Damascus University
Faculty of Agriculture
Department of Food Science

Atomic Energy Commission of Syria
Department of Molecular Biology
and Biotechnology

Isolation and Identification of Phytase Producing Bacteria from Syrian Soil and Characterization of this Enzyme

Dissertation thesis submitted as a partial fulfillment for Master degree of
BIOTECHNOLOGY

Prepared by

Rafan Abd-Alhadi (Agric. Eng.)

Supervised by

Dr. Amal Korieh

Department of Food Science
Faculty of Agriculture
Damascus University

Dr. Bassam Al-Balaa

Department of Molecular Biology
and Biotechnology
Atomic Energy Commission of Syria

Damascus, Syria - 2015

Declaration

I hereby declare that the present research work described in this thesis entitled

"Isolation and Identification of Phytase Producing Bacteria from Syrian Soil and Characterization of this Enzyme"

Has not been, and is not actually submitted to obtain any other university and that all experiments and results indicated are of my own realization under the direction of my scientific supervisors.

Sources of any other data, methods or results included have been clearly mentioned in the text and in the references list.

Candidate

Rafan Abd-Alhadi

Damascus, 2015

Certificate

This thesis "Isolation and Identification of Phytase Producing Bacteria from Syrian Soil and Characterization of this Enzyme" was submitted by Eng. Rafan Abd-Alhadi in conformity with the requirements for the degree of Master of Biotechnology and authorized on 9/6/2015.

Supervisors:

Dr. Amal Korieh

Dr. Bassam Al-Balaa

Judgment Committee

Member: **Prof. Dr. Muhammad Muhammad**, Damascus University.

Member: **Prof. Dr. Mahmoud Abou Ghorrah**, Damascus University.

Head of Committee: **Dr. Amal Korieh**, Damascus University.

Abstract

Isolation and Identification of Phytase Producing Bacteria from Syrian Soil and Characterization of this Enzyme

Abstract:

Phytase is an important enzyme in the food and feed industry. It catalyzes the hydrolysis of phytate, an anti-nutrient compound present in cereals and grains, to release orthophosphate and Myo-inositol hexakisphosphate with lower degrees of phosphorylation. Phytic acid is a strong chelator capable of complexion with a variety of metal ions under neutral and alkaline conditions, as well as with proteins and starch under acidic conditions. Treatment with phytase increases not only the bioavailability of inorganic phosphorus but also the digestibility of proteins. Moreover, it improves absorption of minerals from food and feed. The action of phytase also contributes towards reducing the pollution in surface and ground water caused by the phytate and phosphorus run-off from manure in intensive livestock regions.

Phytase has become an important industrial enzyme and is the object of extensive research. The objective of the present study was to isolate a potential phytase producing bacterial isolates from soil samples of Damascus countryside and characterize the extracellular phytase of the best phytase activity bacterial isolate. Different soil samples were collected from various regions of Damascus countryside and were used as a source material for isolation of phytase producing bacteria. Fifteen colonies showed clear zones of hydrolysis around them on PSM plates were re-plated and hydrolysis efficiency calculated. Three isolates showed the best efficiency were investigated for phytase production in submerged fermentation using LPSM media. (C4) isolate was found to produce significantly high phytase activity than other isolates during (72^{th}) h of incubation at (37) °C with the pH of (7.0). The isolate (C4) with high potential for phytase production was selected and Preliminary identified by microscopic and biochemical tests (API 50 CH) system as *Bacillus sp*. Further, the identification of the strain was confirmed by subjecting it to 16S rDNA sequencing followed by BLAST analysis. The isolate (C4) showed high similarity to *Bacillus subtilis* strain X3. Effect of incubation time on phytase production had been investigated to be found that (72^{th}) h of incubation at (37) °C and pH number of (7.0) was the best. Effect of pH and temperature on phytase activity was determined. The temperature at (37) °C and pH (6.0) was found to be the best condition for phytase activity. Phytases produced can be used further for various applications.

Keywords: *Bacillus* sp., Phytase, Sodium phytate.

عزل وتعريف بكتيريا منتجة لإنزيم الفايتاز من تربة سورية وتوصيف هذا الإنزيم

الملخص:

يعتبر الفايتاز إنزيما" هاما" في مجال تغذية الإنسان والحيوانات أحاديات المعدة، لإنزيم الفايتاز ركيزة يعمل عليها هي حمض الفايتيك الذي يخزن في الحبوب والبقول والبذور الزيتية كمصدر أولي للفوسفور والأينوسيتول والذي يعتبر مضادا" غذائيا" (Antinutrient) لقدرته على تشكيل معقدات مع الشوارد المعدنية والعناصر النادرة مع الكثير من الإنزيمات الهاضمة والنشاء. يقوم إنزيم الفايتاز بتفكيك حمض الفايتيك إلى فوسفات أحادية لا عضوية و أينوسيتول مؤديا" لتحطيم المعقد الغذائي بحيث تصبح مكوناته متاحة للإمتصاص الهضمي في الحيوانات أحاديات المعدة والإنسان مما يؤدي إلى دعم نموها الطبيعي وضبط كمية الفوسفور المطروحة في مخلفاتها وبالتالي التخفيف من تلوث البيئة باعتبار الفوسفور هو العامل الأكثر تلويثا" للبيئة.

يعد الفايتاز إنزيما" صناعيا" هاما" وهدفا" للعديد من الأبحاث، ومنه هدفت هذه الدراسة إلى عزل بكتريا منتجة لإنزيم الفايتاز من (30) عينة تربة متنوعة جمعت من مناطق مختلفة في ريف دمشق. حيث تم الحصول على (15) عزلة بكترية منتجة لإنزيم الفايتاز بطريقة الطبق الصلب الحاوي على الوسط المغذي لإنتاج الإنزيم PSM. أجريت غربلة أولية للعزلات اعتمادا" على قطر الهالة المتشكلة واختيرت العزلات (R5-C2-C4) ذات أفضل كفاءة حلمهة على الطبق الصلب ليجرى عليها الغربلة الثانوية في الوسط السائل لإنتاج الإنزيم LPSM فأبدت العزلة (C4) أعلى فعالية إنزيمية (0.818) وحدة إنزيمية/مل. فتم تحديد هوية العزلة (C4) بواسطة الاختبارات المورفولوجية والحيوية الكيميائية (API 50 CH) على أنها من النوع *Bacillus SP.* ثم بالاختبارات الجزيئية بأنها السلالة *Bacillus subtilis* Strain X3. ودرس تأثير زمن التحضين على فعالية إنزيم الفايتاز الخام على درجة الحرارة (37) °س ورقم الpH (7.0) ليكون زمن التحضين (72) ساعة هو الأفضل لعمل الإنزيم. ثم تم توصيف إنزيم الفايتاز الخام ووجد أن رقم الpH المثالي لفعالية الإنزيم هو (6.0) في حين درجة الحرارة المثالية لفعالية الإنزيم كانت (37) °س. ليصار إلى استخدام الإنزيم المنتج من هذه العزلة في تطبيقات مختلفة.

الكلمات المفتاحية: .*Bacillus* sp، فايتاز، فايتات الصوديوم.

Table of Contents

Table of Contents

List of Figures

List of Figures

List of Tables

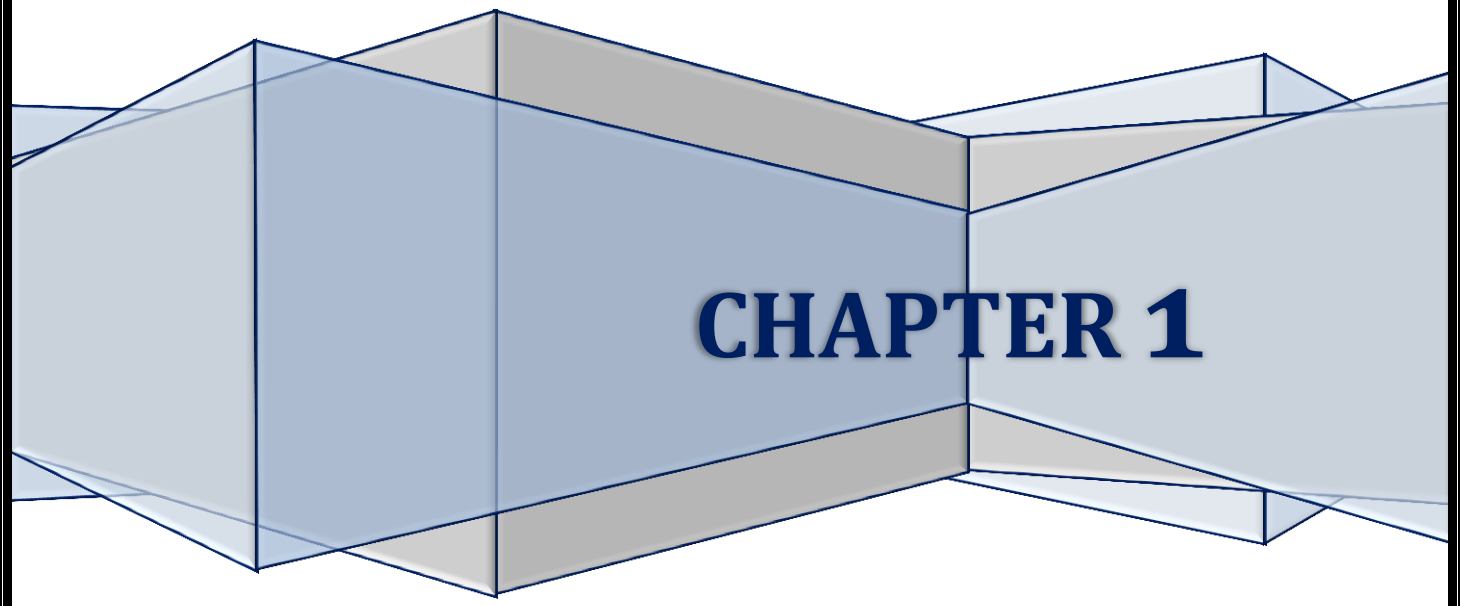
List of Abbreviations

Abbreviation	Meaning
ANFs	Anti-Nutritional Factors
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BPP	β-Propeller Phytase
BSA	Bovine Serum Albumin
ddI	Double Distilled water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetracetic Acid
et al;	and others
EtBr	Ethidium Bromide
FDA	Food and Drug Administration
FPLC	Fast Protein Liquid Chromatography
g	Gram
GRAS	Generally Regarded As Safe
GIT	Gastro Intestinal Tract
h	Hour
HAP	Histidine Acid Phosphatase Phytase
IPPs	Inositol Polyphosphate
IP1,IP2,IP3,IP4,IP5	Inositol mono-, bis-, tris-, tetrakis-, pentakis-Phosphate
IP6	Phytic Acid/ Myo-inositol hexakisphosphate
IUB	International Union of Biochemistry
IUPAC	International Union of Pure and Applied Chemistry
kb	kilo Base (pair)
kDa	Kilo Dalton
L	Liter
LB	Luria – Bertani
mg	Milli Gram
µg	Micro Gram
min	Minute
ml, µl	Milli Liter, Micro Liter
M, mM	Molar, Milli Molar

List of Abbreviations

µmole	Micro Mole
NCBI	National Center for Biomedical Informatics
ng	Nano Gram (10^{-9})
nm	Nano Meter
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PAP	Purple Acid Phosphatase Phytase
PCR	Polymerase Chain Reaction
PDB	Protein Database Bank
PH	Power of Hydrogen
Pi	Inorganic Phosphate
PKa	Symbol for the acid dissociation constant at logarithmic scale
pmol	Pico Mole
PSM	Phytase Screening Media
PTP	Protein Tyrosine Phosphatase Phytase
RNA	Ribonucleic Acid
RPM	Round Per Minute
RT	Room Temperature
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
sec	Second
TAE	Tetra Asetic Acid EDTA
TCA	Tri Chloro Acetic acid
UV	Ultra violet
V	Volt
℃	Degree of Celsius

Amino acid name	**Code**	**Amino acid name**	**Code**	**Amino acid name**	**Code**	**Amino acid name**	**Code**
Alanine	A	Glutamic acid	E	Leucine	L	Serine	S
Arginine	R	Glutamine	Q	Lysine	K	Threonine	T
Asparagine	N	Glycine	G	Methionine	M	Tryptophan	W
Aspartic acid	D	Histidine	H	Phenylalanine	F	Tyrosine	Y
Cysteine	C	Isoleucine	I	Proline	P	Valine	V



CHAPTER 1

1.1. Introduction

Microorganisms exhibit great diversity in their physiological activities. The energy necessary for carrying on cell activity and the building materials needed for the formation of new cells during multiplication is secured in different ways. The acquisition of energy and materials in turn is related in large measure to the different enzymes produced by various microorganisms. A lot of these enzymes are important in food science such as, Amylase, Protease and lipase. But during the last 20 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection, and biotechnology [Singh *et al*; 2011]. Phytase has a wide distribution in plants, microorganisms, and in some animal tissues.

As a substrate, Phytate is the major source of phosphorus and energy in some plants for seed germination, however it has been considered as an ANF that needs to be reduced or removed from cereal-based foods and feeds. Most cereals and legume seeds contain 1-2% phytate, which represents more than 60% of their total phosphorus content [Xuan *et al*; 2009]. Under acidic conditions, phytate interacts with positively charged dietary proteins leading to the formation of phytate-protein aggregates and precipitates, which results in a decreased accessibility for proteases, and consequently in inefficient protein digestion [Dahiya *et al*; 2009]. At neutral pH, phytate acts as a strong chelating agent that binds different vital metal ions in foods and feeds in the small intestine of monogastric organisms. This is the explanation for human nutritional deficiencies of calcium, iron and zinc within societies for which the main food ingredient is plant-based [Lassen *et al*; 2001].

In addition, most of the phosphorus in phytate is unavailable to monogastric animals as they lack the enzyme to digest phytate [Lassen *et al*; 2001]. Hence, addition of inorganic phosphorous, a non-renewable and expensive mineral [Lassen *et al*; 2001], to feeds for monogastric animals is a common practice, which incurs costs and also contributes to water pollution. The price of feed-grade phosphorus has increased more than four-fold in the last few years [Turner *et al*; 2005]. Unutilized phytate from vegetable feed is excreted by animals, and is becoming an environmental pollutant in areas of intensive livestock production [Lassen *et al*; 2001]. Although phosphorus is not harmful

in drinking water, its presence in water bodies can be of considerable concern and environmental significance. Addition of phosphorus - even in small quantities - to aquatic bodies is causing eutrophication of the aquatic system which leads to accelerated growth of algae and other aquatic vegetation [Kim *et al;* 2006].

Phytase is an enzyme that catalysis the sequential hydrolysis of phytate (Myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate; IP6) to less phosphorylated Myo-inositol derivatives with concomitant release of inorganic phosphate [Applegate and Angel, 2004].

Requirements for better animal feed, environmental protection, lower food/feed cost and human health have prompted the fast development of research on phytases and their applications [Singh *et al*; 2011]. Recent researches have shown that microbial phytases are most promising for a biotechnological application. Although phytases from several species of bacteria, fungi and yeast have been characterized, commercial production currently focuses on the soil bacteria. The phytases, which exhibit desirable activity profile over a broad pH range, excellent thermal stability, and broad substrate specificity, are more promising for commercial exploitation.

1.2. Research Justifications

As phytase is increasingly used worldwide, science and technology related to the enzyme have evolved to a new exciting field at a fast pace.

By the reaction it leads, phytase has a wide range of applications, phytase improves the nutritional value of plant-based foods by enhancing protein digestibility and mineral availability through phytate hydrolysis during digestion in stomach or during food processing, phytase also improves dietary phytate phosphorus utilization by food producing animals supporting its normal growth performance and bone strength and reducing environmental pollution of phosphorus from animal waste in areas of intensive animal production. In addition, phytase is used for plant growth improvement and as an effective biological agent during pulp and paper processing.

Potentials of phytase in improving human nutrition and health and in developing specific phytic acid or inositol-derived products have increasingly been gained attention [Haefner *et al;* 2005] and will expand as a new direction of phytase. Genetic engineering techniques could be employed for the generation of consensus phytases with improved and desirable properties for its potential applications.

Because of its significant current and potential applications, access to local bacterial isolates capable of producing phytase enhances the production of this enzyme on a commercial scale locally, Thereby eliminating the need for imported at high cost on the one hand and the strengthening of the nutritional value of plant foods consumed frequently in Syria (Cereals and Legumes) and increasing the yield of feed for animals on the other.

