Production, characterization of bacterial Phytase and Its applications

Project report submitted in partial fulfillment of the requirement for the degree of

Bachelor of Technology in Biotechnology

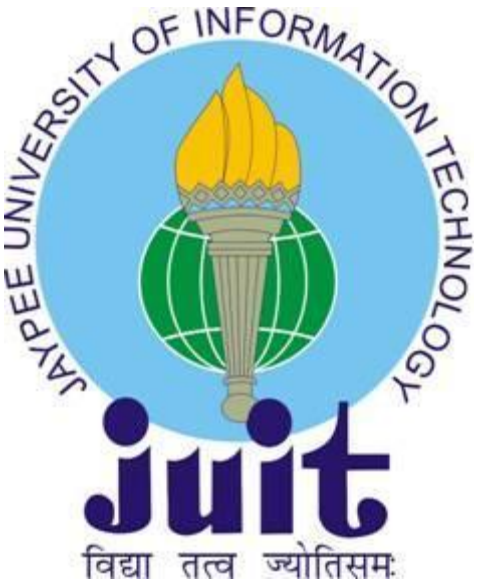
By

Muskan Sharma (201811)

Under the supervision of

Dr. Saurabh Bansal

To



Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology Waknaghat, Solan-173234, Himachal Pradesh

Certificate

This is to certify that the work reported in the B.Tech Project report **"Production, characterisation of Bacterial Phytase and its applications"** submitted by **Ms Muskan Sharma** at Jaypee University of Information Technology, Waknaghat, India, is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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Date : 20 May, 2024

Candidate's Declaration

I hereby declare that the work presented in this report entitled " **Production, characterization of bacterial Phytase and Its applications**" in partial fulfillment of the requirements for the award of the degree of Bachelor of Technology in Biotechnology submitted in the Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology Waknaghat is an authentic record of my own work carried out over a period from **August 2023 to June 2024** under the supervision of **Dr. Saurabh Bansal (Associate Professor (Biotechnology & Bioinformatics)**. I also authenticate that I have carried out the above mentioned project work under the proficiency Stream "**Medical Biotechnology**". The matter embodied in the report has not been submitted for the award of any other degree or diploma.

Muskan Sharma, 201811

This is to certify that the above statement made by the candidate is true to the best of my knowledge.

Dr. Saurabh Bansal

Associate Professor

Department of Biotechnology & Bioinformatics

Acknowledgement

I want to sincerely thank my supervisor, Dr. Saurabh Bansal, for his invaluable advice and assistance in seeing my project through to completion. He was there to help me at every stage, and it was his motivation that made it possible for me to complete my project successfully. I also want to express my gratitude to all the other support staff members who helped me by providing the tools I needed to complete this project successfully. Without their help, I would not have been able to complete my project efficiently.

I also like to express my gratitude to the Jaypee University of Information and Technology for approving my project in my area of interest. As I worked on this project, I'd also like to thank my parents and friends for their encouragement and support.

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Abstract

The anti-nutritional activity of phytic acid is eliminated by reducing it with the aid of phytases, an enzyme that hydrolyses phytic acid. Because of this characteristic, phytases can be utilised as a variety of feed and food additives. Phytase can be obtained from various sources like animals, plants and microbes.

In this study the bacillus that was isolated from the environment was studied. The culture was revived, enzyme production was done and some tests were done in order to characterize the species of bacteria that produced the enzyme phytase. After the enzyme was produced its protein concentration was estimated using Bradford reagent assay.

The bacterial species came out to be gram positive and it produced maximum enzyme when pH was 6 and the growth conditions were 35^0C and 150 RPM.

Chapter 1: Introduction

Phytic acid

Phytic acid, sometimes referred to as inositol hexaphosphate, or IP6, is a substance that is present in whole grains, legumes, plant seeds and nuts. It serves as these plants primary phosphorus storage form, making it vital to their development and advancement.

Phytic acid, however, has also generated a great deal of discussion because of its possible effects on the body's absorption of minerals. Because of its capacity to bind to minerals and decrease their bioavailability, some regard it as an antinutrient; yet, others are aware of its possible health advantages. This article explores the characteristics, impact on nutrient absorption, and possible health implications of phytic acid, delving into its complexity. Phytic acid is made up of six phosphate groups that are joined to an inositol ring. These phosphate groups provide it the capacity to chelate minerals, especially divalent cations including iron, zinc, calcium, and magnesium. Phytic acid creates insoluble compounds with minerals that are difficult for the human digestive system to absorb. Concerns with the use of plant-based foods rich in phytic acid have been increased due to the lower bioavailability of minerals, especially in areas where dietary intake of these vital nutrients is inadequate.

Cereal grains, pollen spores, oilseeds, nuts, legumes tubers and pollen spores contain phytic acid. It serves as the main source of phosphorus, making up as much as 85% of the total P content of cereals and legumes. The majority of total P (60–97%) in cereal grains, oilseeds, and legumes is made up of phytate P; in roots and tubers, phytates may make up 21–25% of total P. Phytic acid can range in concentration and form from 0.4% to 10.7% by weight. Phytic acid can be used in the process of seed germination. By meeting the growing tissues' needs for biosynthesis, it promotes the growth of seedlings. Myo-inositol, one of the byproducts of PA hydrolysis, is used by young seedlings to form their cell walls. The availability of phosphorus and the amount of phytic acid in cereal grains can be influenced by a variety of factors. Genetics, climatic variations, sites, irrigation settings, soil type, time of year, and fertiliser application can all affect phytates. The amount of P2O5 given to the plant raises the percentage of total P that is found as phytic P. Phytic acid appears to be the plant's way of storing excess P when it gets more than it needs. When seeds develop, they accumulate phytic acid, which varies in concentration as the kernels ripen and reaches its peak when the kernels are fully developed (1).

As a result of its capacity to bind to minerals, especially iron, zinc, calcium, and magnesium, phytic acid has caused worries that it may lessen the bioavailability of these vital nutrients. These minerals are less easily absorbed by the body when they are linked to phytic acid. People who consume a diet high in foods high in phytic acid or who have minimal mineral intakes may find this to be especially problematic. High phytic acid consumption has been linked to decreased absorption of iron and zinc, which may raise the risk of deficiencies, according to studies. These inadequacies may lead to negative outcomes such as compromised immune system, compromised cognitive function, and heightened vulnerability to infections. Despite being classified as an antinutrient, phytic acid may have a number of health advantages, according to recent studies. It has been demonstrated that phytic acid possesses antioxidant activity, scavenging dangerous free radicals that may be linked to chronic illnesses and cellular damage. Research suggests that phytic acid may possess anticancer characteristics, with the potential to inhibit tumour growth and metastasis. By lowering LDL (bad) cholesterol and preventing atherosclerosis, or the accumulation of plaque in the arteries, phytic acid may help to maintain heart health.

