ISOLATION AND CHARACTERIZATION OF PHYTASE PRODUCING BACTERIA OF HIMACHAL PRADESH ORIGIN

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DECLARATION

I do hereby declare that this dissertation and titled "**ISOLATION AND CHARACTERIZATION OF PHYTASE PRODUCING BACTERIA OF HIMACHAL PRADESH ORIGIN"** submitted towards fulfillment 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 submitted anywhere for any additional degree or diploma. Therefore the declaration made by the candidate is true and genuine.

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CERTIFICATE

This is to certify that the work titled "**Isolation and Characterization of Phytase Producing Bacteria of Himachal Pradesh Origin**" submitted by **Mr. Lalit Prakash Sharma** and **Mr. Shivam Sharma** 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:

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List Of Abbreviations

DNA Deoxyribo nuclic acid

HAP Histidine acid phosphatases

BPP β-propeller phytases

PAP Purple acid phosphatases

CP Cystein phosphatases

IUB International Union of Biochemistry

TCA Trichloroacetic acid

NA Nutrient agar

PSM Phytase screening media

LB Luria broth

ABSTRACT

Reduction of phytic acid with the help of phytases as a hydrolysing enzyme is used to eliminate the anti-nutritional activity of phytic acid. This property of phytase enables phytases to be used in various feed and food additive. Effectiveness of phytases is highly dependent on the physical as well as cultural conditions used during production such as temperature and pH. The sources of phytases range from micro organisms to higher organisms like plants. Usually the bacterial phytases show maximum activity in the range of 25 ^{o}C to 70 ^{o}C and the pH ranges from 4 to 9. Phytases have applications in the food industry such as in bread making industries, as an additive, in plant growth promotion and also helpful in overcoming phosphorous pollution.

This study shows maximum bacterial phytase activity is found to be at 35 ^{o}C and pH 5. Optimum incubation period came out to be 24 hours at the agitation speed of 150 rpm. Optimum carbon source and optimum nitrogen sources for the maximal phytase production observed to be wheat bran and ammonium nitrate respectively. In the current study, the overall tenfold increase in phytase production was obtained through the OVAT approach.

CHAPTER-1

INTRODUCTION

1.1 Phytic acid

Formula: $C_6H_{18}O_{24}P_6$

Molar mass: 660.04 g/mol

Phytic acid often serves as a reserve of phosphorous in many different plants and plant derived food and it comprises 1-5% by weight of edible seeds, edible legumes, cereals, tubers, nuts and pollens in varying amounts (IUPAC-IUB, 1977). It inhibits the absorption of divalent dietary minerals like Calcium, Magnesium, Iron, and Zinc as it has strong affinity to bind to these minerals. Formation of insoluble precipitates occurs when phytic acid binds to Iron and zinc which makes them less absorbable in the intestines which may be a cause of mineral deficiencies in people with a particular diet [1]. Phytic acid binds to amino acids and inhibits digestive enzymes; therefore hydrolysis of phytic acid is preferable [2]. Phytic acid being the principal storage form of phosphorus in plants has many functions such as acting as phosphorus and an energy store, source of cations. It also plays role in plant cell wall formation containing myo-inositol which is a cell wall precursor.

1.1.1 History

In 1855, Hartig discovered phytic acid from seeds of various plants as small grains or particle [3]. These grains are differentiated into three groups [4].

1. Calcium oxalate crystals

2. A protein substance

3. Globoids in which there is no reaction for protein, fat, or inorganic salts.

In 1903, Posternak named this compound phytin on the basis of its chemical and physical properties.

In 1963 Cosgrove commercialized phytic acid which contains lower phosphate derivatives than hexaphosphate [5]. In 1966 Cosgrove showed that phytic acid can be dephosphorylated by phosphatase enzymes commonly called phytases and also by heating in acid or alkaline solutions. In 1969 Angyal and Russell questioned the presence of these lower esters or they are chemically degraded product due to the isolation procedure [6].

In 1971 Blank and his coworkers stated that in 2-position the phosphate group is equatorial while others are axial. Anderson structure of phytic acid is widely accepted.

1.1.2 Occurrence and distribution

Phytic acid occurs mostly as the salts of monovalent and divalent cations in grains, legumes and cereals. In form of salt phytic acid is called as phytate which comprises 50-80% of phosphorus [7]. It shows that phytic acid is a huge pool of phosphorus that can be consumed by plants, animals and microorganisms. Legumes and cereals gather phytic acid in the phytin crystals and aleurone particles, respectively whereas during ripening, it settles in grains and seeds with other substances like starch and lipids [8]. In soyabean it is located in the protein bodies present in the seeds while in generally, legume seeds deposits phytate in the cotyledons. Compared to the soyabean, rapeseeds are more concentrated in seed tissues and there is no particular location of rapeseed [9]. In commercially produced crop seeds and fruits about 50 million tons of phytic acid is isolated [10]. So, through the use of mineral fertilizers to agricultural land the amount of phosphorus in phytic acid is comparable to approximately two-thirds of the phosphorus used per year.

Following are the contents of phytate in different plant food stuffs-

Table 1.1 List of plant food and ingredients and the percentage of phytate.

Plant food/ingredients	**Phytate (%)**
Cereals	
Cornflakes	0.04–0.15
Flour bread (70% wheat, 30% rye)	0.04–0.11
French bread	0.02–0.04
Maize bread	0.43–0.82
Oat flakes	0.84–1.21
Oat porridge	0.69–1.02
Rice	0.12–0.37
Rye bread	0.19–0.43

Sorghum	0.59–1.18
Sourdough rye bread	0.01–0.03
Wheat bread	0.32–0.73
Legume-based food	
Black beans	0.85–1.73
Chickpea	0.29–1.17
Cowpea	0.39–1.32
Green peas	0.18–1.15
Kidney beans	0.83–1.34
Lentils	0.21–1.01
Peanuts	0.92–1.97
Soybeans	0.92–1.67
White beans	0.96–1.39
Oilseed meals	
Cotton seed meal	0.786
Groundnut meal	0.46
Soybean meal	0.56
Sunflower meal	0.45
Miscellaneous	
Amaranth grain	1.12–2.34
Buckwheat	0.92–1.62
Sesame seeds (toasted)	3.93–5.72
Soy protein concentrate	1.12–2.34
Soy protein isolate	0.24–1.31

1.2 Phytase

In the recent years, phytases has been identified as an important enzyme in the scientific world as well as in the marketing field due to its applications in nutrition, biotechnology and bioremediation. Phytase (EC 3.1.3.8 and EC 3.1.3.26) is a type of phosphatase enzyme which catalyses the hydrolysis of phytic acid through a series of myo-inositol phosphate and eliminates its anti-nutritional properties.

1.2.1 History

Plant Source - Suzuki and his coworkers discovered plant Phytase during 1907 from rice bran and it was commercialized by Gist-Brocades in Europe during 1993-94.

Animal Source - McCollum, E.V; Hart, E.B discovered animal phytase in 1908 from calf's liver and blood.

BASF (a German chemical company) was the first to commercialize phytase as an animal feed nutrient additive under the name Natuphos. This commercialization was brought about due to the first partially cloned phytase gene phyA from *Aspergillus niger* in 1991. Use of bacterial phytases preferred over the fungal phytases is because *E. coli* bacterial phytases were found to be more effective than *Aspergillus niger.*

1.2.2 Types of Phytase

Phytase enzyme belongs to the class of phosphomonoesterases which includes myo-inositol hexakisphosphate 3-phosphorylase and myo-inositol hexakisphosphate 6-phosphorylase.

3-phytase (EC 3.1.3.8) - It is also known as1-phytase. It starts dephosphorylation of ester bond at 3-position on the inositol ring of phytic acid. Current commercially available 3-phytase products are derived from *Aspergillus niger*.

6-phytase (EC 3.1.3.26) - It is also known as 4-phytase. It starts dephosphorylation of ester bond at 6-position on the inositol ring of phytic acid. Majority of the products in the market belonging to this group are derived from *E. coli* and *Peniophora lycii* [11].
There are two major classes of phytases based on pH- Acidic and alkaline. Fungi are the source of most acidic phytases and bacteria and some of the plants are source of alkaline phytases.

Characterization based on structure and catalytic function differentiates phytase into four classes. These four classes include histidine acid phosphatases (HAPs), β-propeller phytases (BPPs), purple acid phosphatases (PAPs), and cystein phosphatases (CPs) [12]. BPP includes most bacterial phytases belonging to *Bacillus* spp. Most of the phytases reported from *Bacillus* spp. are metal dependent. HAP includes bacterial phytases belonging to *E. coli, Klebsiella sp*. and *Pseudomonas syringae*. There are various sources of phytases like plants, animals, bacteria, yeast and fungi; however. In commercial scale microbial sources are more promising [13].

Phytase is widely spread in nature. There are various sources of phytases like plants, animal tissues, bacteria, yeast and fungi; however, in commercial scale microbial sources are more promising.

Phytic acid being an inhibitor of digestive enzymes needs to be hydrolysed into soluble forms. Ruminants have this capability of digesting phytic acid catalysed by phytases produced by the anaerobic bacteria and fungi present in the gut of the ruminants in their rumenal microflora.

