PRODUCTION OF NOVEL FUNGAL PHYTASE AND IT's APPLICATIONS

A Major Project Report submitted in partial fulfillment of the Bachelor of Technology degree requirement in

Bachelor of Technology in Biotechnology

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CERTIFICATE

This is to certify that work title **"Production of novel fungal phytase and it's applications"** by **Megha Tushir, Shubham Dogra and Abhinav Dharmani** during the end semester in June 2022 in fulfillment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has been submitted partially or wholly to any other University or Institute for the award of any degree or appreciation.

Signature of Supervisor
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DECLARATION

I do hereby declare that this dissertation is titled "**Production of a novel fungal phytase and its applications**" submitted towards attainment for the award of degree of Bachelors of Technology in Biotechnology under the guidance of Dr. Saurabh Bansal, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, is wholly based on the study and results carried out. Also till now this work has not been proposed anywhere for any additional degree or diploma. Therefore the declaration made by the candidate is true and genuine.

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LIST OF ABBREVATIONS

Phy	Phytase
v/v	Volume by volume
Arg	Arginine
His	Histidine
HAP	Histidine Acid Phosphatases
BBP	β propeller phytase
PAP	Purple Acid Phosphatase
PTP	Protein Tyrosine Phosphatase-like phytase
µl	microlitre
w/v	Weight by volume
ml	milliliter
SSF	Solid-state fermentation
SmF	Submerged fermentation

ABSTRACT

This study aims to extract the phytase enzyme from wheat bran after the Solid-state fermentation by growing a particular strain of *A. niger* NT7. Crude phytase was removed, and its concentration was measured using the Bradford method. The reaction is used to measure the concentration of protein in a sample.

The phytase production was achieved using wheat bran as a substrate in Solid-state fermentation. Previously optimized physiological parameters for the phytase production were used. A 13×10^6 spores/ml of 3 days older inoculum was used to inoculate a 5 g of wheat bran having moistening agent, distilled water (1:2). The substrate was further supplemented with various biochemicals like sugar mannitol, nitrogen ammonium sulfate and detergent Tween 80. The solid-state fermentation is carried out at medium pH 5.0, and 35° C temperature. After fermentation, the phytase was extracted out using centrifugation at 7000 rpm at 4 °C. To find the protein concentration, Bradford assay was done. The assay works on the principle of binding Coomassie dye with protein molecules under acidic conditions resulting in a color change from brownish green to blue. The absorbance was taken at 595 nm; the concentration comes to 0.862 mg/ml, which seems to be a good concentration.

Chapter 1
INTRODUCTION

1.1 Hydrolytic enzymes

They are the types of enzymes that split different groups of given molecules like proteins, fats etc., into the most straightforward forms required [1].

1.2 Phytic acid

It is a chemical found in plants that occurs naturally. It became relevant when studies were conducted on its absorption properties, mainly mineral absorption, as findings prove that it somehow blocks or prevents the absorption of certain minerals like calcium, zinc etc. IP6 is widely distributed in plants and plays an important role in nutrition as the main storage form of phosphorus in many plant tissues, including bran and seeds. It is a saturated cyclic acid. It is partially ionized at physiological pH and has a high charge density due to its six phosphate groups balanced by cations, mainly Na+ ions [2].

1.3 Phytase

It is a phosphatase enzyme that accelerates the breakdown of phytic acid, thus the breakdown of enzymes responsible for blockage or absorption of some minerals. So it helps in the absorption of minerals. Mainly found in oil seeds and certain grains. It is an essential enzyme for the digestive system and also a key enzyme for bone health [3].

- **History**

It first came to notice when the composition of wheat rice and maize bran was carried out. In the later 1960s, it was taken as a good feed source for animals with good nutritional value; then, microorganism studies were carried out to find suitable organisms for its production.

Mold is widely used in the production of phytase. Based on their catalytic activity and structure, the first and most studied groups of phytases are classified as histidine acid phosphatases (HAPs) separated from filamentous fungi, bacteria, yeast, and plants [4]. Fungal phytase production is achieved under three different fermentation processes including solid, semi-solid-state, and underwater fermentation. Agricultural residues and other waste products have been used as test substrates for the production of enzymes in the fermentation process. [5]

Aspergillus is widely used in the production of phytase. Thus, the first generation of commercially available fungal phytase found in *A. niger* was sold in 1991 and has been

used for various industries since then, such as human food and animal feed and myo-Inositol phosphates processing [6].

1.4 Production of phytase

Phytases are usually produced in many animal and plant tissues and even in microorganisms, mainly in fungi. Most studies are conducted on its microbial production. The following methodologies have produced the production of phytase by microbial means.

- **Surface fermentation**

In this method, microorganisms are grown on a solid structure without a minute amount of free water; and at the same time the process is moist to support the growth of microorganisms. Production is usually carried out with the help of different substrates or sometimes agricultural excess components to compensate for its productive outlay.

- **Submerged fermentation**

This fermentation, as compared to the above process, has plenty of water supply and is relatively easy, cost-efficient, and more effective [7].

1.5 Applications

- Increase mineral absorption thus treating mineral deficiency
- Help in infant development
- Accelerates child growth
- Utilized in feed supplements
- Help in plant growth
- Used as food additive used as therapeutic

CHAPTER -2
REVIEW OF LITERATURE

2.1 *Aspergillus niger*

Aspergillus niger is a group of 15 species of *Aspergillus*, all with black conidia. *Aspergillum* (Latin word) was the origin for its name, which means holy water sprinkler (because of the appearance when viewed under a microscope). It is very thermotolerant. Therefore, it can grow in a wide range of temperatures, including extremely cold and extremely hot conditions. It lives as a prophylaxis in decaying plants, plant parts such as leaves and stored grain and is found mainly in the soil. Humans, birds, and animals can all be hosts because the fungus is opportunistic, but this is highly rare. Mycotoxins, such as ochratoxin A, and isoflavone orobol inhibitor, are produced by some strains of *Aspergillus niger.*

Morphology

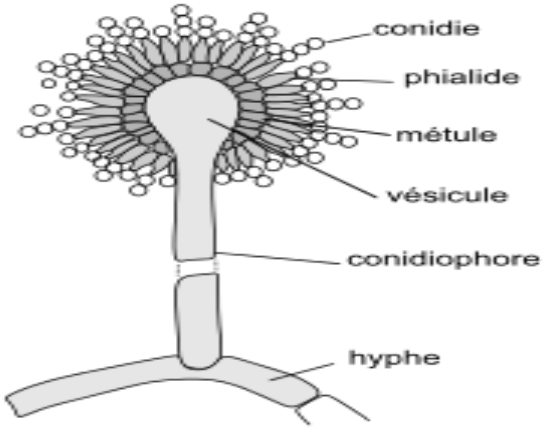


Figure 1.Structure of *A. niger* [8]

1. *Aspergillus niger* is a haploid filamentous fungus.
2. Mycelia are the plant.
3. Aspergillus niger grows white at first but darkens after a few days, producing conidia seeds, according to a large study. Radial fissures appear on the border of the colony, appearing pale yellow.
4. Conidiophores present here are pigmented and when observed experimentally through microscope production of conidiophores are because of septate and hyaline hyphae.