Phytase

Legumes, nuts, plant seeds contain phytic acid, which is broken down by the enzyme phytase. In addition to binding to minerals like iron, zinc, magnesium and calcium, to reduce their availability for cellular absorption, phytic acid is a plant-based phosphorus store. Because it increases these vital minerals bioavailability, phytase is an important component of nutrition.

Phytic acid can be hydrolyzed by phytases to produce myo-inositol and inositol phosphates (IP1–IP5). Phytases come in two kinds: 3-phytase, which is unique to microorganisms, and 6-phytase, which is present in higher plants' seeds. Phytase's activities are all influenced by pH, with the maximum activity occurring at a slightly acidic pH of about 5.1. Because there is not enough moisture in dry cereals for the phytases to be activated, they are mainly found in the aleurone layers of the cereal grains.

Most phytases are exclusively present in microbes and plants. On the basis of chemical properties, they are further divided into groups such as cysteine phosphatases, purple acid phosphatases, histidine acid phosphatases and β-propeller phytase; additionally, they are classified according to the stereospecificity of phytate hydrolysis (alkaline or acid phytases).

One of the biggest developments in poultry nutrition in recent years has been praised: exogenous phytases. By reducing phosphorus excretion in manure, exogenous phytases can minimise environmental phosphorus pollution, improve phosphorus and calcium (Ca) utilisation, reduce the anti-nutritional impact of phytate, and improve nutrient digestibility [[1], [2], [3]].

Plants and microorganisms like fungi, bacteria and yeast have been found to contain phytase genes and proteins. The most effective, environmentally benign, economically stable, and prospective bioinoculants are microbial phytases. They are also the phytase determinant that is most frequently used. The sort of substrate, the desired final product, and the surrounding conditions are taken into consideration when screening microorganisms. Well-known phytase-producing microbes with unusual dietary requirements or complex requirements are known as thermophilic fungi [[4], [5], [6]]. Filamentous fungi are regarded as advantageous sources of phytase for industrial production due to their notable production of phytase [7]. The development of science might lead to fresh perspectives on the large-scale extraction and synthesis, purification, characterization, and use of phytases. By substituting chemical additives for increased yield, modular genetic engineering tools could promote sustainability.

Chapter 2: Literature Review

2.1 Phytic acid

Phytic acid, also referred to as myo-inositol-1,2,3,4,5,6-hexakisphosphoric acid (IP6), is a common component of plants and the main means of storing inositol and phosphate in plant seeds and grains that build up during process of maturity.[8] The twelve ionizable protons in phytic acid provide a distinct structure that gives it its distinctive qualities, particularly the capacity to form chelates with polyvalent metal ions, including calcium, zinc and iron which produce phytates, which are insoluble salts.[9,10] Other substances like pentaphosphate (IP5), tetra-(IP4), and inositol tri-(IP3) are also referred to as he phytates.

Phytic acid is sometimes called an anti-nutrient because it chelates the micronutrients in food, making them less bioavailable and consequently not able to be absorbed.[11] Nonetheless, diets that are both high in phytic acid and low in trace elements are primarily associated with this effect. The micronutrients' ability to bind with other food ingredients may also affect bioavailability; additionally, the molar ratio of metal ions to phytic acid should be taken into account.

Because it can bind iron to form an entirely inactive chelate, phytic acid has a strong antioxidant effect that can stop iron catalysis from forming hydroxyl radicals (•OH). Since it is crucial to the preservation of living systems like plant seeds, it has been demonstrated through the use of in vitro biochemical systems.

Sources and Structure

Phytic acid, or myo-inositol-1,2,3,4,5,6-hexakisphosphate, is one of the many derivatives of myo-inositol (Ins) phosphorylation. It is characterised as a cyclic alcohol that provides the carbon backbones needed for phytic acid production.[12] Phytic acid's molecular formula is $C_6H_{18}O_{24}P_6$, and its molar mass is 660.04 g mol^{-1}. X-ray crystallographic study revealed that the phosphate groups are equatorial connected to carbon 2 and along the axis bonded to carbons 1, 3, 4, 5, and 6.[13] Phytic acid is an extremely stable and inert chemical that does not break down when left as a solid in neutral or alkaline fluid solutions for months or even years.[10]

Water-insoluble phytate salts are produced when such cations are chelated by phytic acid.[14] The most prevalent cations are K^+, Mg^{2+}, and Ca^{2+}, which are mono and divalent.[15] Protein and starch can bind to phytic acid either directly or indirectly. For a

while, phytic acid was thought of as an antinutrient. However, numerous investigations using both human and animal models have shown that phytic acid can prevent a variety of diseases.

Decreased myo-inositol concentrations containing fewer phosphate groups (IP1–5) are essential for controlling essential biological processes like cellular differentiation, exocytosis and endocytosis. Many eukaryotic species contain large amounts of phytic acid [16].

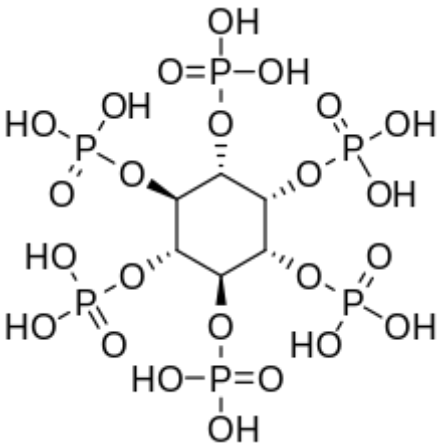

Fig1. Phytic acid

Phytic acid can be found in most nuts, pollen, oilseeds, cereals and legumes [10]. It accounts for 50% to 80% of the total phosphorus content in seeds.[16] In nurturing seeds, phosphorus accumulates at a rate greater than that required for small cellular functions. A number of plants produce phytic acid by utilising the extra phosphorus.[12] Certain vegetables and fruits, like bananas and carrots, have been found to have a lower phytic acid content. The most phytic acid concentrations, however, are found in the ground cereal fractions, particularly in rice bran, in which the phytic acid content is noticeably higher than in brown and polished rice.

Germ, endosperm, and bran make up whole grains like brown rice.[17] 5% to 8% of the weight of all rice grains is made up of the starchy endosperm, germ, aleurone, testa, mesocarp, and epicarp, which together make up the bran 1.2% of the organic phosphorus in rice bran is found in the endosperm, 7.6% in the germ, and 80% in the pericarp and aleurone.[18] In other cereal analyses, the main locations for phytic acid are the germ (88%),

endosperm (3.20%), and hull (0.40%) of maize; in wheat, the main locations are the germ (12.9%), endosperm (2.20%), and aleurone (87%).[19]

Chemical reaction

Phytic acid can undergo certain chemical reactions like:

Hydrolysis: The enzyme phytase has the ability to hydrolyze phytic acid, releasing inositol and dissolving the phosphate groups. The release of phosphorus from plant seeds during germination depends on this reaction.