CHAPTER-2

REVIEW OF LITERATURE

2.1 Phytic acid

Phytic acid (*myo*-inositolhexakis phosphate) is a phosphate ester of inositol. It has six phosphate groups, making it naturally a source of inorganic phosphorus and is found in plants and plant based food products [14-16]. As phosphorus is a special need for all the animals as a part of their diet for right growth, therefore instead of phytic acid, phytate could be used as source of phosphorus in animal feed and food products as phytates are phytic acid bound to a mineral group.

This is because of the anti-nutritional properties of the phytic acid. It inhibits the absorption of divalent dietary minerals like Calcium, Magnesium, Iron, and Zinc by chelating these metal ions as it has strong affinity to bind to these minerals [17]. On chelating these ions, formation of insoluble precipitates occurs when phytic acid binds to Iron and zinc which makes them less absorbable in the intestines. The chelating properties of the phytic acid can be is because of the presence of negative ions that are mainly its free hydrogen atoms. These negative ions bind to the positive moieties such as present in divalent atoms which were mentioned before. It also affects the diet by binding to amino acids and inhibits digestive enzymes and interferes with their activities, it then becomes very important that hydrolysis of phytic acid is performed [18]. But hydrolysis of phytic acid is a difficult task to accomplish as it is a chemically stable compound which is due to its structure. Phytic acid is easily digested in the gut of the ruminants as they host anaerobic microorganisms that produce phytase which makes it easier to digest phytic acid present in their feed [19]. But on the other hand non-ruminants like pigs, turkeys, chickens, fishes and humans lack such anaerobes in their gut and also phytases which makes it difficult for them to digest and breakdown the phytic acid. This means that phytate phosphorus is not available to the non-ruminants in sufficient amounts. Also the non-digested or the poorly digested phytic acid is excreted by the non-ruminants which is the cause of eutrophication which causes environmental phosphorus pollution and is a serious environmental issue which needs to be tackled [20, 21].

Therefore, to hydrolyse phytic acid many chemical methods and physical methods been employed but these methods have been found to degrade the nutrition quality of the feed they treat [22, 23]. This is the prime reason why biological methods are used to hydrolyse phytic acid like the enzymatic treatment of phytic acid using enzymes like phytase have gained more

prevalence and recognition in the scientific world has been a subject of research in the recent years.

Enzymatic method not just maintains the nutritional value of the feed but also improve it by reducing the phytic acid [24, 25]. But the applications of phytases are not just limited in the food and feed industry; they have applications in bread making, in plant growth promotion, preparation of myo-inositol phosphates and combating phosphorus pollution in the environment.

2.2 Phytase

The past few decades has seen a new surge in research of Phytase and it has been identified as an important enzyme in the scientific world as well as in the commercial field due to its applications in nutrition, biotechnology and bioremediation. Phytase (EC 3.1.3.8 and EC 3.1.3.26) is a type of phosphatase enzyme which catalyses the hydrolysis of phytic acid through a series of myo-inositol phosphate and eliminates its anti-nutritional properties. This enzymatic treatment used to reduce phytic acid has contributed to its applications in various fields which we shall discuss later.

2.2.1 Classification of phytase

Phytase enzyme has been classified into two different classes by the International Union of Biochemistry (IUB) (1979). They are 3-phytase (E.C.3.1.3.8) and 6-phytase (E.C.3.1.3.26). the position of the inostol ring where the dephosphorylation initiates i.e. either 3 or 6 is the basis of this classification. Three phytase is produced as metabolites mostly in fungi whereas the six phytase is commonly produced by plants and bacterial sources [26, 27]. Phytase is also classified into three types on the basis of their catalytic functions, structure and their active site geometry. These are the following classes-

Histidine acid phosphatase

In prokaryotes and eukaryotes a large number of phytase is present. All the members of histidine acid phosphatases are designated by conserved active site hepta-peptide motif RHGXRXP at N-terminal end and HD- at C-terminal end [28-30]. These are the group of enzymes that are active at pH and in two steps process these enzymes catalyses phytic acid hydrolysis. No co-factor is necessary for optimal activity to the family of HAP. Generally, HAPs are not catalytically active as phytases [31-33].

Most commonly studied HAP in eukaryotes is obtained from *A. niger* and *A. fumigatus*. In prokaryotes HAP is most commonly obtained by *E. coli*. According to HAPs catalytic properties they are distinguished into two classes-

- Broader surface specificity with lesser specific activity for phytate.
- Narrow substrate specificity with higher specificity for phytate.

β propeller phytase (BPP)

BPP displays no homology to any familiar phosphatases and includes mainly 3 propeller sheet and favours a 6 bladed propeller [34, 35]. BPP is also known as alkaline phytase as it is active at alkaline pH requiring calcium ions for its thermostability and activity. 90-97% of sequence identities have been shared by BPP but they are unconnected to HAP and other phosphates.

BPPs have been produced from *Bacillus subtilis* and *Bacillus amyloliquefaciens* which display an odd mechanism for hydrolysing its substrate [36, 37]. A 3D structure of its molecule shows a basic form similar to a propeller with 6 blades. The dependence on binding calcium ions for thermostability [38, 39] and catalytic activity categorizes phytase from other subclasses of phytases. BPP contains two phosphate binding sites [40].

Purple acid phosphatase

From the cotyledons of germinating soybeans PAP has been obtained [41] GmPhy (soybean phytase) has the active site sequence motif of PAP and distinguished by seven hoarded residues in the five hoarded motifs – **D**XG, G**D**XX**Y**, G**N**H (D/E), VXX**H** and G**H**X**H** [42]. This class of metalloenzymes having two ferric and ferrous ions, zinc ions, magnesium ions and manganate ions catalytic centre displays decreased activity of catalysis. Molecular mass have been recommended by the approximated size of purified soybean phytase. Nonetheless, soybean phytase is a partly recognized PAP reported to have momentous phytase activity.

2.2.2 Sources of phytase

Phytase have been shown to be found in various bacteria like *Aerobacter aerogenes, Enterobacter spp., E. coli. B. subtilis and B. subtilis var. natto* were only bacteria that produce extracellular phytases. Ca^{2+} ions and phytate initiates the phytase production by bacteria. Under

carbon restriction in the presence of phytic acid cytoplasmic phytase from *K. terrigena* get activated. In anaerobic conditions intracellular *E. coli* activity got increased in the stationary phase. In 1996 Nys et al reported a variation of activity in phytases in the seeds of different varieties of plants. Wheat, rye, and barley have been reported as maximum levels of phytase production.

Table 2.1 List of phytase producing bacteria.

Microorganism	***Location of the Enzyme***	***Reference***
Bacteria		
Aerobacter aerogenes	*Cell bound*	*Greaves et al., 1967*
Bacillus pantothenticus	*Extracellular*	*Anis Shobirin et al., 2009*
Bacillus amyloliquefaciens	*Extracellular*	*Ha et al., 1999*
Bacillus cereus ASUIA260	*Extracellular*	*Anis Shobirin et al., 2009*
Bacillus circulans	*Extracellular*	*Anis Shobirin et al.,2009*
Bacillus licheniformis	*Extracellular*	*Joseph and Raj, 2007*
Bacillus megaterium	*Extracellular*	*Kumar et al., 2013*
Bacillus nealsonii ZJ0702	*Extracellular*	*Yu and Chen, 2013*
Bacillus sp. KHU-10	*Extracellular*	*Choi et al., 1999*
Bacillus sp., DS11	*Extracellular*	*Kim et al., 1998*
Bacillus sp.C43	*Extracellular*	*Sreedevi and Reddy, 2012*
Bacillus subtilis	*Extracellular*	*Kerovuo et al., 1998*
Bacillus subtilis	*Extracellular*	*Powar and Jagannathan,1982*
Bacillus subtilis (natto) N-77	*Extracellular*	*Shimizu, 1992*
Bacillus subtilis MJA	*Extracellular*	*EL-Toukhy et al., 2013*
Citrobacter braakii	*Intracellular*	*Kim et al., 2003*
Enterobacter sp.4	*Extracellular*	*Yoon et al., 1996*
Escherichia coli	*Cell bound*	*Greiner et al., 1993*
Escherichia coli BL21	*Extracellular*	*Sunitha et al., 1999*
Klebsiella aerogenes	*Cell bound*	*Tambe et al., 1994*
Klebsiella oxytoca MO-3	*Cell bound*	*Jareonkitmongkol et al., 1997*

