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Immobilization of Phytase Produced by *Bacillus licheniformis*

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by

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DECLARATION

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

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Certificate

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TABLE OF CONTENTS

List of Figures i

List of Tables ii

List of Abbreviations ..... **Error! Bookmark not defined.**

Abstract iv

1. Introduction 1

1.1. Aim and Objectives 6

2. REVIEW OF LITERATURE 7

2.1. Phytic Acid 7

2.1.1. Occurrence and Functions of Phytic acid 7

2.1.2. Anti-nutritive effects 7

2.2. Phytase (Phytate-degrading enzyme) 8

2.2.1 Enzymatic catalysis of Phytate 10

2.2.2. Biological Sources of Phytase 10

2.3. Thermostable Enzymes 12

2.3.1. Strategies to Develop Thermostable and Recoverable Enzyme 13

2.4. Immobilization of Enzyme 14

2.4.1. Types of Immobilizations 15

2.5. Recent Developments 20

2.5.1. Combi-CLEAs and Catalytic Cascade Processes 20

2.5.2. Enzyme-Immobilized Micro Channel Reactors for Process Intensification ... 20

2.5.3. Immobilization with Nanofibrous polymers, Nanoparticles and Nanoporous gold (nanobiocatalysis) 21

2.6. Bentonite 22

2.6.1. Types of Bentonite 22

2.6.2. Efficacy of Bentonite as Aflatoxin binders in Poultry 22
2.7. Factors affecting the stability and performance of extracellular enzyme in Bentonite 24
3. Materials and Methods 25
3.1. Study Area 25
3.2. Chemicals 25
3.3. Screening of Bacterial Isolates for Phytase Production 25
3.4.1. Substrate Preparation 25
3.4.2. Inoculum Preparation 26
3.4.3. Shake flask Fermentation for Phytase Production 27
3.5. Assay to Quantitate Phytase Activity 27
3.5.1. Unit of Enzyme 27
3.5.2. Characterization of Assay for Quantitative Estimation of Phytase 28
3.6. Standard curve for Phosphorous 30
3.6.1. Reagents 30
3.6.2. Procedure 31
3.7. Total protein Estimation 33
3.7.1. Required Reagents 33
3.7.2. Procedure 34
3.8. Standard curve for Bovine Serum Albumin 35
3.8.1. Procedure 35
Table 3.7. BSA standard curve. 35
3.9. Specific Activity Estimation 36
3.10. Optimization of Phytase Production from *Bacillus licheniformis* using Shaken flask Fermentation 37
3.10.1. Effect of change in incubation time on phytase production 37

3.10.2. Effect of change in pH on phytase production 37
3.10.3. Effect of change in temperature on phytase production 37
3.11. Batch Fermentation 38
3.12.1. Enzyme Extraction 38
3.12.2. Acetone Precipitation 38
3.12.3. Pelleting 39
3.13. Characterization of Partially Purified Free Enzyme 39
3.13.1. Effect of temperature on activity of free enzyme 39
3.13.2. Effect of pH on proteolytic activity of free enzyme 39
3.14. Immobilization of Alkaline Phytase 40
3.14.1. Immobilization without Optimized Conditions 40
3.15. Characterization of Immobilized Enzyme 41
3.15.1. Effect of temperature on immobilized enzyme 41
3.15.2. Effect of pH on immobilized enzyme 42
3.16. Desorption of enzyme to estimate Adsorption 42
4.1. Characterization of Assay for Quantitative Estimation of Phytase 43
4.1.1. Effect of different ratio of enzyme and substrate on activity of enzyme 43
4.1.2. Effect of different Incubation times of assay on activity of enzyme 43
4.2. Optimization of Phytase Production from *Bacillus licheniformis* using Shaken Flask Fermentation 44
4.2.1. Effect of change in incubation time on enzyme production 44
4.2.2. Effect of change in pH on phytase production 46
4.2.3. Effect of change in temperature on phytase production 48
4.3. Precipitation of Alkaline Phytase 50
4.4. Characterization of Partially Purified Free Enzyme 51

4.4.1. Effect of temperature on activity of partially precipitated free enzyme 51
4.4.2. Effect of pH on activity of partially precipitated free enzyme 53
4.5. Immobilization without optimized conditions 56
4.6. Characterization of Immobilization of Partially Precipitated Phytase on Bentonite 57
4.6.1. Effect of pH on immobilization process 57
4.7. Characterization of Immobilized Enzyme 58
4.7.1. Effect of temperature on activity of immobilized enzyme 58
4.7.2. Effect of pH on activity of immobilized enzyme 61
4.8. Desorption 63
5. Discussion 64
6. Conclusions 69
7. Future Prospects 70
8. References 71

LIST OF FIGURES

3.1	Phosphorous standard curve.	33
3.2	BSA standard curve.	36
4.1	Effect of change in incubation time of production media on enzyme activity	45
4.2	Effect of change in incubation time of production media on specific activity of phytase	46
4.3	Effect of change in pH of production media on enzyme activity	47
4.4	Effect of change in pH of production media on specific activity of phytase	48
4.5	Effect of change in incubation temperature of production media on specific activity.	49
4.6	Effect of change in incubation temperature of production media on activity of alkaline phytase	50
4.7	Effect of change in temperature on the activity of partially precipitated free alkaline phytase	52
4.8	Effect of change in temperature on specific activity of partially precipitated free alkaline phytase.	53
4.9	Effect of change in pH on the activity of partially precipitated free alkaline phytase	55
4.10	Effect of pH on specific activity of partially precipitated free alkaline phytase	56
4.11	Effect of change in temperature on activity of alkaline phytase after immobilization	60
4.12	Effect of change in temperature on specific activity of alkaline phytase after immobilization.	60
4.13	Effect of change in pH of buffer on enzyme activity (U/ml) after immobilization	62
4.14	Effect of change in pH on specific activity (U/mg) after immobilization	62

LIST OF TABLES

3.1	Composition of production media	26
3.2	Composition of media for inoculums preparation.	26
3.3	General scheme of the enzyme assay for alkaline phytase.	27
3.4	Scheme for optimization of enzyme substrate ratio for quantitative assay.	29
3.5	Scheme for optimization of incubation time of quantitative assay.	30
3.6	Phosphorous standard curve.	32
3.7	BSA standard curve.	35
4.1	Effect of different ratio of enzyme and substrate on activity of enzyme.	43
4.2	Effect of different incubation times of assay on activity of enzyme.	44
4.3	Effect of different incubation times on enzyme production.	45
4.4	Effect of change in pH on the production of phytase.	47
4.5	Effect of change in temperature on production of phytase	49
4.6	Results for precipitation of alkaline phytase using acetone.	50
4.7	Effect of change in temperature on activity of partaillt purified free alkaline phytase.	51
4.8	Effect of change in pH on activity of partially purified free alkaline phytase.	54
4.9	Immobilization without maintaining the optimized conditions.	57
4.10	Effect of change in pH on immobilization	58
4.11	Effect of change in temperature on activity of immobilized alkaline phytase	59
4.12	Effect of change in pH on activity of immobilized alkaline phytase	61
4.13	Desorption of phytase from bentonite	63

LIST OF ABBREVIATIONS

g	Gram
Mg	Milligram
Ml	Millilitre
Rpm	Revolution per minute
°C	Degree centigrade
nm	Nanometre
Min	Minutes
$CaCl_2$	Calcium chloride
B.licheniformis	*Bacillus licheniformis*
TCA	Tri-chloro-acetic acid
BSA	Bovine Serum Albumin
$FeSO_4$	Ferrous sulphate

ABSTRACT

The aim of present study was to prepare immobilized product for poultry industry. For this purpose phytase enzyme from previously isolated *Bacillus licheniformis* was immobilized on poultry feed bentonite. As phytase is of great importance in poultry because it degrades hazardous phytic acid so immobilization of phytase would allow us to make the enzyme cost-effective. First of all phytase production from *Bacillus licheniformis* using raw substrate wheat bran was optimized. Maximum production was obtained at pH 8, temperature 50°C and incubation period 5 days. After successful production enzyme was extracted by centrifugation. This extracted enzyme was precipitated with the help of acetone. As a result semi-purified enzyme was obtained and there was 4 folds increase in enzyme activity. Characterization of assay to quantitate phytase was carried out. Ideal conditions were obtained with 0.2 ml enzyme and 0.8 ml substrate and incubation time of 10 minutes. After assay characterization partially purified free enzyme, immobilization process and immobilized enzyme were characterized. The partially purified free enzyme showed maximum activity (88.5 U/mg) at pH 8 and 50°C temperature, even at 60°C there was no significant decrease in activity. The effect of incubation time and pH was studied on immobilization process. There was no significant effect of incubation time was observed while immobilization yield was maximum at pH 8 (95%) at pH 7 yield was (89%) while there was significant decrease in immobilization yield at pH 5 and pH 9. After immobilization optimization, effect of temperature and pH was studied on immobilized enzyme. This study revealed that immobilized enzyme was stable at higher temperature than free enzyme i.e. free enzyme showed maximum activity at 50°C and immobilized enzyme showed maximum activity at 70°C which is a beneficial character from industrial point of view. Moreover, only 14% activity was lost at pH 8 after immobilization. Desorption was carried out after adsorption. There was no significant loss of activity in wash 1, 2, 3 and 4. The loss in activity was 8.2 U/mg, 1.06U/mg, 2.5 U/mg for wash 1, 2 and 3, respectively and there was no activity found in wash 4. Therefore, it was concluded that immobilization of thermostable phytase from *Bacillus licheniformis* on bentonite can be carried out successfully for application in poultry industry.

INTRODUCTION

1. INTRODUCTION

Enzymes collectively signify highly special types of receptors comprising several drugs with proteins targets and also as therapeutic agents. Mechanisms for enzymatic actions vary extensively in different classes. Enzymes are biocatalysts with hallmark of acting upon a specific substrate and its conversion to a specific product. Almost all the biochemical reactions involve enzymatic catalysis (Copeland RA, 2004) .

This thesis is associated with study of immobilization of phytase produced by *Bacillus licheniformis.*

Phytases (myo-inositol hexaphosphate phosphohydrolases) are member of special group of acid phosphatases which can carry out the hydrolysis of phytate, phytates are the major storage forms of phosphate in legumes and cereal's mature seeds (Reddy et al., 1989). A wide range of phytases are present in nature (Irving GCJ, 1980, Nayini NR and Markakis P, 1986), for example in animals, plants and microbes.

On the basis of their various catalytic properties and structural differences (Mullaney EJ and Ullah AH 2003), phytases are subdivided into three major classes, including

- members of histidine acid phosphatases (HAP)
- beta-propeller phytases (BPP)
- purple acid phosphatases (PAP).

Both bacterial and fungal phytases have been isolated, cloned and expressed in different hosts and their characteristics have been observed also (Ha NC, et al., 2000; Choi YM, et al., 2001, Tye AG et al., 2002; Mukesh P, et al., 2004; Cao L, et al., 2007, Lee J, et al., 2007, Soni SK and Khire JM, 2007, Fu et al., 2008a,b,c, Guo MJ, et al., 2008, Hang HF, et al., 2009; Mullaney EJ et al., 2010; Tian YS, et al., 2010).

Phytases (myo-inositol(1,2,3,4,5,6) hexakisphosphate phosphohydrolases) are usually used as additives in animal feed for poultry and swine. The use of phytase as a feed

additive has been approved as GRAS (Generally Regarded as Safe) in 22 countries (Wodzinski RJ, Ullah AHJ, 1996). Phytase has been found in variety of bacteria, e.g. *Aerobacter aerogenes* (Greaves MP, et.al, 1967), *Pseudomonas* sp. (Irving GCJ, Cosgrove DJ, 1971), *Bacillus subtilis* (Powar VK, Jagannathan V, 1982), *Klebsiella* sp. (Shah V, Parekh LJ, 1990) and *Enterobacter* sp. (Yoon SJ, et.al., 1996). Corn-soyabean is a diet for pigs and it contains added phytases which convert almost one-third of unavailable form of phosphate to its available form (Cromwell GL, et.al., 1993). Micro-organisms, plants and some animal tissues are known to contain phytases (Konietzny U, Greiner R, 2002, Haefner S, Knietsch A, 2005).

Phosphorous is one of the major feed ingredients and is supplied to animals in required amounts through raw material and added phosphates. 50-80% of phosphorous is bound in phytates, which cannot be broken down by endogenous enzymes in poultry (Maga JA, J Agric Food Chem , 1982). Vegetable sources provide phosphorous which is poorly digested and can't fulfill requirement of nutrition of poultry regardless the fact that legumes, cereals and oilseed plants have only 1-5% phytate. Therefore, there should be an enzyme which can hydrolyze phytate to inositols and inorganic phosphates which are easily and readily adsorbed in tract of digestive system (Annison G, Choct M, 1993).

As organisms have been proved to be important source for phytase production therefore bacterial strains have been isolated from various sources in order to find any phytase activity. In recent, three bacterial strains were isolated from the Malaysian maize plantations with the ability of phytase production (Anis Shobirin, et.al., 2007). Activity of phytase is also found in vegetables like radish, white mustard, spinach, lettuce, potato and it is also found in grass and lilly pollens. Thermostable phytases are of great economical importance especially in paper pulp industry where eco-friendly and thermostable phytases are used to degrade phytic acid (Liu BL, et.al., 1998).

Phytate is taken in large amount by vegetarians, people consuming cereals in large quantity (unbalanced food), people living in undeveloped countries who take unleavened bread as food and by those babies who eat soy-based infant formulas (Simell M, et.al., 1989). Phytase obtained from *A.niger* is added to flour containing wheat bran in order to

increase the absorption of iron in humans as iron, calcium, zinc and maganesium are not properly absorbed in the presence of undigested phytate present in the small intestine. Undigested phytate also responsible for poor digestibility of dietary proteins and inhibition of digestive enzymes (Sandberg AS, et.al., 1996). The most important reason for intensive study on phytases now-a-days is their ability to reduce phytate content of food consumed by humans and animal feedstuffs because phytate makes insoluble complexes with metal ions which are nutritionally important, these metal ions include zinc, magnesium, iron, and calcium. This complex formation decreases their bioavailability (Harland BF and Oberleas D, 1999), phosphorous is released in the environment where microbes act upon this released phosphorous and causes pollution (Baruah H et al., 2004). The breakdown of phytate is majorly carried out by the activity of phytase which trigers the stepwise excretion of phosphate from phytate (Konietzny U and Greiner R, 2004).

Enzyme purification from crude enzyme is an important step after enzyme production and extraction as other proteins are also formed in the same media which may hinder the activity of required enzyme. Enzyme purification or semi-purification can increase the activity of purified enzyme by several folds. Precipitation using organic solvent is one of the fast and successful method for enzyme semi-purification. Hong SW, et al, in 2011 used ethanol precipitation and chromatography for purification of phytase after phytase production and extraction.

Following are few characteristics of phytase which are of practical significance.

Substrate specificity: Substrate specificity and affinity of various phytases has been extensively studied. Microbial phytases (*A. niger, E. coli, Bacillus* sp.) prove to have a high affinity to phytic acid, while phytases obtained from plants and fungus A. fumigatus have a wider substrate specificity (Wyss M, et al., 1999) and can degrade the lower inositol phosphate. Although most phytases are able to degrade phytic acid to the monophosphate ester of inositol as the final product (Greiner et al., 2002, Ullah AHJ, Phillippy BQ. 1994, Wyss M, et al., 1999), phytases from *Bacillus* sp. hydrolyze preferentially every second phosphate over the adjacent ones and degrade the phytic acid

molecule to inositol triphosphate [IP3 either as IP3(2,4,6) or IP3(1,3,5)] as the final product (Kerovuo J. et al., 2000).

pH and temperature optima: Most phytases which are isolated have their pH optima in the range of 4.5–6. But, phytases from *Bacillus* sp. have neutral or alkaline pH optima (Choi YM et al., 2001, Kim Y. et al., 1998). The pH profile of *A. niger* phytase (phyA) is featured by two pH optima at 2.5 and 5.5, respectively, and a dip in activity between these two points (Han YM, Lei XG. 1999). (Mullaney EJ, et al., 2002) used site directed mutagenesis to remove the drop of activity in the pH range 3–5 for maintaining a high specific activity at the stomach pH. The temperature optima of most plant and microbial phytases range from 45 to 60°C. These relatively high optimal temperatures preclude a full activity of phytases at the stomach temperatures of swine or poultry (37–40°C), and result in even poorer performance of phytases in fish.

Thermostability: Since commercial nourishes or feeds are regularly pelleted, a procedure utilizing high temperature (60–80°C) and steam, every single food enzyme should be heat stable in order to ignore substantial loss of activity during this procedure. Thermostability of any given phytase, just like other proteins, is decided by its ability to resist heat denaturation such as in the case of hyperthermophilic organisms and (or) its ability to refold appropriately into the native-like, fully active conformation after heat denaturation (Wyss M, et al., 1998).