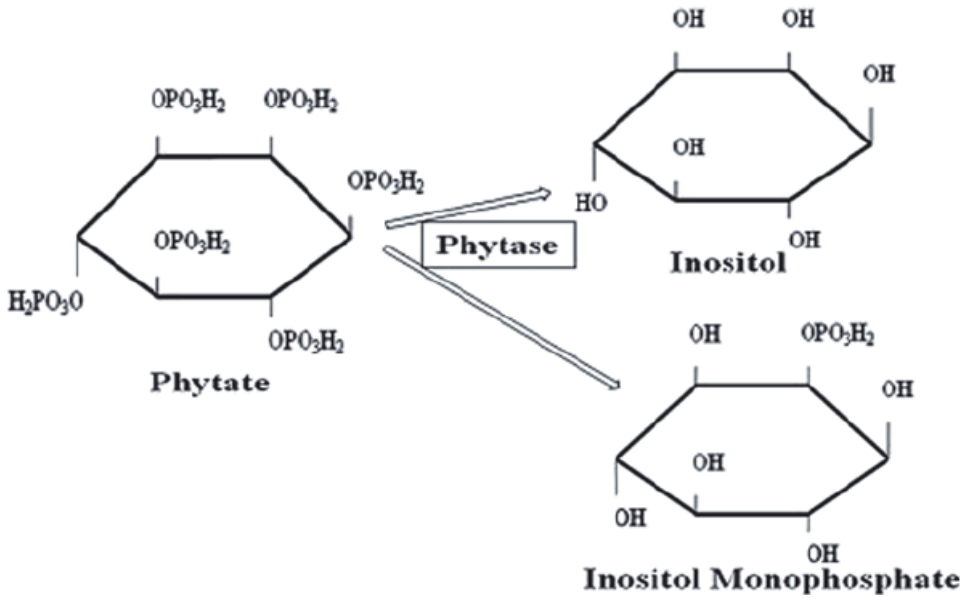

Fig2. Hydrolysis by phytic acid

Oxidation: Reactive oxygen species (ROS) have the ability to oxidise phytic acid and produce inositol phosphates. Antioxidant and anticancer qualities are present in these inositol phosphates.

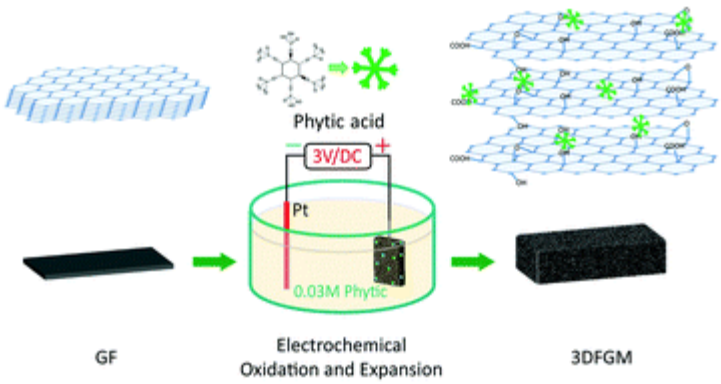

Fig3. Oxidation by phytic acid

Complexation: Phytic acid can combine with iron, zinc, calcium, and magnesium to form complexes. These complexes may lessen the minerals' bioavailability, which would hinder the body's ability to absorb them.

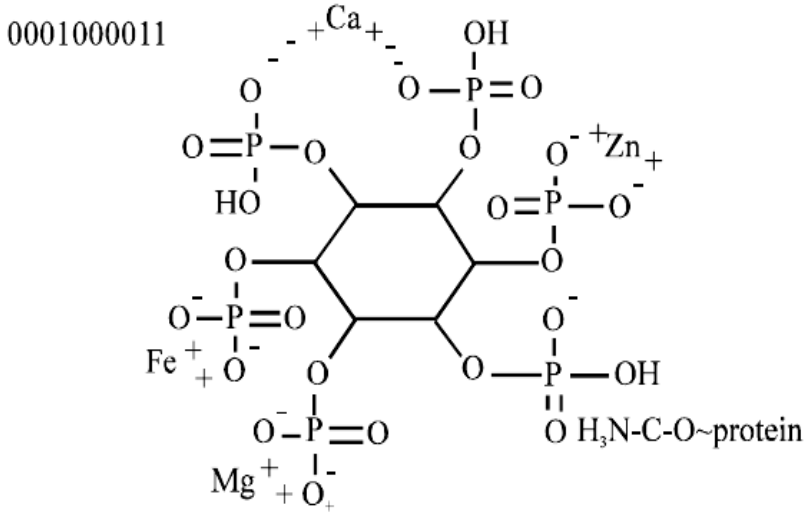

Fig4. Complexation by phytic acid

Decarboxylation: Inositol penraphosphate (IP5), inositol tetraphosphate (IP4), inositol triphosphate (IP3), inositol bisphosphate (IP2), and inositol monophosphate (IP1) can all be produced by decarboxylating phytic acid. These inositol phosphates are involved in cell division and signalling pathways, among other biological processes.

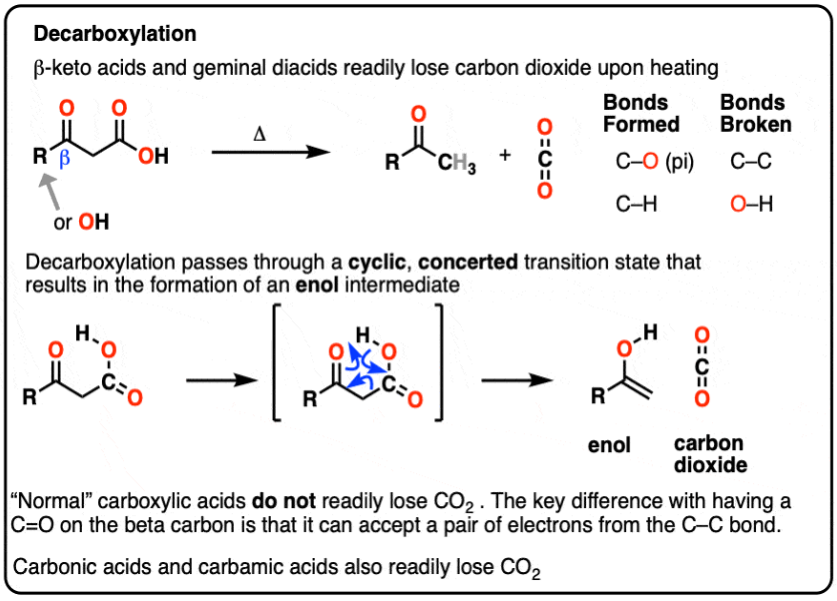

Fig5. Decarboxylation by phytic acid

Biosynthesis of phytic acid:

Many factors can affect how phytic acid is biosynthesised in grains and seeds, including the location of the crop being grown, differences in the climate and environment, type of soil, use of fertilisers, irrigation conditions, and genotypic variation in the plants [8,11]. They discovered that during seed maturation, the concentration of phytic acid was 8.8 mg germination time may have an impact on the amount of phytic acid present,[24]. In particular, they discovered that the phytic acid content of chickpea seeds decreased from 1.01% to 0.60% following 48 hours of germination. Phytic acid biosynthesis and development can be seen a few days after flowering and proceed through seed growth and maturity.[20] Protein bodies contain globoids that hold the synthesised phytic acid, along with its salt and protein complexes.

Myo-inositol-3-phosphate synthase (MIPS) catalyses the conversion of glucose 6-phosphate to myoinositol-3-phosphate (InsP3s), which is the initial step in the synthesis process.[20] There are two possible pathways for synthesising phytic acid in the subsequent steps: lipid-independent and lipid-dependent [14], with the lipid-independent pathway being the most prevalent and found in legumes and cereals.[20] In this course, inositol tris/tetrakisphosphate kinase (ITPK), also called inositol-1,3,4-triskisphosphate 5/6-kinase (ITP5/6 K), catalyses the conversion of inositol-3,3,4,5,6-5-phosphate [Ins(1,3,4,5,6)P5] from inositol-3s and myo-inositol-4-phosphate (InsP4s).[20] Lastly, the enzyme inositol

pentakisphosphate 2-kinase (IPK1) converts Ins(1,3,4,5,6)P5 into phytic acid in the last stage of phytic acid synthesis.

Myo-inositol-(1,4,5)-3-phosphate synthesis from phosphatidylinositol-4,5-bisphosphate is catalysed by phospholipase C (PLC) in the lipid-dependent pathway. Enzymes inositol 1,4,5-trisphosphate kinase (IPK2) and inositol 1,3,4,5,6-pentakisphosphate 2-kinase (IPK1) then catalyses the production of phytic acid.[21]

2.2 Phytase:

The hydrolysis of phytic acid, an indigestible type of phosphorus present in plant-based feedstuffs, is catalysed by the enzyme phytase. Phytase releases inorganic phosphorus, which animals can easily absorb and use, by dissolving phytic acid. The nutrition and general health of animals will greatly benefit from this.

Classification:

Phytases are a broad class of enzymes which can be categorised depending on several elements, including substrate specificity, pH optimal values, and catalytic mechanism.

Substrate specificity:

Phytases can also be categorised according to how specific they are for different types of phytic acid, a property known as substrate specificity. Based on substrate specificity, phytase can be divided into two major categories:

3. Phytases: These enzymes specifically break down the phosphate group that is affixed to the inositol ring's third carbon in phytic acid.

6-Phytases: These enzymes specifically break down the phosphate group that is affixed to the inositol ring's sixth carbon in phytic acid.

pH optimal values:

Phytases can also be categorised according to the pH range in which they are most engaged, or according to their ideal pH. According to pH optimal values, phytase falls into three major categories:

Acid Phytases: Acid phytases work best in environments that are acidic, usually with a pH of 4.0 to 5.5.

Neutral Phytases: Neutral phytases are most active at pH values that are neutral, which are normally between 6.0 and 7.5.

Alkaline Phytases: Usually found in alkaline conditions with a pH of 8.0 to 9.5, alkaline phytases work best in these conditions.

Catalytic mechanism:

Based on their catalytic mechanism, phytases can be divided into four main classes:

Histidine Acid Phosphatases (HAPs): The most prevalent class of phytases, HAPs catalyse the hydrolysis of phytic acid by using histidine residues in their active sites.

β-Propeller Phytases (BPPs): BPPs have an exclusive catalytic mechanism that involves a β-barrel active site, and they have a β-propeller protein structure.

Purple Acid Phosphatases (PAPs): PAPs work in acidic environments and are distinguished by their purple colour. They hydrolyze phytic acid using a binuclear metal ion active site.

Protein Tyrosine Phosphatase-like Phytases (PTP-like Phytases): These phytases use a conserved aspartic acid-cysteine-glutamic acid catalytic motif and are structurally similar to protein tyrosine phosphatases.

Sources:

Phytases can be obtained from animals, plants, microbes.

Animals: Small intestinal mucosa, gut associated micro floral

Plants: *Avena sativa, Secale cereale, Triticum aestivum, Hordeum vulgare*

Microbes:

Bacteria: *Bacillus species, Pseudomonas sp., Raoultella sp., Citrobacter braakii, Enterobacter sp., Selenomonas ruminantium, Megasphaera elsdenii, Prevotella sp., Mitusuokella multiacidus, Mitsuokella jalaludinii*

Fungi: *Trichoderma reesei, Aspergillus oryzae, Aspergillus niger, Penicillium funiculosum, Pichia pastoris, Mucor racemosus, Thermoascus aurantiacus, Sporotrichum thermophile.*

Yeasts: *Pichia anomala, Schwanniomyces castellii, Arxula adeninivorans, Candida krusei, Kodamaea ohmeri, Cryptococcus aureus.*

Fermentation:

The most common and long-lasting technique for producing phytase is fermentation. It has been reported that three different fermentation processes—solid-state fermentation (SSF), semi-solid fermentation (SSF), and submerged fermentation (SmF)—are successful [22,23]. Furthermore, microbes that use SSF, SSSF, and SMF are commonly used to produce exogenous phytases that are available for purchase [24].

Solid state fermentation:

Agricultural waste and other inexpensive natural resources are used as substrate by microorganisms growing on a solid material surface with little to no free water but sufficient moisture to support microbial growth (SSF) [18,25]. *A. nigrum, A. ficuum, A. tubingensis, A. flavus,* and *R. oryzae* are a few of the filamentous fungi that are cultivated using SSF.

SSF does, however, have certain disadvantages, including low biomass growth and poor nutrient action. This results from a build-up of heat and moisture loss during the fermentation process, in addition to a low free water content.

Common substrates that are important in promoting fungal development and their natural metabolism (secreted enzymes) include citrus peels, rice bran, soybean meal, wheat bran and maize cobs [26, 27]. *A. niger* uses triticale, which includes barley, malt, and other grains, as a substrate for phytase production [20,28], whereas *Penicillium purpurogenum* uses maize cob and maize bran as substrates for phytase production.