Klebsiella pneumoniae 9-3B	*Extracellular*	*Mopera et al., 2012*
Klebsiella pneumoniae ASR1	*Extracellular*	*Sajidan et al., 2004*
Klebsiella sp. DB-3	*Extracellular*	*Mittal et al., 2011*
Lactobacillus amylovorus	*Extracellular*	*Sreeramulu et al., 1996*
Lactobacillus fructivorans	*Extracellular*	*De Angelis et al., 2003*
Lactobacillus plantarum	*Extracellular*	*Saribuga et al., 2014*
Lactobacillus sanfranciscensis	*Extracellular*	*De Angelis et al., 2003*
Megasphaera elsdenii	*Extracellular*	*Yanke et al., 1998*
Mitsuokella jalaludinii	*Extracellular*	*Lan et al., 2002*
Mitsuokella multiacidus	*Cell bound*	*Yanke et al., 1999*
Prevotella ruminicola	*Extracellular*	*Yanke et al., 1998*
Pseudomonas putida	*Extracellular*	*Richardson et al., 1997*
Pseudomonas sp.	*Cell bound*	*Irving and Cosgrove, 1971*
Selenomonas ruminatium	*Cell bound*	*Yanke et al., 1999*
Serratia marcescens	*Extracellular*	*Mukesh Kumar et al., 2011*
Serratia plymuthica	*Extracellular*	*Shedova et al., 2008*
Weissela confuse	*Extracellular*	*De Angelis et al., 2003*
Hanseniaspora uvarum WZ1	*Extracellular*	*Hirimuthugoda et al., 2006*
Hanseniaspora uvarum WZ1	*Cell bound*	*Hirimuthugoda et al., 2006*
Hanseniaspora valbyensis	*Extracellular*	*Nakamura et al., 2000*
Hansenula polymorpha	*Extracellular*	*Mayer et al., 1999*
Issatchenkia orientalis	*Intracellular*	*Suyal and Tewari, 2013*
Issatchenkia orientalis YF04C	*Extracellular*	*Hirimuthugoda et al., 2006*
Kluyveromyces fragilis	*Extracellular*	*Lambrechts et al., 1992*
Kluyveromyces lactis	*Extracellular*	*Nakamura et al., 2000*
Kodamaea ohmeri BG3	*Extracellular*	*Li et al., 2008*
Metchnikowia pulcherrima	*Extracellular*	*Nakamura et al., 2000*
Pichia anomala	*Cell bound*	*Vohra and Satyanarayana, 2001*
Pichia anomala J121	*Extracellular*	*Olstorpe et al., 2009*
Pichia anomala J379	*Extracellular*	*Olstorpe et al., 2009*

Pichia ohmeri	*Extracellular*	*Nakamura et al., 2000*
Pichia rhodanensis	*Extracellular*	*Nakamura et al., 2000*
Pichia spartinae	*Extracellular*	*Nakamura et al., 2000*
Pichia wickerhamii	*Extracellular*	*Nakamura et al., 2000*
Pichia anomala	*Extracellular*	*Nakamura et al., 2000*
Pichia gluermondii	*Intracellular*	*Suyal and Tewari, 2013*
Pichia kudriavzevii	*Extracellular*	*Hellstrom et al., 2012*
Rhodotorula gracilis	*Extracellular*	*Bindu et al., 1998*
Saccharomyces cerevisiae	*Extracellular*	*Nakamura et al., 2000*
Saccharomyces cerevisiae J122	*Extracellular*	*Olstorpe et al., 2009*
Saccharomyces kluyveri	*Extracellular*	*Nakamura et al., 2000*
Saccharomyces cerevisiae	*Cell bound*	*Howson and Davis, 1983*
Saccharomyces cerevisiae	*Intracellular*	*Nayini and Markakis, 1984*
Saccharomyces cerevisiae	*Extracellular*	*Ries and Macedo, 2011*
Saccharomyces cerevisiae CY	*Extracellular*	*In et al., 2007*
Schizosaccharomyces octoporus	*Extracellular*	*Pable et al., 2013*
Schwanniomyces castellii	*Extracellular*	*Lambrechts et al., 1992*
Schwanniomyces occidentalis	*Extracellular*	*Segueilha et al., 1992*
Schwanniomyces occidentalis	*Extracellular*	*Nakamura et al., 2000*
Torulaspora globosa	*Extracellular*	*Nakamura et al., 2000*
Torulaspora pretoriensis	*Extracellular*	*Nakamura et al., 2000*
Torulaspora delbrueckii	*Extracellular*	*Nakamura et al., 2000*
Torulopsis candida	*Extracellular*	*Lambrechts et al., 1992*
Williopsis saturnus	*Extracellular*	*Pable et al., 2013*
Yarrowia lipolytica YF08	*Extracellular*	*Hirimuthugoda et al., 2006*
Yarrowia lipolytica W2B	*Extracellular*	*Hirimuthugoda et al., 2006*
Zygosaccharomyces bisporus	*Extracellular*	*Pable et al., 2013*

Fungi	***Location of the Enzyme***	***Reference***
Aspergillus amstelodami	*Extracellular*	*Howson and Davis, 1983*
Aspergillus candidus	*Extracellular*	*Howson and Davis, 1983*
Aspergillus carbonarius	*Extracellular*	*Al-asheh et al., 1994*
Aspergillus carneus	*Extracellular*	*Ghareib, 1990*
Aspergillus chevalieri	*Extracellular*	*Howson and Davis, 1983*
Aspergillus ficuum	*Extracellular*	*Ullah et al.,1988*
Aspergillus ficuum	*Extracellular*	*Ullah & Dischinger 1993a; Ebune et al., 1995; Kim et al., 1999a; Kim et al., 1999b*
Aspergillus ficuum NTG-23	*Extracellular*	*Zhang et al., 2010*
Aspergillus flavus	*Extracellular*	*Shieh and Ware,1968*
Aspergillus fumigates	*Extracellular*	*Pasamontes et al.,1997a*
Aspergillus fumigates	*Extracellular*	*Wyss et al., 1998;1999 a and b*
Aspergillus fumigates	*Extracellular*	*Rodriguez et al., 2000*
Aspergillus japonicas	*Extracellular*	*Moreira et al., 2014*
Aspergillus niger	*Extracellular*	*Skowronski, 1978*
Aspergillus niger	*Extracellular*	*Shieh and Ware,1968*
Aspergillus niger	*Extracellular*	*Phillipy and Mullaney, 1997*
Aspergillus niger	*Extracellular*	*Wyss et al.,1999; Kostrewa et al.,1999*
Aspergillus niger	*Extracellular*	*Papagianni et al., 2000*
Aspergillus niger 113	*Extracellular*	*Xiong et al., 2009*
Aspergillus niger 11T25A5	*Extracellular*	*Da Silva et al., 2005*
Aspergillus niger 11T53A9	*Extracellular*	*Greiner et al., 2009*
Aspergillus niger 551	*Extracellular*	*Anita et al., 2012*
Aspergillus niger ATCC 9142	*Extracellular*	*Casey and Walsh, 2003*
Aspergillus niger NCIM 563	*Extracellular*	*Soni et al., 2007*
Aspergillus niger ST-6	*Extracellular*	*Tahir et al., 2010*
A.niger syn A. ficuum	*Extracellular*	*Howson and Davis, 1983*

Aspergillus niger USM AI1	*Extracellular*	*Ibrahim and Lim, 2014*
Aspergillus niger van teigham	*Extracellular*	*Vats et al., 2002*
Aspergillus niger var. phoenicis	*Extracellular*	*Nascimento et al., 2013*
Aspergillus niveus	*Extracellular*	*El-Gindy et al., 2009*
Aspergillus oryzae AK 9	*Extracellular*	*Chantasartrasamee et al., 2005*
Aspergillus repens	*Extracellular*	*Howson and Davis, 1983*
Aspergillus sp. L117	*Extracellular*	*Lee et al., 2005*
Aspergillus syndowi	*Extracellular*	*Howson and Davis, 1983*
Aspergillus terreus	*Extracellular*	*Yamada et al., 1968*
Aspergillus terreus	*Extracellular*	*Mitchell et al., 1997*
Aspergillus terreus 9A1	*Extracellular*	*Mitchell et al., 1997*
Aspergillus wentii	*Extracellular*	*Howson and Davis, 1983*
Botrytis cinerea	*Extracellular*	*Howson and Davis, 1983*
Emericella nidulans	*Extracellular*	*Pasamontes et al., 1997b*
Emericella nidulans	*Extracellular*	*Pasamontes et al., 1997b; Wyss et al., 1999a*
Fusarium verticillioides	*Extracellular*	*Marlida et al., 2010*
Geotrichum candidum	*Extracellular*	*Howson and Davis, 1983*
Lichtheimia blakesleeana	*Extracellular*	*Neves et al., 2011*
Mucor indicus MTCC 6333	*Extracellular*	*Gulati et al., 2007*
Mucor piriformis	*Extracellular*	*Howson and Davis, 1983*
Mucor racemosus	*Extracellular*	*Howson and Davis, 1983*
Myceliophthora Thermophila	*Extracellular*	*Mitchell et al., 1997*
Myceliophthora Thermophila	*Extracellular*	*Pasamontes et al., 1997; Wyss et al., 1999a*
Myceliophthora thermophila	*Extracellular*	*Hassouni et al., 2006*
Pencillium sp.	*Extracellular*	*Shieh and Ware, 1968*
P. purpurogenum GE1	*Extracellular*	*Awad et al., 2014*
Penicillium sps.	*Extracellular*	*Eida et al., 2013*