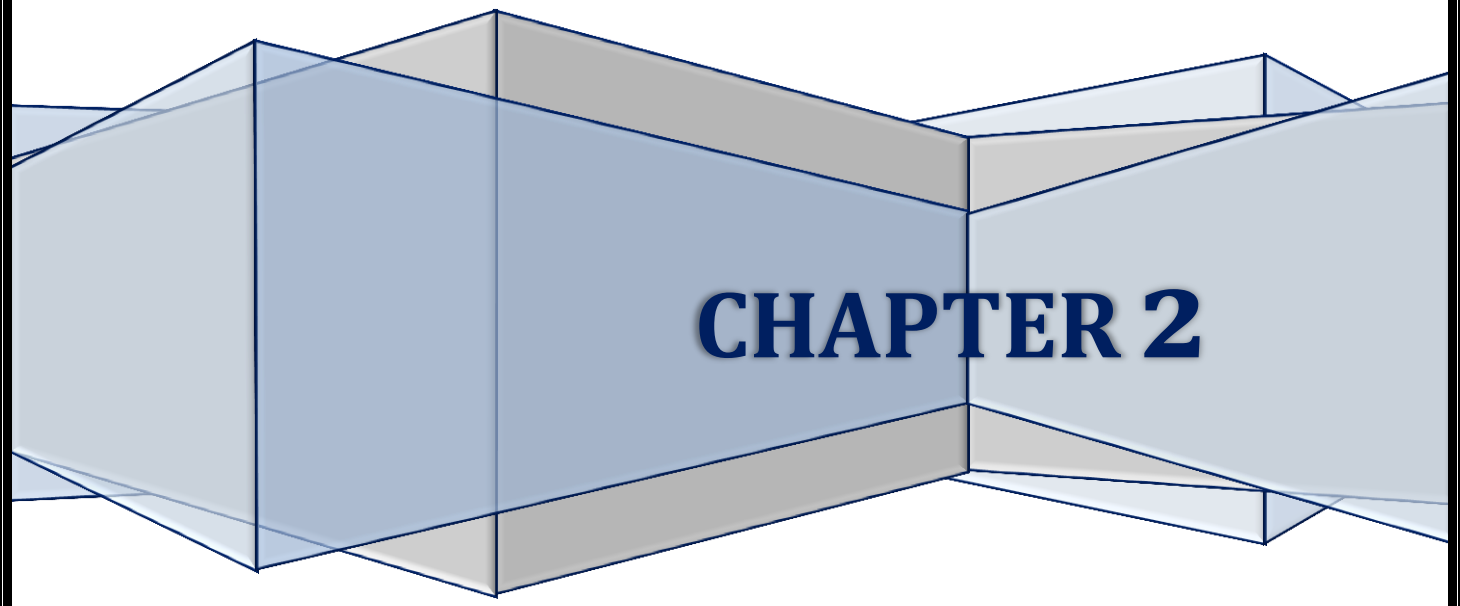
As phytases, which exhibit desirable activity profile over a broad pH range, excellent thermal stability, and broad substrate specificity, are more promising for commercial exploitation, this study aimed to characterize the extracted phytase by Measurement of phytase activity (PH and Temperature assay).

1.3. Research Objectives

According to the significant current and potential applications of phytase, access to local bacterial isolates capable of producing this enzyme is of great importance, to achieve this goal:

- Isolation of phytase producing bacteria from Syrian soil.
- Identification of the high phytase activity bacterial isolate.
- Enzymatic assays and Characterization of phytase.

* * * * *



CHAPTER 2

As the main phosphorus storage in plant seeds, phytate plays an important role in ripening, germination and signaling. However, it is considered as an anti-nutrient factor which needs to be degraded by phytase enzymes in food and feed.

2.1. Phytate

The terms phytic acid, phytate and phytin refer to the free acid, salt and calcium/magnesium salt, respectively [Almedia, 2010]. About 75-85% of the total phosphorus in plant seeds is in the form of phytate which is the main storage form of phosphorus in cereals, legumes and other plant tissues.

2.1.1. Historical background, chemical structure and properties

Phytate was isolated for the first time by Hartig in 1855 and 1856 as small, non-starch particles from the seeds of various plants. Later on, numerous researchers have isolated phytate in the form of a (globoid) and confirmed its main chemical content to be Calcium, phosphorus and metal ions [Kerovuo, 2000]. The name (inositol-phosphoric acid) was suggested by Winterstein in 1897, and revised as (Myo-inositol 1, 2, 3, 4, 5, 6 Hexakisdihydrogen phosphate) by IUPAC-IUB in 1968.

Structure of phytic acid has been intensively discussed the chemical society for many years during the twentieth century. The two structures suggested by Neuberg and Anderson are the most accepted (Figure 2.1). The controversy concerning these two structures has been the isomeric conformation of the phosphate groups within the compound and whether three strongly bound water molecules are incorporated into the structure [Tran, 2010]. Several studies including elemental analysis, titration of sodium phytate with metal ions, X-ray crystallography, and nuclear magnetic resonance (NMR), support each of these structures.

Several investigations on the phytate structure using NMR, crystallography and other methods have suggested that the phosphate at the C-2 position is axially placed while the others (C-1, C-3, C-4, C-5, and C-6) are equatorial. On the other hand, proposed only an equatorial position of C-2 whereas the other C positions were said to be axial according to single X-ray analysis.

By combining ^{13}C NMR, ^{31}P NMR, and Raman spectroscopic analyses, [Coulibaly *et al;* 2011] concluded that Myo-inositol hexakisphosphate exists in two conformations in aqueous solution depending on the pH: Under acidic conditions, a 1-axial/5-equatorial conformation exists, while strong alkaline conditions cause the inverted conformation, 5-axial/1-equatorial, to prevail.

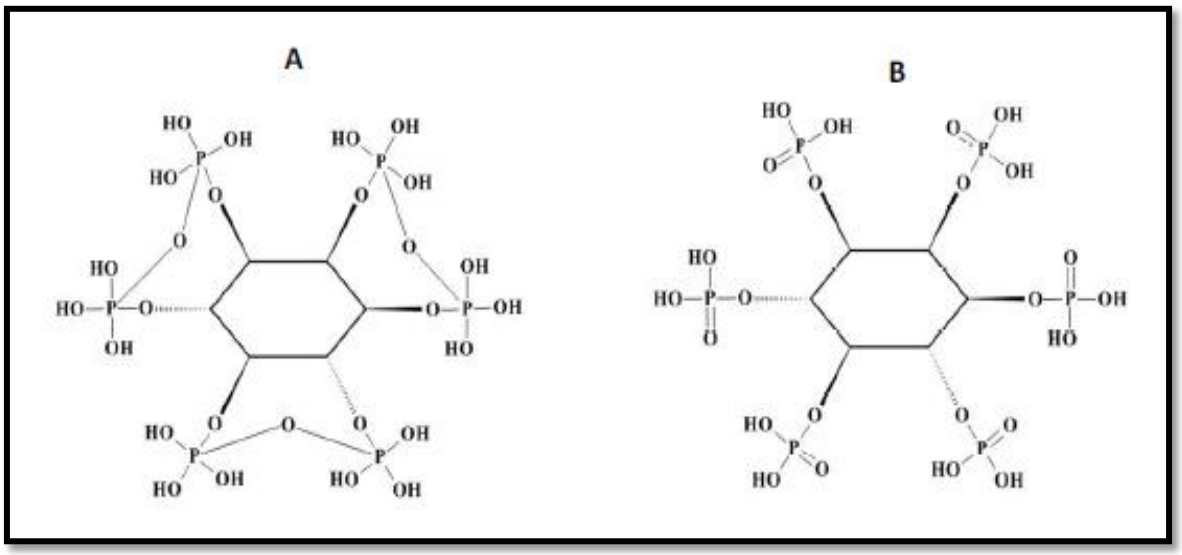


Figure 2.1. Structure of phytic acid proposed by **(A)** Neuberg 1908 and **(B)** Anderson 1914 [Tran, 2010].

Phytic acid shows several levels of negative charge in a wide PH range since its twelve ionizable hydrogen atoms have varying dissociation constants, six of them have a PKa of 1.84 (strongly dissociable), two have a PKa of 6.3 (weakly dissociable) and the remaining four have a PKa of 9.7 (very weakly dissociable). This feature makes IP6 a very strong chelating agent [Kerovuo, 2000]. The negative charges of IP6 can complex with positively charged moieties of a number of metal ions, peptides, proteins and starch.

It has been reported that IP6-metal ion complexes are formed under neutral or slightly alkaline conditions. On the other hand, the IP6-protein complexes are formed under acidic conditions where most of the proteins originating from plants are positively charged. IP6-metal ion-protein complexes have also been suggested to be formed under neutral and slightly alkaline conditions.

2.1.2. Natural distribution and physiological functions

Phytate is primarily found in seeds; however, its location within the seed differs among plants (Table 2.1). For instance, 90% of the phytin in corn is in the germ portion of the kernel, whereas the endosperm of wheat and rice kernels is almost devoid of phytate, as it concentrates in the germ, aleuronic layers of the cells of the kernel and the outer bran [Reddy, 2002]. While 99% of the phytate in dry peas was in the cotyledons and 1% in the embryo taxis.

Phytin in oilseeds and grain legumes is associated mostly with protein and concentrated within globoids, the subcellular inclusions, which are distributed throughout the kernel. On the other hand, Phytate in soybean seeds appears to have no specific location [Kerovuo, 2000]. Phytate has also been found in roots and tubers, fruits and vegetables, nuts, as well as pollen of various plant species.

Among several plant feedstuffs, sesame, pumpkin/squash, and flax (linseed) have the highest IP6 content on a dry weight basis (3.7 to 4.7%). Other plant feedstuffs comprise between 1 and 3% dry weight of IP6. The highest amount of phytate among cereals is found in maize (0.83 - 2.22%) and among legumes in dolique beans (5.92 - 9.15%).

Phytate is one of the most abundant organic phosphorous compounds in nature, which it's component makes approximately up to 75-85% of phosphorus in plant seeds and 1-5% of total weight in most of foodstuff seeds such as legumes, cereal grains and oilseed meals [Tuyet *et al;* 2004]. In dormant seeds phytate represents 60 to 90 % of the total phosphate. Phytate is regarded as the primary storage form of both phosphate and inositol in plant seeds and grains [Greiner *et al;* 2006]. The IP6 molecule contains 28.2% P [Hidvegi and Lasztity, 2003]. It is normally found as a potassium magnesium salt in rice, wheat, broad beans (Vicia faba), and sesame seeds; and as a calcium-magnesium-potassium salt in soybeans (Glycine max) and Great Northern beans (Phaseolus vulgaris). Phytate rapidly accumulates in grains and seeds during their ripening period and maturation, accompanied by other substances such as starch, proteins, and lipids [Khan *et al;* 2007]. In cereals and legumes phytic acid accumulates in the aleuronic particles and globoid crystals, respectively.

Table 2.1. Phytic acid content in seeds and grains (g/100g)
[Afinah *et al;* 2010].

Plant	Part	Phytic acid %	Plant	Part	Phytic acid %
Sesame	Dry seed	4.71	Beans	Dry seed	1.41
Pumpkin/Squash	Embryo	4.08	Watermelon	Seed Only	1.36
Flax(Linseed)	Dry seed	3.69	Kiwi Fruit	Fleshy Fruit	1.34
Rapeseed(Canola)	Dry seed	2.50	Broad Beans	Dry seed	1.11
Sunflower	Embryo	2.10	Cucumber	Immature Seed	1.01
Mustard	Dry seed	2.00	Sorghum	Dry grain	1.06
Cashew	Embryo	1.97	Cocoa Beans	Dry grain	1.04
Peanut seed	Shell	1.70	Barley	Dry grain	1.02
Tomato	Seed only	1.66	Oats	Dry grain	1.02
Soy bean	Dry seed	1.55	Wheat	Dry grain	1.02
Almond	Dry seed	1.42	Peas	Dry grain	1.02
Calculated phytic acid content assuming 28.20% phosphorus in the molecule.					
The phytic acid content is influenced by cultivar, climatic conditions and year.					

The role of phytate in seeds has been proposed by [Almedia, 2010] to be: as a phosphorus and Myo-inositol (a cell wall precursor) reserve, for energy storage, as a competitor for adenosine triphosphate during the rapid biosynthesis of phytin near seed maturity when seed metabolism is inhibited and dormancy is induced, as an immobilizer of divalent cations required for the control of cellular processes and that is released during germination upon the action of intrinsic plant phytases, and as a regulator of the readily available seed Pi level.

The role of phytic acid as a natural antioxidant in seeds during dormancy was suggested by [Reddy, 2002]. The antioxidant property of phytic acid is based on the assumption that phytic acid effectively blocks iron-driven hydroxyl radical formation. IP6 has been suggested to play an anti-fungal role in the field, preventing aflatoxin production in soybean seeds by making zinc unavailable to the mold [Minihane and Rimbach, 2002].

IP6 has been found in nucleated erythrocytes of birds, fresh water fish and turtles, as well as in organic soils. Lower degrees of phosphorylation of inositol (IP2, IP3, IP4, IP5) and even IP6 has been recognized as components of cell signaling and phosphate transfer systems present in possibly all animal and plant tissues[Tran, 2010].Phytic acid has been shown to exert an antineoplastic effect in animal models of both colon and breast carcinomas. The presence of undigested phytic acid in the colon may protect against the development of colonic carcinoma [Kerovuo, 2000]. Although plants routinely synthesize IP6, there is evidence that human also sparsely synthesizing IP6 from D-glucose by the action of a phosphorylase on inositol-phosphate which is derived from the cyclization of glucose-6-phosphate. Several mammalian tissues such as testes, mammary glands, brain, liver and kidneys can synthesize about 4 g IP6 per day.

2.1.3. Anti-nutritional effects

From a plant nutrient perspective, phytate is important for growing seedlings and promoting good crop yields [Raboy, 2009]. In many areas of the world with large populations, the availability in soil of phosphorus to plants is limited, causing an increased use of fertilizers which supplement Pi to plant soil.

However, from nutrition point of view (Table 2.2), phytate is considered as an anti-nutritional compound due to its strong chelating properties, it behaves in a broad pH-region as a highly negatively charged ion and has therefore a tremendous affinity for food components with positive charge(s), such as minerals, trace elements and proteins [Greiner *et al;* 2006].

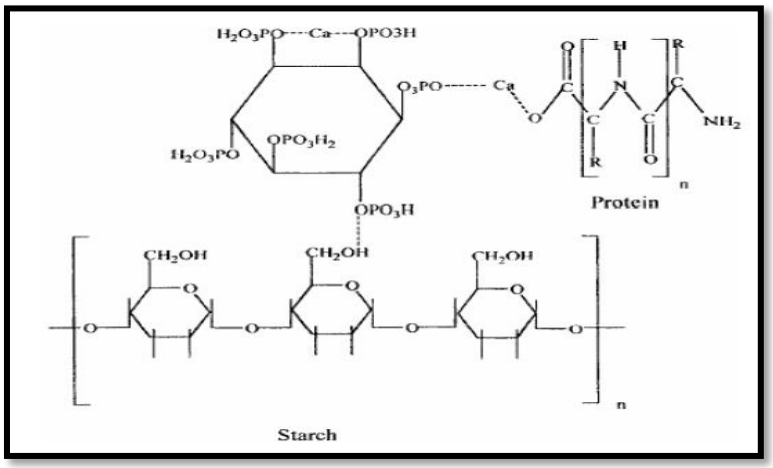


Figure 2.2. Interaction of phytic acid with metals, proteins and carbohydrate [Singh *et al;* 2011].

Phytate forms complexes with numerous divalent and trivalent metal cations (Zn^{+2}, $Fe^{+2/+3}$, Mg^{+2}, Mn^{+2} and Cu^{+2}), Stability and solubility of the metal cation phytate complexes depends on the individual cation, the pH-value, the phytate cation molar ratio, and the presence of other compounds in the solution [Greiner *et al;* 2006]. Many essential metal ions (Ca^{+2}, Zn^{+2}, Fe^{+2}…) present in food/feed are bound to IP6 and form precipitates under neutral or slightly alkaline conditions. The stability of the complexes formed between IP6 and metal ions is in the order (Zn^{+2}> Cu^{+2}> Co^{+2}> Mn^{+2}> Ca^{+2}), whereas at PH 7.4 this order is reported to be (Cu^{+2}> Zn^{+2}> Ni^{+2}> Co^{+2}> Mn^{+2}> Fe^{+2}> Ca^{+2}). The insolubility of these complexes is regarded as the major reason for the reduced bioavailability of minerals in diets with high levels of phytic acid. The simultaneous presence of two different cations raises the proportion of the IP6-metal complex that precipitates [Coulibaly *et al;* 2011].

Table 2.2. Phytate content in plant-derived food [Greiner and Konietzny, 2006].

Food types	**Phytate (mg/g)**	**Food types**	**Phytate (mg/g)**
Cereals		**Legume-based food**	
Rice (polished, cooked)	1.2–3.7	Green peas (cooked)	1.8–11.5
Rice(unpolished, cooked)	12.7–21.6	Soybeans	9.2–16.7
Maize bread	4.3–8.2	Tofu	8.9–17.8
Unleavened maize bread	12.2–19.3	Lentils (cooked)	2.1–10.1
Wheat bread	3.2–7.3	Peanuts	9.2–19.7
Unleavened wheat bread	3.2–10.6	Chickpea (cooked)	2.9–11.7
Rye bread	1.9–4.3	Cowpea (cooked)	3.9–13.2
Sourdough rye bread	0.1–0.3	Black beans (cooked)	8.5–17.3
French bread	0.2–0.4	White beans (cooked)	9.6–13.9
Flour bread (70% wheat, 30% rye)	0.4–1.1	Kidney beans (cooked)	8.3–13.4
Flour bread (30% wheat, 70% rye)	0–0.4	**Miscellaneous**	
Cornflakes	0.4–1.5	Sesame seeds (toasted)	39.3–57.2
Oat flakes	8.4–12.1	Soy protein isolate	2.4–13.1
Pasta	0.7–9.1	Soy protein concentrate	11.2–23.4
Sorghum	5.9–11.8	Buckwheat	9.2–16.2
Oat porridge	6.9–10.2	Amaranth grain	10.6–15.1

IP6 also interacts with positively charged dietary proteins leading to the formation of IP6-protein aggregates and precipitates, which decreases their accessibility to proteases [Kerovuo, 2000], thus resulting in inefficient protein digestion. The lowering of the protein solubility, results from the masking of positive charges on the protein molecules by phytic acid, which changes the isoelectric points of the proteins [Singh *et al;* 2011]. Complexion with IP6 has been reported for several proteins from wheat and oat bran, beans, corn germs, soybean flakes, and sesame meal, soybeans and peanuts as well as cotton seed. There is thus strong evidence that phytic acid-protein interactions negatively affect protein digestibility. Phytate interactions with proteins are pH-dependent [Greiner *et al;* 2006]. The IP6-metal ion-protein complex formed under neutral and alkaline conditions was suggested to be fairly labile, but it could lead to the synergistic effect of mineral deficiency and inefficient protein digestion.