In order to put phytases in commercial application few upstream processing of these enzymes are necessary to enhance their stability, bioactivity and shelf life for storage. Most of commercially available enzymes specially those in poultry are available in dry formats immobilized over some inert media. Various preferred protocols are being followed preceding commercialization .

One of these protocols suggests immix of steam and enzyme pellet immobilized over feed and then it is subjected to high pressure die and molding machines maintaining the moisture between 12 to 20 percent at elevated temperature (above 60°C and below 95°C) along with suitable stabilizer to prevent enzyme inactivation for example magnesium

sulphate and other bivalent cations containing inorganic compounds or salts. Granulation, absorption and adsorption of phytases are also proposed formulating procedures prior to their commercial packaging and poultry application (Le XG, et al., 2013).

To develop stable format of enzymes on such a matrix that is edible and non toxic to living cells there are few available materials like spores of Probiotic *Bacillus* strains. These bacilli spores are being employed as support matrix for immobilization of *E.coli* phytases which is responsible to degrade anti nutrient phytin and phytate (Herzallah S, et al., 2008) . Since spores are heat resistant and are reluctant to drought conditions thus they are likely to be a sole candidate for phytases immobilization.

Bacillus Polyfermenticus spores immix with phytase enzyme cause them to get immobilized over the surface. Estimated amount of enzyme that get attached to spores was 28.2 mg/g with specific activity of 41,120 u/g of spores with 2 to 10 folds increase in half life. When such spores are exposed to saline solution, 10 % formic acid solution, urea, sodium dodecyl sulphate then it results in desorption of immobilized enzymes (Cho EA, et.al., 2011). Thus the possible interaction between spore surface and phytases were proposed to be either electrostatic or hydrophobic interaction. Safety with respect to feed and food grades is the main feature of *Bacillus* spores to be implied for immobilization (Dvořáková J, 1998).

Blackburn studied commercially available phytases from two microbial sources *A.niger* and *E. coli* by immobilizing their respective phytases on nano clay. Characterization and Optimization of phytases immobilization was the main aim of their studies. Naturally available clay materials from volcanic glass (montmorillonite) and synthetic Fe-allophanes were materials of choice for immobilization of phytases. Patterns observed for immobilization was slightly different for two enzymes at different pH. High residual phytases activity was observed when conditions were slightly acidic along with improvement in thermostability and proteolysis resistance. Natural and synthetic support matrix were proved to be good in case of *E.coli* phytases although for *Aspergillus niger* they were less favourable (Menezes-Blackburn D, et al., 2011).

1.1. Aim and Objectives

In this work the aim was to produce, characterize and immobilize phytase on bentonite. The strain used was *Bacillus licheniformis* which proved to be potential producer of phytase.

1. First of all production of phytase by *Bacillus licheniformis* at best optimum conditions using wheat bran as raw substrate.
2. Precipitation of crude enzyme to get semi-purified enzyme.
3. Characterization of partially purified free enzyme.
4. Characterization of immobilization of phytase on bentonite.
5. Characterization of an immobilized enzyme.
6. Desorption of adsorbed enzyme.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Phytic Acid

Phytic acid was discovered in 1903, it is a saturated cyclic acid and serves as a principle storage form of phosphorous in legumes, cereals and oilseeds. By forming complexes with minerals and proteins, phytic acid not only performs many physiological functions but also notably influences the nutritional and functional properties of legumes, oilseeds and cereals (and these are the source of food and feed). Salts of phytic acid are called phytates. Myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate is the correct chemical name of phytic acid (IUPAC-IUB, 1977).

2.1.1. Occurrence and Functions of Phytic acid

There are many physiological functions of phytic acid, it serves as (i) storage house of phosphorous (ii) source of cations (iii) store of energy (iv) myo-inositol (a cell wall component) source and (v) undergoes dormancy initiation. Moreover, phytic acid perhaps performs many other undiscovered functions in seeds (Reddy NR, et al., 1989). Phytic acid performs the function of natural antioxidant during dormancy in seeds (Graf et al., 1987). Phytic acid has also been noticed to have an antineoplastic influence on animal models of breast and colon carcinomas. Colonic carcinoma can be avoided by the presence of phytic acid (undigested) within the colon (Dvorakova J. 1998). Inositol hexaphosphate has gained attention due to it's hypocholesterolaemic effect and role in cancer therapies. Intermediates of inositol phosphate especially inositol triphosphate helps in cell funtion's regulation and signal transduction in plant as well as in animal cells.

2.1.2. Anti-nutritive effects

Phytic acid has strong anti-nutritional impacts (Pallauf J, Rimbach G. 1996). These anti-nutrition effects are due to it's unwanted molecular structure. The insoluble complexes of phytate-mineral is formed in the intestine which prevents absorption of minerals. This

decreases the bioavailibility of minerals which are essential to be absorbed (Davies NT, 1982). The total number of phosphate groups in phytic acid is six, after complete breakdown these six groups have twelve total negative charges. That's why phytic acid readily binds with different monovalent, divalent, trivalent cation and their mixtures as a result insoluble complexes are formed (Reddy NR, et al., 1989).

The powerfull chelating, related with six receptive phosphate groups of phytic acid, is the primary reason of anti-nutritive effect of phytic acid. The intake of phytate with diet by people in China was lower than in developing countries and higher than those in developed western countries. Perhaps, the bioavailability of calcium, iron and zinc is impaired by dietary phytate present in the diet of people in China. Apparantly, Zn^{2+} absorption and life weight gain was badly affected by gradual supplementation of phytic acid in growing rats, showed by (Rimbah G, Pallauf J. 1992). Also, some functional properties like seed's cookability and protein's pH-solubility are influenced by phytic acid presence (Cheryan M. 1980).

Phytic acid forms very stable chelates with almost all multivalent cations and at pH 6 to 7 these chelates are not soluble. As a result of direct electrostatic interaction, phytate-protein complexes are formed at low pH and low concentration of cations, however, at pH greater than 6 to 7, a ternary phytic acid-mineral protein complex is formed. This complex breakdowns at high Na^{+} concentrations. Complex minerals are less bioavailible due to these complexes moreover, at low pH these complexes are more resistant to proteolytic digestion (Cheryan M. 1980).

2.2. Phytase (Phytate-degrading enzyme)

Phytate-degrading enzymes have been gained attention of scientist because of their beneficial ability of decreasing phytate quantity in feed of animal and human food as well. Initially phytate-degrading enzymes were treated as additives for animal feed because by releasing orthophosphate they enhance the plant material importance in feed of an animal. In addition, phytate degrading enzymes have also been considered to decrease phosphate pollution in the area of life stock management. Several studies have

already proved that addition of phytases improves phosphate uptake from phytate and immensly decreases orthophosphate release (Cromwell GL, et al., 1995).

Comparatively, strong phytate breakdown and powerful phytase activity was observed in chyme present in the stomach of pigs which were given controlled diet. Inositol phosphates were the predominant products of phytate hydrolysis, characteristically made by plant phytases. Regardless of the diet given, appreciable activity was shown by alkaline phosphatases while phytases showed low activity in small intestine and colon. Phytate was degraded completely through out the whole gut, similarly for both diets while apparently the availability of total phosphorous was appreciably higher in pigs which were given controlled diet as compare to those given phytase deactivated diet (Schlemmer U et al., 2001).

Phytases present in human intestine do not play a major role in degradation of phytate while endogenous phytases present in bran play a major role in phytate hydrolysis. The technique used for processing the fiber effects the quantity of endogenous phytase activity. Iron and zinc absorption is inhibited by phytate. In case of phytate hydrolyses during the processing of food the availability of minerals is increased. Endogenous enzyme phytase are activated or phytase are added to food, different inositolphosphates having 1-5 phosphate groups per an inositol molecule are produced as a result of phytate degradation. How the products formed as a result of phytate degradation effect the absorption of calcium, iron and zinc is under further investigation. To reduce the content of phytate of vegetables and cereals completely certain food processes like germination, fermentation and soaking are followed (Sandberg AS. 1991).

Phytase clearly plays a beneficial role in zinc and iron absorption from foods rich in phytate. Phytase has the ability to enhance the absorption of calcium, phosphorous and magnesium in areas like Southeast Asia where mineral deficiency is common (Troesch B, et al., 2013).

2.2.1 Enzymatic catalysis of Phytate

Phytate-degrading enzymes catalyze the stepwise hydrolysis of myo-inositol hexakisphosphate to orthophosphate and lower myo-inositol phosphates. The reaction intermediates are released from the enzymes and serve as substrates for further hydrolysis (Vats P, Banerjee UC, 2004).

Phytic acid + H_2O ⟶ p- myo - inositol 1, 2, 4, 5, 6-PKP + Pi

2.2.2. Biological Sources of Phytase

Following are the sources of Phytases in nature.

i. Animal sources.
ii. Plant sources.
iii. Microbial sources.

2.2.2.1. Animal Sources

Monogastric animals produce phytase (Bitar, Reinhold JG, 1972, Copper JR, Gowing AS, 1983; Yang WJ, et al., 1991, Chi H, et al., 1999). In monogastrics phytates derived from food are not significantly digested by phytase produced in intestine. (Craxton A, et al., 1997) cloned and expressed a rat hepatic multiple inositol polyphosphate phosphatase (MIPP) which showed phytase activity. All rat tissues examined contained MIPP mRNA, but strongest expression was observed in liver and kidney. Protozoan *Paramecium* also contained a phytase-like enzyme (Freund WD, et al., 1992).

2.2.2.2. Plant Sources

Plants widely produce phytase. Cereals like wheat, barely, rice, maize, triticale etc. produce phytase. Phytase has also been extracted and characterized from beans such as mung beans, dwarf beans and small white beans of California. Activity of phytase has been found in potato, lettuce, grass, white mustard, lily pollen, radish and spinach (Dvorakova J. 1998). Phytase has been purified as well as characterized maize

germinating seedlings (Zea mays) by (Laboure AM, et al., 1993), moreover, coding of cDNA was also done for Zea mays phytase (Maugenest S, et al., 1997). Genomic library of maize was screened using this cDNA library two different genes were isolated as well as sequenced.

2.2.2.3. Microbial Sources

Microbial enzymes are preferred over animal and plant sources because of some economic advantages i.e. bulk production, easy extraction and not dependent on sessional variation and on technical advantages i.e. single source for variety of enzymes, stability of enzyme, short generation time, easy genetic manipulation and production on raw substrate (Shanmugam S. 2009, Mishra S, Behera N. 2008). From the history of ancient Greek, microbial enzymes play vital role in many important processes such as brewing, baking, cheese making and alcohol production (Malakar R, et al., 2012). With the passage of time and advancement in technologies role of enzyme in chemical, pharmaceutical beverage and food industries are also investigated (Liu XD, Xu Y, 2008, Mrudula S, et al., 2011). According to report published in 2011, the global market of industrial enzymes was closed to a billion dollars in 1990 and crossed 2.0 billion dollar in 2005. The market has been estimated at 3.3 billion dollar in 2010 and is expressed to expected to reach 4.4 billion dollar by 2015. Among them, animal feed (6%), baking (8%), starch (11%), textiles (12%) and detergents (37%) are the main industries, which use about 75% of industrially produced enzymes (Singh S. et al., 2011, Harwood CR. 1992, Schallmey M. et al., 2004, Ferrari E. et al., 1993).

Phytase producing bacteria were isolated in media (phytase specific medium) with phytin and glucose as the only sources of phosphate carbon, from soil and poultry faeces. Out of five strains isolated from poultry faeces and soil, one identified as *Pseudomonas* sp. and showed high phytase activity (Hosseinkhani B, et al., 2012). *Bacillus subtilis* synthesis significant quantity of phytase during 48th hour of incubation at pH of 6.5 and 32°C. *Klebsiella* sp. produces acido-thermophilic phytases which have immense significance for feed industry (Mittal A et al., 2011)

Aspergillus niger, a filamentous fungus, have most frequently used for commercial synthesis of extracellular phytase (Yoon SM, et al., 2011). Different cultivation methods like submerged or solid-state fermentation have been used for production of phytase from fungus (Jafari-Tapeh H, et al, 2012). Inspite of several bacterial strains, fungi and yeast have been used for phytase production, *Aspergillus ficcum* and *A. niger* are the two strains of *Aspergillus* sp. which have been most commonly employed for phytase production on commercial scale (Jafari-Tapeh H, et al., 2012). *Serratia marcescens* are inhabited in soil legumes and secrete phytase. *Serratia marcescens* help enhance plant growth by making phytate phosphorous for plants when phosphates are in low concentrations (Mukesh Kumar DJ, et al., 2011).

2.3. Thermostable Enzymes

Extremophilic microbes can survive and grow in extreme conditions, they can grow in environments having extremely high temperatures (thermophiles) as well as in environments having extremely low temperatures (psychrophiles), high salt concentration (halophiles) and at places with extreme pH conditions (acidophilic and alkalophilic) and also in marine environments at the depth dealing with pressure of several hundred of bar (barophiles) (Van Den Burg B 2003). Extremophiles have structural adaptation at the molecular level to survive in such extreme conditions (Bertoldo C, Antranikian G. 2002, Dermirjian DC, et al., 2001). The most extensively studied microbes so far are those found at high temperature. Temperature is one of the most important factors for growth, survival activities and for development of organism. Multicellular organisms has complex body system so find difficulties in survival in extreme conditions while unicellular organisms or microorganisms have the ability to thrive in extreme conditions like in high temperature (Bertoldo C, Antranikian G. 2002). Thermophilic organisms are those growing at high temperatures from 45-85°C, with optimum growth in the temperature range of 50-60°C. Thermostable enzymes are those which have been mainly isolated from thermophilic microbes. Thermostable enzymes have found a number of commercial applications because of their significant ability in different industrial processes like they prevent contamination by pathogens especially by mesophiles, easier to purify by heat treatment, overall inherent stability, reduce medium viscosity, high

concentration of substrate are applicable, reduce reaction time and reduce processing cost (Haki GD, Rakshit SK, 2003). Therefore, thermophilic organisms have gained the attention of many researchers, because they are the source of thermostable enzymes. Until now, many phytase have been produced, identified and characterized from many microbes like thermophiles, hyperthermophiles and mesophiles. Therefore, enzymes from extremophiles are being explored and initiated to use in various applications in different industries like in the production of detergent, in sugar industries, lipid and oil processing industries and in food processing industries (Dermirjian DC, et al., 2001). Further are discussed few strategies to make thermostable enzymes recoverable and reusable.

2.3.1. Strategies to Develop Thermostable and Recoverable Enzyme

i. The first way to obtain bio-catalysts for a variety of industrial processes is to isolate enzymes from microbes which naturally exist in extreme environments, such as thermophiles from hot springs, fuel tanks and salt saturated waters of Dead Sea (Haki GD, Rakshit SK, et.al., 2003). Some major advantages offered by thermo-stable enzymes in industrial processes are increase in the rate of reaction, higher substrate solubility, decrease in viscosity of liquids and less chances of microbial contamination. However, in order to obtain individual hyper-thermo-stable enzymes in the amounts required for their use in industry, new bio-reactor concepts and processing protocols must be developed to provide suitable conditions to the isolated extremophilic microbes.

ii. The second way of obtaining naturally stable enzyme involves genetic engineering which involves the isolation of all or part of the genome of a thermophillic microbe followed by its introduction into the genetic machinery of a suitable mesophile. This general approach is well established and has found applications in the production of pharmaceutically valuable products.

iii. The third strategy of stabilizing enzymes from mesophiles is considered to be the best way to obtain stable bio-catalyst sand this is achieved by immobilization, protein engineering and chemical modifications. Protein engineering has been used to produce thermo stable enzymes that differ from their native counterparts only in one or several

predefined amino acids. This approach (protein engineering) has brought the possibility of stabilizing mesophilic enzymes by changes in amino acid sequences (Declerck S, et.al., 2003).

iv. Immobilization by associating the enzyme with an insoluble matrix, so that it can be retained in proper reactor geometry for its economic reuse under stabilized conditions. Immobilization has revolutionized the field of biotechnology because these enzymes provide an alternative tool to traditional chemical technologies (Iqbal HMN, Asgher M. 2013, Asgher M, et.al., 2012, Asgher M, Iqbal HMN.2013).

The industrial applications of biocatalysts depend on the development of effective and stable immobilized enzymes. The immobilization greatly increases the stability and eases the burden of enzyme cost and thus, is widely pursued for efficient, selective and environmentally friendly catalysis. Immobilized enzymes have ability to catalyze the reactions in wide environmental conditions. Solid support like xerogels, sand, clay or soil are required for the attachment of enzyme without posing any kind of environmental risks and thus are beneficial for many of the industrial application (Iqbal HMN, Asgher M. 2013). In modern reactor sections it permits to decouple the catalyst area from the stream of the fluid conveying the reagent sand items. Immobilization helps in the improvement of consistent procedures permitting more monetary association of the operations, computerization, decline of work, and venture/limit proportion.