Peniophora lycii	*Extracellular*	*Lassen et al., 2001*
Rhizoctonia sp.	*Extracellular*	*Marlida et al., 2010*
Rhizopus oligosporus	*Extracellular*	*Howson and Davis, 1983*
Rhizopus oligosporus	*Intracellular*	*Sutardi and Buckle, 1988*
Rhizopus oligosporus	*Extracellular*	*Sabu et al., 2002*
Rhizopus oligosporus	*Extracellular*	*Casey and Walsh, 2004*
Rhizopus oligosporus 556	*Extracellular*	*Haritha and Sambasivarao,2010*
Rhizopus oryzae	*Extracellular*	*Howson and Davis, 1983*
Rhizopus oryzae	*Extracellular*	*Rani and Ghosh, 2011*
Rhizopus stolonifer	*Extracellular*	*Howson and Davis, 1983*
Sporotrichum thermophile	*Extracellular*	*Ghosh, 1997*
Sporotrichum thermophile	*Extracellular*	*Javed et al., 2010*
Sporotrichum thermophile	*Extracellular*	*Singh and Satyanarayana, 2012*
Talaromyces thermophilus	*Extracellular*	*Pasamontes et al., 1997b*
Thermomyces lanuginosus	*Extracellular*	*Berka et al., 1998*

2.2.3 Structure and chemical properties of phytate

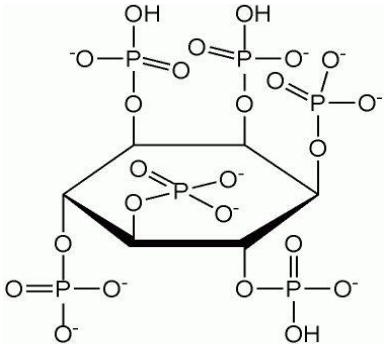

Fig. 2.1 Structure of phytate

In a wide pH range phytic acid have some negative charges because of its twelve ionisable hydrogen atoms that change the dissociation constants [43]. This may feature phytic acid to be a

very strong chelating agent. The negative ions phytic acid can bind with positive ions moieties of a number of divalent metal ions, proteins peptides and starch.

2.2.4 Chemical reaction containing phytase as hydrolyzing agent

A phytase is a type of phosphatase enzyme that catalyzes the hydrolysis of phytic acid. Phytic acid is considered as anti-nutritional component of food diet due to its ability to form complexes with metal ions, digestive enzymes. Phytase generates free myo-inositol phosphate and inorganic orthophosphate by hydrolysis of phytic acid as shown in fig.2.2

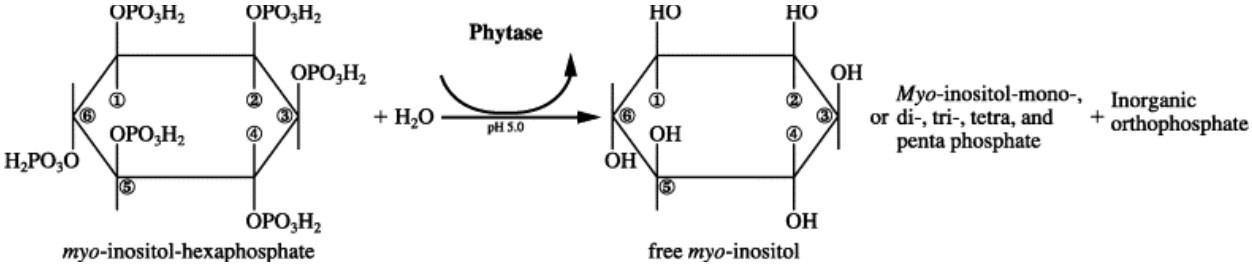

Fig.2.2 Hydrolysis of phytic acid by microbial phytase

2.3 Fermentation

For higher phytase production each organism or stain has its own conditions and special requirements. The fermentation process acquires 2 methods for the production of phytase.

1. Submerged fermentation

2. Solid state fermentation

Submerged fermentation is the most commonly used to the production of bacterial phytase. Solid state fermentation is also another method for bacterial phytases. Researchers are now making solid state fermentation target for production of bacterial phytase over submerged fermentation as solid state has advantages over submerged fermentation.

2.3.1 Submerged fermentation

The production of the microbial metabolites is greatly affected by the culture conditions and the type of fermentation method opted. For optimal production of the microbial metabolite every factor needs to be individually optimized. These factors include the following factors-

Physical Conditions

Microbial phytases are significantly affected by physical parameters such temperature, pH, agitation speed, and pressure among others.

Most of the bacterial phytases are produced at slightly acidic to neutral pH range and fungal sources show optimal growth in the acidic pH. Just like pH, temperature also affects microbial growth, for most of the bacteria producing phytase the optimal range is mesophilic conditions, but many thermophilic bacteria also have been found to show growth at higher ranges. Same is the case in fungal strains have been reported showing phytase production. Dissolved oxygen is a major parameter affecting the growth. Bacteria have been reported to show submerged fermentation in different agitation speeds ranging from 115 rpm to 200 rpm.

Chemical Factors

Chemical factors like carbon and nitrogen source, their ratio, metal ions, inorganic phosphate affect the microbial growth and metabolism and hence the phytase production. These factors affect the microbial growth similarly as the physical conditions. A crucial step in media composition is its carbon and nitrogen sources, various sources of carbon have been employed such as wheat and rice bran, molasses, sucrose, glucose etc [44]. Similarly various nitrogen sources both organic and inorganic sources have been reported to have shown growth of phytase producing microorganism such as Ammonium Nitrate, Ammonium Sulphate, peptone, urea and their different combinations have also been reported [45].

Table 2.2 General characteristics of phytase from various microbial sources

Source	***Molecular Weight (kDa)***	***Optimal Temperature (ºC)***	***Optima pH***	***Reference***
Aspergillus niger NCIM 553	*264*	*55*	*2.5*	*Bhavsar et al (2013)*
Rhizopus oryzae	*34*	*45*	*1.5-5.5*	*Rani & Ghosh (2011)*
Rhizopus oligosporus	*124*	*65*	*5.0*	*Casey & Walsh (2004)*
Aspergillus niger ATCC 9142	*84*	*65*	*5.0*	*Casey & Walsh (2003)*
Aspergillus oryzae	*120*	*50*	*5.5*	*Shimizu (1993)*
Aspergillus ficuum (Phy B)	*68*	*63*	*2.5*	*Ehrlich et al (1993)*
Aspergillus ficuum (Phy A)	*85*	*58*	*2.5*	*Ullah & Gibson (1987)*
Arxula adeninivorans	_	*75*	*4.5*	*Sano et al (1999)*
Schwanniomyces castellii	*490*	*77*	_	*Segueilha et al (1992)*
Saccharomyces cerevisiae	*120*	*60*	*5.5*	*Han et al (1999)*
Pichia pastoris	*95*	*60*	*2.5-5.5*	*Tai et al (2013)*
Citrobacter braakii	*47*	*50*	*4.0*	*Kim et al (2003)*
Pseudomonas syringae	*45*	*40*	*5.5*	*Cho et al (2003)*
Klebsiella terrigena	*40*	*58*	*5.0*	*Griener & Carlsson*
Escherichia coli	*42*	*60*	*4.5*	*Griener et al (1993)*
Enterobacter sp.	-	*50*	*7.0-7.5*	*Yoon et al (1996)*
Bacillus sp. DS11	*44*	*70*	*7.0*	*Kim et al (1998)*
Bacillus subtilis	*38*	*60*	*6.5*	*Kerovuo et al (2000)*

2.3.2 Solid state fermentation

As solid state fermentation is more advantageous than submerged fermentation which include less requirement of energy, less yield of waste water, uncomplicated transfer of oxygen, less contamination of bacteria etc. increased the interest of researchers for obtaining commercially products. Consequently it is very necessary to upgrade the performance of the systems and to enlarge yield of the product without expanding the cost via optimization process [46]

The list of common substrates important for solid state fermentation is:

- Crop remnant - wheat bran, wheat straw, orange peel, banana pulp. rice straw, soybean, hay etc.
- Fruit processing industries remnant – apple and grapes baggase, pineapple waste, orange peel etc.
- Vegetable waste – sugarcane baggase, potato waste, sweet potato waste etc.
- Coffee processing industries – coffee husk, coffee pulp etc.
- Oil processing industries – mustard cake, soyabean cake, peanut cake etc.

Mckinney et al. showed five time increased production of phytase at 37^{o}C by *E. coli* with wheat bran over submerged fermentation. There are some researches that have reported high phytase production by using *Bacillus* sp. T4 with corn flour, *A. oryzae* AKA with rice flour, *S. thermophile* with sesame oil, maximum production of phytase by *A. ficuum* SGA01 and *A. niger* CFR335 with both rice and wheat bran, at 45^{o}C in 120 hr high level of production of phytase in thermophilic mould *S. thermophile* was achieved and at 30^{o}C in 96 hrs in *R. oligosporus* was achieved.

