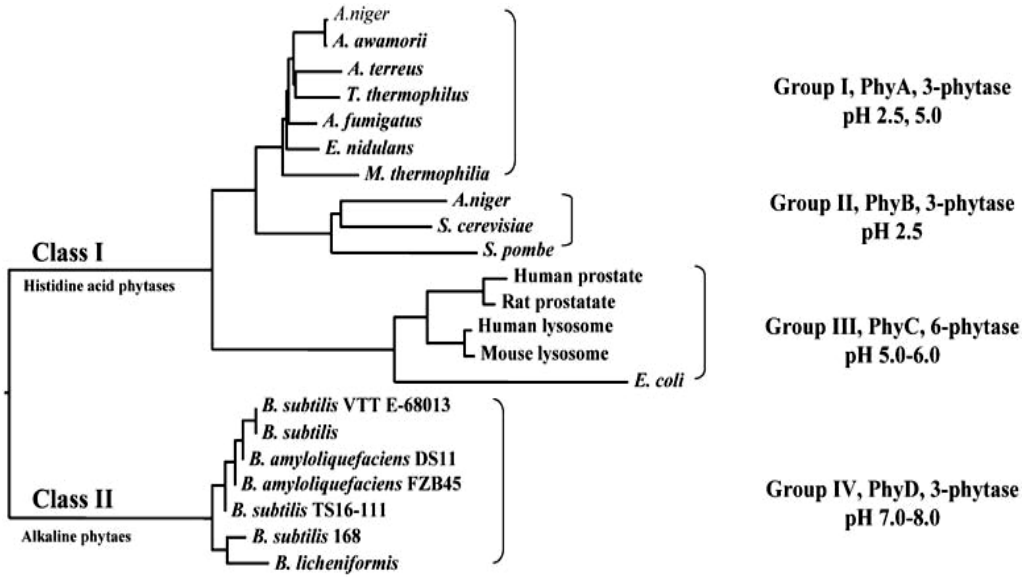

Fig. 1.6: Phylogenetic analysis of various HAPs and alkaline phytases (Oh *et al.,* 2004).

1.2.4 Occurrence and Sources of Phytase

Phytase is widely distributed among living beings including plants, microorganisms and animal cells. Phytase activities have been reported in variety of seeds and have been shown to play an important role in the hydrolysis of phytate, the major form of phosphate for plant seeds. Microorganisms include bacteria, yeast and many species of filamentous fungi. Phytases from several species of bacteria, yeast and fungi have been characterized (Konientzny *et al.,* 2002). Microbial sources are more promising for commercial production. In plants, phytase can be found in cereals, legumes, seeds and pollens. In animals, phytase exists in the erythrocytes and plasma of various species of vertebrates and in the mammalian small intestine (Cooper and Gowing, 1983; Martin and Lugue, 1985; Zyta, 1992). Lactic acid bacteria isolated from sour dough's exhibit a significant phytate degrading capacity (De angelis *et al.,* 2003).

1.2.4.1 Plant Sources

Plants express high levels of phytase in storage compartments such as grains, seeds (Chang, 1967; Eskin and Wiebe, 1983; Gibson and Ullah, 1990; Laboure *et al.,* 1993), cereals for the purpose of inorganic phosphate utilization and energy requirement for the growth of a germinating plant. Phytases have been isolated and characterized from a number of plant sources including rice and wheat bran (Suzuki *et al.,* 1907). They also isolated inositol as a product of the reaction. Plant phytases from wheat, mung bean and soya bean cotyledon phytases (Mandal *et al.,* 1972; Maiti *et al.,* 1974; Nagai *et al.,* 1975; Gibson and Ullah, 1988, Philippy, 1989), rye (Greiner *et al.,* 1998) spelt (Konietzny *et al.,* 1994) and barley (Sandberg and Thomas, 2002) were also purified, characterized and studied well. Root phytase has been proved as one of the mechanisms of plants to improve utilization of solid phosphate. In recent years, much focus has been given on

transgenic plants that express phytase with higher activities. The most well known PhyA phytase from *Aspergillus niger* was transformed into tobacco (Ullah *et al.,* 1999) and alfalfa (Ullah *et al.,* 2002), with the heterologously expressed and purified phytase exhibiting remarkable activities.

1.2.4.2 Microbial Sources

Microbial phytases are considered very valuable in upgrading the nutritional quality of plant foods. Phytate degrading activity is detected in numerous microorganisms. Microbial phytase may be intracellular or extracellular. For food application or fermentation, extracellular phytases are the most important. There are myo-inositol hexakisphosphate hydrolyzing enzymes that are secreted to the surroundings, aimed at efficient degradation of phytate as a source of phosphorous. The secretory microbial phytase are sometimes the case of common bakers yeast, fairly unspecific and many degrade other organic phosphorous sources. Most microbial phytases studied so far show their optimum activity in the acidic pH range (Pandey *et al.,* 2001; Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004; Singh and Satyanarayana, 2009; Rao *et al.,* 2009).

1.2.4.3 Bacterial Sources

Quite a lot of bacteria have expressed phytase activity. In bacteria, phytase is an inducible enzyme. Phytase production was studied in detail in *Bacillus subtilis* (Powar and Jagannathan, 1982; Shimizu, 1992), *Escherichia coli* (Greiner *et al.,* 1993), *Pseudomonas* sp. (Irving and Cosgrove, 1971) and *Klebsiella* sp. (Shah and Parekh, 1990). Some of these bacteria can degrade phytate during growth and hence produce extracellular phytases. Yoon *et al.,* (1996) isolated a bacterial strain that produced extracellular phytase from soil close to the roots of leguminous plants and this was identified as *Enterobacter* P4. *Escherichia coli* enzymes have been reported to be periplasmic and constitutively produce alkaline phosphatase (homodimeric periplasmic protein).

Strains of lactic acid producing bacteria lack extracellular phytase activity. Studies show that *Lactobacillus amylovorus* has the ability to improve the nutritional qualities of cereal and pulse based food fermentation.

1.2.4.4 Fungal Sources

Fungi are another important source of phytases. Among mycelia fungi, the most explored genus is *Aspergillus* from which the commercial by product phytase has originated. Howson and Davis. (1983) surveyed 84 fungi from 25 species for phytase production. The incidence of phytase production was highest in *Aspergilli*. Genes have been cloned and research has been undertaken to further improve the *Aspergillus* enzymes by protein engineering (Wyss *et al.,* 1999). Extracellular phytase activity has also been observed in filamentous fungi (Ricardson and Hadobus, 2001). Also in yeasts, the ability to synthesize and secrete phytase seems to be almost universal. Common bakers yeast is considered the best because of its "generally regarded as safe" (GRAS) status. Moulds commonly used in oriental food fermentations show both extracellular and intracellular phytase activity. Moulds generally studied for phytase activity include *Mucor* sp. *Rhizopus oligosporus, Aspergillus oryzae* and *Actinomucor*.

1.2.4.5 Animal Tissue

It has been reported that phytate degrading enzymes are seen in liver, mucosa extracts and blood of animals. Investigations of animal phytases are limited. The first report on these enzymes found in liver and blood of calves was given a century ago (McCollum and Hart, 1908). It was detected in the blood of lower vertebrates such as birds, reptiles and fishes (Rapoport *et al.,* 1941). The presence of phytate degrading activity in the gastro intestinal tract of various animals was investigated. Phytase activity has been detected in the mucosa extracts of the small intestine of rats, rabbits, guinea pigs, chicken, calves and humans (Bitar and Reinhold, 1971; Cooper and Gowing, 1983; Iqbal *et al.,* 1994).

Phytase activity of the mammalian intestine has been attributed to the action of nonspecific intestinal alkaline phosphatases (Davies and Motzok, 1972; Davies and Flett, 1978), intestinal phytase activity does not play a significant role in digestion of dietary phytate in monogastric (Iqbal *et al.,* 1994) but dietary phytate degrading enzymes have shown to be an important factor (Lantsch *et al.,* 1992). Degradation of phytate in monogastrics is also caused by microbial flora of the large intestine. However, ruminants can digest phytate through the action of phytase produced by microbial flora in the rumen. The inorganic phosphate produced by splitting of phytate is utilized by both the microbial flora and ruminant host (Vohra and Satyanarayana, 2003).

1.2.5 Properties of Phytases

Phytate degrading enzyme (phytase) often be found in various sources and is considered to be a special kind of phosphatase capable of releasing orthophosphate from phytate as well as other diversified phosphorylated compounds. It has distinct molecular features as well as catalytic properties depending on the source of origin. Only a few phytate degrading enzymes have been purified to homogeneity or near homogeneity (Konietzny and Greiner, 2002).

1.2.5.1 Biochemical Properties of Phytases

Phytases have a wide distribution and recent research has shown that phytases are most promising for many biotechnological applications. Phytases from several species of bacteria, yeast and fungi have been well characterized (Konietzny *et al.,* 2002; Vohra *et al.,* 2003).

Phytase activity has been found most frequently in fungi, such as *Aspergillus terreus* (Yamada *et al.,* 1968), *Aspergillus ficcum* (Gibson,1987) and *Aspergillus niger* (Shieh and Ware, 1968). Phytase is also produced by Gram positive bacteria such as *Bacillus* and Gram negative bacteria like *Aerobacter aerogenes* (Greaves *et al.,* 1967), *Pseudomonas* sp (Cosgrove, 1970), *Escherichia coli* (Greiner *et al.,* 1993) and *Klebsiella* (Shah and Parekh, 1990; Tamble *et al.,* 1994; Greiner *et al.,* 1997; Jareonkitmongkol *et al.,* 1997).

Generally phytases from Gram negative bacteria are intracellular enzymes, whereas phytase from Gram positive bacteria and fungi are extracellular (Powar and Jagannathan, 1982; Shimizu 1992; Kerouvuo *et al.,* 1998; Kim *et al.,* 1998 a; Choi *et al.,* 2001).

All phytase are monomeric proteins except for phytase B from *Aspergillus niger* which is a tetramer. The molecular weight of phytases from bacteria is rather variable within the range from 35 to 50 kDa, except the one from *Klebsiella aerogenes* which has two inducible molecular forms (Tambe *et al.,* 1994). Phytases from eukaryotic organisms (yeasts, fungi, plants and animals) are often glycosylated and have higher molecular weights: 85-150 kDa for fungal phytases, around 500 kDa for yeast phytases, and 50-150 kDa for phytases from plants and animal tissues.

Glycosylation may have an effect on the catalytic properties, the stability or the isoelectric point of an enzyme and has least effect on the specific activity and thermostability of phytases. The higher molecular weights of fungi and yeast enzymes are due to glycosylation of the enzymes by the host organism (Wyss *et al.,* 1996). However, a complete deglycosylation of the phytases from *Aspergillus niger* resulted in a 34% reduction in thermostability (Han *et al.,* 1999). Fungal and some plant phytate-degrading enzymes have been found to be glycosylated. The N-linked mannose and galactose of enzyme from *Aspergillus niger* NRRL 3135 account for 27.3% of the molecular mass (Ullah, 1987). Most phytases have an optimal temperature of 44-60^{o}C. Phytases from *Aspergillus fumigatus* and *Bacillus amyloliquifaciens* have an optimum temperature of about 70^{o}C (Table 1.1). To use phytases as feed additives, thermostability of the enzyme is a highly desirable property during the feed pelleting process (80-100^{o}C). Alkaline phytase from *Bacillus* are quite stable at the high temperature range of 80-95^{o}C (Kim *et al.,* 1998; Choi *et al.,* 2001; Tye *et al.,* 2002) while other phytases are rapidly inactivated above 60^{o}C (Liu *et al.,* 1998; Wyss *et al.,* 1999a; Tomschy *et al.,* 2002).

Table 1.1: Biochemical properties of phytases from various organisms (Oh *et al.,* 2004).

Source	Origin	Molecular Mass (kDa)	Optimum temperature		Heat inactiv-ation (°C)	Reference
			(°C)	pH		
Fungi	*Aspergillus niger* (Phy A)	85	58	2.5, 5.0	60	Ullah (1987)
	A.niger (Phy B)	85-100	60	2.5	60	Ullah and Phillippy (1994)
	A.fumigatus	85-100	58	5.0	60	Ullah et al., (2000)
	A.terreus	214	70	4.5	60	Yamamoto et al., (1972), Wyss et al., (1998)
	A.oryzae	120-140	50	5.5	60	Shimizu (1993)
	A.nidulans	77.8	55	5.5	60	Wyss et al., (1999a)
Yeast	*Sacccharomyces cerevisiae*	-	45	4.6	40	Nayini (1984)
	Schawanniomyces castellii	490	77	4.4	65	Segueilha et al., (1992)
Bacteria	*Bacillus subtilis*	37	60	7.5	-	Powar and Jagannathan (1982)
	B.Subtilis (natto)	38	60	6.0-6.5	-	Shimizu (1992)
	B.Subtilis	42	55	7.0 -7.5	60	Kerovuo et al., (1998)
	B.amyloliquefaciens	44	70	7.0-7.5	80	Kim et al., (1998b), Oh et al., (2001)
	Escherichia coli	42	55	4.5	60	Greiner et al., (1993)
	Klebsiella oxytoca	40	55	5.0-6.0	50	Jareonkitmongkol et al., (1997)
	K.terrigena	40	58	5.0	50	Greiner et al., /1997)
	Pseudomonas sp.	-	-	5.0	-	Irvine and Cosgrove (1971)
Plant	Canola seed	-	50	5.2	-	Houde et al., (1990)
	Cucurbita maxima	66.5	48	4.8	-	Goel and Sharma (1979)
	Lilium longiflorum	36,88	55-60	8.0	-	Scott and Loewus (1986)
	Legume seeds	-	-	8.0	-	Scott (1991)
	Maize	76	55	4.8	-	Laboure et al., (1993)
	Mung beans	158	57	7.5	-	Mandal et al., (1972)
	Soybean seeds	119	60	4.5-4.8	-	Sutardi and Buckle (1986); Gibson and Ullah (1988)
	Spelt	60	55	6.0	-	Konietzny et al., (1995)
	Typa latifola L.	-	-	8.0	-	Hara et al., (1985)
	Wheat bran	-	47	5.0	-	Nelson et al.,(1968); Lim and Tate (1973); Nagai et al.,(1975)

1.2.5.2 Molecular Properties

Majority of the so far characterized phytases behave like monomeric proteins with molecular masses between 35 and 70 kDa (Table 1.2). However, some phytase appear to be built up of multiple subunits. The phytases from *Schwanniomyces castellii* was identified as a tetrameric protein with one large subunit (M_r 125 kDa) and three identical small subunits (M_r 70 kDa) (Segueilha *et al.,* 1992) and that of the rat intestine was suggested to be an heterodimer consisting of 70kDa and 90kDa subunit (Yang *et al.,* 1991b). As both subunits were expressed differentially during the development of the rat (Yang *et al.,* 1991a), these subunits may represent two different enzymes.

The phytases isolated from maize roots (Hubel and Beck, 1996), germinating maize seeds (Laboure *et al.,* 1993), tomato roots (Li *et al.,* 1997b), soybean seeds (Hegeman and Grabau, 2001) and *Aspergillus oryzae* (Shimizu, 1993) were reported to be homodimeric proteins, whereas a homohexameric structure was proposed for the *Aspergillus terreus* enzyme (Yamamoto *et al.,* 1972). In other studies there was no indication of an oligomeric structure of the phytases from *Aspergillus terreus* (Wyss *et al.,* 1999b) and soybeans (Gibson and Ullah, 1988).This conflicting observation was suggested to be the result of the use of gel filtration or native PAGE (polyacrylamide gel electrophoresis) for molecular mass determination.

Table 1.2: Molecular properties of phytate degrading enzymes (Courtesy: Konietzny and Greiner 2002)

Source	Origin	Molecular Mass (kDa)	Optimum temperature		Heat inactiv-ation	Reference
			(°C)	pH	(°C)	
Fungi	*Aspergillus niger* (Phy A)	85	58	2.5, 5.0	60	Ullah (1987)
	A.niger (Phy B)	85-100	60	2.5	60	Ullah and Phillippy (1994)
	A.fumigatus	85-100	58	5.0	60	Ullah et al., (2000)
	A.terreus	214	70	4.5	60	Yamamoto et al., (1972), Wyss et al., (1998)
	A.oryzae	120-140	50	5.5	60	Shimizu (1993)
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	Escherichia coli	42	55	4.5	60	Greiner et al., (1993)
	Klebsiella oxytoca	40	55	5.0-6.0	50	Jareonkitmongkol et al., (1997)
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Plant	Canola seed	-	50	5.2	-	Houde et al., (1990)
	Cucurbita maxima	66.5	48	4.8	-	Goel and Sharma (1979)
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	Maize	76	55	4.8	-	Laboure et al., (1993)
	Mung beans	158	57	7.5	-	Mandal et al., (1972)
	Soybean seeds	119	60	4.5-4.8	-	Sutardi and Buckle (1986); Gibson and Ullah (1988)
	Spelt	60	55	6.0	-	Konietzny et al., (1995)
	Typa latifola L.	-	-	8.0	-	Hara et al., (1985)
	Wheat bran	-	47	5.0	-	Nelson et al.,(1968); Lim and Tate (1973); Nagai et al.,(1975)

1.3 Application of Phytases

Phytases are enzymes that have multifaceted applications ranging from human to animal, of plant and environmental benefits (Iti *et al.,* 2013).

1.3.1 Animal Feed Industry

Enzymes in animal nutrition have important roles in current farming systems. Enzymes are added to feed in order to promote beneficial effects such as degradation of anti-nutritional factors in feed that might interfere with the digestive processes, making available/increasing the availability of nutrients otherwise assimilated poorly through endogenous digestive enzymes, thereby decreasing the polluting effect of animal manure (Bedford, 2000).

With the advancement of animal feed industry, demand for enzymes has also increased rapidly having significant role in the production system. The feed enzymes available commercially are 3-phytase, 6-phytase, subtilisin, α - galactosidase, glucanase, xylanase, α-amylase and polygalacturonase (Selle *et al.,* 2007).Phytases have been mainly used as animal feed additive in diets largely for cattle, swine and poultry and to some extent fish. Plant raw materials including cereals, legumes, oil seeds, pollens and nuts are used for animal diet production. These materials contain phytic acid or phytate as their major storage of phosphorus (Reddy *et al.,* 1982). However, this form of phosphorus is unusable by animals like porcine and poultry which lack phytate digesting enzyme (Nelson, 1967) Furthermore, phytate chelates several important minerals, such as Fe^{2+}, Cu^{2+} and Zn^{2+}, and makes them unavailable to be assimilated in animal gastrointestinal tract. For phytate that is not absorbed, it is disposed with the feces to the environment (Mullaney *et al.,* 2000). The phosphorus released is thus transported into the water bodies causing

eutrophication which results in oxygen depletion due to excessive algal growth (Bali and Satyanarayana, 2001).

The problem caused by phytate can be resolved by the addition of microbial phytase to the animal feed which contain phytase-rich cereal diet (Nelson, 1967). The enzyme minimizes the need for supplementation of inorganic phosphorus due to its ability to improve the utilization of organic phosphorus in poultry. Furthermore, reduction of the excretion of phosphorus in manure also occurs (Mohanna and Nys, 1999). Apart from hydrolyzing phytate, the addition of phytase to feed at 250 to 1000 U/kg can fully replace phosphorus supplementation to the animal feed formula (Golovan *et al.,* 2001).

Nevertheless, the use of phytase as feed additive is limited by the enzyme cost and the loss of activity during feed pelleting at high temperatures up to 80^{o}C or during storage (Vohra and Satyanarayana, 2003). *Aspergillus niger* phytase was denatured at high temperature during feed pelleting (65^{o}C to 95^{o}C). Although the enzyme was added after pelleting process, the production cost was still high (Mullaney *et al.,* 2000). Such problems could be overcome by using thermotolerant phytase. For example, *Aspergillus fumigatus* phytase incubated at 90^{o}C or even 100^{o}C for 20 min resulted in only a minor loss of activity (10%), and after exposure to 90^{o}C for 120 min, 70% of the initial activity still remained (Pasamontes *et al.,* 1997b).

Although, *Myceliophthora thermophila* (Mitchell *et al.,* 1997) and *Thermomyces lanuginosus* (Berka *et al.,* 1998) are thermophilic fungi, their phytase are fully inactivated by high-temperature incubation. Therefore, from the characteristic property of thermophilic phytase gene, *Aspergillus fumigatus* phytase was cloned and expressed in *Aspergillus niger* NW205 (Pasamontes *et*

al., 1997b) and *Pichia pastoris* (Rodriguez *et al.,* 2000), the transformant phytase still showed the thermostability at 65^{o}C and 90^{o}C. Moreover, the use of compounds to enhance thermostability of phytase has been investigated.

1.3.2 Aquaculture

Aquaculture is the rapidly growing food industry. Fishmeal, which is usually used in fish feed manufacturing, turns out to be expensive and needs substitutes. Several studies have been conducted on the use of soybean meal or other plant meals in aquaculture (Mwachireya *et al.,* 1999). By substituting a more expensive protein source, with lower-cost plant protein, a significant cost reduction could be achieved. It also facilitates making available the minerals and nutrients to fish, which ultimately helps in reducing aquatic pollution (Gabriel *et al.,* 2007). Eventhough, there has been enhanced interest in the potential of feed enzymes in aquaculture, commercially viable versions have not been produced (Ringo *et al.,* 2010, 2014). However, there has been enthusiastic approach to the development of heat stable enzymes with reliable assays (Choct, 2006).

The replacement of fishmeal with plant or grain by-products is an effective low-cost option for fish feed manufacturing. The phosphorous content in plants and grains in the form of phytic acid/phytate (anti-nutritional factor) is not available to monogastric or agastric aquatic animals (NRC, 1993). Hence the better option is to supplement the fish diet with microbial phytase. This could increase the availability of phosphorus from plant ingredients to fish (Cain and Garling, 1995; Eya and Lovell, 1997; Li and Robinson, 1997; Sugiura *et al.,* 2001; Liebert and Portz, 2005). In addition, it helps in the

reduction of phosphorus discharge into the aquatic environment, thereby causing less pollution.

Studies revealed that dietary phytase improves the nutritive value of canola protein concentrate and decreases phosphorus output in the case of rainbow trout (Forster *et al.,* 1999). Related studies have been also reported for different species like channel catfish (Li and Robinson, 1997), African catfish (Weerd *et al.,* 1999) and *Pangasius pangasius* (Debnath *et al.,* 2005b). It has been reported that 20% and 40% of phytate can be released by the phytase addition of 500 and 1000 U/kg respectively in carps fed with soybean meal based diet (Schaefer and Koppe, 1995). In soybean meal based diet for crucian carp *Carassius carassius* 60% and 80% of phytate-P were released by the phytase addition of 500 and 1000 U/kg, respectively (Yu and Wang., 2000). Baruah *et al.* (2007) reported that phytase improves nutrient digestibility and growth performance of fish fed on plant protein sources supplemented with 750 FTU kg^{-1} level of phytase in *Labeo rohita* fingerlings. Higher growth was observed in juveniles of *Labeo rohita* when supplemented with microbial phytase in combination with different protein levels (Baruah *et al.,* 2004). Recent report by Hussain *et al.* (2011) proved that supplementation of corn gluten meal based diets with 750 FTU kg^{-1} of phytase is highly beneficial in developing cost effective and environment friendly aqua feed for major carps.

1.3.3 Food Industry

The plant origin food contains phytic acid or phytate that can cause the unavailability of nutrient; i.e. trace elements, vitamins and protein in human. There is also a great potential for the use of phytases in the processing and manufacturing of food for human consumption.

1.3.3.1 Probiotics

Probiotics are dietary supplements containing useful bacteria or yeasts. They are live microorganisms beneficial to the host organism, commonly consumed as part of fermented foods with specially added active live cultures. Phytase activity of some of the yeasts and bacteria which are generally regarded as safe for human and animal consumption is very well documented. Microorganisms such as *Saccharomyces cerevisiae* can be considered to be used as probiotic in various food formulations for improving phosphate utilization (Nayini and Markakis, 1984). *Candida tropicalis,* known to produce phytase, could not be included directly as probiotic as it was a common pathogen in humans (Hirimuthugoda *et al.,* 2007). However, phytase (phy) genes from microorganisms could be cloned in to those organisms such as *Lactobacillus* sp., *Brevibacterium casei* and *Brevibacterium epidermidis* regarded safe for human consumption (Iti *et al.,* 2013).

1.3.3.2 Baking and Food Fermentation

The chemical composition of flour used for bread-making depends on the percentage of the cereal grain removed by the milling process. Preference for the consumption of whole grain bread is increasing due to its nutritional benefits like high fibre content. Whole grain flour contains high concentrations of iron and phytate that originate from the bran (Brune *et al.,* 1992).

Phytases have known applications in bread-making processes (Haros *et al.,* 2001). The addition of phytase significantly reduces the phytate content in dough as well as shortens the fermentation time. Apart from the reduction of phytate content, it also improves the bread shape, volume and softness of the crumb. The fermentation time could also be shortened by phytase addition

without affecting the dough pH. Other texture parameters such as gumminess and chewiness were also decreased. These improvements in bread quality have been suggested to be associated with an in direct impact of phytase on α-amylase activity. Phytases enhances the bioavailability of essential minerals like Ca, Mg, Zn, Fe, etc. by acting on phytate, which has a trend to form complexes with these metal ions.

Steeping is a process required in wet milling of maize to obtain valuable corn steep liquor (Caransa *et al.,* 1988). Microbial phytase can alter steeping process, improve the separation of germ and starch from fiber and give a higher yield of starch and gluten as well as improve the properties of corn steep liquor.

Traditional food processing methods such as soaking and germination of grains and seeds have been reported to increase phytase activity and to be efficient for reduction of phytic acid (Tabekhia and Luh 1980, Ologhobo and Fetuga 1984; Beal and Mehta, 1985; Laboure *et al.,* 1993). The potential to produce phytic acid free weaning foods using phytase naturally occurring in grains and seeds needs to be evaluated and conditions optimized for such methods.

Moulds commonly used in oriental food fermentation have been examined for their ability to produce phytase. Tempeh is a popular oriental fermented food made from soya beans inoculated with moulds in the koji process (Wang *et al.,* 1980; Fujita *et al.,* 2003a; Fujita *et al.,* 2003b). Convincing evidence shows that not only the digestibility, vitamin content and flavors of soya bean are improved by this mould fermentation, but phytate content was also reduced to one-third.

1.3.4 Transgenic Plants

Microbial Phytase encoding genes are introduced into plant seeds like sesame, soya bean, canola, rice, wheat etc. to produce transgenic plants. An alternative to the addition of exogenic phytases to increase phytate-degrading activity during food processing is achieved by the incorporation of plants with a high phytase activity into the plant derived raw material. Phytase expressing transgenic seeds were discussed as a novel feed additive for improved utilization in animal agriculture (Pen *et al.,* 1993) and showed comparable results to feed supplemented with microbial phytase in terms of phosphorous (P) utilization. Transgenic plants have been assessed as bioreactors for the production of recombinant phytases to meet the industrial demand (Iti *et al.,* 2013). Transgenic *Arabidopsis* expressing phytase gene from *Aspergillus niger* (Mudge *et al.,* 2003) and transgenic soybean plants expressing phytase gene from *Aspergillus ficuum* (Li *et al.,* 2009) secreted phytase in the roots and they markedly enhanced the phosphorus nutrition of crop plants. This strategy has the ability to improve the efficiency of phosphorus fertilization in agricultural systems.

1.3.5 Paper & pulp industry

One of the main problems faced by the pulp and paper industries is the aging of papers. A thermostable phytase have a potential as a novel biological agent for degrading phytic acid during pulp and paper processing. Aging of paper can be prevented by carrying out the enzymatic degradation of phytic acid. The enzymatic degradation of phytic acid will not produce toxic by-products, suggesting its environment friendliness. (Ciofalo *et al.,* 2003). Therefore, application of phytases will turn out paper and pulp mill industry

eco-friendly and assist in the development of cleaner technologies (Liu *et al.,* 1998).

1.4 Purification and Characterization of Phytases

To study the properties of phytase, purification is a vital step. Purification of the enzymes included ammonium sulphate/acetone precipitation, gel filtration, ion exchange chromatography, affinity chromatography and hydrophobic interaction. One major problem in the purification of phytase from plant sources is their separation from non-specific acid phosphatases (Konietzny *et al.,* 1995).

In addition, purification of the enzymes derived from plant involves extended germination times. Purification of phytase from plant source is difficult due to its limited stability in comparison with that of the microbial enzymes. Ullah and Gibson. (1987) purified a phytase from *Aspergillus niger* NRRL 3135 to homogeneity in three steps by using ion exchange chromatography and chromatofocusing, the obtained enzyme was purified 22 fold with a recovery of 58%. Nagashima *et al.* (1999) purified a phytase from *Aspergillus niger* 3K-57 in four steps by using ion exchange chromatography (two types), gel filtration and chromatofocusing.

Greiner *et al.* (1993) purified two periplasmic phytases from *Escherichia coli* by 16000 fold with a recovery of 7 and 18% respectively using a five step purification protocol including ammonium sulphate fractionation, ion-exchange chromatography and hydrophobic interaction.

Purification of phytase from oat (Greiner and Lasson Alminger, 1999) and faba beans (Greiner *et al.,* 2001) respectively could be achieved using a seven-step purification protocol which included ammonium sulphate

fractionation, acetone precipitation, ion exchange chromatography and gel filtration. The enzyme from oat was purified 5380 fold with a recovery of 23% and that of faba beans by 2190 fold with a recovery of 6%.

For purification of intestinal phytate degrading enzyme of rat, a six-step protocol, including butanol extraction, ethanol precipitation, ion exchange chromatography and gel filtration was adapted. In this process purification of 1136 fold with 19% recovery was achieved (Yang *et al.,* 1991).

Certain microbial phytases have been accessible not by extraction and purification of the enzymes from wild type organisms, but by cloning and heterologously expressing the corresponding genes. The recombinant phytases from *Aspergillus terreus* (Wyss *et al.,* 1999a), *Aspergillus fumigatus* (Psamontes *et al.,* 1997b; Wyss *et al.,* 1998; 1999) as well as from thermophilic fungi *Thermomyces languinosus* (Berka *et al.,* 1998), *Myceliophthora thermophilia* and *Talaromyces thermophilus* (Psamontes *et al.,*1997a; Wyss *et al.,* 1999) have been biochemicaly characterized.

Reverse phase C18 high-performance liquid chromatography (HPLC), a method which is capable of detecting the reduction in phytate during enzymatic hydrolysis has been developed for separation and quantitative determination of phytate and lower myo-inositol phosphates (Skoglund *et al.,* 1997).

1.5 Bioprocess

Bioprocess optimization is essential to make the enzyme industry competitive and sustainable (Gavrilescu *et al.,* 2005). Various novel bioprocess strengthening strategies are being put to use to considerably enhance productivity (Chisti and Moo-Young, 1996). Among them optimization of media composition and culture conditions dominate (Kennedy *et al.,* 1994).

1.5.1 Optimization

Conventional method of medium optimization is by employing 'one factor at a time' technique where one factor is varied at a time keeping all others constant. Its disadvantage is the time required for completion of the analysis which does not bring about the effect of interaction of various parameters (Cochran and Cox, 1992).

Meanwhile, statistical approach of optimization using Response Surface Methodology (RSM) could be performed to tackle this problem. RSM is a combination of statistical and mathematical techniques commonly used to evaluate the effects of several factors that influence the responses by varying them simultaneously in limited number of experiments. Factorial design and RSM are the most important tools to study the effect of both the primary factors and their mutual interactions on the concerned response and thus determine the optimal process conditions (Box *et al.,* 1958). Experimental designs for optimization have been widely used for the optimization of multiple variables with minimum number of experiments (Leiro *et al.,* 1995; Kalil *et al.,* 2000; Montgomery, 2008).

1.5.2 Fermentor

Microbial fermentation for the commercial production of enzymes and has gained tremendous importance. (Weiss and Edwards, 1980; Strohl, 1997; Leeper, 2000; Liese *et al.,* 2000; Schreiber, 2000).A typical fermentation process (bioprocess) involving microbial cells can be divided into three stages;

Stage I: Upstream processing which involves preparation of liquid medium, separation of particulate and inhibitory chemicals from the medium, sterilization and air purification

Stage II: Fermentation, the conversion of substrates to the desired product with the help of biological agents such as microorganisms

Stage III: Downstream processing which involves separation of cells from the fermentation broth, purification and concentration of the desired product and waste disposal or recycling of waste

Depending on the type of the product, the concentration levels it produces and the purity desired, the fermentation stage might constitute anywhere between 5-50% of the total fixed and operating costs of the process. Therefore, optimal design and operation of a bioreactor frequently dominates the overall technological and economic performance of the process. This process can be operated either in a batch or continuous mode.

1.5.3 Downstream Processing

Likewise production, downstream processing also is an integral part of any product development as the final cost of the product largely depends on the cost incurred in extraction and purification. The conventional technique of filtration for solid - liquid separation is not suitable for the bio-separation, in which the size of the microorganism to be separated is small, especially when the cells are broken to release the intracellular components causing increase in the viscosity of the system (Huggins, 1978; Mosqueira, 1981). Downstream processing techniques such as precipitation and column chromatography are not only expensive but also result in lower yields. Centrifugation and other modern methods like electrophoresis have enormous scale-up problems, making them too costly unless the product is of high value.

Hence, there is a need to develop efficient, cost effective, fast, simple, and eco-friendly downstream processing methods for recovery of biomolecules

produced by biotechnological means. Extraction using Aqueous Two-Phase Systems (ATPSs) is one such method. Though this technique was developed by, Albertsson (1956), its importance and applications have been realized only in the recent years.

Liquid-liquid extraction using organic-aqueous phase systems have been now extensively used in the chemical industry. Aqueous two-phase extraction (ATPE) has been successful to a large extent in overcoming the limitations of conventional organic-aqueous extraction, since it employs two aqueous phases. ATPE has been recognized as a superior and versatile technique for the extraction and purification of biomolecules (Walter *et al.,* 1985; Albertsson, 1986; Zaslavski, 1995).

Aqueous two-phase systems are very well known for their utility in the extraction and purification of biological materials such as enzymes/proteins, nucleic acids, viruses, cell organelles etc.(Kula *et al.,* 1982; Walter *et al.,* 1985; Albertsson, 1986; Diamond and Hsu, 1992; Zaslavski, 1995; Raghavarao *et al.,* 1998).

1.6 Present Level of Demand for Phytase

The global market for feed enzymes is definitely one promising segment in the enzyme industry. It was estimated at around $344 million in 2007, and expected to reach $727 million in 2015 (Frost and Sullivan 2007). Global enzyme market is estimated to rise 7 percent at a healthy pace to $8.0 billion in 2015. With the advancement of the animal production industries, demand for enzyme preparations increases rapidly. The Chinese government has been making plans for the use of phytase in recent years, about 20-40% of concentrated feed (excludes ruminant feed), to achieve better utilization of total

plant phosphorus. The use of phytase in animal nutrition has an important role in current farming systems. Feed enzymes can increase the digestibility of nutrients, leading to greater efficiency in feed utilization. Rapid developments in biotechnology over the past decade have enabled advanced discovery, optimization and manufacturing techniques that are driving development across all aspects of the enzyme industry.

1.6.1 National Status of Phytase

The only one particular enzyme, which has really caught on in India, is phytase. The usage of phytase in Indian diets started only in recent years. India has the highest cattle population in the world. The present cattle and buffalo population in the country is estimated to be around one hundred million. The potential demand for phytase in the cattle and poultry feed is around 4000 tons per annum (Gunashree and Venkateswaran, 2008).The potential demand, as stated above for phytase in India, is estimated to be 4000 tons per annum with the actual demand of 200 tons (Vats and Banerjee, 2004). The demand is expected to grow at a pace of 10 to 12% per annum in the coming years. It has to be realized that the phytase usage is confined to only around 5% of the requirement, and all future demand has to be satisfied from the production within the country itself.

New and emerging applications have enhanced greater demand for phytase, and the industry is responding with a continuous stream of innovative products. The phytase is now under the threshold of major research and development (R&D) initiatives, resulting in the development of a number of new products, improvement in the processes and performance of several existing products. Biotechnological interventions play a vital role not only in

the production but also in environmental protection and sustainability. Genetically modified feeds can improve water and soil quality by reducing the level of phosphorus and nitrogen in animal waste.

With the existing huge market for animal feed, India is not in any way near to satisfy the required demand of phytase from the country's production system. At present most of the enzyme products in India are directly imported or the individual enzymes are imported and repacked. These enzymes attract import duty, making their usage expensive. There are very few companies producing enzymes in India, specifically due to lack of viable technology as well as appropriate seed organisms. India needs to look into these impediments and put up new improvised methods for enzyme production units (Singh *et al.,* 2008). Precisely, there is tremendous opportunity for India to be a leading exporter to become very competitive in a world of globalised trade.

1.6.2 International Status of Phytase

The US Government's interest in improving health of animals has increased over the years resulting in registering faster growth of animal feed additive industry. The environmental benefits of using feed enzymes motivated producers in Europe and Asia, China, United States and Canada to treat animal feed routinely with enzymes. Feed enzymes were first introduced in China in 1989, and it became widely accepted in 2000 by many in the animal farming industry and it achieved rapid growth in 2003. Among the enzymes, growth of phytase experienced sharp increase in 2004 internationally, making it the most sought after feed additive (Research and markets reports, 2007). The worldwide phytase potential market size is US$ 500 million, accounting US $ 200 million for China alone (Petry and Bugang, 2009). Rapid developments in biotechnology

over the past decade have enabled advanced optimization and manufacturing techniques that have been driving developments across the enzyme industry.

