Production and partial purification of phytase from novel *Bacillus* strain

Bachelors of Technology in Biotechnology

A Project Report

Submitted By

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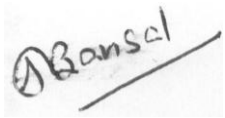
DECLARATION

I do hereby declare that this dissertation and titled "**Production and partial purification of phytase from novel *Bacillus* strain"** 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.

Arsh Tyagi (161838)

CERTIFICATE

This is to certify that the work titled "**Production and partial purification of phytase from novel *Bacillus* strain**" submitted by **Mr. Arsh Tyagi** in partial fulfillment for the award of degree of **Bachelor of Technology** in **Biotechnology** from Jaypee University of Information Technology, Solan 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 of diploma.



Signature of Supervisor

Name of Supervisor: Dr. Saurabh Bansal

Date: 15-06-2020

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Arsh Tyagi

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List of Abbreviations

ASP	Ammonium sulphate precipitation
AP	Chilled acetone precipitation
U/ml	Unit per millilitre
DNA	Deoxyribo nuclic acid
RNA	Ribonuclic acid
GI	Gastro intestinal
SSF	Solid state fermentation
SmF	Submerged fermentation
Temp	Temperature
Min	Minutes
Hrs	Hours
RPM	Revolution per minute
º C	Degree Celsius
PSM	Phytase screening media
µl	microlitre
w/v	Weight by volume
ml	millilitre
HAP	Histidine Acid Phosphatases
BBP	β propeller phytase
PAP	Purple Acid Phosphatase
PTP	Protein Tryosine Phosphatase-like phytase

Abstract

The Phytate also called phytic acid is known to be storage component of the phosphorus in seeds and plants. Phytate have anti-nutritional properties such as it shows more affinity towards many metal ions and chelates them. It is the organic form of phosphorus that is not digestible in the gut without the help of an enzyme called phytase, this enzyme phytase hydrolyze the phytic acid and forms inorganic phosphorus that is helpful in digestion. In this study, we have achieved the production of phytase enzyme using the fermentation technique called submerged fermentation technique, which uses aqueous solution mixed with other essential chemicals for the production. The production with the highest activity of 0.267 U/ml of the phytase enzyme was achieved with the 48 hrs incubated culture, the activity was calculated through differential agar media. Side by side the partial purification of the produced enzyme was done by the two different methods which were ammonium sulphate precipitation (ASP) and chilled acetone precipitation (AP). Among these two precipitation technique, the chilled acetone precipitation was more successful because after precipitation it gave phytase activity of 0.471 U/ml which is more than the ASP i.e. 0.411 U/ml.

CHAPTER- 1

INTRODUCTION

During the exponential era of industries the demand of microorganisms is of the utmost need as microbe plays crucial part in the producing intracellular and extracellular enzymes at large scale (1). These are mostly in demand because the microorganisms are easily available in environment and their presence unfailingly affects the surroundings while being beneficial (2). Bacteria being the most common among microbes are certainly useful and present in large resources like; soil, water, air. Sometimes they lend us aid through curdling of milk in yogurt, helping in digestion while sometimes they are dangerous causing diseases like pneumonia. The bacteria are subdivided into two categories based on presence and absence of outer cell wall called gram positive and gram negative (3, 4).

Bacillus are the gram positive facultative anaerobes and aerobic spore forming rod shaped bacteria that are widely distributed in the environment though there primary habitat is soil, these spores are resistant to radiation, heat and cold(5). These species have significant usage as microorganisms like; enzyme production, antibiotics, agriculture and pharmaceutical industries.

1.1Hydrolytic enzymes

Hydrolytic enzymes are the type of enzymes that acts as biochemical catalysts that break chemical bond using water resulting in division of larger molecule to smaller molecule by ionisation of water and splitting of molecule. Some examples of hydrolytic enzymes are: phosphatase, lipase, protease, glycosidase, amylase, xylanase, chitinase, phytase. Hydrolytic enzymes are valuable because of their degrading properties, which include catalytic conversion of starch to glucose, phytase catalyses hydrolysis of phytic acid (6).

1.2 Phytic acid

Phosphorus is known to be the 2nd most rich mineral that is found in the body of animals and humans, which is used by microbes to integrate different molecules like RNA, DNA. This is stored in the plants as phytic acid (inositol-6phosphate). Phytate is a six fold dihydrogen phosphate ester of inositol, which is especially present in bran, oil seeds, grains, legumes, cereals, etc. Phytic acid is a nondigestible, phosphorus in organic form that is not biologically accessible to non-ruminant organisms because of non availability of digestive enzyme phytase. Because of indigestibility of phytic acid in the GI tract, the level of phosphorus increases in the

manure from the animals which leads to environmental problems like: eutrophication (7). Since phytic acid have strong binding affinity toward zinc, iron, dietary minerals and calcium resulting in inhibiting their absorption (8).

1.3 Phytase

The enzyme phytase is gradually making its way into the enzyme industry because of its action of catalysis hydrolysis of phytic acid to produce inorganic phosphorus that is easily digested by animals. Phytase enzyme is also known as (myo-inositol hexakisphosphate phosphohydrolases) (9). Its presence is known to humans for a long time but it is not synthesized by the body itself but it is formed by certain microbes present in the gut of body. This enzyme is isolated from the organisms by certain techniques and methods to commercially produce it. It is commercially produced as this is added to the animal feed and act as food supplement, that when ingested helps in the digestion process and hydrolyze the phytic acid and convert organic phosphorus into inorganic form (10). The problems created by the phytate can overcome by the phytase enzyme as it is produced by some microbes located in the gut of ruminant organisms (sheep, cattle), while absent in non ruminant animals (dogs, pigs, birds, humans), hence in research it is found that phytase can be supplemented in the feed and act as animal feed supplement and enhancing the nutritive values.

