PHYTASE FROM *BACILLUS* SPP. – PRODUCTION, AND CHARACTERIZATION FOR AQUAFEED SUPPLEMENT

Thesis submitted in partial fulfilment of the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

Submitted By:

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Enrolment number- 207822

Under the guidance of:

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(MAY-2022)

SELF - DECLARATION

I, **Harshita,** student of M.Sc. Biotechnology, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh do hereby declare that the project entitled "**Phytase from *Bacillus* spp. – Production and Characterization for aquafeed supplement**" submitted towards partial fulfilment for the award of the degree of **Master of Science in Biotechnology** of Jaypee University of Information Technology is based on the results of the research work carried out by me and written by me under the guidance and supervision of **Dr. Saurabh Bansal.,** This project or no part of this has been submitted elsewhere for awarding any degree or diploma.

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CERTIFICATE

This is to certify that the work titled "**Phytase from *Bacillus* spp. – Production and Characterization for aquafeed supplement**" submitted by **Harshita (207822)** is in partial fulfilment for the award of the degree of Master of Science in Biotechnology of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

_____

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Harshita

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ABSTRACT

Aqua feed is primarily composed of canola meal, soya meal, and fish meal, which are the sources of carbohydrates, vitamins, and minerals required for fish in aquaculture. These meals are majorly plant-based products; therefore, the phosphorus content of these aqua feeds is mainly present as phytate. Phytate (also known as myoinositol-1,2,3,4,5,6-hexakisphosphates) remains nutritionally unavailable to aquatic animals due to the unavailability of a phytate degrading enzyme, i.e., phytase. Phytic acid is stored as phytate or phytin, is highly negatively charged and is found in the aleurone layer of seeds or grains. Due to the unavailability of phytase enzymes in aquatic animals, phytate is excreted into the water and causes pollution and eutrophication. It combines with proteins and vitamins to form insoluble complexes and has a negative impact on their utilization efficiency, activity, and digestibility. It also inhibits enzyme activity of pepsin, amylopsin, and amylase and adversely impacts the absorption and bioavailability of essential minerals (Ca, Zn, Mg, and Fe), thus affecting the growth of commonly cultured aquatic animals. Dietary phytase can solve the problems arising from phytate (a phosphorus source) by hydrolysing phytate (myoinositol hexakisphosphate) into inositol and inorganic phosphorus, thus increasing nutrient availability to aquatic animals and reducing direct phytate excretion in water bodies. This present study is focused on the production of phytase enzyme from a bacterial source, i.e., *Bacillus* spp., followed by its characterization. The crude phytase from *Bacillus* spp showed its optimal enzyme activity at pH 7.0 and temperature at 50°C.

Chapter-1

Introduction

Canola meal, soya meal, and fish meal are main components of aqua feed, which are the sources of carbohydrates, vitamins, and minerals required for fish in aquaculture. Being majorly plant-based products, these aqua feeds have their phosphorus content mainly in form of phytate. Myoinositol-1,2,3,4,5,6-hexakisphosphates (Phytate) remain nutritionally unavailable to aquatic animals due to the unavailability of a phytate degrading enzyme, i.e., Phytase. Phytic acid is stored as phytate or phytin, is highly negatively charged, and is found in the aleurone layer of seed or grain. Due to the unavailability of phytase enzymes in aquatic animals, phytate is excreted into the water and causes pollution and eutrophication. It combines with proteins and vitamins to form insoluble complexes and has a negative impact on their utilization efficiency, activity, and digestibility. It also inhibits enzyme activity of pepsin, amylopsin, and amylase and adversely impacts the absorption and bioavailability of indispensable minerals (Ca, Zn, Mg, and Fe), thus affecting the growth of commonly cultured aquatic animals. Dietary phytase can solve the problems arising from phytate (a phosphorus source) by hydrolysing phytate, i.e., myoinositol hexakisphosphate into inositol and inorganic phosphorus thus increasing its availability to aquatic animals and reducing direct phytate excretion in water bodies. Most of the phytase enzyme studies have been focused on fungal sources. None of the single phytases from the known phytases has all the characteristics like thermostability, wide pH range and broad substrate specificity. Therefore, searching for a new bacterial isolate producing phytase with desired characteristics is an area of continued research interest. Despite global phytase manufacturing, North America and China remain the economic leaders in phytase and other feed enzyme production. India is dependent on imports of feed enzymes from other nations.

Phytase has received a lot of interest from academics and enterprises in the sectors of food and feed, pollution control, and biotechnological applications over the last 25 years. Phytases have several applications, including in the feed industry and the generation of myoinositol phosphates. It's also utilized in the partial synthesis of peroxidases, the pulp and paper industry, as a soil conditioner, and as a plant growth stimulant [1]**.** As a result of their capacity to increase the nutritional status of feed by decomposing antinutritive phytic acid, phytases are extremely important in the nutritional business.

Chapter-2

Review of Literature

Concerns about the amount of phosphorus and nitrogen released into the water have been raised worldwide. Their increasing levels of discharge into the water are one of the significant causes of aquaculture pollution. It is all because of the phytate complex (the storing form of phosphorus) in nearly all plants. These plant products (as phosphorus sources) form a major component of commonly used aqua feeds. This phytate is hydrolysed by a phosphatase enzyme known as 'Phytase', which is not found in the gastrointestinal tract of aquatic animals.

The environmental impact of aquaculture pollutants has pushed the phytase biotechnological advancements and their use in aquatic animal feeding. Research findings on enzymatic hydrolysis of animal feed employing microbial phytase resources have already shown that this approach boosts the protein and critical mineral bioavailability and promotes growth performance comparable to or higher than phosphate supplements in farm and poultry animals. Phytase enzyme supplementation in aqua feed promotes nutritional availability of inorganic phosphorus (Pi) and other minerals by hydrolysing their substrate, i.e., phytate or phytic acid. The world's phosphate stocks are not replenishable. Also, phosphorus supplementation is very costly, resulting in an overall feed cost increase. As a result, phytate-phosphorus can be effectively transformed into available phosphorus by phytase as an alternative and cost-effective phosphorus source. In the last two decades, phytase has become more popular and widespread in fish feed.

1. IMPORTANCE OF PHOSPHORUS IN AQUACULTURE

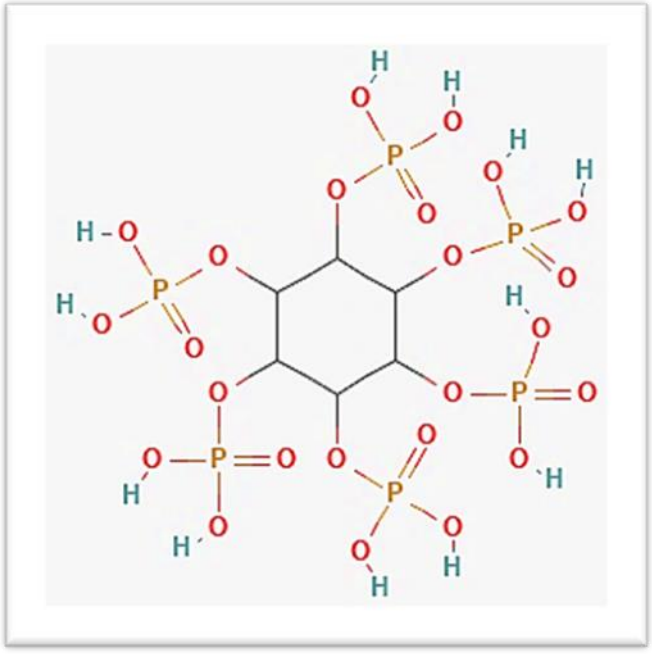
Phosphorus, along with calcium, are crucial for the formation and functioning of the skeletal system, as well as many other biological roles such as maintaining acid-base equilibrium in fish. Phosphorus is stored in skeletal tissue as tricalcium phosphate [$Ca_3(PO_4)_2$], which crystallises to form hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, which is then incorporated into matrix material during mineralization [2]. Besides acting as a supporting system, the skeleton also acts as a P and Ca store house. Phosphate (HPO42-) is an important component of cell function in addition to skeletal tissue metabolism. P is an essential signalling molecule, a constitutional element of the cell wall, as well as a constituent of energy molecules like Adenosine monophosphate (AMP), Adenosine diphosphate (ADP), Adenosine triphosphate (ATP) and needed for the helical structure of RNA and DNA [2]. Because fresh water and sea water are low in phosphate, feed is the primary source of P in fish. Because fish must successfully intake, assimilate and conserve phosphorus salts in both

fresh water and marine water settings, phosphate regulation is regarded as more important than calcium regulation [2].

2. PHYTIC ACID

Phytic acid (PA) also called myoinositol-hexaphosphoric acid or inositol polyphosphate or inositol hexakisphosphate (IP6), is present in all plant-based feed products in the form of its salt, phytate. Other related information is listed in Table 2.1.

Table 2.1: Additional information of phytic acid.

S. No.	Properties	Value
1.	Molecular formula	C6H18O24P6
2.	IUPAC nomenclature	(2,3,4,5,6-pentaphosphonooxycyclohexyl) dihydrogen phosphate
3.	Structure	 **Figure 2.1: Structure of phytic acid**
4.	Molecular Weight	660.04g/mol
5.	European Community (EC) Number	201-506-6
6.	Synonyms	• Acid, Phytic • Hexakisphosphate, Inositol

		• Hexaphosphate, Inositol • Inositol Hexakisphosphate • Inositol Hexaphosphate • Phytate • Phytic Acid • Phytin

Phytate comprises inositol, which is hexa-hydroxy cyclohexane with six phosphate ester linkages in a chair configuration [3]. Phosphoric acid has six arms that quickly bind to minerals like Fe, Mg, Ca, and Zn and chelate them. As a result, minerals become inaccessible and therefore, phytate is thought to be anti-nutritional. This antinutritional property of phytate results in decreased bio-availability of several nutrients. Consequently, it negatively impacts the overall growth performance of fishes and other aquatic animals in aquaculture.