5. Conidiophores are smooth and hyaline and range in length from 400 to 3000 micrometers.
6. The vesicle is protected by the metulae and phialides.
7. Oil globules are reserved food materials.

2.2 Enzymes

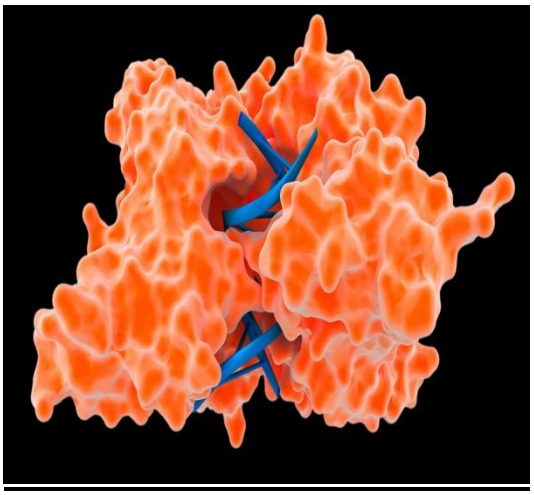


Figure 2. Structure of an Enzyme [9]

These are the elements. In general, organisms synthesize proteins that act as biocatalysts, causing certain biochemical events. These interact with substrates and transform them into molecules known as products. To maintain a balanced life, almost all metabolic pathways within cells require rapid enzymatic catalysis. Enzymes, such as catalysts, affect the rate of a reaction by increasing or decreasing its initiation capacity. Inhibitors and activators of molecules that affect the activity of enzymes; Inhibitors, like other drugs and toxins, affect their function by inhibiting the activity of enzymes. Enzymes are also affected by certain conditions, such as pH, and temperature, and can lose structure and catalytic structure.. Enzymes are being used as biocatalysts due to significant advancements in industrial areas such as optimization, characterization, and gene identification. Hydrolases account for more than 80% of enzymes used in industries. The enzyme phytase is one of the most widely utilized enzymes in animal feed supplements, inositol phosphate production, and transgenic plants.

2.3 Phytic Acid

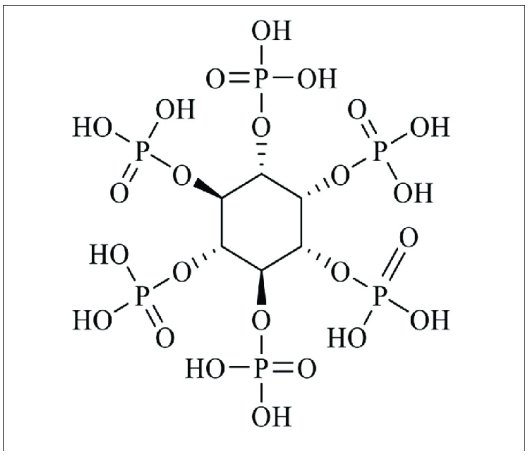


Figure 3 Structure of phytic acid [10]

Phytic acid, commonly known as inositol hexakisphosphate (IP6) or inositol polyphosphate, is a six-fold dihydrogen phosphate ester of inositol. The phosphates are partially ionized at physiological pH, resulting in the phytase anion. In 1903, phytic acid was discovered. Pepsin, amylase, and trypsin, among other digestive enzymes, have been inhibited by phytates. In the stomach, pepsin breaks down proteins, amylase breaks down starch into simple sugar, and trypsin is required for protein digestion in the small intestine. It is usually seen in seeds [11].

It is considered to be the storage place in the seed for phosphorus sources. Phytate is broken down, and phosphorus is released when seeds sprout. Phytic acid in whole grains acts as an anti-nutrient, as studies reveal that it blocks calcium, zinc, magnesium, iron, and copper in many people [12]. It forms a phytate when the "arm" of the phytic acid molecule "chelates "or grabs onto these other molecules. The young plant will use phosphorus. Because of its antioxidant qualities, it is frequently employed as a preservative in commercial products. When phytic acid is ingested, it forms phytates by binding to other minerals. Because you don't have any enzymes to break down phytates, you won't be able to absorb their nutrients.

Table1:- Food items with amount of phytase content

Food	Amount of phytic acid
Almonds	0.4-9.4%
Bran	0.6-2.4%
Hazelnuts	0.2-0.9%
Maize Corn	0.7-2.2%
Peanuts	0.2-4.5%
Rice	0.1-1.1%
Rice bran	2.6-8.7%
Sesame seeds	1.4-5.4%
Soybeans	1.0-2.2%
Walnuts	0.2-6.7%
Wheat	0.4-1.4%
Wheat Bran	2.1-7.3%