Submerged fermentation:

The culture conditions and the chosen fermentation method have a significant impact on the production of microbial metabolites. Each component must be optimised separately for the microbial metabolite to be produced as efficiently as possible. Among these are the following factors:

- Physical conditions:

Physical factors like pressure, temperature, pH, and agitation speed, among others, have a big impact on microbial phytases.

The majority of bacterial phytases are generated in the pH range of slightly acidic to neutral, and fungal sources grow best in acidic environments. Similar to pH, temperature has an impact on microbial growth. Mesophilic conditions are ideal for the majority of bacteria that produce phytase, but thermophilic bacteria have also been found to grow at higher temperatures. The same is true of fungal strains that have been shown to produce phytase. One important factor influencing the growth is the amount of dissolved oxygen. It has been documented that bacteria can ferment while submerged at agitation speeds varying from 115 rpm to 200 rpm.

- Chemical factors:

Chemical elements that impact microbial growth and metabolism, and consequently the production of phytase, include the source of carbon and nitrogen, their ratio, metal ions, and inorganic phosphate. These elements have a similar impact on microbial growth as do environmental factors. The sources of carbon and nitrogen in the media are important steps in its composition. Various sources of carbon have been used, including molasses, glucose, sucrose, and bran from wheat and rice [29]. Similarly, it has been reported that phytase-producing microorganisms have grown in response to a variety of nitrogen sources, both organic and inorganic. These sources include ammonium nitrate, ammonium sulphate, peptone, urea, and their various combinations [30].

2.3 Applications:

Phytase is currently employed in a number of biotechnological processes related to the production and processing of food for humans and animals. The production and manufacturing of animal feed and food supplements accounts for about 60% of the total enzyme market production, with an annual market capitalization of 350 million USD [31].

- Animal feed supplements:

The primary use is as a supplement to animal feed, which will both raise the availability of phosphorus and lessen its burden on the environment. Phytase is an enzyme that is helpful

to monogastric animals because it helps these organisms absorb different trace minerals and phytate phosphorus, which are not readily available to them [32]. Phytase is an additive found in about 70% of animal feed [33]. Broiler diets that include phytase supplements enhance the overall growth and development of the animals [34], [35], [36]. Phytase increases the digestibility of amino acids, carbohydrates, phosphorus, and calcium while also lessening the nutritional impact of phytate. Because they can lower phosphate levels in water bodies and avert eutrophication, phytases have the potential to be employed as marine pollution regulators [37].

- Food industry:

Phytase increases the quality of the final product and reduces production costs when it is added to food while processing and manufacturing. Bread production, plant protein isolate synthesis, maize wet milling, and cereal bran separation have all improved as a result of the application of phytase in food processing [38, 39, 40, 41]. Wheat and whole grain flour, which are used to make a range of doughs and breads, are rich in phytic acid. Phytase increases bread volume and crumb quality while shortening fermentation times without changing pH. The indirect effect of phytase on a-amylase activity has been linked to these improvements in bread quality.

- Aquaculture:

Enhanced Phosphorus Digestibility: To help fish and prawns meet their nutritional needs, phytase is also used in aquaculture feeds to improve the phosphorus digestibility of plant-based ingredients. This is necessary for aquaculture methods that are both economical and sustainable.

- Biofuel Production:

Enhanced Nutrient Release in Plant Biomass: Plant biomass can be converted into biofuels by the enzyme phytase. Phosphorus and other nutrients are released when plant materials that contain phytic acid are broken down; these nutrients can then be added to the overall nutrient content of biofuel byproducts.

- Soil fertilization:

Increased Phosphorus Availability: Phosphorus can be made more readily available to plants in agriculture by adding phytase to the soil. This can be especially helpful in phytic acid-rich soils, where plants have less access to phosphorus.

- Wastewater treatment:

Phosphorus Recovery: Phytase's possible use in the treatment of wastewater has been investigated. It is feasible to break down organic phosphorus compounds in wastewater by adding phytase, which makes it easier to recover phosphorus from wastewater streams.

- Pharmaceuticals:

Phytase has been studied for its possible application in medication formulations, specifically to increase the bioavailability of some minerals in pharmaceuticals.

- Environmental Remediation:

Soil Phytoremediation: In order to help plants access phosphorus in contaminated soils and aid in the removal of pollutants, phytase can be applied in conjunction with plants.

Chapter 3: Materials and Methods

3.1 Revival of culture:

The culture was revived by inoculating bacterial culture in nutrient broth. It was then incubated at 37^0 C for 24 h. The growth culture was then observed.

3.2 Streaking:

Nutrient agar was prepared and autoclaved. This agar medium was then poured onto petri plates, a loop of grown culture was taken and then streaking was done. These plates were then incubated at 37^0C for 24h. This was done in order to check for the purity of the culture.

3.3 Gram staining:

Gram staining was done in order to confirm the shape of the bacteria and whether the bacteria is gram positive or gram negative.

3.4 Glycerol stock:

Glycerol stocks are a common method used in microbiology and molecular biology to preserve bacterial, yeast, or other microbial cultures for long-term storage. So, glycerol stocks were prepared in order to preserve the revived culture for future use.

3.5 Enzyme production:

- Using Nutrient Broth as production medium:

The enzyme production from the bacteria was done. In order to produce enzyme; 2.5 % wheat bran, 1.5% ammonium sulphate were inoculated with 24 h old 1% inoculum with production medium pH 6 and incubated for 24 h at 35°C, rpm 150 where ammonium sulphate was taken as nitrogen source and wheat bran was taken as the carbon source.

- Using PSM media as production medium

The enzyme production from the bacteria was done. In order to produce enzyme; 2.5 % wheat bran, 1.5% ammonium sulphate were inoculated with 24 h old 1% inoculum with production medium pH 6 and incubated for 24 h at 35°C, rpm 150 where ammonium sulphate was taken as nitrogen source and wheat bran was taken as the carbon source.

Table1: Constituents of PSM (phytase screening agar medium) media:

Media components	Volume (g/l) (W/V)
Ammonium nitrate	5
Potassium chloride	0.5
Ferrous sulphate	0.01
Magnesium sulphate	0.5
Manganese sulphate	0.01
Calcium chloride	0.01
Sodium Phytate	4
Glucose	15

pH – 6

3.6 Phytase assay method:

3.6.1: Standard curve:

Potassium dihydrogen phosphate (KH_2PO_4) was used as the standard stock solution for the standard curve. A 1 ml dilution series ranging from 50 to 500 µg/ml of KH_2PO_4 was prepared from the stock solution. A 1 ml solution was mixed with 250 µl of 10% Trichloroacetic acid (TCA), and then a freshly made 1 ml colouring reagent (distilled water acidified with 5% sulfuric acid, plus 1% ammonium molybdate and 7.20% ferrous sulphate) was added. After vortexing the mixture, it was incubated in the dark for ten minutes to develop a blue-coloured complex. In a spectrophotometer, absorbance was measured at 750 nm in relation to a blank.