Table 2.3 Statistical optimization of different microorganisms for phytase production in SSF

Microorganism	***Statistical design***	***Fold Improvement***	***References***
Aspergillus niger NCIM563	Plackett-Burman and RSM	36.67	Buddhiwant et al. (2015)
Aspergillus niger NCIM563	Plackett-Burman and RSM	3.08	Bhavsar et al. (2011)
Aspergillus ficuum NRRL3135	Plackett-Burman and RSM	1.67	Bogar et al. (2003b)
Aspergillus ficuum PTCC5288	Plackett-Burman and RSM	20	Jafari-Tapeh et al. (2012)
Mucor racemosus	Plackett-Burman and RSM	1.75	Bogar et al. (2003a)

Penicillium purpurogenum	Box-Behnken	2.6	Awad et al. (2014)
Pichia anomala	Plackett-Burman and RSM	1.75	Vohra and Satyanarayana (2002)
Rhizopus pusillus	Box-Behnken	1.3	Chadha et al. (2004)
Sporotricum thermophile BJTLR50	Plackett-Burman and RSM	11.6	Kumari et al. (2015)
Sporotricum thermophile BJTLR50	Plackett-Burman and RSM	*2.6*	*Singh and Satyanarayana (2008b)*
Sporotricum thermophile BJTLR50	Plackett-Burman and RSM	*2.0*	*Singh and Satyanarayana (2006b)*

2.4 Applications

Phytase is a very important enzyme in the food and feed based productions given its property to hydrolyze phytic acid and improve the products nutritional quality and value, this also of a great commercial value. Its use in animal nutrition also aids to environmental issue of eutrophication caused by high phosphorus levels in animal excreta. Also it has a role in plant growth promotion and in the bread making industry.

2.4.1 Food and feed additive

Monogastric animals like poultry, fish, pigs and human being unable to hydrolyze phytic acid because they lack gut microbes producing phytase like the ruminants do and digest phytic acid and contain inorganic phosphates from the digestion available in their gut. Therefore, it is important for the non-ruminants that phytic acid should be digested which can be achieved by supplementing feed by phytase and phosphorus digestion is aided. The US-FDA has also approved phytase as an additive instead of inorganic phosphorus as a supplementation for the phosphorus requirements.

In the food industry, phytase has applications in the processing and manufacturing processes for human consumption products. This is due to its capability of decreasing phytic acid content in

the food products. Also food products for animals have been supplemented with phytase. Soybean processing is also performed using phytase.

2.4.2 Paper and Pulp industry

This application comes about as the need to remove phytic acid from plants and is employed in the paper and pulp industry. As the degradation of plant phytic acid is non-carcinogenic and nontoxic product producing process it can also be used for environmental treatment of phytic acid as well.

2.4.3 Application in bread making

Phytases have been proclaimed to enhance the making process of bread as well as properties of bread and its nutritional content. The alkaline phytase from *Bacillus amyloliquefaciens* decreased phytate in whole wheat bread formation and enhance the mineral availability of bread.

Expansion with *E. coli* extract and ascorbic acid decline the phytate substance of entire wheat bread and builds the dialysability of iron. *Bifidobacterium catenulatum, B. longum* and *B. brene* with odd phytate debasing protein pre-possessed as activators in the entire wheat mixture maturation process, overwhelming to dynamic decrease in phytic corrosive substance inside a less time of fermentation. In gluten free bread *Lactobacillus sanfranciscensis* and *L. plantarum* enhances the nutritional importance of gluten free bread having more attentiveness of free amino acid and ion like zinc, calcium and magnesium ions.

There is important role of members of different lactic acid bacteria in the bread industry as the phytases isolated have assorted applications like upgrading the volume, surface and tangible nature of the bread and developing sharpness of the bread to reasonable dimension and improving the physical properties and expanding time span of usability of the bread.

2.4.4 Preparation of myo-inositol phosphates

myo-inositol phosphates have various biologically important functions and have been reported to have major health benefits such as having anti-oxidant properties and significant reduction of heart related diseases [47] reduction in the risk of some cancers [48-52] the inositol triphosphates can also act as a pain killer [53] role of esters of these inositol triphosphates in having critical

inhibitory impact against retroviral infections [54]. Lower *myo*-inositol phosphate derivatives have significant capacity in cell communication and this is achieved through balancing calcium ions cannels [55]. Immobilized phytases have been utilized to make different *myo*-inositol phosphates [56].

2.5 Objectives

On the basis of review of literature, the current study have been done to achieve following objectives:

1. To screen and isolate Phytase-producing bacteria from soil samples and identifying a new source of extracellular Phytase.
2. Characterization of Phytase producing bacteria.
3. Optimization of Phytase production using OVAT method

CHAPTER-3

MATERIALS AND METHODS

In carrying out the study a number of experiments were designed according to resources available and were conducted using many different materials, instruments, samples and chemicals. The soil samples were collected from various regions of Himachal Pradesh (Shimla, Solan, and Mandi) in sterile polythene bags/bottles, and brought to the laboratory for further processing. The soil samples from these regions were collected from poultry farms of the respective regions. The poultry farm soil was collected because it contains the excreta of poultry birds in these cases being the hens and chicken which contain poorly digested or non-digested phytic acid. This is also a great area to find phytase producing bacteria, which is aimed to produce phytase for commercial use that may be added to the animal feed and reduce the phytic acid content in their excreta. The main laboratory instruments used in the whole study are mentioned along the methods used.

3.1 Isolation of bacteria

3.1.1 Isolation and upkeep of bacteria

Starting screening was finished utilizing serial dilution technique. Suspension of 1 gram soil sample and 100 mL of normal saline (0.85 % NaCl) was prepared and incubated at 30^{o} C and 200 rpm for 1 h. Nutrient agar plates were prepared of these serially diluted samples. The pH was set about, pH 6.8. These nutrient plates were then incubated at 37ºC for 24h.

3.2 Screening for phytase

3.2.1Qualitative screening

For the screening the following procedure was followed. This involves inoculation of the bacterial isolates on PSA- phytase screening agar medium at set at pH of 5.5. The plates were incubated at 37ºC for 24h. Hydrolytic zone was developed by colonies by making a clearance zone. This zone was taken as a positive/negative test for phytase (Chaiharn and Lumyong, 2009). Glycerol Stock was prepared for maintenance of the cultures on nutrient agar (NA) plates -20ºC.

3.3 Enzyme assay and analytical procedure

3.3.1 Preparation of standard curve of KH_2PO_4

Phosphorous concentration was determined using the standard curve of KH_2PO_4 prepared using dilutions in the range of 10-200 µg/mL. To 1 mL of the solution, 1 mL of 10 % tricholoroacetic acid (TCA) was added followed by addition of 1 mL distilled water and 0.5 mL 9N H_2SO_4 and allowed it to stand for 30 min at room temperature. After this 0.5 mL of 6 % ammonium molybdate was added, thoroughly mixed and mixture was allowed to stand for 30 min in the dark. Later 0.5 mL of ferrous sulphate solution (prepared by addition of 2 g $FeSO_4.7H_2O$ in 20 mL of distilled water and 0.5 mL 0.9N H_2SO_4 solution and final volume made upto 25 mL) was added in each tube, thoroughly mixed and incubated for 30 min in the dark. Absorbance was recorded at 750 nm in spectrophotometer. Standard curve was plotted against absorbance and phosphorus concentration.

3.3.2 Measurement of phytase activity

Phytase assay was done to determine the amount of phosphate liberated using calcium phytate as the substrate. The reaction mixture consisted of 0.9 mL acetate buffer (0.1 M, pH 5.0 containing 2.0 mM calcium phytate) and 100 µL of crude enzyme. After incubation for 10 min at 37°C, the reaction was stopped by adding 250 µL of 10 % TCA. Coloring agent is freshly prepared by adding 1.875g of ferrous sulphate and 0.25g ammonium molybdate and 1.25 mL sulphuric acid and distilled water was added to make up volume to 25 mL. The phytase activity was determined spectrophotometrically at 750 nm.

3.3.3 Selection of potent phytase producing bacterial isolates

On the basis of quantitative screening, the phytase production by each selected bacterial isolates was measured and the two most potent phytase producer isolates were selected and identified by morphological, biochemical and at molecular methods.

3.4 Identification and characterization of the bacterial isolates

After quantitative screening, these isolates were identified and characterized by biochemical tests. For Species level identification by molecular method using 16S rRNA amplification and sequencing was carried out.

3.4.1 Gram's staining

Thin smears of bacterial isolates were arranged on clean glass slides and fixed by heating method. The smears were engulfed with crystal violet for one minute and splashed with distilled water. Scarcely any drops of the severe (Gram's iodine arrangement) were added to the smear and left for 1 min. In the wake of flushing with refined water, the decolorizer (ethanol) was included drop wise for 10-15 sec. Smear was then counter recolored with safranin for 30 sec, washed with refined water, air dried and saw under the magnifying lens in oil submersion.

3.4.2 Catalase test

Organisms producing catalase enzyme breaks H_2O_2 into water and oxygen. Catalase positive organisms produce bubbles when mixed with H_2O_2. For catalase test, a loopful bacterial culture was mixed with 3 % H_2O_2 on the surface of a glass slide and observed for effervescence.