1.7 Objectives of the Present Work

Considering the requirement of Global phytase industry, the work entitled **"**Bioprocess optimization and characterization of phytase from an environmental isolate *Bacillus* MCCB 242**"** was undertaken with the following objectives:

1) Screening, isolation and identification of phytase producing microorganisms from environment.
2) Molecular characterization and phylogenetic analysis of the phytase producers.
3) Production optimization of phytase from *Bacillus* MCCB 242.
4) Purification and characterization of phytase.
5) Downstream process and purification of the enzyme.

Chapter 2

SCREENING, ISOLATION AND IDENTIFICATION OF PHYTASE PRODUCING MICROORGANISMS FROM ENVIRONMENT

Contents

2.1 Introduction

Since the first recognition of microorganisms, Scientists have devised classification schemes with the goal of systematically identifying species in an evolutionary or phylogenetic context (Clarke, 1985). Biologically active enzymes can be extracted from any living organism. A very wide range of sources are used for commercial enzyme production ranging from *Actinoplanes* to *Zymomonas*, from spinach to snake venom. Microorganisms are well known for their ability to excrete enzymes into the environment. Thus there is an increasing interest in isolating new enzymes and new enzyme-producing strains for their use in biotechnology (Tayyab *et al.,* 2011; Yoo *et al.,* 2011). The extracellular enzymes derived from the bacteria serve for many purposes in the industry. A wide range of microorganisms including bacteria and fungi are capable of producing phytases. Most phytase-producing microorganisms from nature were isolated from soils (Shieh and Ware 1968; Howson and Davis, 1983; Tseng *et al.,* 2000) and the rumen flora (Raun *et al.,* 1956; Yanke *et al.,* 1998). Yet several natural sources of phytase producing organisms remain unexplored (Bae *et al.,* 1999). Very few reports exist on phytase-producing microbes from India and they

include the bacterium *Lactobacillus amylovorus* (Sreeramulu *et al.,* 1996) and the yeast, *Pichia anomala* (Vohra and Satyanarayana 2001, 2002).

Soil is a good source of novel enzyme-producing strains because they are nutrient rich environment where there is increased proliferation of microorganisms. The diversity of these microorganisms rely on nutrient availability and several physicochemical properties related to the climate and type of soil, texture, pH, temperature, solar irradiation, aeration, water content, mineral composition etc. Many soil microorganisms are highly active in nutrient recycling, mainly in the degradation of vegetable polysaccharides and therefore possess highly active enzymatic machinery (Atlas and Bartha, 1998; Uroz *et al.,* 2011).

Shieh and Ware. (1968) first reported the isolation of phytase producing microorganisms using selective phytase screening medium. They prepared insoluble calcium phytate containing turbid screening media, which turned transparent, owing to solubilization, by diffused phytases from the isolates. After that, culture enrichment technique was used to isolate phytase producing microorganisms. Phytases have been detected in different bacteria (Gram positive, Gram negative rods and cocci) viz. *Aerobacter aerogenes* (Yoon et a1., 1996), *Escherichia coli* (Greiner, 2000), *Klebsiella aerogenes* (Sajidan, 2004) and *Pseudomonas* sp. (Kim *et al.,* 2003).

2.1.1 Techniques in Bacterial Identification and Characterization

The advent of molecular techniques in genomics and proteomics has shifted the traditional microbiological procedures for bacterial identification to elucidate specific gene sequences or molecular components of a cell. These new methods can provide a rapid, multidimensional data output with taxonomically relevant molecular information on both individual strains and whole populations (David *et al.,* 2008). Accurate identification of bacterial isolates is

an essential task in microbiology. Precisely, microorganisms are identified based on phenotypic and genotypic traits which are complementary.

The screening of various microorganisms to determine their capability to produce phytase is evaluated in this chapter. To isolate the phytase producing microorganisms, soil samples were chosen for the study. Enrichment culture media containing sodium phytate as the sole phosphorus were used for the primary screening in order to find microorganisms with a phytase producing capacity. An attempt was made to isolate phytase-producing microorganism among hundred kinds of molds, actinomycetes, bacteria, etc

2.2 Materials and Methods

2.2.1 Study Area

Soil samples were collected from different natural sources for the isolation of phytase producing heterotrophic bacteria.

1) Mud from paddy field (farm land soil from Kollam & Pathanamthitta (geological coordinates $8^{o}52'$N $76^{o}35'$E, $9^{o}15'$ N $76^{o}49'$E respectively) districts of Kerala),
2) Marine sediment from the beach sands at Fort Kochi (Geological coordinates- $9^{o}57'$ N $76^{o}14'$ E), Ernakulam District, Kerala and
3) Coir retting area in Alappuzha (Geological coordinates – $9^{o}29'$ N 76°19' E) District, Kerala.

The soil samples taken for the screening of microorganisms for phytase were collected in sterile zip-lock plastic covers maintaining aseptic conditions, stored at 4°C and marked accordingly to their source and location. The collected samples were brought to the laboratory for isolation of phytase producing bacteria and for further analysis.

2.2.2 Preparation of Soil Samples

One gram each of the soil samples were serially diluted in diluent composed of 0.1% peptone and 0.5% NaCl in distilled water. The diluted samples were pour plated on to humic acid agar medium & soil extract agar.

2.2.3 Isolation of Microorganisms on Humic Acid Agar Medium (HV Medium)

Isolation of the phytase-producing microorganism was carried out in Humic acid agar medium of the following composition.

Humic acid agar medium

Humic acid	1.0 g
Na_2HPO_4	0.5 g
KCl	1.7 g
$MgSO_4.7H_2O$	0.05 g
$CaCO_3$	0.02 g
B-vitamin	0.01 g
Distilled water	1000 mL
Agar	18.0 g

The medium was adjusted to pH 7.2 prior to autoclaving and amended with filter sterilized vitamin after sterilization and cooling. Aliquots of 1ml each from each dilution were plated in duplicates by pour plate method and incubated for 72 hrs at 37^{o}C. From the plates colonies were isolated and streaked on to fresh plates and incubated to obtain pure cultures.

2.2.4 Isolation of Microorganisms on Soil Extract Agar

Soil extract was prepared by sieving 20 grams of soil through a porous sieve (1 mm) and boiling the same with 200 ml of water for 3 minutes. After the mixture has cooled and settled, the supernatant was carefully decanted and

filtered through Whatman no.1 filter paper. The filtrate was then sterilized by autoclaving at 121°C for 15 minutes and stored in sterile bottles at 4°C.

Soil Extract Agar Medium

Soil Extract	100 mL
Peptone	5g
Beef Extract	5g
NaCl	5g
Agar	20g
pH	7.5± 0.3
Distilled water	900 mL

Aliquots of 1ml each from each dilution were plated in duplicates by pour plate method and incubated for 72 hrs at 37° C. From the plates colonies were isolated and streaked onto fresh plates and incubated to obtain pure cultures.

2.2.5 Purification of Isolates by Streaking

The isolates were purified by streaking on the respective plates and accordingly 135 pure cultures could be obtained. Purity was confirmed by Gram staining and microscopic observation

2.2.6 Primary Screening

Primary screening was performed by spot inoculating the isolates on soil extract agar plates supplemented with 0.5% sodium phytate and incubated for 5 days at 37°C. Disappearance of precipitated calcium or sodium phytate was considered as the indication of enzyme activity. Subsequent to growth of colonies on the plates a two-step counter staining procedure was followed on the plates by flooding with 2% (w/v) aqueous Cobalt Chloride solution, incubation for 5 minutes at room temperature, and replacing it with freshly prepared solution containing equal volumes of 6.25% aqueous Ammonium Molybdate and 0.42% Ammonium MetaVanadate. After 5 minutes of incubation, the mixture of aqueous

Ammonium Molybdate / Ammonium MetaVanadate was removed and the plates were examined for zones of clearing (Bae *et al.,* 1999).

2.2.7 Cultivation of the Segregated Phytase-Producing Bacteria

The segregated phytase producing bacteria were cultivated in Tryptone Soya Broth (TSB) at 37° C. overnight. Aliquot of 0.1 ml of the culture was inoculated into 50 ml Phytase Screening medium (PSM) (broth) and incubated at 37° C. for 4 days on a rotary shaker at 100 rpm.

Tryptone Soya Broth (TSB)

Casein peptone (pancreatic)	15 g
Soytone	5.0 g
NaCl	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Glucose	2.5 g
Distilled water	1000mL
pH	7.3±0.2

Phytase Screening Medium (PSM)

Glucose	15 g
NH_4NO_3	5.0 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0. 01 g
$MnSO_4.7H_2O$	0. 01 g
Na-phytate	10.0 g
Agar	20.0 g
Distilled water	1000mL
pH	7.0 ±0.2

Phytase was assayed at regular intervals for 96 hrs.

2.2.8 Phytase Assay

Known methods for assaying phytase activity in solution are based on detection of the orthophosphate released from phytate in the enzyme-catalyzed hydrolysis reaction. Phosphate is detected spectrophotometrically after reaction with molybdate in sulphuric acid (Heinonen and Lahti, 1981). The isolate was cultivated 50 ml PSM broth containing 1% Na-phytate at 37^{o}C for 4 days. The culture was centrifuged at 10000 *g* for 5 minutes. The supernatant saved as enzyme stored at 4^{o}C. Phytase activity was measured using sodium phytate as the substrate. The Phytase activity of the isolates was detected under 2 different Hydrogen ion concentration (pH 5.5 & pH 2.5) using Sodium Acetate buffer & Glycine HCl buffer. The absorbance was measured using Cary 50 probe UV-Visible spectrophotometer and read at 750 nm.

One phytase unit is defined as the amount of enzyme that liberates 1 µmol of inorganic phosphorus per minute from 0.01 mol L^{-1} sodium phytate at standard assay conditions at pH 5.5 & 2.5 and temperature 40^{o}C.

For the detection of the enzyme activity following reagents were used:

a. 0.25 M sodium acetate buffer (pH 5.5).
b. 0.25M glycine-HCl buffer (pH 2.5)
c. 10% trichloroacetic acid
d. Color developing agent (hydrated ferrous sulphate & ammonium heptamolybdate tetra hydrate)
e. Sodium Phosphate (NaH_2PO_4)

"Phytase substrate" refers to a compound which is capable of being transformed by a phytase-catalyzed reaction to release inorganic phosphorus. In

this study sodium phytate was used as the substrate and two different buffers were used to dissolve the same.

2.2.8.1 Preparation of Enzyme Substrate

1. Dissolved 1.64 g Na-phytate in 0.25 M sodium acetate buffer (pH 5.5).
2. Dissolved 1.64 g Na-phytate 0.25M glycine-HCl buffer (pH 2.5).

An aliquot of 1 ml enzyme was mixed with 1 ml substrate and the mixture was incubated at 40^{o}C in water bath for 60 minutes. The reaction was stopped by adding an aliquot of 1 ml 10% trichloroacetic acid (TCA) into the mixture. Subsequently, 2 ml color developing agent (3.66g $FeSO_4 7H_2O$, 0.5g ammonium heptamolybdate tetrahydrate, 1.6 ml conc. H_2SO_4 made up to 50 ml by adding double distilled water) was added into the mixture. The liberated inorganic phosphate was detected spectrophotometrically at an absorbance of 750 nm. Assays were conducted at 24, 48, 72 and 98 hours after inoculation. In order to quantify the phosphate released a calibration curve was made using NaH_2PO_4. One phytase unit is defined as the amount of enzyme that liberates 1 μmol of inorganic phosphorus per minute from 0.01 mol L^{-1} sodium phytate at standard assay conditions (pH 5.5 and temperature 40^{o}C).

2.2.9 Estimation of Protein of the Enzyme

Total protein of the enzyme preparation was determined following Lowry's method (1951) by using Bovine Serum as standard.

Reagents for protein estimation

Solution A- 1% $CuSO_4$

Solution B- 2% Sodium Potassium tartrate

Solution C- 4% Na_2CO_3 and 0.2 N NaOH

Solution D- Folin Ciocalteu 1 N prepared in 1:1 ratio

Working Solution

Aliquot of 98 ml of solution C and 1 ml of Solution A & B were added. This mixture was used as the working solution.

Procedure

Solutions of bovine serum albumin having concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6mg were used as standard. Aliquots of 0.2ml sample were diluted to 1 ml with double distilled water and added 5 ml working solution of the reagent, kept for 10 minutes incubation in dark. Added 0.5 ml Folin Ciocalteu reagent diluted to 1:1 ratio and mixed well. The tubes were kept for 30 minutes incubation in dark. A reagent blank was prepared in distilled water. The colour developed was read against the reagent blank at 640 nm.

2.2.10 Phenotypic Characterization of the Isolates

The morphological, cultural, and biochemical characteristics of the isolated phytase producers were evaluated according to Bergey's Manual of Determinative Bacteriology (1984).

2.2.10.1 Gram Reaction

Gram staining was performed following standard Gram's staining technique and observed under oil immersion (100X magnification) objective.

2.2.10.2 Motility Test

Motility was observed following hanging drop method and stabbing in soft agar tubes. The soft agar was composed of peptone 0.5%, beef extract 0.5%, NaCl 0.5% and agar 0.3% at pH 7.5. Rhizoidal growth from the line of inoculation towards the periphery was considered as the sign of motility.

2.2.10.3 Biochemical Characterization

The isolates were biochemically characterized following standard protocols for catalase and oxidase production, indole production, methyl red and Voges Proskauer reactions, citrate utilization, urease activity, H_2S production, arginine, lysine and ornithine decarboxylase reactions,, phenylalanine deamination and nitrate reduction. Their ability to produce acid from Dextrose, Fructose, Ribose, Arabinose, Galactose, Lactose, Sucrose, Maltose, Mannose, Rhamnose, Raffinose. Xylose, Trehalose, Sorbose, Dulcitol, Inositol, Mannitol, Esculin, Salicin, Cellobiose, Melibiose, and Adonitol was investigated.

- **Catalase** activity was tested by adding a few drops of 3% H_2O_2 to a test tube containing over nightly grown culture in nutrient broth. Effervescence indicated positive result.
- To perform **Kovac's oxidase** test (Kovac, 1956), the organisms were freshly grown on nutrient agar slants. A platinum loop was used to pick a bit of inoculum and prepare a compact smear on filter paper moistened with 1% aqueous Tetramethyl-p-phenylene diamine dihydrochloride (TPDD). A positive result was recorded when the smear turned violet within 10 seconds, indicating the formation of indophenol.
- To test the production of **Indole** the culture was grown in growth medium containing tryptophan, the amino acid containing indole ring. After, 48 hours of growth, 3-4 drops of Kovac's reagent (concentrated Hydrochloric acid 25 ml, p-Dimethyl amino benzoic acid 5 g, Amyl alcohol 75 ml) was added to 5 ml culture and agitated. Formation of red colored ring indicated Indole positive reaction.

- Glucose phosphate peptone broth was used for **Methyl red and Voges-Proskauer tests**. Aliquots of 4ml medium, composed of Glucose 0.5%, Peptone 0.5% and K_2HPO_4 0.5% was inoculated and incubated for 3days at 30°C. To 2ml of the culture, 4 drops of Methyl Red reagent was added, a positive reaction was indicated by the development of strong red colouration. To perform Voges-Proskauer test Barritt's reagent A and B (1ml of 40% KOH and 3ml of 5% α-Naphthol in absolute ethanol) was added and the preparation agitated. Positive test was indicated by eosin pink color within 2-5 minutes.

- Test for **nitrate reduction** was carried out using nitrate broth containing 1.0% KNO_3 in nutrient broth. To the tubes (3ml aliquots) after inoculation and incubation, 0.5 ml each of Sulphanilic acid and α-Naphthylamine were added and a positive result was indicated by red colouration.

- **Hydrogen Sulfide (H_2S)** producing capacity of the cultures was checked by stab inoculating Gelatin Iron Agar medium (Peptic digest of animal tissue 2.50%, Meat extract 0.75%, Sodium chloride 0.50%, Gelatin 12.0%, Ferrous chloride 0.05%, Agar 1.00%. Final pH 7.0±0.2 at 25°C). Ferrous chloride present in the medium generated black precipitate of Fe S by entering in to reaction with H_2S produced by the organism.

- Simmon's (1926) demonstrated the ability of bacteria to **utilize Citrate** in Simmon's citrate agar (Ammonium dihydrogen phosphate 0.1%, Magnesium sulphate 0. 02%, Dipotassium phosphate 0.10%, Sodium citrate 0.20%,Sodium chloride 0.50%, Bromo thymol blue 0.008%, Agar 1.50%. Final pH 6.8±0.1 at 25°C). Positive result was indicated by color change of the slant from green to blue.

- Urea broth base (Monopotassium phosphate 0.91%, Dipotassium phosphate 0.95%, Yeast extract 0. 010%, Phenol red 0. 010%. Final pH 6.8±0.2 at 25°C) along with 40% urea solution in the ratio of 19:1 was inoculated to observe **Urease production**. Production of pink color in the broth indicated positive reaction.

- **Phenylalanine deaminase** activity of the cultures was investigated using Phenylalanine agar slants (Yeast extract 0.30%, Sodium chloride 0.50%, DL-Phenylalanine 0.20%, Disodium phosphate 0.10%, Agar 1.50%. Final pH 7.3±0.2 at 25°C). Positive result was indicated by the appearance of green color on addition of a few drops of 10% aqueous ferric chloride to the slant grown cultures.

- **Amino acid decarboxylation** by the cultures was assessed in Decarboxylase Test Medium Base Falkow, (Peptone 0.5%, Yeast extract 0.3%, Dextrose 0.1%, Bromo cresol purple 0.002%. Final pH 6.8 ± 0.2 at 25°C) prepared in distilled water. The amino acids used were L-arginine-hydrochloride, L-ornithine-hydrochloride, and L-lysine-hydrochloride added independently supplemented with glucose. The control consisted of the basal medium without amino acid, but with glucose. While the medium turned yellow in the control tubes, in tests, the pH indicator turned to purple due to the production amines.

- **Production of acid from sugars / carbohydrate** through fermentation was investigated using Phenol Red Broth Base (Peptone 1%, NaCl 0.05%, Phenol red 0.0018%) supplemented with 25mg sugars in the form of discs (Sigma Aldrich Inc.). The sugars used were Dextrose, Fructose, Ribose, Arabinose, Galactose, Lactose, Sucrose, Maltose, Mannose, Rhamnose, Raffinose, Xylose, Trehalose, Sorbose, Dulcitol, Inositol, Mannitol, Esculin, Salicin, Cellobiose, Melibiose and Adonitol.

The tubes were inoculated and incubated for 24-48 hours. Acid production was recorded from the colour change of pH indicator, phenol red, from purple to yellow.

2.2.11 Morphological Characterization using Scanning Electron Microscopy (SEM)

The phytase producing bacteria were taken up for scanning electron microscopic studies for observing the morphological features. Preparation of samples for SEM involved three steps; fixation, dehydration, and coating with heavy metals. Bacterial suspension at the appropriate dilution (1:100) was made from an over-night grown culture. An aliquot of 15 µl culture was centrifuged at 8000*g* in a refrigerated centrifuge for 15 minutes. The sample was washed three times with PBS and fixed with 0.1% glutaraldehyde for 30 minutes. The samples were washed four times with PBS and double distilled water repeatedly (Bayer *et al.,* 1974). The cells were spread on SEM stubs dried in critical point drying apparatus, platinum coated and observed under JEOL Analytical Scanning Electron Microscope, JSM 6390 LV, Tokyo, Japan (Sophisticated Analytical Instrument Facility, Cochin University of Science and Technology, Kochi).

2.2.12 Molecular Characterization

DNA based identification of the bacterial cultures was performed employing the following techniques.

2.2.12.1 Sequencing 16S rRNA Gene

16S rRNA gene sequencing plays a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria. The 16S rRNA gene sequencing involved the following steps:

2.2.12.1.1 DNA Extraction

For the extraction of DNA, a sample of 2 ml bacterial cell suspension (18 h old bacterial cell suspension grown in Luria–Bertani broth) was centrifuged at 15000 g for 10 min at 4°C. The pellet was resuspended in 500µl TNE buffer (10mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.15 mM NaCl) and centrifuged again at 15000 *g* for 10 min at 4°C. Subsequently, the pellets were resuspended in 500 µl lysis buffer (0.05 mM Tris-HCl, pH 8.0, 0.05 mM EDTA, 0.1 mM NaCl, 2%, SDS, 0.2 % PVP and 0.1% Mercaptoethanol) (Lee *et al.,* 2003), and 10µl Proteinase K (20mg/ml) was added and incubated initially for 1 h at 37°C and then for 2 hrs at 55°C . Further extraction was carried out by phenol-chloroform method (Sambrook and Russell, 2001). The sample was deproteinated by adding equal volume of Phenol (Tris - equilibrated, pH 8.0), Chloroform and Isoamyl alcohol mixture (25:24:1). The phenol and the aqueous layers were separated by centrifugation at 15000 *g* for 15 min at 4°C. The aqueous phase was pipetted out into a fresh tube and the process was repeated once more. Following this, an equal volume of Chloroform: Isoamyl alcohol (24:1) mixture was added, mixed by gentle inversion and centrifuged at 15000 *g* for 15 min at 4°C to separate the aqueous phase. This aqueous phase was then transferred to a fresh tube and DNA was precipitated by incubation at -20°C overnight after adding equal volume of chilled absolute ethanol. The precipitated DNA was collected by centrifugation at 15000 *g* for 15 min at 4°C and the pellet was washed with 70% ice cold ethanol. Centrifugation was repeated once more and decanted the supernatant and the tubes were left open until the pellet got dried. The DNA pellet was dissolved in 100µl MilliQ (Millipore) grade water.

The isolated DNA was quantified spectrophotometrically (A_{260}) and the purity of DNA was assessed by calculating the ratio of absorbance at 260 nm

and 280 nm (A_{260}/A_{280}). Electrophoresis was done using 1% agarose gel. An aliquot of 5 µl from each genomic DNA was analysed by 0.8% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light, documented using a gel documentation system (Gel Doc XR+, Bio-Rad, USA).

Concentration of DNA (µg/ml) = OD at 260 nm×50×dilution factor.

2.2.12.1.2 PCR Amplification of the Extracted DNA

Amplification of 16S rRNA gene was performed according to Reddy et al. (2000) using universal primers 16S F (GAG TTT GAT CCT GGC TCA) and 16S R (ACG GCT ACC TTG TTA CGA CTT). The amplification was performed using DNA Thermal cycler (Eppendorf). Reaction mixture (final volume 25µl) contained 2.5µl 10 X buffer, 1µl 10 pmol each of oligonucleotide primer, 1.5µl DNA template, 2.5µl 2.5 mM each Deoxynucleoside triphosphate, 1µl Taq polymerase, and the remaining volume was made up with sterile Milli Q water. The amplification profile consisted of initial denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 94°C for 20s, annealing at 58°C for 30s and extension at 68°C for 2 min followed by a final extension at 68°C for 10 min. The PCR product was analyzed by 1% Agarose gel electrophoresis prepared in 1X TAE buffer and stained with Ethidium bromide and visualized under ultraviolet light, documented using a gel documentation system (Gel Doc XR+, Bio-Rad, USA)..

2.2.12.1.3 Cloning into pGEM-T Easy Vector

The amplified PCR product of 16S rRNA gene was purified using QIAEX II gel purification kit (Qiagen) and was used for cloning into pGEM-T Easy vector (Promega, USA). The pGEM®-T Easy vector (Fig.2.1) is a linearized vector with a single 3′-terminal thymidine at both ends. The T-overhangs at the

insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (eg. *Taq* DNA polymerase). The pGEM®-T Easy vector is a high-copy-number vector, containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates.

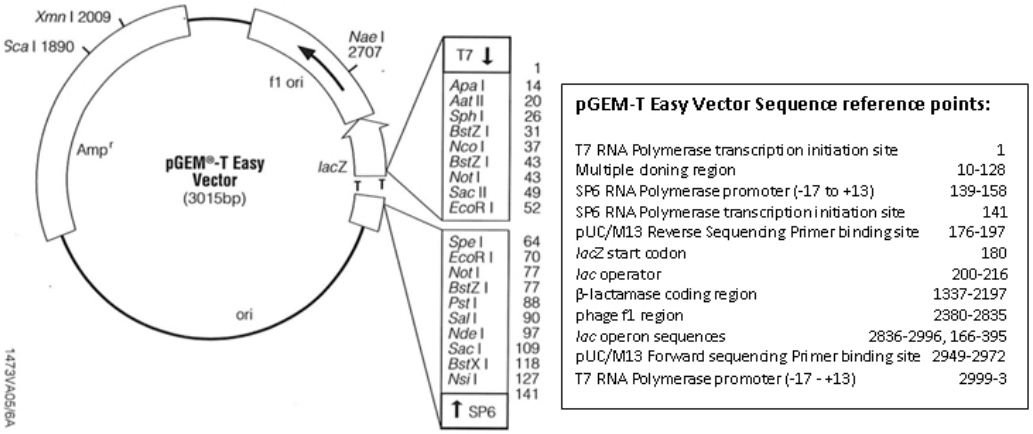


Fig 2.1 The pGEM®-T Easy vector Map

2.2.12.1.4 Transformation into E. coli JM 109

Escherichia coli JM 109 competent cells prepared using Calcium Chloride method. The ligation mix (10µl) consisted of 5µl ligation buffer (2X), 0.5µl vector (50ng/µl), 3.5µl PCR product and 1µl T4 DNA ligase (3U/µl). The ligation mix was incubated overnight at 4°C. The entire ligated mix was used to transform The ligation mix was added to 10 ml glass tube previously placed on ice to which 50µl of competent cells were added and incubated on ice for 20 min, a heat shock at 41.5°C was given for 90s and immediately the tubes were placed on ice for 2 min and then a 600µl sample of SOC media was added and incubated for 2 hr at 37°C in an incubator shaker at 250 rpm. The

transformation mixture (200µl) was spread on Luria-Bertani (LB) agar plates supplemented with Ampicillin (100µg/ml), IPTG (100mM) and X-gal (80µg/ml). The plates were incubated at 37°C overnight. The colonies were selected using blue/white screening. White colonies were selected and streaked to purify on LB-Amp+X-gal+IPTG plates and incubated overnight at 37°C.

2.2.12.1.5 PCR confirmation of gene insert in the selected clones

To confirm the insert, colony PCR of the white colonies were carried out using the vector primers T7 F (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 R (5'-GATTTAGGTGACACTATAG-3'). White colonies (template) picked up from the plate were dispensed into the PCR mix (25µl) containing 2.5µl 10X PCR buffer, 2.0µl of 2.5 mM dNTPs, 1µl of 10 pmol/µl of T7 and SP6 primers, 0.5U of Taq polymerase and the remaining volume was made up with sterile MilliQ water. The amplification profile consisted of initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 15s, annealing of primers at 57°C for 20s, primer extension at 72°C for 60s followed by a final extension at 72°C for 10 min. An aliquot of 10µl of PCR product was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light, documented using a gel documentation system connected with Quantity One® software (Gel Doc XR+, Bio-Rad, USA).

2.2.12.1.6 Plasmid Extraction and Purification

Plasmids from positive clones were extracted using the 'Gen Elute HP' plasmid miniprep kit (Sigma Life Sciences, USA). An aliquot of 2 ml culture of recombinant *E.coli* after overnight incubation was pelletised at 12,000 *g* for 1 min. and subjected to a modified alkaline–SDS lysis procedure followed by adsorption of the plasmid DNA onto a Miniprep binding column. The binding column was inserted into a microcentrifuge tube and centifuged at 12,000 *g* for 1 min. Plasmid DNA bound to the column was washed twice with wash

solution to remove the endotoxins, salt and other contaminants. The column was transferred to a fresh collection tube to elute the plasmid DNA, 100 µl 10 mM Tris-Cl was added and centrifuged at 12,000 *g* for 1 min. The plasmid DNA was stored at -20 ºC. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance at 260/280 nm in a UV-VIS spectrophotometer (U2800, Hitachi, Japan) and fluorometrically using Qubit® fluorometer (invitrogen™, USA).

2.2.12.1.7 Sequencing and Analysis

Nucleotide sequencing was performed using ABI 3730xl DNA Analyzer at Scigenom Kochi. Sequenced DNA data were compiled and analyzed. The sequence obtained was first screened for vector regions using 'VecScreen' system accessible from the National Centre for Biotechnology Information (NCBI). After removing the contaminating vector regions the sequences were matched with homologous sequence obtained from the GenBank database using the BLAST algorithm (Altschul *et al.,* 1990) available from the NCBI website (http://www.ncbi.nlm.nih.gov).

2.2.13 Phylogenetic Analysis

Most phylogenetic trees are built from molecular data, DNA or protein sequences. In this study the phylogenetic analysis of the 16S rRNA gene sequence was done by Molecular Evolutionary Genetics Analysis (MEGA 5.10) software and the tree was constructed by using the Unweighted Pair Group Method with Arithmetic mean (UPGMA). This is user-friendly software for mining online databases building sequence alignments and phylogenetic trees [Tamura *et al.,* 2011]. Construction of a phylogenetic tree from the aligned sequences by the UPGMA method of MEGA permits selecting the most suitable substitution model. Finally the constructed tree can be presented to reveal the relevant information (Hall, 2013).

2.2.14 Statistical Analysis

Data were represented as the mean value ± SD in triplicates using Microsoft Excel 2007 software.

2.3 Results

2.3.1 Screening and Isolation of Phytase Producing Bacteria

Soil samples from 4 locations were inoculated on to humic acid and soil extract agar plates and 135 cultures were isolated for the study. Very few colonies grew on humic acid medium plates even in the lowest dilution (Fig. 2.2a, b). Soil extract agar plates supported sufficiently large number of colonies even at the highest dilution (Fig. 2.3a, b).

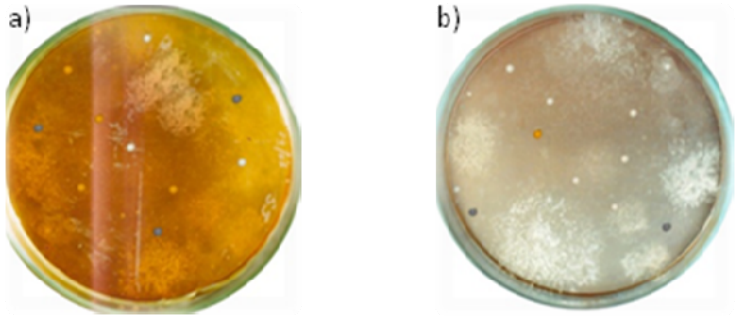


Fig. 2.2 Humic acid agar plates: a) 10^{-1} dilution, b) 10^{-2} dilution

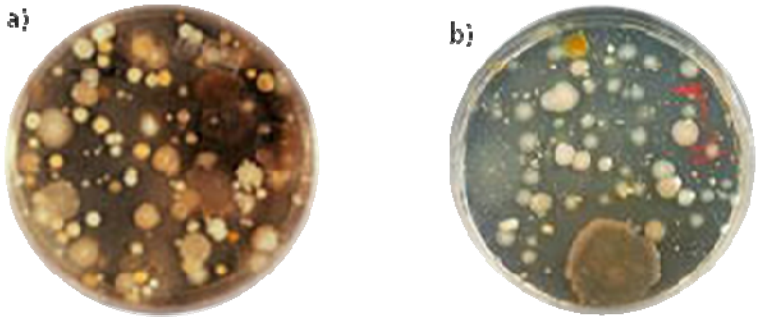


Fig. 2.3 Soil extract agar plates: a) 10^{-2} dilution, b) 10^{-5} dilution

2.3.2 Qualitative Screening

Isolated colonies were screened for phytase activity in differential agar medium containing sodium phytate (phytate agar). They expressed phytase as

clearing zones on the differential agar medium on two step staining employing aqueous cobalt chloride and aqueous ammonium molybdate/ammonium metavanadate solution. Disappearance of precipitated Sodium Phytate indicated enzyme activity (Fig 2.4 a,b). Among 135 bacterial isolates screened on phytate agar plates for the detection of phytase activity, only three could solubilize sodium phytate and generate clearing zones.

These isolates were selected for further identification. The culture obtained from paddy field was named as **'Isolate 1'**, the second one obtained from marine sediment as **'Isolate 2'** and the third one obtained from coir retting ground as **'Isolate 3'**.

Fig 2.4 a, b) Zones of clearing on phytate agar plates indicating phytase activity c) Phytate agar plates showing no activity

2.3.3 Phenotypic Characterization of the Phytase Producing Isolates

On nutrient agar plate the isolate 1 formed dry, flat, and irregular colonies, with lobate margin. Colonies of isolate 2 was translucent to opaque with glossy surface. Isolate 3 formed shiny, mucoid slightly raised colonies with entire margin. Isolate 1 was Gram positive spore forming rod and isolate 2 and 3 Gram negative rods.

2.3.3.1 Biochemical Tests

All the three isolates were subjected to biochemical tests. Table 2.1 represents the morphological and biochemical characteristics of the three selected cultures.

Table 2.1: Morphological and biochemical characteristics of the selected cultures

Characteristics	Isolate 1	Isolate 2	Isolate 3
Colour	Creamy round	Creamy round	Shiny white
Gram reaction	Gram positive	Gram negative	Gram negative
Motility	Motile	Motile	Motile
Catalase	+	+	-
Oxidase	-	-	-
Indole	-	-	-
Methyl red	-	+	+
Voges-Proskaeur	+	-	-
Citrate	+	+	-
Urease	+	+	-
H_2S	-	+	-
Gelatin	+	+	-
Nitrate reduction	+	+	+
Phenylalanine	-	+	-
Arginine	-	+	-
Lysine	-	-	+
Ornithine	-	+	+
L- Arabinose	+	-	+
D-Ribose	+	+	-
D- Xylose	+/-	+	+
Adonitol	-	-	-
Galactose	-	+	+
Dextrose	+	+	+
Fructose	+	-	+
Sorbose	-	-	+
Mannose	+	+	+
Rhamnose	-	+	+
Dulcitol	-	-	+
Inositol	+/-	-	-
Mannitol	+	+	+
Esculin	-	-	-
Salicin	+	-	+
Cellobiose	-	+	-
Maltose	+	+	+
Lactose	-	+	+
Sucrose	+	-	-
Melibiose	+/-	-	+
Trehalose	+	-	+

On the basis of colony morphology, Gram's staining and biochemical tests the isolate 1 was identified as *Bacillus* sp., isolate 2 as *Citrobacter freundii* and isolate 3 as *Escherichia coli*.

Phytase activity of the three isolates was detected under 2 different Hydrogen ion concentration (pH 5.5 & pH 2.5) using Sodium Acetate buffer & Glycine HCl buffer based on previous literature. The absorbance was measured using Cary 50 probe UV-Visible spectrophotometer and read at 750 nm. It was found that phytase was most active at pH 5.5.

2.3.4 Determination of Phytase Activity

Upon enzyme assay the Isolate 1 showed 448U ml^{-1} enzyme activity, isolate 2 showed 321U ml^{-1} and isolate 3 with 244 U ml^{-1} activity at pH 5.5 after 24 hrs of inoculation (Fig. 2.5).

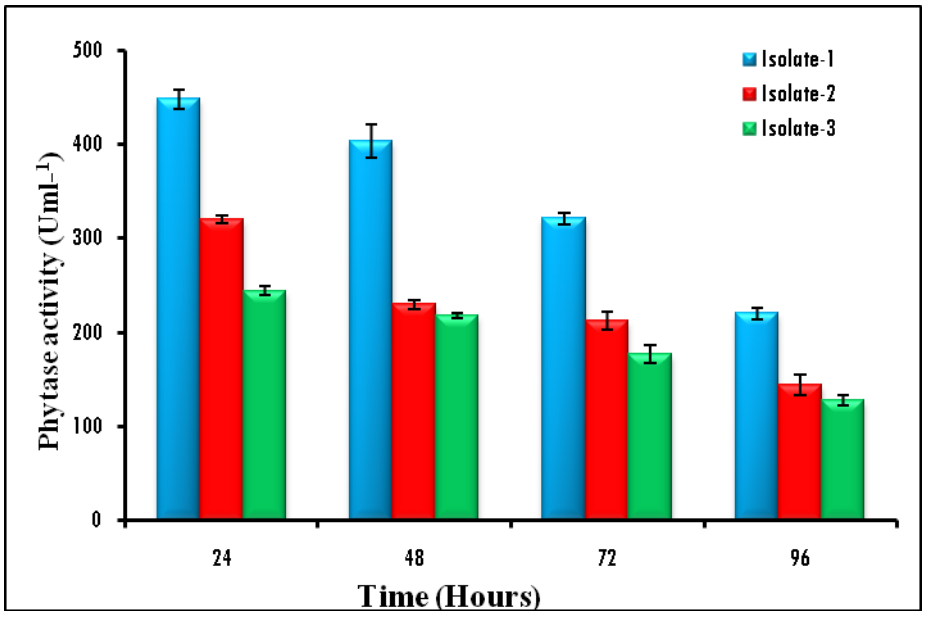


Fig.2.5 Phytase activity of the 3 isolates at pH 5.5

Isolate 1 showed 249U ml^{-1} enzyme activity, isolate 2 showed 220U ml^{-1} and isolate 3 with 144 U ml^{-1} activity at pH 2.5 after 24 hrs of inoculation (Fig. 2.6).