By chelating metal ions, IP6 indirectly impairs the function of some digestive enzymes which need metal ions for their activity and/or stability [Kerovuo, 2000]. A number of metallo-enzymes such as pepsin, trypsin, α-amylase, β-galactosidase, carboxy-peptidases, leucine amino-peptidase, alkaline phosphatase, and amino-peptidases containing either zinc or calcium are present in the small intestine and are essential for the degradation of food/feed stuffs. IP6-Cu has been reported to cause more than 95% decrease in activity of carboxy-peptidase A by exchanging Cu^{+2} with Zn^{+2} in enzyme molecules at PH 7.5.

IP6 demonstrated the same inhibition level as EDTA on the activity of α-amylase [Ali *et al;* 2010]. Calcium is known as a contributing factor in autocatalysis of trypsinogen to trypsin, and also as a stabilizer of trypsin. Hence, when IP6 chelates calcium, the trypsin production and stability are affected. Depletion of intestinal alkaline phosphatase and phytase in the gastro-intestinal tract (GIT) of monogastric animals has been ascribed to high dietary calcium which forms IP6-Ca complexes [Tran, 2010]. Strong inhibition of the non-digestive enzyme thymidine kinase is related to zinc deficiency symptoms by high levels of dietary phytate. The binding of IP6 to starch occurs as a result of hydrogen bond formation which might lead to an inefficiency of starch digestion resulting in blood glucose reduction [Singh *et al;* 2011].

2.1.4. Environmental perspectives

It is well known that plants need inorganic phosphorus Pi for their growth and that they accumulate phytate in their seeds, roots, and other tissues during ripening. IP6 needs to be degraded to return Pi to the soil - thereby closing the phosphorus cycle. However, excess Pi, especially in surface-water, can cause eutrophication [Almedia, 2010], which is a kind of unbalanced growth of algae and other aquatic vegetation leading to oxygen depletion, in turn resulting in anaerobic aquatic environments. Hence, an imbalance of the aquatic ecosystem is the main environmental impact of the release of excess phosphorus into water bodies.

Phytate, which is consumed in food/feed by human, swine, poultry, and other monogastric animals, often end up in excrement. The manure disposal problems associated with intensive animal farming operations are now widely recognized [Tran, 2010]. It has been shown that human and monogastric animals do not possess any intestinal phytase, and that the hydrolysis of phytate occurring in the GIT is due to a non-specific action of intestinal phosphatases or of gut bacteria. Hence [Angel *et al;* 2010], Pi supplementation in feeds to fulfill the nutritional requirements of animals is common practice, incurring an extra expense to the feed and increasing IP6 and Pi excreted to litter. Microorganisms present in soil and water which possess a wide range of phytate hydrolytic enzymes have contributed to the phytate hydrolysis in livestock litter [Kerovuo, 2000]. When IP6 and Pi enter into rivers, they give rise to cyano bacterial blooms, hypoxia and death of water-borne organisms. Additional problems with respect to odor and taste are also common.

2.1.5. Therapeutic potential

In spite of the negative impact on food/feed nutrition and environment, phytic acid and inositol intermediates have been implicated in the treatment of several diseases as Parkinson's diseases, Alzheimer's disease, multiple sclerosis and certain types of cancer [Phillippy and Wyatt, 2001]. Phytate has been reported to block the formation of free radicals (•OH), suppress Iron-catalyzed oxidative reactions, hence providing antioxidant and anti-carcinogenic benefits

[Singh *et al;* 2011]. Other advantages of phytate have been reported, such as improving the glucose response, decreasing plasma cholesterol and triglycerides, decreasing renal stone formation, detoxification of heavy metals such as cadmium and lead, as well as therapy for calcium urolithiasis and against dental caries.

The position of the phosphate groups on the Myo-inositol ring is of a great significance for their physiological functions. The IPPs D-Myo-inositol (1, 3, 4, and 5) tetrakisphosphate and D-Myo-inositol (1, 4, and 5) triphosphate have been found to stimulate intracellular Ca^{+2} release which affects cellular metabolism and secretion [Coulibaly *et al;* 2011]. Specific inositol triphosphates have been suggested to prevent or alleviate diseases or conditions associated with abnormal levels of Neuropeptide Y. Among others, these include inflammatory diseases such as arthritis and respiratory diseases such as asthma [Kerovuo, 2000]. The use of specific inositol triphosphates as pain killers has also been proposed. Surprisingly, the esters of inositol triphosphates have been shown to exert significant inhibitory effects against retroviral infections including HIV.

It has been mentioned that Inositol as the basis for a number of signaling and secondary messenger molecules, is involved in a number of biological processes, including: Insulin signal transduction, Cytoskeleton assembly, Nerve guidance (Epsin), Intracellular Ca^{+2} concentration control, Cell membrane potential maintenance, Serotonin activity modulation, Breakdown of fats and reducing blood cholesterol, and Gene expression [Almedia, 2010].

2.2. Phytase

2.2.1. Phytase sources

Phytase is widely distributed in nature, occurring in microorganisms, plant, and animal tissues [Oh and In, 2009]. The physicochemical characteristics and catalytic properties of phytases from various sources indicate to be ester-hydrolyzing enzyme with an estimated molecular weight of 35-50 kDa for bacteria, 85-150 kDa for fungal phytases, around 500 kDa for yeast and 50-150 kDa for plants and animal tissues [Pandey *et al;* 2001]. Most of the known phytases have optimum temperatures in the range of 45-80 °C, and are active within a PH range of 4.5–6.0 at 45–60 °C.

2.2.1.1. Microbial source

Phytase is normally produced in bacteria, fungi and yeast [Pandey *et al;* 2001]. Fungi are known for their abilities to secrete large amounts of phytase, also Molds metabolize phosphor more effectively than bacteria [Tahir *et al;* 2010]. Generally, the phytases produced by fungi are extracellular, whereas the enzymes from bacteria are mostly cell associated. The only bacteria showing extracellular phytase activity are those of the genera *Bacillus* and *Enterobacter* [Konietzny and Greiner, 2004]. The phytases of *E. coli* have been reported to be periplasmatic enzyme and phytase activity in *Selenomonas ruminantium* and *Mitsuokella multiacidus* was found to be associated with the outer membrane. Some yeast, such as *Saccharomyces cerevisiae*, *Candida tropicalis*, *Torulopsis candida*, *Debaryomyces castelii*, *Debaryomyces occidentalis, Kluyveromyces fragilis* and *Schwanniomyces castelii*; [Kerovuo, 2000] have also been shown to produce phytase. Most phytases are monomers; with the exception of phytase from *Aspergillus niger* and phytase from *Penophora lycii* those are tetrameric and dimeric proteins, respectively [Tran, 2010]. Generally, the phytases from bacteria have optimum PH in neutral to alkaline range while in fungi optimum PH range is 2.5–6.0.

2.2.1.2. Plant source

Phytase occurs widely in the plant kingdom, phytate degradation occurs mostly in grains, seeds and pollen of higher plants, such as cereals, legumes, oilseeds and nuts [Konietzny and Greiner, 2002]. High levels of phytase activity have been found in wheat, rye, triticale, barley and malt sprouts (500-5000 U/kg), while maize, oats, sorghum grain, and legume seeds contain almost no phytase activity at all. Low phytase activity is found in plant roots.

A rapid increase of phytase activity was monitored in plant seeds during their germination [Rodríguez and Fraga, 2000]. The phytase activity of grains, seeds and pollen has been shown to be responsible for phytate degradation during germination, to make phosphate, minerals and Myo-inositol available for the purpose of plant growth and development, it is assumed that during seed germination, phytate after decomposition by phytase is utilized in the

form of phosphate and inositol [Tran, 2010]. Some authors ascribed the rise in phytase activity to phytase synthesis during germination while others attribute it to a rise of an already existing phytase activity. So, it was concluded that seeds contain both constitutive and germination inducible phytases.

2.2.1.3. Animal source

Phytase in calf liver and blood was the first report on animal phytases. Phytase is normally produced (endogenous phytase) in ruminant animals. On the other hand, the Intestinal phytase of non-ruminant animals (monogastric animals) like poultry, swine and fish does not play a significant role in feed-derived phytate digestion [Pandey *et al;* 2001]. Phytases has been purified from rat and human intestines and reported that the activity of human intestines phytase was 30 times lower than that of rats [Tran, 2010]. Moreover, it did not play a significant role in phytate digestion in the GIT. Phytase has also been purified and characterized from the protozoan *Paramecium*.

2.2.2. Reaction mechanism of phytase

Phytase (Myo-inositol hexakisphosphate hydrolase) is a special class of phosphatase enzymes that is able to catalyze the sequential hydrolysis of phosphate ester bonds of Myo-inositol hexakisphosphate (Phytic acid) [Mullaney and Ullah, 2003]. This yields Pi and a series of Myo-inositol lower phosphoric esters and in some cases to free Myo-inositol (Figure 2.3).

The enzyme reaction is likely to proceed through a direct attack of the metal-binding water molecule on the phosphorus atom of a substrate and the subsequent stabilization of the pentavalent transition state by the bound calcium ions [Vats and Banerjee, 2004]. The enzyme has two phosphate binding sites, the “cleavage site”, which is responsible for the hydrolysis of a substrate and the “affinity site”, which increases the binding affinity for substrate containing adjacent phosphate groups. The existence of the two nonequivalent phosphate binding sites explains the formation of alternately dephosphorylated Myo-inositol triphosphates from phytate and the hydrolysis of Myo-inositol mono phosphates.

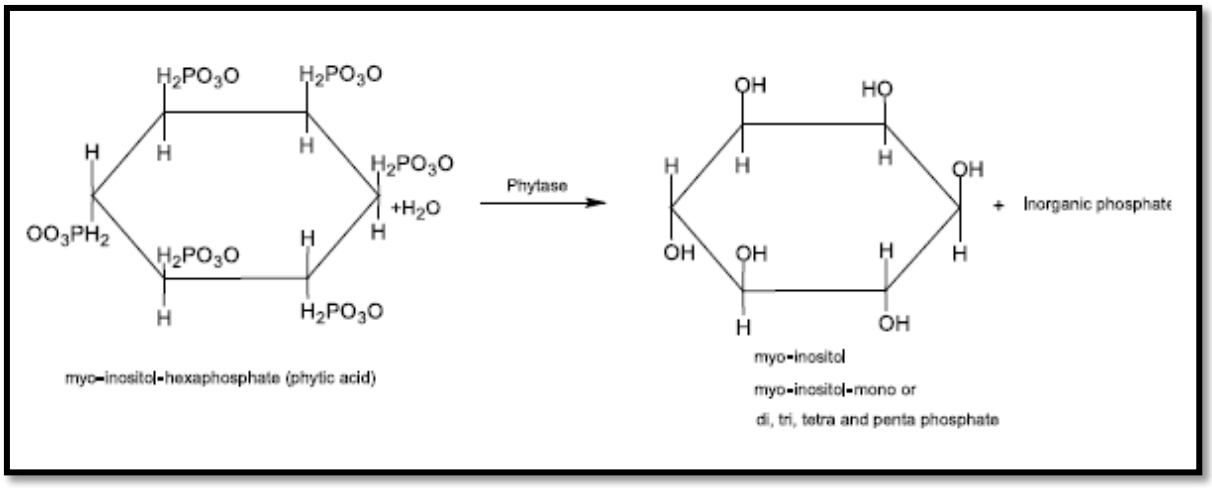


Figure 2.3. Reaction catalyzed by the phytate degrading enzyme (phytase) [Meor Hussin *et al;* 2010].

By the hydrolyzing process of phytase, all phytic acid chelated metal ions; proteins and digestive enzymes would be available for monogastric animals and human that lack phytase [Applegate and Angel, 2004]. In biological system, Hydrolyzing phytic acid by phytase is an important reaction for energy metabolism, metabolic regulation and signal transduction pathways [Vats and Banerjee, 2004]. Phytase can be distinguished from acid phosphatase; the second one is incapable of degrading phytate.

2.2.3. Determination of phytase activity

An ideal supplemental phytase should have stability at low pH and at high temperature which allows it to survive the feed pelleting process, food processing conditions, and adequate gastric performance. Most of the phytase activity assays developed so far is end point measurement of the color formed between IP6 or Pi and the color reagents after certain reaction times. Methods to quantify IP6 are quite complicated, comprising numerous steps of extraction, precipitation with FeCl3, centrifugation, and washing [Tran, 2010]. They are also expensive since they require the use of HPLC analysis or infrared spectroscopy (FT-IR). Hence, a more convenient phytase assay involves measurements of Pi release after stopping the enzymatic reaction.This method is based on the formation of a colored complex between Pi and ammonium molybdate [Arjula *et al;* 2010]. The end point measurement of Pi has been widely used to determine phytase activity of several commercial phytases.

2.2.4. Diversity of phytase

Since the first phytase was found in 1907, many different phytases from a variety of sources have been discovered and described [Kerovuo, 2000].

The enzyme nomenclature committee of the IUB distinguishes between two classes of phytase enzymes: a 3-5phytase and a 6-phytase (4-phytase assuming d-configuration), depending on the position (d-3 or l-6) on the inositol ring where the de-phosphorylation of IP6 is initiated. The 3-phytases (EC 3.1.3.8) yield 1, 2, 4, 5, 6-pentakis-phosphate, while the 6-phytases (EC 3.1.3.26) give 1, 2, 3, 4, 5-pentakis-phosphate as the first product along with Pi. The 3-phytases do not always completely dephosphorylate IP6, whereas the 6-phytases do [Tran, 2010]. The 3-phytases have been found to dominate among microorganisms and are the largest group of phytases while 6-phytase has been considered to be characteristic of the seeds of higher plants. However, there are some exceptions: *E. coli* has been shown to produce a 6-phytase [Greiner *et al;* 1993] and soybean a 3-phytase [Phillippy *et al;* 1998].

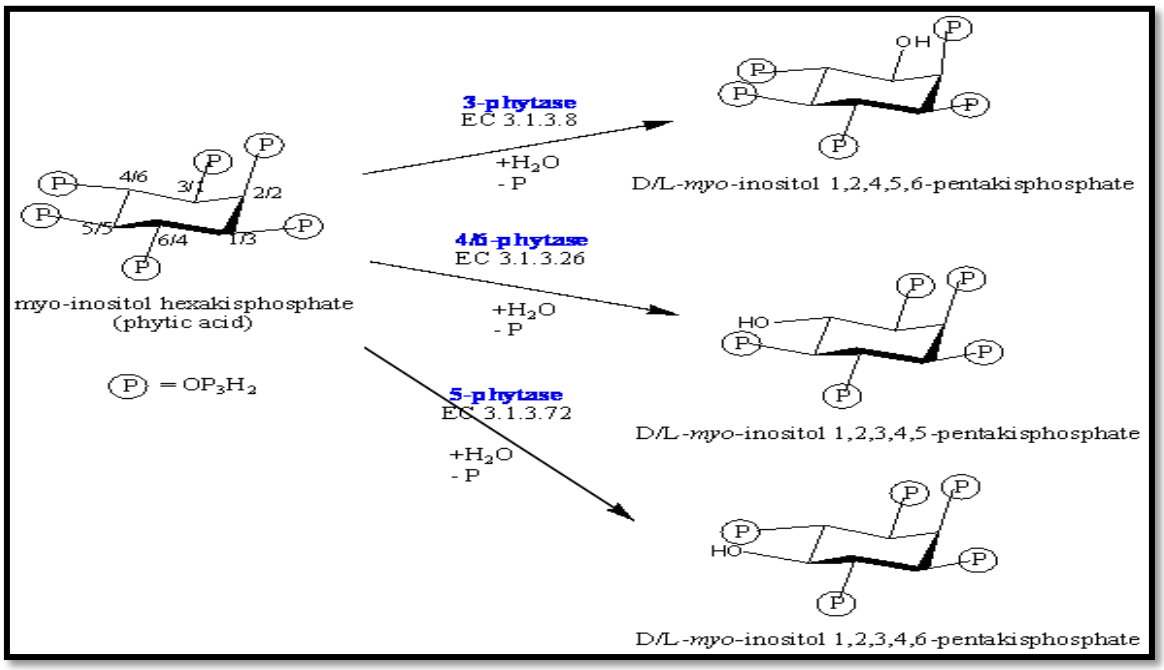


Figure 2.4. De-phosphorylation of phytic acid [Bohn *et al;* 2008].

Based on the PH optimum for catalysis, phytases can be designated as acid, neutral or alkaline. [Oh and In, 2009] have used the phytases´ amino acid sequences and their biochemical properties to divide them into two major groups (HAP and alkaline phytases) with four sub groups (PhyA, B, C, D) (Figure 2.5); however, plant phytases were not included in their classification.