3.4.3 Estimation of soluble proteins

4-fold dilutions of a 2 mg/mL BSA test was set up by including 50 µl of 2 mg/mL BSA to 150 µl of distilled water to make 200 µl of 0.5 mg/mL BSA. Test sample for the reference cell, clear, BSA measures and the protein test were produced to be tried by Table in expendable cuvettes. Each sample was permitted to brood at room temperature for 10-30 minutes. Absorbance of each sample was estimated at 595 nm utilizing an UV-unmistakable spectrophotometer. Absorbance of each BSA standard as an element of its hypothetical focus was plotted.

3.5 Optimization of the growth parameters of the selected bacterial culture

3.5.1 Selection of best carbon source

Phytase production medium supplemented with different carbon sources (1 %) like rice bran, wheat bran, fruit pulp, glucose, sucrose, citrus peel and was incubated at 37 ºC and 150 rpm for 24 h. After incubation, culture filtrate was used in phytase assays. Each 250mL Erlenmeyer flask

containing 25mL of the selected production medium (pH 6.0) containing each substrate. The cell-free culture filtrates were used in phytase assay.

3.5.2 Effect of different concentration selected carbon source wheat bran

Effect of different concentrations of wheat bran (0.5- 3 %) was studied on phytase production under the same condition as above and cell-free culture filtrates were used in phytase assay.

3.5.3

Selection of best Nitrogen source

Phytase production medium supplemented with different nitrogen sources (1 %) including both organic and inorganic sources.

Organic sources like Peptone and Malt extract and inorganic sources like ammonium nitrate, ammonium sulphate, urea, and asparagine and was incubated at 37ºC and 150 rpm for 24 h.

3.5.4 Effect of different concentration selected nitrogen source Ammonium Nitrate

Effect of different concentrations of ammonium nitrate (0.5-3%) was studied on phytase production under the same condition as above and cell-free culture filtrates were used in phytase assay.

3.5.5 Selection of suitable substrate for phytase production

Different residues like rice bran, wheat bran, fruit pulp, glucose, sucrose, citrus peel were used for phytase production in submerged fermentation

.**3.5.6 Effect of temperature**

Erlenmeyer flasks containing production medium inoculated with bacterial culture were incubated at different temperatures ranging from 25^{o}C to 40^{o}C. Cell free culture filtrate was analyzed for phytase activity.

3.5.7 Effect of pH

Effect of pH on phytase production was studied by cultivating the bacterial inoculums in a set of media prepared by adjusting the pH ranging from 3.0-9.0 at 37^{o}C and 150 rpm for 24 h. After incubation, cell free culture filtrate was used for phytase assays.

3.5.8 Effect of inoculum age

Bacterial inoculum of various ages ranging from 12-72 h was prepared and used to inoculate the medium and cell-free culture filtrate was assayed for phytase activity.

3.5.9 Effect of inoculum size

Various inoculum levels in the range of 0.5% - 5% were used to inoculate the medium. After incubation, culture supernatant was analyzed for phytase activity.

3.5.10 Effect of agitation on phytase production

Erlenmeyer flasks (250 mL) containing 25 mL medium were inoculated and incubated at 37 ^{o}C for 48 h in an incubator shaker agitated at different speeds of 50, 100, 150, 200 and 250 rpm. Cell free culture filtrate was analyzed for phytase activity.

CHAPTER-4

RESULTS AND DISSCUSIONS

4.1 Biochemical Testing

The above mentioned methods were gave the following results. The procedures that followed were in a consequence of the results of the prior experiments. These in result lead us to classification of the bacterial cultures.

4.1.1 Gram staining

Table 4.1 Results of Gram staining of samples

Samples	**Results**	**Shape**
A1	Gram Positive	Coccus
D1	Gram Positive	Coccus
D2	Gram Positive	Coccus
D3	Gram Negative	Coccus
E2	Gram Positive	Coccus
G2	Gram Positive	Coccus

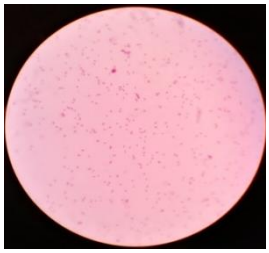

Fig.4.1 Gram staining of isolate D2 (Gram positive)

4.1.2 Catalase Test

Samples A1 and D2 gave negative result and samples D1, D3, E2 and G2 gave positive result by giving effervescence.

Table 4.2 Result showing catalase test of bacterial isolates

Samples	**Results**
A1	Negative
D1	Positive
D2	Positive
D3	Negative
E2	Positive
G2	Positive

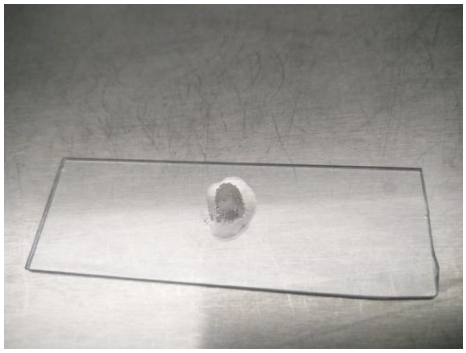

Fig.4.2 Isolate D2 showing bubble formation

4.1.3 Standard curve of KH_2PO_4

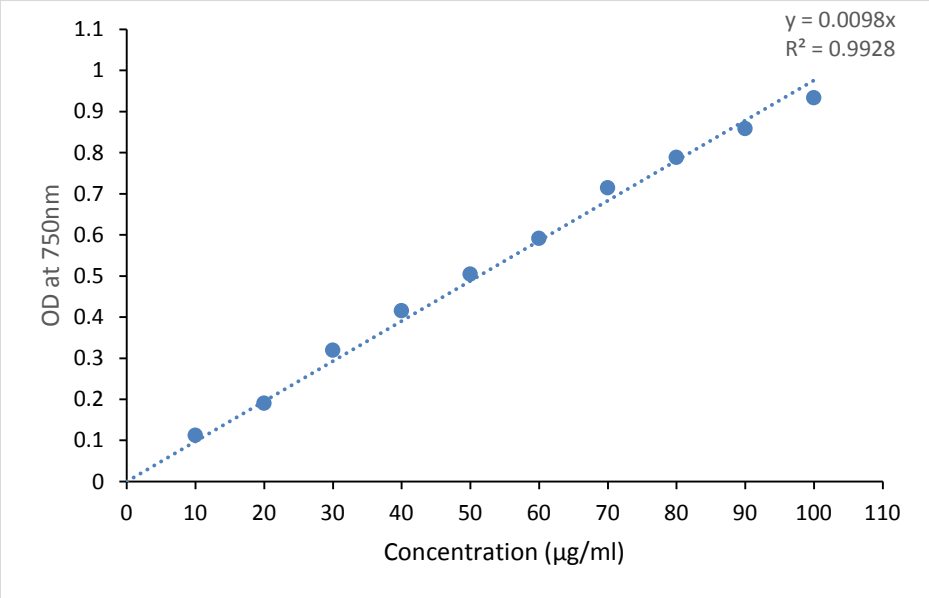

Fig 4.3 Standard curve of KH_2PO_4

Table 4.3 Phytase activity of samples (U/ml)

Samples	Phytase Activity (U/ml)		
	24 hrs	48 hrs	72 hrs
A1	0.165	0.290	0.088
D1	0.338	0.465	0.194
D2	0.301	0.437	0.200
D3	0.270	0.381	0.139
E2	0.290	0.364	0.113
G2	0.163	0.265	0.128

These readings exhibits the result that sample D2 shows maximum phytase activity and sample A1 shows minimum activity.

4.1.4 Protein concentration

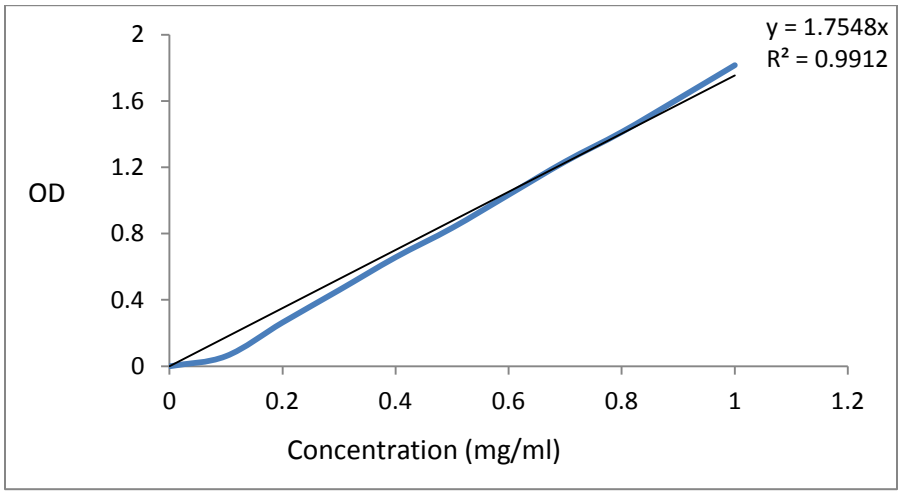

Fig 4.4 Standard curve for protein concentration (Bradford Assay)

Table 4.4 Protein Concentration of samples (mg/ml)

Samples	Protein Concentration (mg/ml)		
	24 hrs	**48 hrs**	**72 hrs**
A1	0.218	0.339	0.133
D1	0.463	0.572	0.298
D2	0.346	0.407	0.296
D3	0.281	0.322	0.173
E2	0.291	0.325	0.185
G2	0.232	0.282	0.152

Based on the above results we selected 3 samples, D1, D2, and E2, for carrying out several biochemical tests.