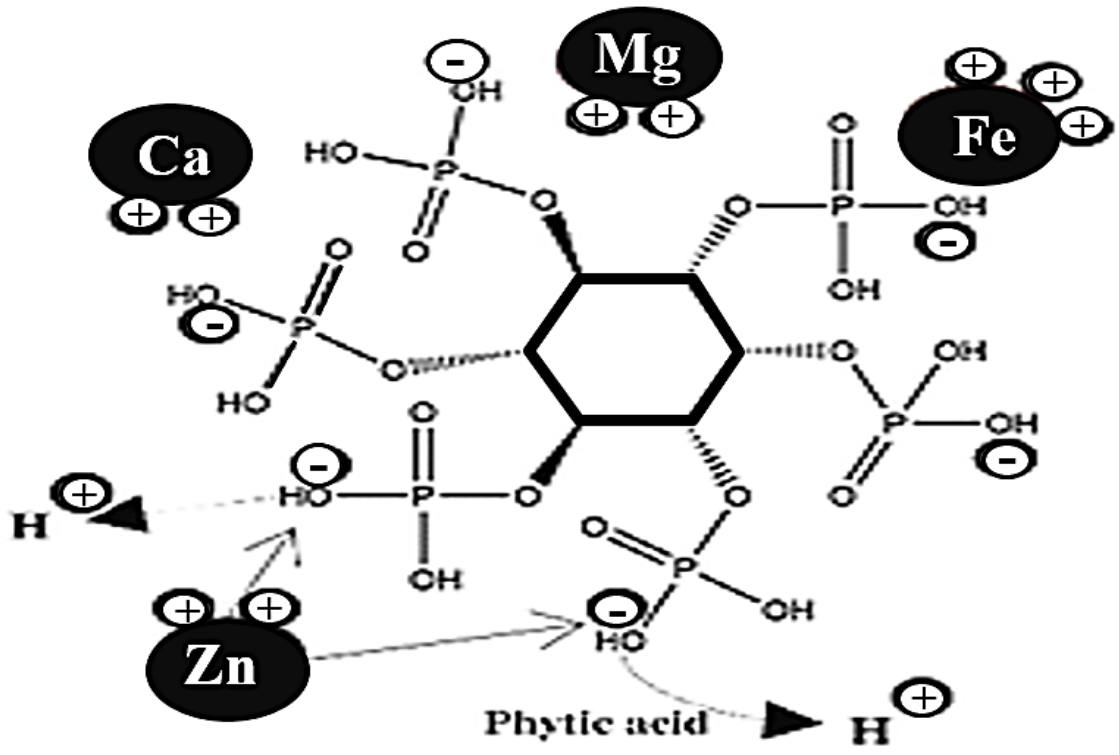


Figure 2.2: Chelation property of phytic acid.

In all oil seed crops like maize and soybean and grain crops like wheat, rye, oats etc., phytate is the major phosphorus storage form [4]. In grains and plant seeds, phytate

amounts vary between 0.5 and 5% [5]. Cereal by-products have the largest percentage (73-84%), followed by oil seed meals (51-82%), cereals and millets (60-73%) [5]. In small-grain cereals, the aleurone contains around 90% of the seed phytate, while the scutellum contains the remaining 10%. On the other hand, the scutellum of maize contains 90% phytate, whereas the aleurone has 10%. Phytate is stored in the endosperm of dicotyledons and monocotyledons [5]. Cosgrove and Irving proposed in 1980 that phytate functions in seeds as a phosphorus reserve, an energy storehouse, and a challenger for ATP during the quick biosynthesis of phytic acid around the time of the seed's full growth when seed metabolism gets suppressed; inactivity gets prompted. It also functions as an immobiliser of divalent cations, which are essential for controlling cellular activities that are liberated during germination by constitutive plant phytases, and as a controller of the inorganic phosphorus level in seeds.

Phytate is mainly found as a complex of + and +2 charged cations like K^{+}, Mg^{2+}, and Ca^{2+}. It happens when plant seeds and grains mature or germinate. Phytic acid has negative charges in acid, neutral, and alkaline pH environments [6], which can get fixed to the positive charge on its molecule in the diet and in-house GIT discharges such as hydrolytic enzymes and other mucosal secretions at all pH levels observed in the GIT, decreasing mineral digestion and incrementing in-house nutrient discharge. Positively charged amino acids interact with phytic acid at lower pH levels, reducing protein solubility and digestibility [7].

The effect of dietary phytate has been reported in several animals. In hens, the withholding of the positively charged minerals increased with increment in diet supplemented non-phytate P level when the dietary phytic-acid amount was 0.71% and showed a reduction with increment in the diet supplemented non-phytate P level when diet supplemented phytic acid amount was 0.57% [8]. Dietary phytic acid significantly reduced calcium and zinc absorption in rats [9]–[12]. Apparently, the impact of phytic acid on mineral digestion in pigs and broilers has been studied by Cowieson and Woyengo [13]. In poultry, endogenous losses of minerals like calcium, iron, sodium and sulphur have also been investigated [13]**.**

In fishes, phytate causes precipitation of trace elements through chelation. It makes them unavailable to fishes [14], and itself is excreted out in the faecal matter, therefore, contributing to the enhancement of nutrient levels in aquatic ecosystems. In young blackmouth fish, it has been reported that when Na-phytate was supplemented in its feed, it resulted in a great reduction of zinc availability and the development of cataracts, decreased

growth, decreased protein conversion and thyroid functioning [15]. In channel catfish, increased phytate content in feed led to an increased dietary requirement of zinc to prevent deficiency signs, decreased weight gain and feed efficiency [16]**.** Similar effects were observed in carp. An increase in dietary Ca and Mg levels in the presence of phytate decreased the bioavailability of Ca, Mg, Zn, Fe and Cu, resulting in abnormalities in the epithelial layer of the intestine due to the occurrence of insoluble phytate-mineral complexes [17]. Rainbow trout experienced a reduction in protein digestibility, feed conversion and overall growth when supplemented with phytic acid-containing diets [18].

3. PHYTASE

- **INTRODUCTION**

Phytases are phospho-hydrolytic enzymes, also known chemically as myoinositol (1,2,3,4,5,6)-hexakisphosphate phosphohydrolase. They have been identified in plants, microorganisms, and some animal tissues. They represent a subgroup of phosphatases that begin the removal of phosphate groups from myoinositol hexakisphosphate in a stepwise manner. As an intermediate, the enzyme uses phosphate groups of myoinositol ring from PA to give rise to free iP and a chain of small esters with phosphate (inositol-5P to -1P) [19], as shown in figure 2.3.

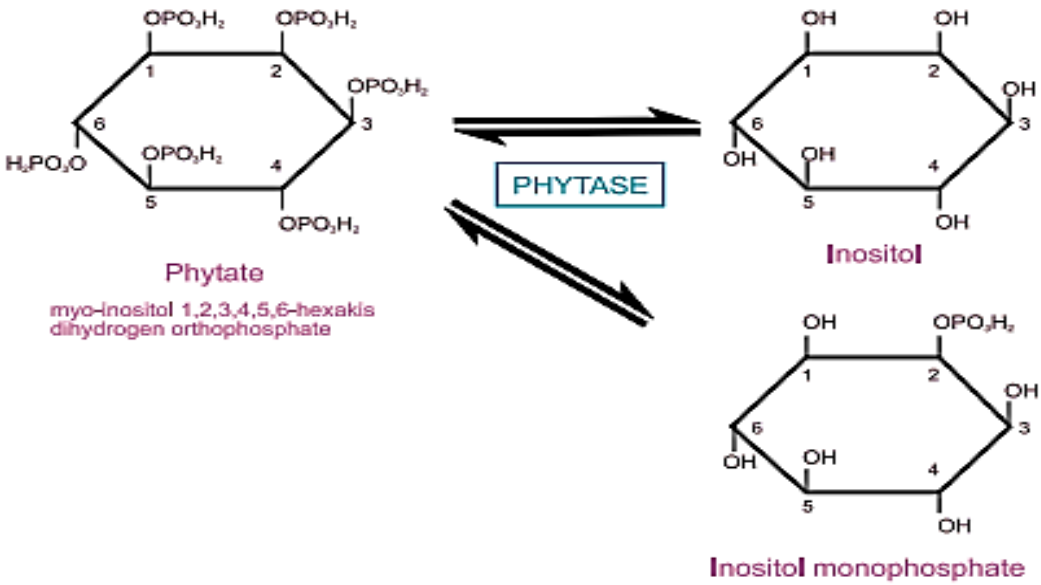


Figure 2.3: Phytase action on phytate.

Thus, by letting go of the bonded P in plant-derived meal and feed constituents, phytase increases the availability of P to fuel biological processes like bone formation and membrane firmness. As previously stated, phytase liberates P from plant-derived foods and enhances the bio-availability of Ca, Mg, protein, and lipids **(Figure 2.4)**.