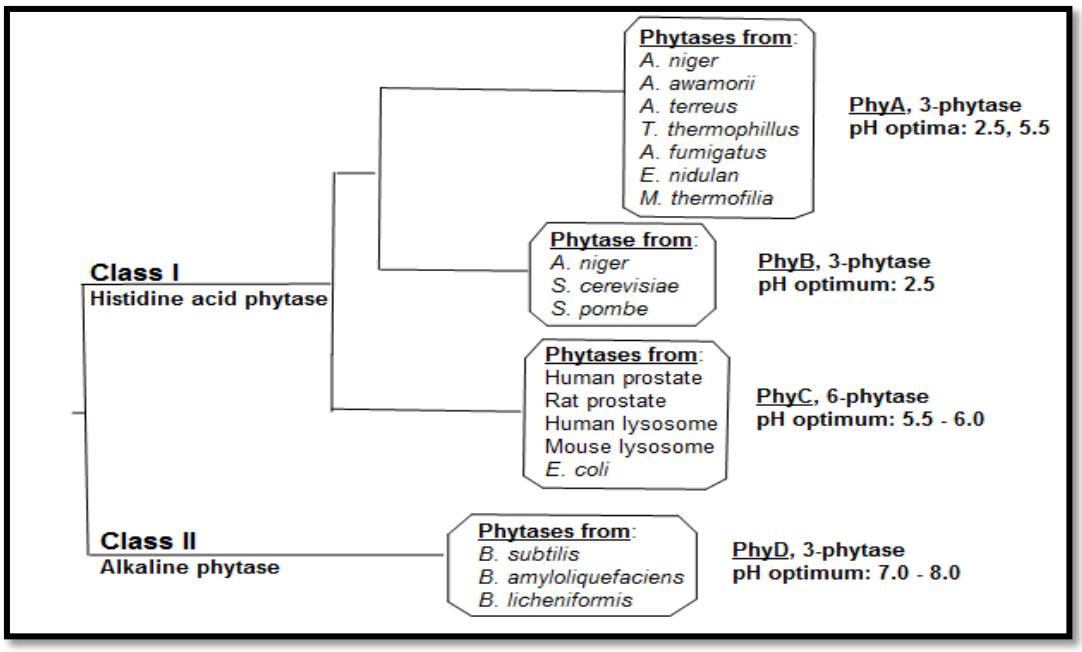


Figure 2.5. Classification of phytases based on their sequence analysis and biochemical properties [Oh and In, 2009].

Based on their active site geometry and catalytic mechanism, phytases are classified into histidine acid phosphatase phytases (HAP), β-propeller phytases (BPP) and Cysteine phosphatases or purple acid phosphatase phytases (PAP) [Bohn *et al;* 2008] and most recently, protein tyrosine phosphatase (PTP)-phytase [Chu *et al.,* 2004].

2.2.4.1. Histidine acid phosphatase (HAP)-phytase

The histidine acid phytase (HAP) group has been the most extensively studied, that it has a high specific activity for phytic acid [Tran, 2010]. Members of this group are found among prokaryotes (appA phytase from *E. coli*) and eukaryotes (PhyA and B from *Aspergillus sp.*, HAP phytases from yeast and

plants). All HAP phytases share a common active site motif, RHGXRXP, at the N-terminal end and a HD motif at the C-terminal end in their DNA sequences.

This allows a two-step mechanism in the hydrolysis of phosphor-monoesters, which has been investigated with site-directed mutagenesis studies and crystal structures of transition-state complexes. These results indicate that:

- The positive charge on the guanidinium group of arginine in the tri-peptide RHG interacts directly with the phosphate group of the substrate [Kerovuo, 2000], making it more susceptible to nucleophilic attack.
- The histidine residue serves as a nucleophile in the formation of a covalent phosphor-histidine intermediate.
- The aspartic acid residue from the C-terminal HD sequence motif protonates the leaving group. The PhyA and PhyB from *Aspergillus niger* and appA from *E. coli* are the representatives which have been the most characterized.

Three-dimensional structures of PhyA from *Aspergillus niger* and appA from *E. coli* are available in the protein data base (PDB). The proteins contain a large α/β-domain and a smaller α-domain with a relatively large and positively charged active site at the interface of these two domains [Baker and Augspurger, 2003]. Six residues related to substrate-specific sites of PhyA phytase were found to encircle the active site cavity.

On the other hand, four subunits of PhyB from *Aspergillus niger* are comprised of two identical dimers, where each monomer consists of a large α/β-domain and a smaller α-domain, forming an active site like the PhyA molecule. The substrate-specific sites of PhyB comprise only two acidic residues rendering the electrostatic surface at the active site of PhyB less positively charged than that of PhyA [Tran, 2010]. This has also been suggested to be the reason for the difference in PH profile of these two HAP phytases despite that they have the same RHGXRXP active site motif.

The DNA sequence analyses of yeast and maize phytases [Tran, 2010] has revealed that they also belong to the HAP phytase group with both RHGXRXP and HD conservative motifs.

2.2.4.2. β-propeller phytase

β-propeller phytases are not related to any other phytases or phosphatases in sequence databanks, nor do they contain the conserved HAP phytases active site sequence motif RHGXRXP. Unlike HAP phytases, these enzymes are dependent on Ca^{+2} ions for stability and activity, implying a different mode of IP6 hydrolysis [Kerovuo, 2000]. X-ray diffraction data of β-propeller phytase has revealed a six-bladed propeller structure which has been found in a range of proteins and exhibits a notable functional diversity [Ha *et al;* 2000]. There are six calcium-binding sites in each protein molecule. Calcium ions facilitate the binding of substrate by providing a favorable electrostatic environment [Lei and Porres, 2003]. β-propelle phytases have two phosphate binding sites, a cleavage site and an affinity site [Caoa *et al;* 2007]. The phosphate in the cleavage site is hydrolyzed, while the affinity site enhances binding of substrates containing adjacent phosphate groups, such as IP6.

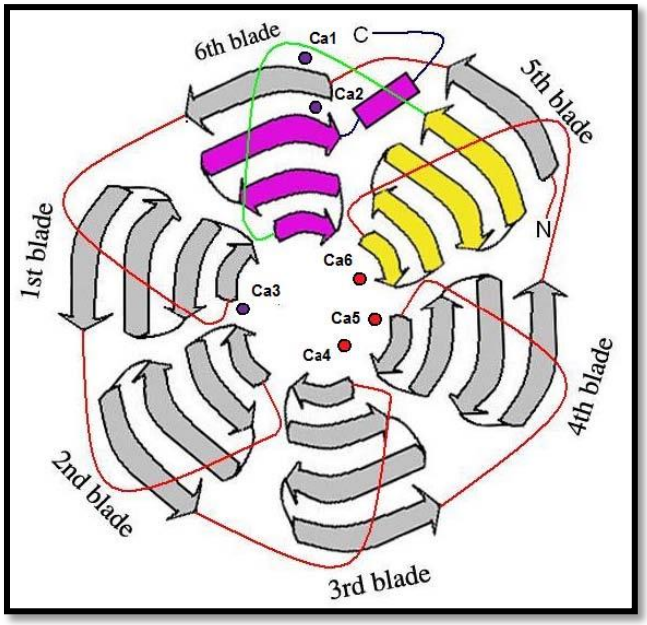


Figure 2.6. Scheme of the structure of β-propeller phytase [Caoa *et al;* 2007].

Most HAP phytases have pH-optima in the range of 4.5-6.0, and the β-propeller phytases have PH optima between PH 6 and 7.5 [Tran, 2010]. Hence, HAP phytases can work in the upper part of an animal's intestinal tract where acidic conditions prevail, whereas β-propeller phytases can work in the lower parts, such as the small intestine, where neutral and alkaline conditions are predominant.

2.2.4.3. Purple acid phosphatase (PAP)-phytase

PAP phytases share conserved metal-ligating residues which form binuclear Fe (III)-Me (II) centers, where Me is (Fe, Mn, or Zn). The metal ions are coordinated by seven invariant amino acids, which are essential features of the active site found in all PAP sequences. A metal-bridging or metal coordinated hydroxide ion in the metal centers has been suggested to directly attack the phosphorus atom of the substrate [Lei *et al;* 2007]. Recent mutagenesis experiments have begun to identify the roles of key active site residues of PAP phytases [Noorbatcha *et al;* 2009] to determine its precise catalytic mechanism.

2.2.4.4. Protein tyrosine phosphatase (PTP)-phytase

The phytase from *S. ruminantium* shares no sequence homology and structural similarity with other microbial phytases. The crystal structures of this phytase revealed the presence of two domains with a dual-specificity phosphatase fold. The large domain is having a protein tyrosine phosphatase (PTP)-like fold and contains a slightly twisted five-stranded β sheet (β2, β3, β11, β4, and β10) that is sandwiched by two (αB and αD) and three helices (αE, αF, and αG) on the two sides, and the other small domain contains a five-stranded β barrel (β8, β7, β1, β6, and β5) and a short β strand (β9), and the active site is located near a conserved cysteine-containing (Cys241) P loop. It was also suggested by [Chu *et al;* 2004] that this enzyme has different phosphatase mechanism.

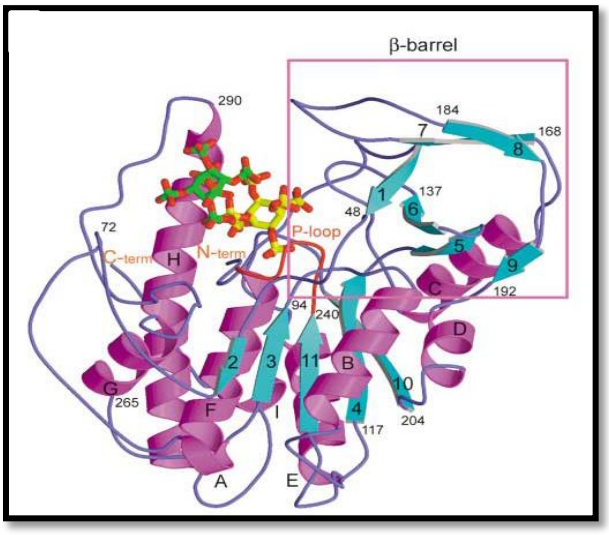


Figure 2.7. Overall structure of *Selenomonas ruminantium* phytase (the small domain is indicated with a box) [Chu *et al;* 2004].

2.2.5. Applications of phytase

2.2.5.1. Application in feed

Despite of the presence of phytase in the GIT of monogastric animals (poultry, pigs, fish…), phytate phosphorus is still poorly available to these animals and is largely excreted into feces, since their intestinal enzymes are depressed by high levels of dietary IP6 , calcium, magnesium and Pi in the feed.

Thus, an addition of phytase instead of di-calcium phosphate to the feed is increasing [Turner, 2005].

Phytase has mainly been used as a feed supplement in diets for poultry, swine and to some extent for fish. This leads to a reduction of the costs of adding di-calcium phosphate to the feeds, as well as in phosphorus run-off from livestock manure to water streams, which creates the environmental problem of eutrophication in water bodies.

[Kim *et al;* 2006] estimated that 250 g of phytase could replace 10 kg of di-calcium phosphate in animal feeds, and that the actual demand of phytase enzyme for livestock was approximately 200 tons per annum. It was suggested from laboratory experiments and field trials that 500-1000 phytase units could replace approximately 1g of inorganic phosphorus supplementation and reduce total phosphorus excretion by 30-50% [Godoy *et al;* 2005].

The addition of microbial phytases to feeds has led to increase phytate phosphorus utilization in turkeys and chickens. [Bharathidhasan *et al;* 2009] found an increase in bone ash of one-day-old chickens fed with a corn-soya diet with phytase from *Aspergillus niger* together with the increase of available phosphorus 60% and the decrease of phosphorus in droppings 50%.

In addition, adding phytase to feed also increases the absorption of minerals and other nutrients (Ca, Fe, Zn, Mn, and Cu), which support growth performance and feed conversion ratio for the animals [Marlida *et al;* 2010].

The FDA has approved the phytase preparation as GRAS [Kerovuo, 2000]. Phytase in feeds can be inactivated by temperature during feed processing, by the low PH or pepsin in the upper part of the GIT of an animal.

Phytase for feed applications should therefore be heat tolerant, pepsin- and pancreatin-resistant [Tran, 2010], have a tolerance to low pH and work well in a broad PH range in both the upper and lower part of an animal's GIT and at the animal's body temperature [Marlida *et al;* 2010]. The neutral and slightly alkaline conditions in the lower part of the GIT have been reported to decrease the solubility of IP6-metal complexes thus lowering the efficacy of phytase.

2.2.5.2. Application in food

Besides a major application in the feed industry, phytase has also been found to be increasingly interesting for use in processing and manufacturing of foods for human consumption [Kerovuo, 2000].

Adults consume about 1 g of inositol per day from both animal and plant sources. This value could be as high as 4.5 g/day depending on the amount of plant-derived foods; an average intake of phytate is 2-2.6 g/day for vegetarian diets and inhabitants of rural areas in developing countries [Greiner and Konietzny, 2006]. An increased dietary consumption of cereal fibers, legumes and soy protein leads to a higher intake of phytate. Vegetarians eating mostly wholegrain products and extruded cereals, elderly people consuming unbalanced foods with large amounts of cereals, people in developing countries who eat unleavened bread and babies eating soy-based infant formulas take in large amounts of phytate [Tran, 2010].

Although phytate has been reported to be an antioxidant with anticancer activity, its negative effect related to mineral deficiency [Nielsen *et al;* 2007] is much more severe in undeveloped and developing countries, small amounts of phytate (< l µmole/g) had a strong negative effect on iron solubility. Different strategies have been developed to reduce the risk of mineral deficiency in vulnerable groups such as child-bearing women, strict vegetarians, inhabitants of developing countries, particularly fast-growing children, but none has been very successful [Olempska-Beer *et al;* 2006]. Some suggestions for reducing the phytate in foods have been given by [Greiner and Konietzny, 2006], include producing low phytate mutants in maize, barley, rice and soy bean, enhancing levels of seed phytase by gene transfer, eating more germinating seeds, using yeasts, lactic acid bacteria and other microorganisms producing phytase for bread making and fermented foods, and employing non-enzymatic hydrolysis

of phytate efficiently during food processing (such as soaking, discarding the cooking water). Since only a very low phytate-degrading activity occurs in the human intestines, supplemental phytases to degrade phytate during food processing and in the GIT has been suggested [Singh et al; 2011].

Phytase has a wide range of applications in human nutrition, The addition of phytase can improve the nutritional value of plant-based foods by enhancing mineral availability and protein digestibility through phytate hydrolysis during digestion in stomach or during food processing [Meor Hussin *et al;* 2009]. The phytase for food applications would need to be thermo stable enough to withstand cooking and also resistant to the acidic conditions of the stomach as well as to the digestive enzymes; otherwise it would work only before the food processing and during the storage time.

[Sandberg and Svanberg, 2006] reported that supplement of phytase to flour containing wheat bran would help to increase the iron absorption in human. When inositol hexa and penta phosphates of wheat bran and rye flour were completely hydrolyzed by activating endogenous phytase, iron solubility under simulated physiological conditions increased from 3 to 53% (wheat) and 5 to 21% (rye). It has also been proposed by [Sandberg and Svanberg, 2006] that addition of wheat phytase to uncooked oatmeal increases iron solubility from 4 to 11% and in precooked to 18%, while endogenous phytase of uncooked oatmeal has less effect on phytate digestion and iron solubility. Addition of phytase during bread making not only lowers the phytate level, but also releases calcium from the IP6-Ca complex for α-amylase, hence providing an indirect improvement of the bread quality [Meor Hussin *et al;* 2009]. Phytases from plant and GRAS (generally regarded as safe) microorganisms such as baker's yeast, lactobacilli and bifidobacteria are preferred since they generally do not cause allergic responses in human [Sanz-Penella *et al*; 2009].

In order to improve mineral availability, degradation of inositol hexakis- and pentakis-phosphates is essential [Kerovuo, 2000]. However, positive effects of phytate and its lower phosphate derivatives for health (discussed in 2.1.5) should be considered in the diet of inhabitants in developed countries. Feeding phytase or low-phytic acid ingredients to food producing animals does not likely cause any health problems [Agostini *et al;* 2010] since animals live for a

relatively short period of time and do not normally receive high levels of dietary iron. However, low-phytic acid diets may have potentially adverse effects on human health, in particular for people with high iron stores caused by elevated dietary intakes of highly available iron from animal products or high dietary intakes of fruits that greatly enhance the absorption of non-heme iron. The presence of undigested phytate in the colon may provide protection against the development of colonic carcinoma [Mullaney and UIlah, 2007].

Phytase has the potential as an exceptional bread making improver. The supplementation of phytase (EC 3.1.3.8) in the dough ingredients containing fiber formulation leads to an acceleration of the proofing, an improvement of the bread shape, a slight increase of the specific volume, and also confers softness to the crumb [Afinah *et al;* 2010].

Recently, novel bacterial phytate-degrading enzymes from bifidobacterial strains are incorporated in wheat dough as a fermentation starter replacing the common lactic acid bacteria [Ozusaglam, 2009].

2.2.5.3. Preparation of Myo-inositol phosphates

The growing list of pharmaceutical applications of Myo-inositol phosphate derivatives has increased interest in the preparation of these compounds.

The chemical synthesis of Myo-inositol phosphates includes difficult protection and de-protection steps, and is performed at extreme temperatures and pressures. The separation of Myo-inositol phosphates isomers has also been reported to be difficult with most analytical approaches [Billington, 1993]. Since phytases hydrolyze Myo-inositol hexakis-phosphate sequentially, the production of Myo-inositol phosphate derivates and free Myo-inositol using phytase is a potential alternative to chemical synthesis.