2.4. Immobilization of Enzyme

Immobilization is carried out by associating or coupling the enzyme with an inert and insoluble support matrix, in this way enzyme can retain it's proper and original reactor geometry for it's economical reuse in sustained conditions (Iqbal HMN, Asgher M. 2013). Biocatalysts have a wide range of applications in industries and efficiency of enzymes depends upon their stability and recovery without any change in its structure. Such enzyme can be attained as a result of immobilization of that enzyme. Thus, immobilization has greatly decreased the enzyme cost and therefore this technique is widely used for selective, efficient and environmentally friendly catalysis.

2.4.1. Types of Immobilizations

Following are the types of immobilization discussed below.

i. Covalent Binding.
ii. Entrapment
iii. Carrier free immobilization using cross-linking.
iv. Smart polymers.
v. Adsorption.

2.4.1.1. Covalent Binding

In this sort of responses the catalyst is covalently attached to a matrix through a concoction response. There takes an immediate tying of compound with strong backing by covalent linkages or utilizing a cross connecting reagent that ties the catalyst at one side and immobilization matrix on other side (Rekuc A, et. al., 2009, Wang M, et. al., 2011, Cao L, et. al., 2003). This technique is by a wide margin the most effective and catalyst can be immobilized by multipoint covalent connection of compound with immobilization help to build action, security and reusability of proteins. This procedure guarantees that the coupling site does not cover the protein's dynamic site, the activity of the catalyst is just influenced by fixed nature. However, the resoluteness of the covalent bonds blocks the self-recuperating properties displayed by chemo-adsorbed self-gathered monolayers. Utilization of a spacer atom like poly(ethylene glycol) serves to decrease the steric deterrent by the substrate.

2.4.1.2. Entrapment

In this strategy the catalysts can be immobilized by entrapment in sol-gel frameworks shaped by hydrolytic polymerization of metal alkoxides. Immobilization in silica sol gels arranged by hydrolytic polymerization of tetraethoxysilane, in the vicinity of the chemical, was initially utilized by Avnir and collaborators (Braun S, et. al., 1990) also, has been utilized for the immobilization of a tremendous scope of compounds (Avnir D,

et. al., 1994). It must be demonstrated out that the morphologies of the silica sol-gels rely on upon the system for drying.

2.4.1.3. Carrier-Free Immobilization by Cross-Linking

In the mid 1960s, investigations of strong stage protein science prompted the revelation that cross-linkage of broke up catalysts by means of response of surface NH_2 bunches with a two way useful substance cross-linker,e.g. glutaraldehyde, managed insoluble cross-connected compounds (CLEs) that hold catalytic activity (Quiocho FA, Richards FM. 1966). However, this system for delivering cross-linked catalysts (CLEs) had a few downsides, for example, low action maintainance, poor reproducibility, low mechanical strength, and troubles in taking care of the thick CLEs. Mechanical strength and simple taking care of could be enhanced by cross-linkage of the compound in a gel framework or on a bearer yet this prompts the disadvantageous weakening of action specified previously. Accordingly, in the late 1960s, laid accentuation to bearer bound compounds, which turned into the most generally utilized mechanical technique for catalyst immobilization for the following thirty years.

2.4.1.4. Smart Polymers

The most recent systems to immobilization of compounds is by means of covalent connection to boost responsive or 'smart polymers' which experience vast conformational changes in light of little changes in their surroundings, e.g., temperature, pH and ionic quality (Galaev IY, et. al., 1999)

2.4.1.5. Adsorption (Support binding)

Adsorption is the simplest technique of immobilization but weak bonding is present in adsorption as compared to other techniques. Immobilization of phytase on bentonite did not need very strong binding forces, moreover, adsorption is simple and economical technique from industrial point of view, therefore, in this work adsorption was chosen for immobilization of phytase on bentonite.

In adsorption the protein holds fast to the external surface of a non-responsive or latent network. This strategy experiences the connection of a chemical by surface-tying on alginate beads, framework or glass (Fan L et al., 2009).

Support binding can be ionic, covalent or physical (such as van der Waals and hydrophobic interactions) in nature. Physical bonding is usually so weak to strongly fix the enzyme with the carrier in industrial conditions i.e. in the presence of elevated concentrations of product and reactants as well as elevated ionic strength. Ionic binding is usually stronger than physical bonding and covalent binding is even stronger than ionic binding, which is advantageous because the enzyme can't be leached from surface of the support material. But, there is also a disadvantage: in case of enzyme's irreversible deactivation both the support (which is often costly) and the enzyme become unusable.

The supports are usually of two types.

i. Synthetic resin.
ii. inorganic polymer or a biopolymer like mesoporous silica or zeolite. (Sheldon RA. 2007)

2.4.1.5.1. Synthetic Organic Polymers

i. Acrylic resins

Eupergit=C is a sort of acrylic tar extensively utilized as bolster material for immobilization. It is a macroporous copolymer of N,N'- methylene-bi-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide with normal molecule size of 170 mm and a pore measurement of 25 nm (Katchalski-Katzir E, et. al., 2000).

It is stable and highly hydrophilic, both mechanically and chemically, in a wide pH range from 0 to 14, and it does not shrink or swell even in case of significant pH changes within this range. It binds at alkaline or neutral pH as a result of reaction of it's attached oxirane groups with proteins, covalent bonds are formed as a result of this reaction which provide

stability within a wide pH range, pH 1-12 (Kallenberg AI, van Rantwijk F, et. al., 2005, Katchalski-Katzir E, et al., 2000)

So also, different permeable acrylic gums, for example, Amberlite XAD-7, are utilized to immobilize compounds by means of straight forward adsorption without covalent connection. For instance, the broadly utilized compound C. Antarctica lipase B (CaLB) (Kirk O, Christensen MW, 2002) is industrially accessible in immobilized frame as Novozym 435 which comprises of the catalyst adsorbed on a macroporous acrylic tar. A disservice of immobilization along these lines is that, in light of the fact that it is not covalently bound, the chemical can be drained from the backing in a watery medium (Petkar M, et. al., 2006).

ii. Biopolymers

Distinctive sorts of biopolymers, basically polysaccharides which are water-insoluble like cellulose, agarose, starch and chitosan (Krajewska B. 2004) what's more, proteins like egg whites and gelatin have been widely utilized as bolster materials for chemical immobilization. The primary modern utilization of an immobilized compound in a biotransformation is the Tanabe procedure was the first mechanical use of an immobilized catalyst in a biotransformation (Chibata I, et. al., 1992) first time marketed before over 40 years and I-amino acids was delivered by racemic acylamoni corrosive determination utilizing aminoacylase of *Aspergillus oryzae*. DEAE-Sephadex comprising of cellulose adjusted by diethylaminoethyl functionalities was utilized for immobilization of chemical utilizing technique for ionic adsorption and this procedure was done in a settled bed reactor in persistent operation. This technique is still generally utilized, e.g., in the immobilization of a recombinant epoxide hydrolase from *Aspergillus niger* (Karboune S, et. al., 2005).

iii. Hydrogels

Immobilization of enzyme is also carried out using natural or synthetic hydrogels or cryogels e.g. Polyvinyl alcohol (PVA) cryogels. These (PVA) cryogels are formed using freeze-thawing method.

Hydrogels are usually defined as three-dimensional network of polymers which when come in contact with water or any other aqueous solution, swell several hundred times as compared to dry network mass. Hydrogels have different characteristics e.g. they are insoluble in water, hydrophilic, elastic, soft and swell with water. They swell in a way that they maintain there shape but increase in size until physical and chemical equilibrium is reached. These properties of hydrogels depend upon different factors e.g. chemical composition of the polymer chains, interactions of liquid present around and network, degree of crosslinking. Hydrogels have different applications e.g. they are used for membrane preparation in purification and separation of water, contact lenses, catheters also in food industry, biotechnology, agriculture and medicine (Rosiak JM, Olejniczak J, 1993, Lin SH, 1993). Different hydrophilic groups are present in hydrogels, for example, carboxylic acids, amides and alcohol etc. these functional groups are responsible for hydrophilicity of hydrogels. As per the kind of the useful gathering the properties of hydrogels can be changed with pH and temperature [3±5]. Hydrogels may be arranged by treating the watery arrangement of hydrophilic polymers and monomers with c-beams, warmth and synthetic initiators. Among these strategies, c-beam actuated concurrent polymerization and crosslinking has favorable circumstances over the others. This system is by and large termed a "clean procedure", not obliging any additional chemicals and abandoning some undesirable buildups. It can be connected at any temperature and measurement rate. The expulsion of dyestus from material waste waters is a genuine natural issue which is troublesome by traditional system (Lin SH, 1993).

2.4.1.5.2 Inorganic Supports

Distinctive number of inorganic solids can be utilized for the immobilization of catalysts, similar to alumina, silica (Petri A, et.al., 2005) zeolites (Diaz JF, et.al., 1996, Yan X, et.al., 2002) and mesoporous silicas (Moelans D, et.al., 2005, Takahashi H, et.al., 2001, Wang P, et.al., 2001, Borole A, et.al., 2004) for example, MCM-41, and SBA-15. One of the least demanding and best strategies to immobilize a compound is by silica granulation (Kirk O, Christensen MW. 2002) It is utilized, for instance, to figure chemicals for cleanser powders which discharge the protein into the washing fluid during washing. Granulation innovation was utilized to immobilize CaLB lipase on silica granules, by

first adsorbing the lipase on silica powder took after by agglomeration (Kirk O, Christensen MW. 2002). Recent developments in immobilization have also taken place, few of them are discussed here.

2.5. Recent Developments

2.5.1. Combi-CLEAs and Catalytic Cascade Processes

The most recent system in ecological and financial effectiveness is a mix of iota productive, synergist, ventures into an one-pot, reactant course transform without the requirement for division of diverse steps (Bruggink A, et. al., 2003). Catalytic cascade processes have various potential points of interest: more quick witted unit operations, less reactor volume, and higher volumetric out put efficiencies, shorter process durations and less waste era. Moreover, by coupling steps together unfavorable balance can be driven towards item. On a basic level, this can be accomplished by co-precipitation and cross-connecting of two or more proteins in combi CLEAs. Case in point, combi CLEAs have been readied from catalase in mix with glucose oxidase or galactose oxidase. The response serves to catalyze the quick debasement of the hydrogen peroxide framed in the oxygen consuming oxidation of glucose and galactose, separately, catalyzed by these enzymes..

2.5.2. Enzyme-Immobilized Micro Channel Reactors for Process Intensification

Small scale reactor innovation is an interdisciplinary field that has picked up prominence recently (P. Watts, et. al., 2005). The substance response improvement through the utilization of microchannel reactors (small scale fluidic gadgets) has numerous points of interest as contrasted and customary group process advancements, for example, quick mass and warmth exchange and huge surface territory to volume proportions. These are famous elements for leading synergist responses in smaller scale reactors containing the chemical immobilized on their internal walls. Maeda and collaborators (Honda T, et. al., 2006, Miyazaki M, Maeda H. 2006, T. Honda, et. al., 2005) built up a smaller scale reactor in which compounds are immobilized as a enzyme polymer film on the inward dividers of the micro scale channels (Honda T, et. al., 2005).

2.5.3. Immobilization with Nanofibrous polymers, Nanoparticles and Nanoporous gold (nanobiocatalysis)

Presently a days, nanobiocatalytic methodologies have developed as simple,efficient, and dependable strategy for the immobilization of enzymes (Chigome S, et. al., 2011). In addition, the utilization of nanoparticles as immobilization backing is accomplishing an expanding significance, as the nanoparticles versatility increments (Garcia-Galan C, et. al., 2011). Poly(lactic-co-glycolic acid) (PLGA) is anamorphous, nontoxic, biocompatible manufactured polymer that has been generally utilized as a backing for chemical immobilization as system for giving better reactant steadiness, consistent operation, and simple impetus reusing. This methodology could prompt an extraordinary diminishment of operational expenses for different business forms (Lee JW, et.al 2006). Contrasted and other nano organized backings (e.g., nanotubes, nanoparticles, and mesoporous silica), nanofibrous polymers have numerous focal points including their natural high particular surface range, entomb fiber porosity, a low deterrent for mass exchange, simple taking care of, and great mechanical quality (Asghar M, et.al., 2014). However, restricted studies have been accounted for on the immobilization of ligninolytic on nanofibrous polymers (Ignatova MG, et .al., 2010). Immobilization of enzymes on permeable inorganic materials like nanoporous gold (NPG) is additionally essential for biocatalysis and biotransformation (Qiu H, et.al., 2009). Enzyme immobilization on to chitosan covered attractive nanoparticles (Fe_3O_4-CS) by adsorption or covalent tying in the wake of actuating the hydroxyl gatherings of chitosan with carbodiimide, methanediimine, or a cyanuric chloride has been accounted for to upgrade their reusability (Kalkan NA, et. al., 2012).

In past different support materials like silica, glass and sand etc. have been used for adsorption. In this piece of research a novel support material i.e. Bentonite is used for adsorption of enzyme. Bentonite is discussed briefly here.

2.6. Bentonite

Deposits of Benton Shale near Rock River, Wyoming were named 'bentonite' by Wilbur C. Knight in 1898. Bentonite is originally found as 'mineral soap' or 'soap clay'.

Bentonite clay is made up of aged and weathered volcanic ash. Wyoming and Montana have most active and largest deposits of bentonite clay. Bentonite includes any natural material dominantly composed of clay minerals in the smectite group (Hosterman JW, Patterson SH, 1992).

Bentonite clay contains a high quantity of montmorillonite (a clay mineral) which is formed as a result of decomposition of ash of volcano. Bentonite has high plasticity, it is highly water absorbent and has high shrinkage and swelling characteristics.

2.6.1. Types of Bentonite

There ae basically 2 types of bentonite.

i. Sodium Bentonite

Sodium bentonite, also known as Wyoming bentonite is highly swelling clay.

ii. Calcium Bentonite

Calcium bentonite is a low or non-swelling variety.

2.6.2. Efficacy of Bentonite as Aflatoxin binders in Poultry

(Miazzo R et al., 2005) incorporated 0.3 % sodium bentonite in diets containing 2500 µg AfB1/kg fed to chickens for fattening from day 23 to day 52. According to (Miazzo R et al., 2005) sodium bentonite sufficiently decreases the inhibitory effects of AfB1 present in the poultry diet. When sodium bentonite was added to the diet, the weight gain of observed organs like liver, spleen, kidney was found to be less than the weight gained when AfB1 was fed alone.

Sodium bentonite 0.5% found to decrease serum phosphorus. Adverse effects associated with aflatoxicosis were decreased by 0.5 and/or 1 % bentonite in following studies: (Pasha TN, et al., 2007) (100 µg Af/kg) (Keçeci T, et al., 1998) (2500 µg/kg, 83% AfB1), (Desheng Q, et al., 2005) (200 µg AfB1/kg) and (Phillips TD, et al., 1988) (7500 µg Af/kg). In a five-week study on chickens for fattening, (Manafi M, et al., 2009) could show a reduction of the adverse effects of AfB1 (500 µg/kg; significant reduction of body weight, feed consumption and antibody titres against Newcastle disease (ND) and Infectious Bursal Disease (IBD); increases in the relative liver weight) by 1 % bentonite in the diet. The adverse effects of aflatoxin (254 µg Af = 200 µg AfB1/kg diet) on antibody production against IBD were reduced by 0.5 % bentonite in the diet of chickens (254 µg Af = 200 µg AfB1/kg diet; (Ghahri H, et al., 2009), on antibody production against ND by up to 0.6 % bentonite in the diet (2500 µg AF = 81 % AfB1/kg diet; (Ibrahim IK, et al., 2000).

(Eraslan G, et al., 2005) watched that aflatoxin diminished levels of calcium and phosphate in chicken's blood, which was less stamped when bentonite at 0.25 or 0.5 % was added to the eating regimen. Usage of supplements, discouraged if there should be an occurrence of aflatoxicosis, was enhanced by bentonite at consideration rates of 0.15 and 0.3 % in the eating regimen (Chaturvedi VB, Singh KS. 2004).

Zootechnical parameters of chickens for fattening (day 30 to day 52) supressed by aflatoxin (5000 µg/kg) were restored in an average amount by inclusion of 0.3 % sodium bentonite. However, as liver steatosis associated by Aflatoxin did not come back to normal, therefore it was finally concluded by the author that the sodium bentonite showed no positive effect. In another study by (Shi Y, et al., 2009), 0.3 % montmorillonite demonstrated no assurance against aflatoxin instigated (110 µg AfB1/kg) depression of weight pick up and food to pick up proportion and increment in the weights of liver, kidney, spleen and pancreas; be that as it may, a nanocomposite of montmorillonite at the same consideration rate was successful.