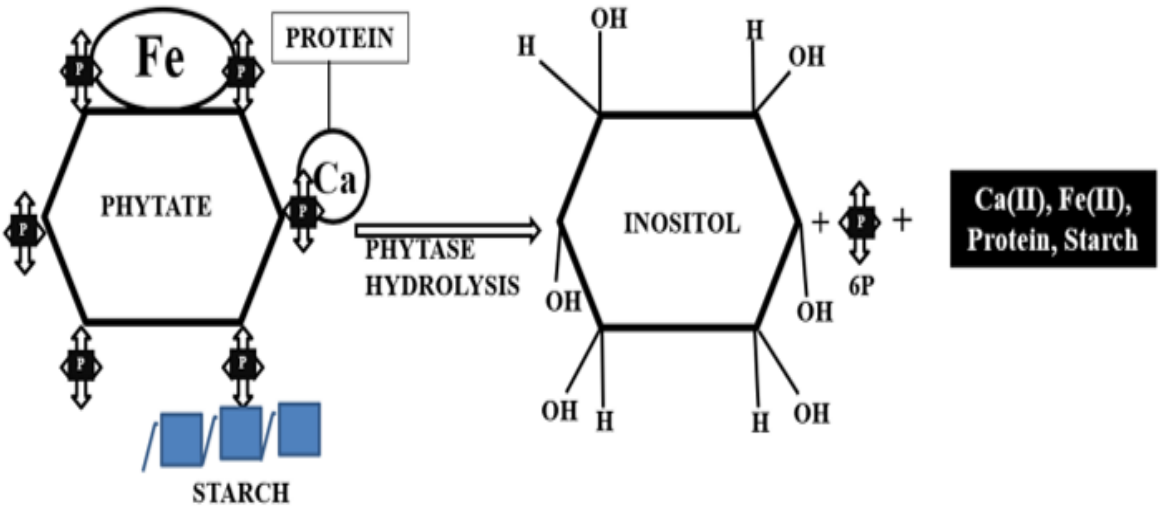


Figure 2.4: Hydrolysis of the phytate-mineral complex.

- **HISTORICAL BACKGROUND**

Phytase was discovered for the first time by Suzuki and others [20] from rice bran as an enzyme which catalysed phytate hydrolysis [21], followed by the calves' blood [22]. Its presence was later confirmed in various genera, including those from prokaryotes, yeast, fungi, and plants. Since humans and most monogastric animals do not have the phytase enzyme in their gastrointestinal tract; they must rely on duodenum or microbial enzymes found within the colon for phytate hydrolysis or upon non-enzymatic digestion by a gastro-intestinal acidic environment. Compared to plant and microbial phytase, in-house phytase activity in humans and animals is insignificant.

Natuphos, a commercially available phytase derived from the fungus *Aspergillus niger*, was the first to hit the world market in 1991 [23]. As a result, it began to gain popularity as an animal feed supplement worldwide. Furthermore, its potential for human [24] and animal nutrition [25] is being thoroughly investigated. It was discovered in 1999 that some phytases

found in bacteria, such as those produced by *E. coli*, were high functionally active compared to those made by fungus [26]–[28]. In 1997, the first phytase, "Consensus", was developed from a fully artificial gene expressed in *Hansenula polymorpha*. The enzyme produced was heat resistant to a temperature of 90◦C and showed activity comparable to that of *A. niger* phytase [29], [30]. Since then, many phytases have been discovered and their properties have been described by various authors [31]–[35].

3.1 Types of phytases

Phytase is a phosphomonoesterase, an extracellular enzyme that can catalyse the hydrolytic breakdown of phytate to remove the inorganic phosphate groups. These are found in a wide variety of organisms, including plants, animals and microorganisms [32], [36], [37]. There are numerous phytases, each with unique structural and other properties like catalytic mechanisms. Phytases are classified as alkaline or acidic phytases based on their optimal pH. Fungi, plants, and most bacteria can produce acidic phytases, while only a few bacteria and plants produce alkaline or neutral phytases [38]. Phytases are classified into three types based on their successive phosphorylation sites: 4/6 - phosphatase, 5 - phosphatase, and 3 - phosphatase. There are different types of phytases, as well as different sources. Phytases are classified into four types based on their catalytic functions, 3-D structures, and specific sequence properties: cysteine(C). phytase (CPhy), histidine-acid (HA) phosphatase (HAPhy), β-propeller(BP) phytase (BPPhy) and purple-acid (PA). phosphatase (PAPhy) (Figure. 2.5). BPPhy is the only phytase that retains enzyme activity in neutral or alkaline conditions [39]–[43]. BPPhy serves many functions in various species, which are determined by their very different biochemical properties. BPPhy is thermally stable, protease-resistant, and absolutely substrate-specific [41], [44], [45]. After being treated at 95 ℃ for 10 minutes, BPPhy produced by Bacillus licheniformis retained 80% of its enzyme activity and showed almost no action on phosphates other than those of phytate [46].

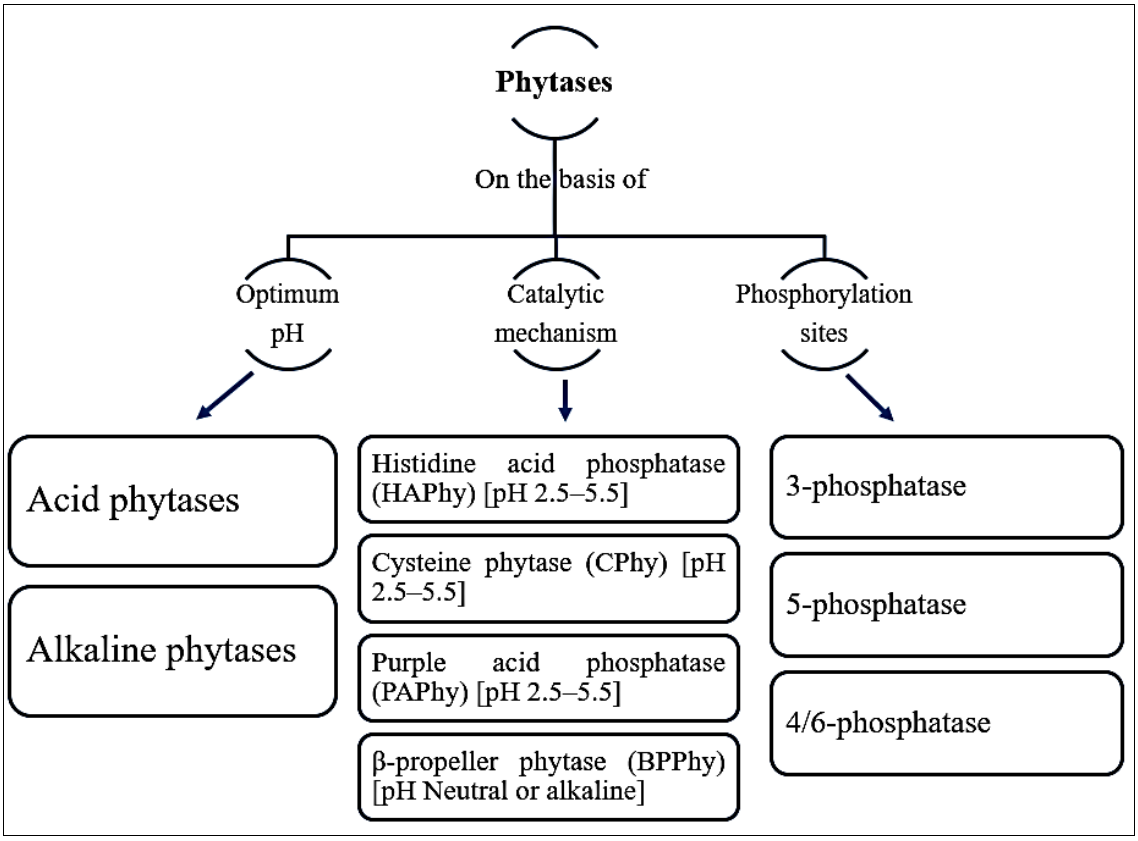


Figure 2.5: Classification of Phytases.

3.1.1 On the basis of optimum pH

Phytases are classified as acidic, neutral, or alkaline phosphatases based on their optimal pH of activity. These groups' optimal activity has been observed at pH 5.0, 7.0, and 8.0, respectively [47]. The majority of microbial-sourced phytate-digesting enzymes are acidic (typically histidine acid phosphatases) [48]. Similarly, the majority of plant phytases have an optimal pH of 5.0. The bio-efficacy of plant and microbial phytases from diverse sources can fluctuate depending on pH. Furthermore, because of their applicability in human food and broad substrate specificity, acidic phytases have received more attention than alkaline phytases.

3.1.2 On the basis of phosphorylation site

The International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology (IUPAC- IUBMB) approved the division of phytase enzymes into three classes based on the site where phytases start dephosphorylation on phytate molecules:

- 6 - phytase (EC 3.1.3.26)

- 5 - phytase (EC 3.1.3.72)
- 3 - phytase (EC 3.1.3.8)

The 3-phytases begin by removing the phosphate at the 3rd carbon position, thus giving (1,2,4,5,6) pentakisphosphate as the product. The 5-phytases start by removing the phosphate in the 5th carbon position, giving (1,2,3,4,6) pentakisphosphate as a product. In contrast, the 6-phytases begin by removing the phosphate in the 6th carbon place, giving (1,2,3,4,5)pentakisphosphate as a product, coupled with Pi (inorganic phosphorus). Microorganisms synthesise the 3-phytase while plants produce the 6-phytase [7]. It has been recognised that every type of phytase differs in terms of structural properties as well as a mechanism for hydrolysing phosphate from phytate. Also, it has been discovered that the majority of phytases show strong stereospecificity and prefer groups of equatorial-phosphate over groups of axial-phosphate [49].

3.1.3 On the basis of catalytic mechanism, 3-D structures and specific sequence properties

Phytases are classified into four types based on their catalytic mechanism, 3D structures, and specific sequence properties: (1) Histidine acid (HA) phosphatase (HAPhy), (2) Cysteine (C) phytase (CPhy), (3) Purple-acid(PA) phosphatase (PAPhy), and (4) β-propeller(BP) phytase (BPPhy) shown in figure 2.6.