The preparation of D-Myo- inositol 1, 2, 6-trisphosphate, D-Myo-inositol 1, 2, 5-tris-phosphate, L-Myo-inositol 1, 3, 4- tris-phosphate and Myo-inositol 1, 2, 3-tris-phosphate by enzymatic hydrolysis of phytic acid by *Saccharomyces Cerevisiae* phytase has been described by [Siren, 1986]. Immobilized phytases have been used to produce various Myo-inositol phosphates. Starting with immobilized phytase from *E. coli*, [Greiner and Konietzny, 1996] prepared

inositol 1, 2, 3, 4, 5-pentakis-phosphate, inositol 2, 3, 4, 5-tetrakis-phosphate, inositol 2, 4, 5-tris-phosphate and inositol 2, 5-bis-phosphate.

Naturally, the advantages of enzymatic hydrolysis are stereo-specificity and mild reaction conditions [Dvorakova, 1998].

2.2.5.4. Pulp and paper industry

The removal of plant phytic acid is important in the pulp and paper industry. A thermo stable phytase could have potential as a novel biological agent to degrade phytic acid during pulp and paper processing. The enzymatic degradation of phytic acid would not produce carcinogenic and highly toxic by-products [Kerovuo, 2000]. Therefore, application of phytases in the pulp and paper process could be environmentally friendly and would assist in the development of cleaner technologies.

2.2.5.5. Combating environmental phosphorus pollution

Phytase improves phosphor utilization efficiency in monogastrics, reducing the phosphor excreted by manure (the most effective factor in environment contamination); the phosphorus thus released is transported into the water bodies causing eutrophication [Musapuor *et al;* 2006]. This results in oxygen depletion due to excessive algal growth. So phytases are very well known to reduce pollution caused by excess of phosphorus accumulation in soil and water, helping in environmental balance and maintenance.

2.2.6. Potential biotechnological applications of phytase

Because of the potential value of phytases for improving the efficiency of phosphorus use, biotechnology has led the rapid development of the field to its current stage. With the development of heterologous gene expression, large amounts enzymes could be produced at relatively low cost.

2.2.6.1. Microbial expression systems

A transgenic approach was proved to be a powerful tool for expressing specific phytase genes on a large scale in yeast strains, which was verified to be an elite expression system for heterologous genes. Several studies have already investigated the use of various yeast expression systems as an alternative to

the current phytase production method using overexpression in filamentous fungi [Singh and Satyanarayana, 2010]. It was found that phytases used in ecotopic expression could be derived from a host of micro-organism sources. The latter expression system offers the possibility of combining phytase with beneficial probiotic lactic acid bacteria [Westers *et al;* 2004].

2.2.6.2. Transgenic plants and animals

Transgenic plants might contain sufficient phytase activity that they could replace additional supplementation of feed and food with microbial phytases, thereby reducing the downstream processing and formulation costs involved in the commercial production of phytases [Tran, 2010]. Alternatively, transgenic plants could be used as bioreactors for the production of phytase as a supplement. Meanwhile, transgenic animals producing endogenous phytase along with other digestive enzymes are known to increase the bioavailability of plant phytate, which in turn leads to reduced phosphorus output in manure.

2.2.7. Regulation of phytase formation

In bacteria, phytase is an inducible enzyme and its expression is subjected to a complex regulation, but phytase formation is not controlled uniformly among different bacteria. Until now, phytase production was studied in some detail only in *Escherichia coli* and *Raoultella terrigena*. In non-limiting media the formation of both the *Escherichia coli* and the *Raoultella terrigena* phytase was turned off in exponentially growing bacteria and started as soon as the cultures entered the stationary phase. Because the synthesis of the enzymes started as soon as the growth rate began to fall, it was suggested that either a nutrient or an energy limitation, known to occur in the stationary phase, could be at the origin of its induction. Among the nutrient limitations tested, only carbon starvation was able to provoke an immediate synthesis of the *Raoultella terrigena* phytase, whereas in *Escherichia coli*, phytase synthesis was triggered, when bacteria were starved for inorganic phosphate, while carbon, nitrogen and sulfur limitation were ineffective. A tight regulatory inhibition of phytase formation by inorganic phosphate levels was generally observed in all microbial phytase producers, including moulds, yeast and bacteria, with the exception of *Raoultella terrigena* and the rumen bacteria. The repression of phytase synthesis by inorganic phosphate seems to be less

significant with higher medium composition complexities. It is not known, however, what components in the complex media account for the reduced repression [Tran, 2010].

In *Escherichia coli*, the primary response to the limitation of a specific nutrient was shown to be an activation of a certain set of genes that allow a better uptake of the nutrient present in low concentration or the utilization of other substances that belong to the same class of nutrients. These nutrient-specific systems include the cyclic AMP (cAMP) and its receptor the catabolite activator protein (CAP) for the use of alternative carbon sources, the NtrB/NtrC/σ54 regulon that is induced under nitrogen limitation, and the PhoB/PhoR regulon that is induced under phosphorus limitation. However, if the environment is totally exhausted for an essential nutrient, the cells enter into the stationary phase. The formation of several dozen proteins is stimulated during transition into stationary phase and a core set of proteins is induced regardless of the class of nutrient for which the cells are starved. As mentioned above, phytase formation in *Escherichia coli* is induced in non-limiting media upon entry into the stationary phase and under anaerobic conditions. The expression of the phytase-encoding gene *appA* was shown to be strongly dependent on the *rpoS*-encoded sigma factor σS, which has been identified as a central regulator for many stationary-phase-responsive genes. Very often σS-dependent genes are regulated by several promoters and only one of them is controlled by σS. Thus, not all genes identified as σS-controlled are entirely dependent on σS for expression. In minimal medium, starvation for phosphate, but not for glucose or ammonia, resulted in a strong stimulation of *rpoS* expression followed by an increase in phytase activity.

In addition, phytase expression depends on the nature of the carbon source used for growth. Glucose, which is known to cause catabolite repression, has been widely used to improve phytase production. That cAMP-CAP, rather than the carbon source itself, are directly involved in this regulation was shown in *Escherichia coli*. Synthesis of the phytases in both *Escherichia coli* and *Raoultella terrigena* have been reported to be negatively regulated by cAMP, which is suggested to be involved in the amphibolic metabolism of glucose and galactose as well as directly or indirectly in controlling the expression of an important stationary growth regulator. For example, *rpoS* transcription in

Escherichia coli was described to be negatively controlled by the cAMP-CAP complex.For several *Raoultella* sp. it was reported that phytate is needed to induce phytase production. Substrate induction was also found in *Mitsuokella jalaludinii*, whereas phytate had no effect on the formation of phytase in *Escherichia coli*. Phytase formation in *Pseudomonas* sp. and *Raoultella aerogenes* was reported to be significantly induced in the presence of *myo*-inositol as the sole carbon source. In the other *Raoultella* sp. studied *myo*-inositol was ineffective [Konietzny and Greiner, 2004].

2.2.8. In vivo function of bacterial phytase

The complex mode of regulation does not shed much light on the role of bacterial phytases. The stationary phase induction suggests that phytase is not required for balanced growth, and that this enzyme may be synthesized in response to a nutrient or energy limitation. This suggestion could also explain why there is, with the exception of sourdough bacteria, no clear evidence for lactic acid bacteria with phytase-producing ability. Lactic acid bacteria are adapted to environments rich in nutrients and energy and therefore, it never may have been an evolutionary selection for lactic acid bacteria with the capability to produce a phytase.

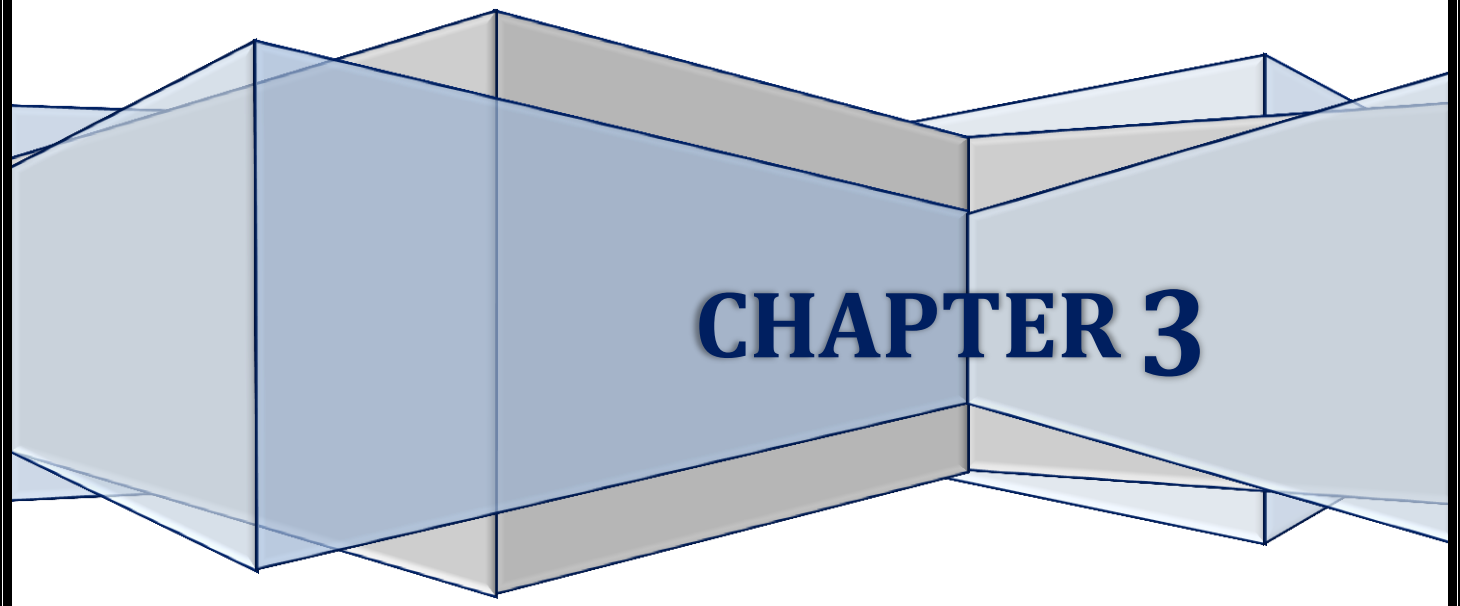
The efficient induction or depression of phytase formation by phosphate starvation in most bacteria raises the question of a possible role in providing the cell with phosphatehydrolysed from molecules such as phytate. This hypothesis is, for example, supported by the identification of a phytase in the stalk of *Caulobacter crescentus*, a gram-negative alphapurple proteobacterium, which is an oligotroph that lives in aquatic environments dilute in nutrients. Phosphate is the limiting nutrient in the environments in which *Caulobacte*r is found and one of the hypothesized functions of the stalk is phosphate uptake. Stalks elongate when phosphate is limiting and increasing the surface area available for phosphate uptake as well as the presence of a phytase would allow the uptake of the organically phosphate by the stalk.

In addition, ruminants seem to digest phytate through the action of phytase produced by microbial flora in the rumen. In contrast to most other bacteria, anaerobic rumen bacteria are capable of tolerating a high level of phosphate without any negative impact on phytase production. This unique ability leads to a more efficient phytate hydrolysis in the rumen, even under the high

phosphate levels in the rumen fluid of ruminants fed concentrated feed. The phosphate generated by splitting of phytate is utilized by both the microbial flora and ruminant host [Tran, 2010].

Because phytase formation was observed when bacterial cells had to adapt to environmental fluctuations imposed before the onset of growth or when actively growing cells are stressed, it was suggested that phytase could be involved in a signal transduction mechanism of metabolic regulation. Several *myo*-inositol phosphates have recently been identified in gram negative bacteria and are probably involved in signal transduction. For instance, *Salmonella dublin* excretes *myo*-inositol polyphosphate 4-phosphatase, which contributes to its virulence by subverting cellular *myo*-inositol phosphate signaling reactions. Furthermore, two proteins with *myo*-inositol monophosphate phosphatase activity implicated in the control of gene expression have also been reported in *Escherichia coli* and *Rhizobium leguminosarum*.

In higher plants phytases occur predominantly in grains, seeds and pollen. These enzymes were reported to be responsible for phytate degradation during germination to make phosphate, minerals and *myo*-inositol available for plant growth and development. A low phytase activity is also found in the plant root. Root phytase has been suggested as one of the mechanisms of plants to improve utilisation of soil phosphate. Organic forms of phosphate generally account for at least 50% of total soil phosphate and it is known that a major component occurs in form of inositol penta and hexakisphosphates. Due to the low phytase activity in roots and the inability of phytase secretion into the rhizosphere, however, phytate appears to be only poorly utilised by plants. Thus, it was suggested, that soil microorganisms colonizing the plant rhizosphere and producing extracellular phytase activity, such as *Bacillus* and *Enterobacter* ssp., could act as plant growth promoting rhizobacteria (PGPR) by making phytate phosphate available to the plant. Recently, the importance of phosphate availability from soil phytate for plant nutrition under phosphate limitation was demonstrated by enabling the plants to utilise phytate phosphate through expression of extracellular secreted *Aspergillus* phytase in the plant root and by adding of purified phytase as well as soil microorganisms expressing extracellular phytase activity to the rooting medium [Konietzny and Greiner, 2004].



CHAPTER 3

Experimental strategy

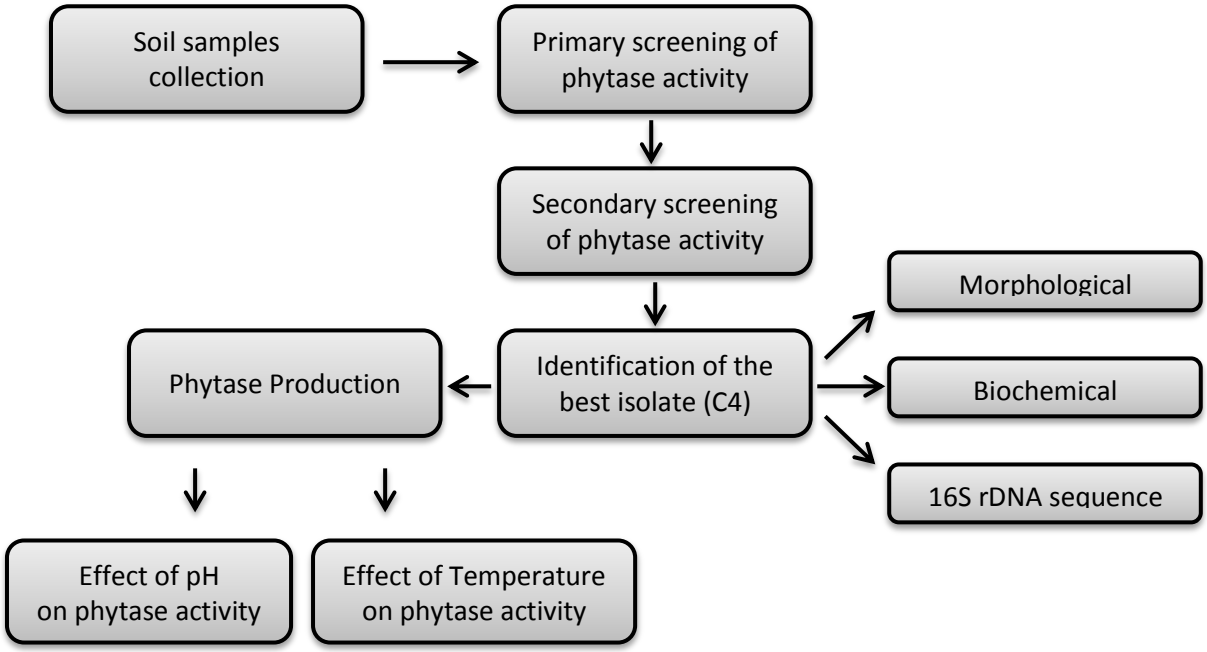


Figure 2.8. Flow chart elucidating the general experimental plan.

* * * * *

Sodium phytate was purchased from Sigma Chemical Corporation.
All other used chemicals were of the analytical grade commercially available.

3.1. Isolation and screening of phytase producing bacteria from soil

3.1.1. Soil samples collection

Total of Thirty [(20) soil samples of Legumes, (5) cattle shed soil samples and (5) poultry farm soil samples] were collected from various regions in Damascus countryside, by sterilized sampling tools and preserved in sterilized plastic bags at (4) °C. One gram of each collected soil sample was suspended in (10) ml of sterile distilled water, and the mixture was shaken at (30) °C for (1) h [Hosseinkhani *et al;* 2007]. Dilutions of (10^{-2}) to (10^{-6}) of each sample were prepared, (3) µl of each dilution was dropped on the LB media plates and then incubated at (37) °C for (24) h, to determine the appropriate dilution of each soil sample to be plated on the selective media later.

3.1.2. Primary screening of phytase activity

To screen phytase producing bacteria effectively, plate agar method was used using phytase screening media [Glucose (1.5) %, $(NH4)_2SO4$ (0.5) %, $MgSO4.7H_2O$ (0.01) %, KCL (0.05) %, NaCl (0.01) %, $CaCl_2.2H_2O$ (0.01) %, FeSO4 (0.001) %, MnSO4 (0.001) %, Sodium Phytate (0.5) %]. The pH was adjusted to 7.0 using 1N HCl and (1.5) % agar was added before autoclaving at (121) °C for (15) min. To insure the effectiveness of the prepared phytase screening medium PSM the following bacterial genera (*Bacillus, Enterobacter, Pseudomonas and Serratia*) had been plated on PSM plates and incubated at (37) °C for (3) days as positive controls (Figure 4.1).

Then (100) µl of each soil sample appropriate dilution was spreaded directly on PSM plates then the inoculated plates were incubated at temperatures of (37) °C for (1-3) days and observed for the clear zones of hydrolysis around the colonies (Figure 4.2) which gave an indication of extracellular phytase production [Shamna *et al;* 2012]. Each such colony was picked up and maintained till further use. Screening for best phytase producing strain microbial colonies capable of hydrolyzing phytate which can be recognized by their surrounding clear halo were obtained by re-plating single colonies.