1.3.1 History

The first phytase was inaugurated in 1907 from the bran of rice and was classified as plant phytase (11). The first animal phytase was found from the blood and liver of calf (12).

1962 was the time when the trial was done to commercially produce phytase for the animal feed. But after so many try and hardships it was discontinued for production on commercial scale. Several different microbial strains were tested and tried for the production of the phytase enzyme but at that time *Aspergillus niger* NRRl 3135 was the most promising strain that could produce the phytase (13). Finally after so many years of research BASF a German company began to sell commercial phytase in 1991 which was used to increase nutrients in animal feed (13).

1.4 Production of phytase

Production of the Phytase is a crucial process including cultivation of strains of organisms, separation, purification, cell disruption, debris removal and precipitation. The process is varied according to the nature of the strain (extracellular or intracellular) (14). For the production of enzymes it is advisable to have the organisms that could produce higher yields, can have inexpensive media, no contamination. While producing the enzymes there are certain factors that must be dealt with caution like: screening of the strains by gram staining that distinguishes between gram negative and gram positive bacteria, mode of cultivation as this provides the inoculation of the microorganism by two methods – Surface culture and Submerged culture (this depends upon the microbe to be inoculated.

1.4.1 Surface fermentation

In surface culture fermentation a biological technique is used, SSF (Solid state fermentation) is a process which is used for growing microbes on a solid medium which contains no or very less amounts of liquid. SSF is an economical and easy process as it requires lower capital and also requirement for the equipment is small, the most economical and immense power rich substrate used in fermentation industry are depicted as cakes of oil and conventional agricultural by-products (brans of wheat and rice, rice husk, etc).

1.4.2 Submerged fermentation

The submerged culture is a technique which includes the inoculation of microbes in the liquid media, this technique offers ease with the control of pH, temperature, composition (15). This method includes the aqueous solution mixed with essential components necessary for the growth of microorganism in the solution. The requirement of oxygen is high, and the production of the enzyme takes place when there is interaction of microorganism with the nutrients present in the broth (16).

1.5 Purification and characterization of phytase

Optimization of characteristics is followed because of the search of the most optimum conditions and compositions for the enzyme production, several characteristics are optimized like: temperature, pH, solvent, agitation speed, incubation temperature, metal ion effect, etc. this

is done so to improve the yield of the phytase. Protein requires the partial purification to remove out the debris of cells and unwanted materials of microbes, hence different methods are developed for the partial purification like ammonium sulphate precipitation by separating out proteins by altering their solubility in higher salt concentrations and chilled acetone precipitation (17). When microbial enzymes of highest purity is required, a step of purification is needed to divorce the protein from non enzyme protein and its component, so enzyme fractionation in aqueous salt solution is generally done which is an effect of electrolyte and non-electrolyte synergy, while the non-electrolytes are soluble in limit in greater salt concentration (18).

1.6 Applications

Phytase play a fundamental role in different industries because of its ample use in food, agriculture, pharmaceutical and paper and pulp industry to command and hasten reactions in view to instantaneously and precisely attain a profitable final product (19). Phytase is used as:-

- Saccharification: In Food industry as diastase enzyme which converts starch in the food into maltose and glucose, generally used in brewing beers and wines. It also plays role in glycolysis to convert sugar in milk into lactic acid for the cheese production.
- In the detergent industry where the enzymes like protease, amylase, cellulose breakdown the dirt.
- Enzyme catalysis has been used for the production of pharmaceutically active chemicals, also has been used as feed supplement.
- Bioconversion of natural gas into liquid fuels.

CHAPTER- 2

REVIEW OF LITERATURE

2.1 Genus *Bacillus*

Bacillus is the gram positive rod shaped like microbes that are aerobic, anaerobic and few are facultative anaerobes also. They are both free-living and parasitic and found mainly in soil and water, when under adverse environment or conditions their cells produce endospores. The endospores are the special type of cell which is formed as instead of binary fission where one cell divides into two cells, this cell forms a new cell inside the older and possesses several outer-layers which provide resistance against chemicals, direct sunlight, radiations, extreme cold and heat. These cell are present for longer periods of time, just like other reproductive spores, endospores germinates and forms vegetative cell which will thrive again until appropriate nutrients and conditions are available and if not then again endospores can be formed. Out of the bacteria of *Bacillus* species present, some of them are good and some are bad for the humans and environment. For example, *B. cereus* is responsible for spoilage in the canned foods and may cause food poisoning, *B.Subtilis* is another species that are common containment of laboratories, similarly, *B. anthracis* causes anthrax in humans and animals. Though most strains are non pathogenic for humans and provide benefits to the environment like *B. thuringiensis* causes infection to the insects by producing a toxin called BT toxin and protects cotton plant from infection, several strains of *B. amyloliquefaciens* are discovered to synthesize different antibiotic substances from the plants. Apart from general advantages it performs huge role in the industrial applications.