Table 2: Standard plot for Bradford assay:

	KH_2PO_4 **(µl)**	dH_2O **(µl)**	TCA **(µl)**	Colouring Reagent (ml)	Incubation
Blank	---	1000	250	1	10 minutes at room temperature
1.	50	950	250	1	
2.	60	940	250	1	
3.	70	930	250	1	
4.	80	920	250	1	
5.	90	910	250	1	
6	100	900	250	1	

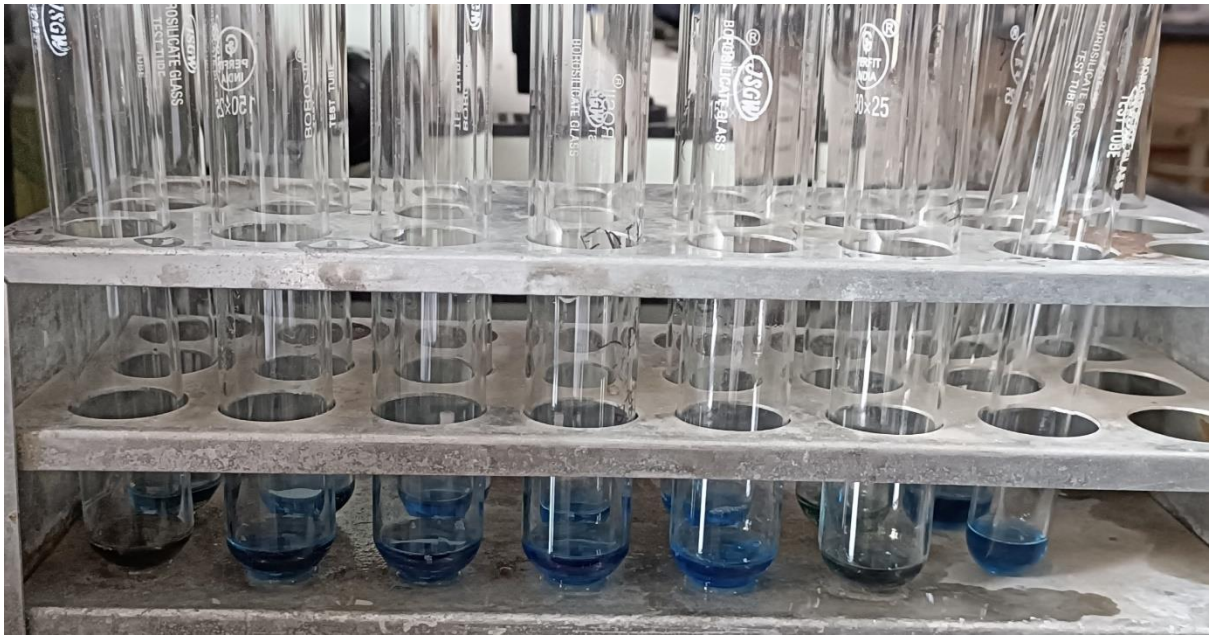

Fig6. Standard curve using KH_2PO

3.6.2: Phytase assay:

The phytase assay was performed using 0.1 ml of enzyme and 0.9 ml of acetate buffer (0.1 M; pH 5.0) with 2.0 mM wheat bran as a substrate. After 30 minutes of incubation at 50 °C, 250 µl of 10% TCA was added to stop the reaction. After adding 1 millilitre of colouring reagent and letting it sit in the dark for 10 minutes, the colour was developed. The absorbance was determined using spectrophotometry at 750 nm. Next, using a KH_2PO_4 standard curve, the amount of liberated free phosphate was determined.

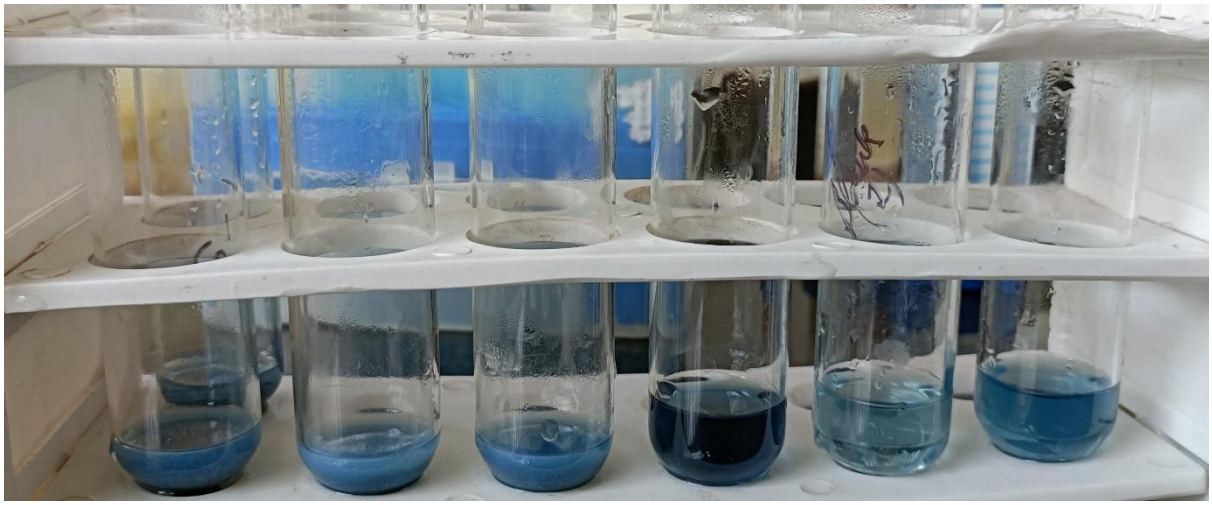

Fig7. Phytase assay

3.7 Biochemical testing:

3.7.1: Catalase test:

Onto a glass slide a colony of about 24h growth culture was smeared using sterile loop. Drops of H_2O_2 were dropped on the slide.

The presence for bubbles was determined.

3.7.2: Potassium hydroxide test:

3% KOH was prepared.

Onto a glass slide drops of this prepared KOH were taken.

A colony of 24h old growth culture was spread on the slide and stir to for 60s.

Whether threads were made was determined.

3.8 Protein Concentration:

In order to check for the protein concentration Bradford assay method was used. Different dilutions of BSA from standard solution (1mg/ml) and adjusting to volume of 200µl. 1 ml of bradford reagent was added. Vortexing was done and incubation at room temperature for 10 minutes was done. OD was taken at 595 nm. Standard graph was plotted and concentration was determined.