Inositol phosphates makes a major portion of the endogenous P present in the soil in organic form in a big proportion (Richardson AE, et al., 2005). Extracellular phytases

undergo the mineralization of inositol phosphates and bioavailability of inositol phosphates depends on this mineralization. Interaction between inositol phosphates and phytases in soil is not clearly defined yet.

2.7. Factors affecting the stability and performance of extracellular enzyme in Bentonite

Stability and performance of extracellular enzymes depends upon these factors.

(i) Microbially mediated and proteinase degradation.

(ii) Total or partial deactivation of enzyme due to adsorption on bentonite particles.

(iii) Deactivation by interaction between enzyme polyvalent annions, microbial metabolites and metal ions (Quiquampoix H, Mousain D. 2005).

The effect of these factors on phytase activity varies with soil environment characters, biochemical properties and source of the distinct phytase.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Study Area

The current research work was conducted at the Microbiology Research Laboratory of (MRL), (Department of Microbiology), Quaid-i-Azam University Islamabad during the session of 2014 to 2015.

3.2. Chemicals

All chemicals used for research purpose were obtained from Sigma (USA), Merck (Germany), Acros (Belgium), Fluka (Switzerland), BHD (UK), others were bought from local market.

3.3. Screening of Bacterial Isolates for Phytase Production

Bacterial isolate i.e. *Bacillus licheniformis* was collected from the stock culture of MRL, Department of Microbiology, Quaid-i-Azam University, Islamabad. Same bacterial isolate was previously used for protease production. The bacterial isolates were streaked in the form of slants and stored at 4°C in a refrigerator.

Production of crude enzyme was basically dependent upon 2 steps, discussed below.

3.4.1. Substrate Preparation

Indigenous carbon source i.e. wheat bran was used for biosynthesis of phytase enzyme. Wheat bran was pretreated and then added to production media. The 100g of wheat bran was added to 1000ml of water and was boiled for 1 hour, for obtaining 100ml filtrate. After boiling the filtrate was separated with the help of porous cloth. The wheat bran was obtained from the local market. The crude enzyme production involves two steps.

3.4.1.1. Phytase Fermentation Medium

The fermentation medium used is shown in table given below.

Table 3.1. Composition of production media

Components	Concentration
Ammonium sulphate	0.04g/L
Magnesium sulphate	0.02g/L
Casein	1g/L
Calcium chloride	2g/L
Wheat bran	100g/L
Distilled water	1000ml

3.4.2. Inoculum Preparation

For preparation of 150ml of inoculum, nutrient broth (150 ml) was prepared in 500ml Erlenmeyer flask and was autoclaved at 15 psi pressure and 121°C for 15 to 20 minutes. Dextrose was added to nutrient broth as an additional carbon source for proper growth of strain. The medium was further tested for sterility by keeping it on shelf at room temperature, overnight. Next day, a loop full of respective bacterial strain was transferred to sterile dextrose supplemented medium and was incubated at 50°C and 150 rpm for 24 hours. The composition of inoculum preparation media for 100 ml is given below. Further the phytase production medium was prepared and autoclaved.

Table 3.2. Composition of media for inoculums preparation.

Ingredients	Concentration
Nutrient Broth	1.3g
Dextrose	2g
Distilled water	100ml

3.4.3. Shake flask Fermentation for Phytase Production

The inoculums was added to fermentation medium present in Erlenmeyer flask and was allowed to ferment at optimum temperature, pH and incubation time period. Optimization of these parameters is given under heading number 3.10.

3.5. Assay to Quantitate Phytase Activity

It is a procedure which confirms the production of an enzyme. Specific enzyme act on specific substrate and convert them to different smaller peptide molecules which are named as products and by products. According to Molybdate-Blue Method for determination of phytase activity, substrate sodium phytate was dissolved in sodium acetate buffer and 0.8ml of substrate was added to test tube then 0.2ml of enzyme was added to same tube and incubated for 10 minutes at 50°C. After 10 minutes incubation at 50°C 1ml of TCA (5%) was added which acts as a stop solution. After adding TCA 1ml of color reagent was added and mixed. The change in color detected the presence of phytase activity. Further the sample was centrifuged for 10 minutes at 4,000 rpm and absorbance was taken at 700nm with the spectrophotometer. This process was carried out in duplicates and triplicats.

3.5.1. Unit of Enzyme

One unit of enzyme activity can be defined as the amount of enzyme required to release 1 mg of inorganic orthophosphate in 10 minutes at 50°C, pH 5 under standard assay conditions. The general scheme of opted method is as follows;

Table 3.3. General scheme of the enzyme assay for alkaline phytase.

Components	Enzyme Control (ml)	Substrate Control (ml)	Assay (ml)
Distilled water	0.8	0.2	0
Substrate	0	0.8	0.8
Enzyme	0.2	0	0.2

The assay was performed and attained values were compared with the standard curve of myo-inositolphosphate. Further the different enzyme units were calculated by following the formula given below:

$$\text{Units/ml} = \frac{A \times D.F \times V.A}{T \times V.E.A \times S.G \times 0.18}$$

Where,

A = Absorbance (700nm) of sample.

D.F = Dilution factor

V.A = Volume of assay

T = Time of incubation

V.E.A = Volume of enzyme used in assay

S.G = Slope of graph

3.5.2. Characterization of Assay for Quantitative Estimation of Phytase

Assay used for the quantitative estimation of phytase as mentioned above was optimized for quantity of enzyme, quantity of substrate and time of incubation of assay.

3.5.2.1. Effect of different ratio of enzyme and substrate on activity of enzyme

Effect of enzyme and substrate quantity on assay efficiency was determined with varying quantities of enzyme and substrate used. Water was added instead of enzyme and substrate in case of enzyme blank and substrate blank, respectively. The following table depicts the whole experiment. Incubation time was maintained constant.

Table 3.4. Scheme for optimization of enzyme substrate ratio for quantitative assay.

Enzyme quantity (µl)	Substrate quantity (µl)	Water quantity (µl)
200	800	–
200	–	800
–	800	200
400	600	–
400	–	600
–	600	400
600	400	–
600	–	400
–	400	600

3.5.2.2. Effect of different Incubation times of assay on activity of enzyme

Effect of different incubation time on assay efficiency was studied by varying incubation time of assay, quantity of enzyme and substrate was maintained constant. The table 3.5 depicts the experiment.

Table 3.5. Scheme for optimization of incubation time of quantitative assay.

Incubation time (min)	Enzyme quantity (µl)	Substrate quantity (µl)	Water quantity (µl)
5 min	200	800	–
	200	–	800
	–	800	200
10 min	200	800	–
	200	–	800
	–	800	200
15 min	200	800	–
	200	–	800
	–	800	200
20 min	200	800	–
	200	–	800
	–	800	200
25 min	200	800	–
	200	–	800
	–	800	200
30 min	200	800	–
	200	–	800
	–	800	200

3.6. Standard curve for Phosphorous

3.6.1. Reagents

- Distilled water, Buffer solution include
- Sodium acetate 5.74g
- TritonX-100 0.5g
- Bovine serum albumin 0.5g, were mixed in 900ml of distilled water then pH 5 was adjusted with acetic acid and diluted till 1 liter.

Substrate solution

Sodium phytate (0.577g) and Sodium acetate (0.574g in 90 ml of distilled water). pH was adjusted with acetic acid and diluted to 100ml.

Reaction Stop solution

The 5g of TCA was added in 100 ml of distilled water. Stop solution was added 1ml per sample.

Color mix

Ammonium Heptamolybedate stock solution was prepared as sol A (7.5g in 400 ml of distilled water) then 98 percent sulphuric acid (22 ml) was added slowly and further diluted to 500 ml. This can be kept for one month but at 4°C shielded from light. Ferrous sulphate stock solution was prepared as sol B. The 2.7g of $FeSO_4$ were added to100 ml of distilled water can also be kept at 4°C for one month. Sol A (100ml), Sol B (25ml) were mixed to make color mix. Color mix should be prepared fresh and was added 1ml per sample.

Stock Solution

Potassium di-hydrogen Phosphate (stock solution). Freshly prepared 0.4mM solution of potassium dihydrogen phosphate was used to prepare standard phosphorus curve.

3.6.2. Procedure

For standard curve of phosphorous first of all stock solution of Potassium dihydrogen Phosphate was prepared using buffer solutions. By mixing distilled water with the stock solution various dilutions of about 1ml were prepared as given in table 3.6. it was followed by addition of 1ml of 5% stop solution (TCA) to all tubes. After adding stop solution, 1ml of color mix was added to all test tubes. Centrifuged the content of each tube at 4,000 rpm foe 10 minutes and absorbance was measured using spectrophometer at 700nm.

$Y=1.187X+0.043$

$R^2=0.961$

$X=Y+0.043/1.187$

X is the μg of sample in an unknown sample.

Y is the optical density of sample at 700nm.

Table 3.6. Phosphorous standard curve.

Stock Solution (ml)	**Distilled water (ml)**	**Final concentration (mg/L)**
0.1	0.9	10
0.2	0.8	20
0.3	0.7	30
0.4	0.6	40
0.5	0.5	50
0.6	0.4	60
0.7	0.3	70
0.8	0.2	80
0.9	0.1	90

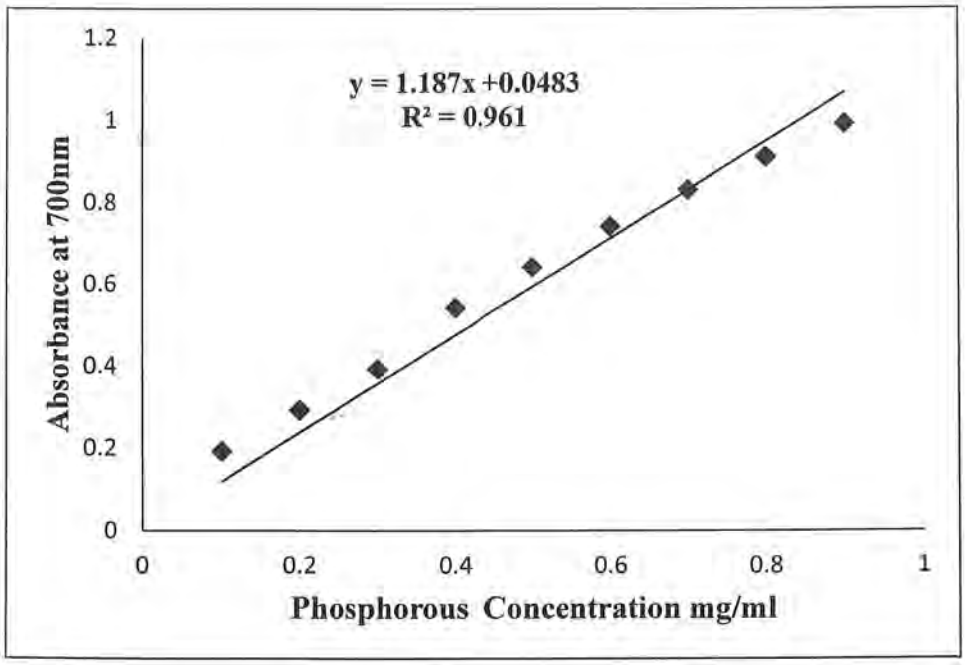

Fig.3.1. Phosphorous standard curve.

3.7. Total protein Estimation

The amount of protein was estimated by Lowr'y method (Lowry OH, et al., 1951). According to this method three different reagents were used for quantitative analysis. BSA was used as a standard.

3.7.1. Required Reagents

i. Test Sample
ii. Analytical reagents

Reagent A

Composition of Lowry reagent A and solution B

Components	Quantity
Na_2CO_3	10g
NaOH	2g
Distilled water	500ml

Reagent B

Components	Quantity
$CuSO_4.5H_2O$	0.05g
K Na tartrate	0.15g
Distilled water	10ml

Reagent C (fresh; 1ml per sample)

Solution A and solution B were mixed in the ratio 50:1 or 100:2, for preparing Lowry solution C. Solution C should be prepared fresh.

Reagent D or Folin Reagent (instant fresh; 0.1ml per sample)

This reagent was prepared in the dilution ratio of 1:1 in distilled water, resulting in 1N folin reagent. The 10ml of Folin and Ciocalteu's Phenol reagent was added in 10ml of distilled water and as a result 20ml of 1N Folin reagent was obtained.

3.7.2. Procedure

Added 900 microlitre of distilled water in the test tube and then 100 microlitre of supernatant was added in the test tube but in control 100 µl of distilled water was added instead of supernatant. Then 1ml of freshly prepared solution C was added to each test tube and incubated for 10 minutes at room temperature. After 10 minutes incubation 100 microlitre of solution D was added to each test tube mixed properly and immediately,

incubated for 30 minutes at room temperature in dark. After 30 minutes incubation blue color appeared in test tubes containing supernatant but no color appeared in control, the optical density was measured at 650nm.

3.8. Standard curve for Bovine Serum Albumin

i. 0.1g of BSA was weighed and was added gradually to 100ml round bottom flask containing 50ml distilled water.
ii. It was stirred gently and after whole amount of BSA was added and dissolved, volume was adjusted to 100ml with distilled water.
iii. Hence final concentration of the stock was 100mg BSA/100ml or 1mg BSA/ml.

3.8.1. Procedure

Table 3.7. BSA standard curve.

Volume of distilled water (ml)	Volume of BSA stock (ml)	Final Concentration (mg/L)
1	0.0	0
0.9	0.1	10
0.8	0.2	20
0.7	0.3	30
0.6	0.4	40
0.5	0.5	50
0.4	0.6	60
0.3	0.7	70
0.2	0.8	80
0.1	0.9	90
0.0	1	100

After preparing dilutions of BSA, 1ml of freshly prepared Lowry Solution C was added to each test tube sample and each sample was incubated for 10 minutes at 37°C. After 10 minutes incubation at 37°C, 0.1ml of freshly prepared Folin reagent was added to each

test tube sample and was incubated for 30 minutes at 37°C. After 30 minutes incubation at 37°C, absorbance was taken for each sample in 1.5ml glass cuvette at 650nm.

$Y=3.462X+0.026$

$R^2=0.981$

'X' is the concentration of protein of unknown protein.

'Y' is the optical density of the sample at 650 nm.

In order to calculate the protein mg/ml the following formula was used:

$$\text{mg of protein/ml} = \frac{\text{Absorbance} \times \text{Dilution Factor}}{\text{Slope of the graph}}$$

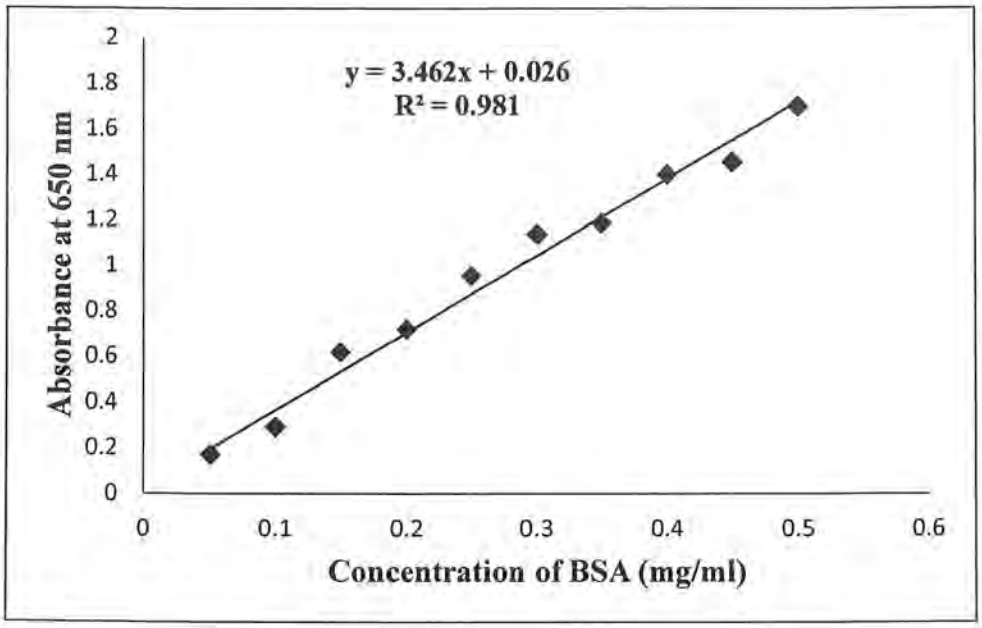

Fig.3.2. BSA standard curve.