2.4 Phytase

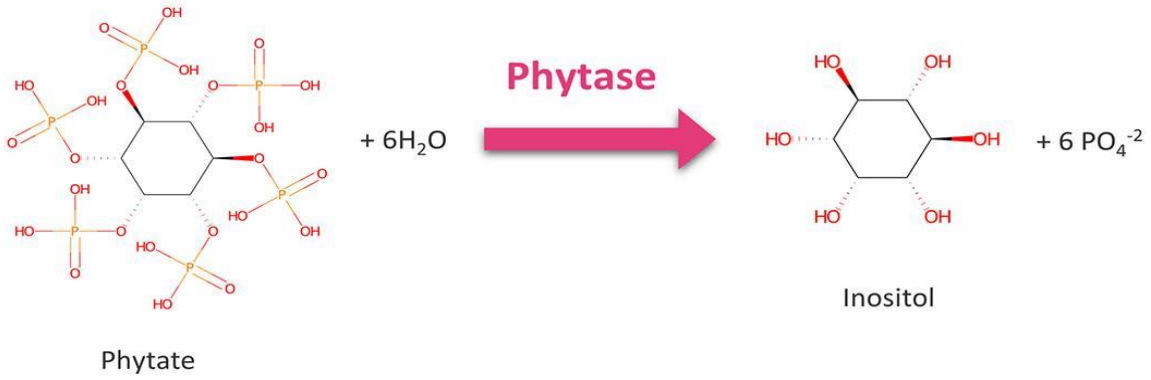


Figure 4: Reaction of Phytase [13]

Phytase is an enzyme that acts directly on phytate, breaking it down to release phosphorus in a way that is available to an animal [14]. This significantly reduces the need for inorganic phosphorus supplementation and improves the nutritional value of the diet. Phytase activity

is expressed as phytase units or FTUs. Phytase was first time discovered in rice bran phytase are found in a variety of sources including plant tissues and microbes and mammals[15].

Types of phytase

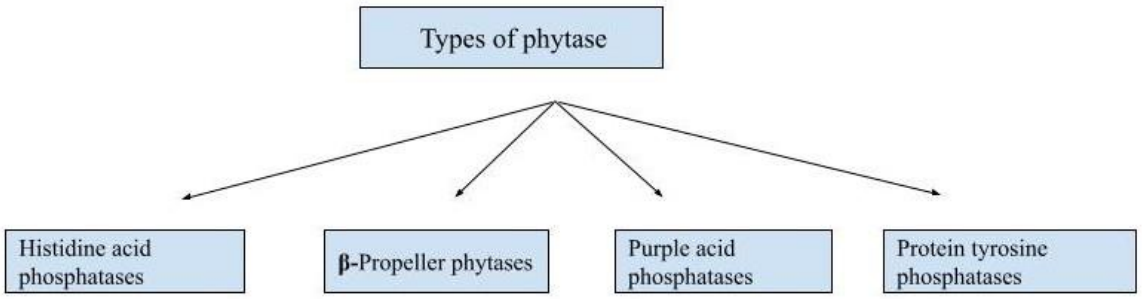


Figure 5 Types of Phytase

There are 4 different types of phytase

1. Histidine acid phosphatases (HAPs)

Most known phytases belong to the histidine phosphatase (HAP) family of enzymes. Mycelium fungi, bacteria, yeast, and plants contain HAP [16].All members of this class of phytase contain the same active site sequence motif (ArgHisGlyXArgXPro) and hydrolyze phytic acid in two steps [17].The fungus *Aspergillus niger* produces phytases, which are well known for their high inertness and commercial role as an animal feed additive to increase the bioavailability of phosphates from phytic acid in poultry and pig grain feeds.

2. β-propeller phytases

B-propeller phytases make up a recently discovered class of phytase. These first examples of this class of enzyme were originally cloned from *Bacillus* species, but numerous microorganisms have since been identified as producing β-propeller phytases [18]. The three-dimensional structure of β-propeller phytase is similar to a propeller with

six blades. Research suggests that β-propeller phytases are the major phytate-degrading enzymes in water and soil, and may play a major role in phytate-phosphorus cycling.

3. Purple acid phosphatases

A phytase with the active site motif of a purple acid phosphatase has recently been isolated from the cotyledons of growing soybeans (PAP)[19].According to genomic database searches, PAP-like sequences have been found in plants, animals, fungi, and bacteria. However, only the PAP from soybeans has been discovered to have any phytase action.

4. Protein tyrosine phosphatase-like phytases

Phytases are members of the protein tyrosine phosphatase super family of enzymes (PTPs). PTP-like phytases, a relatively novel family of phytases, have been identified from bacteria that ordinarily live in ruminant animals' guts. All characterized PTP-like phytases have the same active site sequence motif (His-Cys-(X) 5-Arg), a two-step acid-base dephosphorylating process, and activity against phosphorylated tyrosine residues, all of which are shared by the entire PTP superfamily of enzymes. The specific biological substrates and roles of bacterial PTP-like phytases, like many other PTP superfamily enzymes, are unknown. The ruminal bacteria PTP-like phytases are structurally and sequence-wise similar to the mammalian PTP-like phosphoinositide-inositol phosphatase PTEN [20].

2.5 Sources of phytase

Phytases can be found in a variety of plant and animal tissues, as well as microbes like bacteria, yeast, and fungi. The phytase enzyme was first discovered in rice bran [21, 22].Microbial phytases, notably those produced from filamentous fungi such as *Aspergillus, Myceliophthora, Mucor, Penicillium, Rhizopus, and Trichoderma*, have been the subject of the majority of scientific studies. *A. carbonarius, A. fumigatus, A. niger, A. oryzae, Cladosporium species, Mucor piriformis,* and *Rhizopus oligosporus* are filamentous fungal species that can produce phytase during the fermentation process.

2.6 Production of phytase

Certain strains of species that produce phytase have their own conditions and requirements that allow for higher phytase synthesis. For the synthesis of phytase, two distinct fermentation methods are used:

- Solid-State Fermentation (SmF)
- Submerged Fermentation (SSF)

1 Submerged Fermentation (SmF)

This type of fermentation is usually utilized for the synthesis of bacterial phytase, and it involves submerging microbes in excess of water, alcohol, oil, or nutrient broth, also known as medium, with other necessary nutrients for growth [23]. Since the optimal conditions and procedures, as well as certain other elements, have a significant impact on microorganism development. These are the elements:

Physical parameters such as

Temperature, agitation speed, and pH have an impact on the yield's quality and quantity, as phytase is typically produced at a slightly alkaline pH and approximately 37 degrees Celsius.

Chemical parameters such as

Nitrogen and carbon sources, oxygen rate, and metal ions have a significant impact on production, just as glucose content has an impact on microbial development.