2.2 Enzymes

These are the substances generally proteins that are produced by organisms and act as biocatalysts and bring out specific biochemical reactions. These acts on substrates and converts them into molecules called products. Nearly all metabolic pathways inside a cell need enzyme catalysis in fast rate in order to sustain a balanced life. Just like catalysts, enzymes affect that rate of reaction by increasing or decreasing the activation energy, inhibitors and activators are the molecules that affects the enzyme activity as the name suggests the inhibitors like some drugs and poison inhibits the functioning of enzymes hence effecting its activity, the enzymes are also affected by the certain conditions like pH, temp, heat and may even lose structural and catalytic properties. With rapid development in the industrial aspects like optimization, characterization and gene discovery, enzymes are being used as biocatalysts. More than 80% of enzymes that are

used in the industries belong to hydrolases. The enzyme phytase currently is one of the most usable enzymes as it is used in animal feed supplements, synthesis of inositol phosphates, transgenic plants.

2.3 Phytic acid

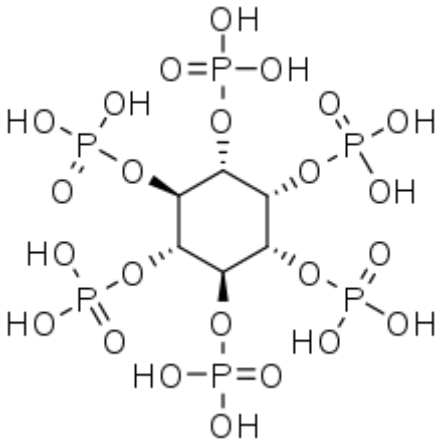

Fig.1 Structure of Phytic Acid

Phytic acid (myo-inositolhexakis phosphate) also known as phytate or phytin is an ester of inositol ring of phosphate, having six fold groups of phosphate, making it an origin source of phosphorus storage in many plant tissues, mainly in bran, legumes, cereals and seeds. Phytic acid has many points of (- ve) charges in expanded pH range because of the 12 ionisable hydrogen atoms. This makes the phytate a substantial chelating agent (20).

Phosphorus is essential ingredient in the diet of animals for their right growth but the phosphorus and inositol in form of phytate is not biologically accessible to the non-ruminant organisms because of the absence of the enzyme phytase that helps to hydrolyse the inositol-phosphate linkages (21). Phytic acid is indigestible, organic form of phosphorus that is not digested by non ruminant animals, because of the indigestibility of the phytic acid in GI tract, the levels of phosphorus increases in the manure of the animals and results in many environmental problems like: eutrophication. For the reason of the anti-nutritional quality of the phytate, it constrains the absorption of many materials like mineral, Ca, Fe and zinc by chelation of ions, since phytate

have an immense affinity towards these materials (22). When chelating the metal ions the insoluble precipitates are formed during binding of phytic acid to zinc or iron that makes them less absorbable in intestine.

Hence, the hydrolysis of phytic acid is necessary to avoid the phosphorus pollution in the environment. Since it is only absorbed in the gut of ruminant animals due to the presence of microorganisms that produce phytase enzymes to hydrolyse the phytate. But on the other hand in the case of non ruminant animals where the phytic acid is not absorbed in sufficient amounts or the phytase enzyme is not produced, then the enzyme is given from outside in the form of animal feed supplement.

Phytic acid is present in many different food items like seeds, grains, pulses, etc. The technique used at home to prepare food might breakdown and reduce the phytic acid. It is often used as food additive in the form of preservative.

Table.1 Food sources of phytic acid:-

Food	**Percentage [%]**
Almonds	1.35
Beans	2.38
Brown rice	0.84
Buckwheat	1.0
Pumpkin seeds	4.3
Sesame seed flour	5.6
Tofu	1.46
Soyabean	1.36
Wheat flour	0.36
Oat meal	2.4

2.4 Phytase

Phytase is a special kind of phosphatase enzyme, with the surge in research about enzymes it has been found out that phytase is quite useful in the fields related to commercial and research because of the application in many different industries like pharma, biotech, nutrition, biomed. Phytases dephosphorly the phytate or phytic acid into the (myo)inositol derivatives and in-organic phosphorus (23). Phytase enzyme was first found in the rice bran by (24). Though phytases are spread all over the place in our environment and is originates by discrete source including plant tissues, microorganisms and animals (25).

2.4.1 Classification of phytase

International Union of Pure and Applied Chemistry (IUPAC) and International Union of Biochemistry and Molecular Biology (IUB) (1979) have acknowledged two different classes of phytase enzyme based on their nature as catalyst function and structural properties. Within the different classes of the phytase, the structure difference can be seen and their mechanism to hydrolyze phosphate from phytic acid is different (26). They are 3 phytase (E.C.3.1.3.8) and 6 phytase (E.C.3.1.3.26) and it is also classified on the basis of the catalytic functions and active site geometry into four different classes.

These are the four classes:-

Histidine acid phosphatases (HAP)

The first and the utmost researched class of phytase is known as HAP that have been isolated from prokaryotes and eukaryotes including filamentous fungus, yeasts, plants, etc (27). Each member in this division of phytase engage with common active site motif sequence (Arg- His- Gly- Arg- X- Pro) and works in 2 phase mechanism that hydrolyze phytate (28). For the optimal activity of the family of HAP the co-factor is not necessary. Phytase enzyme produced by the fungus *Aspergillus niger* is generally mostly studied and greatly acknowledged because of its greater specific activity, among prokaryotes *E.coli* is used for isolation.