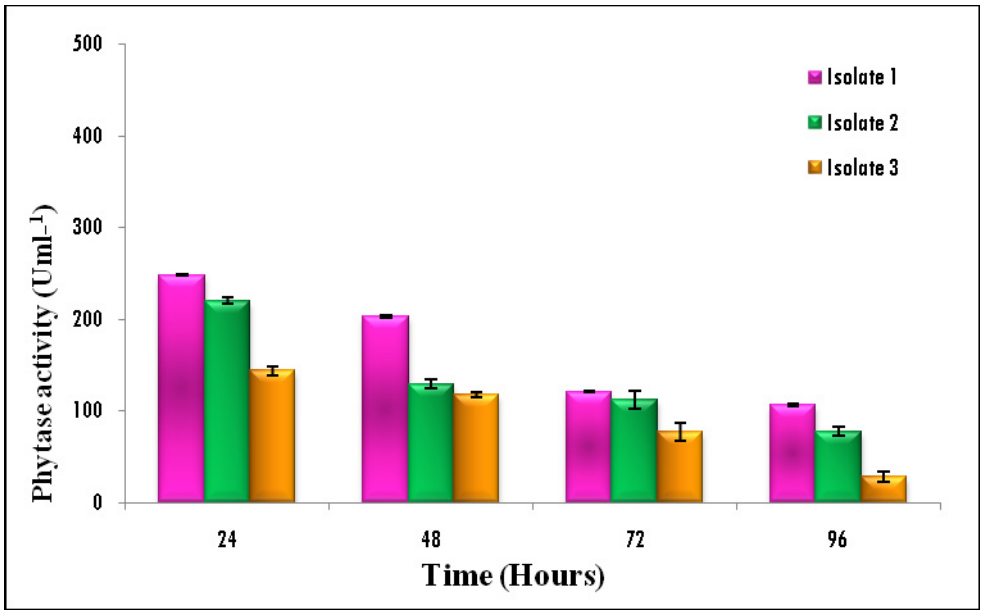
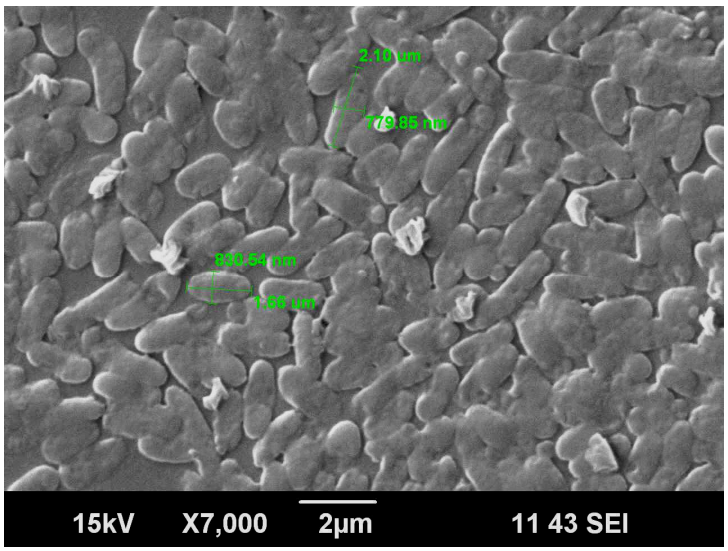


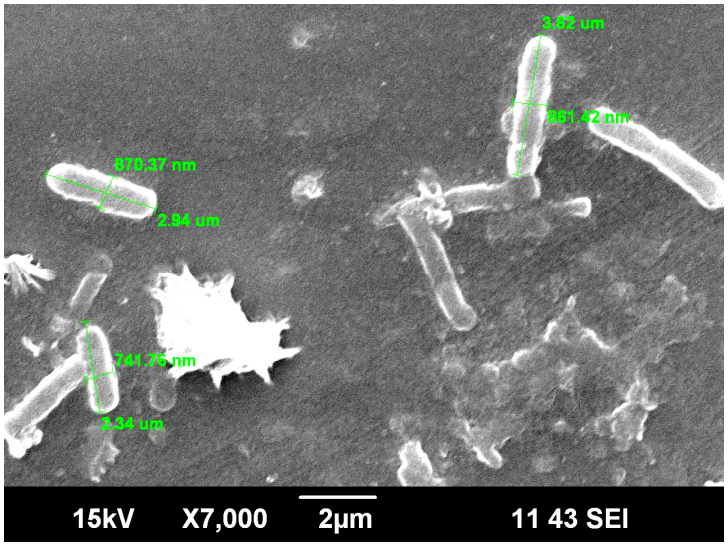
Fig.2.6 Phytase activity of the 3 isolates at pH 2.5

2.3.5 SEM Observation

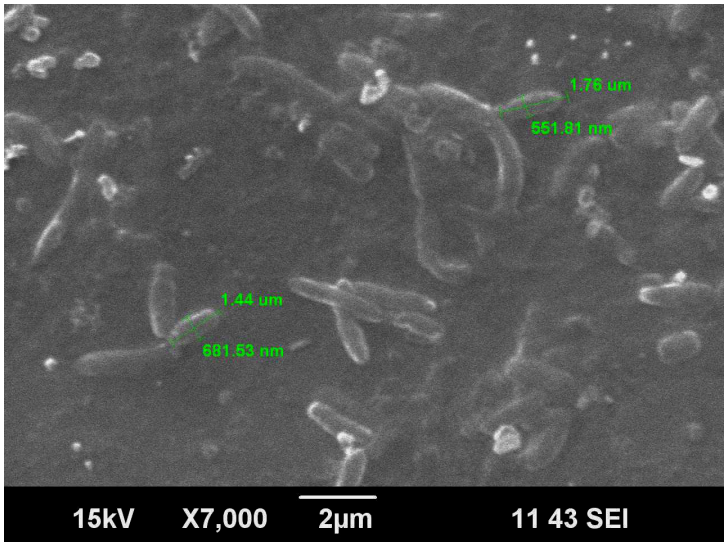
The scanning electron microscope (SEM) results of the 3 isolates showed the cells elongated measuring 2.10µm (isolate 1: *Bacillus*), 3.6µm (isolate 2: *Citrobacter*) and 1.76µm (isolate: 3 *E.coli*) (Fig.2.7a-c).



a)



b)



c)

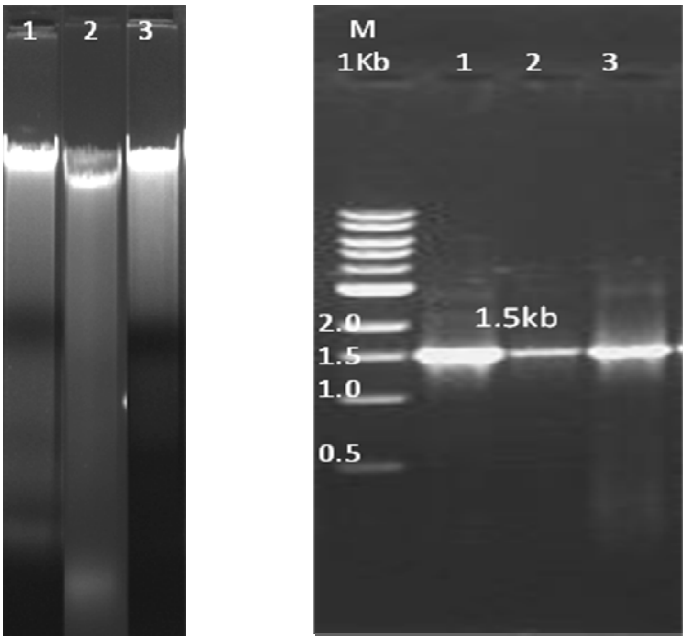
Fig.2.7 a) Scanning electron micrograph of isolate 1 *Bacillus,* b) isolate 2 *Citrobacter* and c) isolate 3 *Escherichia coli*

2.3.6 Molecular Identification by 16S rRNA Gene Sequencing

DNA was isolated (Fig.2.8 a) and amplified using universal primers of 16S rRNA gene yielding a PCR product of 1.5 kb (Fig.2.8 b). The PCR products were cloned into pGEM – T easy Vector and transformed *E. coli* (JM109) cells.

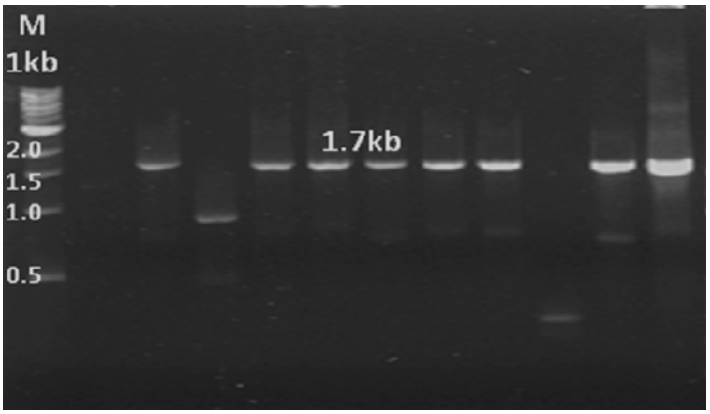
The insert confirmed by colony PCR using T7 and SP6 vector primers produced a product of 1.7 kb (Fig.2.9 a-c). Plasmid (2.5 kb) from the transformed organisms was extracted (Fig.2.10) and partially sequenced using T7 and SP6 vector primers.

Sequenced DNA data were screened for vector regions using 'VecScreen' system (NCBI) to remove vector contamination regions. When compared with GenBank data base using BLAST algorithm available from NCBI (www.ncbi.nlm.nih.gov), the sequence of isolate 1 showed 99% similarity to *Bacillus* sp. JX293291.1, isolate 2 showed 99% similarity to *Citrobacter freundii KF535142.1* and isolate 3, 100% similarity to *Escherichia coli* LM993812.1. The sequences were deposited in GenBank with the accession numbers KM880161, KM880162, KM880163 respectively.

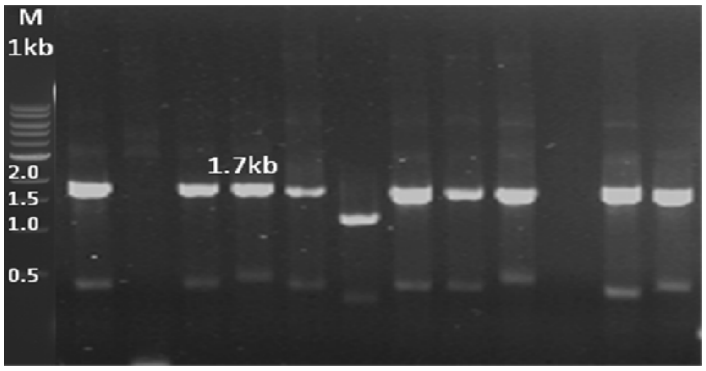


a) b)

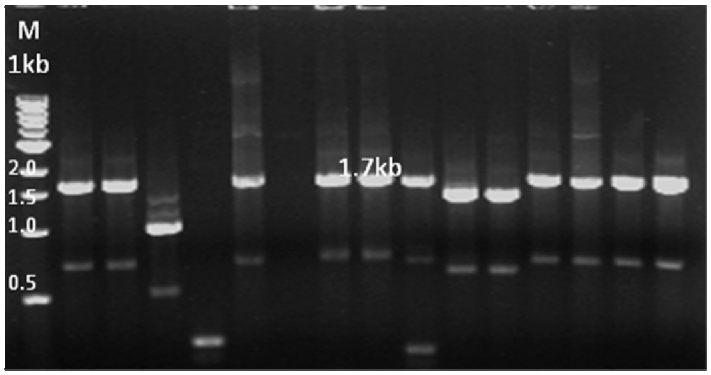
Fig.2.8: a) Genomic DNA b) PCR products of the 3 isolates
M- Molecular marker, Lane 1- Isolate 1, Lane 2- Isolate 2, Lane 3 – Isolate 3



a)



b)



c)

Fig.2.9: Colony PCR of the clones carrying 16S rRNA gene insert of a) Isolate 1, b) Isolate 2, c) Isolate 3 (1.7 kb insert)

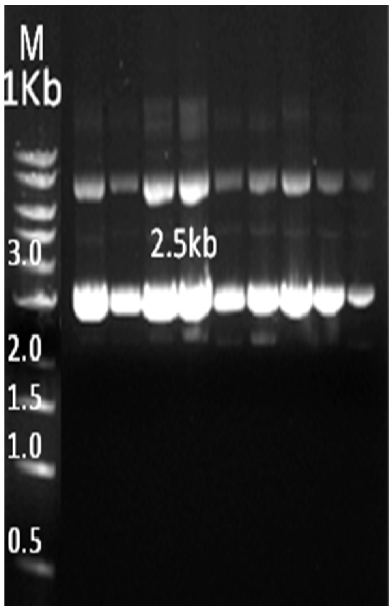


Fig.2.10: Purified plasmid: Plasmid with insert of 16S rRNA gene from isolate 1, isolate 2, and isolate 3

2.3.7 Phylogenetic Tree Construction (MEGA5.10)

The phylogenetic analysis of the three isolates was analyzed using MEGA (5.10) programme. The 16S rRNA phylogenetic tree constructed using UPGMA statistical method and Kimura 2-parameter substitution model with 1000 bootstrap replications clearly showed that isolate 1 (KM880161) is closely related to and branched from *Bacillus amyloliquefaciens* strain (Fig. 2.11).The 16S rRNA phylogenetic tree of isolate 2 (KM880162) showed similarity and branching from *Citrobacter freundii* (Fig. 2.12) while isolate 3 (KM880163) is closely related to *E. coli* 16S rRNA and *E.coli* genome assembly (Fig. 2.13).

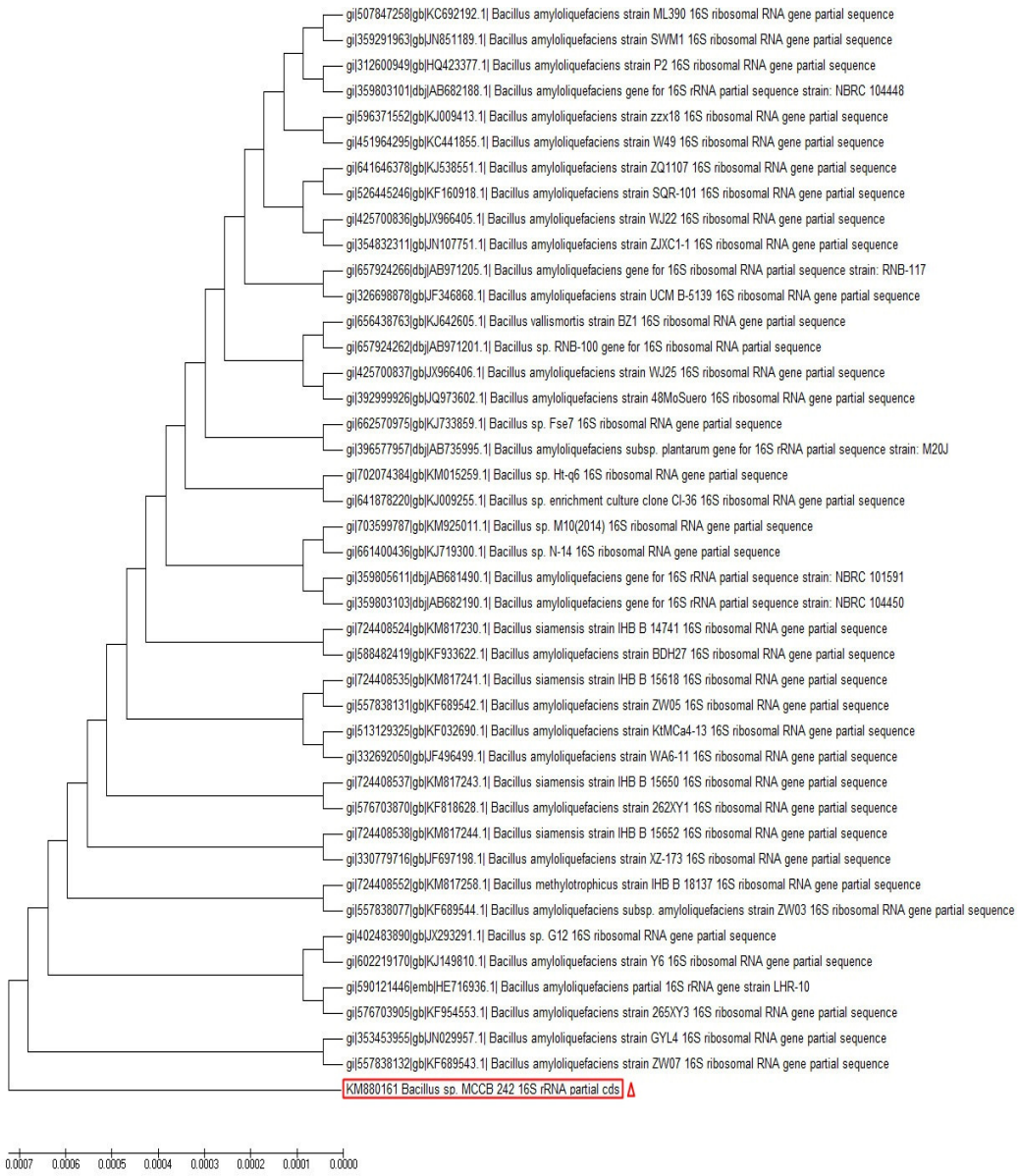


Fig.2.11: Phylogenetic tree based on 16S rRNA gene sequences of isolate 1 *(Bacillus)* compared with the sequences available in GenBank.

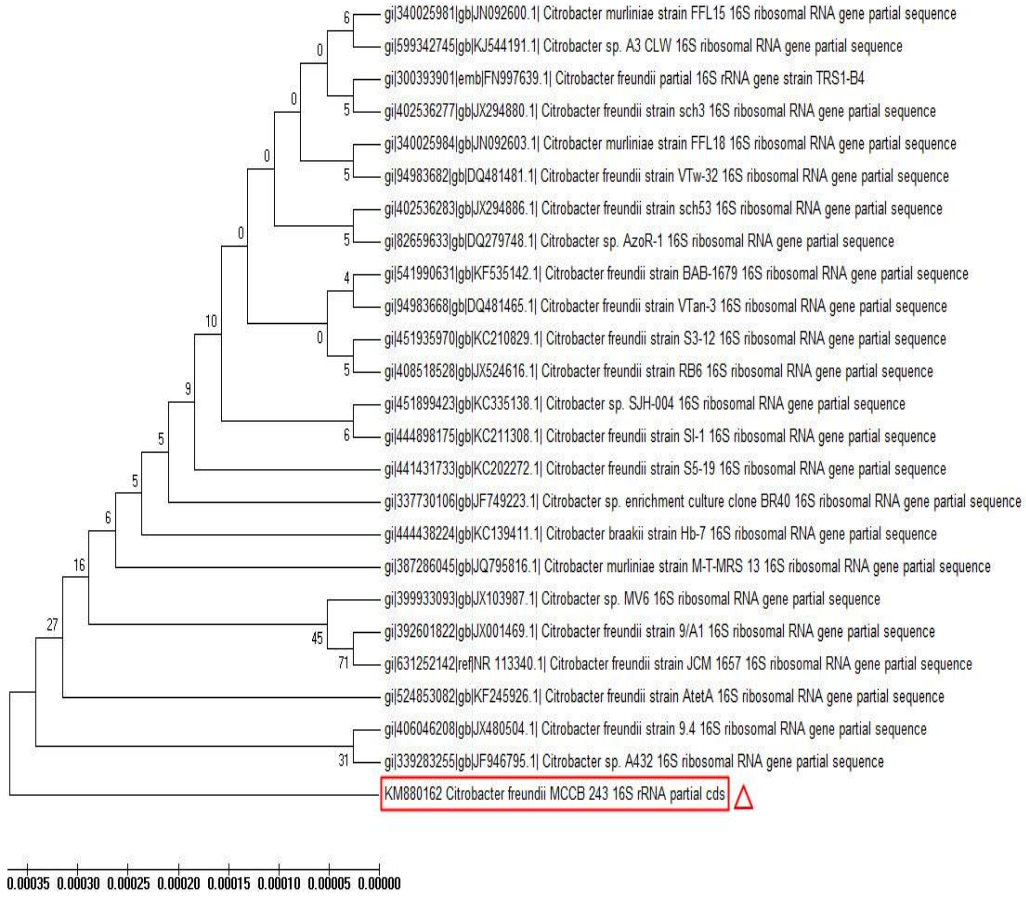


Fig.2.12: Phylogenetic tree based on 16S rRNA gene sequence of isolate 2 (*Citrobacter*) compared with the sequences available in GenBank

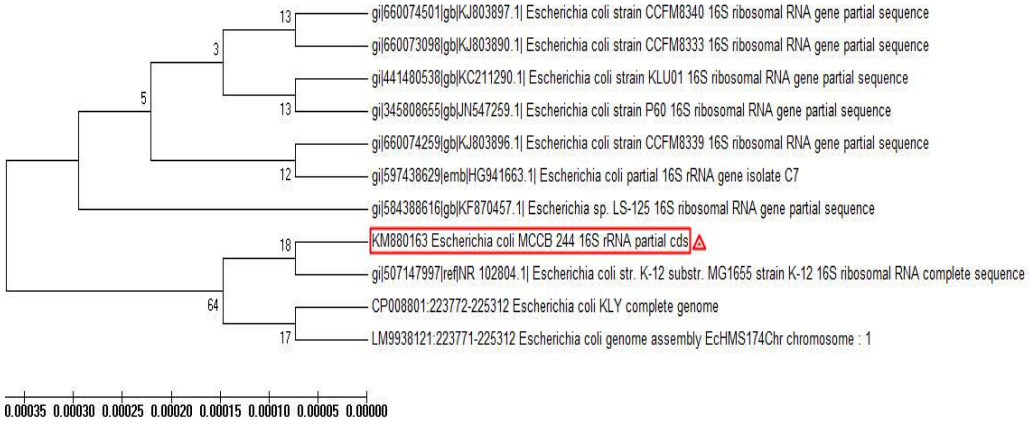


Fig.2.13: Phylogenetic tree based on 16S rRNA gene sequence of isolate 3 (*Escherichia coli*) compared with the sequences available inGenBank.

2.4 Discussion

Phosphatases, such as phytase, are able to augment the rate of the dephosphorylation (hydrolysis) of organic phosphorus compounds. These enzymes are normally present in soils, where they originate from microorganisms. Phytases are very much recognized for their beneficial environmental role in reducing the phosphorous levels in manure and minimizing the need to supplement phosphorous in diets. Focus of the work was to isolate bacteria as producers of extracellular phytase and to identify the potential phytase producing organisms for further investigation. Accordingly three isolates could be obtained to have phytate degrading (phytase producing) capability. They were characterized phenotypically, Scanning electron microscopy and 16S rRNA gene sequence.

This study demonstrated a viable screening strategy for phytase producing bacteria. Several screening strategies have been documented in literature. Lissitskaya *et al*. (1999) screened micro-organisms producing phytase using museum and soil samples. Chen. (1998) developed a bioassay for the screening for extra-cellular phytase-producing micro-organisms. The method consisted of determining the inorganic orthophosphate released on hydrolysis of sodium phytate at pH 5.5 (Engelen *et al.,* 1994). In the present work differential agar medium plates with 0.5% phytate was used for the screening operation. In the counter staining procedure with 2% cobalt chloride, 6.25% aqueous ammonium molybdate and 0.42% ammonium vanadate, clearance zone produced around the colonies confirmed phytase production. The mechanism behind the reaction is that when phytase is produced by the organism, the phytate/phytic acid phosphorus is cleaved and a free hydroxyl group is produced. At this stage when cobalt chloride is added, it binds to the free hydroxyl group and phytate

remains unchanged. When ammonium molybdate and ammonium monovanadate are added they bind to cobalt chloride and produce a clearance zone.

Over the years phytase producing bacteria have been screened, isolated and reported, such as *Pseudomonas* sp. (Richardson and Hadobas, 1997) *Bacillus* sp. (Choi *et al*. 2001) *E. coli* (Greiner *et al*. 1993) and anaerobic rumen bacterium, *Mitsuokella jalaludinii* (Lan *et al*. 2002). Phytate degrading activity was determined by measuring the orthophosphate liberated from phytate by the enzymatic action. The measured levels of phytase activity suggested that soil bacteria are potential source of phytase that could be exploited commercially. A high yield of phytase was found in the isolate 1 (*Bacillus* sp. which was numbered MCCB 242) obtained from farmland soil. The isolate, *Bacillus* MCCB 242, was chosen for further research as it exhibited significantly higher activity than the other two bacterial isolates (*Citrobacter* and *E. coli*). This isolate could exhibit highest activity within 24hrs, an advantageous characteristic required for commercial production. The isolate, *Bacillus* MCCB 242, has been found to be a good source of phytase worth attempting to be exploited at commercial level as feed supplement for monogastric animals and agastric fishes to satisfy the phosphorus nutrition, as well as for combating the problem of environmental pollution. Precisely, the isolate *Bacillus* MCCB 242 could be identified as a promising candidate for the production of phytase at commercial level which is unlike from the *Bacillus amyloliquefaciens* isolated by Kim *et al*. (1998) and Oh *et al*. (2001) with respect to the efficacy of phytase production.

Chapter 3

PRODUCTION OPTIMIZATION OF PHYTASE FROM *BACILLUS* MCCB 242

Contents

3.1 Introduction

Aim of design experiments is primarily to optimize media applications for better product yield or concentration. The intention is to find out better composition of nutrient factors, in order to maximize the production by supplying a well balanced mixture of nutrients which enhances yield. To be economically feasible, it is necessary to engineer optimum culture conditions to minimize the cost and to maximize production. Optimization is carried out to maintain specific ratio between different media components for their total utilization by the microorganism, thereby preventing wastage of nutrients at the end of the process. Composition of fermentation medium has always been a vital component in the optimization of growth conditions, because it affects productivity and overall cost of the production process (Lee *et al.,* 2005). It has been suggested that economical and commercially available media should be investigated to reduce the production cost (Laawford and Rousseau, 1997; Kona *et al.,* 2001).

An appropriate bioprocess technology is crucial to bring forth microbial phytase to application mode at commercial level. For building such a technology the principal requirement is optimization of growth medium

constituents and culture conditions to augment production. Therefore, in order to facilitate large scale commercial production of the enzyme, optimization of carbon and nitrogen sources and other growth factors were to be accomplished. These objectives were achieved by adopting the statistical modeling technique, Response Surface Methodology (RSM).

3.1.1 Bioprocess Design

Of late, considerable progress has been achieved in designing media facilitating commercial production of several microorganisms. Bioprocess development generally comprises three sequential steps, namely screening for novel biological activities and/or metabolic design of the biocatalyst, process development at laboratory scale and scale-up to industrial production (Puskeiler *et al.,* 2005). Optimization of bioprocess in lab and industrial bioreactors demands enormous number of experiments with high cost of operation (Puskeiler *et al.,* 2005; Duetz *et al.,* 2000).

The Conventional method of media optimization is to analyze 'one variable at a time' (OVAT), while keeping the others constant. Major drawback of this approach is that it does not bring about the effect of interaction of various parameters (Stowe and Mayer, 1999; Vohra and Satyanarayana, 2002; Bogar *et al.,* 2003).

Statistical approaches provide efficient optimization of media constituents and culture conditions and has been widely applied in many areas of biotechnology (Farrera *et al.,* 1998; Chakravarti and Sahai, 2002; Lai *et al.,* 2003; Elibol, 2004; Soni *et al.,* 2007; Sen and Swaminathan, 1997; Hujanen *et al.,* 2001). Different statistical design such as response surface methodology (RSM), Taguchi, Plackett-Burman, Quality by design etc., are widely used at an industrial scale. Statistical experimental planning, factorial design, and design-of-experiments (DoE) are more or less the same concepts for investigating the

mathematical relationships between input and output of variables of a system. Although fundamentals of the methodology have been known since early 1900s (Yates, 1937; Pilat *et al.*, 1976; Montgomery, 2008) they have not been widely used in applied in biotechnology.

3.1.2 Factorial Design and Response Surface Methodology

The Design of Experiments (DoE) is a powerful methodology for building a definite set of factorial experiments to evaluate important experimental variables in a bioprocess, or to perform an optimization of them in a biotechnical system. DoE avoids experimental biases and reduces the required number of experiments (Box *et al.,* 1978; Brereton, 2003; Box *et al.,* 2005; Carlson and Carlson, 2005). The two main applications of experimental design are screening, wherein the factors that influence the experiment are identified, and optimization wherein the optimal settings or conditions for an experiment are found. Screening techniques such as factorial designs allow the experimenter to select the factors significant and the level at which they are significant (Grady and Kenneth, 2006). By performing factorial design, consistent results can be achieved with few experiments, after which the best possible direction to move on to find an accurate optimum can be evaluated. As first step in a DoE plan, the most efficient approach to be attempted is screening of important variables of the studied process through a set of experiments proceeding from selected corners in the experimental space (Mandenius and Brundin, 2008).

For identification of the optimum variable ranges (partial), factorial experimental designs are often used and response surface method (RSM) is well established. This statistical modeling technique was initially developed and described by George Edward Pelham Box and K.B Wilson in the year 1951 at Imperial Chemical Industries. They suggested to use a first-degree polynomial model to approximate the response variable. Response surface methodology

optimize a response between several explanatory variables (input variables) and response variables (output variable).This technique is also employed for multiple regression analysis, using quantitative data obtained from appropriately designed experiments to solve multivariable equations simultaneously (Dirk, 2000). The graphical perspective of the mathematical model has led to the term Response Surface Methodology (Bas and Boyaci, 2007).

3.1.3 Plackett-Burman Design

A popular class of screening designs known as Plackett-Burman design (PBD) was developed by the statisticians Robin Plackett and Burman in 1946. It was designed to improve the quality control process that could be used to study the effects of design parameters on the system so that intelligent decisions can be made. PBD is an efficient screening method which capitulate unbiased estimates of all main effects in the smallest design and to identify the active factors using few experimental runs as possible. The main effects have a complicated perplexing relationship with two-factor interactions in Plackett-Burman designs. As a result, these designs should be used to study main effects where it could be assumed that two-way interactions are negligible.

Plackett- Burman design has been extensively used for screening the media components as well as physical factors essential for extracellular phytase production by different microorganisms. In PBD, various number or 'n' factors can be screened in an 'n+1' run design. A distinguishing feature of PBD is that the sample size is always a multiple of four (4k observations with k =1, 2…n). These designs are proposed for more than seven factors and are known as saturated designs. By employing saturated designs a minimum number of observations are required to calculate an effect for a certain factor.

3.1.4 Response Surface Methodology

It is possible to separate a bioprocess optimization study using response surface methodology into three phases. The first phase entails preliminary work, in which the independent parameters are determined and their levels are carried out. In this stage the parameters that have major effects on the biochemical process are identified from among several others affecting it. Screening experiments are useful to identify these independent parameters. Factorial designs may be used for this purpose. The second phase is the selection of the experimental design, prediction and verification of the model equation. The last one is getting the 3 D response surface plot and contour plot of the response as a function of the independent variables and to determine the optimum points (Bas and Boyaci, 2007).

The field of response surface methodology consists of the experimental strategy for exploring the space of the process or independent variables, empirical statistical modeling to develop an appropriate approximating relationship between the yield and the process variables, and optimization methods for finding the values of the process variables that produce desirable values of the response (Raymond *et al.,* 2014). In many cases, either a first-order or a second order model is used.

For the case of two independent variables, the first-order model in terms of the coded variables is

$$\eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2; \quad \text{----------(1)}$$

The type of the first-order model in Equation (1) is sometimes called a main effects model, because it includes only the main effects of the two variables X_1 and X_2. If there is an interaction between these variables, it can be added to the model effortlessly as follows:

$$\eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2; \text{ ----------(2)}$$

This is the first-order model with interaction. Adding the interaction term introduces curvature into the response function.

Often the curvature in the true response surface is strong enough that the first-order model is insufficient. A second-order model will probably be needed in these situations. For the case of two variables, the second-order model is

$$\eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad \text{---------- (3)}$$

Where 'η' is the estimated response, 'β_0' is a constant, 'β_1, β_2, β_3, β_{11}, β_{22}, β_{12}, are the coefficients for each term and 'x_1, x_2 and x_3 are the independent variables.

OR

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\, \beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j, \text{ ----------(4)}$$

Where Y represents the response variable, β_0 represents the interception coefficients, β_i is the coefficient of linear effect, β_{ii} is the coefficient of quadratic effect and β_{ij} is the coefficient of the interaction effect (Bas and Boyaci, 2007; Jian and Nian-fa, 2007; Venil *et al.,* 2009).

The second-order model is extensively used in response surface methodology for several reasons:

1) The second-order model is very flexible. It can take on a wide variety of functional forms, thus it will often work well as an estimate to the true response surface.
2) It is easy to approximate the parameters (the β's) in the second-order model. The method of least squares can be used for this reason.
3) There is significant practical experience signifying that second-order models work well in explaining real response surface problems.

The Central Composite Design (CCD) is a well established widely used RSM design for determining the key factors from a large number of medium components by a small number of experiments (Soni *et al.,* 2007). This design can be easily constructed by increasing the fractional factorial design with center points that are augmented with a group of axial (star) points that allow estimation of curvature of the first-order model (Hanrahan *et al.,* 2005). The central composite design (CCD) which contain imbedded factorial or fractional factorial designs has three groups of design points:

a) Two-level factorial or fractional design points - all possible combinations of the +1 and -1 levels of the factors (2^k).

b) Axial points or star points - all of the factors set to 0, the midpoint, except one factor, which has the value +/- alpha. However, in the case of axial points of face centered central composite design, all the factors are set to 0 (midpoint), except one factor, which is at the +1/-1 value (i.e., the star points is set at the face of the cube portion on the design)

c) Center points - points with all levels set to coded level 0 (midpoint).

Two–level factorial part (the core) of the design consists of all possible combinations of the plus or minus ("-1" or "+1") levels of the factors. Axial points (outside the core), often represented by stars, emanate from the center point, with all but one of the factors set to 0. The coded distance of the axial points is represented as a plus or minus alpha ("-α" or "+α"). The "α" is set default at 1.68179 in coded units, is the axial distance from the center point and makes the design rotatable and provides equally good predictions at points equally distant from the center. Center points are usually repeated to get an estimate of experimental error. Thus the central composite design requires five coded levels of each factor: "-1" or "+1" (factorial points), "-α" or "+ α" (axial points), and all zero level (center point). The variables are coded according to the following equation:

$$\text{Coded value} = \frac{\left[\text{Actual value} - \frac{1}{2}(\text{high level} + \text{low level})\right]}{\left[\frac{1}{2}(\text{high level} - \text{low level})\right]}$$

RSM gives the graphical representation of the model equation and determination of optimal operating conditions. The visualization of the predicted model equation can be obtained graphically by the response surface plot or contour plot. The 3D response surface and the 2D contour plots are the graphical representations of the regression equations, which could be used to describe the individual and collective effect of the test variables on the response and to determine the mutual interactions between the test variables and their subsequent effect on the response. Each contour curve in a 2D plot signifies countless number of combinations of two test variables with all the others at fixed levels. The yield values for different concentrations of the variables can also be predicted from the respective contour plots (Box and Wilson, 1951; Box *et al.,* 1958; Khuri and Cornell, 1987). The main disadvantage with the response surfaces is that the plotting is limited to two variables at a time. The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram (Elibol, 2004). The shape of the contour plot, circular or elliptical, indicates whether the mutual interactions between the corresponding variables are significant or not (Manimekalai and Swaminathan, 1999). If it is circular, the interactions between the variables are negligible and if it is elliptical the interaction between the variables are significant (Liu *et al.,* 2003).

3.1.5 Production Parameters of Microbial Enzymes

A fermentation medium forms the environment in which the microorganisms live, reproduce and carry out their specific metabolic reactions to produce useful products. Two distinct biological requirements are considered in most of the industrial fermentation processes for medium design where the product is something other than the cell mass itself. First, the nutrient has to be

supplied to establish the growth of the microorganism. Second, the proper nutritional conditions have to be provided to maximize the enzyme production.

An efficient system for large-scale production of phytase from *Bacillus* MCCB 242 is intended to be developed by way of optimizing the culture conditions and nutritional parameters. Response surface modeling was applied to optimize the factors which support maximum enzyme production.

3.1.5.1 Physical Conditions

The main significant physical parameters for growth and phytase production are pH, temperature, agitation, dissolved oxygen, pressure and incubation time.

3.1.5.1.1 pH

Different microbial phytases show different level of pH optima for their production and activities. For bacteria, their optimum pH usually range from 6.5 to 7.5 (Vohra and Satyanarayana, 2003). Phytases from *Aerobacter aerogenes* (Greaves *et al.,* 1967), *Pseudomonas* sp. (Irving and Cosgrove, 1971), *E. coli* (Greiner *et al.,* 1993), *Selenomonas ruminantium* (Yanke *et al.,* 1999) and *Lactobacillus amylovorus* (Sreeramulu *et al.,* 1996) showed optimum pH in the range between 4 and 5.5. Phytases that worked well at higher pH have also been reported, for example, those from *Entrobacter* sp.4 (Yoon *et al.,* 1996) and *Bacillus* sp. DS11 (Kim *et al.,* 1998) exhibited the optimal pH for activity in the neutral range; i.e. pH 7.0 to 7.5.