In recent years, SSF has been thought to be more suited for the manufacture of phytase since SmF contains:

- Volumetric yield nominal
- By-product concentration is lower in comparison.
- Increased wastewater production

2. Solid - State Fermentation (SSF)

This type of fermentation uses solid media with very little water. Microbial development is replaced by the free-flowing aqueous medium with grains bran and oilseed cakes. Because of economic and commercial considerations, the usage of SSF for the synthesis of phytase has gradually expanded during the previous decade. SSF is considered to be more advanced than SMF, such as inexpensive substrate, energy needed for the process is less as

compared to SMF, and reduced pollution. Industries desire more efficient procedures that provide more output with less cost input [24].

Advantages

- Reduced cost of preparation and fewer raw material requirements.
- Aeration is simple and requires little energy.
- Final result is so focused therefore less DSP is needed.
- Many types of garbage, both domestic and agricultural, can be used as a solid surface.

2.7 Application of phytase

In the natural environment, phytic acid is a great supply of carbon, nitrogen, and sulphur that can be transformed into a variety of compounds. Because of their ability to break down keratins, phytase enzymes have a wide range of industrial, agricultural, and biotechnological applications.

- **Food Additives**

  In the fermentation process grain and whole wheat bray (also known as flour) for varieties of bread and dough, phytases are used as a food additive in fermentation and a range of bread-related applications, including feeding supplements[25]. For example, the phytase enzyme from *A. ficuum* has been used to dephosphorylate legumes.

- **Feed Supplements**

  As they have the potential to boost phosphorus efficiency and limit phytate concentration in food and livestock feed, phytase enzymes are extremely important in biotechnological applications in animal and human nutrition processing and manufacturing. Ruminant's such as pigs, and fish which deficit, or have insufficient gastrointestinal phytases, prohibiting them from utilizing phytate, a phosphorus compound found in food and livestock feed. As a result, inorganic phosphate supplementation is required to meet their nutritional and growth requirements, rising feed costs and phosphorus pollution levels.

- **Plant Growth Promotional Applications**

  Phosphorus is an important and necessary component of cells that helps with energy metabolism, acid generation, and cell membrane manufacturing [26].This plays an important role in plant development and growth. Lack of phosphorus in the soil is a major problem for agricultural farmers around the world. Total soil P, which can be found in both organic and inorganic forms, is abundant in the majority of soils. Phytic acid is a major source of biological phosphorus in the soil, accounting for 10 to 50% of all accessible organic phosphorus [27, 24]. As a result, the phytase enzyme is required for digestion.

- **Therapeutic Applications**

  Low levels of healthy red blood cells (anemia) due to iron deficiency [28]. Previous studies show that taking phytase in a diet high in iron increases iron absorption in the diet. But it is unclear whether taking phytase can help prevent or treat iron deficiency. Zinc deficiency. Previous studies show that taking phytase with a zinc-containing diet increases zinc absorption in the diet [29].But it is unclear whether taking phytase can help prevent or treat zinc deficiency, aging skin, and movements of the eyelids and also in development of infants or children.

- **Other Commercial Phytase Enzyme Products**

  Phytases are enzymes that help animals digest their food. They had the largest revenue share in the industry, with 83.6%. Approx 60% of the herbivores animal diet is supplemented with phytases. The first industrial phytase, classified as a 3-phytase, was discovered in *A. niger*. *Peniophora lycia* was later used to make a 6-phytase industrial product. Several companies have been developing and marketing fungal phytase products for years. Commercially, phytase enzyme is made using either phytate-producing fungus or recombinant DNA technology.

CHAPTER 3
Materials and Methods

Several experiments and methodologies were devised in line with the available resources to carry out the research, and a range of substances, tools, samples, and equipment were used to achieve the results [30]. A colleague previously already obtained the unique *Aspergillus niger* NT7 strain used in the study, which was then regenerated and used in further experiments.

3.1 Raw Material

Wheat bran was purchased from a nearby market. Medium-sized bran particles were extracted; the wheat bran was first sieved with a pore size of 600 microns and dried at 55 °C in a hot air oven to remove all moisture. Further experiments were conducted with this medium-sized, moisture-free bran.

3.2 Microorganism and inoculum preparation

A novel strain of *Aspergillus niger NT7* species was procured from the previously isolated culture collection[31].It was revived on the potato dextrose agar plates by streaking method and plates were kept at 37 degree C for fungal growth. To conduct out fermentation, three-day-old inoculums was harvested in normal saline and counted by hemocytometer to a level of 13×10^6 spores per ml.

3.3 Production of phytase through SSF

5 g wheat bran was poured into a 250-ml Erlenmeyer flask and 10 ml distilled water was added to provide moisture to the content, during which it was supplemented with 0.1 g Mannitol (0.5 percent) and 0.025 g ammonium sulfate (0.5 percent). Fermentation was carried out for 4-5 days at 35°C with a pH of 5 maintained through.

3.4 Extraction of phytase

To extract crude phytase from *A. niger* NT7, moldy wheat was extracted [31]. After the fermentation was completed, the moldy wheat bran was soaked in 0.1 percent (500 microliter) Tween 80 (100 microliter per gram of wheat) and stored in a shaking condition at 30°C for 1 hour [32]. Solid moldy particles were emptied through a double-layered muslin cloth and placed in the appendix, followed by centrifugation at 7000 rpm for 20 minutes at 4°C and storage for 24 hours to get clear supernatant, which was then used as crude phytase enzyme.

3.5 Phytase assay

By measuring inorganic phosphorous release from sodium phytate, phytase activity was evaluated spectrophotometrically at 750 nm. The reaction mixture contains one mM sodium phytate produced in 0.1 M acetate buffer (pH 5.5) and 50 µl crude phytase enzyme. To determine released phosphorus, KH_2PO_4 was used as a standard [27]. One unit was defined as the quantity of enzyme necessary to release 1µmol of inorganic phosphorus per minute under the test conditions. Units of phytase activity per gram of dry substrate ($Ugds^{-1}$) were used to measure phytase production.

Coloring reagent formation:-

50ML : Made it inside the coloured bottle take 47.5ml distilled water add conc.H_2SO_4 2.5ml then addition Ferrous sulfate $FeSO_4.7H_20$ 3.6gm mix well at last addition of Ammonium Molybdate 0.5gm also make it fresh when you use.