Table 4.5 The results of various biochemical tests for the isolates were which shows utilization of following sugars by bacteria.

Tests	**D1**	**D2**	**E2**
Inulin	Negative	Negative	Negative
Sodium gluconate	Positive	Positive	Positive
Glycerol	Positive	Positive	Positive
Salicin	Positive	Positive	Negative
Dulcitol	Negative	Positive	Positive
Inositol	Negative	Positive	Positive
Sorbitol	Negative	Positive	Positive
Mannitol	Positive	Positive	Positive
Adonitol	Positive	Positive	Positive
Arabitol	Positive	Positive	Negative
Erythritol	Positive	Negative	Negative
α-methyl-D-glucoside	Positive	Positive	Negative

Fig. 4.5 D1

Fig.4.6 D2

Fig.4.7 E2

Table 4.6 Results of various biochemical tests for the isolates

Tests	**D1**	**D2**	**E2**
Rhamnose	Positive	Positive	Negative
Cellobiose	Negative	Positive	Negative
Meleritose	Negative	Negative	Negative
α-methyl-D-mannoside	Negative	Negative	Negative
Xylitol	Positive	Negative	Negative

ONPG	Positive	Negative	Positive
Esculin	Positive	Negative	Positive
D-Arabinose	Positive	Positive	Positive
Citrate	Positive	Positive	Positive
Malonate	Negative	Positive	Positive
Sorbose	Negative	Negative	Negative

Fig.4.8 D1

Fig.4.9 D2

Fig.4.10 E2

4.2 Optimization of phytase production by using (OVAT) 'ONE VARIABLE AT A TIME' approaches in submerged fermentation:

4.2.1 Selection of suitable carbon sources:

Different carbon sources were used for production of phytase by isolate D2 like glucose, sucrose, starch, wheat bran, orange peel, fruit pulp. Wheat bran was found to be the best among all carbon sources.

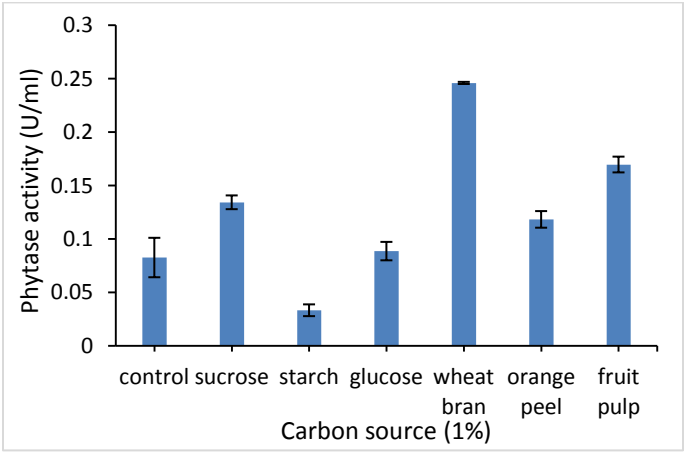

Fig. 4.11 Effect of different carbon sources (%) on phytase production

4.2.2 Effect of different concentration of wheat bran

With the increase in concentration of wheat bran there is gradual increase in phytase production. Concentration of wheat bran at 2.5% found to be the optimum for phytase production

Fig. 4.12 Effect of different concentration of wheat bran (%) on phytase production

4.2.3 Nitrogen Source

Ammonium nitrate was proved to be the best among all nitrogen sources tested for phytase production.

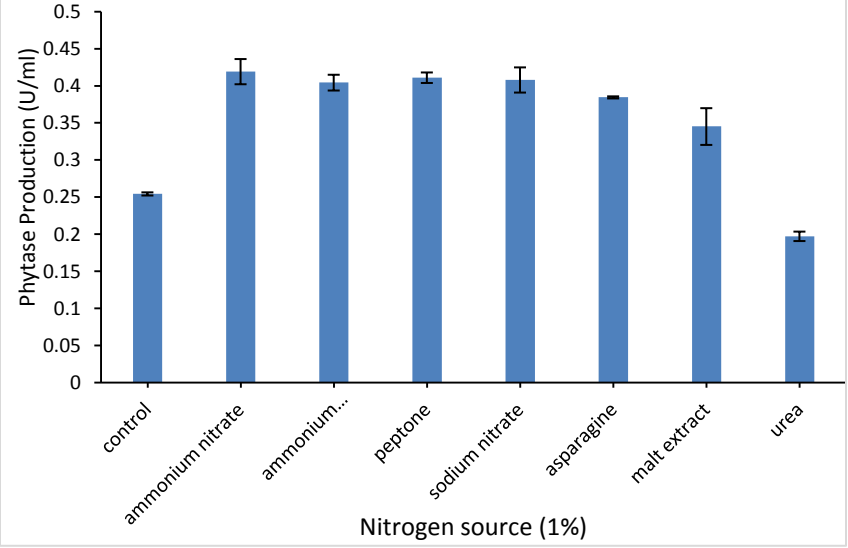

Fig. 4.13 Effect of different nitrogen sources (%) on phytase production

4.2.4 Ammonium nitrate concentration

Ammonium nitrate concentration when supplemented with 1.5% resulted in highest phytase production.

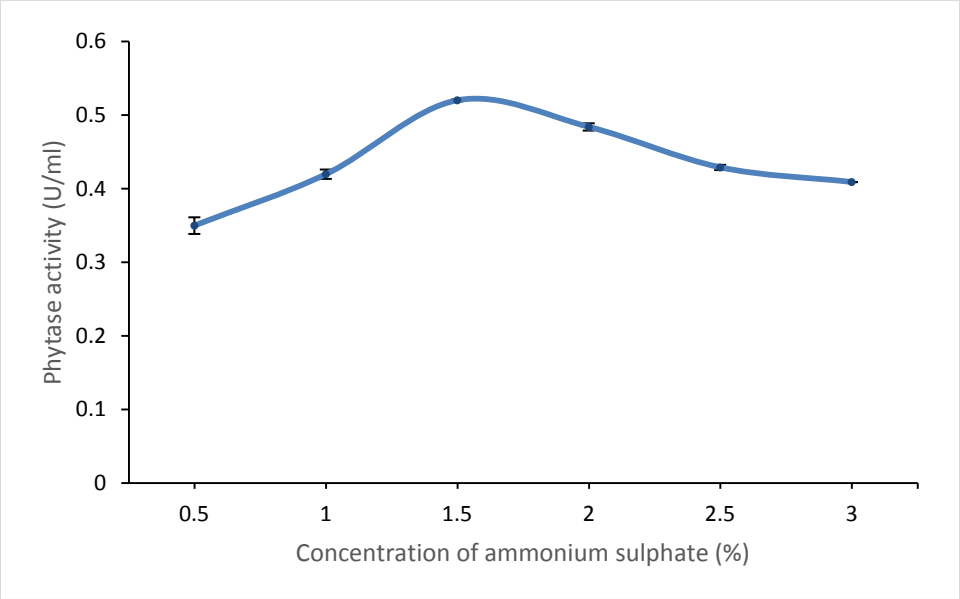

Fig. 4.14 Effect of different concentration of nitrogen sources (%) on phytase production

4.2.5 Temperature

Phytase production was achieved highest at temperature 35 ºC and below and above this temperature phytase activity declined.

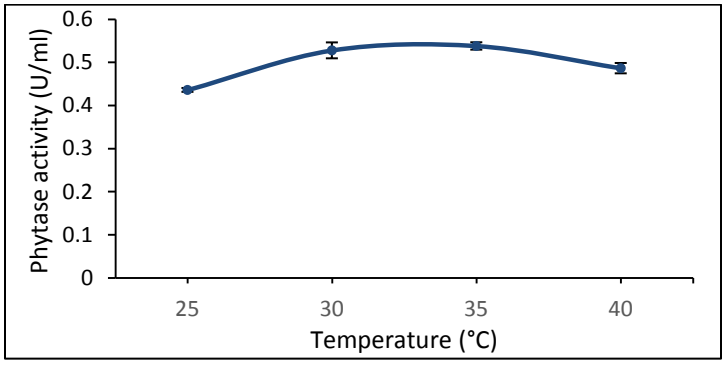

Fig. 4.15 Effect of temperature (°C) on phytase production

4.2.6 Inoculum size

Phytase production was achieved highest at 1% inoculums size and below and above this percentage phytase activity declined.