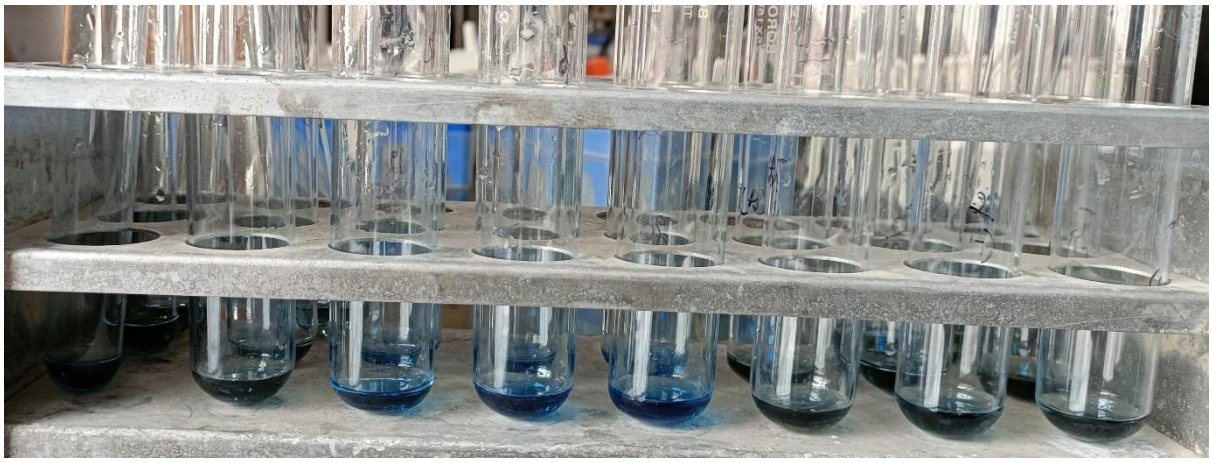

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Fig8. Protein concentration using Bradford reagent

Chapter 5: Results and discussions

Streaking:

Fig9. Streaking to confirm pure culture

Gram staining:

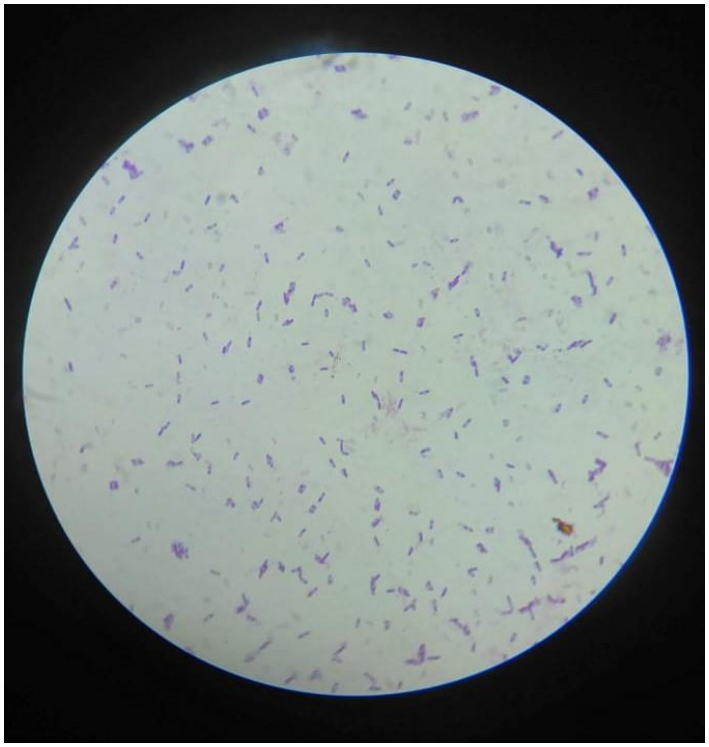

Fig10. Gram staining

Glycerol stock:

Fig 11. Glycerol stock

Enzyme production:

Nutrient Broth Medium:

Fig12.Supernatant and pellet (Nutrient Broth medium)

PSM medium:

Supernatant **Pellet**

Fig 13. Supernatant and pellet (PMS media)

Phytase assay:

Table 3: Phytase enzyme activity assay

	Buffer (ml)	Substrate (ml)	Enzyme (ml)	Incubation	TCA (µl)	Colouring reagent (ml)	Incubation
Blank	**0.9**	**---**	**0.1**	**30 minutes at 50^0C**	**250**	**1**	**10 minutes at room temperature**
Control	**0.1**	**0.9**	**---**		**250**	**1**	
Test sample	**---**	**0.9**	**0.1**		**250**	**1**	

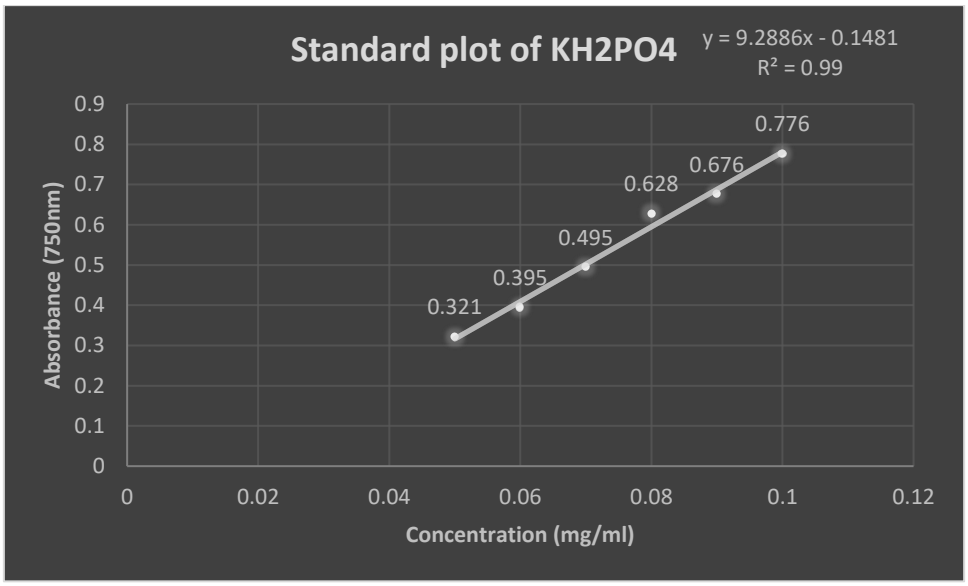

Fig14. Standard curve (Plot)

Enzyme Activity: $\frac{\text{Amount of product formed(mg/ml)} \times \text{Total reaction volume}}{\text{Inoculation time} \times \text{Volume of enzyme}}$

- For Nutrient Broth media:

$$= \frac{0.3069 \times 10^3 \times 1}{94.97 \times 30 \times 0.1}$$

$$= 1.077 \text{ U/ml}$$

- For PSM media:

$$= \frac{0.4057 \times 10^3 \times 1}{94.97 \times 30 \times 0.1}$$

$$= 1.423 \text{ U/ml}$$

Biochemical testing:

Catalase test

Fig15. Catalase test

Appearance of bubbles indicate catalase positive bacteria i.e. the bacteria is gram positive.