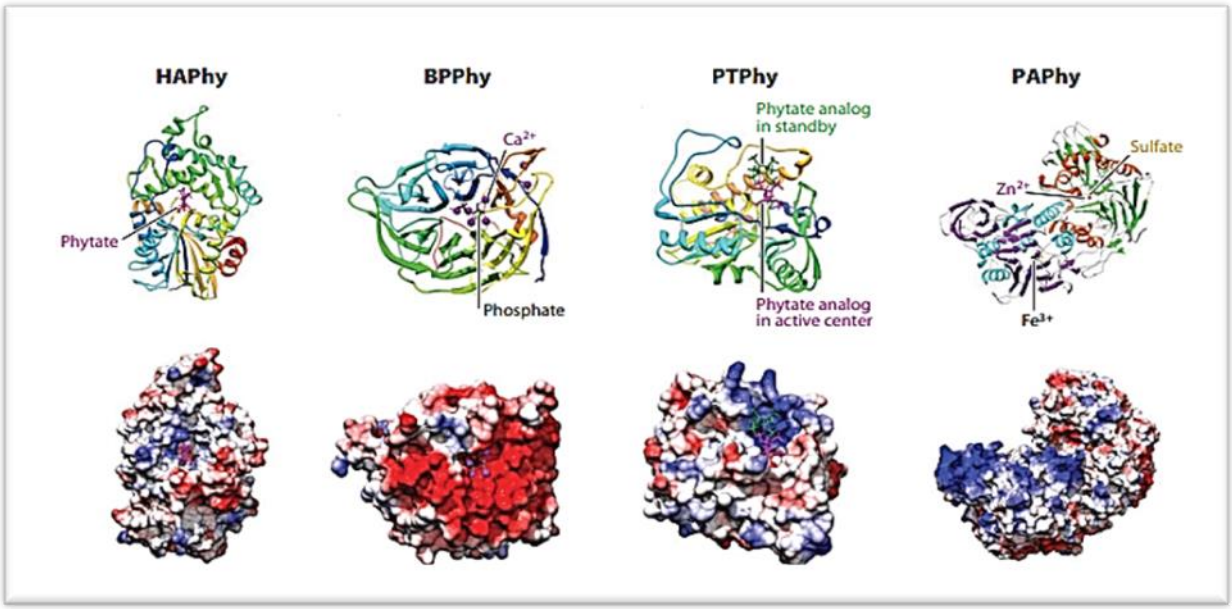


Figure 2.6: Secondary structure of different types of phytases.

Table 2.2: Optimum pH and specific sequence properties different types of phytases

S. No.	Type of phytase	Specific Sequence Properties	Active pH Range	References
1.	HAPhy	• RHGXRXP motif Conserved at active site • Cysteine motif Conserved • CAP having catalytic C-terminal dipeptide (HD)	2.5 –5.5	[29], [50], [51]
2.	CPhy	• α-helix and β-fold domain • 2 α-helices (near the C-terminal)	2.5 –5.5	[33], [52]
3.	PAPhy	• Motif of AA sequences GNH [E / D], GDXXY, DXG, VXXH and GHXH	2.5 –5.5	[53], [54]
4.	BPPhy	• P-loop containing AA sequence HCXXGXXR • WPD loop	Around 7 and >7	[55], [56]

- **Histidine acid phosphatase (HAPhy)**

This group is a widely researched group because of its high specific activity for phytic acid. This group is found in both prokaryotes (*E. coli* app A phytase) and eukaryotes (phyA and phyB from Aspergillus species, and yeasts). The conservative active site 6-peptide motif (RHGXRXP) and also metabolically active 2-peptide motif (HD) are shared by all HAP [57]. The enzymes of this group result in phytic acid formation in 2 steps: first nucleophilic approach on the P atom by the histidine of its active site, followed by catalysis of the [P-histidine] intermediate [58]. They do not require any co-factor to function optimally [28]. All the information regarding the active sites is related to disulphide bond formation and thermal stability and is used for binding of substrate and releasing product [42], [59]–[61].

- **Cysteine phytase (CPhy)**

CPhy comprises two domains: one large with a β-sheet and three α-helices, and one smaller towards the C-terminal with two α-helices that help in binding with the substrate.

- **Purple acid phosphatase (PAPhy)**

They are usually in dimer form having some VXXH, GNH [E/D], GDXXY, XG and GHXH like phosphomonoesterase motifs and 7 primary amino acids residues for metal ion bonding [62]–[64]. Plant PAPhy is mostly a homo-dimeric protein with an iron ion Fe^{3+} linked to a Zn or Mn ion, whereas animal PAPhy has a binuclear metal centre [$Fe^{3+}Fe^{2+}$] [63], [65].

- **β-propeller phytase (BPPhy)**

The BPPhy groups have been found in a wide variety of microorganisms. Six blades form the three-dimensional structure of BPPhy. BPPhy are the most important phytate digesting enzymes in water and soil, which play a significant function in the phytate-phosphorus pathway. There are 2 PO_4^{2-}-binding sites on it [66]. Alkaline PhyAsr (also known as protein tyrosine-phosphatase (PTP)-like phytase) is a ruminant phytase that exists in the rumen and has a sequence that is quite similar to the rest of the bacterial phytases. The active core has a phosphate binding ([HCXXGXXR], P-loop) and a [WPD] loop, which together create the tyrosine phosphatase substrate-binding area (pocket) [67]–[69]. They exist in many microorganisms like archaea, bacteria, cyanobacteria etc. These are frequently reported in *Bacillus.* BPPhy of *Bacillus* displays an enzyme action in the neutral or weak-alkaline range along with high thermal stability [70].

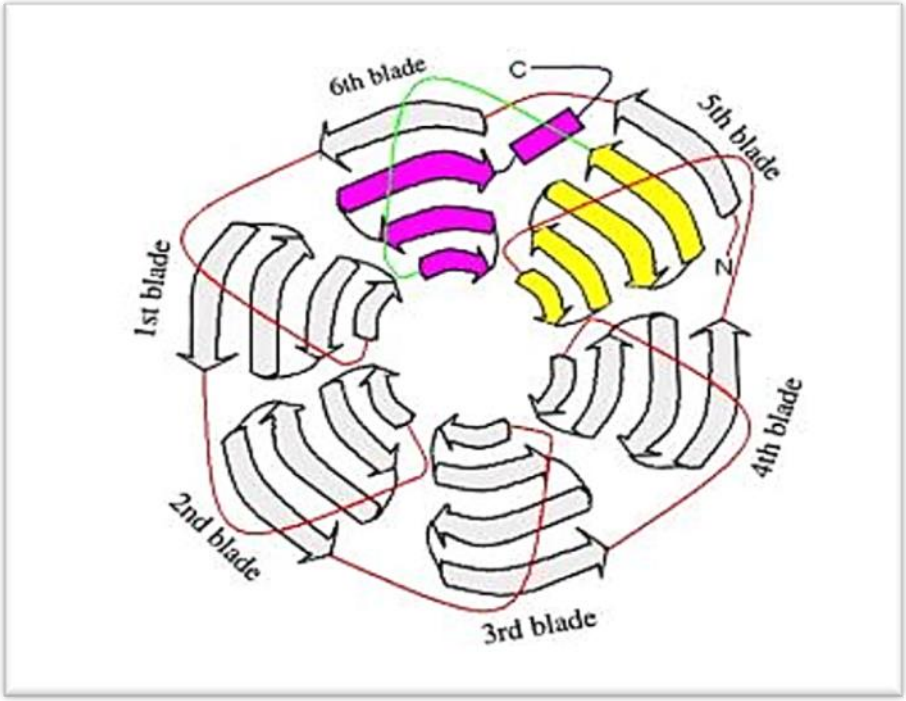


Figure 2.7: Structure of β-propeller(BP) phytase [71]**.**

3.2 Sources of phytases

Microbes and plants produce Phytases more abundantly and less abundantly by vertebrates [72]. Fungi, bacteria, and yeast are the primary phytase producers [72]. The majority of them in this group are 3-phytases [6], with only a few being 6-phytases [73]. Around 2000 microbes isolated from soil were examined for phytase-producing properties [74].

3.2.1 Plants

Most plant phytases are either acidic or have an optimal pH of approximately 5.5 or basic with an optimal pH of approximately 8.0. Plant phytases are found in nearly all germinating seeds. Plant phytase levels have been reported to increase by many degrees during germination. The seeds from several different species of plants were examined and they were found to have phytase activity in *Phoenix dactylifera* (date), hard wheat, soft wheat, oats, barley, *Bromus pratensis, Dactylis glomerata, Ricinus communis,* radish white mustard, *Citrus nobilis, C. vulgaris, C. aurantium, Pistacia atlantica, Faba vulgaris, Phaseolus lunatis, Lens esculenta* and *Cicera rietinum* [75]. Wheat and barley showed highest activity, with hard wheat more active than soft wheat. Dates showed the least activity and the activity in all seeds increased on germination. Phytase has also been detected in the spinach leaf, tobacco leaf [76], orange juice and in the roots of higher plants [77], [78].

3.2.2 Microbes

All microorganisms, including bacteria, fungus, and yeast, have been found to have phytase activity. Microbial sources are much more feasible for industrial enzyme production as well as commercial level manufacturing [7], [72].

i) Fungi

Fungal sources appear more viable for commercially producing phytases [1], [72]. Fungi phytase is extracellular in nature and formed in enormous quantities [1], [35]. Many fungi phytases studies have already been published on species of genera *Aspergillus* such as *A. ficuum, A. niger, A. oryzae,* and *A. fumigatus* [79]–[82]. The manufacture of fungal phytase has been accomplished via production methodologies such as SSF(solid-state fermentation), which is particularly cost-effective and uses diverse agro-industrial residues as substrates, or submerged fermentation [1]. Many other enzymes (amylase, cellulose, xylanase, and so on) are secreted by these fungi, which enhance the nutritional value and digestion of animals' feed [83]–[86].

ii) Bacteria

Bacteria-released enzymes are primarily cell-associated, and the only bacteria that create extracellular phytases are *Bacillus* [87]–[89]. Bacterial phytases are found in a variety of bacteria, including *Bacillus, Pseudomonas, E. coli, Klebsiella*, and *Lactobacillus* [72], [80], [89]–[92]**.** Bacterial phytases are typically synthesised through submerged fermentation at pH 7.0. A few bacterial phytases, particularly those belonging to the genus *Bacillus*, *Enterobacter*, have optimum pH around 6.0 - 8.0 [80]. As a result, they are more advantageous for application as aquaculture feed additives because their pH optima are close to the working physiological pH of the fishes.

iii) Yeast

Yeast has been discovered to be a significant source of phytase. In SmF systems, yeast phytase production was carried out using strains such as *Schwanniomyces occidentalis, S. castellii, H. polymorph, A. adeninivorans*, and *R. gracilis* [72]. When phytic acid or PO_4^{2-} concentration increased, phytase production decreased in *S. castellii* [72]. Using *Hansenula polymorpha*, an effective approach for pocket-friendly phytase manufacturing was refined. During fermentation, the predominant carbon source was glucose or glucose syrups. Compared to the glycerol method, glucose resulted in a greater than 80% reduction in raw materials cost. Furthermore, extremely high quantities of the activated enzyme were achieved in production media, having phytase accounting for more than 97 % of the total accumulated protein [72].