3.9. Specific Activity Estimation

The specific activity was calculated by the given formula:

$$\text{Specific activity} = \frac{\text{Enzyme units/ml}}{\text{mg of protein/ml}}$$

3.10. Optimization of Phytase Production from *Bacillus licheniformis* using Shaken flask Fermentation

Phytase was produced by *Bacillus licheniformis* using shake flask method. Phytase production was optimized by optimizing three important parameters i.e. incubation time, temperature and pH. Method followed for observing effect of these parameters on phytase production from *Bacillus licheniformis* are further discussed.

3.10.1. Effect of change in incubation time on phytase production

150ml sterilized production media was taken in 500ml glass flask and was inculated with 3ml inoculum. This flask was put at 50°C in shaker for 8 days. The 2ml of inoculated production media was taken from this flask under sterilized conditions after every 24 hours. The sample was further centrifuged and supernatant was assayed for estimation of protein produced and enzyme activity.

3.10.2. Effect of change in pH on phytase production

Effect of pH on enzyme production was studied at pH 5, 6, 7, 7.5, 8 and 9. All these pH values were maintained in separate glass flask and these flasks were put into shaker incubator at 50°C for 5 days. All parameters other than pH were maintained constant. After 5 days enzyme was extracted via centrifugation and samples were assayed for enzyme activity and protein estimation.

3.10.3. Effect of change in temperature on phytase production

Effect of temperature on phytase production was observed at 25°C, 30°C, 37°C, 45°C, 50°C, 55°C. Equal amount of production media was put in different flasks. After inoculation each flask was put in shaker incubator at different temperature for 5 days. All parameters other than temperature were kept constant. After 5 days fermentation in

shaking incubator enzyme was extracted and each sample was assayed for enzyme activity and protein estimation.

3.11. Batch Fermentation

After optimization of production parameters, phytase was produced in larger amount by shake flask fermentation method. Two 5000 ml Erlenmeyer flasks were filled with 2500ml production media each then both flasks were inoculated with fresh inoculums and were put into shaking incubator at 50°C and 150 rpm for 5 days. pH of production media was maintained 8.

3.12. Down Stream Processes

Downstream processing comprises of following steps.

3.12.1. Enzyme Extraction

The required product which is extracellular alkaline phytase was produced in fermentation flask. This enzyme was extracted by centrifugation. It is known from previous knowledge that centrifugation machine works on the principle of sedimentation. The centrifugation was carried out at the speed of 6000 rpm for 20 minutes at 4°C. After centrifugation biomass was separated and settled at the base of centrifugation tubes as pellet. Supernatant containg desired enzyme was carefully poured into another tube while biomass pellet was discarded.

3.12.2. Acetone Precipitation

Alkaline phytase was precipitated out by adding an organic acid known as acetone. Four volumes of chilled acetone were added to filtrate and incubated for 1 hour at -20°C. After 1 hour incubation at -20°C precipitates were separated by centrifugation at 13000-14000 rpm for 15 minutes. After centrifugation enzyme was separated as a pellet at the bottom of the centrifugation tubes. The supernatant containing acetone was separated carefully. The centrifugation tubes containing pellets were placed open at room temperature for 30

minutes in order to evaporate remaining acetone from the pellets. After evaporating acetone pellets were further dissolved in 0.02M sodium acetate buffer of pH 5 for evaluating the presence of required enzyme. The precipitation was carried out in acetone resistant tubes and pellets dissolved in buffer were stored at -20°C.

3.12.3. Pelleting

The precipitated enzyme was further transferred to the centrifuge tubes and centrifuged at the speed of 6000 rpm for 20 minutes. The pellets were transferred to the buffer an protein estimation was performed along with the enzyme estimation. The enzyme quantification of supernatant was also performed. Further the pellets were stored at -20°C.

3.13. Characterization of Partially Purified Free Enzyme

For characterization effect of temperature and pH on activity of partially purified enzyme was studied.

3.13.1. Effect of temperature on activity of free enzyme

The protein pellets were obtained, each 0.2g of pellet was dissolved in 5ml of 0.02 molar sodium acetate buffer and kept at 4°C for 24 hours. The effect of temperature was observed while keeping all other factors constant. Only the temperature range was 20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C. the protein estimation and enzyme assay were performed for total protein and enzyme activity determination.

3.13.2. Effect of pH on proteolytic activity of free enzyme

The pH factor was studied while keeping other factors as constant such as molarity and temperature. The protein pellets were obtained by precipitating several 2mls of filtrate separately. The 0.2g of pellet was added to each 5ml of buffers present in separate test tubes. The range of pH under study was 2, 3, 4, 5, 6, 7, 8, and 9. pH 2, 3, 4, 5, 6 were studied with sodium acetate buffer and pH 7, 8 and 9 were studied with tris-

hydroxymethyl aminomethane/HCl buffer as these buffers are stable only at these pH values. The enzyme assay and protein estimation were performed in order to calculate total protein and enzyme activity, respectively.

3.14. Immobilization of Alkaline Phytase

Two different techniques of immobilization were followed to find out the most cheap and effective method for immobilization onto bentonite. First method is a simple adsorption technique in which the precipitated enzyme pellets were suspended in the appropriate buffer under maintained specific conditions such as pH, molarity, temperature and buffer type. Further the enzyme preparation was spread over untreated bentonite in 1:1 proportion. In this technique each factor was studied by keeping other factors constant and the combined effect was also studied.

In the second technique bentonite is treated before adsorption. It is treated with 1% sulphuric acid and washed several times, properly. The treated oven was dried in oven after washing with distilled deionized water. The 0.2g of weighed pellet of alkaline phytase was added to 5ml of 0.1M tris-HCl buffer of pH 8. The preparation was added to 5gm of dried treated bentonite. The preparation was incubated at 4°C for 30 minutes. Added 8ml of cold acetone and incubated for 1 hour keeping temperature 4°C. About 15ml of cold acetone was added for 30 minutes washed. The preparation was centrifuged and washed several times to remove excessive acetone along with the unbound protein. The remained pellets were suspended in buffer and enzyme activity and protein content was measured.

3.14.1. Immobilization without Optimized Conditions

The 0.2gm of alkaline phytase pellets were added to 5ml of distilled water and palced in fridge at 4°C overnight. After 24 hours poured preparation to 5gm of Bentonite and placed at room temperature under fan for drying. After drying the product was crushed slightly with the help of mortar and pestle. Performed protein assay and enzyme activity assay.

3.14.2. Characterization of Immobilization of Phytase on Bentonite

Two parameters were studied for immobilization of phytase on bentonite

3.14.2.1. Effect of different incubation time on immobilization

The 0.2g of pellet was dissolved in each 5ml of 0.1M tris-(hydroxymethyl)aminomethane/HCl buffer in separate test tubes. These test tubes were incubated at 4°C overnight. After 24 hours incubation poured preparation to 5gm of bentonite. Each preparation was incubated for different time period at room temperature i.e; one was placed under fan for drying and other was placed on a shelf overnight for drying. After drying the products were crushed slightly with the help of mortar and pestle. Performed protein estimation and enzyme activity assay.

3.14.2.2. Effect of pH on immobilization

Effect of pH on immobilization of phytase on bentonite was studied at pH 5, 7, 8 and 9. 0.2g of enzyme pellet was dissolved in 5ml of 0.1M buffer of different pH in separate test tubes. These test tubes were incubated at 4°C. After 24 hours incubation poured preparation to 5gm of bentonite. Each preparation was fan dried. After drying the products were slightly crushed with the help of pestle and mortar. Each sample was assayed for enzyme activity and protein estimation. pH 5 was maintained using sodium acetate buffer of pH 5 and for pH 7-9 tris-(hydroxymethyl)aminomethane/HCl buffer was used. All factors other than pH were kept constant.

3.15. Characterization of Immobilized Enzyme

For characterization of an immobilized enzyme, two parameters i.e. temperature and pH were studied.

3.15.1. Effect of temperature on immobilized enzyme

5g of product of immobilization was dissolved in 5ml of 0.1 molar sodium acetate buffer of pH 6 and kept at 4°C for 24 hours. The effect of temperature was observed while

keeping all other factors constant. Only the temperature range was 20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C. Enzyme assay and protein estimation were performed.

3.15.2. Effect of pH on immobilized enzyme

The pH factor was studied while keeping other factors as constant such as molarity and temperature. The 5g of product were added to each 5ml of 0.1M stable buffers present in separate test tubes and incubated at 4°C . The range of pH under study was 2, 3, 4, 5, 6, 7, 8, and 9. pH 2, 3, 4, 5, 6 were studied with sodium acetate buffer and pH 7, 8 and 9 were studied with tris-(hydroxymethyl) aminomethane/HCl buffer as these buffers are stable only at these pH values. Protein estimation and enzyme assay were performed.

3.16. Desorption of Enzyme to Estimate Adsorption

The immobilized dried product was suspended in buffer for 10 mins and centrifuged at 6000 rpm. The process was repeated until most of the protein is being separated from the dried product. Each wash was estimated and the amount of the total protein and enzyme activity quantified. Almost the product was washed four times and at the end the product was also assayed for the estimation of the protein and enzyme activity.

RESULTS

4. RESULTS

The sample of *Bacillus licheniformis* under observation was obtained from the culture bank of Quaid-e-Azam University, Islamabad.

4.1. Characterization of Assay for Quantitative Estimation of Phytase

4.1.1. Effect of different ratio of enzyme and substrate on activity of enzyme

Phyatse assay was optimized for enzyme and substrate ratio. Enzyme and substrate ratio was varied step wise and enzyme activity, protein quantity and specific activity were calculated. Results are given below in tabular form.

Table.4.1. Describes results for effect of different ratio of enzyme and substrate on activity of enzyme.

Enzyme (µl)	Substrate (µl)	Enzyme activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Standard deviation
200	800	8.093677	0.404968	19.98596	0.070711
400	600	8.673302	0.404968	21.41724	0.000707
600	400	9.867681	0.404968	24.36656	0.000707

Ratio of 200 µl enzyme and 800 µl substrate was selected because it was most economical option as least amount of enzyme is degrading most of the substrate and there was no significant decline in activity of enzyme at this ratio as compared to other ratios.

4.1.2. Effect of different Incubation times of assay on activity of enzyme

Substrate and enzyme were incubated for different time period. The time range followed was 5-30 minutes, with interval of 5 minutes. Results are depicted in table given below.

Table 4.2. Describes results for effect of different incubation times of assay on activity of enzyme.

Incubation time (minutes)	Enzyme activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Standard deviation
5	9.90281	0.404968	24.4533	0.000707
10	10.7459	0.404968	26.53517	0.009192
15	11.76464	0.404968	29.05077	0.003536
20	11.17096	0.404968	27.58478	0.001414
25	10.90749	0.404968	26.9342	0.000707
30	10.78806	0.404968	26.63927	0.00495

Enzyme was stable almost from 5 to 30 minutes. Maximum specific activity was found at 15 minutes. After 15 minutes specific activity started decreasing gradually but no significant decline was observed. This means activity would be calculated lesser at 30 minutes interval as compared to actual activity. Although activity was maximum at 15 minutes but 10 minutes incubation was considered ideal as there was no significant decrease at 10 minutes as compared to 15 minutes.

4.2. Optimization of Phytase Production from *Bacillus licheniformis* using Shaken Flask Fermentation

4.2.1. Effect of change in incubation time on enzyme production

The *Bacillus licheniformis* was inoculated to growth medium. After 24 hours the medium was transferred to production medium which was placed in incubator for 8 days at 50°C. 2ml of production medium was taken from the flask everyday under sterilized conditions. After centrifugation the sample was assayed for the protein estimation and alkaline phytase activity.

Table.4.3. Descriptive of effect of different incubation times on enzyme production.

Incubation time for production media (hours)	Enzyme activity (U/ml)	Protein estimation (mg/ml)	Specific activity (U/mg)	Standard deviation
24	0.28	0.22	1.261903	0.014142
48	0.84	0.20	4.148944	0.014142
72	1.22	0.18	6.528462	0.077782
96	1.89	0.18	10.07248	0.084853
120	12.34	0.701	17.60342	0.008485
144	1.26	0.099	12.72725	0.070711
168	0.70	0.099	7.070693	0.028284

Table 4.3. shows that maximum phytase activity was observed after 120 hours incubation (12.34 U/ml). maximum specific activity was also observed after 120 hours incubation (17.60 U/mg). Maximum protein had also been produced after 120 hours incubation (0.701 mg/ml). Results are also shown in graphs.

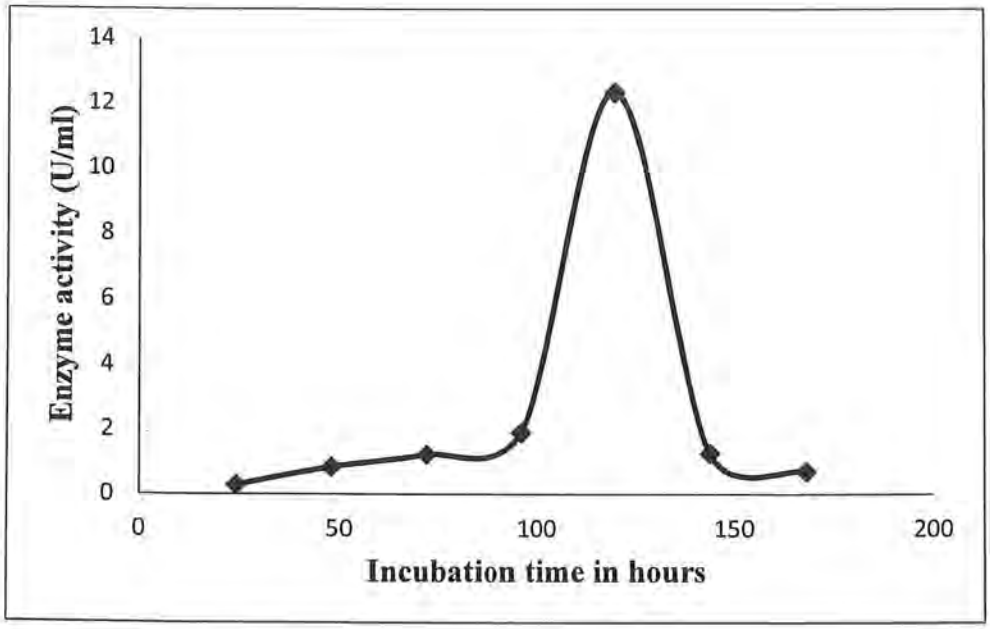

Fig.4.1. Effect of change in incubation time on enzyme production while all other parameters were constant.

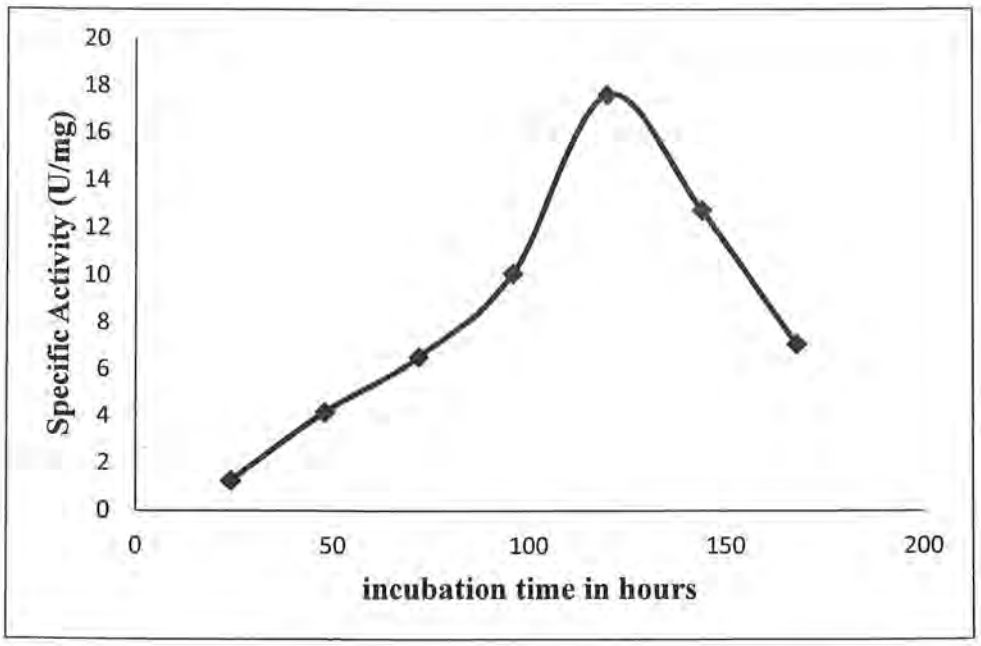

Fig.4.2. Effect of change in incubation time for production media on specific activity while other parameters were maintained constant. Maximum phytase activity was found in production media after 5 days of incubation. However, maximum protein was already produced after 24 hours.