The halo (Z) and colony (C) diameters were measured after (3) days of incubation at (37) °C. Hydrolysis efficiency of all the isolates was determined by the formula Z-C/ C [Fitriatin *et al;* 2009] [Fitriatin *et al;* 2011]. Then all the obtained phytase producing isolates had been selected and stored at (-20) °C to be used in this study.

Storage procedure for bacterial isolates

Preservation of the selected bacterial isolates had been done by the following steps:

- Growing the selected isolate in LB Broth with incubation at (37) °C for (24) h.
- Sloping (1) ml of the obtained culture in an Eppendorf tube.
- Performing centrifugation at (5000) rpm for (5) min.
- Adding (500) µl of LB Broth and (500) µl of Glycerol (80) % to the culture precipitance with vortex.
- Preserving the obtained bacterial stock at (-20) °C or (-80) °C.

3.1.3. Crude enzyme preparation and phytase activity assay

Enzyme sample preparation

The production of enzyme was carried out in LPSM medium without addition of agar LPSM using Shaken flask fermentation method.

The inoculum of the selected strain was produced during (24) h using LB Broth. (10^6) cell/ml of inoculum was inoculated in (30) ml of production medium taken in (100) ml conical flask.

The flask was then incubated at (37) °C for (3) days at shaken condition at (200) rpm for better aeration and growth of organism.

The fermented broth from the flask was transferred every day into centrifuge tubes and centrifuged at (4000) rpm for (15) min at (4) °C.

The supernatant was then transferred into clean test tube and stored at (4) °C to be used in this study as crude enzyme solution [Gulati *et al;* 2007] for determination of phytase activity according to standard phytase activity assay.

Determination of phytase activity (Molybdate-Blue Method)

Determination of phytase activity is based on the colorimetrical quantification at (700) nm of free phosphorus released by the hydrolysis of phytate using ammonium molybdate as color reagent.

The enzymatic activity was measured by a modification of the Heinonen-Lahti method using sodium phytate as substrate [Heinonen and Lahti, 1981].

One unit (U) of phytase was defined as the amount of enzyme required to liberate one micromole inorganic orthophosphate per minute under test conditions [pH (7.0), temperature (37) °C, substrate concentration (0.0051) mol/L].

The used materials:

- Water: Distilled water, or equivalent.
- Buffer solution (0.1) mol/L: Dissolve (5.742) g sodium acetic acid, (0.5) g Triton (100X) and (0.5) g bovin serum albumin in (900) ml water, adjust to pH (5.0) with acetic acid (100) %, and dilute to (1) L with water.
- Substrate solution: Dissolve (577.4) mg sodium phytate ($C_6H_6O_{24}P_6Na_{12}$) from rice and (574.2) mg sodium acetic acid in (90) ml water, adjust the pH to (5.0) with acetic acid (100) %, and dilute to (100) ml with water. Prepare this solution fresh daily.
- Reaction stop solution: Trichloroacetic acid (5) %.
- Ammonium hyptamolybdate stock solution (Solution A): Dissolve (7.5) g ammonium heptamolybdate ($N_6H_{24}Mo_7O_{24}.4H_2O$) in (400) ml distilled water, slowly add (22) ml sulfuric acid (98) %, and dilute to (500) ml with water. This solution may be kept at (4) °C shielded from light for (1) month.
- Ferrous sulfate stock solution (Solution B): ferrous sulfate (2.7) %. This solution may be kept at (4) °C shielded from light for (1) month.
- Color mix: mix (100) ml solution A and (25) ml solution B. Prepare this solution fresh daily.
- Potassium dihydrogen phosphate stock solution: Prepare potassium dihydrogen phosphate to constant weight at (60) °C before dissolving it to final concentration of (4) mmol/L using buffer solution.
  Prepare this solution fresh daily.

Method of work:

- Preparation of standard solutions and curve:

Prepare working standards of (0.0, 0.8, 1.6, 2.4, 3.2, 4.0) mmol/L potassium dihydrogen phosphate solution by serial dilution of stock solution. Carry out the procedure as described in (Table 3.1) and then plot the absorbance differences of the standard solutions (X-axis) against the corresponding exactly calculated amount of potassium dihydrogen phosphate (Y-axis). Draw the best fitting curve through the origin and give the regression equation (Y=KX+B).

- Preparation of sample:

Dilute the weighted sample in duplicate (sample and blank) with (0.03-0.08) FTU/ml.

A standard sample with exactly calculated activity is recommended to be determined as the same procedure to test the accuracy.

- Assay:

The assay is carried out as the following procedure (Table 3.1). In this procedure, interval of adding reagents to every tube should be completely coincident after the substrate is added to the reaction mixture.

Table 3.1. Procedure of phytase activity assay.

Procedure	Sample, Standard	Sample, Blank	Standard, Blank
Sample Solution (ml)	0.2	0.2	-
Buffer Solution (ml)	-	-	0.2
5 min at 37 °C	✓	✓	-
Substrate Solution (ml)	0.8	0.8 (the second step)	0.8 (the second step)
Mixing	✓	✓	✓
60 min at 37 °C	✓	-	-
Stop Solution (ml)	1.0	1.0 (the first step)	1.0 (the first step)
Mixing	✓	✓	✓
Color Reagent (ml)	1.0	1.0	1.0
Mixing	✓	✓	✓
Total Volume (ml)	3.0	3.0	3.0

Centrifuge all the tubes for (10) min at (4000) rpm before standing for (10) min at room temperature. Measure the absorbance of sample (A) and its blank (A_0) at (700) nm with standards blank of. Determine the enzyme activity by reading the corrected absorbance for the sample ($A-A_0$) and calculating the released phosphorus. Enzyme activity is expressed in activity units (FYU).

(1) FYU is the amount of enzyme that liberates (1) µmol inorganic orthophosphate per minute under test conditions (pH 7.0, temperature 37 °C, and substrate concentration at 0.005 mol/L).

- Calculation and expression:

Activity of sample (U) is calculated as follows:

[$U= K*(A-A_0)/S*m*60 \times F$]

U: slop of sample, U/g;

F: Dilution multiple;

S: Determination amount of sample, S= 0.2 (ml) in (Table 3.1);

m: Sample weight, g;

60: Time of reaction, min.

The final result is from two average values, and should be expressed by whole number.

Determination of protein concentration

The protein assay, which is based on the method of Bradford [Bradford, 1976] is a simple and accurate procedure for determining the concentration of solubilized proteins. It involves the addition of coomassie blue dye that binds to primarily basic and aromatic amino acid residues, in which a differential color change of a dye occurs in response to various concentrations of protein. Color variations are measured at (595) nm wave length with a spectrophotometer or a microplate reader.

Comparison to a standard curve, using known BSA concentration, provides a relative measurement of protein concentration.

The used materials:

- BSA (1) µg/µl.
- (5X) dye reagent Bradford (BioRad).
- Whatman #1 filter (BioRad).

Method of work:

- Dye reagent Bradford (1X) was prepared by diluting (1) part concentrated dye reagent with (4) parts ddH_2O. Then it was filtered through Whatman #1 filter to remove particulates.
- Five dilutions of a protein standard were prepared, which are representative of the protein solution to be tested. The linear range of the assay for BSA is (0.2) to (1) µg/µl.
- (10) µl of each standard and sample solution was added into a clean, dry microplate. Protein solutions are normally assayed in duplicate.
- (200) µl of diluted dye reagent were added to each well and mixed well, and then the microplate was incubated at RT for at least (5) min.
- Absorbance will increase over time thus samples should be incubated at RT for no more than (1) h.
- Absorbance was measured at (595) nm using (Thermo Labsystem/well wash 4MK2).

3.1.4. Secondary screening of phytase activity

A promising isolates (R5-C2-C4) showed the best hydrolysis efficiency on PSM plate were selected to investigate for phytase production in LPSM. (10^6) cell/ml of inoculum was inoculated on (30) ml of production medium taken in (100) ml conical flask. The flask was then incubated at (37) °C for (72) h at shaken condition at (200) rpm. The culture broth was sampled with (24) h intervals for determination of phytase activity. Since growth study was essential for the production of extracellular enzymes, the effect of incubation time had been studied by shaken flask fermentation method on the selected isolate (C4) for (96) h by proceeding in the same previous mentioned experiment conditions. The culture broth was sampling for the first time after incubation for (18) h then with (24) h intervals for determination of phytase activity.

3.2. Identification of the highest phytase activity bacterial isolate

Taxonomic characteristics of strain (C4) were determined for the morphology and biochemical test as well as 16S rDNA sequencing analysis.

3.2.1. Morphological characteristics and Gram staining

The isolate (C4) was identified based on the identification scheme in Bergey's Manual of Systematic Bacteriology. The strain was initially examined for cell morphologies and cell arrangement by gram staining, presence or absence of spores and capsules and motility using microscopy [Singh *et al;* 2013].

3.2.2. Biochemical properties

3.2.2.1. Indole Test

Principle of Indole Test

Indole test is used to determine the ability of an organism to spilt amino acid tryptophan to form the compound indole. Tryptophan is hydrolysed by tryptophanase to produce three possible end products (one of which is indole). Indole production is detected by Kovac's or Ehrlich's reagent which contains 4 (p)-dimethylamino benzaldehyde, this reacts with indole to produce a red coloured compound.

Two methods are in used;

- A spot indole test, which detects rapid indole producing organisms and
- A conventional tube method requiring overnight incubation, which identifies weak indole producing organisms.

Procedure for Indole Test

- Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth.
- Incubate at (37) °C for (24-28) hours in ambient air.
- Add (0.5) ml of Kovac's reagent to the broth culture.
- Positive:

Pink colored rink after addition of appropriate reagent.

- Negative:

No color change even after the addition of appropriate reagent.

3.2.2.2. Nitrate Reduction Test

Principle of Nitrate Reduction Test

This test determines the production of an enzyme called nitrate reductase, resulting in the reduction of nitrate (NO_3): the test is also called the nitrate reduction test. With this enzyme, nitrate is reduced to nitrite (NO_2). It then forms nitrous acid that reacts with the first reagent sulfanilic acid, and that reacts with the other reagent naphthylamine to form a red color.

Reduction of nitrate is generally an anaerobic respiration in which an organism derives its oxygen from nitrate.

The objective is to identify the different ways that nitrate can be reduced by bacteria.

Procedure for Nitrate Reduction Test

- Inoculate nitrate broth with an isolate and incubate for (48) hours.
- Add two nitrate tablets to the sample.
- If the bacterium produces nitrate reductase, the broth will turn a deep red within (5) minutes at this step.
- If no color change is observed, then the result is inconclusive.
- Add a small amount of zinc to the broth.
- If the solution remains colorless, then both nitrate reductase and nitrite reductase are present.
- If the solution turns red, nitrate reductase is not present.

3.2.2.3 Catalase Test

Principle of Catalase Test

Catalase is the enzyme that breaks hydrogen peroxide (H_2O_2) into (H_2O) and (O_2). Hydrogen peroxide is often used as a topical disinfectant in wounds, and the bubbling that is seen is due to the evolution of (O_2) gas. (H_2O_2) is a potent oxidizing agent that can wreak havoc in a cell, because of this, any cell that uses (O_2) or can live in the presence of (O_2) must have a way to get rid of the peroxide. One of those ways is to make catalase.

Procedure for Catalase Test

- Place a small amount of growth from your culture onto a clean microscope slide.
- Add a few drops of (H_2O_2) onto the smear. If needed, mix with a toothpick. DO NOT use a metal loop or needle with (H_2O_2), it will give a false positive and degrade the metal.
- A positive result is the rapid evolution of (O_2) as evidenced by bubbling.
- A negative result is no bubbles or only a few scattered bubbles.
- Dispose of your slide in the biohazard glass disposal container. Dispose of any toothpicks in the Pipet Keeper.

3.2.2.4. Oxidase Test

Principle of Oxidase Test

Basically, this is a test to see if an organism is an aerobe. It is a check for the presence of the electron transport chain that is the final phase of aerobic respiration. Normally, oxygen is the final electron acceptor for this system. In the oxidase test, an artificial final electron acceptor (N, N, N', N'-tetramethyl phenylenediamine dihydrochloride) is used in the place of oxygen. This acceptor is a chemical that changes color to a dark blue/purple when it takes the electron from the last element (cytochrome oxidase) in the electron transport chain.

Procedure for Oxidase Test

- To open a new reagent dispenser: Hold reagent dropper upright and point tip away from yourself. Grasp the middle with thumb and forefinger and squeeze gently to crush the glass ampule inside the dropper. Tap the bottom on the tabletop a few times. Invert the ampule and squeeze gently for drop-by-drop dispensing.
- With a sterile swab, obtain a small amount of organism from an agar slant or plate.
- Place one drop of reagent onto the culture on the swab.
- Positive reactions turn the bacteria violet to purple immediately or within (10) to (30) seconds. Delayed reactions should be ignored.

3.2.2.5. Urease Test

Principle of Urease Test

Many organisms have a urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

Procedure for Urease Test

- Streak the surface of a urea agar slant with a portion of well isolated colony. Alternatively, slant can be incubated with (1-2) drops of overnight brain-heart infusion broth.
- Leave the cap on loosely and incubate the test tube at (35) °C in ambient air for (48) hours to (7) days.
- If organism produces urease enzyme, the color of the slant changes from light orange to magenta. If organism does not produce urease the agar slant and butt remain light orange (No color change).

3.2.2.6. H_2S Production

Principle of H_2S Production Test

Hydrogen sulfide production from thiosulfate is indicated by a blackening of the butt as a result of the reaction of H_2S with the ferrous ammonium sulfate to form black ferrous sulfide.

The black precipitate indicates that the bacteria were able to produce hydrogen sulfide H_2S from sodium thiosulfate.

Because H_2S is colorless, ferric ammonium citrate is used as an indicator resulting in the formation of insoluble ferrous sulfide.

Formation of H_2S requires an acidic environment; even though a yellow butt cannot be seen because of the black precipitate, the butt is acidic.

The results would be recorded as acid over acid (A/A), H_2S positive.

Procedure for H_2S Production Test

- An inoculum from a pure culture is transferred aseptically to a sterile triple sugar iron agar (TSIA) slant.
- The inoculated tube is incubated at (35-37) °C for (24) hours and the results are determined.
- Present in TSIA is an iron compound.
- The iron ions (Fe2+) have a high affinity (strong attraction) for sulfide ions.
- The result is that H_2S combines with the iron to make FeS, a black compound. In tubes of TSIA containing bacteria producing hydrogen sulfide, the agar turns black from the FeS.

API is a standardized system combining biochemical tests that offer wide spread capabilities. The API consists of micro-tubes used to study fermentation of substrates belonging to the carbohydrate family and its derivatives (heterosides, polyalcohols, uronic acids). The isolate (C4) was examined for its biochemical properties using API 50 CH System (API, BioMerieux). API kit was used according to manufacturer's instructions.

API 50 CH Procedure:

- Prepare an incubation box (tray and lid) and distribute about (10) ml of distilled water into the honeycombed wells of the tray to create a humid atmosphere.
- Prepare the inoculum in the appropriate medium by solving the bacterial colonies in saline solution (0.85) % and make a dense suspension with turbidity greater than (2) McFarland.
- Distribute the bacterial suspension using a sterile pipette into the API tubes.
- Incubate the strips at the (37) °C in aerobic conditions for (48) h.
- After the end of the incubation, the results were read.
- The results were integrated using the API Web software.

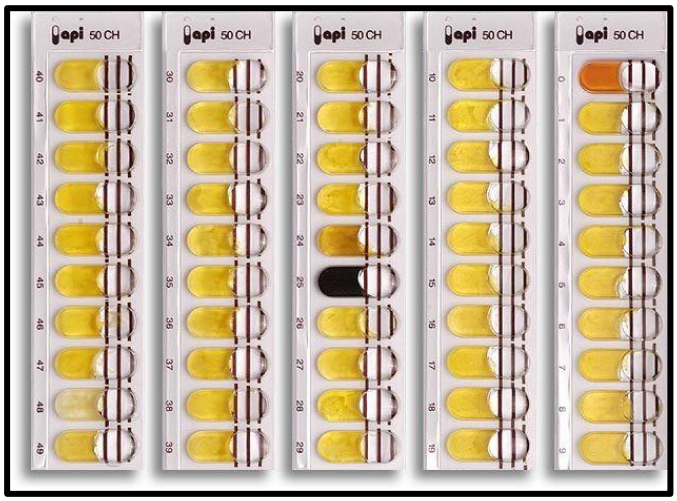


Figure 3.1. API 50 CH Strip.

3.2.3. 16S rDNA sequence analysis

For the sequence analysis [Singh *et al;* 2014], bacterial genomic DNA was extracted and purified using CTAB method.

The used materials:

- TE buffer.
- (10) % (W/V) SDS (Sodium Dodecyl Sulfate).
- (20) mg/ml Proteinase K [stored at - (20) °C].
- (5) M NaCl.
- CTAB/NaCl Solution [weight (4.1) g of NaCl in (70) ml H_2O then add (10) g CTAB slowly while heating and stirring, heat to (65) °C to dissolve if necessary, at the end adjust final volume to (100) ml by H_2O].
- 24:1 Cloroform/ Isoamyl alcohol.
- 25:24:1 Phenol/ Chloroform/ Isoamyl alcohol.
- Isoprobanol.
- (70) % Ethanol.