3.3 Phytase in aquafeed

The nutritional importance of phytate-degradation (myoinositol hexakisphosphate, IP6) is that phytate binding with the metal ion and other nutrient capacity showed decrement and its solvability show increment when PO_4^{2-} are cleaved from the myoinositol ring, it results in increment in bio-availability of necessary diet supplemented nutrients [9], [93]–[95]. Plant phytates are similarly inaccessible to fishes and other monogastric organisms lacking intestine phytases [96]. Therefore, the principal P supply in the aquatic ecosystem only comes from diet. The incorporation of bacterial phytase into aquafeed has been shown to enhance phytate-P utilisation in Freshwater salmon (*Oncorhynchus mykiss*) [97], [98] and Carpe commune (*Cyprinus carpio)* [99], [100]. The enriched experimental feed of *Ictalurus punctatus* had a varying amount of bacterial phytase and observed that bone powder, phosphorus, body growth, and food intake were increased and the feed-transformation ratio

was decreased for aquafeed diets having phytase in comparison to the control group. It was also reported that the P content in faeces decreased straightaway as the degree of phytase addition rose, suggesting the efficiency of phytase in enhancing phytate phosphorus bioavailability.

The supplementation of 250U of microbial phytase/kg of food enhanced phytate-P bioavailability in catfish. Furthermore, fish fed with $CaHPO_4$ had less bone-P concentration than fish provided with microbial phytase, suggesting that iP supplementation in catfish food may be eliminated [101]. A post-pelleting application of 250 FTU/kg food phytase could successfully substitute the $CaPO_4$ addition in Catfish feed without impacting growth, food efficacy, or bone-phosphorus accumulation [102]. It was also said that using phytase as a supplement in catfish food was less expensive than using Pi additions.

Several studies have found that phytase addition improves total Phosphorus availability in fish. These are summarized in the table 2.3.

Table 2.3: Studies showing P availability in fishes with or without phytase.

Organism name	Fish-feed from plant-source	P available (- phytase)	P available (+ phytase)	References
Rainbow trout (*Oncorhynchus mykiss*)	• Canola-meal • Solvent abstracted soybean-meal • Full fat soybean • Peanut-meal • Corn-gluten meal • Cottonseed meal	4.8% (−13.4%) 8.4% 22.1% 30.7% NA	46.2% 46.6% 64.4% 75.6% 76.8% 56.3%	[103]
	• Canola-meal • *Hordeum vulgare* • *Triticum sativum*	12.2% 79.4% 61.6%	41.8% 82.7% 64.6%	[104]
	• Crude soybean • Removed soybean • Crushed full-fat soybean	21.2% NA 12.5%	NA 31.7% (81.3%, 92.2%, 89.7%,	[105]

			95.2%, 93.9%)	
	• *Brassica napus* meal • Soyabean-meal • Corn-gluten meal • *Helianthus* meal • Corn-gluten meal • Lupin-seed meal	(-1.0%) 48.3% 61% (-0.9%) 45% 65.2%	53.8% 85.2% 118% 45.7% 72% 84.6%	[106]
	• Soy protein concentrate • Pea-meal • Faba bean meal	29.9% 74.1% 47.8%	46.9% 80.3% 69.9%	[104]
Nile Tilapia (*Oreochromis niloticus*)	• Soybean-meal • Palm-kernel cake • Rice-bran • Corn-maize • Cassava	47.9% 25.5% 35.2% 23.6% 72.4%	76.9% 50.4% 59.5% 58.3% 92.6%	[107]
Sea bass (*Dicentrarchus labrax*)	• Isolated soy-protein • Soybean-meal • Corn-gluten meal • Wheat-middlings	48% 59% 52% (−10%)	74% 87% 70% 11%	[108]

- **<u>Increased Bioavailability of other nutrients and minerals</u>**

In fish, Bone powder and Phosphorus are a measure of levels of Phosphorus. This is due to fact that the P necessity for maximal bone-mineralizatiom exceeds the maximal body mass growth. Inadequate Phosphorus intake causes P migration from bones and its translocation to fibrous tissues and muscles and metabolism functions [109]. The rise in bone powder in fishes fed with phytase-supplemented diets indicates that dietary manipulation considerably boosted mineral bioavailability [47], [110]**.** A food consuming assessment on Carp fish demonstrated the result of pre-pelletizing moist-incubation of diet plant foodstuffs having 2 distinct phytases, PhyN (Natuphos) and PhyR (Ronozyme), on nutrient-digestion and accumulation in the fishes [111]. Pre-treated foodstuffs with PhyN and PhyR at 4000 FTU/kg levels in both types of feed increased apparent mineral digestion and mineral-accumulation in

aquatic animals, particularly fishes. It enhanced nutrient utilisation, resulting in an increment in fish growth and feed efficiency. Phytase addition in the feed also improves the digestion of minerals linked with phytate. Evidently, the supplementation of phytase to a partial-purified feed comprising fifty percent soybean-meal fed to freshwater salmon considerably enhanced Zn digestibility, but no quantity-level effect was found [112]**.** Furthermore, it has been demonstrated that phytase supplemented in feed increases the overall accessibility of proteins, bone mass, Ca^{2+}, Cu^{+}, Mg^{2+}, $Fe^{+/2+}$, Sr, and $Zn^{+/2+}$ in low mass diets while having minimal outcomes in high mass diets [113].

- **Increased growth performance**

Phytase-enzyme supplemented diets have shown an overall positive impact on the growth performance of fishes observed and demonstrated by several authors summarized in table 2.4. These researchers showed phytate breakdown in plant-based meals using phytase, as well as improved fish growth and mineralization.

Table 2.4: Studies done on fishes reported increased growth in fishes after supplementation with phytase enzyme.

S. No.	Fish name	References
1.	Channel-catfish	[99]
2.	African-catfish	[114]
3.	Striped seabass	[108]
4.	Rainbow-trout	[115]
5.	Pangus catfish	[110]
6.	*Nile tilapia*	[107]
7.	Common-carp	[111]
8.	Rohu	[47]

- **Decreased water pollution**

High quantities of soluble P discharged from aquaculture systems into open freshwater ecosystems encourage phytoplankton development, resulting in broad variations in dissolved-

O_2 (DO) concentrations [101]. Studies showing reduced P excretion from various fishes after phytase-feed supplementation are tabulated below.

Table 2.5: Studies done on fishes reported decreased pollution due to aquaculture after supplementation with phytase enzyme in aquafeed.

S. No.	Fish name	Meal given to fishes	References
1.	Japanese seabass	Diet + Phytase(200FTU/kg)	[116]
2.	Sea bream	Soybean meal + Phytase	[117]
3.	Rainbow trout	Soyabean protein concentrated diet + Phytase	[106], [113]
4.	Atlantic salmon	Soy protein concentrate diet + Phytase	[109]
5.	Juvenile catfish (*Ictalurus punctatus*)	Diet + phytase	[15]

Chapter – 3

Objectives

- None of the phytases from the known ones has all the ideal characteristics like good thermostability, wide pH range and broad substrate specificity; therefore, the search for a new bacterial isolate producing phytase with desired characteristics is an area of continued research interest. Keeping these significant things in view, the objectives were designed as follows:

1. **Revival and maintenance of *Bacillus* spp. Culture.**
2. **Production of extracellular Phytase under optimised conditions.**
3. **Characterization of phytase enzyme.**

Chapter – 4

Materials and Methods

1. MATERIALS AND CHEMICALS

All the materials utilized in the study are given in Table 4.1.

Table 4.1: List of materials utilized in the present study

S. No.	Products	Source
1.	Sodium phytate	Sigma-Aldrich, USA
2.	Ammonium persulphate, agar-agar, sodium chloride, D-glucose, potassium chloride, ferrous sulphate, manganese sulphate, magnesium sulphate, glycerol, ammonium sulphate, potassium dihydrogen phosphate, trichloroacetic acid (TCA), ammonium molybdate, sodium acetate, copper sulphate, sodium potassium tartrate, crystal violet, safranin, malachite green, glucose, Luria broth, calcium chloride, sucrose, ammonium nitrate, tris buffer, sodium hydroxide, glycine, hydrogen peroxide, ferric chloride, methanol, glacial acetic acid, nutrient broth, nutrient agar	Hi-Media, India
3.	Concentrated sulphuric acid, Concentrated hydrochloric acid	Merck Millipore

2. EQUIPMENT USED

Table 4.2: List of equipment used during the study.

Products
1. Autoclave
2. BOD Incubator
3. Microscope
4. pH Meter
5. Spectrophotometer

6. Weighing Balance
7. Ice Machine
8. Microwave
9. Laminar Air Flow
10. Micropipettes
11. Petri Plates

3. MEDIA PREPARATION

3.1 Nutrient Broth

Composition: For 1L

Table 4.3: Composition of NB.

S. No.	Components	Amount used(g/L)
1.	Peptone	5.00
2.	NaCl	5.00
3.	Malt Extract B#	1.50
4.	Yeast Extract	1.50

Methodology: For 100ml media preparation, 1.3g was suspended in 100ml distilled water. The media was then slightly heated to allow complete dissolution. The media was then autoclaved at 121°C, 15psi pressure for about 20 minutes.