3.1.5.1.2 Temperature

Changes in growth conditions such as temperature represent a key factor that influences cell growth and survival of *Bacillus* spp. in their surroundings. *Bacillus subtilis* has the ability to sustain growth in a wide range of temperature from around 11ºC (Nicholson *et al.,* 1996) to 52ºC (Holtman and Bremer,

2004). In animal feed industry, phytase with thermotolerant property is preferred since the pelleting process involves a step of exposure to high temperature up to 80 - 85°C for a few seconds (Vohra and Satyanarayana, 2003). Some bacterial phytases were found to have optimal temperature for activities at relatively high levels. For examples, phytases from *Bacillus subtilis* (Powar and Jagannathan, 1982), *E. coli* (Greiner *et al.,* 1993), *Klebsiella aerogenes* (Tambe *et al.,* 1994), *Enterobacter* sp.4 (Yoon *et al.,* 1996), *Klebsiella oxytoca* MO-3 (Jareonkitmongkol *et al.,* 1997) and *Selenomonas ruminantium* (Yanke *et al.,* 1999) were optimally active at temperatures ranging from 50 to 60°C. *Bacillus* sp. DS11 phytase showed maximum activity at 70°C (Kim *et al.,* 1998). These phytases would be able to tolerate high level of temperature for a few seconds during the pelleting process in feed production. In the contrary, phytase of *Aerobacter aerogenes* had an optimal temperature for its activity at a lower level of 25°C, and this kind of phytase could only be used for pre-treatment of raw material prior to the feed formulation process (Greaves *et al.,* 1967).

3.1.5.1.3 Aeration and Agitation

Aeration and agitation are extremely important for uniform distribution of the medium composition, microbial cells and oxygen. These parameters vary among different organisms. For example, in *Saccharomyces castellii*, cultivation was carried out with an aeration rate of 1 vvm (volume of air per volume of liquid per minute) and agitation at 600 rev/min (Segueilha *et al.,* 1992). Phytase was maximally produced by *Bacillus* sp. DS11 with shaking at 230 rpm (Kim *et al.,* 1998a). However, the lower agitation speed of 180 rpm was optimum for *Bacillus subtilis* phytase production in a fed-batch culture (Kerovuo *et al.,* 2000a).

3.1.5.2 Nutrients

Apart from the culture conditions, nature of the substrate and availability of the nutrients are critical factors affecting the yield of phytase production. Nutrients such as carbon, nitrogen, K, Ca, Mg, Fe and microelements play important roles in microbial physiology. Proper utilization of these nutrients increases phytase production.

3.1.5.2.1 Carbon Source

Glucose has been a common carbon source for the use as substrate for phytase production (Vohra and Satyanarayana, 2003). In bacteria, 1% of glucose was optimal for *Lactobacillus amylovorus* (Sreeramulu *et al.,* 1996) and *Enterobacter* sp.4 producing phytase. Glucose at 2% was suitable for *Bacillus subtilis* producing phytase (Kerovuo *et al.,* 1998). Other kinds of carbon sources have also been reported. For example, wheat bran at 6% was used as carbon source for phytase production in *Bacillus* sp. DS11 (Kim *et al.,* 1998a), while for glucose as sole carbon source, high concentration of glucose up to 5.2% was found to promote phytase synthesis from *Aspergillus ficuum* in SSF (Ebune *et al.,* 1995). However, higher glucose concentration, i.e. 9.8 and 17.8%, showed an adverse effect (Vohra and Satyanarayana, 2003).

3.1.5.2.2 Nitrogen Source

Both organic and inorganic forms of nitrogen have been used extensively for the production of phytase. For organic nitrogen sources, peptone was used for phytase production from *Aerobacter aerogenes* (Greaves *et al.,* 1967) and *Klebsiella oxytoca* (Jareonkitmongkol *et al.,* 1997). In the case of *Klebsiella oxytoca* phytase production, the medium was also supplemented with 1% yeast extract (Jareonkitmongkol *et al.,* 1997). For *Arxula adeninivorans*, 1% each of yeast extract and peptone served as good nitrogen sources (Sano *et*

al., 1999). As the inorganic nitrogen sources, ammonium sulfate at 1% (w/v) was used for phytase production from *Pseudomonas* sp. (Irving and Cosgrove, 1971), *Enterobacter sp*. 4 (Yoon *et al.,* 1996) and *Saccharomyces castellii* (Lambrechts *et al.,* 1992). In some cases, both forms of nitrogen sources such as casein hydrolysate at 1% and $(NH_4)_2SO_4$ at 0.1% were used for phytase production from *Bacillus subtilis* (Powar and Jagannathan, 1967).

3.1.5.2.3 Requirement of Trace Elements and Vitamins

Trace elements and vitamins are required to promote growth and phytase production from yeast (Galzy, 1964; Segueilha *et al.,* 1992). However, these were not needed for phytase production from either bacteria, such as *Bacillus subtilis* (Powar and Jagannathan, 1967), *Bacillus* sp. DS11 (Kim *et al.,*1998a), *Escherichia coli* (Greiner *et al.,* 1993), or from fungi such as *Aspergillus niger* ATCC 9142 and *Aspergillus ficuum* NRRL 3135 (Shieh and Ware, 1968).

3.2 Materials & Methods

3.2.1 Phytase Producing Microorganisms

Out of the three phytase producers screened, only one isolate was selected in the present study. The isolate was obtained from farmland soil of Pathanamthitta region, Kerala and maintained at National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology, Cochin, Kerala, India under the code *Bacillus* MCCB 242. The other two organisms isolated also formed part of the microbial culture collection of NCAAH under the code *Citrobacter freundii* MCCB 243 and *Escherichia coli* MCCB 244 respectively.

3.2.2 Optimization of Growth Factors for Phytase Production

Composition of phytase screening medium used for phytase was optimized to maximize its production using Plackett & Burman design for

initial screening. This is then followed by Response Surface Methodology (RSM) based on Central Composite Design (CCD) to optimize significant parameters and to establish a theoretical model of phytase production.

3.2.2.1 Culture Medium Used

Phytase Screening Medium (PSM broth) (50 ml) was used for phytase production (Table 3.1) from *Bacillus* MCCB 242. PSM broth was prepared in 250 ml flasks and sterilized by autoclaving at 121ºC for 15 min. Glucose was filter sterilized separately and added to the medium before inoculation.

Table 3.1: Composition of Phytase Screening Medium

Ingredients	Concentration
Glucose	15g
NH_4NO_3	5g
KCl	5g
$MgSO_4.7H_2O$	5g
$FeSO_4.7H_2O$	0.01g
$MnSO_4.7H_2O$	0.01 g
Na-Phytate	10 g
Distilled water	1000 mL
pH	7

3.2.2.2 Preparation of Inoculum

Bacillus MCCB 242 was inoculated onto nutrient agar slant and incubated at 28ºC. From the slant, a loop full of culture was inoculated into the phytase screening medium and incubated on a rotary shaker at 25ºC at 120 rpm overnight (18 hrs). This was treated as the mother culture.

3.2.3 Experimental Design

3.2.3.1 Shake Flask Experiments

To find the suitable concentration of medium components for maximum yield of phytase by *Bacillus* MCCB 242, initial screening was carried out by one-variable-at-a-time approach (Table 3.2 & Table 3.3). All experiments were done in triplicate (250ml) with 50ml phytase screening medium.

Table 3.2: Nutrients and the concentrations selected for initial optimization

Ingredient	Concentration (gL^{-1})
Glucose	0, 1, 2.5,5, 7.5, 10, 20, 30, 40 and 50
NH_4NO_3	0, 0.5,1, 2.5, 5, 10, 15, 20, 25 and 30
KCl	0, 0.5, 1, 2.5, 5, 10, 15, 20 and 30
$MgSO_4.7H_2O$	0, 0.5, 1, 2.5, 5, 10, 15, 20 and 30
Na phytate	0, 0.5, 1, 2, 5, 10, 15 and 20
$FeSO_4.7H_2O$	0.0 1
$MnSO_4.7H_2O$	0.0 1

Table3.3: Physical parameters selected for initial optimization

Parameter	Values
pH	2.5,3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5
Temperature	20, 25, 30, 35, 40,45 and 50°C
Agitation	0, 50, 100, 150, 200 and 250 rpm
Inoculum size	0.02, 0.04, 0.08 and 0.1 O.D
Inoculum age	18h, 1,2,3 days

3.2.3.2 Optimization of Glucose

Phytase screening medium (broth) was prepared at 50 ml aliquots having different concentrations (0, 1, 2.5, 5, 7.5, 10, 20, 30, 40 and 50 g/L) of glucose. The flasks were inoculated with 0.1ml mother culture having absorbance of 0.1 at Abs_{600} and incubated at 28±1°C for 24-48 hrs on rotary shaker at 120 rpm.

Phytase activity was measured following the method of (Heinonen and Lahti, (1981) as described in section 2.2.8.

3.2.3.3 Optimization of NH_4NO_3

Phytase screening medium (broth) was prepared at 50 ml aliquots having different concentrations of NH_4NO_3 (0, 0.5, 1, 2.5, 5, 10, 15, 20, 25 & 30gL^{-1}). The flasks were inoculated with 0.1ml mother culture having absorbance of 0.1 at Abs_{600} and incubated at 28±1^oC for 24-48 hrs on rotary shaker (Orbitek, Scigenics Biotech. (Pvt.) Ltd. India) at 120 rpm and measured phytase activity as detailed earlier.

3.2.3.4 Optimization of KCl

Phytase screening medium (broth) was prepared at 50 ml aliquots having different concentrations of KCl (0, 0.5, 1, 2.5, 5, 10, 15, 20 & 30gL^{-1}). The flasks were inoculated with 0.1ml mother culture having absorbance of 0.1 at Abs_{600} and incubated at 28±1^oC for 24-48 hrs on rotary shaker at 120 rpm and measured phytase activity as detailed earlier.

3.2.3.5 Optimization of $MgSO_47H_2O$

Phytase screening medium (broth) was prepared at 50 ml aliquots having different concentrations of $MgSO_47H_2O$ (0, 0.5, 1, 2.5, 5, 10, 15, 20 & 30gL^{-1}). The flasks were inoculated with 0.1ml mother culture having absorbance of 0.1 at Abs_{600} nm and incubated at 28±1^oC for 24-48 hrs on rotary shaker at 120 rpm, and measured phytase activity as detailed earlier.

3.2.3.6 Optimization of Na phytate

Phytase screening medium (broth) was prepared at 50 ml aliquots having different concentrations of Na phytate (0, 0.5, 1, 2, 5, 10, 15 & 20gL^{-1}). The flasks were inoculated with 0.1ml mother culture having absorbance of 0.1 at

Abs_{600} nm and incubated at 28±1°C for 24-48 hrs on rotary shaker at 120 rpm and measured phytase activity as detailed earlier.

3.2.3.7 Optimization of pH

Phytase screening medium (broth) was prepared in 50 ml aliquots having optimum concentrations of glucose, NH_4NO_3, KCl, $MgSO_47H_2O$ and Na phytate. pH was adjusted to 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 using 1N HCl and 1N NaOH, inoculated with 0.1ml mother culture having absorbance of 0.1 at Abs_{600} nm and incubated at 28±1°C for 24-48 hrs on rotary shaker at 120 rpm and measured phytase activity as detailed earlier.

3.2.3.8 Optimization of Temperature

Phytase screening medium (broth) was prepared in 50 ml aliquots having optimum concentrations of glucose, NH_4NO_3, KCl, $MgSO_47H_2O$ and Na phytate and at pH 7.0. The flasks were inoculated with 0.1ml mother culture having absorbance of 0.1 at Abs_{600} nm, incubated at different temperatures such as 20, 25, 30, 35, 40, 45 and 50ºC in a temperature controlled rotary shaker (Julabo SW23, Julabo GmbH, Germany) and measured the phytase activity as detailed earlier.

3.2.3.9 Optimization of Agitation

Phytase screening medium (broth) was prepared in 50 ml aliquots having optimum concentrations of glucose, NH_4NO_3, KCl, $MgSO_47H_2O$ and Na phytate and adjusted the pH to 7.0 and inoculated with 0.1ml mother culture having absorbance of 0.1 at Abs_{600} nm and incubated at 28±1°C on temperature controlled rotary shaker at 0, 50, 100, 150, 200 and 250 rpm and measured the phytase activity as detailed earlier.

3.2.3.10 Optimization of Incubation (fermentation) Period

Phytase screening medium (broth) was prepared in 50 ml aliquots having optimum concentrations of glucose, NH_4NO_3, KCl, $MgSO_47H_2O$ and Na phytate and adjusted the pH to 7.0., inoculated with 0.1ml mother culture having absorbance of 0.1 at Abs_{600} and incubated at 28±1°C on temperature controlled rotary shaker at 120rpm for 3 days**.** Samples were aseptically withdrawn from the flasks at 24 hrs intervals, Cells separated by centrifugation at 10000 *g* for 10 min at 4°C and measured the phytase activity in the supernatant as detailed earlier.

3.2.3.11 Optimization of Inoculum Size

Phytase screening medium (broth) was prepared in 50 ml aliquots having optimum concentrations of glucose, NH_4NO_3, KCl, $MgS0_47H_2O$ and Na phytate and adjusted the pH to 7.0, inoculated with 0.1ml mother culture having absorbance of 0.02, 0.04, 0.08 and 0.1 at Abs_{600} and incubated at 28±1°C on temperature controlled rotary shaker at 120 rpm for 3 days and measured the phytase activity in the supernatant as detailed earlier.

3.2.4 Phytase Assay

Phytase activity was assayed in a reaction mixture containing 0.5% w/v sodium phytate (Sigma Aldrich) prepared in 0.2 M sodium acetate buffer (pH 5.5) and suitably diluted enzyme. One phytase unit is defined as the amount of enzyme that liberates 1 μmol of inorganic phosphorus per minute from 0.01 mol L^{-1} sodium phytate at standard assay conditions (pH 5.5 and temperature 40°C).

The phytase activity was measured at 750nm in a UV-Vis double beam spectrophotometer using sodium phytate as the substrate.

3.2.5 First Step Optimization

After the initial screening of medium components and growth conditions in shake flasks by one-variable-at-a-time approach, one - dimensional screening of 11 factors (7 nutritional factors and 4 physical parameters) was carried out to find out the range of each parameter to be used for further optimization.(Glucose 5.0-15g, NH_4NO_3 1.0-5.0 g, KCl 0.5 – 2.0g, $MgSO_4.7H_2O$ 0.5 – 2.0 g, $FeSO_4.7H_2O$ 0.001 – 0.1 g, $MnSO_4.7H_2O$ 0.00 – 1.00 g, Na phytate 0.1-10 g, pH – 2.5-8.5,Temp- 25 –50^oC, Agitation 75-150rpm, Inoculum size- 0. 05- 0.4 ml). The optimum ranges of each of the medium components were subjected for analysis using Plackett- Burman design.

pH of the medium was monitored using narrow range pH paper during incubation and adjusted by aseptic addition of sterile 1mol L^{-1} NaOH and 1 mol L^{-1} HCl. Temperature of 25^o C was provided on incubating at room temperature on rotary shaker, and 50^oC in a temperature controlled shaking water bath. Agitation of 50 rpm and 150 rpm was provided using the same instruments. All flasks were inoculated with 0.1ml mother culture having absorbance of 0.1 at Abs_{600}.

3.2.5.1 Estimation of Growth

Growth was determined spectrophotometrically by measuring absorbance of the culture at Abs_{600} in UV-Visible spectrophotometer (Varian Cary 50 probe UV Visible Spectrophotometer).

3.2.5.2 Estimation of Phytase Production

The culture broth was centrifuged at 10000*g* for 15 min at 4ºC. The cell free supernatant obtained was assayed for phytase activity as described earlier.

3.2.6 Experimental Design and Data Anlaysis

3.2.6.1 Plackett & Burman design: A First-Step Optimization

Twelve run 2 level Plackett & Burman design was used to screen medium components (Glucose, NH_4NO_3, KCl, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnSO_4.7H_2O$, Na-phytate) and growth conditions such as pH, temperature, agitation, inoculum size, denoted as A-L respectively to identify those having significant effects on phytase production (Table 3.4). A total of 11 parameters were included for selection with each variable represented at two levels, high (+1) and low (-1). The design consisting of a set of 12 experiments was used to determine the relative importance of 11 factors that influenced the enzyme production (Table 3.5). Response value was measured in terms of phytase activity. The design was developed using Design Expert version (7.1.2), Stat-Ease Inc, Minneapolis, MN. All the trials were carried out in triplicates and the average phytase activity of each trial was used as the response variable. Regression analysis determined the variables that had significant (p<0.05) effect on phytase activity and these variables were evaluated for further optimization.

Table 3.4: Levels of variables tested in Plackett-Burman Design

Variables	Coded levels	
	-1 (Low)	*+1 (High)*
Glucose	5.0	15.0
NH_4NO_3	1.0	3.0
KCl	0.5	2.0
$MgSO_4.7H_2O$	0.05	2.0
$FeSO_4.7H_2O$	0.0	0.1
$MnSO_4.7H_2O$	0.0	1.0
Na phytate,	1.0	5.0
pH	2.5	8.5
Temperature	25.0	50.0
Agitation	75.0	150
Inoculum	0.05	0.4

Table 3.5: Plackett-Burman design matrix for media components

Run	A	B	C	D	E	F	G	H	J	K	L
1	1(15)	-1(1)	1(2)	-1(0.05)	-1(0.1)	-1(0)	1(5)	1(8.5)	1(50)	-1(75)	1(0.4)
2	1(15)	1(3)	-1(0.5)	1(2)	-1(0.1)	-1(0)	-1(1)	1(8.5)	1(50)	1(150)	-1(0.05)
3	-1(5)	1(3)	1(2)	-1(0.05)	1(0)	-1(0)	-1(1)	-1(2.5)	1(50)	1(150)	1(0.4)
4	1(15)	-1(1)	1(2))	1(2)	-1(0.1)	1(1)	-1(1)	-1(2.5)	-1(25)	1(150)	1(0.4)
5	1(15)	1(3)	-1(0.5)	1(2)	1(0)	-1(0)	1(5)	-1(2.5)	-1(25)	-1(75)	1(0.4)
6	1(15)	1(3)	1(2)	-1(0.05)	-1(0.1)	1(1)	-1(1)	1(8.5)	-1(25)	-1(75)	-1(0.05)
7	-1(5)	1(3)	1(2))	1(2)	-1(0.1)	1(1)	1(5)	-1(2.5)	1(50)	-1(75)	-1(0.05)
8	-1(5)	-1(1)	1(2))	1(2)	1(0)	-1(0)	1(5)	1(8.5)	-1(25)	1(150)	-1(0.05)
9	-1(5)	-1(1)	-1(0.5)	1(2)	1(0)	1(1)	-1(1)	1(8.5)	1(50)	-1(75)	1(0.4)
10	1(15)	-1(1)	-1(0.5)	-1(0.05)	1(0)	1(1)	1(5)	-1(2.5)	1(50)	1(150)	-1(0.05)
11	-1(5)	1(3)	-1(0.5)	-1(0.05)	-1(0.1)	1(1)	1(5)	1(8.5)	-1(25)	1(150)	1(0.4)
12	-1(5)	1(3)	-1(0.5)	-1(0.05)	-1(0.1)	-1(0)	-1(1)	-1(2.5)	-1(25)	-1(75)	-1(0.05)

(The actual values of factors are given in brackets)

3.2.6.2 Optimization of Phytase Production by Response Surface Methodology

Response surface approach using Central Composite Design (CCD) was applied to find out the optimum levels of significant variables and the effects of their interaction on enzyme production. A 2^3 CCD of 3 factors with an alpha value of 1, including six centre points with a set of 20 experiments were carried out. Each independent variable was studied at three different levels (low, medium and high, coded as -1, 0 and +1, respectively (Table 3.6). The centre point of the design was replicated eight times for the estimation of errors. Each run was performed in triplicate and the average phytase activity was taken as the experimental value of the dependent variable or response (Y), while predicted values of the response were obtained from quadratic model fitting. A multiple regression analysis of the data was carried out to define the response in terms of independent variables. The response surface graphs were obtained to understand the effects of the variables individually and in combination and to determine their optimum levels for maximum enzyme production. The data on phytase production

was subjected to analysis of variance (ANOVA). The soft ware, Design Expert (version 7.1.2, Stat-Ease Inc, Minneapolis, MN) was used for the experimental design, data analysis and quadratic model building.

Table 3.6: Central Composite Design matrixes of the three variables

No	Glucose (gl^{-1})	NH_4NO_3 (gl^{-1})	Na Phytate (gl^{-1})	Phytase (Uml^{-1})
1	-1 (7.50)	-1 (0.50)	-1 (2.00)	
2	1 (10.00)	-1 (0.50)	-1 (2.00)	
3	-1 (7.50)	1 (1.00)	-1 (2.00)	
4	1 (10.00)	1 (1.00)	-1 (2.00)	
5	-1 (7.50)	-1 (0.50)	1 (5.00)	
6	1 (10.00)	-1 (0.50)	1 (5.00)	
7	-1 (7.50)	1 (1.00)	1 (5.00)	
8	1 (10.00)	1 (1.00)	1 (5.00)	
9	-1 (6.6477)	0 (0.75)	0 (3.50)	
10	1 (10.852)	0 (0.75)	0 (3.50)	
11	0 (8.75)	-1 (0.3295)	0 (3.50)	
12	0 (8.75)	1 (1.1704)	0 (3.50)	
13	0 (8.75)	0 (0.75)	-1 (0.9773)	
14	0 (8.75)	0 (0.75)	1 (6.0226)	
15	0 (8.75)	0 (0.75)	0 (3.50)	
16	0 (8.75)	0 (0.75)	0 (3.50)	
17	0 (8.75)	0 (0.75)	0 (3.50)	
18	0 (8.75)	0 (0.75)	0 (3.50)	
19	0 (8.75)	0 (0.75)	0 (3.50)	
20	0 (8.75)	0 (0.75)	0 (3.50)	

3.2.7 Validation of the Model

The statistical model was validated with respect to phytase production under the conditions predicted by the model. Samples were withdrawn at desired intervals and phytase assay was carried out as described earlier. The experiments were carried out in triplicates and results were expressed as mean ± standard deviation. The experimental values were subsequently compared with the predicted values.

3.2.8 Scale up of Phytase Production in 5L Fermenter

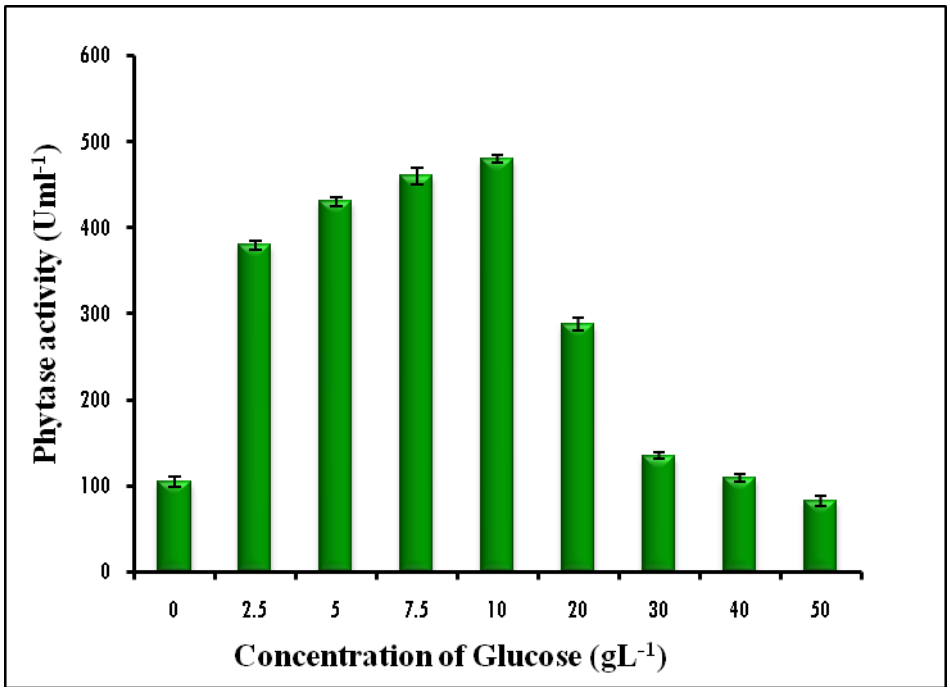
Bacillus MCCB 242 was grown for 18h at 25°C on rotary shaker at 120 rev min^{-1}. The optical density of this culture was adjusted to 0.1 Abs_{600} ($10^7 CFUml^{-1}$) and 0.1ml of the inoculum was transferred to 5L fermenter (Biostat-B-Lite bench top fermenter, Sartorius, Germany) containing 3L optimized medium. Fermentation was carried out at pH 7.0, 25°C and 300 rev min^{-1} and sterile air was supplied at the rate 2.5 l min^{-1} and the pH maintained at 5.5 ± 0.05. Phytase activity was monitored at regular intervals of 6 hrs for 54 hrs.

3.3 Results

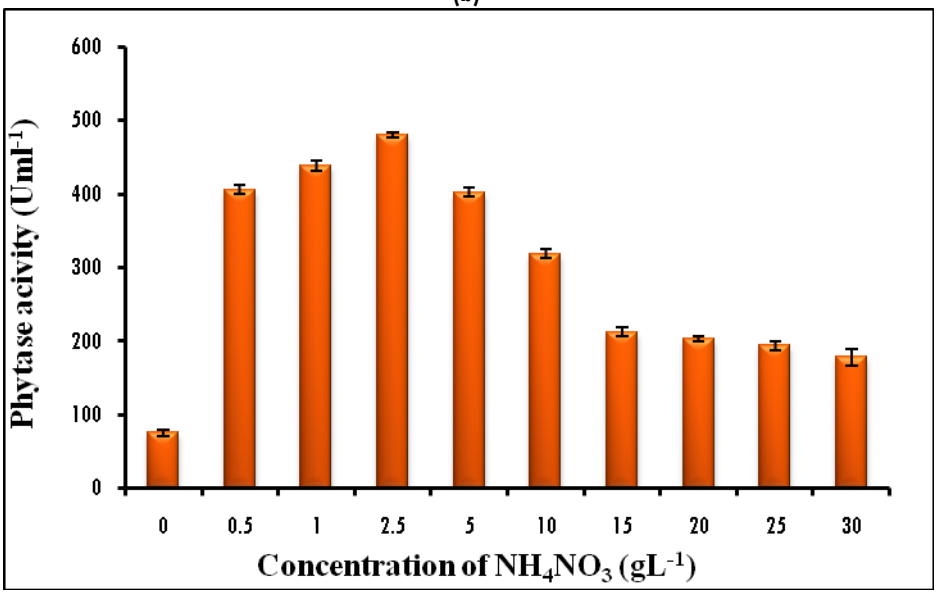
3.3.1 Initial optimization of phytase production

One dimensional screening of 11 factors (7nutritional factors and 4 physical parameters) was carried out to find out the range of each parameter to be used for further optimization experiments. The effect of different nutritional parameters and culture conditions on phytase production is represented graphically in Fig.3.1 (a-j).

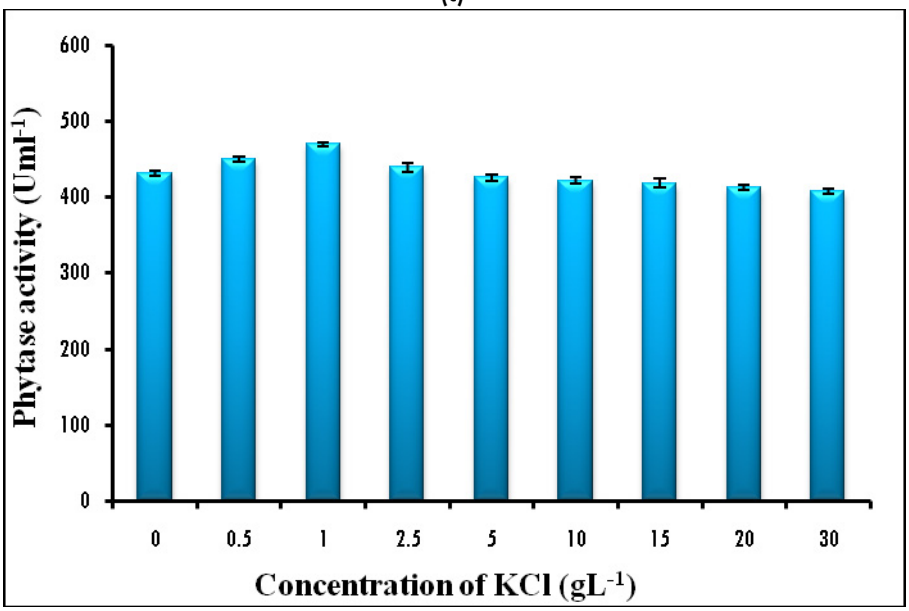
(a)


Phytase activity (Uml-1)
600
500
400
300
200
100
0
0
2.5
5
7.5
10
20
30
40
50
Concentration of Glucose (gL-1)

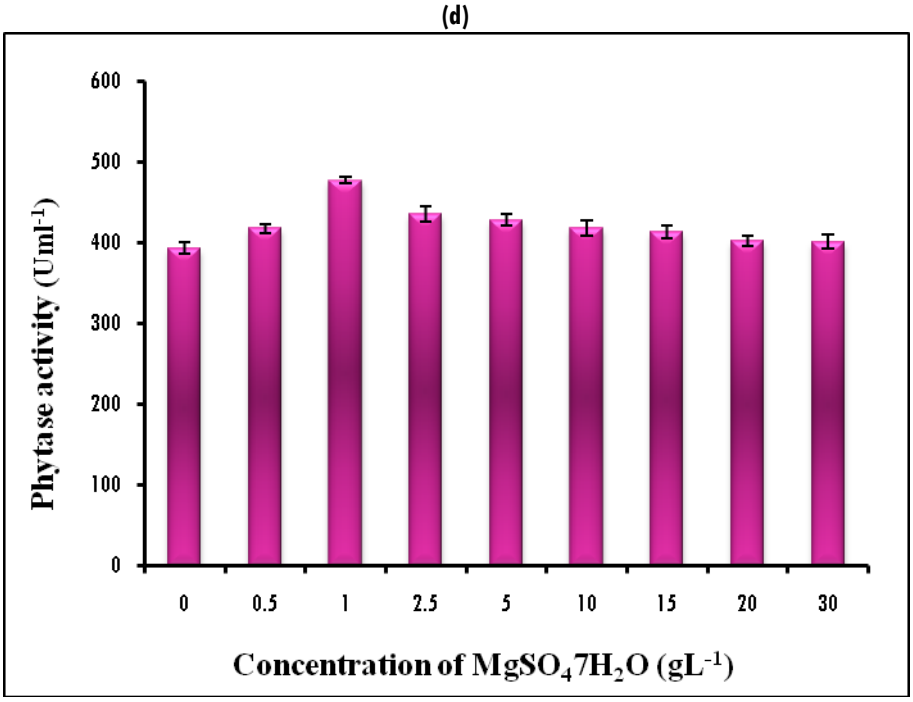
(b)


Phytase acivity (Uml-1)
600
500
400
300
200
100
0
0
0.5
1
2.5
5
10
15
20
25
30
Concentration of NH4NO3 (gL-1)

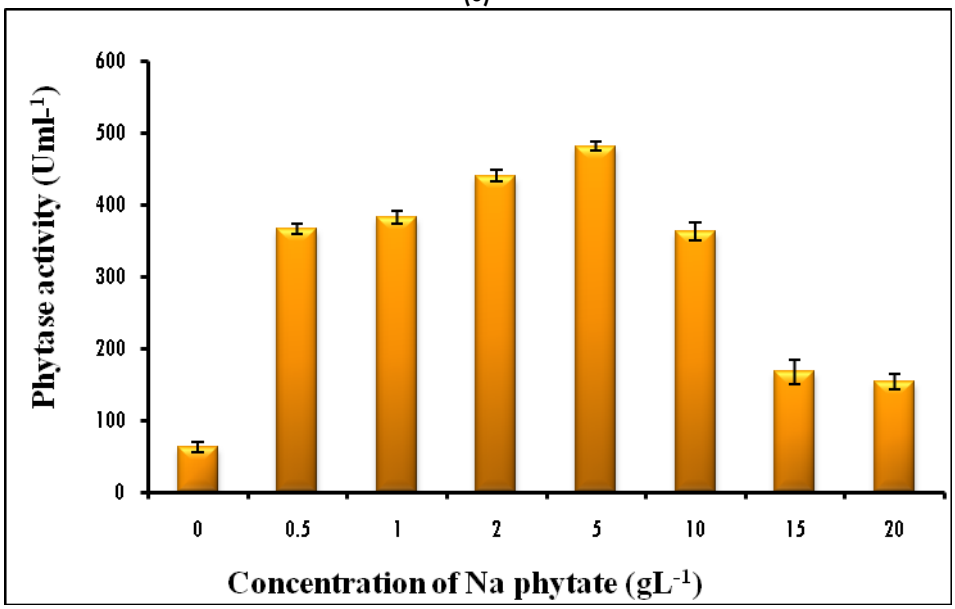
(c)


Phytase activity (Uml-1)
600
500
400
300
200
100
0
0
0.5
1
2.5
5
10
15
20
30
Concentration of KCl (gL-1)

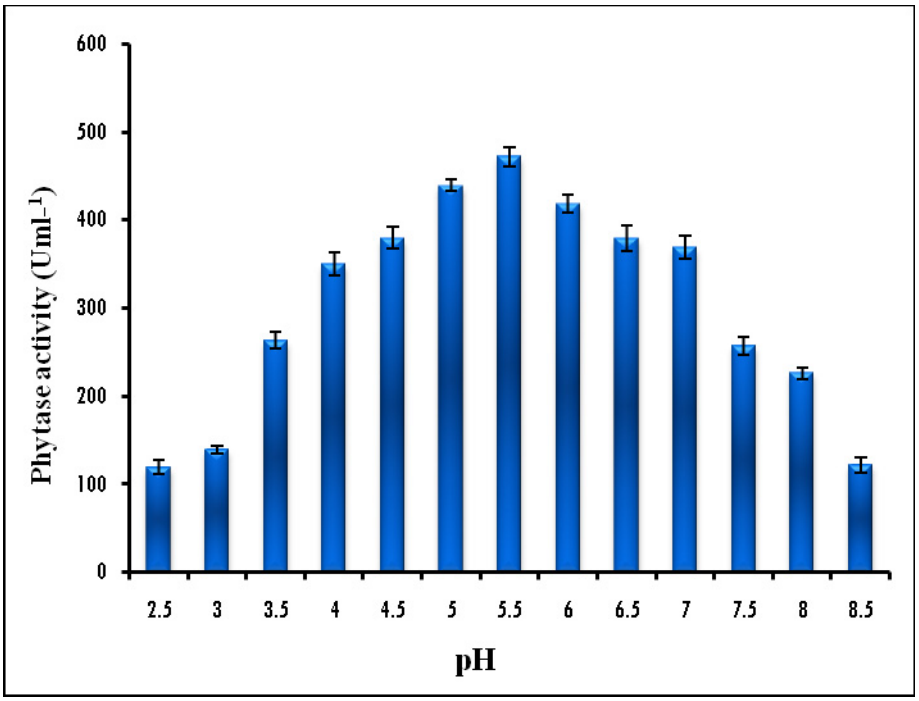
(d)


Phytase activity (Uml-1)
600
500
400
300
200
100
0
0
0.5
1
2.5
5
10
15
20
30
Concentration of MgSO4 7H2O (gL-1)

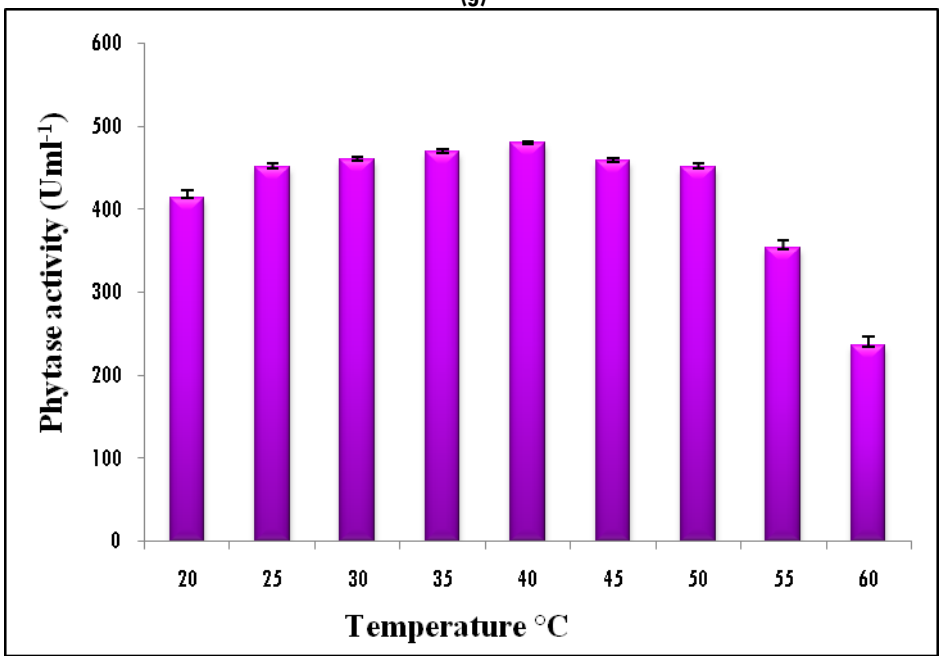
(e)