β propeller phytase (BBP)

This is one of the recently discovered a class of phytase and display none of the homology as familiar to phosphatases and include 3 propeller sheets and favour six bladed propeller (29).

Firstly it was cloned from *Bacillus* species. It has got its name as alkaline phytase because it is activity in alkali pH requiring Ca ion for thermostability. These are dominant phytic acid degenerating enzymes in aquatic body and soil and, play an important aspect in phytate-phosphorus cycling (30).

Purple acid Phosphatase (PAP)

Phytases newly originated by the germinated soybean from cotelydon, with active motif site (31). The genomic databases have disclosed that Purple acid like sequences in fungi, mammals, plants but only the soybean has shown significant phytase activity. Active site motif sequence, 3-D structure and recommended mechanism of catalyze is determined for Purple acid.

Protein Tryosine Phosphatase-like Phytase (PTP)

This is the newest phytase that has been discovered. Only very few known phytases belongs to this family of enzymes. PTP has been recently isolated from the bacteria that are present in the gut of ruminant animals (32). The role and biological substrates have not been identified yet.

2.4.2 Sources of Phytase

The occurrence of phytases is widely seen in the nature in territory of plants tissue and animals, microorganisms like bacteria, fungi, yeast but maximum levels have been seen to be produced in the wheat and rye and barley (33, 34). Majority of the research has been concentrated on the microbial phytases because of abundance. Generally these are obtained from the filamentous fungus like *Aspergillus, Penicillium, Rhizopus*. Phytases have been seen to be occurred in bacteria as well like *Bacillus, E.coli, Aerobacter.*

Table.2 List of microorganism producing phytase:-

Organisms	**Reference**
Bacillus subtilis	Tye et al (2001)
Bacillus licheniformis	Tye et al (2001)
Cirtobacter baraakii	Kim et al., 2003
Escherichia coli	Greiner et al., 1993
Bacillus circulans	Anis Shobirin et al.,2009
Bacillus megaterium	Kumar et al., 2013
Bacillus sp. KHU-10	Choi et al., 1999
Enterobacter sp. 4	Yoon et al., 1996
Lactobacillus amylovorus	Sreeramulu et al., 1996
Lactobacillus plantarum	Saribuga et al., 2014
Pseudomonas putida	Richardson et al., 1997
Selenomonas ruminatium	Yanke et al., 1999
Aspergillus ficuum	Ullah et al.,1988
Saccharomyces cerevisiae	Howson and Davis, 1983
Schwanniomyces castellii	Lambrechts et al., 1992
Aspergillus candidus	Howson and Davis, 1983
Aspergillus carneus	Ghareib, 1990
Botrytis cinerea	Howson and Davis, 1983
Fusarium verticillioides	Marlida et al., 2010
Mucor racemosus	Howson and Davis, 1983
Rhizopus stolonifer	Howson and Davis, 1983
Thermomyces lanuginosus	Berka et al., 1998

2.4.3 Chemical properties and structure of phytase

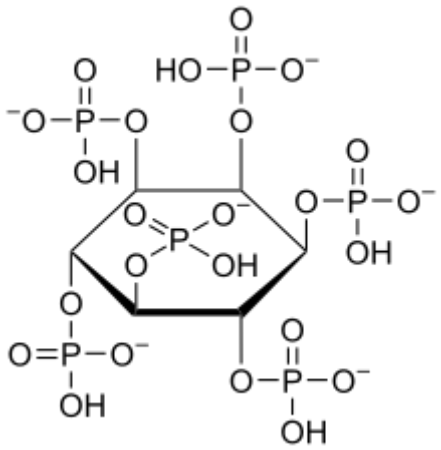

Fig.2 Phytase structure

Phytases are small, acid stable molecule that acts and hydrolyze the phytic acid. Phytase mainly uses histidine in the reaction which is activated by aspartate in the neighbour. Phytic acid have phosphate groups because of this it carries a strong negative charge. Hence, it is positioned by positively charged arginine and lysine.

2.4.4 Chemical reactions including phytase as hydrolyzing agent

Phytic acid being anti-nutritional component of the food material as it has skills of making complex with the metallic ions and inhibiting the activity. Phytate has six-fold groups of phosphate out of which five of the phosphate groups are cleaved off by the phytases by hydrolyzing in the step wise manner and generate inorganic phosphorus.

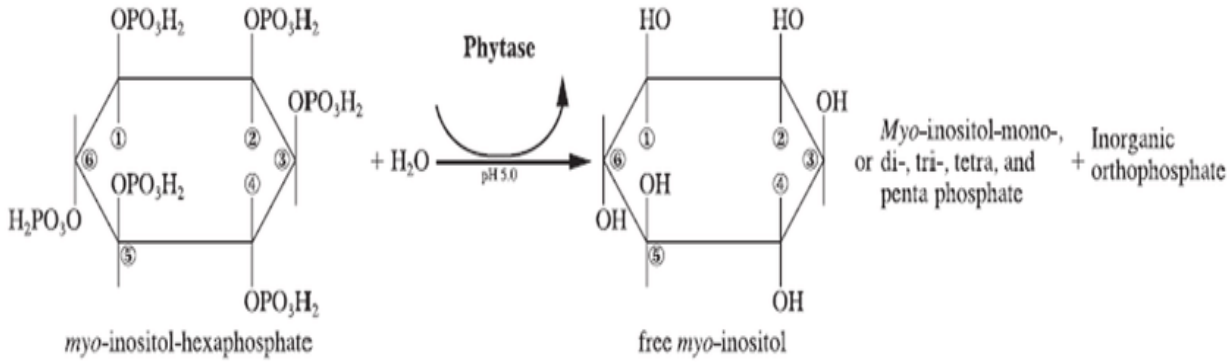

Fig.3 Hydrolysis of phytate by phytase

2.5 Production of phytase

The production of phytase includes certain strains and organisms which have their own condition and requirements that allows higher phytase production. There are two different fermentation approaches are followed for production of phytase which are:

1. Submerged Fermentation (SmF)
2. Solid State Fermentation (SSF)

2.5.1 Submerged Fermentation (SmF)

This is mostly used for production of bacterial phytase, this method of fermentation includes the Submersion of microbes in the excess of water, alcohol, oil or nutrient broth generally known as medium with other additional necessary nutrients required for growth (35). Since, the growth of microorganisms is vastly affected by the optimal conditions and methods with certain additional factors. The factors are:

- Physical conditions- such as temperature, agitation speed, pH affects the quality and the quantity of the yield, as mojorly the phytase is manufactured at slightly alkaline pH, with around 37 ^{o}c of temperature.
- Chemical conditions-such as nitrogen and carbon sources, oxygen rate, metal ions, these have major effect on the production like glucose concentration has affect on the microbial growth.

The recent times, for the production of phytase SSF is considered to be more appropriate because SmF has:

- Nominal volumetric yield
- Comparatively lesser concentration of by-products
- Greater effluent formation

2.5.2 Solid State Fermentation (SSF)

This is a method of production which requires solid medium with minimal water, it helps in microbe growth without free flowing aqueous medium instead making use of grains bran and oil seed cakes. In the last decade the use of SSF for the production of phytase has gradually increased because of economic as well as commercial point of view. Since, industries want highly efficient methods that have less cost input with higher output with greater performance, so, the SSF method is selected over the SmF method due to the more advantages of SSF over SmF like lower energy requirements, very low water wastage, reduce contamination (36).

Advantages of SSF:

- Medium preparation in inexpensive, while lower raw material requirement.
- Lower requirement of energy and easy aeration.
- The final product is highly concentrated hence, less DSP is required.
- Many domestic as well as agriculture waste can be used as the solid surface.

2.6 Purification and characterization of Phytase

In regards to gain information about the structure and functional relationship and biochemical properties of the enzyme in this case about phytase purification is necessary. This is done to achieve the highest degree of purity of phytase. The process of purification is a complex process because of the presence of several different substances all having different properties like size, solubility, polarity, precipitation and affinity towards acetone, ammonium sulphate, ethanol. There are many methods available to gain higher purity levels but the strategy is to make use of the method which is less expensive and simpler to follow up (37, 38). The objective for concluding the strategy for purification is aimed to achieve:

- Greatest final degree of purity.
- Obtaining product with highest catalytic activity.
- Highest yield of product.

The general method used for purification of enzymes in large laboratory scale is ammonium sulphate precipitation because it is highly soluble, have lower density and stabilize protein

structure, this is the reason it is used as precipitant. Ammonium sulphate is a non-organic salt which gets dissociated into (NH_4^+) and (SO_4^{2-}). Since, the solubility of enzymes vary at different ionic strengths therefore at higher ionic strengths in the aqueous solution containing NH_4^+ and SO_4^{2-} gets attracted towards opposite charges and prevents water to attach toward the molecule of phytase, hence, the enzyme gets precipitated and purified (39).

2.6 Applications

Phytase is an extensive hydrolytic enzyme beneficial to food and feed based commercial companies because of its properties to enhance the nutrition of food by reducing phytic acid through hydrolysis. In day to day scenario, the demand of phytase is growing as the use of phytase has been considered in many industries for processes like plant growth, food and feed supplement, paper and pulp industry. It has its immense effects in the improvement of environmental conditions, its helps to degrade the organic phosphorus so that the soil and nature can be protected from eutrophication.

2.6.1 Dephytinization of food ingredients

Dephosphorylation of phytic acid during the food processing and preserving to reduce the phytate content from the food material, this is achieved by gradual increase in inorganic phosphorus liberation and incubation time increase (40). The dephytinization helps in the absorption of zinc and iron present in the food materials.

2.6.2 Food and Feed additive

Phytases are of great concern for the biotechnological industries for the manufacturing and processing the human and animals feed supplements that can enhance the nutritional values of the food and finding the potential to increase the phosphorus usage and decreasing the phytatae in the food content. Non ruminant animals like pigs, fishes, humans, poultry are unable to absorb the phytate present in the organic form of phosphorous in the foods like oilseeds, celery, plants, so, the phytase is being added to the feed and food material so that it can hydrolyze the phytate and can help to convert organic phosphorous into inorganic phosphorous.

2.6.3 Role in Bread making

The supplementation of phytase from *Aspergillus niger* into the dough of the whole wheat bread resulted in increased phytase activity during the proofing time of the bread liberated higher reducing sugar, inorganic phosphorous in comparison to the breads baked using different enzymes (41).

2.6.4 Paper and Pulp industry

The phytic acid produce highly toxic and carcinogenic by-products when it is enzymetically degraded, this is done to achieve the catalytic hydrolyze phytate using a thermostable phytase which can act as a bio operator for degrading phytate during paper and pulp processing (42).