3.2 Nutrient Agar

Composition: Final pH at 25°C = 7.0 ± 0.2

Table 4.4: Composition of NA. (For 1L)

S. No.	Components	Amount used(g/L)
1.	Peptone	5.00
2.	NaCl	5.00
3.	Beef Extract	3.00
4.	Agar	15.0

Methodology: For 100ml media preparation, 2.8g was suspended in 100ml distilled water. The media was then slightly heated to allow complete dissolution. The media was then autoclaved at 121°C, 15psi pressure for about 20 minutes. It was then allowed to cool down to about 45-50°C before pouring.

3.3 PSM (Phytase Screening Media) (pH = 6)

Composition: For 1 L

Table 4.5: Composition of PSM

S. No.	Component Name	% Used
1.	D-Glucose	1.5
2.	Ammonium Sulphate [$(NH_4)_2SO_4$]	0.5
3.	Sodium Chloride [NaCl]	0.01
4.	Potassium Chloride [KCl]	0.05
5.	Ferrous Sulphate [$FeSO_4.7H_2O$]	0.001
6.	Magnesium Sulphate [$MgSO_4.7H_2O$]	0.01
7.	Calcium Chloride [$CaCl_2.2H_2O$]	0.01
8.	Manganese Sulphate [$MnSO_4$]	0.001
9.	Sodium Phytate [Na-P]	0.5

Methodology: The components were weighed and added to a flask and then distilled water was added according to the volume required. Then, a microwave was used to slightly warm the media to dissolve the components. pH was adjusted to 6.0 using a pH meter. A cotton plug was used to close the flask and the media was autoclaved.

3.4 PSA (Phytase Screening Agar) (pH = 6)

Composition: PSM + Agar-Agar Type 1 (2%)

Methodology: The components of PSM were added to the flask along with the required volume of distilled water and slightly warmed to dissolve the components completely. pH

was adjusted to 6 by pH meter using 1M HCl and 1N NaOH. 2% Agar-Agar Type 1 was added and then autoclaved.

3.5 Enzyme Production Media

Composition: For 1 L

Table 4.6: Composition of Enzyme Production Media.

S. No.	Component Name	% Used
1.	Wheat Bran	2.5
2.	Ammonium Sulphate [$(NH_4)SO_4$]	1.5
3.	Sodium Chloride [NaCl]	0.01
4.	Potassium Chloride [KCl]	0.05
5.	Ferrous Sulphate [$FeSO_4.7H_2O$]	0.001
6.	Magnesium Sulphate [$MgSO_4.7H_2O$]	0.01
7.	Calcium Chloride [$CaCl_2.2H_2O$]	0.01
8.	Manganese Sulphate [$MnSO_4$]	0.001

Note: Wheat bran is added last.

4. REAGENT PREPARATION

4.1 Bradford Reagent for Protein Estimation Assay

Composition:

(a) Coomassie Brilliant Blue G-250
(b) Ethanol (95%)
(c) Phosphoric acid
(d) Distilled water
(e) Whatman filter paper

Methodology: 100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol, then 100 ml 85% (w/v) phosphoric acid was added. It was then diluted to 1L when the dye had completely dissolved, and then it was filtered through Whatman filter paper just before use.

4.2 Colouring Reagent for Enzymatic Assay

Composition: For 50ml

Table 4.7: Composition of colouring reagent.

S. No.	Name od Component	Amount/Volume added
1.	Distilled water	47.5 ml
2.	Conc. H_2SO_4	2.5 ml
3.	$FeSO_4.7H_2O$	3.6g
4.	Ammonium Molybdate	0.5g

Methodology: The components were added in the same sequence as in Table 4.7.

4.3 10% Trichloroacetic acid (TCA)

Composition and Methodology: 1.5g of TCA is weighed and dissolved in 150ml distilled water.

5. METHODOLOGY

5.1 Revival of *Bacillus* culture from glycerol stock

Procedure:

Glycerol stock of *Bacillus* spp. was taken out to room temperature in LAF. Four test tubes containing 10 ml NB (Nutrient Broth) were prepared, autoclaved and cooled down to room temperature. Media in each test tube was inoculated with 100µl of glycerol stocked culture of bacteria inside LAF. In an incubator, the test tubes were incubated at 37◦C and under shaking conditions of 150 rpm. After 24 hours, subculturing was done in other autoclaved 10ml NB media test tubes and then incubated at 37◦C and shaking at 150rpm in an incubator shaker.

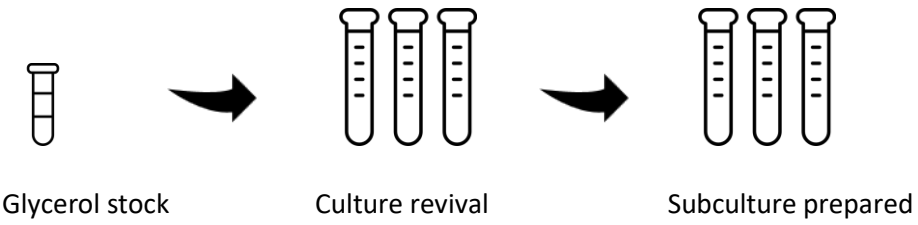
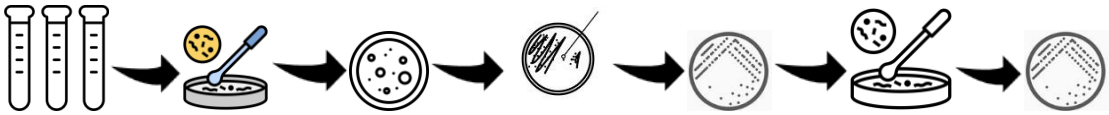


Figure 4.1: Culture revival from glycerol stock

Preparation of Pure culture and Activation of culture in PSM

Procedure:

NA (Nutrient Agar) was prepared, autoclaved and poured into petri-plates inside LAF. The media was allowed to solidify for 10-15 min. 10µl culture was taken from subculture test tubes and spreading was done on the NA plates. The petri-plates were then incubated for about 24 hours at a temperature of 37◦C in the incubator. After 24 hours, the inoculum was taken from spread plates and quadrant streaking was done on other NA plates and incubated at 37◦C in the incubator for 24 hours. Also, **Gram staining** [Thin smears of bacterial isolates were taken on clean glass slides and then heat fixed. The smears were then flooded with crystal violet for 1 min and rinsed out with distilled water. A few drops of the mordant (Gram's iodine solution) were added to the smear and left undisturbed for 1 min. After again rinsing with distilled water, the decolourizer or ethyl alcohol was added dropwise for 10-15 sec. The smear was then counter-stained with safranin for 30 sec, rinsed with distilled water, air-dried and observed under the microscope in oil immersion.] was done for biochemical identification of the *Bacillus* bacteria from spread plates. From the plated with quadrant streaking, single isolated colonies were chosen and transferred to 10ml PSM containing test tubes (pure culture) and then incubated at a temperature 37◦C in the incubator for about 24 hours. The inoculum was taken from these PSM tubes and Quadrant streaking was done on earlier prepared 3 PSA plates. Point inoculation on 1 PSA plate was done for Qualitative screening.



Subculture NA plates PSA plates

Figure 4.2: Pure culture preparation and activation on PSA.

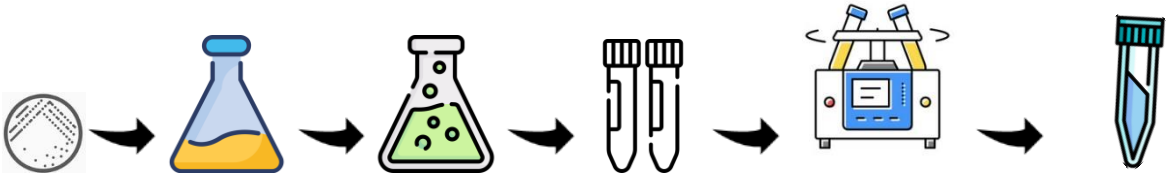
5.2 Production media setup, Enzyme production and extraction

Procedure:

The bacterial culture was grown in a previously optimized submerged fermentation medium (pH-7) having 24-hour old inoculum (having media pH 6), supplemented with 2.5% wheat

bran and 1.5% ammonium sulphate; incubated for 24 hours at 37◦C, 150rpm in BOD incubator and shaker.

Production media was set up in two 250ml flasks containing 50 ml autoclaved production media in each. These were then inoculated with 1% inoculum and incubation was done at 40◦C for 24 hours at 150 rpm in the incubator shaker. After fermentation, the culture was centrifuged at 8000 rpm at 4 ºC for 10 min and cell-free supernatant was assayed for phytase activity. The inorganic phosphate released by phytase was measured using the classic Fiske and Subbarow method [118].



Culture taken | Media | After Fermentation | Centrifugation | Supernatant

Figure 4.3: Production setup and extraction of the enzyme.

5.3 Protein assay (Bradford method)

Procedure:

Eppendorf tubes (2ml) were taken and labelled as blank and 1 to 10. Dilution of protein (BSA) standards from stock solution of concentration 1mg/ml into 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 µg/µl were made by transferring respective amount of BSA and adjusting it to a total volume of 1000 µl by adding distilled water and 1000 µl sample in 3 tubes. 1ml of Bradford reagent was added to each Eppendorf tube, including the blank and other tubes, the content of each tube was mixed thoroughly by vortexing the tubes and then incubated at RT for 10 min. In the spectrophotometer, the wavelength was selected at 595 nm and the absorbance (OD) was recorded, followed by plotting a standard graph of Concentration and absorbance for BSA samples. The protein concentration in the sample was calculated using the standard plot. All tests were carried out in triplicate.