Phytase activity (Uml-1)
600
500
400
300
200
100
0
0
0.5
1
2
5
10
15
20
Concentration of Na phytate (gL-1)

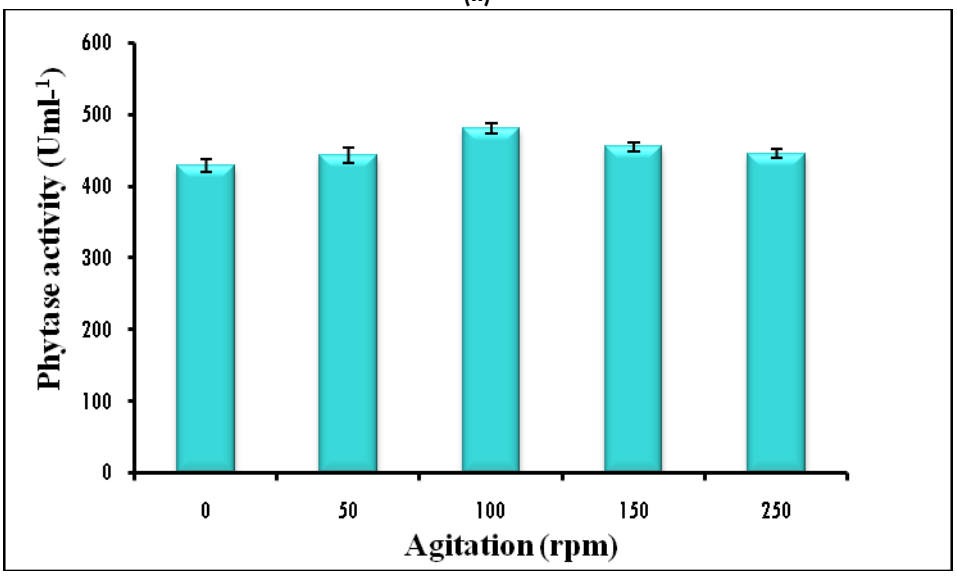
(f)


Phytase activity (Uml-1)
600
500
400
300
200
100
0
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
pH

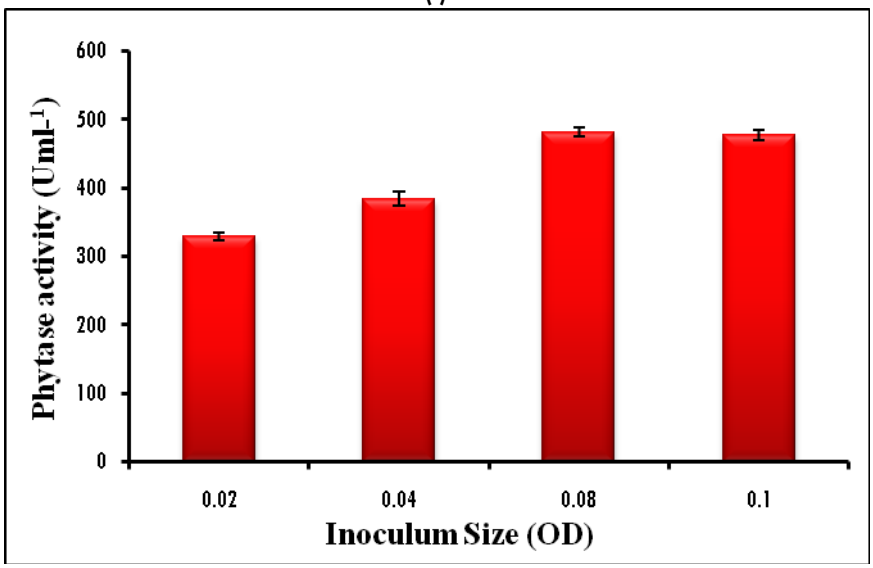
(g)


Phytase activity (Uml-1)
600
500
400
300
200
100
0
20
25
30
35
40
45
50
55
60
Temperature °C

(h)


Phytase activity (Uml-1)
600
500
400
300
200
100
0
0
50
100
150
250
Agitation (rpm)

(i)



(j)

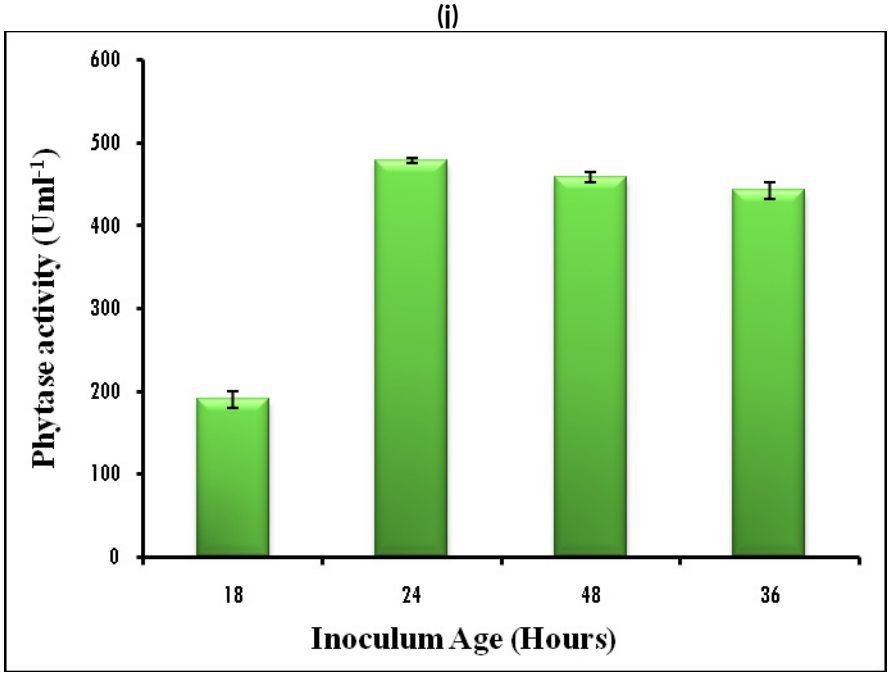


Fig 3.1a-j: One dimensional screening on the effect of physical and nutritional parameters on phytase production

Regression analysis was carried out for all factors and wherever the regression was found highly significant, the factors were taken for further screening by Plackett - Burman design. Table 3.7 shows the significant (p<0.05) ranges medium components selected.

Table 3.7: Medium components

Medium Components	Concentration range
Glucose	(5 -15 g)
NH_4NO_3	1- 3 g
KCl	0.5 – 2 g
$MgSO4.7H_2O$	0.5 – 2 g
$FeSO_4.7H_2O$	0.01 – 0. 1 g
$MnSO_4.7H_2O$	0.01 – 0. 1 g
Na phytate	1-5 g
Distilled water	1000 ml
pH	5-8
Temperature	25 – 50°C
Inoculum size	0. 05- 0.4 ml

3.3.2 Screening of Parameters using Plackett-Burman Design

In order to bring down the number of parameters for further analysis, critical medium components affecting phytase production were screened using Plackett-Burman design (PB). The design used for screening 11 selected variables along with their corresponding experimental and predicted values is shown in Table 3.8

Table 3.8: Plackett-Burman matrix for medium components with the corresponding observed and predicted values of phytase production from *Bacillus* MCCB 242

Run	A	B	C	D	E	F	G	H	J	K	L	Phytase (Uml^{-1})	
												Observed	Predicted
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	440	429
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	426	421
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	180	226
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	250	285
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	465	465
6	1	1	1	-1	-1	1	-1	1	-1	-1	-1	480	452
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	445	407
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	240	259
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	220	220
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	430	435
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	410	432
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	135	84

A: Glucose, B: NH_4NO_3, C: KCl, D: $MgSO_4.7H_2O$, E: $FeSO_4.7H_2O$, F: $MnSO_4.7H_2O$, G: Na phytate, H: pH, J: Temperature, K: Agitation, L: Inoculum size

Table 3.9 shows the effect of each variable, degrees of freedom, standard error and prob>F value. When the value of the tested variable was positive, influence of the variable was higher at higher concentration, and when negative, the influence was higher at lower concentration. A prob>F value less than 0.05 indicated that the model terms were significant. The Model F-value of 15.19 implied that the model was significant. There was only a 0.45% chance that the F-value could occur due to noise. Values of "Prob > F" less than 0.0500 indicated that the model terms were significant. Therefore, considering the prob>F value and the positive effect of A, B and G, glucose, NH_4NO_3, Na phytate were found to be statistically significant affecting phytase production.

Table3.9: Results of statistical analysis under the Plackett-Burman design

Source		Sum of Squares	df	Mean Square	F value	P value	Significant
Code	Variables						
Model		1.674E+005	6	27907.86	15.19	0.0045	
A	Glucose	61776.75	1	61776.75	33.63	0.0021	
B	NH_4NO_3	39790.08	1	39790.08	21.66	0.0056	
F	$MnSO_4.7H_2O$	10150.08	1	10150.08	5.53	0.0655	
G	Na phytate	45510.08	1	45510.08	24.78	0.0042	
H	pH	8060.08	1	8060.08	4.39	0.0903	
J	Temperature	2160.08	1	2160.08	1.18	0.3277	

Table 3.10: Percentage of contribution of Plackett-Burman design

Term Intercept	Variables	Sum Sqr	% Contribution
A	Glucose	61776.8	34.975
B	NH_4NO_3	39790.1	22.5272
C	KCl	216.75	0.122714
D	$MgSO_4.7H_2O$	70.0833	0.039677
E	$FeSO_4.7H_2O$	690.083	0.390692
F	$MnSO_4.7H_2O$	10150.1	5.74649
G	Na phytate,	45510.1	25.7656
H	pH	8060.08	4.56323
J	Temperature	2160.08	1.22294
K	Agitation	5166.75	2.92517
L	Inoculum	3040.08	1.72115

Coefficient of determination (R^2) = 0.9480; Adjusted R^2 = 0.8856; Predicted R^2 = 0.7005

The magnitude of coefficient of each variable indicated the intensity of its effect on the studied response. Greater the magnitude, significance of the variable is higher. Thus glucose had the highest influence on phytase production (34.9%) followed by Na phytate (25.7%) and NH_4NO_3 (22.5%) as represented in Table 3.10.

3.3.3 Second step optimization by Central Composite Design of Response Surface Methodology

The three most important factors from the previous screening experiment (PB design) were used for the optimization by way of Central Composite Design of Response Surface Methodology. On the basis of percentage contribution of the ingredients (factors) for maximum growth the negligible ingredients were avoided from the media in the next round of optimization. Each independent variable was studied at three different levels (low, medium and high, coded as -1, 0 and +1 respectively) in a set of 20 experiments. The central composite design matrix of the variables (Glucose: A, NH_4NO_3: B, and Na phytate: C) along with the experimental (n=3) and predicted values for the phytase production and the actual level of factors is given in Table 3.11.

The experimental data were statistically analyzed by the analysis of variance (ANOVA) and the results shown in Table 3.12. ANOVA of the quadratic regression model indicated that the model was highly significant ($p<0.0001$) by the F-value of 44.40. The 'lack of fit' was insignificant and the goodness of fit of the model was checked by regression coefficient (R^2). The R^2 value should be between 0 and 1. Closer the R^2 value to 1, stronger the model and better it predicts the response. R^2 had a value of 0.9756, indicating that the model could explain up to 97.56% of the variability of the response and the model did not explain 2.44% of the total variation. The value of R^2 indicated a good agreement between the experimental and predicted values of phytase production. For phytase production, A, C, A^2, B^2, C^2, AC were the significant model terms. Adequate precision measures such as signal to noise ratio and a ratio greater than 4 is desirable. The signal to noise ratio (adequate precision) for the model was higher than 4 (15.670) for phytase production, indicating an adequate signal which could be used to navigate the design space (good fit). The predicted R^2 value 0.8384 was in reasonable agreement with adjusted R^2 value 0.9536.

The two dimensional contour plots and their respective three dimensional contour response surface plots demonstrated significant interaction

effects of glucose, NH_4NO_3 and Na phytate on phytase production when all the other parameters were maintained at their optimum level (Figs.3.2.a-c). Fig.3.2.a presents the response surface and contour plots for the interactive factors, glucose and NH_4NO_3, when Na phytate was kept at $0.75gL^{-1}$. In Fig. 3.2.b the response surface and contour plots are brought in for interaction effects of glucose and Na phytate when NH_4NO_3 has been kept at $1.25gL^{-1}$. In Fig.3.2.c the response surface and contour plots are presented for interaction effects between NH_4NO_3 and Na phytate having the concentration of glucose kept at 2.75 gL^{-1}. The optimal values obtained from the contour plots have been almost equal to the results obtained from the regression equation.

Table 3.11: Central composite design matrixes of the three variables along with the experimental and predicted values

No	*Glucose*	*NH_4NO_3*	*Na Phytate*	*Phytase (Uml^{-1})*	
	(gL^{-1})	(gL^{-1})	(gL^{-1})	Observed	Predicted
1	7.5	0.5	2	248	220
2	10	0.5	2	220	198
3	7.5	1	2	278	230
4	10	1	2	234	254
5	7.5	0.5	5	555	505
6	10	0.5	5	233	251
7	7.5	1	5	434	426
8	10	1	5	220	218
9	6.64776	0.75	3.5	343	403
10	10.8522	0.75	3.5	221	203
11	8.75	0.329552	3.5	227	260
12	8.75	1.17045	3.5	235	241
13	8.75	0.75	0.977311	220	257
14	8.75	0.75	6.02269	469	474
15	8.75	0.75	3.5	655	639
16	8.75	0.75	3.5	605	639
17	8.75	0.75	3.5	656	639
18	8.75	0.75	3.5	580	639
19	8.75	0.75	3.5	665	639

Table 3.12: Analysis of variance (ANOVA) for the fitted quadratic polynomial model of central composite design

Source	Sum of Square	df	Mean square	F value	P value	
Model	6.46E+05	9	71815.76	44.4	< 0.0001	significant
A	48419.62	1	48419.62	29.93	0.0003	
B	487.45	1	487.45	0.3	0.5951	
C	56802.94	1	56802.94	35.12	0.0001	
A2	2.03E+05	1	2.03E+05	125.76	< 0.0001	
B2	2.68E+05	1	2.68E+05	165.54	< 0.0001	
C2	1.35E+05	1	1.35E+05	83.33	< 0.0001	
AB	1058	1	1058	0.65	0.4375	
AC	26912	1	26912	16.64	0.0022	
BC	3960.5	1	3960.5	2.45	0.1487	
Residual	16175.39	10	1617.54			
Lack of Fit	13158.06	5	2631.61	4.36	0.0659	not significant
Pure Error	3017.33	5	603.47			
Cor Total	6.63E+05	19				

Df –Degree of freedom, R^2 =0.9756, Adjusted R^2 = 0.9536, Predicted R^2 = 0.8384,

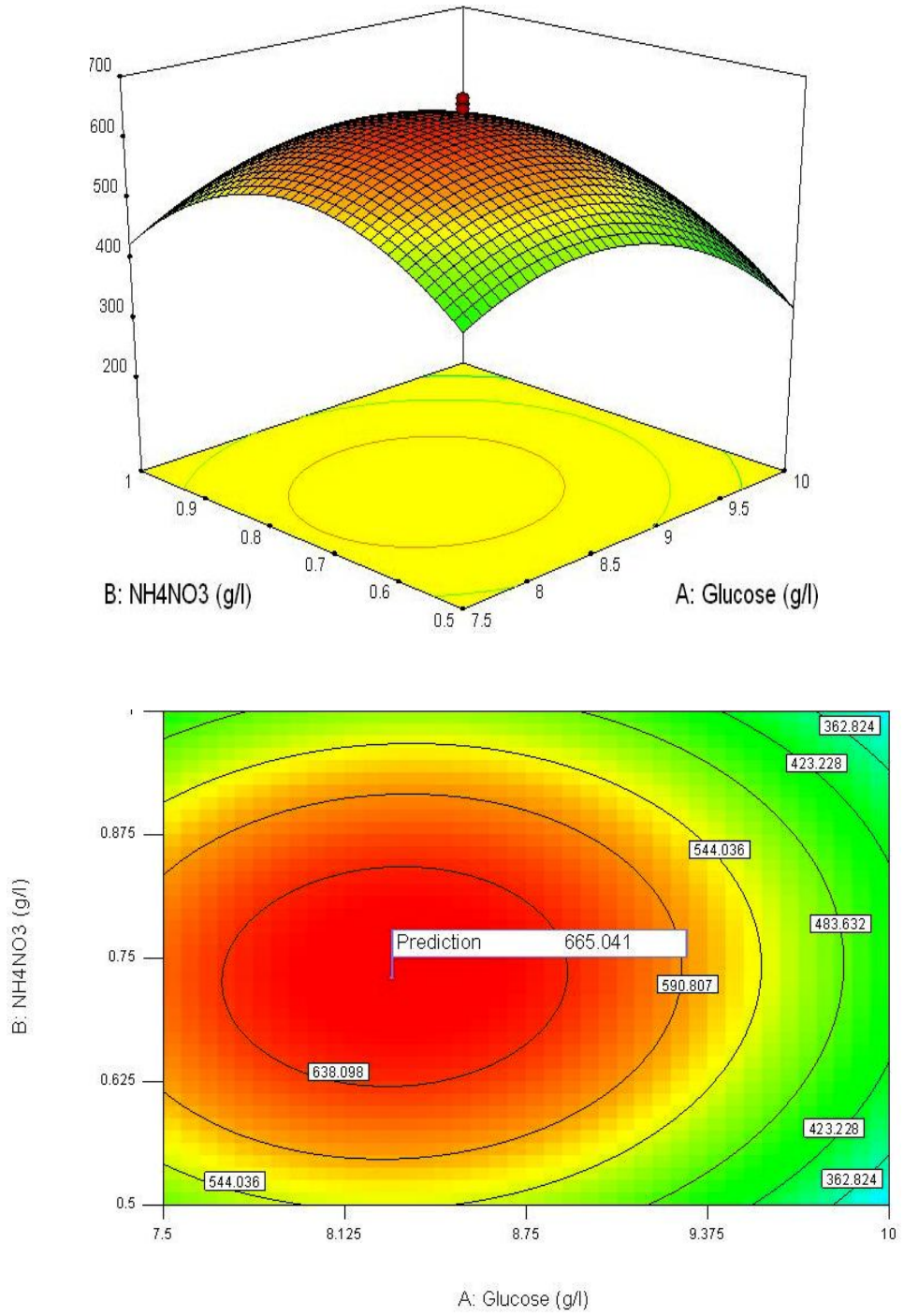


Fig.3.2 (a): Contour plot and its response surface plot of phytase activity of *Bacillus MCCB*242. Interaction of (A) Glucose and (B) NH_4NO_3 at constant Na. phytate content on phytase production

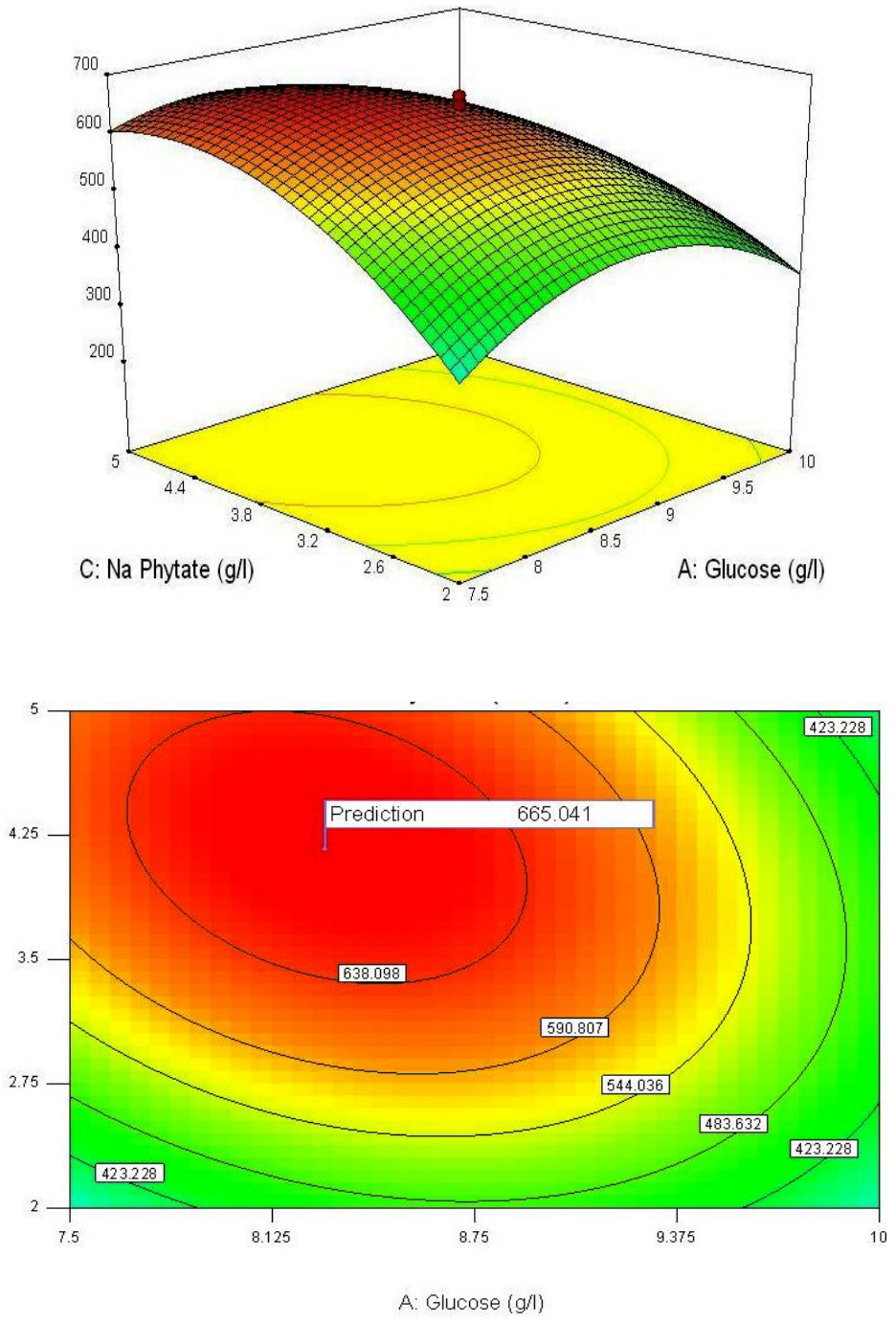


Fig 3.2 (b): Contour plot and its response surface plot of phytase activity of *Bacillus MCCB* 242 Interaction of (A) Glucose and (C) Na phytate at constant NH_4NO_3on phytase production

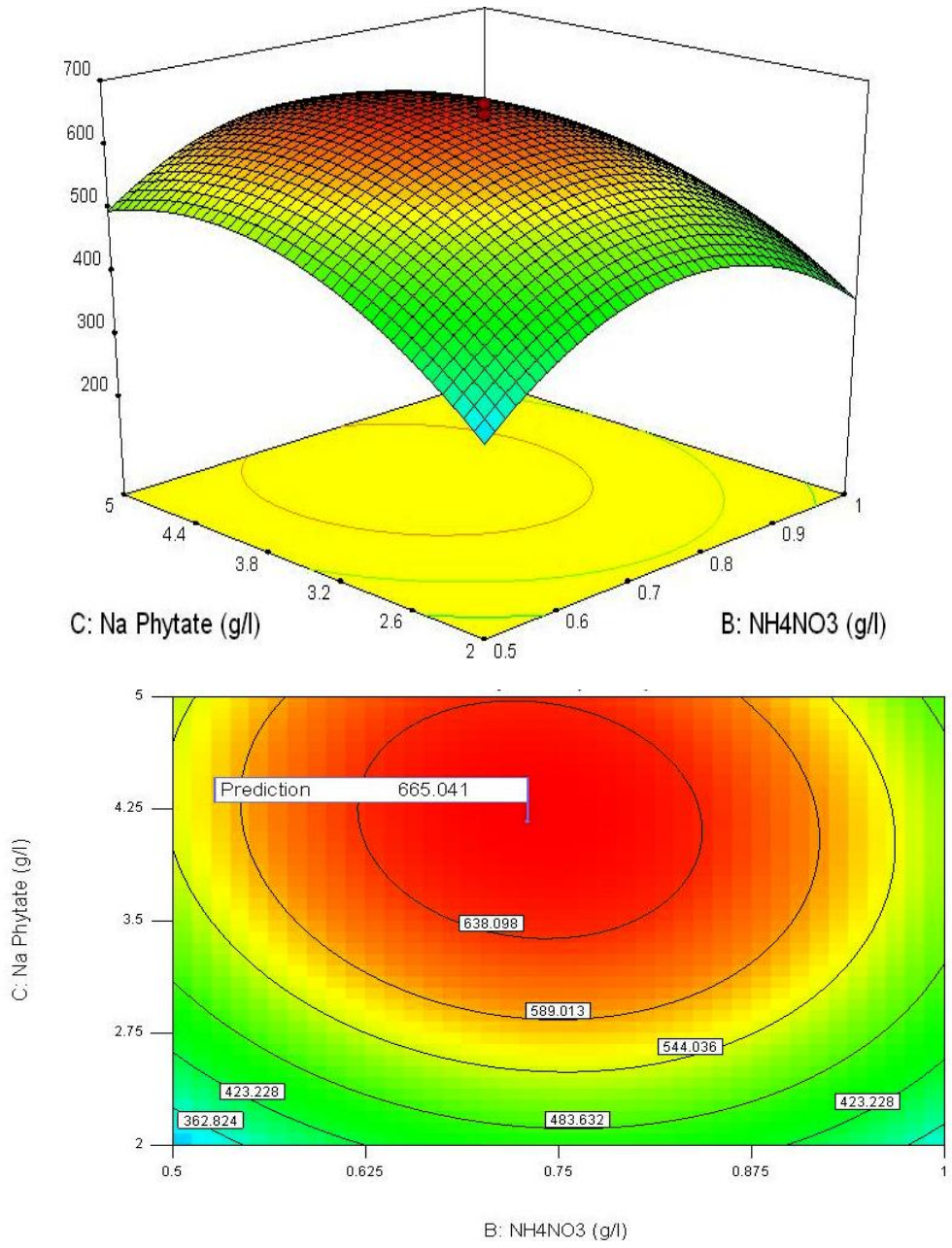


Fig 3.2 (c): Contour plot and its response surface plot of phytase activity of *Bacillus MCCB* 242. Interaction of (B) NH_4NO_3 and (C) Na phytate at constant Glucose content on phytase production

3.3.4 Modified composition of Phytase Screening Medium

Based on the above observations the phytase screening medium was reconstituted (Table 3.13) for maximizing the production of phytase from *Bacillus* MCCB 242. The modified phytase screening medium consists of the ingredients as given below (gL^{-1});

Table 3.13: Modified Phytase Screening Medium

Ingredient (gL^{-1})	
Glucose	8.2
NH_4NO_3	0.73
KCl	1.5
$MgSO_47H_2O$	1.5
$Fe\ SO_47H_2O$	0.01
$MnSO_47H_2O$	0.01
Na phytate	4.2
pH	5.5
Temperature	40°C
Agitation	120rpm
Inoculum size	0.4ml(0.8 OD)

3.3.5 Validation of the Experimental Model

Aim of the optimization was to determine a set of variables (parameters) that would lead to the development of the best model which would maximize production of phytase. The solution obtained from the model is given in Table 3.14. The maximum predicted value of phytase activity was 665.04±6.08 Uml^{-1} with the optimum level of medium components such as glucose, 8.2gL^{-1}, NH_4NO_3, 0.73 gL^{-1} and Na phytate, 4.2 gL^{-1}. The model was validated by

repeating the experiment under optimum conditions in shake flask mode which resulted in 653.3±3.05 Uml^{-1} phytase activity, indicating a good correlation between predicted and experimental values, proving validity of the model.

Table 3.14: Solution predicted by the model for phytase production

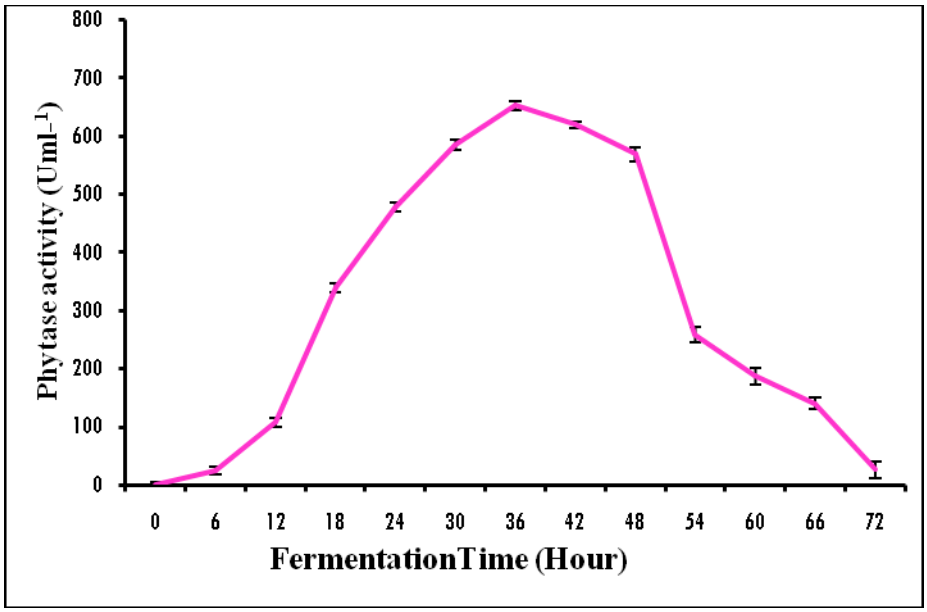
Solution	Glucose	NH_4NO_3	Na Phytate	Predicted Phytase	Desirability
1	8.2 (gL^{-1})	0.73 (gL^{-1})	4.2 (gL^{-1})	665 (Uml^{-1})	1

3.3.6 Fermentation Curve in Shake Flask

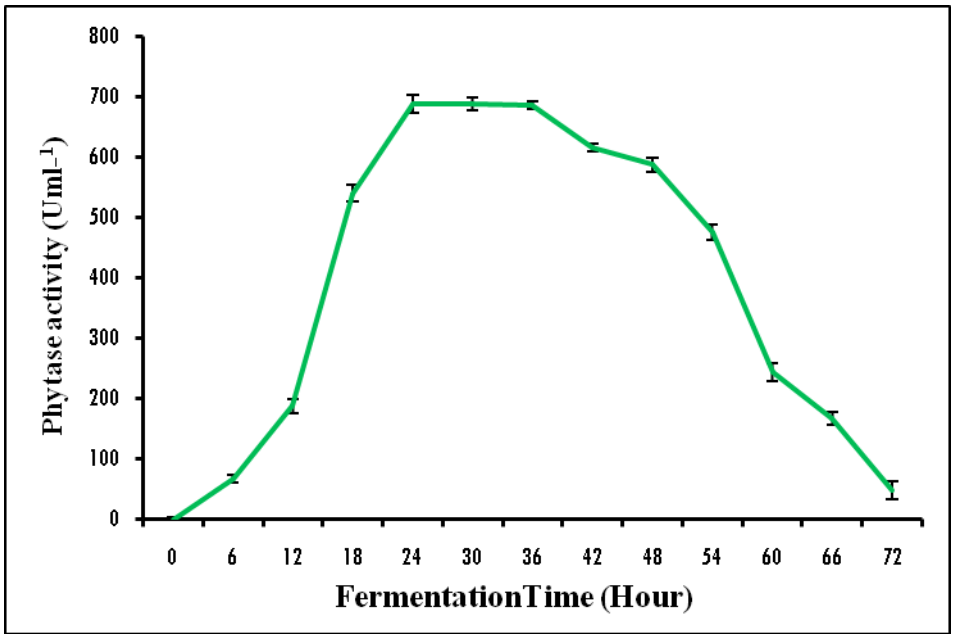
The time course of phytase production by *Bacillus* MCCB 242 in the optimized medium in shake flask is given in Fig. 3.3a. Phytase production reached maximum at 36th h with an activity of 653.3±3.05 Uml^{-1}

3.3.7 Scale up in 5L Fermenter

The time course of phytase production by *Bacillus* MCCB 242 in the optimized medium in 5L fermenter is shown in Fig.3.3b. Phytase production reached maximum at 24th hrs with an activity of 688 ±3.21Uml^{-1} with 1.433 fold increase in production compared to that in the un optimized medium (480 ± 2.89Uml^{-1}).The production and sampling for phytase in 5-L fermenter is shown in Fig.3.4 and 3.5 respectively.



(a)



(b)

Fig 3.3 a,b:Time course of phytase production in shake flask (a) and 5L fermentor (b

Fig 3.4 Phytase production in 5-L fermenter

Fig 3.5 Sample collection at 6 hrs intervals

3.4 Discussion

Development of an appropriate fermentation medium is an absolute requirement for commercial production of enzymes (Quang *et al.,* 2013). Review of existing literature demonstrated the need of optimizing fermentation conditions for phytase production. The enzyme production was depended upon several factors such as capability of the culture, physical and chemical factors of the culture medium and growth conditions provided (Vats and Banerjee, 2004). In the present study, an attempt was made to optimize the medium components and culture conditions to develop a cost effective medium for phytase production by using *Bacillus* MCCB 242. Statistical designs are currently used to find ways to enhance phytase production keeping the production cost minimum. In this process, the use of response surface methodology (RSM) has gained greater importance (Li *et al.,* 2008; Singh *et al.,* 2006).

The conventional one-variable at a time approach is time consuming and requires more experimental data sets and cannot predict the interaction between the components and it does not depict the complete effects of the parameters on the process (Weruster-Botz, 2000; Wang and Lu, 2004; Bas and Boyaci, 2007; Li *et al.,* 2008; Liu *et al.,* 2010). The use of statistical techniques (Placket-Burman and Response Surface Methodology) for the optimization of media components eliminates the limitations of 'one variable at a time' approach (Stanbury *et al.,* 2003). There is very few information available on the statistical optimization of bacterial phytase production. Therefore, the present study on optimization of the phytase screening medium to maximize phytase production by *Bacillus* MCCB 242 using the Central Composite Design of the Response Surface Methodology is of great significance.

In this study, *Bacillus* MCCB 242 phytase exhibited pH optima that are in agreement with other reported *Bacillus* phytases. Optimal activities of the

phytase produced, was found to be in the range of pH 5.5-7.0. The same pH optima for activity were found in *Bacillus* sp KHU-10, (Choi *et al.,* 2001) *Bacillus subtilis* (Kerovuo *et al.,* 1998; Powar & Jagannathan, 1982) and *Bacillus amyloliquefaciens* (Kim *et al.,* 1998; Idriss *et al.,* 2002) while *Bacillus laevolacticus* (Gulati *et al.,* 2006) exhibited optimum activity at neutral to slightly alkaline pH (7.0 - 8.0). Phytase activity at low pH values make *Bacillus* MCCB 242 phytase suitable as feed additives for monogastric animals having stomach pH values of 2.0 - 6.0.

Temperature is one of the most influencing factors in fermentation and production of enzymes. In the present study, *Bacillus* MCCB 242 phytase exhibited highest phytase activity at lower temperature (25^{o}C) but maintained almost the same even in high reaction temperatures up to 50^{o}C, with optimum activity at 40^{o}C. Optimal temperature evolved for phytase production and activity in this study is agreeable to a large extent to those of other microbial origin that ranged from 40 - 60^{o}C in the case of *Bacillus* sp KHU-10, (Choi *et al.,* 2001) 55^{o}C as far as *Bacillus subtilis* (Kerovuo *et al.,* 1998) is concerned and 50^{o}C as far as *Citrobacter braaki*, (Kim *et al.,* 2003). It can be concluded that *Bacillus* MCCB 242 phytase in this study could possibly perform optimal phytate degrading activity in the gastric temperature of monogastric animals.

Through optimization of composition of growth medium and culture conditions by implementing Plackett–Burman and Response Surface Methodology a phytase production medium could be reconstituted having the composition (gL^{-1}) Glucose - 8.2, NH_4NO_3 - 0.73, KCl - 1.5, $MgSO_47H_2O$ - 1.5, $FeSO_47H_2O$ - 0.01, $MnSO_47H_2O$ - 0.01, Na phytate - 4.2, and growth conditions such as pH- 5.5, Temperature - 40°C and Agitation - 120 rpm, Under shake flask condition 653.3±3.05Uml^{-1} phytase activity could be obtained using the medium and employing the specific growth conditions.

This activity is achieved during a fermentation period of 36 hrs. But when this is elevated to 5L fermenter, the production could further be enhanced to 688 ±3.21Uml^{-1}, 1.433 fold increase compared to that in the un optimized medium (480 ±2.89Uml^{-1}). The fermentation time could be brought down to 24hrs. Duration of fermentation required by *Bacillus* MCCB 242 for maximum phytase production in this study is not in agreement with that of other *Bacillus* isolates. According to Choi *et al*. (2001) phytase activity of *Bacillus* sp. KHU-10 increased markedly after the cells had reached the late stationary phase.

The phytase production has to be scaled up to larger fermenters with an efficient downstream process for commercial production.