Standard Assay:-

Table 2:- Dihydrogen Phosphate assay composition

S.No	Conc.(mg/ml)	KH_2PO_4 (µl)	D/W (µl)	10% TCA (µl)	Coloring reagent (µl)
1	0.1	100	900	250	1000
2	0.2	200	800	250	1000
3	0.3	300	700	250	1000
4	0.4	400	600	250	1000
5	0.5	500	500	250	1000
6	0.6.	600	400	250	1000
7	0.7	700	300	250	1000

8	0.8	800	200	250	1000
9	0.9	900	100	250	1000
10	1	1000	0	250	1000
11	Blank	0	1000	0	0

3.6 Protein Content

Bradford method was used to measure the protein content in extracts by using bovine serum albumin as a standard. Bradford reagent was prepared by dissolving 5mg of Coomassie blue in 5ml of methanol then 10 ml of 85% of H_2PO_4 was added followed by addition of solution from step 2 into 50 ml of H_2O and then was mixed properly after that filtration was carried out to remove precipitates at last 35 ml of H_2O was added.11 test tubes were prepared with the composition as shown in the sample and then the OD was calculated at 595 nm and then comparison was made between the standard and sample.

Table 3:- Standard Assay for Protein content

S. No	**Conc. (mg/ml)**	**BSA (µl)**	**Distilled H_2O (µl)**	**Bradford Reagent (µl)**	
1	0	0	1000	1000	**Incubation at Dark for 5- 10 min**
2	0.1	100	900	1000	
3	0.2	200	800	1000	
4	0.3	300	700	1000	
5	0.4	400	600	1000	
6	0.5	500	500	1000	

7	0.6	600	400	1000	
8	0.7	700	300	1000	
9	0.8	800	200	1000	
10	0.9	900	100	1000	
11	1	1000	0	1000	

Table 4:- Assay For Protein content in crude Phytase Enzyme

Crude Phytase (µl)	**Distilled H_2O (µl)**	**Bradford reagent (µl)**	**Incubation in dark for 5-10 min**
1000	0	1000	
500	500	1000	

3.7 Preparation of Phy-CLEA

For the precipitation of phytase molecules, 10 mL of ice-cold acetone (100 percent, v/v) was applied drop wise to crude phytase (1.67 mg/mL). At 4 °C, the precipitation was carried out for 1 hour with continuous stirring at 150 rpm [32].After 2 hours, GA (25 percent v/v) was added to the aggregated phytase molecules at a final concentration of 0.02 percent (v/v). For 4 hours, the cross-linking was done at 4 °C with steady agitation at rpm. Centrifugation of the reaction mixture at 10,000 rpm for 5 minutes at 4 °C yielded the Phy-CLEAs. To eliminate excess GA and unbound enzymes, the CLEAs were washed three times with sodium phosphate buffer (50 mM, pH 7) The CLEAs were subsequently placed on hold. The CLEAs were then suspended in a 50mM sodium phosphate buffer (pH 7) and assayed for phytase activity, followed by storage at 4 °C [33].

CHAPTER- 4
RESULTS AND DISCUSSION

The following results were obtained using numerous different ways in this study; these results were obtained by utilizing the resources available, and several different research articles also contributed to the development of the results. The original fungal strain was successfully re-grown, yielding the following findings

4.1 Fungal Regeneration

The A. niger laboratory strain was re-grown on potato dextrose agar prepared for its revival under the right conditions, and spreading was also done, resulting in a revived strain on the Potato agar plates (fig 6a) similarly fungal growth was done in PDB broth (fig 6b).

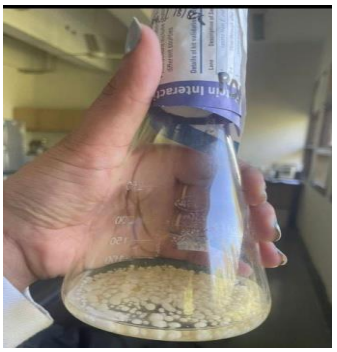


Figure6. (a)A.niger on PDA plates **(b) A.niger on PDB broth**

4.2 Morphological Characters

The fungal staining was done on the unique *A. niger* strain, and when seen under a microscope, it confirms the existence of. The fungal expansion is moderate, as seen by staining at 62 and 86 hour intervals.

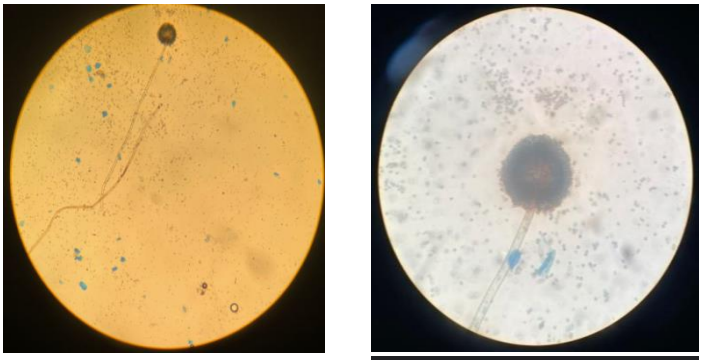


Figure 7.A.niger after Lacto phenol cotton blue staining

4.3 Inoculum preparation

The spore suspension was generated from 96-hour-old PDA slants by collecting spores in normal saline and counting them using a hemocytometer. In the beginning, spore suspension with 13 $\times 10^6$ spores/ml was utilized for inoculation.

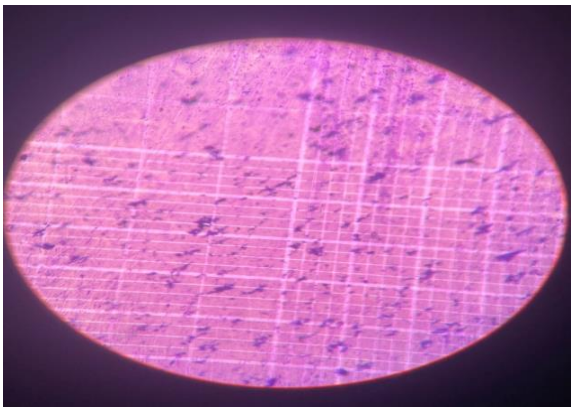


Figure8. Counting No. of spores using hemocytometer

4.4 Solid state fermentation

Surface culture fermentation is a biological approach. SSF (Solid state fermentation) is a process for cultivating microorganisms on a solid media that contains no or very little liquid. SSF is a cost-effective and simple process since it requires less capital and equipment. The most cost-effective and energy-dense substrates utilized in the fermentation sector are oil cakes and standard agricultural wastes (brans of wheat and rice, rice husk etc.