4.2.2. Effect of change in pH on phytase production

The *Bacillus licheniformis* was inoculated to fresh sterilized growth medium. After 24 hours incubation at 50°C the medium was transferred equally to flasks containing equal amount of different pH production medium. All other parameters except pH were kept constant. The pH range selected for pH optimization was 5-9. After enzyme assay and protein estimation following results were obtained.

Table.4.4. Descriptive analysis of the effect of change in pH on the production of phytase.

pH of production media	Enzyme activity (U/ml)	Protein estimation (mg/ml)	Specific activity (U/mg)	Standard deviation
5	6.108899	0.538995	11.33387	0.021213
6	9.449649	0.592432	15.9506	0.00495
7	11.01639	0.644425	17.09491	0.017678
7.5	9.649883	0.538995	17.90348	0.008554
8	11.38876	0.54766	20.7953	0.024042
9	10.637	0.596765	17.82444	0.009899

The table shows that phytase produced at pH 8 has maximum enzyme activity (11.38876 U/ml) and specific activity (20.7953 U/mg). Experiment shows that *Bacillus licheniformis* produces alkaline phytase at pH 8. The effect is shown in graph as well.

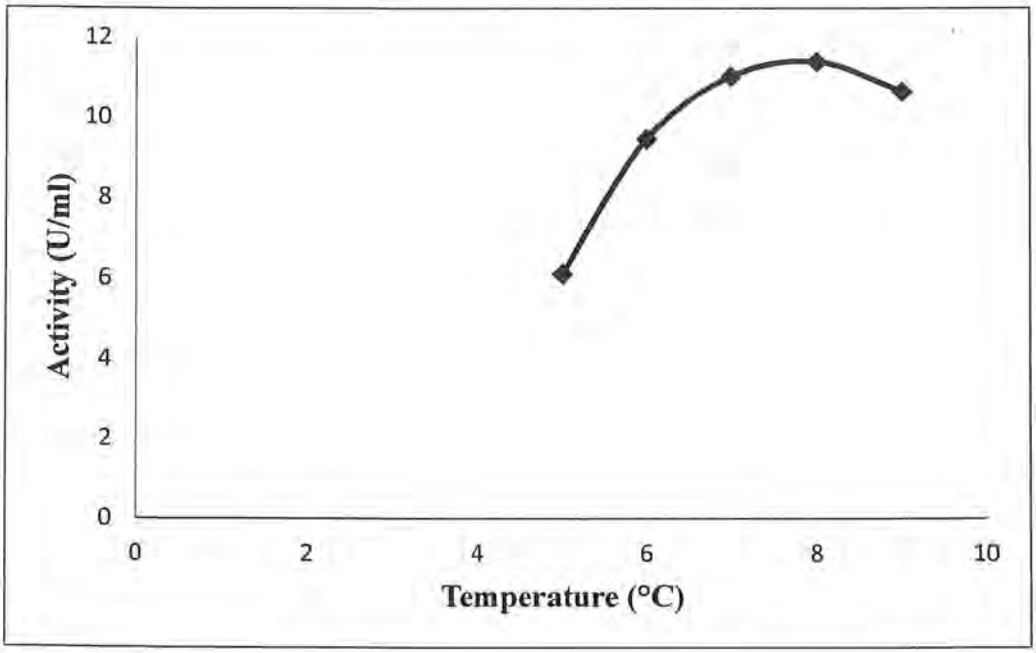

Fig.4.3. Effect of change in pH of production media on enzyme activity after 5 days incubation.

Fig.4.3. shows the effect of change in pH on phytase production from *Bacillus licheniformis*. Production increased as pH increased from pH 5-8 and again started decreasing at pH 9.

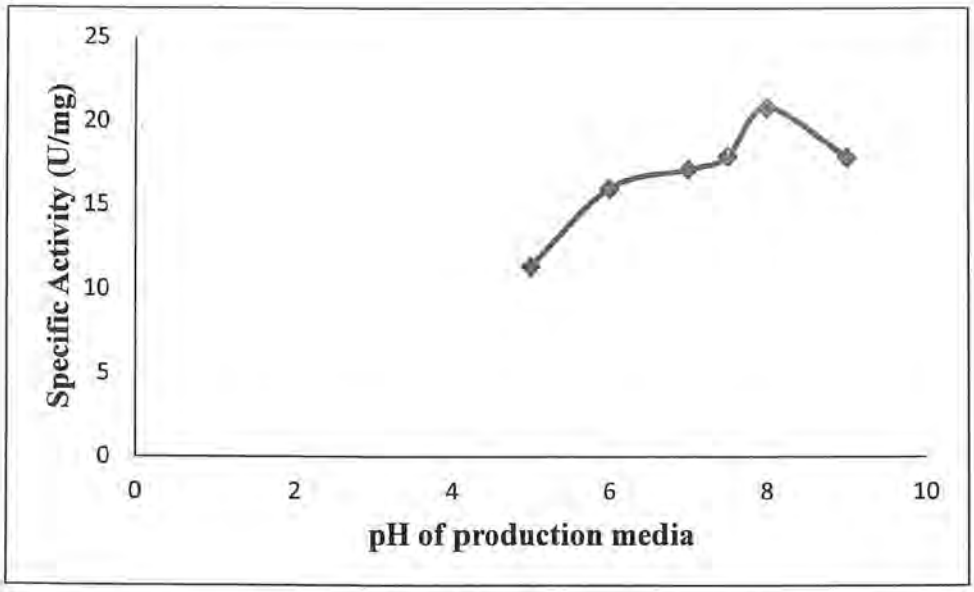

Fig.4.4. Graphical representation of effect of change in pH of production media on specific activity of phytase.

4.2.3. Effect of change in temperature on phytase production

Effect of change in temperature was studied for temperature range 25°C 30°C 37°C 45°C 50°C 55°C. All other parameters i.e; pH and incubation time were kept constant. After 5 days incubation at different temperatures and pH 8 assay for enzyme activity and protein estimation were carried out. Results are shown in Table.4.5.

Table.4.5. Descriptive analysis of the effect of change in temperature on production of phytase from *Bacillus licheniformis.*

Temperature (°C)	Enzyme activity (U/ml)	Protein estimation (mg/ml)	Specific activity (U/mg)	Standard deviation
25	3.144028	0.742345	4.235263	0
30	3.021077	0.683131	4.422397	0.000707
37	4.405152	0.687464	6.407831	0
45	3.354801	0.616696	5.439963	0.000707
50	11.38876	0.54766	20.7953	
55	6.435597	0.512905	12.547347	0.000707

Table.4.5. shows maximum enzyme activity (11.38 U/ml) and specific activity (20.79 U/mg) at 50°C which means *Bacillus licheniformis* produced maximum and most stable alkaline phytase at 50°C. Effect is also shown in graph.

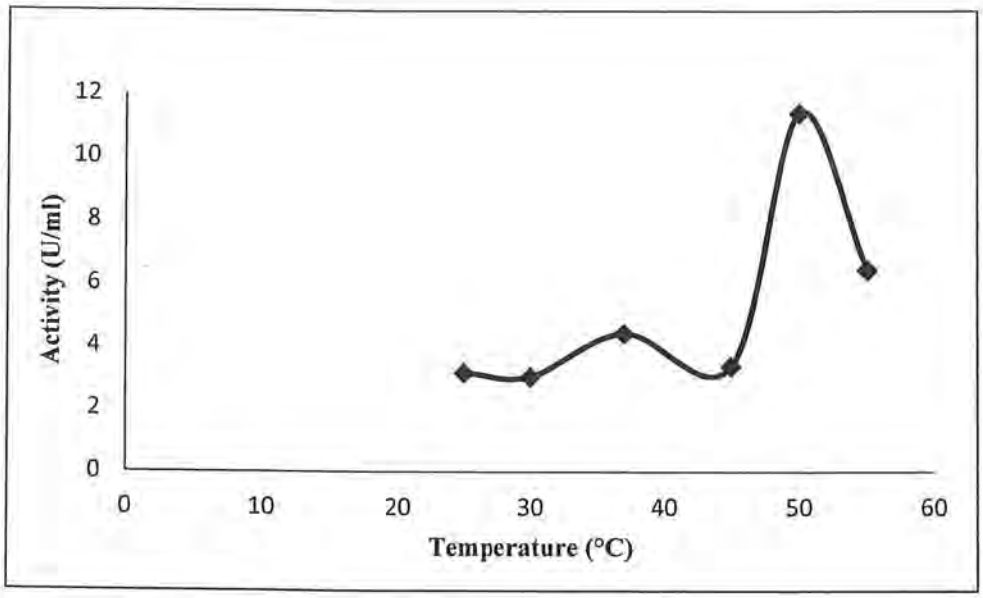

Fig.4.5. Effect of change in incubation temperature of production media on activity of alkaline phytase from *Bacillus licheniformis.*

Fig.4.5. shows that increase in temperature increased production from 25°C-50°C then suddenly dropped at 55°C

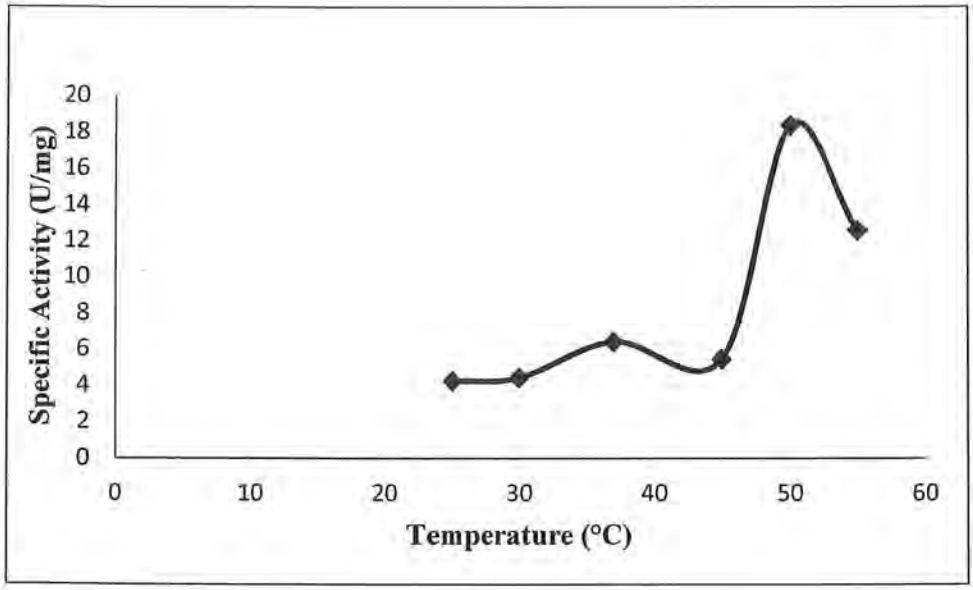

Fig.4.6. Effect of change in incubation temperature of production media on specific activity.

4.3. Precipitation of Alkaline Phytase

Precipitation was carried out with an organic solvent named acetone at -20°C. Pellets obtained were dissolved in 0.02M sodium acetate buffer of pH 5. Further quantified for the protein and enzyme activity as shown in Table 4.6.

Table 4.6. Describes results for precipitation of alkaline phytase using acetone.

Sample	Enzyme activity (U/ml)	Protein estimation (mg/ml)	Specific activity (U/mg)
Pellet	24.614	0.397	62
Supernatant	0.562	0.096	5.854

4.4. Characterization of Partially Purified Free Enzyme

4.4.1. Effect of temperature on activity of partially precipitated free enzyme

Temperature strongly effects the activation energy of enzyme carrying out chemical reaction. The change in temperature causes the increase or decrease in activation energy resulting in increase or decrease in the product formulated as a result of enzyme action. Thus, the effect of temperature was studied upon the alkaline phytase by the temperature range 20, 30, 40, 50, 60, 70, 80°C. The temperature was regulated and observed with an interval of 10°C. All other factors like pH of buffer, type of buffer, molarity of buffer were kept constant. The calculated maximum enzyme activity and specific activity were observed at 50°C, moreover enzyme showed the same activity at 60°C. The results also showed no significant loss of enzyme activity and specific activity even at 70°C as shown in the Table.4.7.

Table.4.7. Descriptive analysis of the effect of change in temperature with the factor of 10°C on the enzyme activity of alkaline phytase.

Assay temperature	Enzyme activity (U/ml)	Protein estimation (mg/ml)	Specific activity (U/mg)	Standard deviation
20	6.973068	0.18472	37.74943	0.017436
30	5.620609	0.157423	35.70376	0.004583
40	7.166276	0.18472	38.79539	0.005033
50	8.606557	0.18472	46.5925	0.038184
60	8.624122	0.18472	46.68758	0.017436
70	8.185012	0.18472	44.31042	0.001
80	7.517564	0.18472	40.69712	0.001155

Table.4.7. shows that alkaline phytase produced by *Bacillus licheniformis* is stable at 50°C and at 60°C as well. Moreover there is no significant decrease in enzyme activity and specific activity of enzyme at 70°C. At 40°C and 80°C specific activity was slightly

decreased. So, alkaline phytase produced from *Bacillus licheniformis* is stable at higher temperatures 50°C-70°C and proved to be a thermophilic enzyme. Graphical representation of above mentioned results is also given.

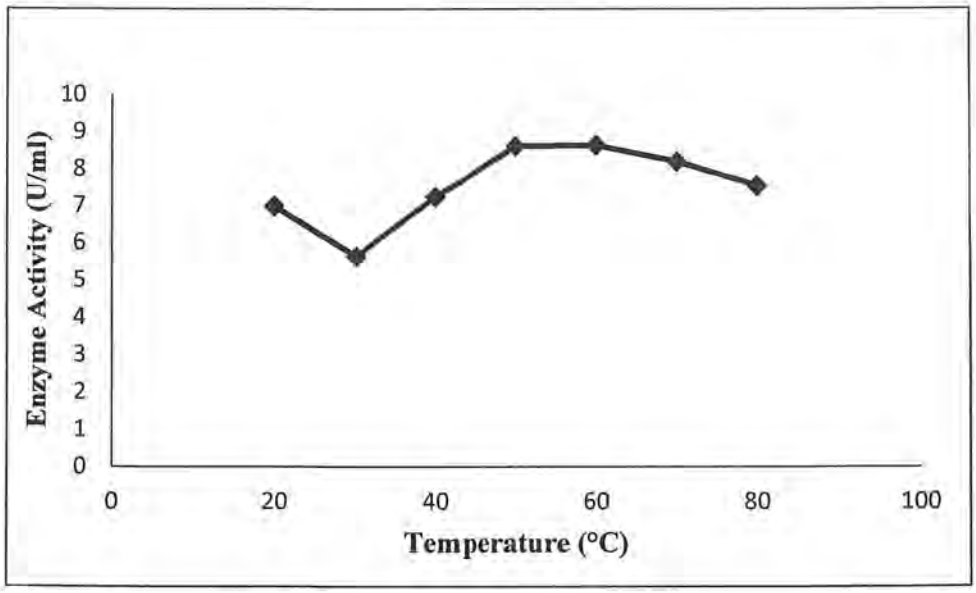

Fig.4.7. Effect of change in temperature with a factor of 10°C on the activity of alkaline phytase

Fig.4.7. shows the effect of changing temperature on the activity (U/ml) of alkaline phytase. The observed trend was that the increase in temperature from 40°C to 80°C did not effect enzyme activity (U/ml) significantly.

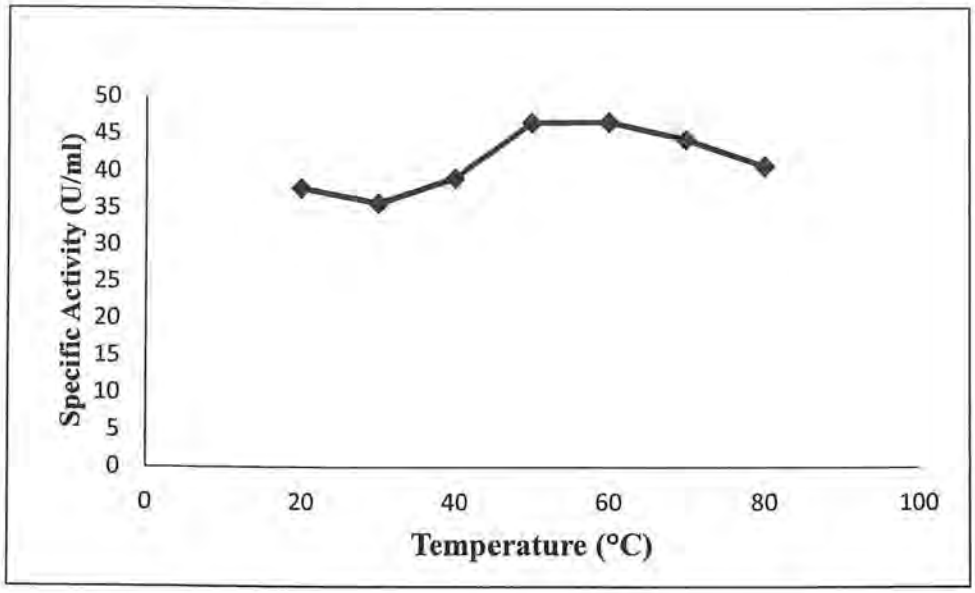

Fig.4.8. Effect of change in temperature on specific activity of alkaline phytase.