Table.3 Industries commercially producing microbial phytases:-

Company	**Country**	**Microbe**
Roal	Finland	*A. awamori*
Novozymes	Denmark	*A. oryzae*
AB enzymes	Germany	*A. awamori*
Fermic	Mexico	*A. oryzae*
BioZymes	USA	*A. oryzae*
DSM	USA	*A. oryzae*
Altech	USA	*A.niger*
BASF	Germany	*A. niger*

Source: (Mittal et al., 2003)

CHAPTER- 3

MATERIAL AND METHOD

In order to conduct the research, different experimentation and procedures were constructed in accordance to the availability of resources at the hand and many different chemicals, tools, samples and instruments were used to get the results. The novel *Bacillus* strain adopted in the research was previously isolated by the fellow researcher that strain was regenerated and further experimentation was carried out.

3.1 Microorganism and Inoculum preparation

A novel strain of *Bacillus* species was procured from the previously isolated culture collection. It was regenerated in the nutrient agar broth. The Nutrient agar with addition of D-glucose was prepared in the form of broth as well as plates. Primary inoculum was prepared by inoculating the *Bacillus* spores into the liquid broth and put to rest for 48 hrs at 37 ^{o}C at shaking speed of 150-155 rpm.

Streaking of the *Bacillus* Strains was done on the agar plate from the primary inoculum prepared and placed for incubation at 37 ^{o}C for 24 hr at agitation speed of 150 rpm.

3.2 Media preparation

The different media were prepared for the processes like screening, assays and growth. The phytase screeing media (PSM) was prepared with the compositions as 2% agar, 0.1% sodium phytate, 0.5% NH_4NO_3, 1% D-glucose, 0.05% $MgSO_4.7H_2O$, 0.01% $MnSO_4.H_2O$, 0.05% NaCl, 0.05% $CaCl_2.2H_2O$, 0.01% $FeSo_4.7H_2O$ and the pH was set to 5.5.

The media for different assays were prepared with the same components mentioned above but the only difference was the inactive enzymes used according to the assay preparation like for xylanase assay instead of sodium phytate, xylan was used.

3.3 Screening for phytase

The screening was carried out by inoculating the strain onto the PSM plates formed, and after inoculating, the incubation was done for 24 hrs at 37 ^{o}C. The zone of clearance was formed on the PSM plate and strains were selected from the hydrolytic zones as this test positive/negative for phytase.

3.4 Morphological characters

The staining of the sub cultures was done to gain knowledge about the morphological characters of the strain. The gram staining was performed as it stains gram positive and gram negative strains differently. 100µl inoculum was taken on a thin glass slide and smear was formed that was fixed with heat. The smear was then submerged with drops of crystal violet, after letting it rest for 1 min, it was cleaned with distilled water and then engulfed in Gram's iodine solution and after 1 min wait washed with ethanol, at last few drops of safranin solution was added for counter colouring and after 1 min it was washed using water and kept in open for air drying. This slide was then observed under 40X and 100X zoom using emersion oil.

3.5 Production of phytase enzyme

The production of enzyme prior to partial purification was done with the method of submerged fermentation (SmF) which included aqueous mixture of different components such as 2.8% glucose, 1.8% KCl, 0.5% $MgSO_4.7H_2O$, 1.5% Kh_2PO_4, 1% $CaCl_2.2H_2O$, 0.04% $(NH_4)_2SO_4$ and the solution was set up at 5.5 pH. The 100 ml solution was suspended in 250 ml Erlenmeyer flask and 250 µl culture was suspended in the flask and put to incubation at 37 ^oC for 48 hrs at shaking speed of 150 rpm.

3.6 Phytase acivity

The activity of phytase is calculated to determine the amount of phosphate liberated. The method used was ammonium molybdate as mentioned by (Heinonen and Lahti, 1981). The known quantity of crude enzyme (100 µl) and 0.01% sodium phytate were used as mixture for assay. The reaction was carried out by the 0.1 M acetate buffer set at pH 5.0. After incubation at 40 ^oC for 10 min, 250 µl of 10% TCA was added for stopping the reaction. Colouring reagent was newly formed by addition of 1.875g $FeSO_4$, 0.25g of $(NH_4)_2MoO_4$ and 1.0 ml H_2SO_4 and for making up the total volume to 25 ml distilled water was used. The phytase activity was taken by spectrophotometer at 540 nm.

3.7 Purification of Phytase

Phytase isolated from the *Bacillus* strain was partially purified by the method ASP and chilled acetone precipitation. The ammonium sulphate precipitation was conducted by taking 40% w/v

dry ammonium sulphate was crushed to fine powder in pestle and mortar, the phytase containing solution was equilibrated at 4 ^{o}C and put on the magnetic stirrer inside the fridge to maintain 4 ^{o}C constantly and then the fine powder of ammonium sulphate was added little by little to the solution over a long period of time. At last the solution was centrifuged to obtain the purified phytase.

The chilled acetone precipitation is performed by weighing 15 ml of chilled acetone and taking enzyme solution in a flask at 4 ^{o}C sitting onto the magnetic stirrer, the chilled acetone is added to the solution drop wise for a period and then is centrifuged to obtain the purified phytase enzyme.

CHAPTER- 4

RESULTS AND DISCUSSION

Several different Methods used in this study have given the following results, these results were obtained by using the resources that were in hand and several different research papers also helped in the accumulation of results. The novel bacterial strain used was re-grown successfully and helped to gain the following results.