Method of work:

- Grow (5) ml bacteria culture to saturation [ON at (37) °C].
- Centrifuge (1.5) ml of the culture (2) min at max speed.
- Re-suspended pellet in (567) µl TE buffer by repeated pipetting or vortexing.
- Add (30) µl of (10) % SDS.

- Add (3) µl of (20) mg/ml Proteinase K, mix, and incubate (1) h at (37) °C with shaking (1000 rpm) in the thermo-mixer machine.
- Add (100) µl of (5) M NaCl and mix thoroughly.
- Add (80) µl CTAB/NaCl solution, mix, and incubate (10) min at (65) °C with shaking (1000 rpm) in the thermo-mixer machine.
- Add equal volume (~700 µl) chloroform/ Isoamyl alcohol, mix and centrifuge (5) min at max speed.
- Transfer the supernatant to fresh tube.
- Add equal volume (~600 µl) Phenol/ Chloroform/ Isoamyl alcohol, mix, and centrifuge (5) min at max speed.
- Transfer the supernatant to a fresh tube.
- Add (0.6) vol isoprobanol (~300 µl) and mix gently until DNA precipitants. [Sample (100) µl + (60) µl Isopropanol].
- Centrifuge (5) min at max speed, and then discard the supernatant.
- Wash with (1) ml of (70) % ethanol, mix and centrifuge (5) min at max, and discard the supernatant.
- Dry for (10) min in speed vac/ (45) °C, and re-suspend in (100) µl TE buffer.
- Read DNA concentration using (2) µl in nanodrop machine with TE as blank.
- Make concentration up to (100) ng/ µl in each sample. TE to be added to obtain the desired concentration =~ (98*conc/ 100) – (98) in ul.
- Store DNA at (4) °C short term, - (20) °C or - (80) °C long term.
- Dilute your DNA to obtain final concentration of (20) ng/ µl (1/5 dilution).
- Take 5 µl of each DNA sample (total of 100 ng) in PCR tube (0.2) ml.

Two primers annealing at the 5′ and 3′ end of the 16S rDNA were

Forward: 5′- AGAGTTTGATCCTGGCTCAG -3′,

Reverse: 5′-TACCTTGTTACGACTT -3′. [Jorquera and Mora, 2010].

PCR amplification was performed in a final reaction volume of (100) µl.

The PCR reaction was run for (35) cycles in a DNA thermal cycler.

Table 3.2. The 16S rDNA PCR amplification conditions.

Parameters	Temperature (°C)	Time (min/sec)
Initial denaturation	95	5 min
35 cycle	-	-
Denaturation	94	40 sec
Primer annealing	52	20 sec
Extension	72	2 min
Final extension	72	10 min

The amplified PCR products were then analyzed in a (2) % (w/v) agarose gel, excised from the gel, and purified.

Agarose gel electrophoresis is used to determine the size of DNA fragments. In this method, DNA is forced to migrate through a highly cross-linked agarose matrix in response to an electric current. In solution, the phosphates on the DNA are negatively charged, and the molecule will therefore migrate to the positive pole. The samples were separated on (2) % agarose gel.

The used materials:

- TAE buffer (50x) [(2) M Tris, (5.7) % Acetic acid, (50) mM EDTA, PH (8.0)].
- Ethidium bromide (1) mg/ml (Sigma)
- Agarose (Sigma)
- (6x) DNA Loading buffer: (0.10) % Xylene cyanol, (0.10) % Bromophenyl blue, (60) % Glycerol, (50) mM Tris, PH (7.6).

Method of work:

- Agarose gel prepared by dissolving (2) g of agarose powder in (100) ml (1x) TAE buffer, the mixture was heated until boiling so all agarose was dissolved in the buffer.
- The agarose was cooled enough (~60) ℃ before it was poured in the gel tray with well comb fixed properly, after adding (~100) µl of (1) mg/ml ethidium bromide.
- When the gel hardens the comb was removed carefully then TAE (1x) buffer was added to cover the gel.

- Samples were prepared by adding (1) µl of (6x) DNA loading buffer to every (5) µl of PCR product and loaded to the gel wells, DNA marker was also added to the first well of the gel.
- The electrophoresis was run at constant voltage of (80) V for (30) min then it was examined under the UV light (Uvetic, Uvtransilluminators).

The amplified DNA sequence was then sequenced. The 16S rRNA gene sequence of the isolates was aligned with reference 16S rDNA sequences of the GenBank using the BLAST algorithm available in NCBI.

3.3. Characterization of crude phytase

3.3.1. Effect of pH value on phytase activity

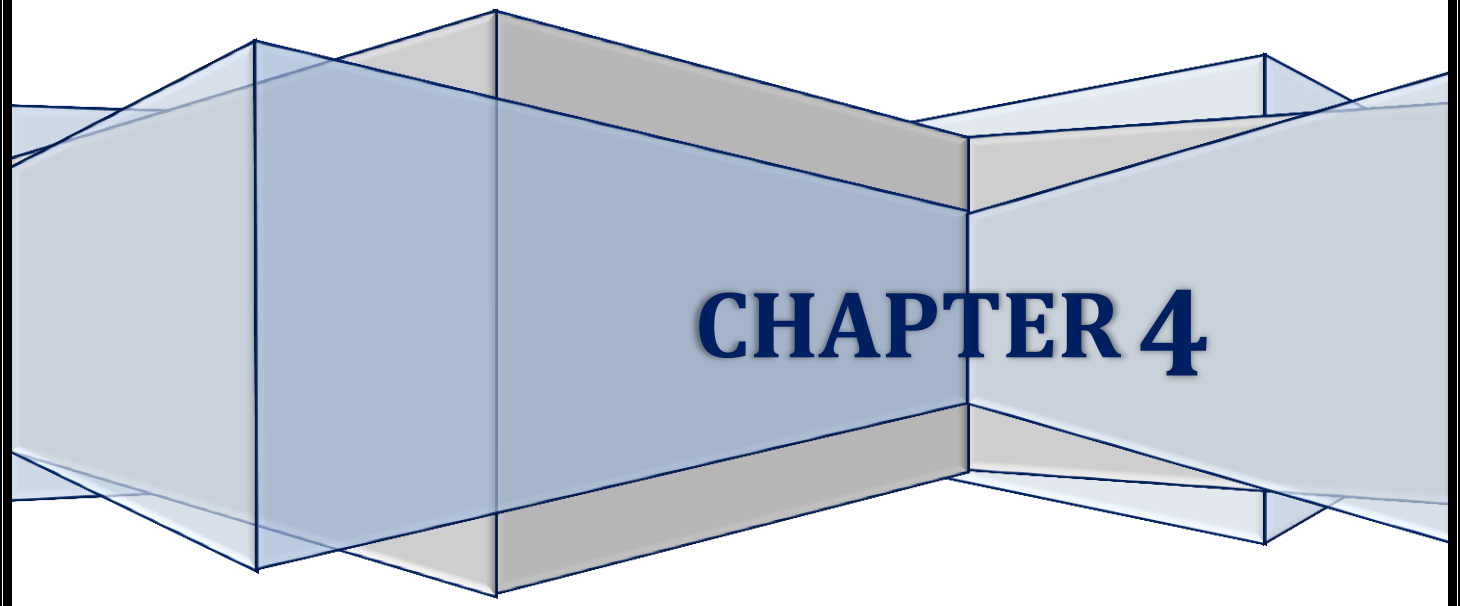
The pH influences the velocity of an enzyme-catalyzed reaction. The active sites on enzymes are frequently composed of ionizable proper ionic form. The effects of pH on the activity of an enzyme must be taken into account in any study of the effect of pH on substrate, binding and catalysis. The decline of pH could result from the formation of an improper ionic form of the substrate or enzyme (or both), or from inactivation of the enzyme, or from a combination of these effects. In the present study, the optimum pH for phytase activity was determined by using NaOAc-HOAc buffer at various pH values, ranging from (4.0) to (6.0) [Pandey *et al;* 2001] for assays at (37) °C, for (60) min and with Tris-HCl buffer at pH value (7.0) for assays according to the previous mentioned standard phytase activity assay.

3.3.2. Effect of temperature on phytase activity

Temperature is one of the vital physical factors that play a role in growth and metabolism of all the organisms. An optimum temperature exists for every activity. This may enhance the metabolic activities.

In the present study, the optimum temperature for phytase activity was determined by assaying the enzyme activity at various temperatures (30) °C, (35) °C, (37) °C and (40) °C [Choi *et al;* 2001] according to the previous mentioned standard phytase activity assay.

* * * * *



CHAPTER 4

Scope of the investigation

The work presented in this thesis focused on the phytase enzyme as an additive for the food and feed industry. The properties of phytate and its roles in food-feed, environment, and therapy together with a summary of phytase sources, applications and assay methods are given in chapter (2).

Isolation of (15) phytase producing bacterial isolates from (30) soil samples, with identifying the best phytase activity bacterial isolate (C4) by morphological, biochemical and molecular methods as *Bacillus subtilis* strain X3. Followed by production and characterization of the extracellular crude phytase obtained from the studied isolate (C4) which produces the significant amount of phytase during (72^{th}) hour of incubation at (37) °C with the pH of (6.0) was investigated in chapter (3) and presented in chapter (4).

* * * * *

4. Results and Discussion

Microorganisms, being a diversified group of organisms, are reliable sources for a number of useful products such as enzymes, amino acids, antibiotics and other bioactive molecules. To date, biotechnologists have turned their attention to bacteria as a source of the enzymes which are cheaper and less time consuming than the other sources.

In earlier studies, phytate known to be degraded by organisms include aerobic, anaerobic bacteria and fungi which isolated from different environmental conditions. However, the present study is concerned with local bacterial isolates capable of producing phytase and characterizes the extracted phytase for ideal supplement.

4.1. Isolation and screening of phytase producing bacteria from soil

In the present study, the appropriate dilution of each soil sample which was plated later on the selective media had been determined according to the (Table 4.1).

Table 4.1. The used dilution of each soil sample for plating on the selective media.

Sample no.	Dilution	Sample no.	Dilution	Sample no.	Dilution
1	10^{-3}	**11**	10^{-3}	**21**	10^{-3}
2	10^{-3}	**12**	10^{-2}	**22**	10^{-3}
3	10^{-3}	**13**	10^{-3}	**23**	10^{-3}
4	10^{-3}	**14**	10^{-3}	**24**	10^{-3}
5	10^{-3}	**15**	10^{-2}	**25**	10^{-2}
6	10^{-3}	**16**	10^{-3}	**26**	10^{-3}
7	10^{-3}	**17**	10^{-3}	**27**	10^{-3}
8	10^{-3}	**18**	10^{-3}	**28**	10^{-2}
9	10^{-3}	**19**	10^{-3}	**29**	10^{-3}
10	10^{-3}	**20**	10^{-3}	**30**	10^{-3}

Figure 4.1. Positive controls testing PSM medium.

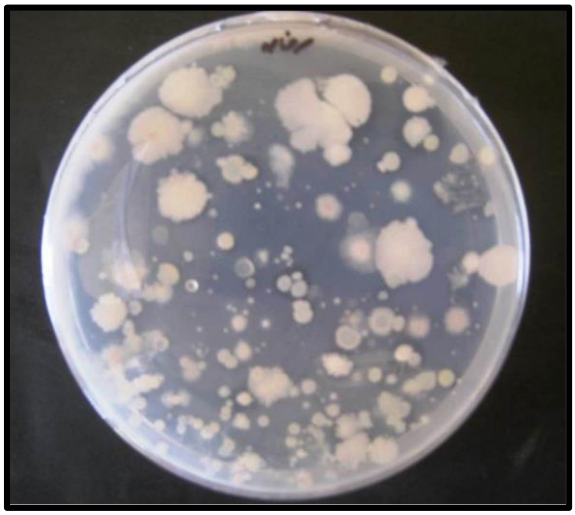


Figure 4.2. Sample of positive PSM plate.

4.1.1. Primary screening of phytase activity

Total of (15) differentiated colonies showed positive for phytase production. Among these, (9) were from soil samples and were designated as R1 to R9 and (4) were from cattle shed soil samples which were designated as C1 to C4 and (2) were from poultry farm soil samples and designated as P1, P2.

All the (15) isolates were re-plated and their halo (Z) and colony (C) diameters were measured after (3) days of incubation at (37) °C (Table 4.2). Hydrolysis efficiency of all the isolates was calculated which ranged from (5) % to (50) %.

The lack of growth could be a reflection of the isolates inability to degrade phytate or perhaps, merely an indication of unfavorable conditions for phytase production.

Table 4.2. Hydrolysis efficiency of isolates.

Isolate.no	Colony diameter, C (mm)	Halo diameter, Z (mm)	Hydrolysis Efficiency, Z-C/C (%)
R1	40	42	5
R2	23	27	17
R3	30	40	33
R4	30	33	10
R5	14	19	36
R6	26	34	31
R7	29	34	17
R8	22	27	23
R9	26	32	23
C1	31	33	6
C2	20	27	35
C3	18	23	28
C4	16	24	50
P1	8	9	13
P2	5	6	20

4.1.2. Secondary screening of phytase activity

A promising isolates (R5-C2-C4) showed the best hydrolysis efficiency on PSM plate were selected to investigate the phytase production in LPSM, in constant conditions of temperature (37) °C and pH number (7.0) during the incubation time (72) h with intervals of (24) h. And as a result the isolate (C4) had been shown the best phytase activity (0.818 U/ml) in the mentioned conditions (Figure 4.3), consequently it had been selected as a promising strain for phytase production and further study.

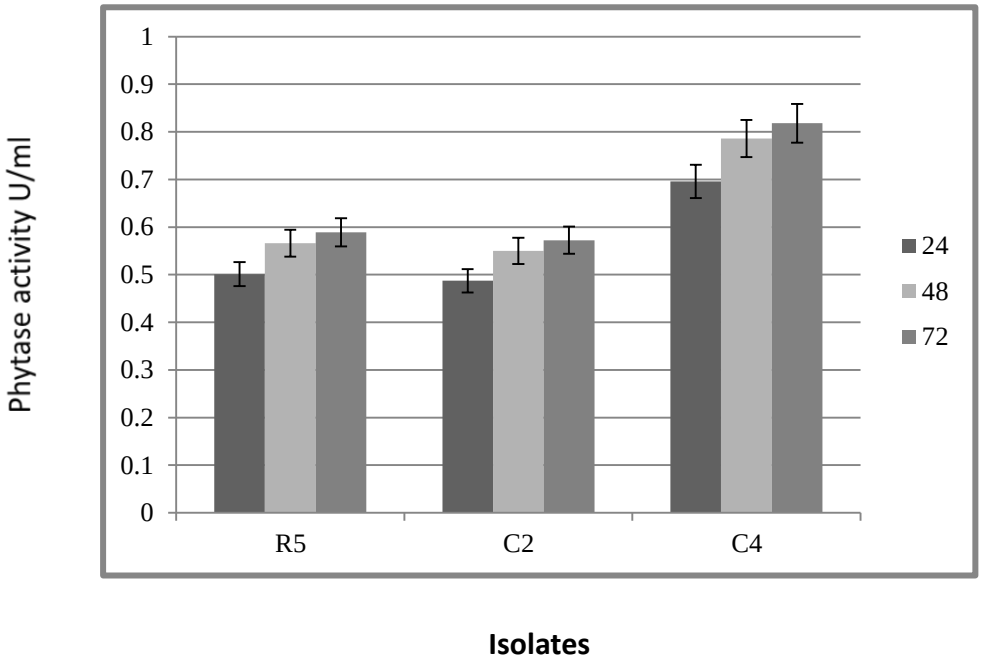


Figure 4.3. Phytase production capability of the selected isolates (R5-C2-C4) at constant temperature (37) ˚C, pH (7.0) and incubation time (72) h.

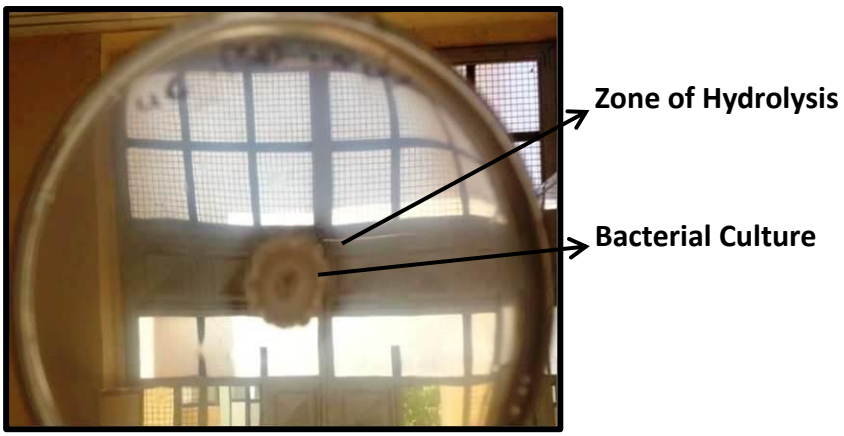


Figure 4.4. Halo formation of the isolate (C4) after incubation on PSM agar at (37)°C-(72)h.

4. Results and Discussion

Phytase is a useful enzyme in commercial interest. This enzyme is used in food and feed to increase the availability of phosphorus. Until now, it has been reported that phytase is produced in a variety of microorganisms. The concept of adding microbial phytase to the food and feed-stuffs was described over years ago and many successful trails have been reported since then. Nowadays, commercial production of microbial phytase for food and feed was broadly applied.

Although large amounts of microbial derived phytase can be now commercially produced with economic feasibility, there still exist technical difficulties, which preclude the widespread use of this enzyme. The main area of concern for the phytase is the low activity and stability under different conditions of pH. The second major obstacle is that the phytase is not thermo-tolerant to food and feed trials. Food processing temperature of (100) ℃ and Pellet temperatures of (80) ℃ or greater lead to unacceptable activity loss [Singh *et al*; 2011]. So, many noble phytase was still screened to find thermo-stable and broad pH applicable phytase.