Figure9: after 5 day's solid-state fermentation conical flask

4.5 Phytase Extraction and enzymatic assay

After centrifugation at 7000 rpm for 20 minutes at 4°C and storage for 24 hours a clear supernatant was achieved, which was then used as crude phytase enzyme.

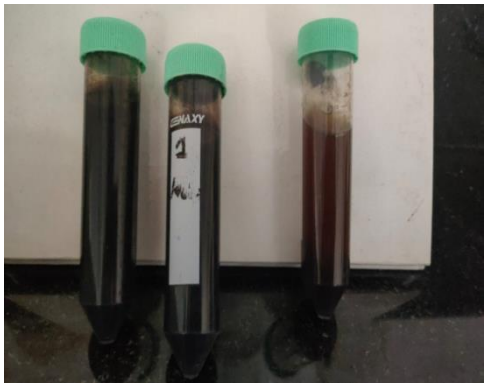


Figure10. Tubes containing crude phytase after centrifugation

Standard Assay:-

Table 5:- Dihydrogen Phosphate Assay composition with absorbance

S.No	Conc.(mg/ ml)	KH_2PO_4 (µl)	D/W (µl)	10% TCA (µl)	Coloring reagent (µl)	OD_1 at 750 nm	OD_2 at 750 nm	OD_{Avg}
1	0	0	1000	0	0	0	0	0
2	0.1	100	900	250	1000	0.125	0.155	0.14
3	0.2	200	800	250	1000	0.243	0.236	0.239
4	0.3	300	700	250	1000	0.315	0.378	0.346
5	0.4	400	600	250	1000	0.451	0.426	0.438
6	0.5	500	500	250	1000	0.536	0.548	0.542
7	0.6.	600	400	250	1000	0.628	0.645	0.636
8	0.7	700	300	250	1000	0.754	0.715	0.734
9	0.8	800	200	250	1000	0.848	0.851	0.849
10	0.9	900	100	250	1000	0.939	0.936	0.937

11	1	1000	0	250	1000	1.093	1.082	1.087

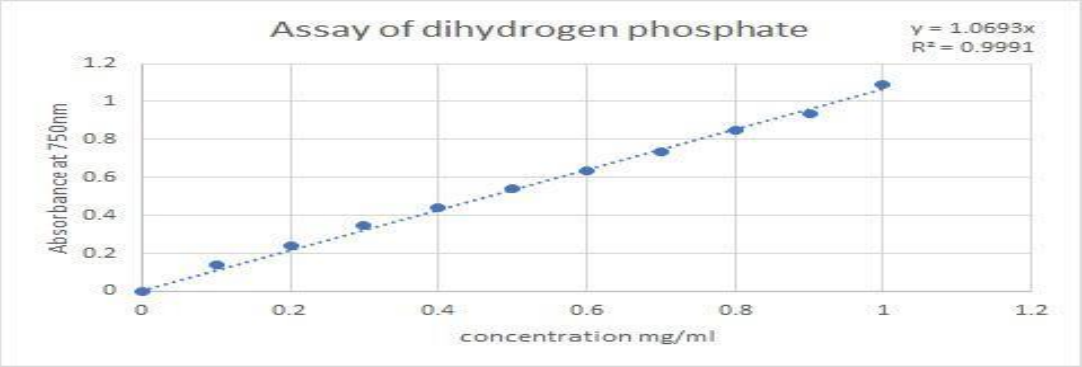


Figure11. Standard curve of Dihydrogen phosphate

Table6:- Standard Assay for Protein content with absorbance

S.No	Conc. (mg/ml)	BSA (µl)	Distilled H2O (µl)	Bradford Reagent (µl)		OD_1 at 595 nm	OD_2 at 595 nm	OD_{Avg}
1	0	0	1000	1000		0	0	0
2	0.1	100	900	1000		0.132	0.13	0.131
3	0.2	200	800	1000		0.256	0.258	0.257
4	0.3	300	700	1000		0.362	0.361	0.361
5	0.4	400	600	1000		0.458	0.46	0.459
6	0.5	500	500	1000	**Incubation at Dark for 5- 10 min**	0.555	0.552	0.553
7	0.6	600	400	1000		0.685	0.688	0.686
8	0.7	700	300	1000		0.736	0.734	0.735
9	0.8	800	200	1000		0.845	0.848	0.846