4.1 Microorganism Regeneration

The *Bacillus* strain that was provided by the laboratory was re-grown in the nutrient broth prepared for its revival by providing suitable conditions, spreading was also performed and that gave us revived culture on the nutrient agar plates. The streaking was also done and smooth pure colonies were obtained.

Fig.4 Primary inoculum of *Bacillus* strain for regeneration

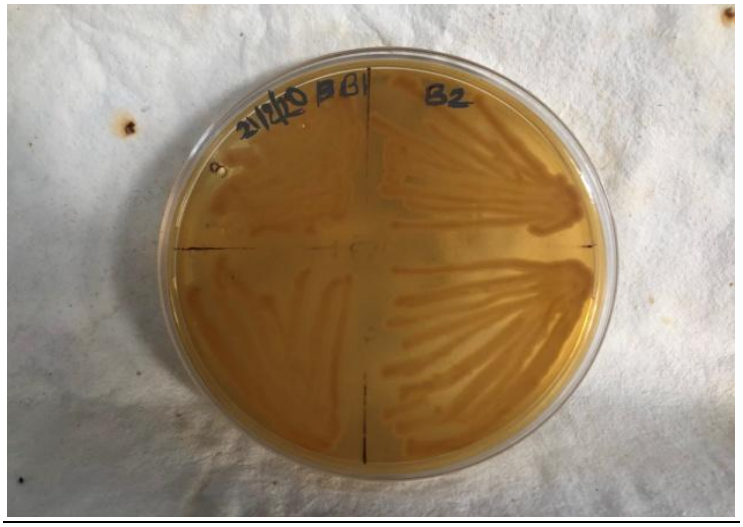

Fig.5 Streaking of Culture to obtain the pure colonies.

4.2 Qualitative screening

The qualitative screening of the phytase enzyme was done on the PSM plate, The method used was helpful in determination of catalysis of dephosphorylation of the phytate by the *Bacillus* strain and forms the clear zone of clearance which tests positive for the phytase enzyme.

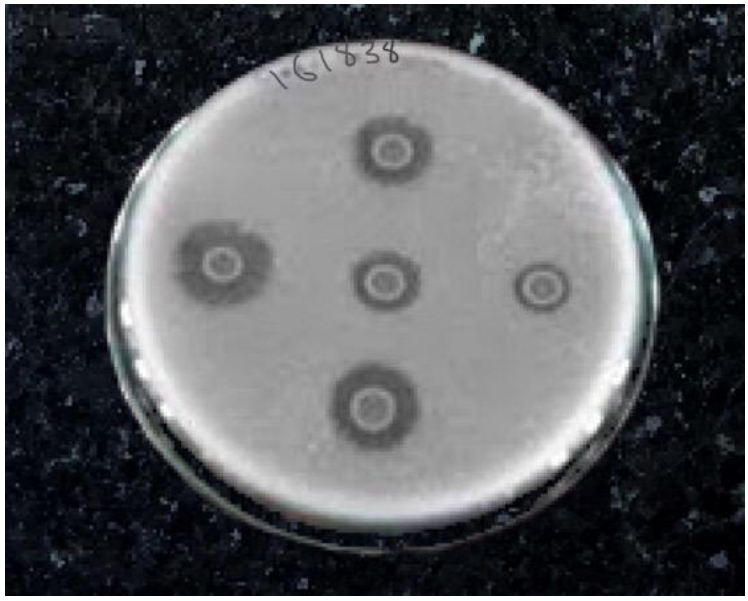

Fig.6 Phytase producing Microbial Screening through zone of clearance on PSM Agar Media

4.3 Morphological Characters

The gram staining was performed on the novel *Bacillus* strain and when observed under the micro scope it shows the presence of purple coloured rod shaped microbes that signifies that the strain used was Gram positive bacteria. The growth of the bacteria is at gradual speed as observed by staining at intervals of 24 hr and 48 hr.

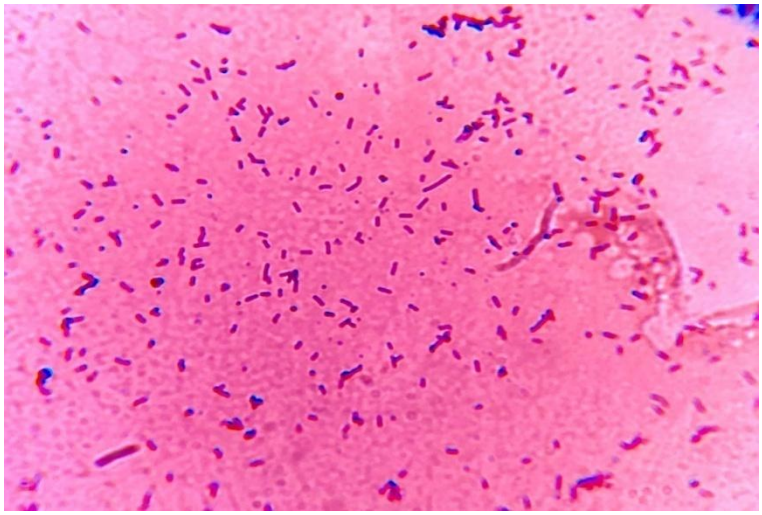

Fig.7 Gram Staining of pure Bacillus culture showing the rod shaped microbes, whose inoculums is incubated for 24 hrs.