5.4 Enzymatic assay (Phytase assay by Fiske and Subbarow method [118])

Procedure:

Eppendorf tubes (2ml) were taken and labelled them as blank and 1 to 10. Make dilution of KH_2PO_4 standards from stock solution of concentration 1mg/ml into 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 µg/µl by transferring respective amount of KH_2PO_4 and adjusting it to a total volume of 1000 µl by adding distilled water. 900 µl of 1mM Na-Phytate in acetate buffer (0.1mM, pH = 5) was taken in 3 tubes and 100 µl of the crude enzyme was added to each. 250 µl of 10% TCA was added, followed by 1000 µl of colouring reagent to each Eppendorf tube, including the blank and other tubes, the content of each tube was mixed thoroughly by vortexing the tubes and then incubated at RT for 10 min. In the spectrophotometer, the wavelength was selected at 750 nm and the absorbance (OD) was recorded and a standard graph of concentration and absorbance for KH_2PO_4 was plotted. Using this standard plot, the concentration of released phosphorus and enzyme activity were calculated. All tests were carried out in triplicate.

5.5 Characterization of phytase

5.5.1 Optimization of pH

The effect of pH on enzyme activity was studied by using buffers (0.1 M) in the reaction mixture of desired pH, glycine-HCl buffer (pH 2.0-3.0), sodium acetate buffer (pH 4.0-5.0), Tris-HCl buffer (pH 6.0-8.0) and Glycine-NaOH buffer (pH 9.0-10.0). The Sodium phytate substrate was prepared in different pH and phytase assay was carried out. All tests were carried out in triplicate.

5.5.2 Optimization of Temperature

To determine the effect of temperature on phytase activity, the reaction was carried out at different temperatures ranging from 30^{o}C-60^{o}C at optimized pH (7) for 30 minutes and carried out the phytase assay. O.D. values were measured at 750nm. All tests were carried out in triplicate.

Chapter – 5

Results and Discussion

1. Revival of bacterial culture from glycerol stock

Inoculum from glycerol stock was cultured in test tubes containing 10ml NB media. From these tubes, subculturing was done in other test tubes containing 10ml NB media. NA plates were prepared and streaked by the subculture prepared.

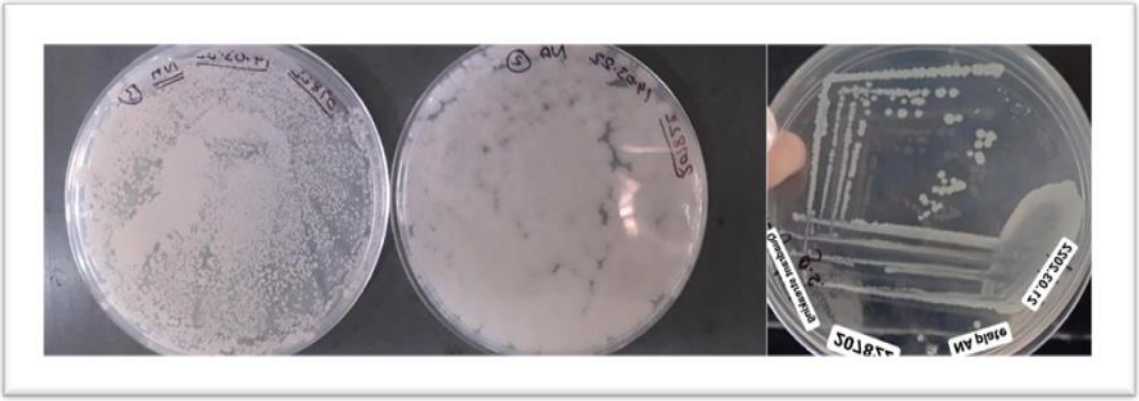


Figure 5.1: Preparation on NA plates (Spread plate and quadrant streaking)

The pure culture was obtained from NA plates and used for further experimentation. Glycerol stocks were also prepared from the pure culture.

2. Identification and Qualitative Screening of bacterial isolate for extracellular phytase production

Based on colony morphology and Gram's staining, it was observed that bacterial colonies are Gram-positive, rod-shaped *Bacilli* having white-coloured colonies firmly adhering to media on plates.

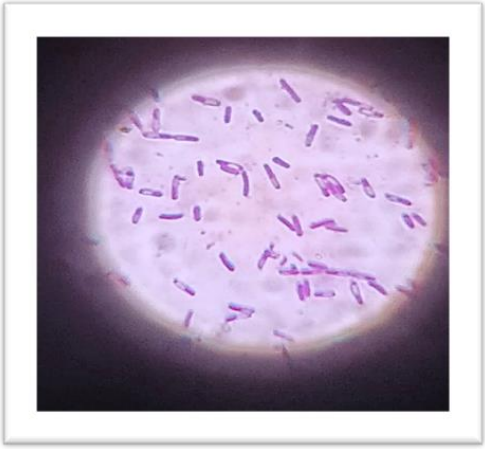


Figure 5.2: Gram staining of the selected bacterial colony under the light microscope showing Gram-positive, rod-shaped bacteria.

The first screening approach used in the screening of phytase producers is critical in distinguishing between phytase-generating and non-producing bacteria. On PSM plates containing sodium phytate, all bacterial isolates were screened.

3. Submerged Fermentation

3.1. Enzyme Production

Submerged fermentation was carried out in two flasks under optimum enzyme production conditions [Production media (pH-7) having 24-hour old inoculum (having media pH 6), supplemented with 2.5% wheat bran and 1.5% ammonium sulphate; incubated for 24 hours at 37◦C, 150rpm in BOD incubator and shaker] resulting in phytase production. After fermentation, the culture was centrifuged at 8000 rpm at 4 ºC for 10 min and cell-free supernatant was assayed for total protein estimation and phytase activity.

Figure 5.3: Cell-free supernatant.

3.2. Total Protein Estimation

Quantitative estimation of soluble protein was done by the method of Bradford using bovine serum albumin (BSA) as standard. After mixing all reagents, the tubes were incubated in the dark for 10 minutes. The absorbance was measured at 595nm in a Multiskan spectrophotometer. The table for the standard reaction is as follows:

Table 5.1: Observed O.D. values corresponding to different concentrations of BSA and crude enzyme samples.

S. No.	Concentration (mg/ml)	O.D. at 595nm			Average O.D. – Blank
		O.D.1	O.D.2	0.D.3	
1.	0	0.25	0.25	0.25	0
2.	0.1	0.29	0.32	0.27	0.04
3.	0.2	0.31	0.31	0.31	0.06
4.	0.3	0.32	0.34	0.33	0.07
5.	0.4	0.34	0.33	0.33	0.08
6.	0.5	0.36	0.34	0.35	0.10
7.	0.6	0.37	0.31	0.38	0.11
8.	0.7	0.38	0.39	0.37	0.13
9.	0.8	0.39	0.41	0.38	0.14
10.	0.9	0.39	0.40	0.41	0.15
11.	1.0	0.38	0.41	0.43	0.16
12.	Sample 1	0.49	0.42	0.46	0.14
13.	Sample 2	0.45	0.37	0.51	0.12

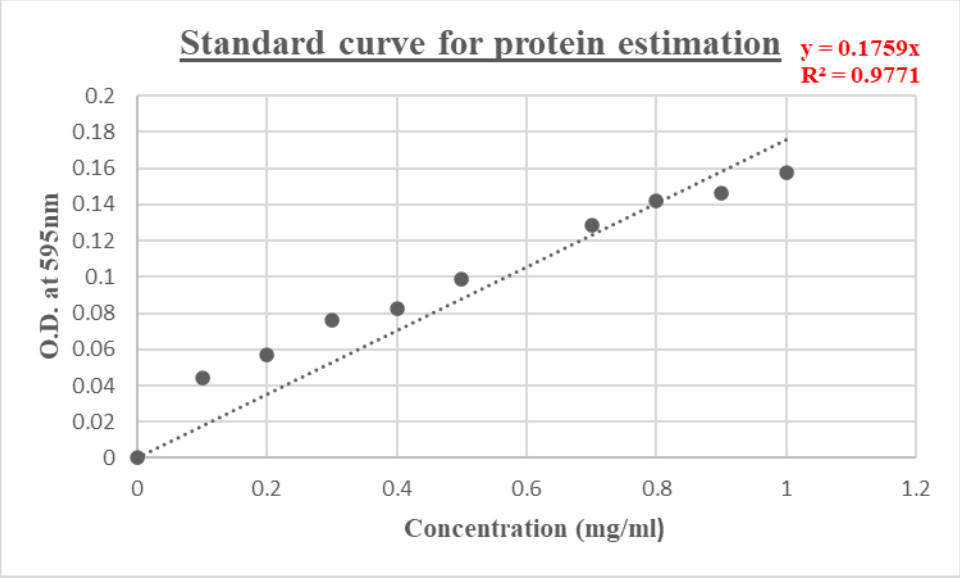


Figure 5.4: Standard graph for protein estimation by Bradford method.

From the standard curve the protein concentration in crude sample came out to be:

Crude enzyme from flask 1 = 0.826 mg/ml

Crude enzyme from flask 2 = 0.657 mg/ml

3. Enzyme Activity and Specific Activity of phytase

The concentration of released phosphorus was determined using the standard curve of KH_2PO_4. The quantitative determination of phytase activity was measured by the release of (Pi) using Na-phytate as the substrate (750-nm). Under the specified circumstances, one unit of phytase activity is defined as the quantity of enzyme necessary to release one µM of inorganic-phosphorus/ml/minute. Phytase activity in SmF is measured in units per volume (U/ml). All tests were carried out in triplicate.

Table 5.2: Observed O.D. values corresponding to different concentrations of KH_2PO_4 and crude enzyme samples.