4.4.2. Effect of pH on activity of partially precipitated free enzyme

The pH impart a special role in increasing or decreasing the activity of the enzyme. The change in the pH alter or might totally inhibit the catalytic reaction occurring due to the activity of enzyme. The change in pH effects both the polar and non-polar intramolecular repulsive and attractive forces that mostly cause the changes in shape of enzyme. Thus a range of pH (2 to 9) was provided to the alkaline phytase and assayed for the activity and protein estimation. The values are represented in Table 4.8.

Table.4.8. Effect of change in pH on the alkaline phytase produced from *Bacillus licheniformis.*

pH	Enzyme activity (U/ml)	Protein estimation (mg/ml)	Specific activity (U/mg)	Standard deviation
3	5.515222	0.134027	41.15022	0.004041
4	5.023419	0.123628	40.63336	0.002517
5	7.429742	0.157423	47.1959	0.005568
6	5.690867	0.116551	48.82721	0.002082
7	8.940281	0.154535	57.85281	0.001528
8	14.42037	0.162912	88.51656	0.002887
8.5	13.45433	0.159012	84.61199	0.001475
9	8.501171	0.165222	51.45289	0.00148

Table.4.8. has clearly explained that at pH 8 the activity was maximum (14.42037 U/ml). The trend increased from pH 3 to 8 and dropped suddenly at pH 9. There was no significant decrease in activity at pH 8.5. The pH 8 showed 0.162912 mg/ml protein and 88.51656 U/mg as specific activity.

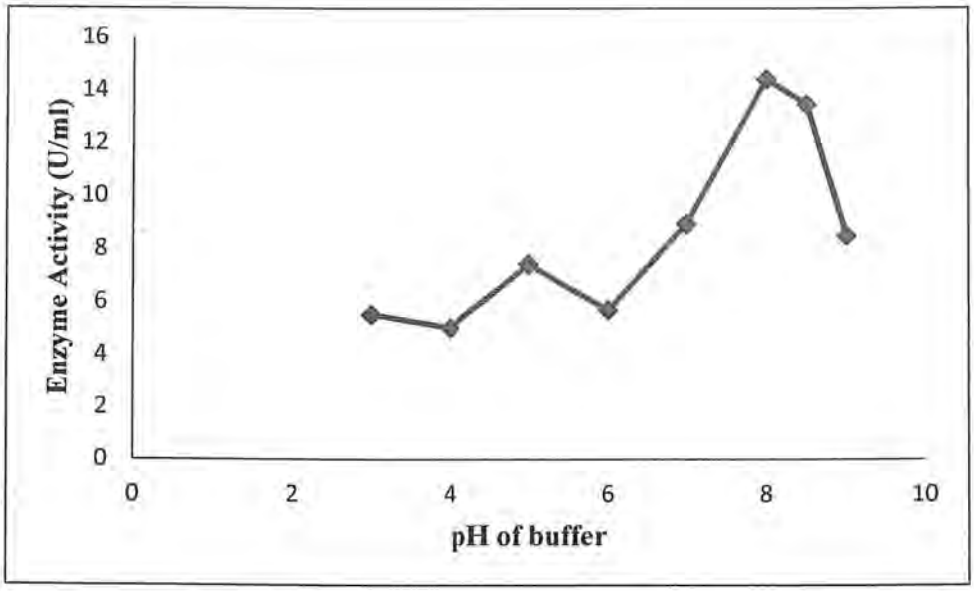

Fig.4.9. Effect of change in pH on the activity of alkaline phytase at 50°C and 10 minutes incubation.

The pH factor significantly effects the enzyme activity of the alkaline phytase. The trend has been shown in Fig. The obtained enzyme activity was maximum at pH 8 (14.42037 U/ml) and suddenly dropped at pH 9.

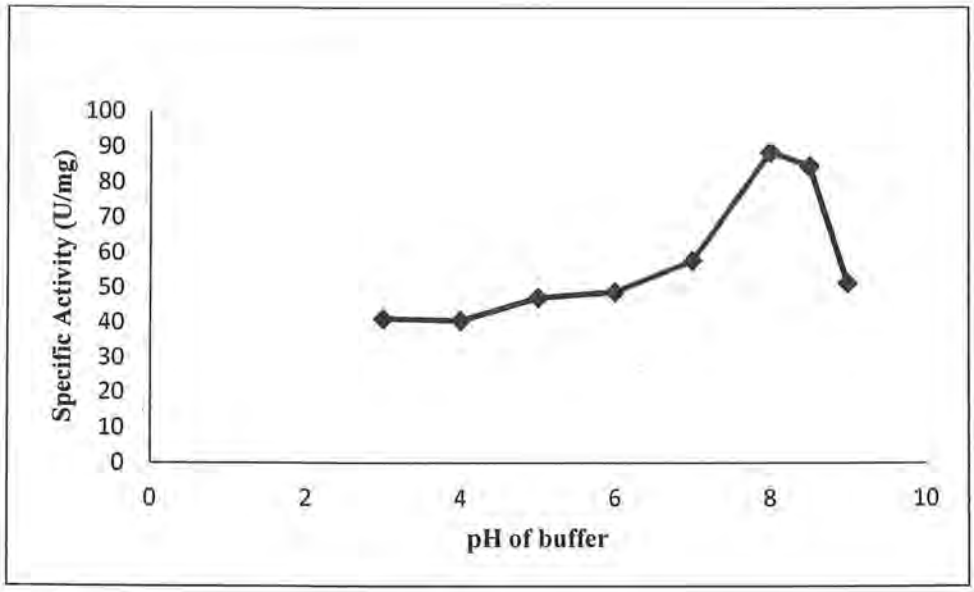

Fig.4.10. Effect of pH on specific activity of alkaline phytase at 50°C and 10 minutes incubation.

The change in pH effects the specific activity in similar manner to the enzyme activity. Initially the specific activity increased and became maximum at pH 8 then dropped suddenly at pH 9. There was no significant change observed at pH 8.5.

4.5. Immobilization without Optimized Conditions

The immobilization or simple adsorption was performed without maintaining any condition such as pH, temperature, type of buffer and molarity of buffer. The enzyme pellets that were suspended in the deionized water and was adsorbed onto the bentonite as solid support. The duplicate was run to obtain mean activity, amount of protein and percentage of immobilization. The product was dried under fan and named as Sid 1.

Table.4.9. Descriptive of the alkaline phytase activity and immobilization without maintaining the optimized conditions.

Adsorbed product	Enzyme activity (U/ml)	Protein estimation (mg/ml)	Specific activity (U/mg)	Percentage of immobilization (%)
Sid 1	17.3676815	2.24725592	7.72839504	15.418

Table 4.9. shows that only 15.4% immobilization was observed after immobilization without optimized conditions. Further conditions were optimized for immobilization of phytase on bentonite.

4.6. Characterization of Immobilization of Partially Precipitated Phytase on Bentonite

Following are the results for optimization of pH for the process of immobilization on bentonite.

4.6.1. Effect of pH on immobilization process

In order to study the effect of different pH on immobilization, equal amounts of pellets were dissolved in equal amounts of different pH tris (hydroxymethyl) aminomethane/HCl buffer of 0.1M molarity. These enzyme pellets, dissolved in buffers of different pH, were adsorbed on equal amounts of bentonite separately. The product was dried and assayed for enzyme activity and protein estimation in order to calculate the specific activity and percentage of immobilization. The product was named as Sid 2. The values are presented in Table 4.10.

Table.4.10. Descriptive effect of change in pH on immobilization of alkaline phytase activity on bentonite.

pH	Enzyme activity (U/ml)	Protein estimation (mg/ml)	Specific activity (U/mg)	Standard deviation	Percentage of immobilization (%)
5	1.31733	0.074523	17.67673	0.006364	44.495
7	6.249415	0.138359	45.168	0.000707	89.651
8	6.056206	0.078278	77.36747	0.001414	95.46
9	3.62178	0.164645	21.99755	0.003082	50.013

The above results of Table.4.9 and Table.4.10 have suggested that there was a markedly great difference between the immobilization with or without maintaining optimized conditions (15.418% immobilization). Maximum immobilization on bentonite was observed at pH 8 as a result dried product was obtained with 95.46% immobilization, 77.36 U/mg specific activity and 6.05 U/ml enzyme activity.

4.7. Characterization of Immobilized Enzyme

4.7.1. Effect of temperature on activity of immobilized enzyme

The effect of temperature on immobilized enzyme was observed while keeping all other factors constant. The temperature range studied was 20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C. Equal amount of semi-purified enzyme was dissolved in equal amount of 0.1M sodium acetate buffer and enzyme activity was observed at different temperatures at pH 6. Protein estimation was also carried out in order to calculate the specific activity at different temperatures. Results are shown in Table.4.11.

Table.4.11. Descriptive of the effect of change in temperature on activity of alkaline phytase after immobilization.

Temperature (°C)	Enzyme activity (U/ml)	Protein estimation (mg/ml)	Specific activity (U/mg)	Standard deviation
40	2.683841	0.271519	9.884541	0.002646
50	4.154567	0.271519	15.3012	0.003055
60	4.154567	0.271519	15.3012	0.003055
70	6.786885	0.271519	24.99599	0.003464
80	5.82904	0.271519	21.46826	0.01097

Table.4.11. shows that after immobilization enzyme shows maximum activity (6.786885 U/ml) and specific activity (24.99599 U/mg) at 70°C. Activity increased as temperature increased by the factor of 10°C from 40°C to 70°C and suddenly decreased at 80°C. However, free enzyme was stable in wider range of temperature as shown in Table. Results are also represented in graphs.

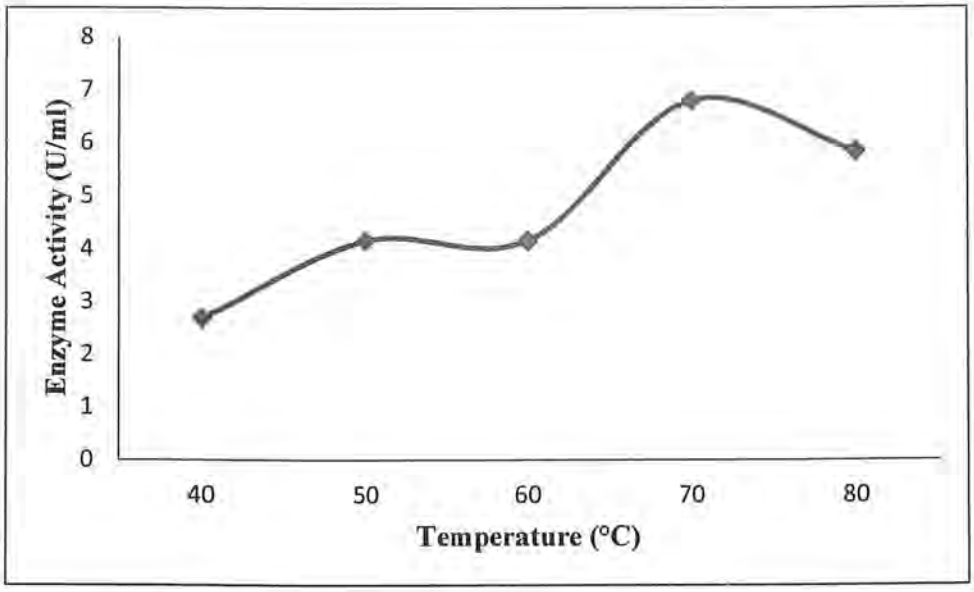

Fig.4.11. Effect of change in temperature on activity of alkaline phytase after immobilization at 50°C and 10 minutes incubation.

The increase in temperature from 40°C-70°C increased the enzyme activity as shown in Fig.4.11. The enzyme activity became maximum at 70°C and then suddenly decreased at 80°C.

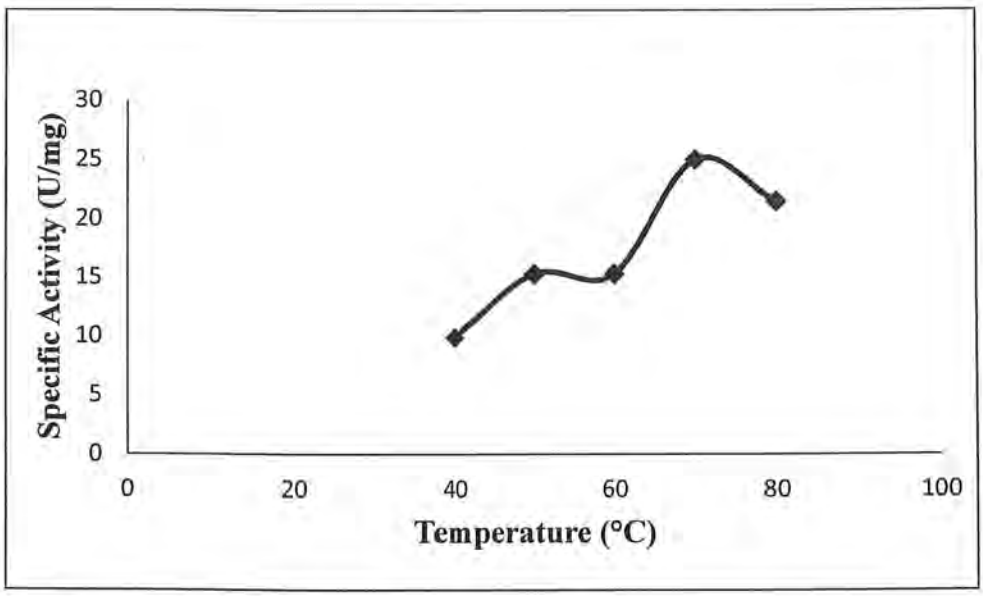

Fig.4.12. Effect of change in temperature by the factor of 10°C on specific activity of alkaline phytase at 50°C and 10 minutes incubation after immobilization.

Fig.4.12. shows maximum specific activity (24.99599 U/mg) at 70°C. Specific activity was also increased as enzyme activity with the increase in temperature from 40°C-70°C then suddenly decreased at 80°C.

4.7.2. Effect of pH on activity of immobilized enzyme

The effect of change in pH was observed in pH range of 3-9 after immobilization of alkaline phytase on bentonite at pH 8. The obtained results are given below.

Table.4.12. Descriptive of the effect of change in pH on alkaline phytase at 50°C and 10 minutes incubation after immobilization.

pH	Enzyme activity (U/ml)	Protein estization (mg/ml)	Specific activity (U/mg)	Standard deviation
3	4.266979	0.240709	17.72674	0.004509
4	4.744731	0.28885	16.42626	0.001528
5	4.142857	0.254188	16.29838	0.388558
6	7.0726	0.271519	26.04823	0.002082
7	12.73536	0.222415	44.98962	0.011015
8	16.91101	0.283073	76.03364	0.002646
9	7.337237	0.28885	25.40151	0.001528

Table.4.12. shows that immobilized alkaline phytase activity has maximum specific activity at pH 8 (76.03364 U/mg).

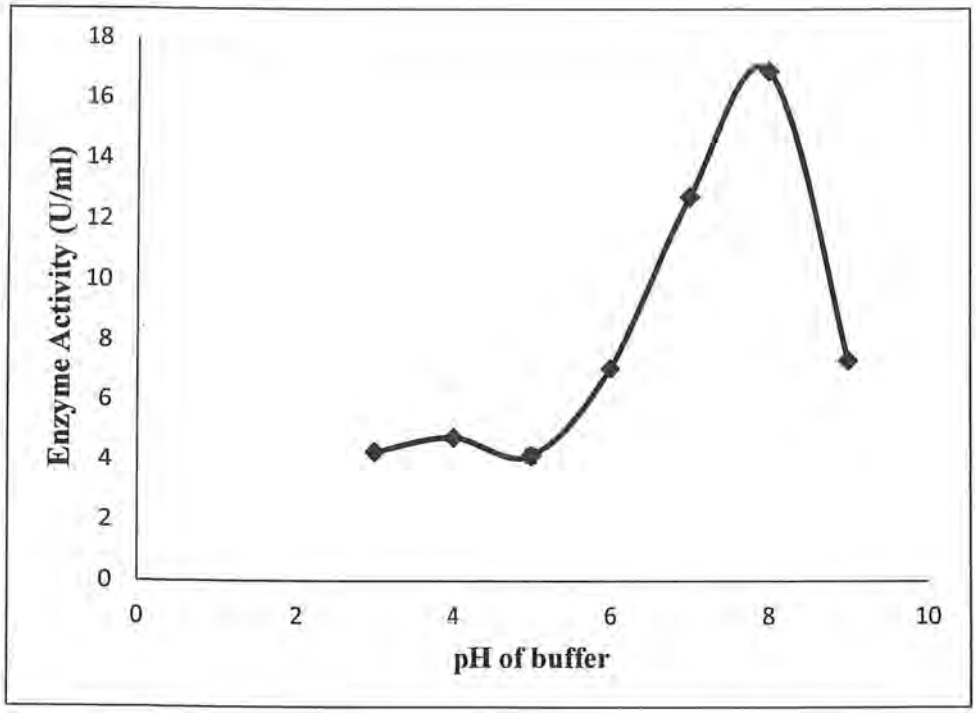

Fig.4.13. Effect of change in pH of buffer on enzyme activity (U/ml) of immobilized enzyme at 50°C and 10 minutes incubation.