10	0.9	900	100	1000		0.945	0.931	0.938
11	1	1000	0	1000		1.04	0.998	1.019

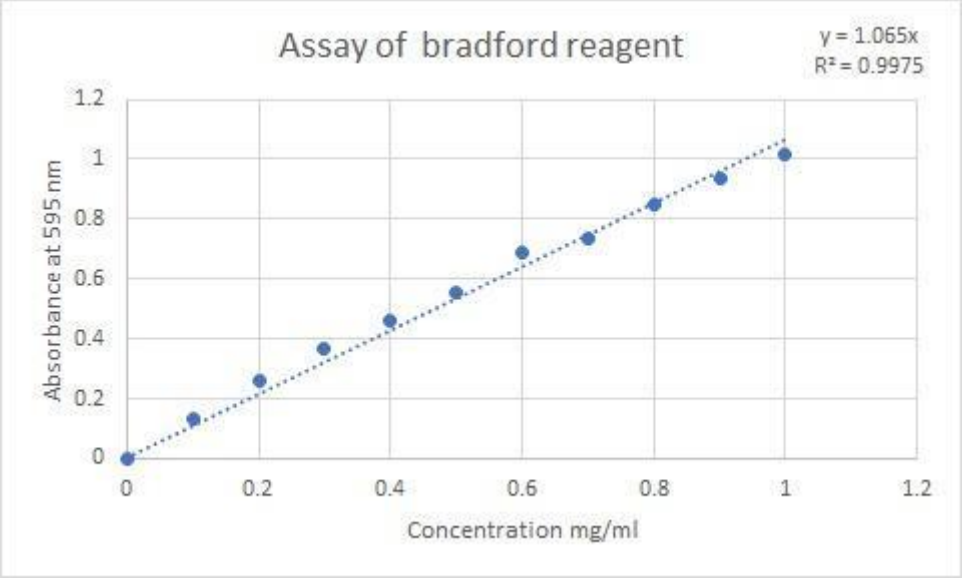


Figure12. Standard curve of BSA using Bradford reagent

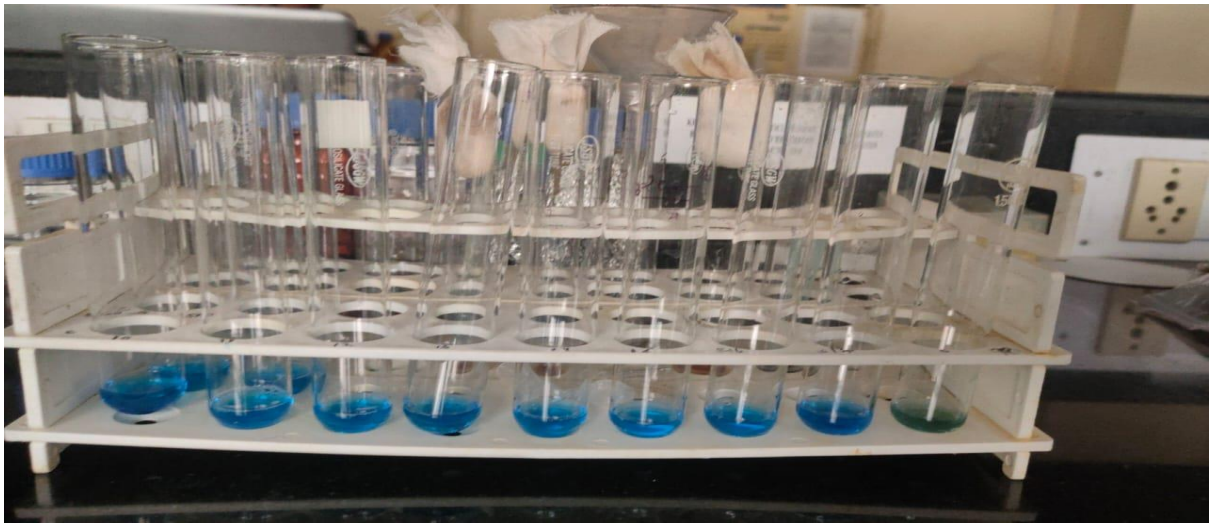
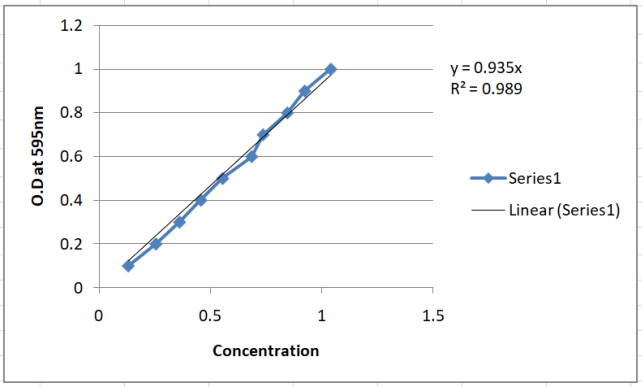


Figure13. Test tubes while doing Bradford test

Table 7:- Phytase Assay for protein content with absorbance

Crude Phytase (µl)	Distilled H2O (µl)	Bradford reagent (µl)	Incubation in dark for 5-10 min	OD at 595 nm	Phytase concentration (mg/ml)
1000	0	1000		0.911	0.8611
500	500	1000		0.491	0.4492

Conc mg/ml	Absorbance at 595nm	
0	0	0
0.1	0.132	0.13
0.2	0.256	0.258
0.3	0.362	0.361
0.4	0.458	0.46
0.5	0.555	0.552
0.6	0.685	0.688
0.7	0.736	0.734
0.8	0.845	0.848
0.9	0.923	0.931
1	1.04	0.998
unknown	0.911	0.491
unknown	0.861179	0.449229



4.6 RESULTS:-

The substrate is still an important feature in SSF since it provides a physical surface for fungal growth as well as nutrients and energy sources [34]. According to our statistical investigation, the kind of substrate has a substantial impact on phytase synthesis. Wheat bran (76.34 0.99 U/gds) was determined to be the most suitable (p-value<0.05) among the various agri-waste evaluated. The age and amount of the inoculum have a direct effect on the metabolic rate and proliferation of the microorganisms [9,35]. Statistical research revealed that both inoculums age and inoculums level had a significant influence on phytase synthesis (P-value <0.05).Control the formation of phytase, Phytase production rises steadily with inoculum age, reaching a peak in the case of a 3-day old inoculum (13×10^6spores/ml). Temperature and period of incubation for SSF remain essential elements since both impact metabolic activities during development and help in the stability of the enzymes [16][5]. Statistical analysis revealed that temperature and incubation time were both important factors in increased phytase production (P value<0.05). We discovered that *A. niger NT7* grew faster at higher temperatures and achieved its maximal production titer at 35 °C. Furthermore; we discovered that phytase synthesis increased with incubation duration and peaked on the fifth day of incubation. However, after 5 days, there was a progressive drop in phytase synthesis, which might be due to nutrient depletion, toxic end product buildup, moisture loss, or a change in medium pH.

Bradford assay was done. The assay works on the principle of binding coomassie die with protein molecules under acidic conditions resulting in a color change from brownish green to blue. Moreover, absorbance was accessed at 595 nm; the concentration comes to 0.862mg/ml, which looks like a good concentration [36].