Chapter 4

PURIFICATION AND CHARACTERIZATION OF PHYTASE FROM *BACILLUS* MCCB 242

Contents

4.1 Introduction

Enzymes are regarded as nature's catalysts and they accelerate all vital biological processes. Most of the known enzymes are proteins but not all proteins are enzymes. If a protein is able to catalyze a biochemical reaction, it is considered as an enzyme. Phytase has a role to catalyze the hydrolytic phosphate splitting of phytic acid (IP6) to lower inositol phosphate esters and inorganic phosphate (Pi). Phytase, the hydrolytic enzyme, have to be purified and characterized for its use in food and feed processing industries. Bacterial phytases are treated to be important because of their high thermal stability, phytate substrate specificity, wide pH profile and proteolysis resistance (Kim *et al.,* 1999; Kerovuo *et al.,* 2000; Oh *et al.,* 2004). Phytase has been detected and described in isolates of *E. coli*, *Bacillus* sp., *Klebsiella* spp., *Pseudomonas* sp., *Enterobacter*, and *Citrobacter* sp. (Kerovuo, 2000). Recent research has focused on the purification of phytases from microbial sources for the reason that these sources are very much promising for phytase production at commercial level.

Phytase has been studied intensely over the years because of its immense importance in hydrolyzing phytate liberating inorganic phosphate in animal feed thereby the content of plant material in the latter could be increased

(Shieh & Ware, 1968; Mitchell *et al.,* 1997). Several studies have proved that the incorporation of phytase augments phosphate utilization from phytic acid and drastically reduces inorganic phosphate excretion (Nelson *et al.,* 1971; Nasi, 1990; Simons *et al.,* 1990).

Proteins are characteristically studied in detail in its pure form. They should be separated from other cellular components such as lipids, nucleic acids, sugars, etc. and isolated to homogeneity. It is always necessary to develop unique approaches for isolation of the target protein of interest. Purifying samples contaminated with different fragments of the same protein is more challenging. Purification strategy will have several stages, each one taking advantage of different characteristics of the protein. The extensively used methods for preparative purification of proteins involve chromatography. Phytase is usually isolated and purified by extraction (with water, buffer or salt solutions), precipitation of the crude extract with ammonium sulfate followed by dialysis or gel filtration and ion exchange chromatography (Reddy *et al.,* 1989).

An enzyme accelerates rate of reactions and phytases (phytate-degrading enzymes) are of importance for producing defined breakdown products of phytate for kinetic and physiological studies. Lineweaver-Burk analysis is one method of linearizing substrate-velocity data so as to determine the kinetic constants, *Km* and *Vmax*. The characteristics, catalytical and physico-chemical properties of phytase, depend on the source and origin. Phytases differing in their pH, temperature optima, specific activity and substrate specificity have been identified and purified to homogeneity or near-homogeneity. Based on the enzyme characteristics and protein sequence homology, the subclass of the family can be identified. In this chapter we report the purification and characterization of a wild type phytase from *Bacillus* MCCB 242 with high

yield and affinity. The kinetic parameters (*Km* and *Vmax*) of this extracellular enzyme were calculated using the Lineweaver-Burk plot.

As presented in the previous Chapter (Chapter 3) production optimization of phytase from *Bacillus* MCCB 242 has been accomplished. This Chapter (Chapter 4) deals with the steps undertaken for its purification employing DEAE cellulose chromatographic techniques with an automated ÄKTA Prime Plus system. Studies on phytase purification using ion-exchange chromatographic methods were reported by Greiner *et al.* (1997, 2009). Gel filtration as a purification method for phytase from different sources was reported by El-Toukhy, 2013 and Greiner *et al.* (1998, 2000). Dialysis and reversed-phase liquid chromatography (RPLC), or ion-exchange chromatography are useful methods for purifying phytase prior to mass spectral analysis.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a widespread analytical tool for peptides, proteins, and most other bio-molecules (including oligonucleotides, carbohydrates, natural products, and lipids). This technique was first introduced in 1988 by Karas and Hillenkamp. MALDI - TOF MS has revolutionized routine identification of bacterial enzymes in the recent years (Pavlovic *et al.,* 2013). In MALDI - TOF MS analysis, the analyte is first co-crystallized with a large molar excess of a matrix compound, usually a UV-absorbing weak organic acid, after which laser radiation of this analyte–matrix mixture results in the vaporization of the matrix which carries the analyte.

MALDI-TOF-MS identification of proteins is carried out by the peptide mass mapping or peptide mass fingerprinting technique. The spectrum of identified peptide masses is unique for a specific protein and is known as mass fingerprint. This extremely efficient approach of protein identification is based on the accurate mass measurement of a group of peptides resulting from a

protein by sequence specific proteolysis. The proteolysis using trypsin, process termed as trypsinization, produces a series of peptide masses, which can be detected by MALDI-TOF-MS. Most of the proteins are identified by searching the selected masses from the fingerprint against databases of known protein sequences.

To deal with the vast data, a collection of automated methods as bioinformatics tools which determine the structure of a protein from its amino acid sequence have emerged. Protein databases contain information derived from the primary sequence databases, protein translations of the nucleic acid sequences and sets of patterns, motifs derived from sequence homology. The protein structural analysis and motif identification is possible using various graphic softwares (PyMOL, Rasmol and Argus lab). There are many programs for visualization of protein structures, which can be freely downloaded from the Internet. Swiss-Prot, NCBI Protein database, RCSB Protein Data Bank PROSITE, PIR-NREF, PMD-Protein Mutant Database, PRF/SEQDB, SwissProt - TrEMBL, Chimera etc. are some of the databases in which protein sequence can be analyzed.

The DEAE cellulose purified phytase was checked for its *in vitro* cytotoxicity. Cytotoxicity can be considered as an important index for evaluating safety of enzymes and its management prior to their administration in feed/aquaculture industry. The purified enzyme from *Bacillus* MCCB 242 was further evaluated for its cytotoxicity. The cytotoxicity assays developed by Weyermann *et al.,* (2005) use different parameters associated with cell death and proliferation.

Phytase induced toxicity in cell lines has not been documented yet. To our knowledge this is the first report on the *in vitro* toxicity studies of phytase

from an environmental isolate on human cell line, Hep-2 and fish cell line, RTG-2.

4.2 Materials and Methods

There are many reports on the purification and characterization of phytase from various microorganisms. In this study *Bacillus* MCCB 242 produced phytase was selected for purification and biochemical characterization.

4.2.1 Enzyme Extraction

Bacillus MCCB 242 was grown in the newly designed phytase screening medium having the composition as described in Chapter -3 (Table 3.13) in a 5L fermentor (Biostat-B-Lite, Sartorius, Germany). Fermentation was carried out at 25°C, pH 5.5 ± 0.05, 120 rpm and supplied with sterile air at the rate 2.5L min^{-1}. For enzyme extraction the culture was centrifuged at 10,000*g* for 15 min at 4°C and the enzyme supernatant was stored in 300 ml aliquots at -20°C for further purification and characterization.

4.2.2 Purification of Phytase

Phytase produced by *Bacillus* MCCB 242 was purified using the standard protein purification protocols, consisting of ammonium sulphate precipitation, dialysis using Amicon UF stirred cells, gel filtration and concentration. All the experiments were done at 4ºC unless otherwise specified.

4.2.2.1 Ammonium Sulphate Precipitation

Ammonium sulphate required to precipitate phytase was optimized by its addition at varying levels of concentrations (20, 40, 60, 80 and 100% saturation), to the crude enzyme in stepwise manner (Englard and Seifter, 1990). To precipitate the protein, ammonium sulphate was slowly added

initially at 20% saturation to the crude extract upward to 100% while keeping in ice with gentle stirring. After complete dissolution of ammonium sulphate, the solution was kept at 4ºC for overnight. The precipitated protein was then centrifuged at 10,000*g* for 15 min at 4ºC and the precipitate was resuspended in minimum quantity of 0.25 M sodium acetate buffer at pH 5.5. To the supernatant collected, ammonium sulphate required for next level of saturation was added, and the procedure repeated. The precipitation was continued up to 100% saturation, precipitates concentrated by centrifugation at 10000*g* for 15 min at 4°C and dissolved in sodium acetate buffer, 0.25 M (pH 5.5). Maximum phytase activity was observed in the fraction of 80% saturation.

4.2.2.2 Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against sodium acetate buffer 0.25 M (pH 5.5) in order to remove ammonium sulphate from the precipitate. Amicon UF stirred cell (Millipore Corporation, USA, Model 8010), with a 10 kDa cutoff membrane (Omega, 25MM, 10K, Pall life sciences) was used for dialyzing the enzyme. Yield and fold of purification were calculated as described in section 4.2.3.

4.2.2.3 Ion Exchange Chromatography

The product obtained after ammonium sulphate precipitation and dialysis (80% fraction) was further subjected to ion exchange chromatography using diethyl aminoethyl (DEAE) cellulose as column material.

4.2.2.3.1 Activation of DEAE Cellulose

A quantity of 5 g DEAE cellulose (Sigma-Aldrich Co.) was soaked in 0.1 M sodium acetate buffer (pH 5.5), stirred properly and kept overnight for swelling. The swollen DEAE cellulose was filtered on a Buchner funnel using

vacuum filtration and incubated with 1 M NaCl for 1 hr. The filtration was repeated until the washings attained pH 5.5.

4.2.2.3.2 Standardization of pH required for binding of phytase to DEAE cellulose

Maximum pH at which phytase binds to the anion exchanger was standardized by eluting the enzyme from the DEAE cellulose column. DEAE cellulose was activated by following the method described in section 4.2.2.3.1, suspended in distilled water and equilibrated to pH using 0.01 M buffers of glycine-HCl (2.5-3.5), sodium acetate (4.0-7.5), Tris-Cl (8.0-9.0) and Carbonate-bicarbonate buffer (pH 9.5-10.5). An aliquot of 1 ml of diluted sample of 80 % ammonium sulphate precipitated fraction was mixed with 2 ml slurry of DEAE cellulose equilibrated to each pH, incubated at 4°C for overnight, and the supernatant decanted and assayed for phytase activity and protein as described in Chapter 2, Section 2.2.8 and 2.2.9. Yield and fold of purification was calculated as described in Section 4.2.3.

4.2.2.3.3 DEAE-cellulose chromatography

The enzyme was loaded on AKTA Prime protein purification system equipped with DEAE cellulose C10/20 column (10mm×20cm) (GE Healthcare Biosciences, Uppsala) equilibrated with 1M sodium acetate buffer, at pH 5.5. The column was washed with the same buffer to remove the unbound proteins and the enzyme was eluted by applying a linear gradient of NaCl from 0-1000 mM at a flow rate of 0.5 ml min^{-1} and fractions of 2 ml were collected. Active fractions were pooled and concentrated by lyophilization.

4.2.3 Calculation of yield of protein, enzyme activity and fold of purification

Yield of protein, enzyme activity and fold of purification of each fraction obtained during purification was calculated as given below:

$$\text{Yield of protein} = \frac{\text{Total protein content of the fraction} \times 100}{\text{Total protein content of the crude extract}}$$

$$\text{Yield of activity} = \frac{\text{Total activity of the fraction} \times 100}{\text{Total activity of the crude extract}}$$

$$\text{Fold of Purification} = \frac{\text{Specific activity of the fraction}}{\text{Specific activity of the crude extract}}$$

4.2.4 Characterization of Phytase

The purified enzyme obtained through ammonium sulfate precipitation, dialysis and ion exchange chromatography were assayed for phytase activity and protein content. The purified enzyme was subjected to electrophoresis for the determination of molecular weight. Effect of pH and temperature for enzyme activity and stability, inhibition and activation of enzyme activity by different metal ions, surfactants, oxidizing agents and reducing agents were investigated.

4.2.4.1 Phytase Assay

Peak fractions from the column were pooled and assayed for phytase activity, protein content and specific enzyme activity. The enzyme substrate (Sodium phytate -1ml) was added to pooled fraction of phytase (1ml) and the mixture was incubated at 40^{o}C in a water bath for 60 min. An aliquot of 1ml 10% trichloroacetic acid (TCA) was added to the mixture to stop the reaction. Subsequently 2 ml color developing agent (3.66g $FeSO_4 7H_2O$, 0.5g ammonium heptamolybdate tetrahydrate, 1.6 ml conc. H_2SO_4 made up to 50ml by adding distilled water) was added to the mixture. The solution was read for liberated

inorganic phosphate spectrophotometrically at an absorbance of 750 nm. A calibration curve was made with the use of NaH_2PO_4 to quantify the phosphate release. One phytase unit is defined as the amount of enzyme that liberates 1 μmol of inorganic phosphorus per minute from 0.01 mol L^{-1} sodium phytate at standard assay conditions (pH 5.5 and temperature 40°C).

4.2.4.2 Protein assay

Quantification of protein was carried out following the method of Lowry (1951) using Bovine Serum Albumin as standard. Details are given in section 2.2.9 of Chapter 2.

4.2.4.3 Specific activity

Specific activity was calculated by dividing the enzyme units with the protein content.

$$\text{Specific activity}\left(\text{U/mg}\right)=\frac{\text{Total unit activity}\left(\text{Uml}^{-1}\right)}{\text{Total protein content}\left(\text{mg ml}^{-1}\right)}$$

4.2.4.4 Relative Activity

It is the percentage enzyme activity of the sample with respect to the sample for which maximum activity was obtained.

$$\text{Relative activity}=\frac{\text{Total unit activity}\left(\text{Uml}^{-1}\right)\times 100}{\text{Maximum activity }\left(\text{Uml}^{-1}\right)}$$

4.2.4.5 Residual Activity

Residual activity is the percentage enzyme activity of the sample with respect to the activity of the control (untreated sample).

$$\text{Residual activity}=\frac{\text{Activity of sample }\left(\text{U}\right)\times 100}{\text{Activity of Control }\left(\text{U}\right)}$$

4.2.4.6 Electrophoretic Techniques

Enzyme collected after ammonium sulphate precipitation and active fractions pooled after DEAE cellulose chromatography were electrophoresed by SDS-PAGE in a polyacrylamide gel following the method of Laemmlli (1970).

4.2.4.6.1 Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE)

The lyophilized active fractions of the enzyme were given a short spin and the supernatant was subjected to 12.0 % reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis. The protein was separated and analyzed using 5 % stacking gel and 12 % resolving gel prepared into 10 x 10.5 cm vertical gel plate of mini VE^{TM} mini vertical electrophoresis unit (Hoefer-Amersham, USA) at a constant current of 12mA. After electrophoresis, gels were stained with 0.025 % Coomassie brilliant blue stain R-250 and then destained in a solution of 5% methanol and 7% acetic acid. Molecular weight of unknown protein was determined by comparing with that of standards (PMWM- Genei, India).

4.2.4.6.2 Zymography

Zymography (substrate gel electrophoresis) was performed according to Bae *et al.* (1999) by soaking the gels first in 1% Triton X-100 for 1 hr at room temperature and then in 0.1 M sodium acetate buffer (pH 5.5) for 1 hr at 4°C. Phytase activity was detected by incubating the gels for 16 hrs in a 1 M sodium acetate buffer (pH 5.5) containing 0.42% (w/v) sodium phytate. Activity bands were visualized by immersing the gel in 2% (w/v) aqueous cobalt chloride solution. After 5 min incubation at room temperature the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of 6.25% (w/v) aqueous ammonium molybdate and 0.42% (w/v)

ammonium metavanadate. Phytase activity was evident as zones of clearing in an opaque background.

4.2.4.7 Effect of pH on Phytase Activity

Effect of pH on the phytase for maximal activity was determined over a pH range of 2.5 -10.5 using buffers of 1 M concentration: Glycine-HCl (2.5 - 3.5), Sodium acetate (4-7.5), Tris-Cl (8-9), Carbonate-bicarbonate (pH 9.5-10.5) for 30 min at 60°C. The substrate, sodium phytate was prepared in respective buffers and assayed as given in section 4.2.4.1 under standard conditions. The relative activities were measured as described in section 4.2.4.4.

4.2.4.8 Effect of pH on Phytase Stability

For the measurement of pH stability, the enzyme was incubated in different buffers for 1 hr at room temperature (25°C) and the residual phytase activity was determined under standard assay conditions as described in section 4.2.4.5. Untreated enzyme was taken as control (100% activity).

4.2.4.9 Effect of Temperature on Phytase Activity

Effect of temperature on phytase activity was tested by carrying out the assay at temperature ranges of 20, 30, 40, 50, 60, 70 and 80°C for 30 min in 1 M sodium acetate buffer (pH 5.5). The relative activities were measured as described in section 4.2.4.4.

4.2.4.10 Effect of Temperature on Phytase Stability

Effect of temperature on phytase stability was examined by incubating the enzyme at temperatures ranging from 20, 30, 40, 50, 60, 70 and 80°C for 1 hr in 1 M sodium acetate buffer (pH 5.5) and the residual phytase activity was calculated as described in section 4.2.4.5 under standard assay conditions. The non-heated enzyme was considered as the control (100% activity).

4.2.4.11 Effect of Surfactants on Phytase Activity

Surfactants such as PEG-6000, Tween- 80, SDS, Triton X-100 and EDTA (0.25% w/v).) were separately added to phytase screening medium. The medium without any surfactant served as control. Effect of these surfactants on phytase activity was measured as detailed in section 4.2.4.1.

4.2.4.12 Effect of Metal ions on Phytase Activity

The effect of various metal ions on the activity of phytase was investigated by including Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Hg^{2+}, Fe^{3+}, Cd^{2+} and Zn^{2+} (5 mM) in the assay mixture, and the relative activity was measured as detailed in section 4.2.4.4. Untreated enzyme was taken as the control (100% activity).

4.2.4.13 Effect of Oxidizing Agent on Phytase Activity

Activity of phytase in the presence of oxidizing agent, hydrogen peroxide (1% v/v H_2O_2, final concentration), was investigated by measuring the activity after 30 min incubation of the enzyme in H_2O_2. The relative activity was calculated as described in section 4.2.4.4.

4.2.4.14 Effect of Reducing Agents on Phytase Activity

The effect of reducing agents on phytase activity was investigated by incubating them in β-mercaptoethanol and cysteine hydrochloride (1% v/v) for 30 min and measuring the relative activities as detailed in section 4.2.4.4.

4.2.4.15 Enzyme Kinetics

Investigations on enzyme kinetics were conducted towards determining Km and *Vmax* values. *Km*, the substrate concentration at which the reaction velocity is half maximum and *Vmax*, the velocity maximum of the enzyme reaction, were determined by incubating the enzyme in the enzyme reaction

buffer containing different concentrations of sodium phytate (0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 mM) as substrate. The kinetic rate constants were determined using Lineweaver–Burk plot. Enzyme assay was carried out as described in section 4.2.4.1. The initial velocity data was plotted as the function of concentration of the substrate by the linear transformation of Michaelis-Menten equation and usual nonlinear curve fitting of Michaelis-Menten equation for calculation of *Km* and *Vmax* of the reaction.

4.2.4.16 Peptide Sequencing

MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time of Flight) of the purified enzyme was carried out at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram using AB SCIEX 3200 QTRAP® LC/MS/MS System for peptide sequence analysis. In-gel tryptic digested mixture was subjected to MALDI-TOF for peptide mass fingerprinting and peptide sequencing. The data thus obtained was searched against the different public databases such as Swiss-Prot/ MASCOT, RCSB Protein Data Bank.

4.2.4.17 Cytotoxicity Analysis of the Phytase Enzyme

Cytotoxicity assays are the *in vitro* bioassay methods used to predict toxicity of substances to various tissues or animals. Potential cytotoxic activity of phytase of *Bacillus* MCCB 242 was assayed in two different cell lines for evaluating the safety of the purified enzyme for its use in animal and fish feed.

4.2.4.17.1 Cell Lines Used

Two cell lines, Hep-2 (Human larynx epithelial cells) and RTG-2 (fish cell line derived from Rainbow trout gonad; *Oncorhynchus mykiss*) were employed in the cytotoxicity studies. Hep-2 was incorporated in the study to test the toxicity in humans so that phytase could be used in food industry and also to assess its toxicity while handling. RTG-2 was used to evaluate the application of

phytase in aquaculture. Cell lines were maintained in Eagle's MEM supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 10% FBS. Hep-2 was maintained at 37^{o}C while RTG-2 at 25^{o} C (Freshney, 2000).

Hep-2 and RTG - 2 were seeded separately in 96 well plates (Greiner Bio-One India Pvt. Ltd., Ahmedabad) at a cell density of 1x 10^{6} cells / well. Purified phytase dissolved in 1X PBS, pH 7.4 at concentrations 0, 1, 3, 5, 10, 25, 50,100, 250 and 500µg ml^{-1} (v/v) was added to the wells in triplicates. A control and solvent control were kept without the enzyme addition. MTT assay was performed after 24 and 48 hrs incubation and the percentage cytotoxicity at each concentration of phytase was calculated.

4.2.4.17.2 MTT Assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay measures the mitochondrial dehydrogenase which reflects the metabolic activity of viable cells. After incubation, the medium was replaced with 50µl MTT (Sigma-Aldrich Co.) having strength of 5 mg ml^{-1} in PBS (720mOsm) and incubated for 4 hrs in dark. The medium was removed and MTT-formazan crystals were dissolved in 100µl dimethylsulfoxide. Absorbance was recorded immediately at 570nm in microplate reader (TECAN Infinite Tm, Austria). Percentage of cytotoxicity was calculated as follows:

% Cytotoxicity= [1- (OD_{570} of treated cells/ OD_{570} of control cells)] x 100

4.2.4.17.3 Statistical Analysis

Data generated were analyzed using Two-way Analysis of Variance (ANOVA). Mean of the results was compared at a significance level of $p< 0.05$. Data are presented as mean ± standard deviation (SD).

4.3 Results

4.3.1 Purification of Phytase

In the present study, phytase was purified from an environmental isolate, *Bacillus* MCCB 242, employing standard protein purification procedures. The enzyme was purified by precipitation with ammonium sulphate followed by dialysis and ion exchange chromatography using DEAE-cellulose. Results of purification are summarized in Table 4.1.

Table 4.1: Yield and fold of purification of phytase produced by *Bacillus* MCCB242

Enzyme	Total Protein (mg)	Total activity (Units)	Specific activity (Units mg^{-1})	Yield of Protein %	Yield activity %	Fold purification
Crude	150	889.8	5.93	100	100	1
Ammonium sulfate precipitation	12.5	620.7	49.65	8.33	69.75	8.41
DEAE column chromatography	2.8	480.56	171.62	1.86	54.0	28.94

Ammonium sulphate fractions from 30-80 % showed phytase activity. Active fractions were pooled and concentrated by ultra filtration using 10 kDa membrane. The pooled fractions were then loaded on to DEAE-cellulose chromatography column. The enzyme was purified with 8.41 fold increase in specific phytase activity. A 28.94 fold purified enzyme was obtained after DEAE cellulose chromatography.

4.3.2 Characterization of Phytase

4.3.2.1 Standardization of Binding pH of Phytase to DEAE-Cellulose

The binding affinity increased from pH 3.0 and showed maximum at pH 5.5. The enzyme eluted from the DEAE cellulose and equilibrated to pH 5.5 showed maximal activities. Hence, the binding pH to DEAE cellulose was maintained as pH 5.5 (Fig. 4.1).

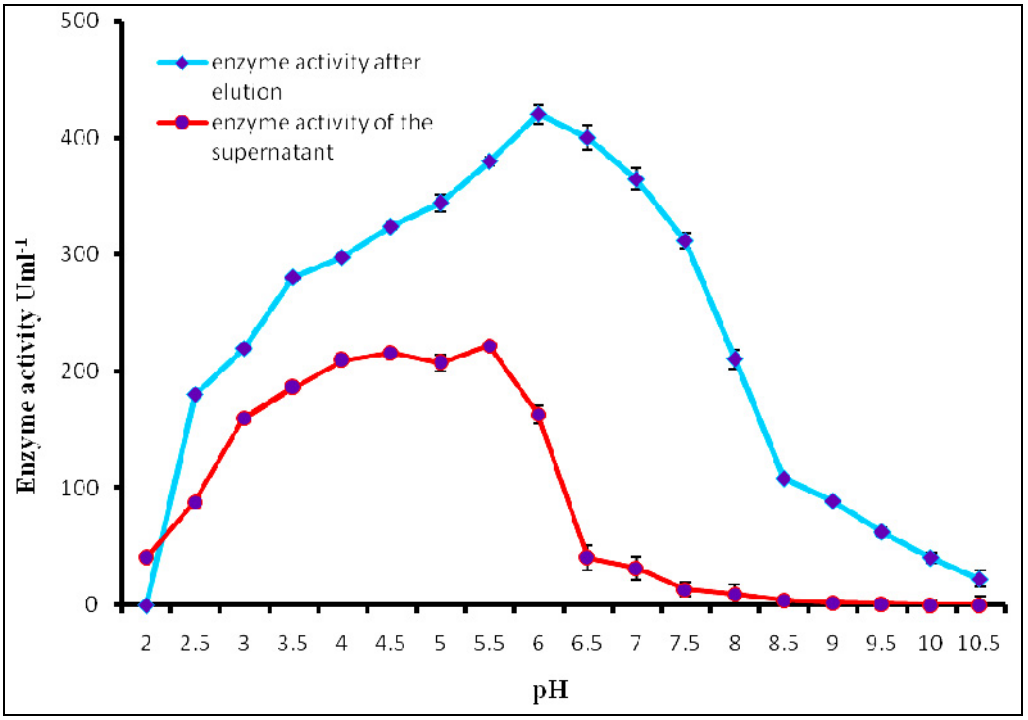


Fig. 4.1: Optimization of binding pH for binding of phytase to DEAE-Cellulose resin

4.3.2.2 Elution Profile of Phytase on DEAE-cellulose Column

The elution profile of phytase on DEAE-cellulose column is shown in Fig.4.2. The chromatogram of gel filtration on DEAE-cellulose showed two protein peaks. The first peak corresponded to the null volume. The phytase got eluted in the second peak between 0.41 M to 0.53 M NaCl (fractions, 41 to 53). These active fractions were pooled and concentrated for further studies. This step resulted in 54 .0 % recovery of phytase (28.94 fold of purification) with a specific activity of 171.62 U mg^{-1} protein (Table 4.1).

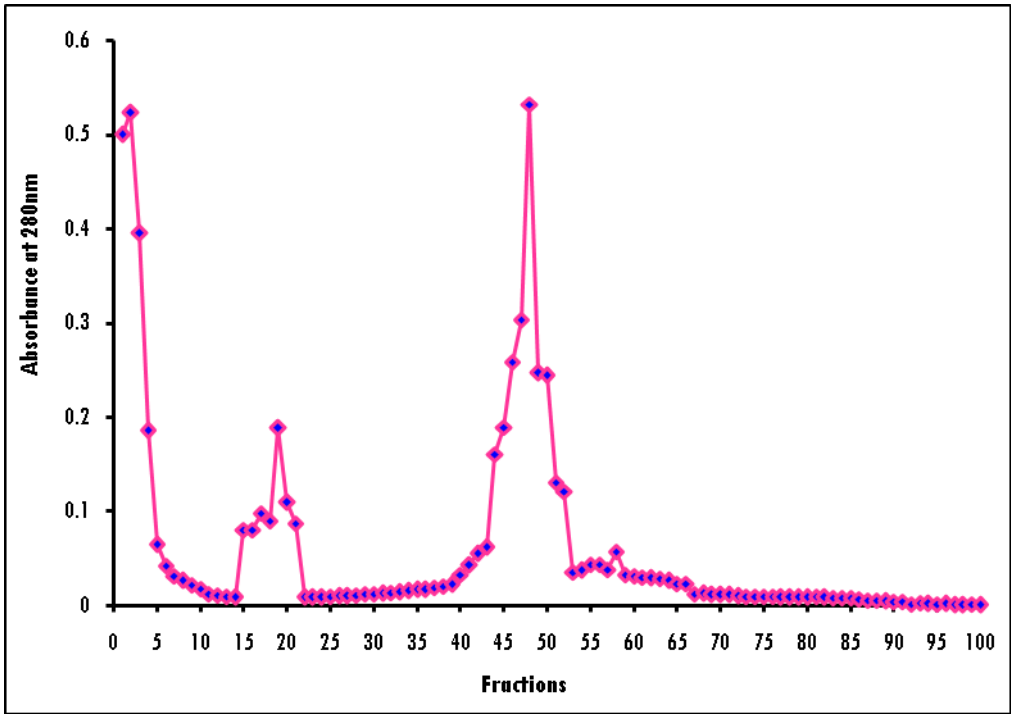
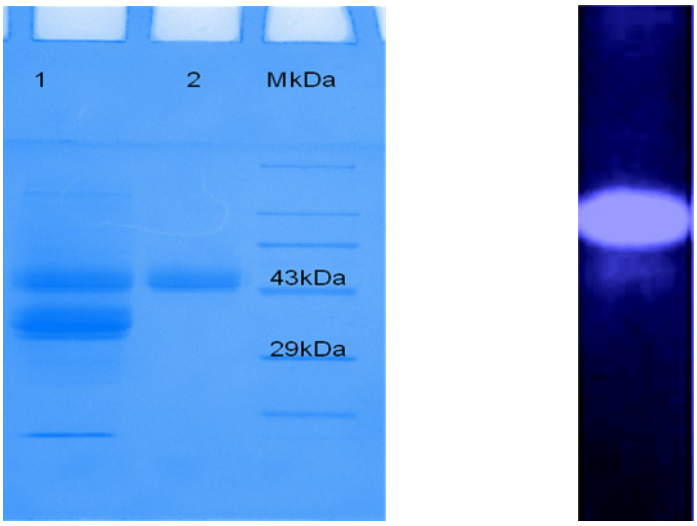


Fig.4.2: Elution profile of phytase through DEAE –cellulose C 16/40 column

4.3.2.3 Sodium dodecyl Sulphate-polyacrylamide gel Electrophoresis and Zymogram Analysis

SDS·PAGE of the purified enzyme yielded a single band suggesting that the purified phytase was homogeneous. The molecular mass of phytase was estimated by comparing with the electrophoretic mobility of marker proteins, which indicated that, *Bacillus* MCCB 242 phytase had an apparent molecular mass of approximately ~ 43 kDa (Fig. 4.3a). The zymogram (substrate gel electrophoresis) also revealed a single band of phytase activity corresponding to the band obtained in SDS-PAGE (Fig.4.3b).



(a) (b)

Fig.4.3: a) SDS–PAGE of phytase of *Bacillus* MCCB 242. Lane 1-Dialysis sample; Lane 2 - Purified sample, Lane 3 - molecular weight marker; b) Zymogram of phytase

4.3.2.4 Effect of pH on Phytase Activity

The effect of pH on phytase activity was determined using buffers in the pH range of 2.5-10.5. The percentage relative phytase activity at different pH is shown in Fig. 4.4. The enzyme was found exhibit phytase activity from pH 4.5 to7.5 with its optimum at 5.5.

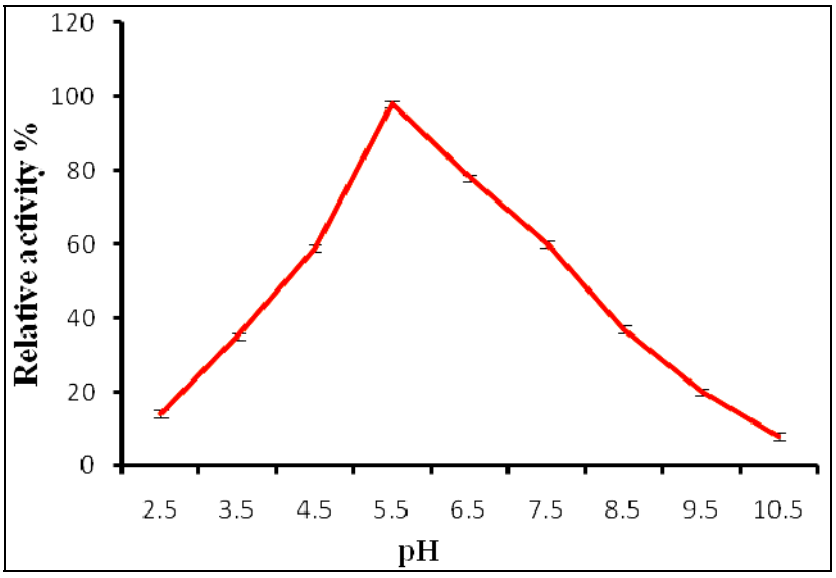


Fig. 4.4: Effect of pH on phytase activity

4.3.2.5 Effect of pH on Phytase Stability

Phytase from *Bacillus* MCCB 242 was found stable at pH 5.0 and 7.0 after pre-incubating for 30 min, whereas it gradually lost its activity at higher (>7.0) and lower pH (<4.0) (Fig. 4.5). Enzyme retained more than 90% residual phytase activity from pH 5.0-7.0.

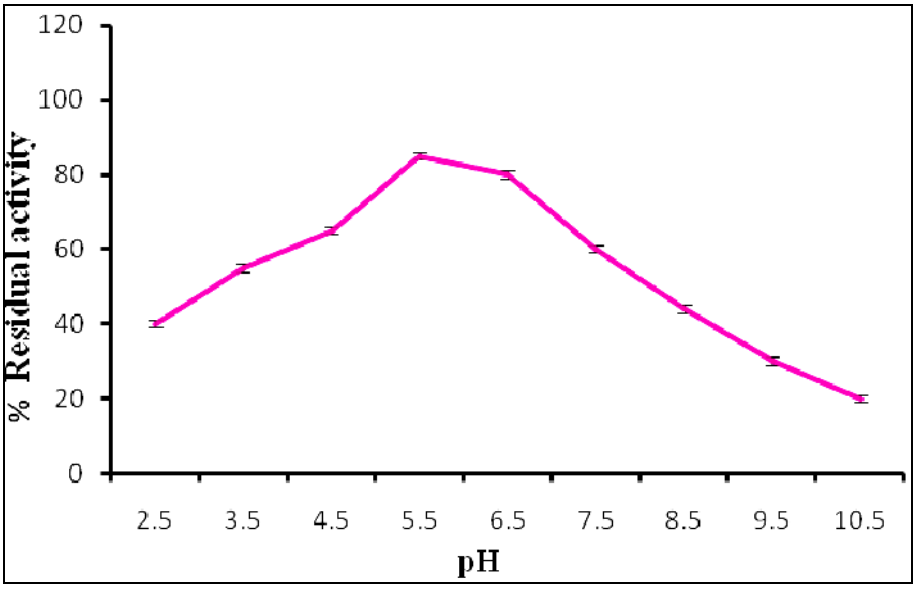


Fig. 4.5: Effect of pH on phytase stability

4.3.2.6 Effect of Temperature on Phytase Activity

Enzyme was active over a broad range of incubation temperature while recording maximal activity at 40°C.Temperatures above 50°C led to a sharp decline in enzyme activity (Fig. 4.6).

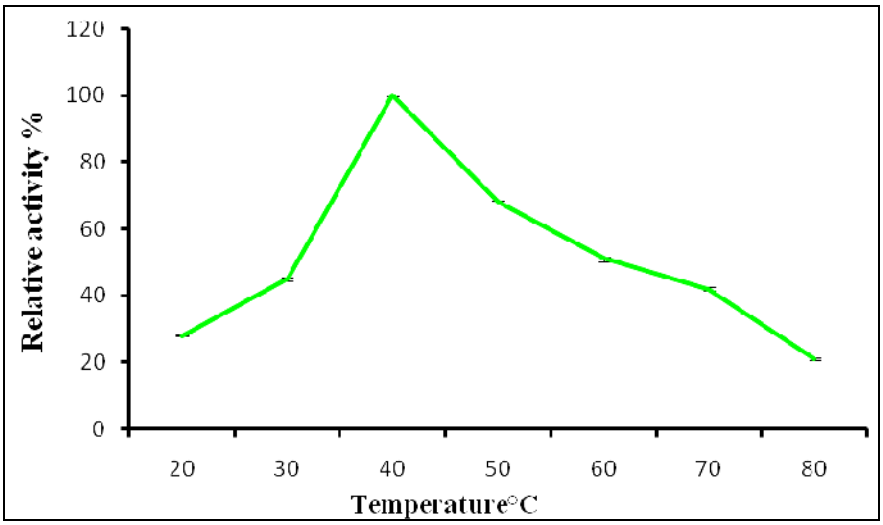


Fig. 4.6 Effect of temperature on phytase activity

4.3.2.7 Effect of Temperature on Phytase Stability

Phytase of *Bacillus* MCCB 242 showed stability over 20-40°C even after 48 hrs. At 40°C, the optimal temperature, the enzyme could retain 98 % phytase activity. Results suggest that at high temperatures, above 50°C, the enzyme loose stability within 30 min of incubation (Fig. 4.7).