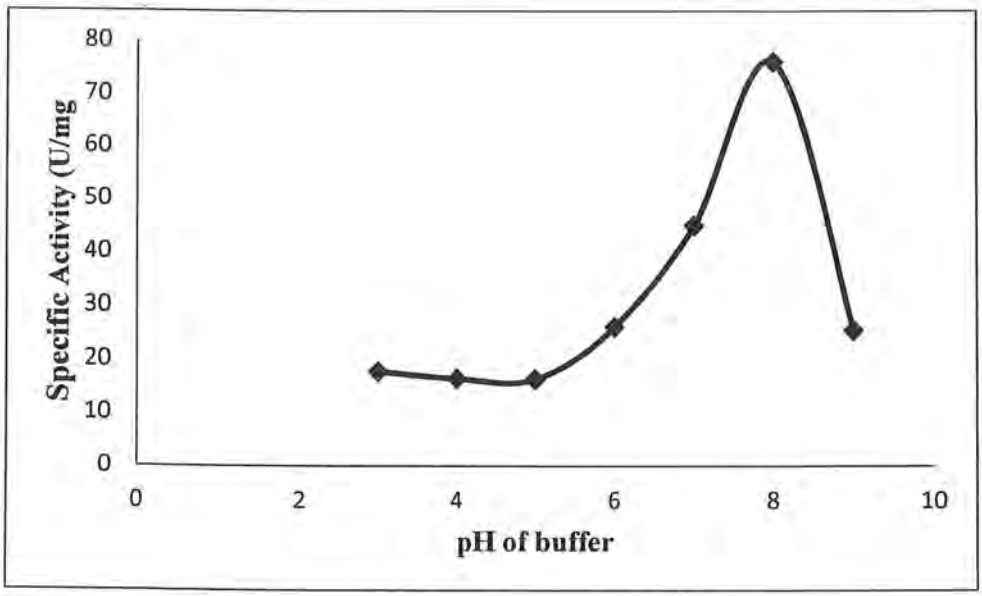

Fig.4.14. Effect of change in pH of buffer on specific activity (U/mg) of immobilized enzyme at 50°C and 10 minutes incubation.

4.8. Desorption

Adsorption and desorption are two opposite processes. An enzyme can be adsorbed in order to reuse same enzyme which makes the processes economical. This technique is mainly used in expensive processes and for expensive enzymes. Here desorption of alkaline phytase was carried out after its adsorption on bentonite in dried form. The adsorbed product was suspended in buffer for 10 minutes and was then centrifuged at 6000 rpm. The supernatant was considered as wash which was further assayed for enzyme activity, protein quantity for calculating specific activity. The process was repeated 3 more times and assay was repeated with each wash. The obtained results are given in table 4.13.

Table 4.13. Desorption of phytase from bentonite.

Wash number	Enzyme Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Standard deviation
1	0.165105	0.02	8.255269	0.002121
2	0.02459	0.023	1.069138	0.000707
3	0.080796	0.032	2.524883	0.000707
4	-0.0281	0.004	-7.02576	0

Desorption of enzyme attached to adsorbed product was found to decrease after each wash. There was no significant loss of enzyme activity after desorption which means phytase was properly adsorbed on bentonite.

DISCUSSION

5. DISCUSSION

The current study was carried out for immobilization of phytase on bentonite. As immobilization is a beneficial and economical technique from industrial point of view. Immobilized enzyme can be recovered and reused. Therefore, the main target of this work is successful immobilization of phytase on bentonite which is used as feed for poultry. Phytase is an important enzyme in poultry industry because it degrades phytic acid which is hazardous for environment.

The *B.licheniformis* was isolated from desert and was optimized for production of crude phytase enzyme. The parameters optimized were incubation time, pH and temperature. Production of phytase from *B.licheniformis* was tested with different incubation times i.e. 24, 48, 72, 96, 120, 144 and 168 hours. The enzyme activity increased till 120 hours (12.34 U/ml) and after 120 hours it started decreasing. Similarly, protein content was also maximum at 120 hours incubation (0.701 mg/ml) and specific activity was 17.6 U/mg. Specific activity was also significant at 96 hours (16.0 U/mg) and 144 hours (12.7 U/mg). Maximum phytase production was found to be at 120 hours incubation (table 4.3).

Another important parameter studied was pH of production media. It was studied at pH 5, 6, 7, 7.5, 8 and 9. The enzyme activity was almost same at pH 6 (9.449649 U/ml), pH 7 (11.01 U/ml), pH 7.5 (9.64 U/ml), pH 8 (11.39 U/ml), pH 9 (10.63 U/ml). Similarly, protein content is also almost similar at all pH but specific activity is maximum at pH 8 (20.79 U/mg). Specific activity at pH 7 and 9 is 3.7 and 2.9 times lesser than that of at pH 8 (table 4.4). So, pH 8 was selected as most suitable pH for phytase production from *B.licheniformis* at 50°C.

Temperature is also one of the significant parameter for enzyme production as enzymes are labile in nature and are very sensitive to change in temperature. Temperature effect on phytase production was studied at temperature 25°C, 30°C, 37°C, 45°C, 50°C, and 55°C. it was observed that maximum protein was produced at 25°C (0.74 mg/ml) and it decreased as temperature increased but phytase activity increased till 50°C (11.39 U/ml) and decreased again at 55°C (6.43 U/ml). This experiment shows that maximum protein

was produced at 25°C but maximum phytase production was at 50°C and other proteins produced were relatively less stable at 50°C and higher temperature as compared to phytase. Enzyme activity at 50°C (11.39 U/ml) was 43.49% greater than activity at 55°C. Similarly, enzyme activity at 50°C was 70.542% greater than activity at 45°C (3.35 U/ml). Moreover, specific activity at 50°C (20.79 mg/ml) was 73.840% and 39.66% greater than specific activity at 45°C (5.43mg/ml) and 55°C (12.54 mg/ml), respectively (table 4.5). So, on the basis of above discussion 50°C was chosen to be the most suitable temperature for phytase production from *B.licheniformis*.

The previously isolated *B.licheniformis* was optimized for phytase production, the optimum conditions obtained were temperature 50°C, pH 8 and incubation time 120 hours (table 4.5, 4.4, 4.1 respectively). The extracted crude enzyme had the enzyme activity 11.38 U/ml and specific activity 20.8 mg/ml (table 4.5). Further the enzyme was precipitated with acetone having enzyme activity 64.160 % (24.61 U/ml) and specific activity (62 U/mg) almost 4 folds greater than crude enzyme (table 4.6). Futher more, enzyme activity left in supernatant was only 0.56 U/ml which means 97.7% phytase activity had been shifted to enzyme precipitates or pellet and specific activity in supernatant was (5.85 U/mg) almost 6 folds less than that of enzyme precipitates. Further effect of change in temperature and pH on semi purified enzyme was studied.

Bacillus sp. strain DS11 phytase (Kim et al., 1998) had a temperature optimum at 70°C, which is higher temperature optimum. It was also very thermostable. 100% residual activity after 10 min incubation at 70°C (in the presence of $CaCl_2$). The enzyme stability of *Bacillus sp.* strain DS11 phytase was drastically reduced above 50°C in the absence of $CaCl_2$, whereas it was rather stable up to 90°C in the presence of $CaCl_2$. After incubation at 90°C for 10 min, the residual enzyme activity was approximately 50% of the initial activity. This indicates that the Ca^{2+} ion has a strong protecting effect on the enzyme against thermal denaturation. According to (Wyss et al., 1998) thermostability is a preferable feature in feed applications, since during the feed pelleting process the feed enzyme is exposed to temperatures of 60°C to 90°C. similarly, this work also depicts that *Bacillus licheniformis* also produces thermostable phytase enzyme which is suitable for use in poultry industry. Therefore, in this work immobilization of phytase from *Bacillus*

licheniformis on bentonite is targeted. The temperature parameter was observed by varying it with a factor of 10°C. The buffer used was 0.02M sodium acetate buffer. The value for enzyme activity (8.61 U/ml) and specific activity was (46.59 U/mg) at 50°C. The enzyme showed no change in enzyme activity (8.62 U/ml) and specific activity (46.69 U/mg) at 60°C, also there was no significant change seen in enzyme activity (8.19 U/ml) and specific activity (44.31 U/mg) at 70°C. Enzyme activity showed significant or stable value for enzyme activity (7.52 U/ml) even at 80°C while specific activity at 80°C was (40.70 U/mg) (table 4.7). As mentioned above $CaCl_2$ added to production media was responsible for enzyme stability at temperatures even above 50°C. Phytase production was also tested with soyabean media without calcium chloride using same strain, pH, temperature and incubation time. This media is also used for protease production from same strain i.e. *Bacillus licheniformis* and phytase activity were also found in this media but phytase did not stable without calcium chloride while protease remained stable. So, we concluded that calcium chloride is responsible for phytase stability at higher temperatures. Scientist are paying, attention in the past decade, towards making maximum phytase and protease yields under similar optimized conditions in order to make phytase production economical and commercial (Hussain ASM, et al., 2010). In addition to temperature pH also plays an important role in decrease or increase of enzyme activity.

Experiments had been performed in order to observe the trend of change in enzyme activity and protein content as a result of change in pH. The pH was studied in pH range 3 to 9 with stable buffers. The activity of alkaline phytase produced from *Bacillus licheniformis* showed a gradual increase till pH 8 (14.42 U/ml) and then decreased on pH 9 (8.50 U/ml). The pH 8 generated 5.91 times greater enzyme activity than that of pH 9 (table 4.8). Similarly, specific activity at pH 8 is 88.51 U/mg which is 37.06 times greater than specific activity at pH 9 (51.45 U/mg).

Immobilization is a unique and beneficial technique which provides an ease to expensive industrial processes as it can be used to recycle expensive enzymes. In this work immobilization is used to recycle phytase. Phytase is immobilized on bentonite which is a poultry feed. Immobilization was carried out both with and without optimized conditions.

Only 15% immobilization was observed without maintaining appropriate conditions and specific activity was 7.72 U/mg (table 4.9). In the second test the experiments were carried out for finding appropriate conditions for adsorption in order to increase yield of immobilization. First of all effect of incubation time on immobilization was studied. Both products obtained after overnight incubation and fan drying showed no significant difference in immobilization yield. So, fan drying was followed in order to save time. Then effect of pH on immobilization was studied by providing different pH i.e. pH 5, 7, 8 and 9. Different immobilization yields were obtained at all chosen pH values. Immobilization yield showed increment till pH 8 (95%) then decreased again at pH 9 (50%). At pH 8 immobilization yield was 45 times greater than at pH 9. Neutral pH 7 also gave significant immobilization yield 89%. Specific activity at pH 8 was 77.36 U/mg. However, specific activity decreased at pH 9 (21.99 U/mg). Specific activity at pH 8 is 55.396 times greater than that observed at pH 9 (table 4.10). So, both experiments showed that immobilization yield was better under optimized and appropriate conditions as compared to inappropriate and without optimized conditions. Sid 2 showed specific activity (77.36 U/mg) 69.639 times greater than specific activity of Sid 1 (7.72 U/mg). Sid 2 showed specific activity 90% greater than Sid 1 (table 4.9 & 4.10).

Effect of temperature was studied by increasing temperature by the factor of 10°C. Immobilized enzyme was most stable at 70°C and showed enzyme activity of 6.79 U/ml, enzyme activity was also almost same at 80°C i.e. 5.82 U/ml. However, there was a slight decrease in specific activity of immobilized enzyme at 80°C (21.46 U/mg) as compared to 70°C (24.99 U/mg). After immobilization specific activity at 70°C is 19.31 times less than specific activity of free enzyme at 70°C (44.31 U/mg). But immobilized enzyme showed more stability at 70°C as compared to 50°C and 60°C (table 4.11) while free enzyme was more stable at 50°C and 60°C as compared to 70°C (table 4.12) which means after immobilization the thermostability of enzyme was increased which is also a beneficial character.

Effect of pH was observed on an immobilized enzyme at pH 3-9. Specific activity increased till pH 8 (76.03 U/mg) which is 50.6 times greater than enzyme activity at

pH 9 (25.40 U/mg). As compared to specific activity of free enzyme at pH 8 (88.51 U/mg) immobilized enzyme lost only 14% specific activity at pH 8 (76.03 U/mg) (table 4.12)

Desorption was carried out to confirm the yield of adsorption. The 4 washes were carried out. Activity was very low in washes i.e. in wash 1, 2, 3 and 4 activity was only 8.25 U/mg. 1.06 U/mg, 2.52 U/mg and -7.02 U/mg, respectively. In wash 1 mazimum specific activity was observed and activity was decreased in wash 2 and 3 while no activity was observed in wash 4. So, concluded that there was no significant loss of activity of an immobilized enzyme which was observed to be 76.03 U/mg.

In this work effect of pH and temperature was also studied on an immobilized enzyme. The immobilized enzyme was more stable at higher temperature i.e. 70°C as compared to free enzyme (table 4.11). Moreover, immobilized enzyme did not loss significant specific activity at optimum pH 8 (table 4.12). Moreover, there was no significant loss of activity after desorption (table 4.13). Thus concluded that immobilization of phytase on bentonite would prove to be beneficial as immobilized enzyme has maximum activity at temperature 70°C while free enzyme has maximum activity at temperature 50°C. Moreover, there is no significant loss in specific activity of partially purified free enzyme after immobilization.

Thus, at the end it can be concluded that immobilization is a technique which can be use to recover and reuse enzyme. It increases the speed and efficiency of process and cut down the cost of expensive industrial processes.

CONCLUSIONS

6. CONCLUSIONS

The conclusions from the present research were as follows:

1. The *Bacillus licheniformis* is an efficient producer for the production of phytase.
2. Wheat bran is an inexpensive, readily available raw substrate for phytase production.
3. Optimum conditions for the production of phytase from *Bacillus licheniformis* are as follow: optimum temperature is 50°C, optimum pH is 8, optimum incubation time is 5 days.
4. *Bacillus licheniformis* produces thermostable alkaline phytase.
5. Phytase enzyme was partially purified using acetone.
6. Characterization of partially purified free and immobilizaed enzyme was carried out. The free enzyme had optimum activity at 50°C and pH 8 while immobilized enzyme had optimum activity at temperature 70°C and pH 8. Thus concluded immobilization made the enzyme more stable at higher temperatures.
7. Characterization of immobilization of phytase on bentonite was also carried out. Phytase showed maximum adsorption on bentonite at pH 8, at pH 7 immobilization yield was also good but yield decrease significantly at pH 5 and pH 9, thus concluded that pH effects immobilization.
8. Desorption was also carried out which concluded that phytase can be successfully adsorbed to bentonite because there was no significant loss of activity after immobilization.
9. It can also be concluded that phytase is a thermostable enzyme and has the potential to be used in industries as most processes run in industries need high temperatures.
10. Thus, it is concluded from the present research work that phytase enzyme from *Bacillus licheniformis* is a best attractive enzyme to be immobilized on bentonite for poultry industry.

FUTURE PROSPECTS

7. FUTURE PROSPECTS

Future Prospects include:

1. Gene isolation, identification and over expression of phytase from *Bacillus licheniformis* in mesophilic host e.g. *E.coli*.
2. Kinetic studies of enzyme with different substrate.
3. Structure to determine the amino acid involved in the enzyme substrate formation.
4. Large scale fermentation of phytase.
5. Production of phytase by merging it's production media with production media of another enzyme like protease from same microbe e.g. *Bacillus licheniformis* as production parameters for both enzymes are similar.
6. Immobilization of phytase on other substrates.
7. Immobilization of phytase and protease simultaneously on bentonite by optimizing conditions as both enzymes are used in poultry industry.

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