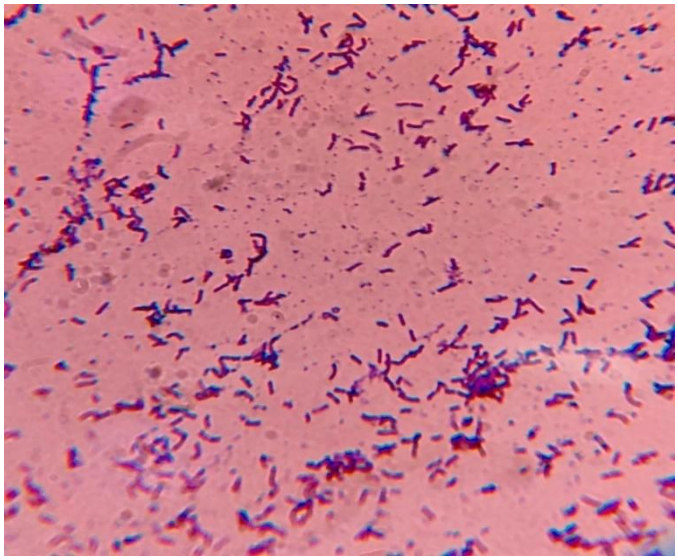

Fig.8 Gram Staining of pure Bacillus culture showing the rod shaped microbes, whose inoculums is incubated for 48 hrs.

4.4 Phytase Activity

The activity of our sample was inspected in the triplicates samples of the same bacterial strain and then the average of the three is taken. The activity was taken at different incubation time of the strains i.e. 24 hrs, 48 hrs.

Table. 3 Activity of phytase of the strain incubated for different time period

Phytase Activity U/ml		
Sample (incubation Time)	OD (avg)	Δ OD
Sample- 24 hrs	0.165	0.071
Sample- 48 hrs	0.363	0.267
Sample- 72 hrs	0.291	0.197

This table shows that the activity of phytase to be 0.267 U/ml, that was highest in the sample that was incubated for 48 hrs.

4.5 Purification of Phytase

The purification of phytase was conducted by the method of ASP and AP. For the comparison the crude enzyme was also precipitated for result. In this method we have taken two different samples which are incubated for 24 hr and 48 hr. The activity of the phytase enzyme is taken after the purification step.

Table. 4 Activity of phytase after the purification of the enzyme

Phytase Activity after Purification U/ml		
Purification step with sample (incubation time)	**OD (avg)**	**Δ OD**
Crude extract	0.891	0.09
ASP- 24 hr Sample	1.037	0.236
ASP- 48 hr Sample	1.212	0.411
AP- 24 hr Sample	1.247	0.346
AP- 48hr Sample	1.272	0.471

Control:- 0.801
Blank:- 0.062

This indicates that the phytase activity determined after the purification resulted in Chilled acetone precipitation with the highest activity.

4.6 Discussion

The importance of hydrolytic enzymes in the industries and enzyme market has shown a great interest in the phytase enzyme due to its properties and role as feed additive in the processing of food supplements for the non ruminant animals. In this study we have used a lot of different techniques for the production and purification of the phytase enzyme, though these have been already listed in the previously done researches. But our focus was to achieve higher yield, and in search of faster process.

The screening of the phytase was done to gain knowledge about whether the strain we are using have the capacity to produce phytase enzyme or not, but with the test we came to a conclusion the after the zone of clearance were formed, it was tested positive for the phytase enzyme.

The production done by the Submerged fermentation tells us that without the bran or oil cakes, the phytase production is faster and is achieved with a little ease only if accurate chemicals are available. The phytase activity among differently incubated samples comes out to be highest in the sample incubated for 48 hrs. This shows that we don't have to incubate our culture for longer period of time.

The purification step is one of the most important steps within the bioprocess of the enzyme. This step eradicates all the unnecessary particles from the solution and provides us with the pure enzyme, this was achieved by the ammonium sulphate precipitation and chilled acetone precipitation. But with purity we have to confirm about the activity also, therefore after the purification the phytase activity was again measured, which resulted that after doing precipitation with chilled acetone the activity was highest. For the production and purification many factors are necessary to be taken care of like temperature, pH, concentration, rpm, etc. as these are the factora that can affect the production rate greatly and may show fluctuation in the phytase activity. We first gained knowledge about these parameters from many different research papers and came to conclusion of using incubation parameters to be 37 ^{o}C, for different time like 24 hrs, 48hrs and shaking speed of 150 rpm. Though different characterizations like SDS-page,

themo-stabilization, HPLC, etc may give many details about the phytase enzyme and may help in dicrovering many new applications like dephytinization, saccharification, hydrolytic profiling.

CONCLUSION

The production of phytase is of utmost importance, if considered by looking at the disputed-nutritional property of the phytate, which make the processing of the food supplement a bigger task with many difficulties hence, lowering its market value. So, the phytase production is very important as it helps in the hydrolysis of the phytic acid can help in overcoming the anti-nutritive values of the phytate.

In this research we have investigated the production of phytases through submerged fermentation, Which is the production using the aqueous phase with additional chemicals essential, through this production method we got results faster and can yield phytase in higher amounts. The purification done in this study used two methods ASP and AP and produced purified enzyme with higher activity of 0.471 U/ml.

Future prospects of this study can be the characterization of the phytase enzyme which will help in gaining the further details of the structure, feasibility and the stability of the enzyme. This may also help in judging different application of phytase enzyme in the industries like sachharification, dephytinization etc.

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