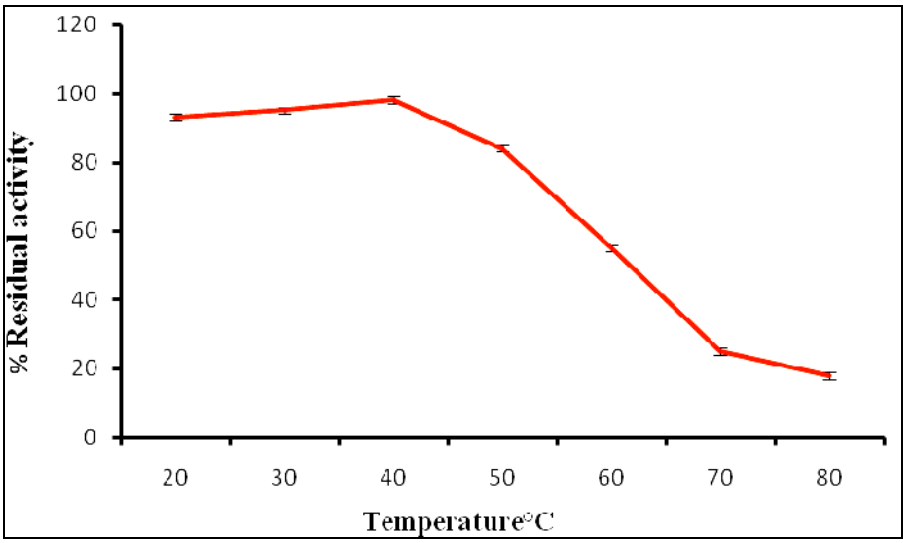


Fig. 4.7: Effect of temperature on phytase stability

4.3.2.8 Effect of Surfactants on Phytase Activity

In the present study PEG 6000, Tween 80 and Triton X-100 showed enhanced phytase production compared to control. Enhancement of 35 % was observed in phytase production with PEG. Sodium dodecyl sulphate (SDS) and EDTA inhibited phytase production by *Bacillus* MCCB 242 (Fig. 4.8).

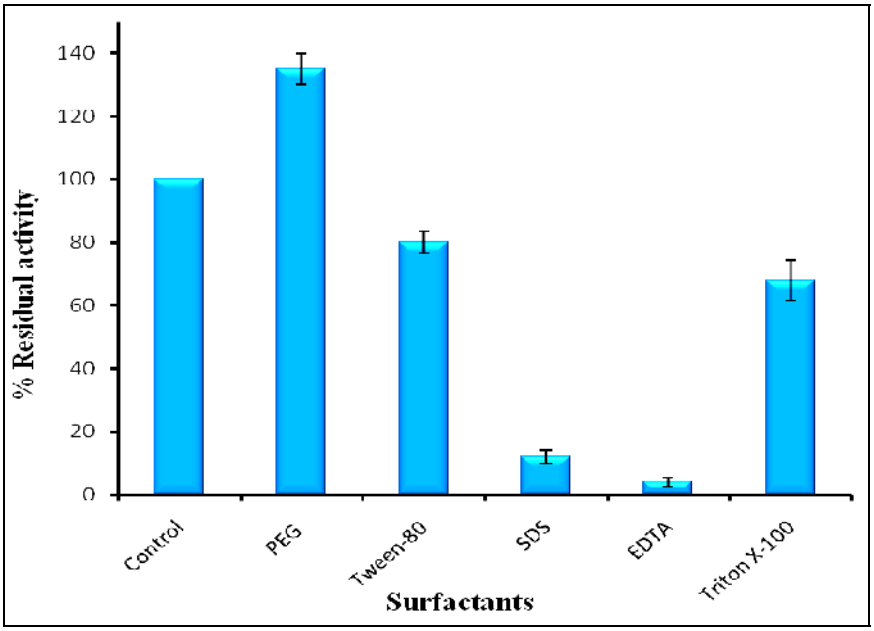


Fig.4.8: Effect of surfactants on phytase activity

4.3.2.9 Effect of Metal ions on Phytase Activity

Metal ions (Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Hg^{2+}, Fe^{3+}, Cd^{2+} and Zn^{2+}) were examined for their effects on phytase activity. Presence of metal ions such as Ca^{2+}, Fe^{3+} Mg^{2+} , Co^{2+} and Mn^{2+} enhanced the activity while heavy metals like Hg^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} inhibited phytase .Presence of Fe^{3+} ion enhanced phytase activity by 98% (Fig. 4.9).

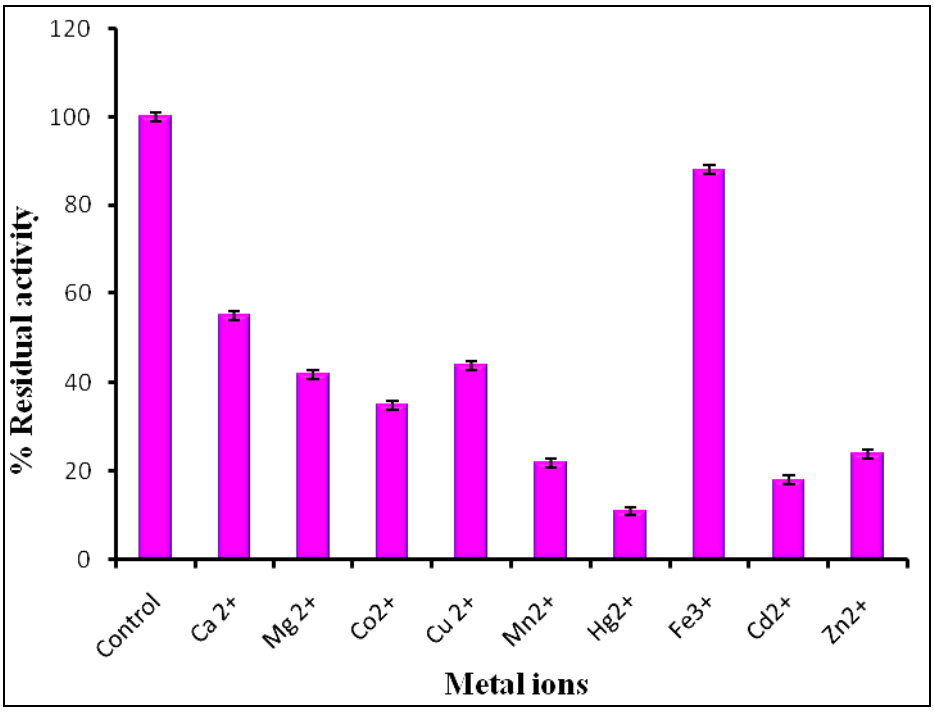


Fig.4.9 Effect of metal ions on phytase activity

4.3.2.10 Effect of Oxidizing Agent on Phytase Activity

Effect of oxidizing agent on phytase activity was investigated using H_2O_2. The enzyme retained 95% phytase activity in the presence of 1 % H_2O_2.

4.3.2.11 Effect of Reducing Agents on Phytase Activity

Effect of reducing agents on phytase activity was evaluated using β-mercaptoethanol and cysteine hydrochloride. β-mercaptoethanol increased the activity considerably whereas only 62% of residual activity was observed with cysteine hydrochloride (Table. 4.2).

Table 4.2: Relative activity of phytase with different reducing agents

Reducing agent	Concentration	(%)Residual activity
β -mercaptoethanol	1%	103
Cysteine hydrochloride	1%	62

4.3.2.12 Kinetic studies

For determination of enzyme kinetics, activity of purified phytase was assayed using sodium phytate. For determination of catalytic parameters, Lineweaver-Burke double reciprocal plots were generated. The kinetic parameters for the hydrolysis of phytate were determined to be *Km* = 182 µmol l^{-1}, *Vmax*= 220µmol min^{-1} mg^{-1} and Kcat = 180 sec^{-1} at pH 5.5 and 40°C (Table 4.3).

Table 4.3: Kinetic parameters of the purified phytase of *Bacillus* MCCB242

Km(µM)	Vmax(µmol $min^{-1}mg^{-1}$)	Kcat(s-1)
182 µmol l^{-1}	220µmolmin^{-1} mg^{-1}	180 sec^{-1}

4.3.2.13 Peptide Sequencing

The data obtained after MALDI TOF analysis was searched against the public database Swiss-Prot/ MASCOT. MALDI-TOF/TOF (MS/MS sequencing) result of *Bacillus* MCCB 242 is shown in Fig.4.10a-d. The protein summary report using the MATRIX SCIENCE MASCOT SEARCH (http://www. matrixscience.com) provided 98% similarity to the existing ones in the database, gi|530614676| ref| YP_008421337.1| phytase [*Bacillus amyloliquefaciens* subsp. plantarum UCMB5113]. The sequences obtained from the purified enzyme using the MALDI-TOF and LC-MS/MS analysis is given in Table 4.4. The *Bacillus* MCCB 242 amino acid sequence obtained was run in the programe developed by the Department of Genome Sciences, University of Washington (http://proteome. gs.washington.edu/cgi-bin/aa_calc.pl) to identify the number of each amino acid and the mono isotopic average mass (Table 4.5). The sequence obtained consists of 383 amino acids with mono isotopic mass of 41835.50956 and average mass

of 41861.3992. Composition of amino acids from *Bacillus* MCCB 242 is shown in Table 4.6.

Table 4.4: Protein sequences

```
         10         20         30         40         50         60
MNHSKTLLLT AAAGLMLTCG AVSSQAKHKL SDPYHFTVNA AAETEPVDTA GDAADDPAIW
        70         80         90        100        110        120
LDPKNPQNSK LITTNKKSGL VVYSLEGKML HSYPTGKLNN VDIRYDFPLN GKKVDIAAAS
       130        140        150        160        170        180
NRSEGKNTIE IYAIDGKNGT LQSITDPDRP IASAIDEVYG FSLYHSQKTG KYYAMVTGKE
       190        200        210        220        230        240
GEFEQYELNA DKNGYISGKK VRAFKMNSQT EGMATDDEYG SLYIAEEDEA IWKFSAEPDG
       250        260        270        280        290        300
GSNGTVIDRA DGRHLTPDIE GLTIYYAADG KGYLLASSQG NSSYAIYERQ GQNKYVADFQ
       310        320        330        340        350        360
ITDGPETDGT SDTDGIDVLG FGLGPEYPFG LFVAQDGENI DHDQKANQNF KMVPWERIAD
       370        380
KIGFHPQVNK QVDPRKLTDR SGK
```

Table 4.5: Amino acid calculation results of *Bacillus* MCCB 242

Amino Acid Sequence				
MNHSKTLLLTAAAGLMLTCGAVSSQAKHKLSDPYHFTVNAAAETEPVDTAGDAADDPAIWLDPKN PQNSKLITTNKKSGLVVYSLEGKMLHSYPTGKLNNVDIRYDFPLNGKKVDIAAASNRSEGKNTIEIY AIDGKNGTLQSITDPDRPIASAIDEVYGFSLYHSQKTGKYYAMVTGKEGEFEQYELNADKNGYISG KKVRAFKMNSQTEGMATDDEYGSLYIAEEDEAIWKFSAEPDGGSNGTVIDRADGRHLTPDIEGLTI YYAADGKGYLLASSQGNSSYAIYERQGQNKYVADFQITDGPETDGTSDTDGIDVLGFGLGPEYPFG LFVAQDGENIDHDQKANQNFKMVPWERIADKIGFHPQVNKQVDPRKLTDRSGK				
C-Terminal Group: Free Acid		Average Mass: 41861.3992		Sequence Length: 383
C-Terminal Group: Free Acid		Average Mass: 41861.3992		
Symbols	**Name**	**Mono Mass**	**Average Mass**	**Count**
Ala A	Alanine	71.03711	71.07880	34
Arg R	Arginine	156.10111	156.1876	10
Asn N	Asparginine	114.04293	114.1039	21
Asp D	Aspartic Acid	115.02694	115.0886	34
Cys C	Cysteine	103.00919	103.1448	1
Glu E	Glutamic Acid	129.04259	129.1155	22
Gln Q	Glutamine	128.05858	128.1308	15
Gly G	Glycine	57.02146	57.0520	37
His H	Histidine	137.05891	137.1412	8
Ile I	Isoleucine	113.08406	113.1595	22
Leu L	Leucine	113.08406	113.1595	25
Lys K	Lysine	128.09496	128.1742	29
Met M	Methionine	131.04049	131.1986	7
Phe F	Phenylalanine	147.06841	147.1766	12

Pro P	Proline	97.05276	97.1167	17
Ser S	Serine	87.03203	87.0782	25
Thr T	Threonine	101.04768	101.1051	24
Trp W	Tryptophan	186.07931	186.2133	3
Tyr Y	Tyrosine	163.06333	163.1760	20
Val V	Valine	99.06841	99.1326	17

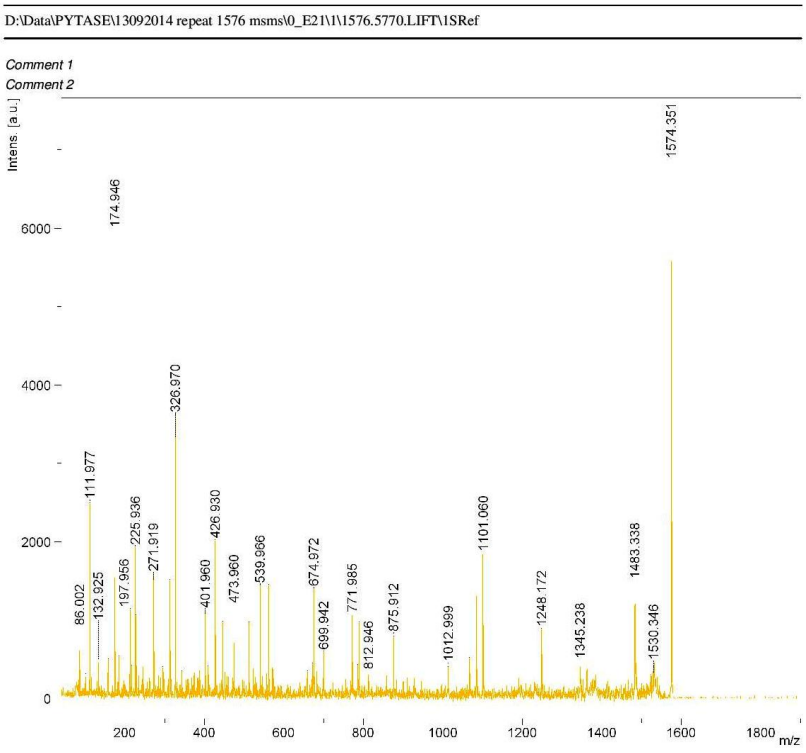
Table 4.6: Amino acid composition of *Bacillus* MCCB 242 phytase protein sequences

Amino acid composition (%)	
Ala (A	8.9%
Arg (R)	2.6%
Asn (N)	5.5%
Asp (D)	8.9%
Cys (C)	0.3%
Gln (Q)	3.9%
Glu (E)	5.7%
Gly (G)	9.7%
His (H)	2.1%
Ile (I)	5.7%
Leu (L)	6.5%
Lys (K)	7.6%
Met (M)	1.8%
Phe (F)	3.1%
Pro (P)	4.4%
Ser (S)	6.5%
Thr (T)	6.3%
Trp (W)	0.8%
Tyr (Y)	5.2%
Val (V)	4.4%
Pyl (O)	0.0%
Sec (U)	0.0%

4.3.2.13.1 Protein Feature View

The Protein Feature View of protein data bank combines annotations from various data sources and provides a graphical summary of any annotations that can be mapped onto the protein sequence or 3D structure. The sequence when searched in RCSB Protein Data Bank, a repository for the three-dimensional structural data of large biological molecules such as proteins and nucleic acids confirmed that it belonged to *Bacillus* species under the gene name phy with 383 protein sequences. Protein feature view of *Bacillus* MCCB 242 mapped to a UniProtKB sequence is shown in Fig.4.11.The main reference (gray track) is the full-length sequence from UniProt. The top of the view (green) provides a summary of important functional motifs plus UniProt sites. By moving the mouse over any of these regions, more information about this region can be viewed. This is followed by annotations from Pfam (yellow), SCOP domain annotations (orange), computationally inferred information, such as protein disorder score or hydropathy. At the bottom, in peach and blue, are the data derived from PDB. A secondary structure track shows helical and strand regions along the protein. In the 'condensed' (default) view representative protein chains are being displayed mapped to the protein sequence. To view the mapping of all available PDB chains the 'expanded' view can be called by pressing the '+' button.

Protein feature view also add functional context, including domain organization, oligomeric structure and catalytic activity. It clearly reveals that the enzyme is class 3-phytase and its catalytic activity is Myo-inositol hexakisphosphate + H_2O = 1D-myo-inositol 1,2,4,5,6-pentakisphosphate + phosphate).The protein model was built up using the Swiss model (swissmodel.expasy.org) and PyMOL (Fig.4.12 a, b).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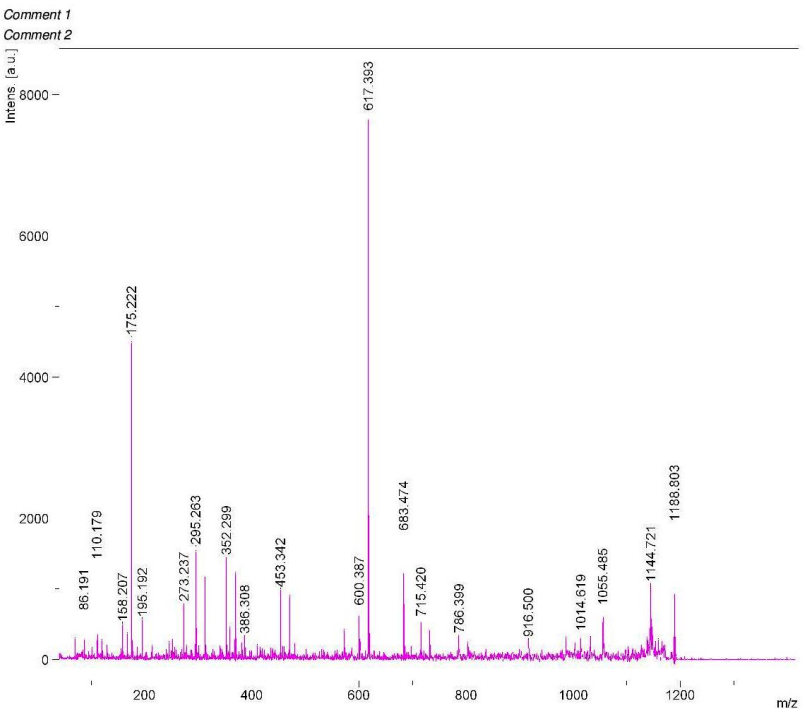
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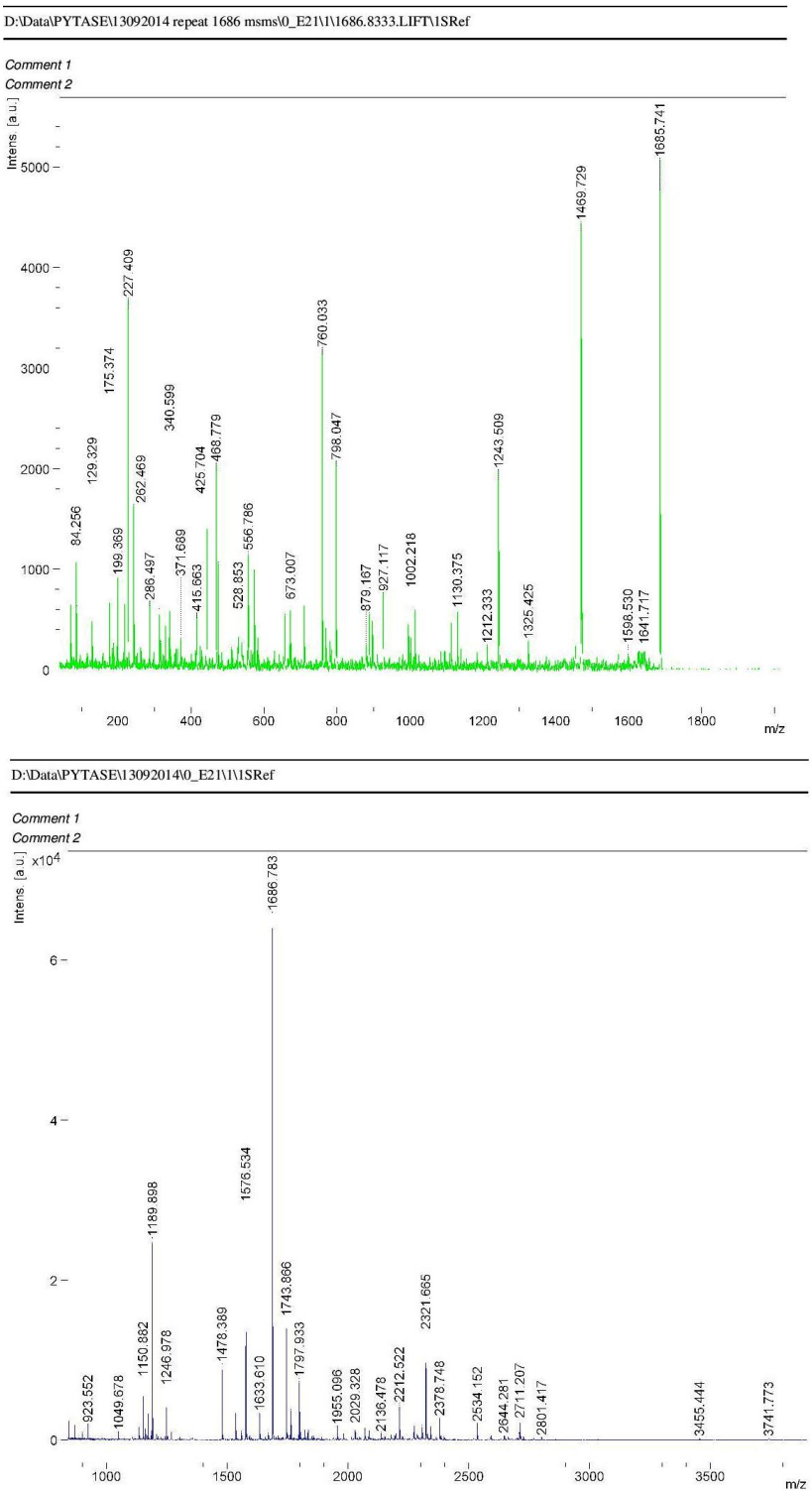


Fig. 4.10 a-d: MALDI-TOF analysis of peptides after in gel tryptic digestion of *Bacillus* MCCB 242

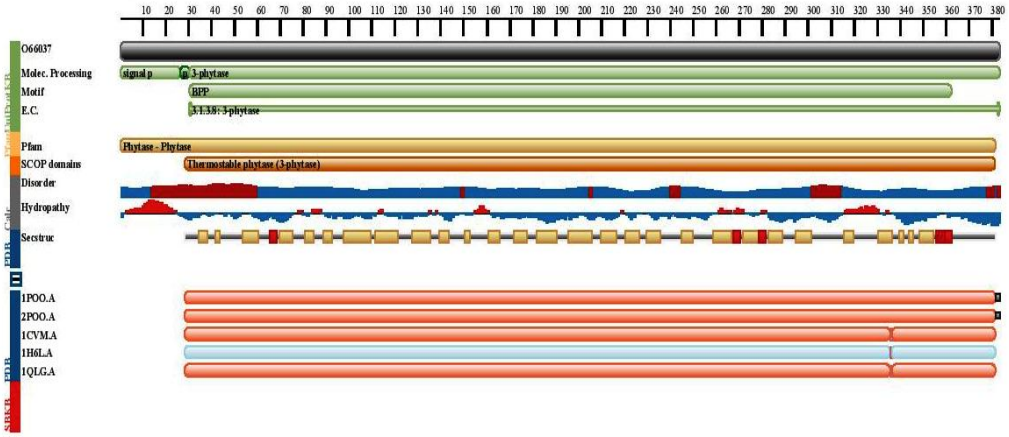
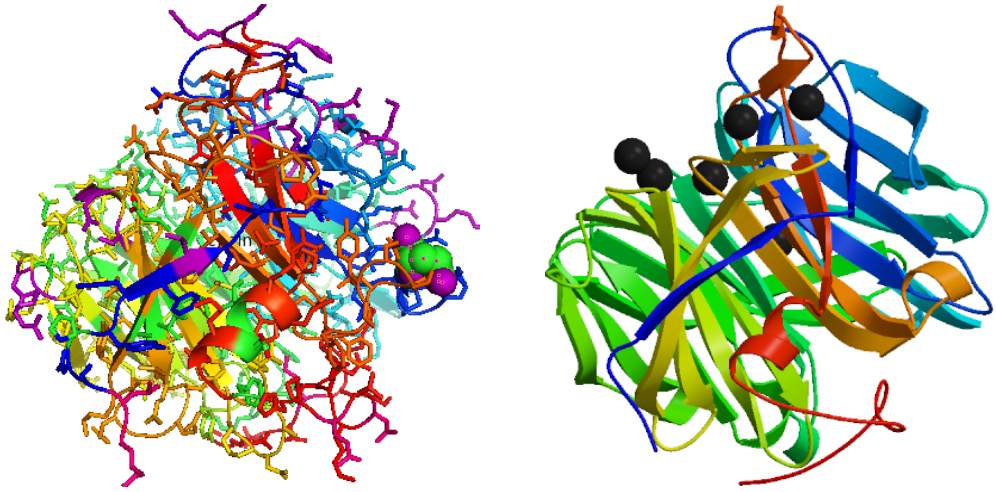


Fig. 4.11: Protein feature view of *Bacillus* MCCB 242 phytase mapped to a UniProtKB sequence



a) **b)**

Fig.4.12 a, b: Structural model of *Bacillus* MCCB 242 phytase created using PyMOL and SWISS-MODEL server

4.3.2.14 Cytotoxicity of purified phytase

The cytotoxicity of *Bacillus* MCCB 242 phytase on Hep-2 cells were studied at different concentrations of enzyme in the range 0, 1, 3, 5, 10, 25, 50,100, 250 and 500µg ml^{-1} enzyme for 24hrs and 48hrs. At concentrations from 1 – 250

µg ml^{-1}, it did not cause any significant change in the cell morphology and the toxicity was undetectable. Results showed that that there was a slight increase in toxicity with increasing concentration. Cytotoxic effects such as cell rounding and cell death were noted partially at 500µg ml^{-1} (v/v) of enzyme. (Fig.4.13 and Fig.4.14).

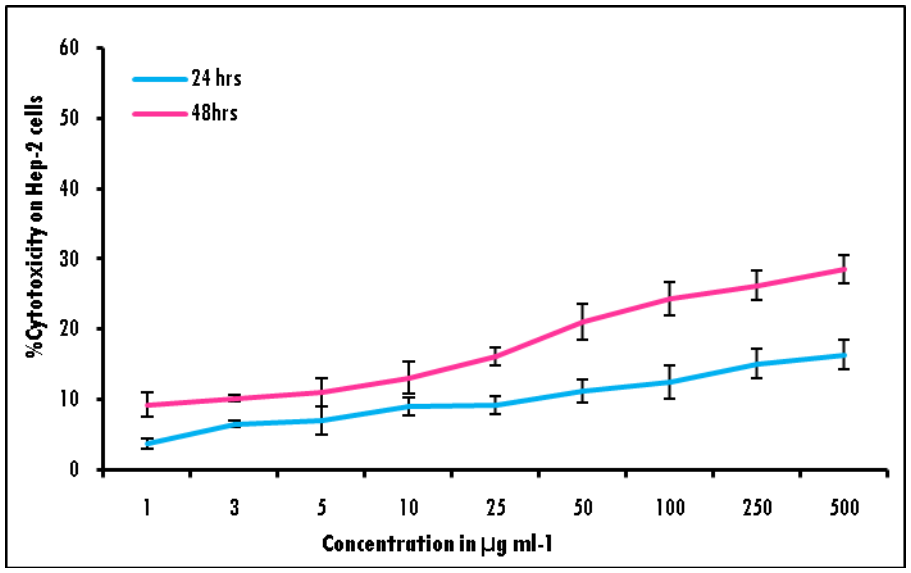
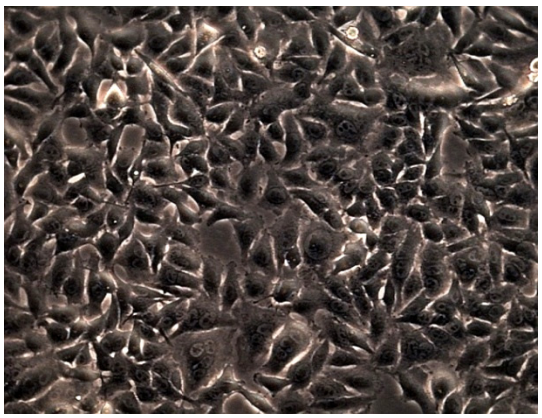
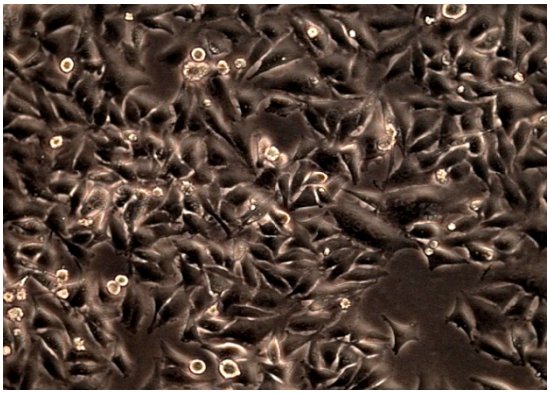


Fig.4.13: Sigmoid curve for cytotoxicity analysis of *Bacillus MCCB 242 phytase* on Hep-2 cells after 24 and 48hrs of incubation



(a)



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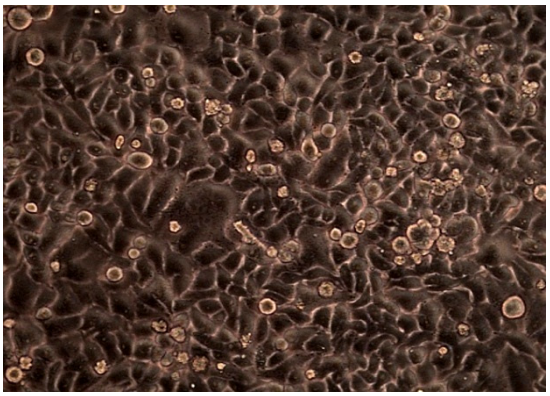


(c)

Fig.4.14 a,b,c: Cytotoxic effects of purified *Bacillus* MCCB 242 phytase on Hep-2 cells
a) Control showing Hep - 2 monolayer,
b) 500µg ml^{-1} phytase showing cell rounding (24hrs),
c) 500µg ml^{-1} phytase showing cell rounding (48hrs)

RTG-2 cell line was exposed to phytase for fixed time intervals of 24 hrs and 48hrs. In RTG-2 cells, *Bacillus* MCCB 242 phytase did not show cell rounding and cell death even at higher concentration of 500µg ml^{-1} (Fig.4.15 and Fig.4.16). The results clearly indicated that phytase did not have toxic effect on RTG-2 cell lines, suggesting that it could be used for supplementing fish feed to enhance nutrition and combating environmental phosphorus pollution.

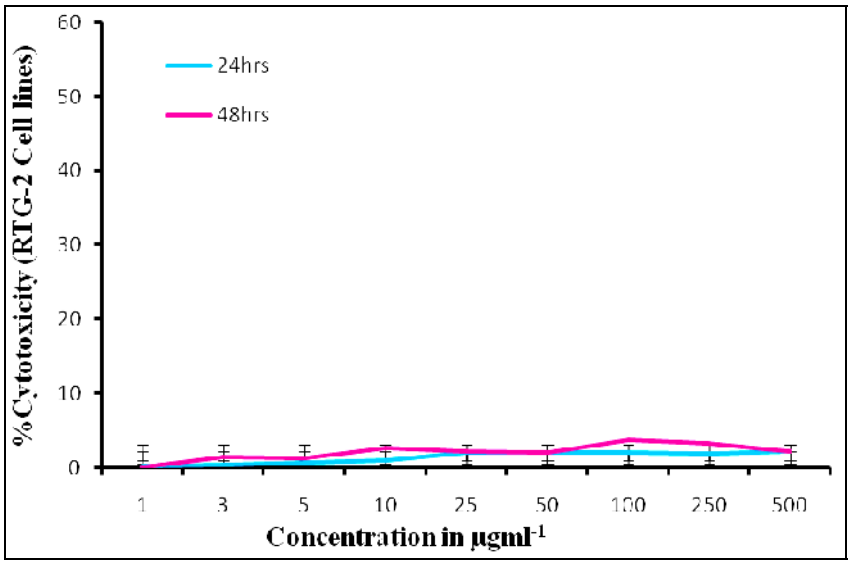
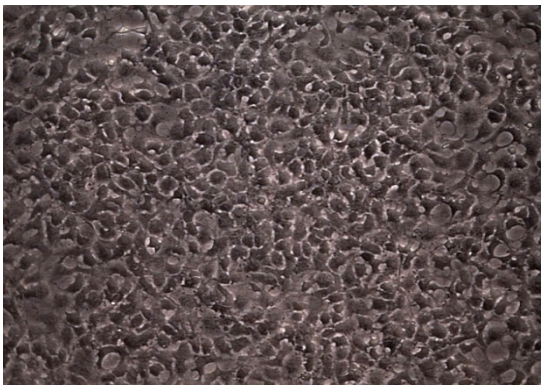
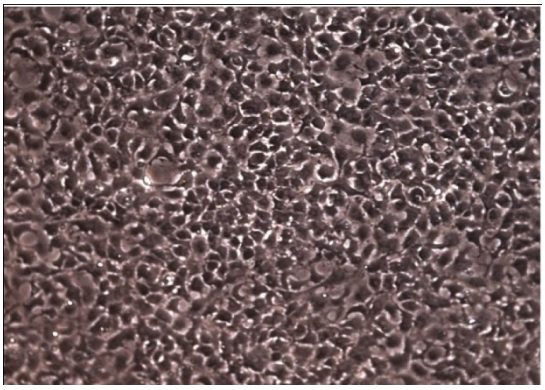


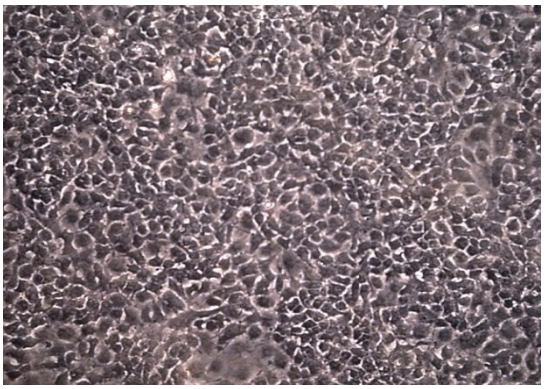
Fig. 4.15: Sigmoid curve for cytotoxicity analysis of *Bacillus MCCB 242 phytase* on RTG-2 cells after 24 and 48hrs of incubation



(a)



(b)



(c)

Fig.4.16 a,b,c: Cytotoxic effects of purified *Bacillus* MCCB 242 phytase on RTG-2 cells. a) Control showing RTG-2monolayer, b) cells after 24hrs, c) cells after 48hrs

4.4 Discussion

Bacterial phytases belong to the group of histidine acid phosphatases, cysteine phosphatases or β-propeller phytases (Hill *et al.,* 2007; Puhl *et al.,* 2008, 2009; Yanke *et al.,* 1999). Each phytase possesses unique properties, such as pH and temperature optima and resistance to heat, proteolysis, acidity, and metals. Kerovuo *et al.,* 1998 were the first to report on enzyme characterization on a phytase of *Bacillus* origin. Phytase have gained attention on cloning and purification shifted to the enzymes originated from *Bacillus* species. Till date only few *Bacillus* phytases were characterized in detail.

Generally, the phytases from bacterial source have optimum pH in neutral to alkaline range while in fungi, optimum pH range is 2.5-6.0 and the stability of phytase decreased dramatically above pH 7.5 and below pH 3.0. This wide range of differences in pH optima could be due to the variation in molecular conformation or stereo-specificity of the protein from different sources. *Bacillus* MCCB 242 showed little activity at acidic pH and is highly active at pH5.5. Reports showed that phytase from *Bacillus* sp. KHU 10 showed maximum activity between pH 6.0 and 8.0 (Choi *et al.,* 2001), *Bacillus*

licheniformis (168 phyA, phyL) had an optimum pH between 4.5-6.0 (Tye *et al.,* 2002), while enzyme from *Bacillus* sp. MD2 was optimally active between pH 6 -7 (Tran *et al.,* 2010). Temperature optimum of most phytases varies from 35 to 80°C. *Bacillus* MCCB 242 fits into this range with two temperature optima at 37°C and 55°C. After incubation for 30 minutes at 40°C, *Bacillus* MCCB 242 phytase retained close to 100% of their initial activity. Purified phytase showed maximum activity at 40ºC with 98% residual activity.

Conventional purification methods used in this study were found to be effective to purify phytase. The enzyme was purified using 80% ammonium sulphate. Also, a single phytase activity peak was found in the elution profile during ion-exchange chromatography on DEAE cellulose.

Purified phytase of *Bacillus* MCCB 242 was resolved on standard Laemmli gels, renatured and incubated with sodium phytate *in situ*. The phytate impregnated gels were exposed to the counterstaining reagent (cobalt chloride, ammonium molybdate and ammonium vanadate). Zone of clearing were seen in the region of active phytase protein bands, in agreeable to the result of Bae *et al.* (1999). Molecular weight of bacterial phytases is reported to be about 42 kDa. In this study phytase from *Bacillus* MCCB 242 was purified by about 28.94 fold to apparent homogeneity with a recovery of 54% referred to the phytase activity in the crude extract and the monomeric enzyme displayed molecular weight of 43KDa. This is in agreement with earlier reports on the purified phytase from *Bacillus subtilis*, *Bacillus subtilis* (natto) N-77, *Bacillus amyloliquefaciens*, *Bacillus* sp. KHU-10 which had an estimated molecular weight of 44, 33.8, 36, and 44 KDa (Powar and Jagannathan, 1982; Shimizu, 1992; Greiner *et al.,* 1993; Kim *et al.,* 1998; Sajidan 2004) respectively. The purified enzyme from *Bacillus* sp. MD2 had a molecular weight of 47.5 KDa (Tran *et al.,* 2010).