Potassium hydroxide test:

Fig16. KOH test

No thread like structure appears i.e. the bacteria is gram positive.

Protein concentration:

Table4. Bradford reagent assay for protein estimation.

Concentration of BSA (mg/ml)	Volume of BSA (µl)	Distilled water (µl)	Bradford reagent (ml)	Incubation
Blank	---	200	1	10 minutes at room temperature
0.02	4	196	1	
0.04	8	192	1	
0.06	12	188	1	
0.08	16	184	1	
0.1	20	180	1	
T_1 (Nutrient Broth media)	14	186	1	
T_2 (PSM media)	14	186	1	

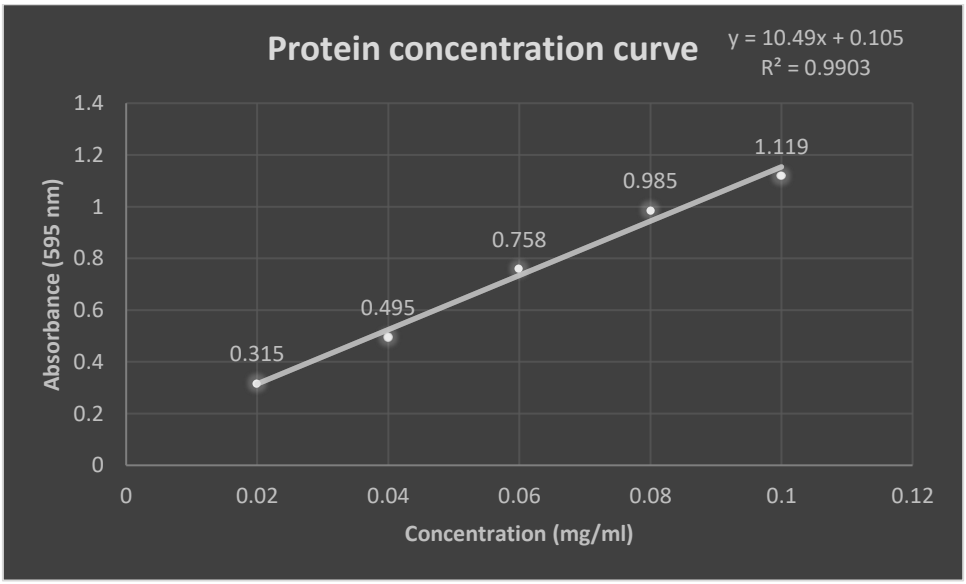

Fig17. Protein estimation (plot)

Protein content in Nutrient Broth medium	Protein content in PSM medium
0.017 mg/ml	0.015 mg/ml

Discussions:

Wheat bran was taken to be the best carbon source and ammonium sulphate as the nitrogen source. The catalase test came out to be positive and KOH test was negative indicating that the bacteria is gram positive. During enzyme production the best pH of the medium was considered to be 6 and the appropriate temperature for enzyme production was 35^0C and rpm was 150.

Chapter 5: Conclusion

Conclusion:

In the twenty-first century, the potential applications of microbial enzymes in industry have grown significantly, and this trend is expected to continue as the world's population grows and natural resources become more scarce. As of right now, phytase is the food enzyme that is most commonly used worldwide.

The impacts of phytic acid in foods have become the major concerns due to its negative effect on mineral bioavailability and protein digestibility in human nutrition. Due to its strong antioxidant properties, it can serve as a natural food preservative in place of the widely used artificial ones. Furthermore, new research has indicated that it has antimicrobial potential. Regulatory agencies from nations that have not yet permitted its use as a food additive should thus provide substitutes, particularly for animal proteins high in fat, like processed meat products. This could increase food quality and stability while also grabbing consumers' attention by using a natural compound in place of artificial preservatives.

Phytase is an adaptable enzyme that finds extensive use in a wide range of industries. This is mainly due to its capacity to improve nutrient availability, especially phosphorus, in diverse settings. Animal feed, aquaculture, the production of biofuel, soil fertilisation, wastewater treatment, the food and beverage sector, pharmaceuticals, and environmental remediation are some of the major uses.

All things considered, phytase's diverse range of uses highlights how crucial it is for tackling issues related to sustainability, nutrition, and the environment. As such, it is an invaluable resource for a number of industries striving for low environmental effect and effective resource use.

Future aspects:

Phytase enzymes have a bright future ahead of them due to continued research and the growing need for effective and sustainable solutions across a range of industries. Research has been going on in looking for the ways to increase the stability and effectiveness of phytase enzymes. In the future, it might be possible to engineer enzyme variants that have higher catalytic activity, a wider range of substrate specificity, and resistance to changes in pH and temperature. An increasing number of people are interested in customising phytase

enzymes for particular uses and target organisms. Tailored enzyme formulations have the potential to maximise nutrient utilisation in aquaculture, plant-based industries, and a variety of animal species, resulting in more efficient and long-lasting results.

Biotechnology advancements such as genetic engineering and synthetic biology have the potential to create new organisms that produce phytase. Potential avenues for sustainable agriculture and animal nutrition could be expanded by using microbial strains optimised for the production of enzymes or engineered crops with increased expression of phytase. The functions of phytase in nutrition recovery and recycling are consistent with the ideas of the circular economy. Phytase may be essential in reducing nutrient losses as societies and industries work towards more environmentally friendly methods, particularly in wastewater treatment and agriculture.

The use of phytase in a variety of applications may be encouraged or required by regulatory frameworks as awareness of its nutritional and environmental benefits grows. These could include requirements for food and pharmaceutical product labelling, environmental standards for nutrient management, and guidelines for formulating animal feed. Academic, industrial, and regulatory groups will probably work together more in the future to address issues pertaining to the broader adoption of phytase and to better understand its potential. Collaboration can result in the creation of best practises and novel applications.

In conclusion, continued progress in enzyme engineering, adaptation for particular uses, incorporation into precision agriculture, and growing environmental roles will define the future of phytase enzymes. These advancements are consistent with the more general objectives of resource efficiency, sustainability, and eco-friendly practises in a variety of industries.

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