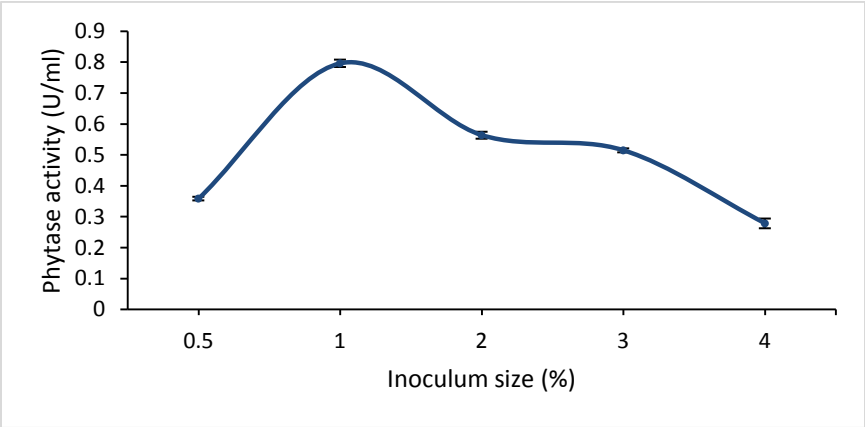

Fig. 4.16 Effect of inoculum (%) size on phytase production

4.2.7 Inoculum age

Inoculum age was varied from 12hrs to 72hrs. Phytase production was achieved highest at 24 h and below and above 24 h phytase activity declined.

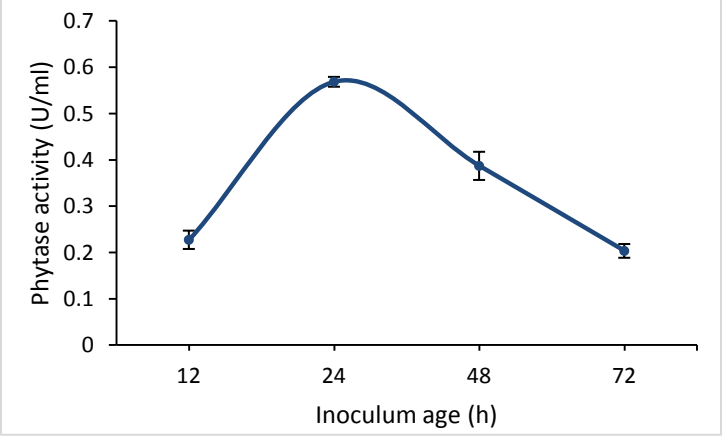

Fig. 4.17 Effect of inoculum age (h) on phytase production.

4.2.8 Incubation period

Incubation period was varied from 12 hrs to 96 hrs. Phytase production was achieved highest at 24 h and below and above 24 h phytase activity declined.

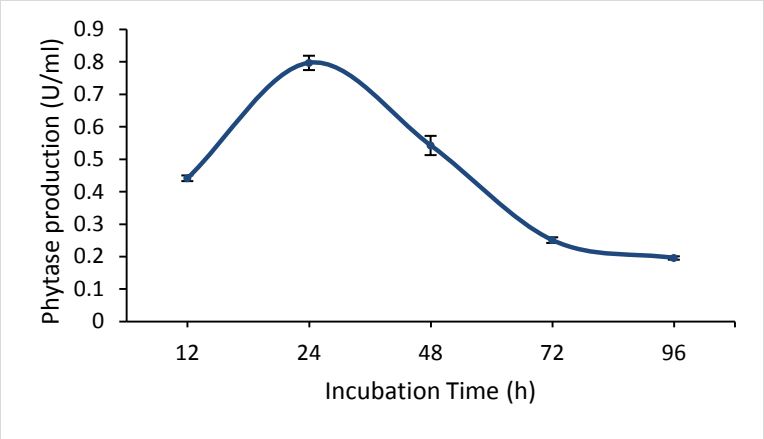

Fig. 4.18 Effect of incubation time (hr) on phytase production

4.2.9 Agitation speed

Agitation speed was varied from 50 rpm to 250 rpm. Phytase production was achieved highest at 150 rpm and below and above 150 rpm phytase activity declined.

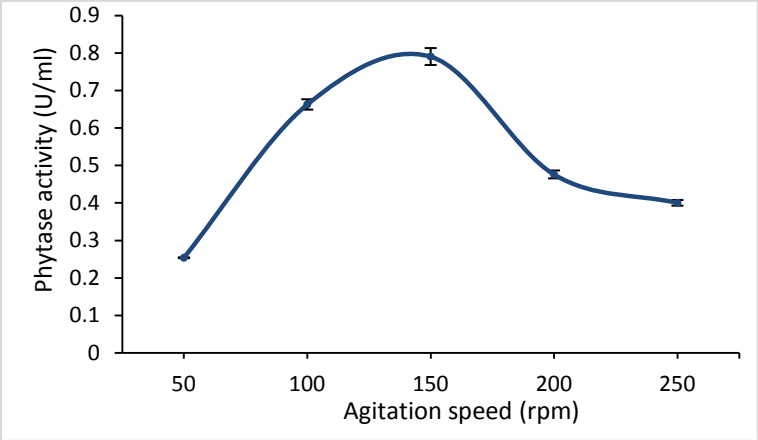

Fig. 4.19 Effect of agitation speed (rpm) on phytase production

4.2.10 pH

Among pH ranged from 3 to 9, pH 5 was found to be the best for phytase production by using wheat bran as substrate. As graph shows pH is low at 3 and then growth start increasing and becomes highest at 5 and then started decrease as the pH was increased.

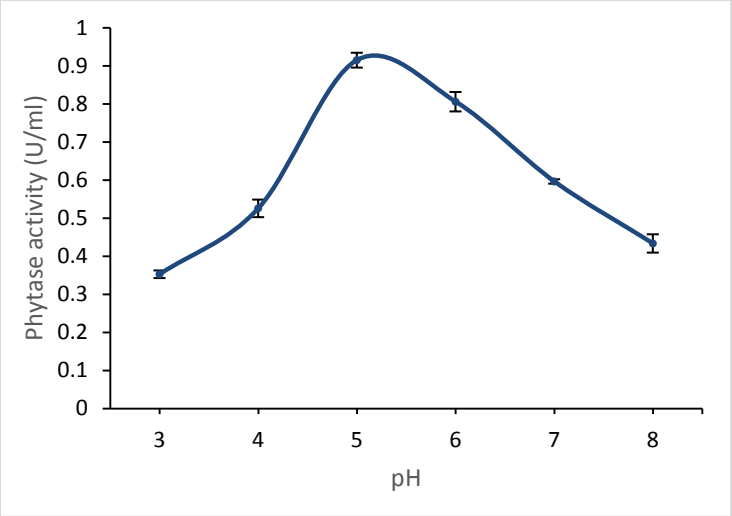

Fig. 4.20 Effect of different pH on phytase production

4.3 Discussions

Enzyme market has obviously demonstrated the importance of development of hydrolyses as feed additive substances for improving the processing and assimilation of ineffectively accessible phosphorus in the diet. According to previous studies rice bran has been the preferred source of carbon. We studied the quality of wheat bran as an alternative carbon source. It can be seen in the results that wheat bran shows excellent phytase activity; therefore wheat bran can also be used as a source for phytase producing bacteria. Wheat bran concentration in media was found to be optimal at 2.5 % by weight. As nitrogen is also an important component for the phytase, different sources like peptone, ammonium sulphate, ammonium nitrate and urea were experimented with. Out of all these, ammonium nitrate at concentration of 1.5% showed highest activity.

Incubation period play an important role in production of bacterial enzyme as growth rate and enzyme production pattern is dependent on it. At an incubation period of 24 hours, highest production of enzyme was observed. After that there was a huge decline in the production. Most of studies also show that at 24 hour there is highest activity. This observation may be due to the fact that after 24 hours, cells enter death phase. The temperature and pH of the production medium also affects the production of enzyme and microbial growth. Usually the bacterial

phytases show maximum activity in the range of 25 ^{o}C to 70 ^{o}C and the pH ranges from 4 to 9. Previous studies show good growth of bacteria at pH 6. According to our study phytase producing bacteria showed best growth and enzyme production at 35^{o}C and in the acidic condition of pH 5. Agitation speed was varied from 50 rpm to 250 rpm. Phytase production was achieved highest at 150 rpm and below and above 150 rpm phytase activity declined.

Both inoculum age and size play an important role in enzyme production. 1% concentration of inoculum showed a wide increase in production and at increased concentration the growth is less. 24 h old inoculum showed a massive growth as compare to other inoculums ages as they showed very less growth.

CONCLUSION

Anti-nutritional properties of phytic acid makes production of animal feed a very difficult and less profitable task. Increasing the production without using any chemical substances is an even greater task. One solution to this problem is the use of hydrolytic enzymes such as phytases which removes the anti-nutritive properties of phytic acid so that the phosphorous released during the catalysis can be utilized. Use of phytases is not just limited to animal feed but has a wide range of uses for animals, humans and environment. Microbial phytases have been found to be isolated from various bacterial, fungal and plant sources.

In this study we investigated soil samples with potential bacterial isolates capable of phytase production from different poultry farms of Himachal Pradesh.

Bacterial isolate D2 was selected on exhibition of best phytase activity. The production of phytase was optimized by OVAT (One Variable At a time) method.

The bacterial isolate in current study shows highest phytase activity at 2.5% wheat bran, 1.5% Ammonium Nitrate as substrate at 35 ºC at pH 5.5 after incubation period of 24 hours kept at agitation speed of 150 revolutions per minute.

Future prospects of the current study could be checking the phytase activity with mixture of substrates and statistical optimization of phytase production by response surface methodology.

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