CHAPTER- 5
REFERENCES

1. Falahi, Parisa, Habte-Michael Habte-Jian, and Valdemar Rossi. "Depolarizing Enzymes in Human Food: Bakery, Dairy Products and Beverages." Enzymes in human and animal nutrition. Academic Press, 2018. 211–237.
2. Bhavsar K, Buddhiwant P, Soni SK, Depan D, Sarkar S, Khire JM (2013) Phytase isozymes from Aspergillus niger NCIM 563 under solid state fermentation: biochemical characterization and their correlation with submerged phytases.Process Biochem 48(11):1618–1625.
3. Bogar B, Szakacs G, Pandey A, Abdulhameed S, Linden JC, Tengerdy RP (2003b) Production of Phytase by Mucor racemosus in solid-state fermentation.BiotechnolProg19(2):312–319.
4. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem72(1–2):248–254.
5. Cherian, Muneer and Joseph J. Rakis. "Phytic Acid Interactions in Feeding Systems."Critical Review in Food Science and Nutrition 13.4 (1980): 297-335.
6. Tai, Hondesh-Ming, and others. "Overreaction and its biochemical properties of Escherichia coli phytase in Pitcheria pastoris." Journal of Agricultural and Food Chemistry 61.25 (2013): 6007-6015
7. Cherian, Muneer and Joseph J. Rakis. "Phytic Acid Interactions in Feeding Systems."Critical Review in Food Science and Nutrition 13.4 (1980): 297-335.
8. Helmenstine, Anne Marie, Ph.D. "Enzyme Biochemistry - What Enzymes Are and How They Work." ThoughtCo, Apr. 14, 2022, thoughtco.com/enzyme-biochemistry-4042435
9. Ogunleye, Akin & Olaiya, Grace. (2015) Isolation identification and mycotoxin production of some mycoflora of dried stockfish(gadus morhua)Academic Journal of Science. 4. 345-363
10. Jatuwong, Kritsana & Suwannarach, Nakarin & Kumla, Jaturong & Penkhrue, Watsana & Kakumyan, Pattana & Lumyong, Saisamorn. (2020). Bioprocess for Production, Characteristics, and Biotechnological Applications of Fungal Phytases. Frontiers in Microbiology. 11. 188. 10.3389/fmicb.2020.00188.
11. Tai, Hondesh-Ming, and others. "Overreaction and its biochemical properties of Escherichia coli phytase in Pitcheria pastoris." Journal of Agricultural and FoodChemistry 61.25 (2013): 6007-6015
12. Mulne, Edward J., Catherine B. Daly, and Abul HJ Ullah. "Progress in Phytus Research." (2000): 157-199.
13. Mulney EJ, Ulla AH (2003). "Phytase contains a wide variety of enzymes". Biochem Biophys Res Commun. 312 (1): 179–184. doi: 10.1016 / j.bbrc.2003.09.176. PMID14630039

14. Cao L, Wang WM, Yang C, Yang Y, Diana J, Yakupitiyage A, Luo Z, Li D (2007) Application of microbial phytase in fish feed. Enzym Microb Technol 40(4):497–507

15. Chadha BS, Harmeet G, Mandeep M, Saini HS, Singh N (2004) Phytase production by the thermophilic fungus Rhizomucor pusillus. World J Microbiol Biotechnol 20(1):105–109.

16. Hegeman, Carla E., and Elizabeth A. Grabou. "Novel phytase with similarity to violet acid phosphatase expressed in cotyledons of soybean seedlings." Plant Physiology 126.4 (2001): 1598-1608.

17. Nakashima, B.A., Encllister, T. A., Sharma, R., & Salinger, L.B. (2006). Diversity of phytases in rumen. Microbial Ecology, 53 (1), 82–88. DOI: 10.1007 / s00248-006-9147-4 33.

18. Amara, A. M. "Interactions between Wheat Properties and Enzyme Replacement in a Broiler Diet." Animal Feed Science & Technology 199 (2015): 1-9

19. Krishna, Chundakkadu. "Solid-State Fermentation - An Overview." Critical Review in Biotechnology 25.1–2 (2005): 1–30.

20. Chantasartrasamee K, Na Ayuthaya DI, Intarareugsorn S, Dharmsthiti S (2005) Phytase activity from Aspergillus oryzae AK9 cultivated on solid state soybean meal medium. Process Biochem 40(7):2285–2289.

21. Chen J, Zhu Y, Nout MJR, Sarkar PK (2013) Solid state fermentation for foods and beverages. CRC Press,

22. Boca Raton Chugh P, Soni R, Soni SK (2016) Deoiled Rice Bran: a substrate for co-production of a consortium of hydrolytic enzymes by Aspergillus niger P-19. Waste Biomass Valoriz 7(3):513–525.

23. Consulting AR (2019) Phytase market global size worth over $1 billion by 2025: Acumen research and consulting. In: Globe News wire News Room. Accessed 21 Jan 2020

24. Das A, Raychaudhuri U, Chakraborty R (2012) Cereal based functional food of Indian subcontinent: a review. J Food Sci Technol 49(6):665–672.

25. Soni SK, Magdum A, Khire JM (2010) Purification and characterization of two distinct acidic phytases with broad pH stability from Aspergillus niger NCIM 563. World J Microbiol Biotechnol 26:2009–2018.

26. Suresh S, Radha KV (2016) Statistical optimization and mutagenesis for high level of phytase production by Rhizopus oligosporus MTCC 556 under solid state fermentation. J Environ Biol 2:253

27. Tian M, Yuan Q (2016) Optimization of phytase production from potato waste using Aspergillus ficuum. 3 Biotech 6:256.

28. Tripathi P, A JL, Kapoor M (2018) Phytase from Citrobacter koseri PM-7: enhanced production using statistical method and application in ameliorating mineral bioaccessibility and protein digestibility of high-phytate food. Prep Biochem Biotechnol 48(1):84–91.

29. Vashishth A, Ram S, Beniwal V (2017) Cereal phytases and their importance in improvement of micronutrients bioavailability. 3 Biotech 7(1):42.

30. Vasudevan UM, Jaiswal AK, Krishna S, Pandey A (2019) Thermostable phytase in feed and fuel industries. BioresourTechnol 278:400–407.

31. Kumari, Neha & Bansal, Saurabh. (2021). Production and characterization of a novel, thermo tolerant fungal phytase from agro-industrial byproducts for cattle feed. Biotechnology Letters. 43. 1-15. 10.1007/s10529-020-03069-8.

32. Linderberg JE, and B Ogle (2002) Digestive physiology of pigs. In: Proceedings of the 8th Symposium. Journal of Animal Physiology and Animal Nutrition 86: 55–56.

33. Makolomakwa M, Puri AK, Permaul K, Singh S (2017) Thermo-acid-stable phytase-mediated enhancement of bioethanol production using Colocasia esculenta. Bioresour Technol 235:396–404.

34. McClenny N (2005) Laboratory detection and identification of Aspergillus species by microscopic observation and culture: the traditional approach. Med Mycol 43(Supplement_1):125–128.

35. McCoy M (1998) Enzymes emerge as big as feed supplement. Chem Eng News 76(18):29–30 Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428