S. No.	Concentration Of KH_2PO_4 (mg/ml)	O.D. at 750nm			Average O.D.	Concentration of P liberated [From standard graph(mg/ml)]
		O.D.1	O.D.2	0.D.3		
1.	0(Blank)	0	0	0	0	
2.	0.1	0.57	0.58	0.56	0.57	
3.	0.2	1.07	1.03	1.05	1.05	
4.	0.3	1.48	1.45	1.51	1.48	
5.	0.4	1.86	1.78	1.82	1.82	
6.	0.5	1.93	1.94	1.92	1.85	
7.	0.6	1.94	1.92	1.93	1.93	
8.	0.7	1.92	1.93	1.94	1.93	
9.	0.8	1.94	1.92	1.93	1.93	
10.	0.9	1.93	1.92	1.94	1.93	
11.	1.0	1.99	2.3	2.1	2.13	
12.	Sample 1	0.63	0.66	0.60	0.628	0.131
13.	Sample 2	0.69	0.64	0.69	0.674	0.141

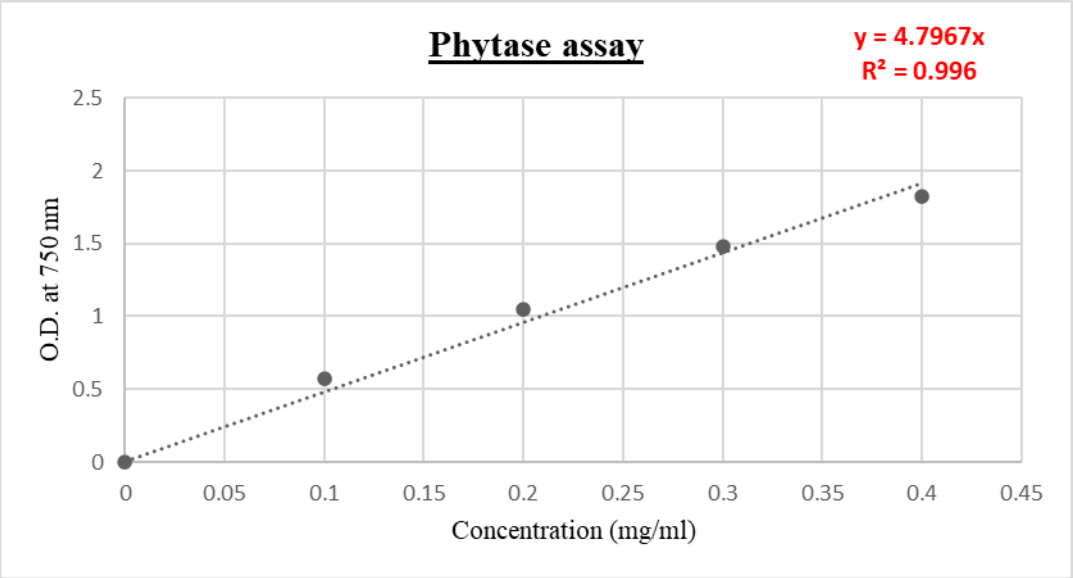


Figure 5.5: Standard graph for Phytase assay by Fiske and Subbarow method.

From the standard curve:

From the standard curve:

Y = m*X (straight line equation)

Therefore, X = Y/m

- **Enzyme activity** (U/ml) =

[X*Reaction volume*1000] / [Molecular weight*Time*Volume of enzyme used]

- **Specific Activity** (U/mg) = **Enzyme Activity** (U/ml) **/ Protein concentration** (mg/ml)

Table 5.3: Enzyme Activity, Protein concentration and Specific Activity of crude enzyme.

S. No.	Crude Sample	Enzyme Activity (U/ml)	Protein Concentration (mg/ml)	Specific Activity (U/mg)
1.	1	0.32	0.83	0.40
2.	2	0.35	0.70	0.53

4. Characterization of Phytase – Enzyme Kinetics

1. Effect of pH on crude phytase activity

The nature of the amino acids in the active site, which undergo protonation and deprotonation resulting in conformational changes generated by ionisation of these amino acids, determines the effect of pH on enzyme activity. Enzymatic properties influence a phytase's capability to metabolise phytate in the gastrointestinal tract. Here, the *Bacillus* Phytase displayed optimal activity at pH 7.0 (Figure 5.6). There was 69.4 % enzyme activity present at pH 8.0. Therefore, it has potential application as aquafeed supplement.

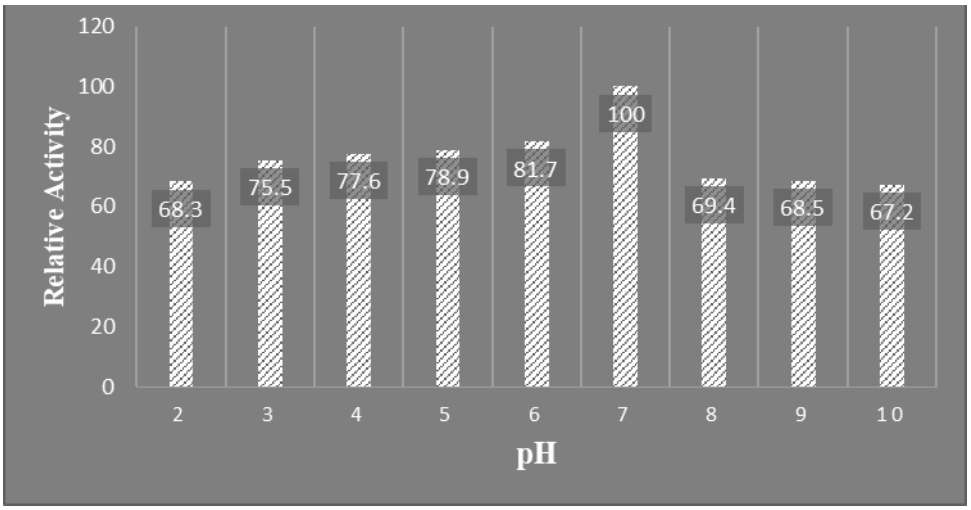


Figure 5.6: Effect of pH on phytase activity.

2. Effect of temperature on crude phytase activity

The activity of phytases is greatly influenced by temperature. In general, phytases can retain high activity at temperatures ranging from 50 to 70 °C. In most instances, their activity is maintained around the 45-60 °C range. *Bacillus* phytase is more thermally stable than acid phytases produced from fungus and *E. coli*. Because of this benefit, Bacillus phytase can be employed as an appropriate aquafeed supplement by preventing enzyme degradation due to high temperatures during feed pelleting or expansion. Here, the *Bacillus* phytase exhibited optimal activity at 50˚C. At 60˚C, about 86.6 % of the activity was retained (Figure 5.7).

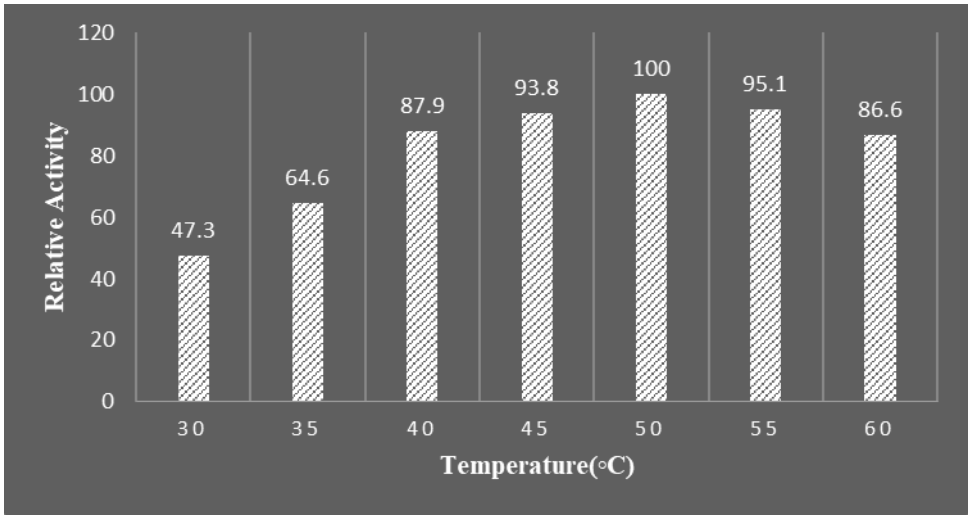


Figure 5.7: Effect of temperature on phytase activity.

Chapter – 6

Conclusion

Phytases catalyze the removal of antinutritional characteristics of phytic acid in various ways, and the liberated phosphorus can be utilised in metabolic processes, resulting in decreased phosphorus pollution. Commercially, microbial phytase synthesis is more promising than non-microbial sources. Because physical and chemical approaches lowered the nutritional content of food and feed during phytate removal, enzymatic hydrolysis using phytases is preferable. Thus, phytase has potential uses in the food and feed sectors for reducing the antinutritional impact of phytic acid while increasing nutrient bioavailability.

In the present study, *Bacillus* spp. was revived from the glycerol stock in nutrient broth. The bacteria were then screened qualitatively by Gram staining and growing on phytase screening media (PSM).

Phytase enzyme was produced from *Bacillus* spp. in production media supplemented with wheat bran (2.5%) by submerged fermentation at 37^{o}C. The protein concentration was then estimated by the Bradford method, followed by enzyme activity and specific activity calculations using Phytase assay (colourimetric method of inorganic phosphorus determination by Fiske and Subbarow), which came out to be approx. 0.3 U/ml and 0.5 U/mg respectively. Crude phytase showed optimum activity at pH 7.0 and temperature 50^{o}C.

From the present investigation the important conclusions are as follows:

The total estimated protein (Phytase enzyme) was 0.826 mg/ml and 0.657 mg/ml from crude enzyme Sample 1 and 2, respectively.

- The crude enzyme from *Bacillus* spp. showed the following enzyme activity and specific activity:

Table 6.1: Enzyme Activity and Specific Activity of crude enzyme sample.

Crude enzyme Sample	Enzyme Activity (U/ml)	Specific Activity (U/mg)
1	0.321	0.388
2	0.345	0.525

- Crude phytase of *Bacillus* spp. was optimally active at pH 7.0 and temperature 50^{o}C.

Chapter – 7

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