Phytase characteristics and potential uses were reviewed and described by Fu *et al.* (2008). *Bacillus* β-propeller phytase is the only known microbial phytase that needs metal ions, and removal of Ca^{2+}ions causes inactivation of the enzyme (Kerovuo *et al.,* 2000b; Simon and Igbasan, 2002). Metal ions play an important role in the catalytic activity of the enzymes and most of the enzymes are dependent on metal ions for maximum catalytic activity. In this study activity was unaffected or moderately stimulated by a range of metal ions. Fe^{3+} and Ca^{2+} exerted stimulatory effect on phytase production. This suggests that the enzyme requires metal ions for its activity similar to most alkaline phytases which require Ca^{2+} (Kim *et al.,* 1998b).

Addition of the chelating agent, EDTA, and SDS (detergent) did not affect the activity, while in the presence of surfactants like polyethylene glycol, Tween-80 and TritonX-100 the enzyme activity was stimulated by 135%, 80% and 68% respectively. PEG 6000 supported enhanced phytase production in the fermented medium compared to control medium. There was about 35% enhancement in phytase production with PEG 6000 compared to control. The detergents not only ease the oil drop dispersion but also attach to protein and induce structural changes that appear to be stimulatory in case of non ionic (Tweens) and inhibitory in the case of anionic (SDS) detergents. Oxidizing agent (H_2O_2) and reducing agents (β-mercaptoethanol and cysteine hydrochloride) had positive effect on *Bacillus* MCCB 242 phytase suggesting good stability.

Results of determination of kinetics, *Km* and *Vmax* values, agree with the results obtained by Greiner *et al.* (1997); Kerovuo *et al.* (1998) and Sajidan (2004). The *Vmax* with sodium acetate buffer and different sodium phytate concentrations as substrates were slightly lower.

Peptide analysis by Mass Spectrometry (MS) MALDI-TOF to those in the protein data bank suggested that there was only one protein. Based on the MALDI– LC–MS/MS identified amino acid sequences of the peptides, *Bacillus* MCCB 242 phytase showed homology with other known phytases. The protein feature view of *Bacillus* MCCB 242 showed that the purified *Bacillus* MCCB 242 phytase was 3- phytase with 98% similarity to other sequences in the data bank. The protein was found to have 383 amino acid residues.

Potential cytotoxic analysis of purified *Bacillus* MCCB 242 phytase in human and fish cell lines favoured its use in feed applications to fishes.

The enzyme characterization indicated that *Bacillus* MCCB 242 phytase had several features satisfying its use in industrial applications. The chemical, physical, and enzymatic characteristics of the purified *Bacillus* MCCB 242 phytase as well as the requirement for calcium and the inhibition by EDTA suggested that the phytase was similar to the one produced by *Bacillus subtilis* (natto) N77 described by Shimizu. (1992) and *Bacillus subtilis* described by Powar and Jagannathan. (1982). It could be assumed that the purified phytase from *Bacillus* MCCB 242 were 98% homologous to that from the reported one by Kerovuo *et al*. (1998), suggesting that the purified enzyme derived from GRAS classified microorganism (*Bacillus*), could be used as feed additive

The extracellular phytase from *Bacillus* MCCB 242 was purified to homogeneity. *Bacillus* MCCB 242 phytase has pH optimum of 5.5, a significant feature, since the enzyme should function in the small intestine of monogastrics (pH 4.5-6.5), where phosphate absorption occurs. Most important feature of any feed enzyme is it's temperature optimum as the enzyme will be exposed to higher temperature during feed pelleting. *Bacillus* MCCB 242 phytase was able to recover 55% of its activity after getting exposed to 60^{o}C for 30 min suggesting its successful application as feed additive.

While evaluating the safety of *Bacillus* MCCB 242 phytase for its use as animal and fish feed additive, it could be demonstrated that it was safe to be applied as feed additive to fishes as it did not cause any toxicity to fish cell line. So far no report has been documented on the cytotoxicity of phytase from an environmental isolate of *Bacillus*, especially in human and fish cell lines.

Based on the results *Bacillus* MCCB 242 phytase is recommended for application as feed additive to fishes after accomplishing *in vivo* studies under laboratory and field conditions.

Chapter 5

DOWNSTREAM PROCESS FOR *BACILLUS* MCCB 242 PHYTASE

Contents

5.1 Introduction

Phytase is used as an important feed additive as it increases the availability of organic phosphate and other nutritionally important minerals for monogastrics by the hydrolysis of phytic acid (Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004; Singh and Satyanarayana, 2006; Bala *et al.,* 2014). In addition, they produce different myo-inositol phosphates that have novel metabolic effects, such as amelioration of heart disease by controlling hypercholesterolemia and atherosclerosis, prevention of renal stone formation, and reduced risk of colon cancer (Koenietzny and Grenier, 2002). The rapidly growing global market for animal feed enzymes is mainly attributed to increased use of exogenous phytases by the feed industry (van Beilen and Li, 2002; Iyer and Ananthanarayan, 2008; Sulabo *et al.,* 2011). It has been reported that 10kg dicalcium phosphate can be replaced by 0.25kg phytase and the worldwide demand for phytase in cattle feed is approximately 4000 tonnes/annum.

Considering the heightened use and demand of phytase more efforts are needed to produce cost effective phytase with fast and economic downstream processing. Downstream processing plays an essential step in the separation of enzymes for its intended use in a commercial scale**.** The main objective for

downstream processing is to minimize the number of unit operations involved in the process, thus reducing overall process and validation costs, simplifying ease and economy of process automation (Brar *et al.,* 2006). The complexity of downstream processes is determined by the required purity and applications of the product. Various stages of processing that occur after completion of fermentation includes separation, purification and packaging of the product.

Employing *Bacillus* MCCB 242, 688±3.21Uml^{-1} phytase could be produced through optimization using response surface methodology and subsequent up-scaling. Phytase production was enhanced by 1.433 times from that by shake flask mode. Downstream processing plays an integral role in enzyme purification and recovery as the cost of the product mostly depends on the cost incurred during extraction and purification. To satisfy the need for efficient, easier, scalable and economically feasible process for phytase production, Aqueous Two-Phase Extraction (ATPE) technology was adopted in this study where clarification, concentration, and partial purification could be incorporated in just one-step. An Aqueous Two-Phase System (ATPS) is formed by the addition of two water-soluble polymers or a polymer and salt to aqueous medium above their significant concentrations. The solution separates into two immiscible phases, and each dissolved component predominates in one or the other phase with water as solvent in both phases.

ATPSs are of two types, Polymer-polymer and Polymer-salt. Among the polymer/polymer systems, the extensively studied one is polyethylene glycol/dextran (PEG/DX) combination. Among the polymer/salt systems, the most popular one is polyethylene glycol/potassium phosphate. For industrial applications polymer/salt systems are preferred over polymer/polymer systems because of the larger droplet size, easier preparation, less time for separation and better selectivity for protein extraction. In addition, recovery of the proteins

from the salt phase can be easily accomplished by simple dialysis. However, phosphate/ sulphate/salts may lead to high phosphate concentration in effluent streams, and are of environmental concern. To reduce the amount of salt discharged into the wastewater these inorganic salts are substituted by citrate which are non-toxic and hence can be discharged directly. In this study poly ethylene glycol / sodium citrate system along with neutral salt such as NaCl was used. NaCl improves the hydrophobic resolution of the system and generates an electrical potential difference between two phases. Factors that affect the formation of aqueous two phase systems include concentration of the polymer, molecular weight, hydrophobicity, type and concentration of salts and temperature. Physical properties such as density, viscosity and interfacial tension also affect aqueous two-phase systems. These determine the phase separation rate and partitioning of bio-molecules (Raghavarao *et al.,* 1995).

Partitioning in Aqueous Two-Phase Systems (ATPS) is a powerful versatile downstream process and has great potential for large scale continuous separation of proteins and removal of contaminants from fermentation broths, since they produce an initial purification rapidly (Hart and Bailey, 1991). ATPS also finds applications in many other fields namely, extraction and purification of intracellular and membrane proteins, concentration and purification of viruses, nucleic acids and plant proteins, partitioning and separation of microbial cells as well as animal cells. In food industry, for the clarification of cheddar whey and isolation of high phytin containing particles from rice bran, ATPS is used.

Purification by aqueous two phase extraction technique is considered superior to any other process as it can be used commercially at low cost. In this study, a simple and cost effective method for large-scale purification and

recovery of phytase was attempted hitherto not reported for recovery and purification of *Bacillus* phytase.

5.2 Materials and Methods

5.2.1 Materials

Poly ethylene glycol with molecular weight of 6000 was used as the polymer and the salt used for the study was sodium citrate. These chemicals were procured from Sigma-Aldrich. All other chemicals were of analytical grade and Millipore water was used in all experiments.

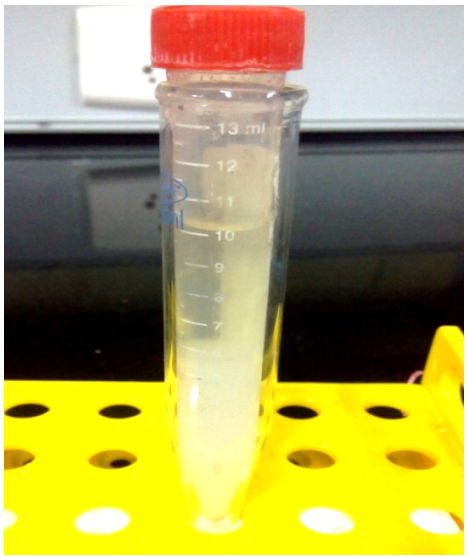
5.2.1.1 Preparation of Aqueous two Phase system

The Aqueous Two-Phase Systems (ATPS) were prepared on a w/w% basis by mixing the required quantities of phase forming solutes in crude enzyme extract. ATPS consisting of 12% (w/w) polymer, polyethylene glycol (PEG) and 15.2 % (w/w) salt (sodium citrate) were taken in 15ml graduated conical tubes and mixed with 1 ml crude phytase (Fig.5.1a). Millipore water was added to obtain 5g of the final weight. pH was set using NaOH or HCl. The contents were mixed thoroughly for 1hr using magnetic stirrer for equilibration and was allowed to stand for phase separation. Sodium chloride (2%) was added to give the required ionic strength and to increase the hydrophobicity of ATPS. After clear phase separation, the top and bottom phases were collected and analyzed for protein concentration and enzyme. All experiments were performed at room temperature in triplicate. Large-volume experiments were conducted using 100 g ATPS in a 1000 ml separating funnel (Fig.5.1b).

5.2.2 Phytase Production

Bacillus MCCB 242 was grown in the newly designed modified phytase screening medium having the composition as described in Chapter - 3 (Table: 3.13) in a 5L fermentor (Biostat-B-Lite, Sartorius, Germany). Fermentation was

carried out at 25°C, pH 5.5 ± 0.05, 120 rpm and supplied with sterile air at the rate 2.5 l min^{-1} (Fig.5.2). For enzyme extraction the culture was centrifuged at 10000 rpm for 15 min at 4°C and the supernatant stored in 300 ml aliquots at -20°C, and used for further purification and characterization.



(a)

(b)

Fig.5.1 a-b: a) Microscale experimental setup for aqueous two phase extraction of *Bacillus* MCCB 242 phytase; b) Macroscale experimental setup for aqueous two phase extraction of *Bacillus* MCCB 242 phytase in 1L separating funnel.

Fig. 5.2: Production of *Bacillus* MCCB 242 phytase in 5L bench top laboratory scale fermentor

5.2.3 Phytase Assay

Volumes of top and bottom phases were measured and were assayed for phytase activity and protein as detailed in section 4.2.4.1 of Chapter 4.

5.2.4 Protein Assay

Quantification of protein was carried out according to Lowry *et al.* (1951) as detailed in section 2.2.8 of Chapter 2.

5.2.5 Partition Parameters

The partition coefficient, purification factor, activity recovery and phase volume ratios were calculated as described by Bhavsar *et al.* (2012).

5.2.5.1 Partition Coefficient

Partition coefficient (*Ke*) for phytase activity in the aqueous two-phase system was defined as the activity of the enzyme in the crude extract (A_i) to that in the bottom phase (A_b).

$$K_e = \frac{A_b}{A_i}$$

Since proteins were not partitioned in top phase but precipitated at the interface the partition coefficient (*Ke*) is therefore calculated using the activities of protein and crude extract.

Therefore, $Ke = A_b/A_i$, where A_b and A_i are the activities of phytase (Uml^{-1}) in bottom phase and crude extract respectively.

5.2.5.2 Purification Factor

The purification factor of phytase is defined as the ratio of specific activity of phytase in bottom phase to that of crude extract. Specific activity is defined as the ratio between enzyme activities in sample and protein concentration in the same sample and is calculated by the following equation:

$$\text{Purification factor}\left(\text{PF}\right) = \frac{A_b \times P_i}{A_i \; P_b}$$

where, Pi and P_b are the protein concentrations in the crude extract and bottom phase, respectively and A_i and A_b are the activities of phytase (Uml^{-1}) in crude extract and bottom phase, respectively.

5.2.5.3 Percentage Enzyme Activity Recovery

Recovery of enzyme in bottom phase is determined as % yield. The formula for calculating % yield of enzyme is:

$$\text{Enzyme activity recovery } (\%) = \frac{A_b\ V_i}{A_i\ V_b} \times 100$$

where, A_i and A_b are the phytase activities (Uml^{-1}) in crude extract and bottom phase, respectively and V_i and V_b are the corresponding volumes.

5.2.5.4 Phase Volume Ratio

The phase volume ratio is defined as the ratio of volume of the top and bottom phases.

$$\text{Phase volume ratio } (\text{Vr}) = \frac{V_t}{V_b}$$

5.2.6 Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE)

The enzyme obtained from ATPS was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis. After electrophoresis, gels were stained with 0.025 % Coomassie brilliant blue stain R-250 and then distained in a solution of 5% methanol and 7% acetic acid. The details have been given in Chapter 4, section 4.2.4.6.1.

5.3 Results

ATPS can be considered as an integrated technique where extraction, concentration and primary purification of biomolecules are in a single unit operation. Several interdependent parameters, such as type of phase forming components, system pH, molecular weight of phase forming polymers, phase composition and additives like neutral salts are responsible for governing partitioning behavior of the enzyme (Kula, 1985). In order to understand the partitioning behavior of phytase, the influence of the parameters described by Kula, (1985) was investigated and the results given.

5.3.1 Purification and extraction of phytase in aqueous two phase system (ATPS)

Extraction of *Bacillus* MCCB 242 phytase was carried out to increase the purity of phytase from the ATPS at pH 5.5. ATPS having 10.5% PEG/15.2% citrate was prepared for the extraction (Table 5.1). An attempt was made to achieve differential partitioning of the enzyme and extract in ATPS at 1000 ml, which can be easily adapted for large-scale operations. The comparative chart of the purification steps of *Bacillus* MCCB 242 phytase using ion-exchange chromatography and aqueous two-phase system is shown in Fig. 5.4.

Table 5.1: Purification of phytase from *Bacillus* MCCB 242

Fraction (Uml^{-1})	Partition coefficient *(Ke)*	Total enzyme (Units)	Total protein (mg)	Specific Activity (Umg^{-1})	Yield of protein %	Purification Factor	% Recovery	Yield activity %
Crude extract	-----	820	128.0	6.41	100	1.0	61.29	100
DEAE Cellulose Chromatography	-----	480.5	2.8	171.62	1.86	26.77	62.4	54.00
ATPS	0.78	640	17	37.65	13.28	5.88	78.53	78.05

5.3.1.1 Effect of Sodium Citrate Concentration on Phytase Partitioning

In order to identify the citrate concentration suitable for purification of phytase, ATPS was employed by adding predetermined quantity of PEG 6000 and different concentrations of sodium citrate to crude phytase (1ml) making the total weight of the system 5g in 15ml centrifuge tube. The results are shown in Table 5.2. Phytase was selectively partitioned to bottom phase and the activity recovery was 88.9% with a purification factor of 3.63. Due to the high molecular weight of PEG and molecular exclusion mechanism, phytase was preferentially driven to the salt phase while the other proteins precipitated at the interface as, the solubility in PEG-rich phase was low.

Table 5.2: Sodium citrate concentration on phytase partitioning

Citrate concentration (%)	Partition coefficient (*Ke*)	Specific activity (Umg^{-1})	Activity recovery (%)	Purification factor (*PF*)
25	0.86	16.0	76.0	2.54
20.0	0.77	13	86.8	1.63
18. 0	0.71	14.3	71.0	1.35
15.2	1.6	16.8	88.9	3.63

5.3.1.2 Effect of pH on Partitioning of Phytase

The pH has an effect on the partitioning of enzymes either by changing the charge of the solute or by altering the ratio of the charged species. To study the influence of pH on phytase partitioning for the selected aqueous two phase system (PEG 6000-citrate), experiments were performed in the pH range 5.0-6.5 and the results are presented in Table 5. 3. These pH values were chosen so that the aqueous phase is neither too acidic nor basic because the solutions could not be discharged to the environment without further treatment.

Table 5.3: Effect of pH on phytase partitioning

pH	Specific activity (Umg^{-1})	Activity recovery (%)	Purification factor (*PF*)
5.0	10.5	93.7	1.85
5.5	16.8	98.5	3.05
6.0	14	90.2	1.40
6.5	7.5	55.0	0.87

5.3.1.3 Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE)

The purity of phytase obtained from ATPS was compared to that from the chromatographic separation. The SDS-PAGE of phytase obtained by ATPS is shown in Fig. 5.3. The purified enzyme from ATPS in bottom phase appears in single band, corresponding to a molecular mass of 43kDa. The top phase of ATPS did not show any protein band.

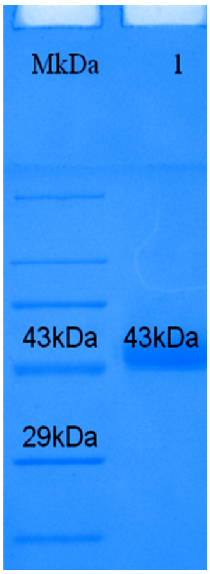


Fig.5.3: SDS page of phytase obtained by ATPS

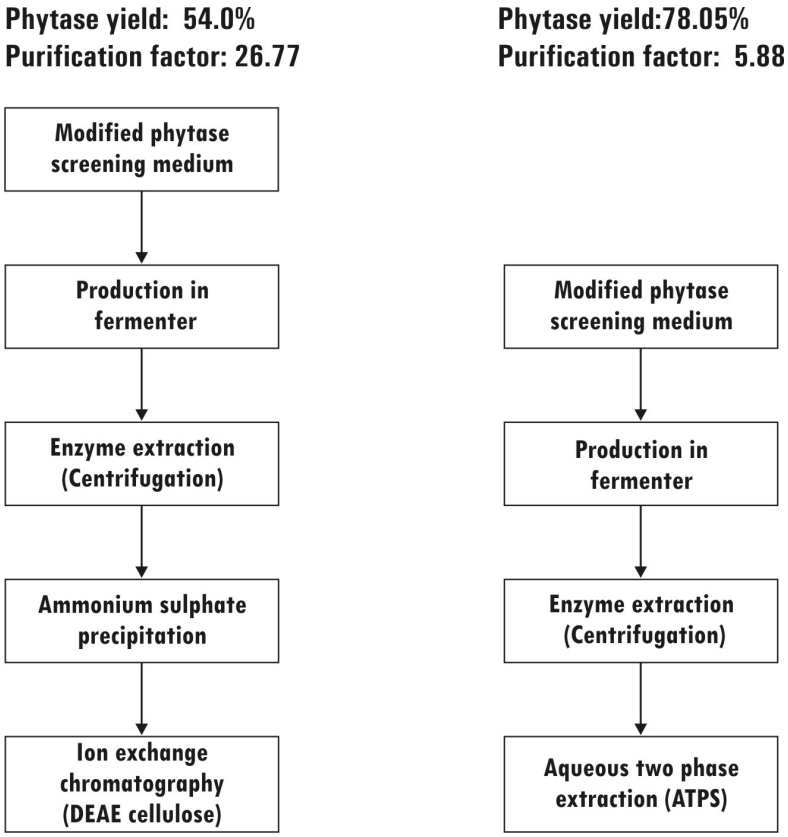


Fig.5.4: Comparison of purification steps of *Bacillus* MCCB 242 phytase: a) by ion-exchange chromatography; b) aqueous two-phase processes

5.4 Discussion

Considering the high commercial potential of phytase, several methods have been attempted to obtain a highly active enzyme suitable for industrial applications. Several traditional purification processes have been employed to purify phytase from microbial sources. However, most of these purification protocols involve a number of steps. The scaling-up is difficult and also very expensive. The conventional technique of filtration for solid liquid separation is not suitable for the bio-separation, in which the size of the microorganisms to be separated is small, especially when the cells are broken to release the intracellular components causing increase in viscosity of the system (Huggins, 1978; Mosqueira, 1981).

Aqueous Two-Phase System (ATPS) is an emerging technique which has applications in partitioning and purification of various bio-molecules. ATPS is considered to be one of the most effective and economically feasible downstream processes for bio-molecule recovery. ATPS for the separation and purification of phytase has been attempted in this study. The partitioning of bio-molecules in the system is dependent on the properties of the protein as well as on the two aqueous phases. ATPS for the downstream process resulted in one-sided partitioning of phytase in to bottom phase with a purification factor involving salt precipitation and column chromatography. The potential trend in the application of ATPS for the recovery of biological products will extend to the non-protein products including food and cosmetic industry.

High phytase recovery of 78.5% within a short span was achieved by ATPS, whereas only 62% recovery had been obtained in 96 hrs by

chromatography. Accordingly, ATPS was found to be an effective alternative for simultaneous partitioning and purification of phytase.

Influence of NaCl concentration on partition coefficient of phytase in ATPS was investigated. The lowest partition coefficient was observed with NaCl concentration of 2%. Generally, addition of neutral salts (additives) to ATPS alters the phase diagram of the system and also the properties of the partitioning solute (Abbot and Hatton, 1988).

Salts are added to aqueous solution of enzymes to help buffer the solution and stabilize the bio-molecules. Larger ions such as phosphate, citrate and sulfate partition more unequally than monoatomic ions such as chloride and thus give large potential (Johansson, 1970; Zaslavski *et al.,* 1983). Advantageously, phytase which has high affinity for citrate is selectively partitioned to the bottom phase with high recovery of 88.9%.

Through aqueous two-phase extraction system, the degree of enzyme purification (purification factor = 5.88) could be enhanced while considerably reducing the volume of the crude sample. The extraction conditions employed has resulted in the enrichment of enzyme specific activity (37.65), which is due to the differential partitioning of the desired enzyme and contaminating enzymes/proteins to opposite phases. DEAE cellulose chromatography of the fraction from salt-rich bottom phase of the ATPS showed marked increase in the degree of enzyme purification and yield.

Compared to other separation techniques, ATPS has many advantages such as low processing time, low energy consumption, biocompatible environment and the relative ease of its scaling-up (Albertsson, 1986). The

effectiveness of ATPS in downstream processing has been confirmed on the extraction, separation, concentration and primary purification of different enzymes including invertase (Yucekan and Onal, 2011); lipase (Lucena *et al.,* 2010); lysozyme (Dembczynski *et al.,* 2010); protease (Nalinanon *et al.,* 2009); xylose reductase (Faria, 2009); α-galactosidase (Nagangouda and Mulimani, 2008); trypsin and α-chymotrypsin (Tubio *et al.,* 2007); polyphenol oxidase (Vaidya *et al.,* 2006) and β-glucosidase (Gautam and Simon, 2006).

Bhavsar *et al.* (2012) described a downstream process for extracellular phytase from *Aspergillus niger* by simultaneous partitioning and purification. No previous report on *Bacillus* phytase purification, extraction and recovery using aqueous two phase system has been found in literature. In this context, outcome the present study is promising in developing an industrial process for phytase production.

Through this study, Polyethylene Glycol/Sodium Citrate (PEG/Na citrate) Aqueous Two-Phase System (ATPS) could be demonstrated as an efficient downstream process suitable for industrial application for the commercial production of *Bacillus* MCCB 242 phytase.

Chapter 6

SUMMARY AND CONCLUSIONS

Contents

Bacterial phytase has considerable potential in animal feed technology and in environmental protection, well studied over the years. The current annual global phytase market is estimated to be approximately $350 million. Phytase is known to reduce the anti-nutrient effects of phytate in animal diets by degrading it, thereby, increasing the availability of energy, minerals and amino acids. The increasing public awareness of environmental protection, along with a concurrent price rise and supply shortage of conventional feed phosphorus sources, leads to a greater demand for phytase.

In this context a study was undertaken to isolate phytase producers from environment and to segregate the most highly efficient phytase producer and to develop a bioprocess technology for commercial application. During this process, a potential phytase producer *Bacillus* MCCB 242 was isolated and characterized phenotypically and genotypically. Subsequently, phytase production was optimized, the enzyme purified and characterized and an appropriate downstream process also could be standardized.

Following were the objectives of the investigation:

1. Screening, isolation and identification of phytase producing microorganisms from environment
2. Molecular characterization and phylogenetic analysis of the phytase producers.

3. Production optimization of phytase from *Bacillus* MCCB 242.
4. Purification and characterization of phytase.
5. Downstream process standardization.

The salient findings are given below:

- Three potent phytase producing bacterial isolates from soil water systems were identified based on phenotypic and molecular characterization. The 16S rRNA gene sequence on comparison with GenBank database showed 99% similarity to *Bacillus* sp. JX293291.1, 99% similarity *to Citrobacter freundii KF535142.1* and 100% similarity to *Escherichia coli* LM993812.1. The sequences were deposited in GenBank with the accession numbers KM880161, KM880162, KM880163 respectively.
- The 16S rRNA phylogenetic tree showed that isolate 1 (KM880161) is closely related to and branched from *Bacillus amyloliquefaciens* strain, isolate 2 (KM880162) showed similarity and branching from *Citrobacter freundii* and isolate 3(KM880163) is closely related to *Escherichia coli* 16S rRNA and *E.coli* genome assembly.
- Subsequently, the isolate *Bacillus* MCCB 242 was selected for further investigations as it possessed significantly higher phytase activity than the other two bacterial isolates.
- *Bacillus* MCCB 242 was found capable to produce phytase which could be exploited as feed additive to monogastric animals and agastric fishes.
- Optimization of total phytase production could be accomplished by employing the Plackett-Burman and Central Composite Design of Response Surface Methodology.
- Altogether, 20 different experiments (2^3 full factorial points, 6 axial points and 6 center points) were suggested by the software for optimization of medium ingredients and culture conditions.

- Analysis of variance (ANOVA) of the quadratic model showed that the model was adequate with no significant lack of fit (prob> F = 0.0659).
- The observed R^2 value 0.9756 explained that the fitted model could explain 97.56% of the total variation and the model did not explain 2.44% of the same, thus indicating the good response prediction.
- An adequate precision value of 15.67 suggested that the polynomial quadratic model was of an adequate signal, and could be used to navigate the design space.
- The coefficient of variation (CV=10.07%) indicated a good precision and reliability of the experiment.
- In this study A, C, AC, A^2, B^2, C^2 were significant model terms.
- The model implied that three factors such as Glucose: A, NH_4NO_3: B, and Na phytate: C significantly influenced the phytase production in *Bacillus* MCCB 242.
- The elliptical nature of the contour plots in the case of phytase activity of *Bacillus* MCCB 242 indicated that the interaction effects between glucose and NH_4NO_3, glucose and Na phytate and NH_4NO_3 and Na phytate were found to be most significant for phytase activity.
- Statistical techniques such as Plackett-Burman design and Response Surface Methodology helped in reducing the cost of production and thus made the process more economical.
- Phytase production by *Bacillus* MCCB 242 carried out in the modified medium reached maximum at 24^{th} hrs with an activity of 688 ±3.21 Uml^{-1} in fermenter.

- The optimized variables using response surface analysis gave the best conditions for maximum phytase production.
- An increase of 1.433 fold in phytase production was observed in the optimized medium compared that in the un optimized one (480 ±2.89Uml^{-1}).
- The model when validated by repeating the experiments under shake flask conditions resulted in 653.3±3.05 Uml^{-1} phytase production, indicating good correlation between predicted and experimental values proving validity of the model.
- Phytase production by *Bacillus* MCCB 242 carried out in the newly designed medium proved to be an economical medium for large scale phytase production.
- Optimum pH and temperature for phytase activity were found to be 5.5 and 40°C, respectively. Effect of pH on phytase stability showed that the enzyme was stable from pH 5.0 and 7.0 after pre-incubating for 30 minutes. The enzyme retained more than 90% residual phytase activity between pH of 5.0-7.0.
- *Bacillus* MCCB 242 phytase was stable over 20-40°C even after 48 hrs of exposure. At 40°C, the enzyme could retain 98 % phytase activity.
- Presence of metal ions such as Ca^{2+}, $Fe^{3+}Mg^{2+}$, Co^{2+} and Mn^{2+} enhanced phytase activity while heavy metals such as Hg^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} inhibited. Presence of Fe^{3+} ion enhanced the phytase activity by 98%.
- Surfactants such as polyethylene glycol, Tween-80 and TritonX-100 stimulated the enzyme activity. PEG 6000 yielded enhanced phytase production in the fermented medium compared to that in the control.
- *Bacillus* MCCB 242 phytase retained 95% activity in the presence of 1 % H_2O_2.

- Kinetics of phytase were determined as *Km* = 182 µmol l^{-1}, *Vmax*= 220µmolmin^{-1} mg^{-1} and *Kcat* = 180 sec^{-1}at pH 5.5 and 40°C
- Purified phytase of *Bacillus* MCCB 242 was resolved on standard SDS-PAGE, renatured and incubated with sodium phytate *in situ*.
- The phytate impregnated gels were exposed to the counterstaining reagent (cobalt chloride, ammonium molybdate and ammonium metavanadate). Zone of clearing were seen in the region of active phytase protein bands.
- The molecular mass of *Bacillus* MCCB 242 phytase was found to be 43 kDa.
- The enzyme was purified with 8.41 fold increase in specific phytase activity. A 28.94 fold purified enzyme was obtained after DEAE cellulose chromatography.
- Peptide analysis by Mass Spectrometry (MS) MALDI-TOF to those in the protein data bank suggested that there was only one protein.
- The sequence obtained consisted of 383 amino acids with mono isotopic mass of 41835.50956 and average mass of 41861.3992.
- The enzyme belonged to alkaline class 3-phytase and its catalytic activity was defined as Myo-inositol hexakisphosphate + H_2O = 1D-myo-inositol 1,2,4,5,6-pentakisphosphate + phosphate
- *Bacillus* MCCB 242 phytase on Hep-2 cells did not cause any significant change in cell morphology on testing with concentrations of 1- 250 µg ml^{-1} enzyme, however, cell rounding and cell death could be noticed at higher concentrations (500µg ml^{-1}(v/v)). On MTT assay there recorded marginal increase in toxicity on Hep-2 cells with increase in concentration of phytase.

- In RTG-2 cells, *Bacillus* MCCB 242 phytase did not show cell rounding and cell death even at higher concentrations (500µg ml^{-1}). MTT assay did not show toxicity of phytase on RTG-2 cell lines even at higher concentrations.
- Based on the results of test of cytotoxicity it was concluded that *Bacillus* MCCB 242 phytase could be used for supplementing fish feed to enhance nutrition and to curtail release of phosphorus to environment from fish excreta.
- Economical and effective technique for recovery and purification of *Bacillus* MCCB 242 phytase was achieved through single step aqueous two phase extraction. ATPS has markedly increased the degree of enzyme purification.
- The purified enzyme from ATPS in bottom phase gave a single band in SDS – PAGE, corresponding to a molecular mass of 43kDa.
- Compared to ion exchange chromatography, aqueous two phase system improved purification of *Bacillus* MCCB 242 phytase by a factor of 5.88.
- By this process its yield could be increased by 24% compared to that by DEAE cellulose chromatography.

Precisely, through this work an environmental isolate *Bacillus* MCCB 242 could be brought out as phytase producer for commercial application. The enzyme production could be optimized and characterized, and an appropriate downstream process standardized. Cytotoxicity studies revealed the enzyme safe for feed application, especially in fish.

6.1 Suggestions for Future Course of Action

- ✓ Application and evaluation of *Bacillus* MCCB 242 phytase as feed additive to finfish and shellfishes under laboratory and field conditions.
- ✓ Production standardization in larger fermenters (100 litres and above) and evaluation of the standardized downstream process for cost effective commercial production.
- ✓ Recombinant production of phytase in an appropriate expression system and development of large scale production systems.

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APPENDIX

Phytase producer from environmental isolate *Bacillus* sp. MCCB 242 16S rRNA, partial cds

LOCUS : KM880161 1394 bp DNA linear 08-OCT-2014

DEFINITION : *Bacillus* sp. MCCB 242 16S rRNA, partial cds.

ACCESSION : KM880161

SOURCE : *Bacillus* sp. MCCB 242

ORGANISM : *Bacillus* sp. MCCB 242

REFERENCE 1 : (bases 1 to 1394)

AUTHORS : **Sareen,S.J., Bright Singh,I.S. and Vrinda,S.**

TITLE : Phytase producer from environmental isolate

JOURNAL : Unpublished

REFERENCE 2 : (bases 1 to 1394)

AUTHORS : Sareen,S.J., Bright Singh,I.S. and Vrinda,S.

TITLE : Direct Submission

Isolation source: "Farm land soil" **Country**: "India"

Collected by :"Sareen Sarah John"

JOURNAL : Submitted (07-OCT-2014) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

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 181 gcattagcta gttggtgagg taacggctca ccaaggcaac gatgcgtagc cgacctgaga
 241 gggtgatcgg ccacactggg actgagacac ggcccagact cctacgggag gcagcagtag
 301 ggaatcttcc gcaatggacg aaagtctgac ggagcaacgc cgcgtgagtg atgaaggttt
```

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481 ggtggcaagc gttgtccgga attattgggc gtaaagggct cgcaggcggt ttcttaagtc
541 tgatgtgaaa gcccccggct caaccgggga gggtcattgg aaactgggga acttgagtgc
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1141 catcatgccc cttatgacct gggctacaca cgtgctacaa tggacagaac aaagggcagc
1201 gaaaccgcga ggttaagcca atcccacaaa tctgttctca gttcggatcg cagtctgcaa
1261 ctcgactgcg tgaagctgga atcgctagta atcgcggatc agcatgccgc ggtgaatacg
1321 ttcccgggcc ttgtacacac cgcccgtcac accacgagag tttgtaacac ccgaagtcgg
1381 tgaggtacct cgta

Phytase producer from environmental isolate *Citrobacter freundii* MCCB 243

16S rRNA, partial cds

LOCUS : KM880162 1389 bp DNA linear 08-OCT-2014

DEFINITION : *Citrobacter freundii* MCCB 243 16S rRNA, partial cds.

ACCESSION : KM880162

SOURCE : *Citrobacter freundii* MCCB 243

ORGANISM : *Citrobacter freundii* MCCB 243

REFERENCE 1 : (bases 1 to 1389)

AUTHORS : Sareen,S.J., Bright Singh,I.S. and Vrinda,S.

TITLE : Phytase producer from environmental isolate

JOURNAL : Unpublished

REFERENCE 2 : (bases 1 to 1389)

AUTHORS : Sareen,S.J., Bright Singh,I.S. and Vrinda,S.

TITLE : Direct Submission

Isolation source : "Soil" **Country**: India"

Collected by : "Sareen Sarah John"

JOURNAL : Submitted (07-OCT-2014) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

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   1 cctggctcat gaagtcgtaa cagggcagtg gtacggggat gttggacggg tgagtaatgt
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 121 gtcgcaagac caaagagggg gaccttcggg cctcttgcca tcggatgtgc ccagatggga
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 241 tgaccagcca cactggaact gagacacggt ccagactcct acgggaggca gcagtgggga
 301 atattgcaca atgggcgcaa gcctgatgca gccatgccgc gtgtatgaag aaggccttcg
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1261 tccatgaagt cggaatcgct agtaatcgtg gatcagaatg ccacggtgaa tacttttcct
1321 gctccttgta cactcaagtt gtaccaagct agcgatcggt gagtatgatc ctggctcatg
1381 aagtcgtaa

Phytase producer from environmental isolate *Escherichia coli* MCCB 244

16S rRNA, partial cds.

LOCUS : KM880163 1353 bp DNA linear 08-OCT-2014

DEFINITION : *Escherichia coli* MCCB 244 16S rRNA, partial cds.

ACCESSION : KM880163

SOURCE : *Escherichia coli* MCCB 244

ORGANISM : *Escherichia coli* MCCB 244

REFERENCE 1 : (bases 1 to 1353)

AUTHORS : Sareen,S.J., Bright Singh,I.S. and Vrinda,S.

TITLE : Phytase producer from environmental isolate

JOURNAL : Unpublished

REFERENCE 2 : (bases 1 to 1353)

AUTHORS : Sareen,S.J., Bright Singh,I.S. and Vrinda,S.

TITLE : Direct Submission

Isolation source : "Marine Sediment" **Country** : "India"

Collected by : Sareen Sarah John"

JOURNAL : Submitted (07-OCT-2014) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

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