In the screening of phytase, initial screening method is very important to distinguish phytase producing microorganisms and nonproducing microorganisms. PSM agar plate has been used for screening bacteria, which give a visual indication of extracellular phytase production by making clear zone. In the liquid assay system, LPSM media had been used to culture the phytase-producing bacteria. And the method of [Heinonen and Lahti, 1981] was sensitive and accurate enough to determine the phytase activity.

Many microorganisms were isolated from the soil samples such and primary screened using the plate screening method. Microorganisms isolated from the cattle soil sample largely made the clear zone. The size of clear zone in the plate screening medium was a greatly relative to a phytase activity in the liquid assay.

The isolate C4 was selected a phytase producer, although its phytase activity is relatively moderate. This phytase activity is likely to be increased by genetic manipulation.

4.2. Identification of the selected phytase producing bacterial isolate

Identification of the isolate C4 was carried out using microscopic examination, biochemical tests, and then 16S rDNA sequence analysis was performed to confirm the results of biochemical tests.

4.2.1. Morphological characteristics and Gram staining

Cell morphology of the isolate C4 was determined using routine laboratory staining protocols. The stained cells were observed under oil immersion objective of phase contrast microscope (Olympus, Germany). Cell shape and arrangements were recorded.

The selected isolate C4 was identified by morphological characteristics Table (4.3). Microscopic study revealed that C4 was Bacilli shaped. The shape of colony was a typical *Bacillus* type with round and milk-white color (Figure 4.5). Spore was observed after one day of incubation, the bacteria had a spore with mobility. It was stained as Gram-positive (Figure 4.6). Based on the identification scheme in Bergey's Manual of Systematic Bacteriology [Krieg and Holt,1984], C4 was identified as *Bacillus* sp.

Table 4.3. Morphological and biochemical identification of the isolate (C4)**.**

Microscopic Characteristic	**Result**
1) Form	Bacilli
2) Gram Staining	Gram-positive
3) Motility	+
4) Spore	+
5) Capsule	-
Biochemical Characteristic	**Result**
1) Indole Test	-
2) Nitrate Reduction Test	+
3) Catalase Test	+
4) Oxidase Test	+
5) Urease test	-
6) H2S Production	-

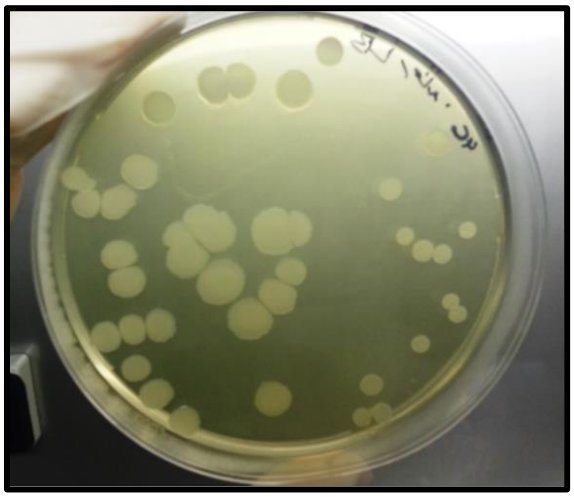


Figure 4.5. Colony form of the isolate (C4) after incubation on LB agar at (37) °C- (24) h.

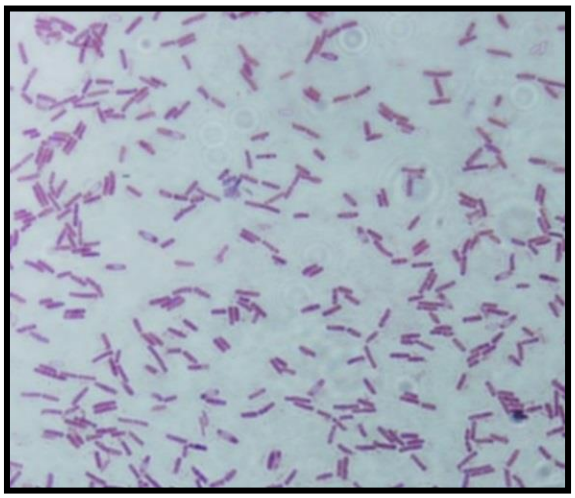


Figure 4.6. Gram positive staining of the isolate (C4) after incubation on LB agar at (37)°C-(24)h.

4.2.2. Biochemical properties

The biochemical properties of the isolate C4 were carried out using the API 50 CH kit (Figure 4.7). To identify in detail, the result of API test was summarized in (Table 4.4). Results of API 50 CH indicate that the isolate C4 belong to *Bacillus subtilis* with expectancy of 95.5%.

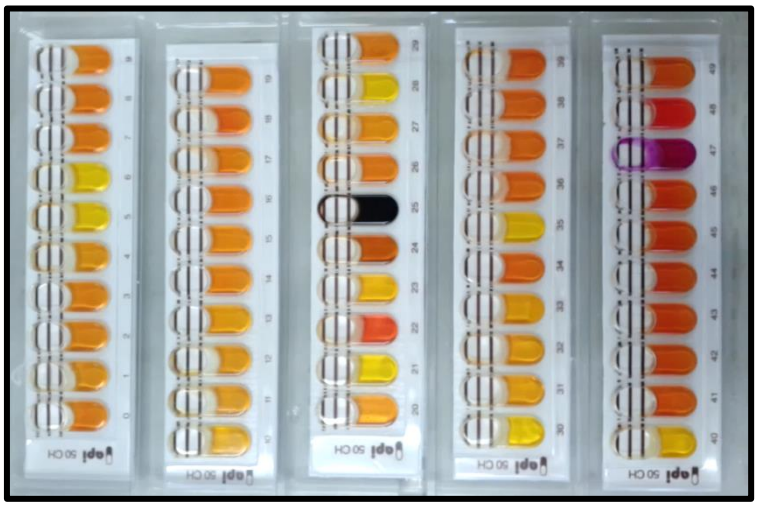


Figure 4.7. Results of isolate (C4) identification by API 50 CH.

Table 4.4. Characterization of the isolate (C4) using API 50 CH.

1.GLY	+	8.ADO	-	15.RHA	-	22.NAG	-	29.LAC	-	36.AMD	+	42.DFUC	-
2.ERY	-	9.MDX	-	46.DUL	-	23.AMY	±	30.MEL	+	37.GLYG	+	43.LFUC	-
3.DARA	-	10.GAL	-	17.INO	+	24.ARB	±	31.SAC	+	38.XLT	-	44.DARL	-
4.LARA	+	11.GLU	+	18.MAN	+	25.ESC	+	32.TRU	+	39.GEN	-	45.LARL	-
5.RIB	+	12.FRU	+	19.SOR	-	26.SAL	±	33.INU	+	40.TRU	-	46.GNT	-
6.DXYL	+	13.MNE	-	20.MDM	-	27.CEL	±	34.MLZ	-	41.LYX	-	47.2KG	-
7.LXYL	-	14.SBE	-	21.MDG	±	28.MAL	±	35.RAF	+	42.TAG	-	49.5KG	-

Terms: GLY: Glycerol, ERY: Erythritol, DARA: D-Arabinose, LARA: L-Arabinose, RIB: D-Ribose, DXYL: D-Xylose, LXYL: D-Xylose, ADO: D-Adonitol, MDX: Methyl-ßD-Xylopyranoside, GAL: D-Galactose, GLU: D-Glucose, FRU: D-Fructose, MNE: D-Mannose, SBE: L-Sorbose, RHA: L-Rhamnose, DUL: Dulcitol, INO: Inositol, MAN: D-Mannitol, SOR: D-Sorbitol, MDM: Methyl-αD-Mannopyranoside, MDG: Methyl-αD-Glucopyranoside, NAG: N-Acetyl Glucosamine, AMY: Amygdalin, ARB: Arbutin, ESC: Esculin ferric citrate, SAL: SALicin, CEL: D-Cellobiose, MAL: D-Maltose, LAC: D-Lactose (bovine origin), MEL: D-Melibiose, SAC: D-Saccharose (sucrose), TRE: D-Trehalose, INU: Inulin, MLZ: D-Melezitose, RAF: D-Raffinose, AMD: Amidon (starch), GLYG: Glycogen, XLT: Xylitol, GEN: Gentiobiose, TUR: D-Turanose, LYX: D-Lyxose, TAG: D-Tagatose, DFUC: D-Fucose, LFUC: L-Fucose, DARL: D-Arabitol, LARL: L-Arabitol, GNT: Potassium Gluconate, 2KG: Potassium 2-Ketogluconate, 5KG: Potassium 5-Ketogluconate.

4.2.3. 16S rDNA sequence analysis

Hence, the identification of the strain was further done by 16S rRNA gene sequencing analysis. Comparison of the 16S rRNA gene sequence of the isolate C4 with those in GenBank using BlastN showed (99) % sequence homology with *Bacillus subtilis* strain X3. The sequence is deposited under accession no. KM187652.1 in GenBank.

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method.

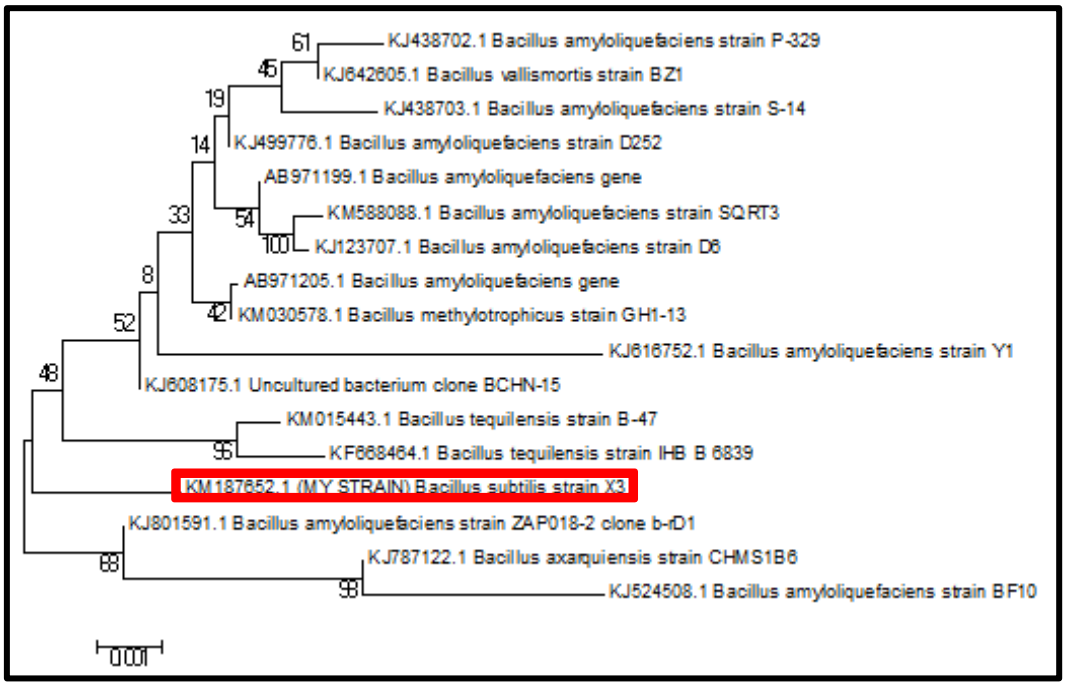


Figure 4.8. Phylogenetic trees of bacterial strain (C4) and its related species.

In diagnostic morphological and biochemical characteristics, isolate C4 was classified to a *Bacillus* genus. And there was not a difference in the classification of species using API test and Biologic test.

The isolate C4 was identified *B. subtilis* by the sequence homology of (99) %. Comparing these results with the proposed criteria that a prokaryotic organism whose 16S rDNA sequence is more than (97) % identical to any other sequence should considered the same species [Mardigan *et al;* 2000]. A phylogenetic tree was constructed for the detailed and confirmative identification, using the 16S rDNA sequence of five authentic species of the genus *Bacillus* deposited in GenBank. The phylogenetic tree shows the isolate is also closely related to the same group as *Bacillus subtilis* strain X3.

4.3. Effect of incubation time on phytase production

Hence it is necessary to optimize the fermentation parameters for the maximum production of phytase with a view to develop economically feasible technologies [Noorbatcha *et al;* 2009]. Incubation time plays an important role in maximum enzyme production [Tahir *et al;* 2010].The present results showed that the significantly high level enzyme activity was obtained during (72th) h of incubation at (37) °C with the pH of (7.0) (Figure 4.9). Extending the fermentation resulted in a slight decrease in phytase activity, which might be due to proteolytic degradation of the enzyme [Vats and Banerjee, 2004].

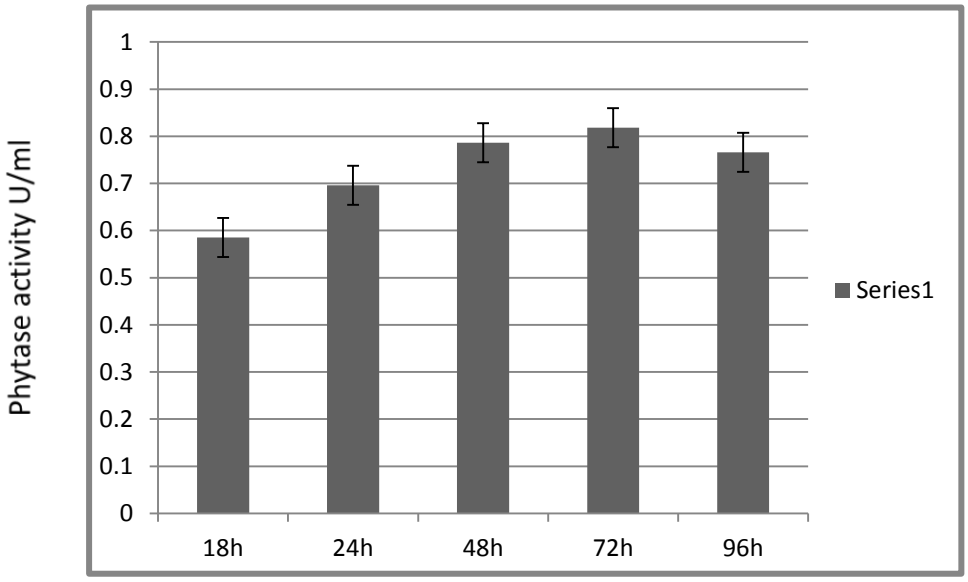


Figure 4.9. Effect of Incubation time on phytase production by the isolate (C4).

The achieved result may be due to protease activity released from cell during growing. Some studies reported that proteases were ubiquitous in living cell. As soon as cells were disrupted, proteases were released and quickly degraded any protein and this could drastically reduce the yield of protein during isolation and purification. In addition, other studies also suggested that the enzymes may be digested by protease and may also degrade other enzymes and reduce the effectiveness of enzyme production during the examination of the protease effect on production of cellulase, xylanase, pectinase, hemicellulase, glucanase, and phytase.

4.4. Characterization of crude phytase

4.4.1. Effect of pH value on phytase activity

Enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on charge distribution on both, substrate and, in particular enzyme molecules, especially active site. To study the effect of pH on phytase activity, the crude enzyme was incubated in various buffers ranging from pH 4.0 to 7.0. The crude phytase had a single pH optimum at pH 6.0 (Figure 4.10).

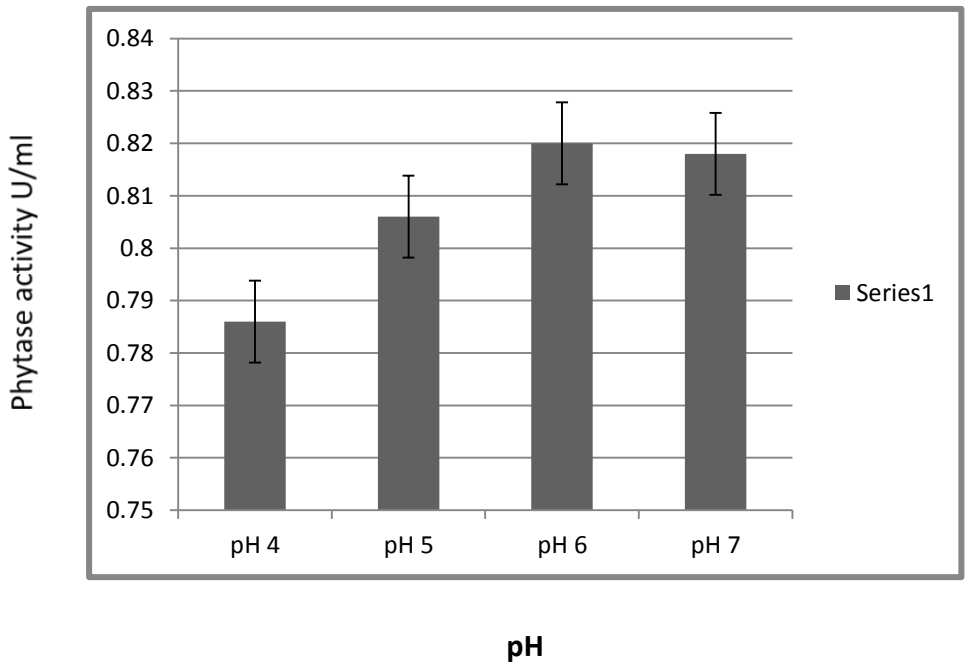


Figure 4.10. Effect of pH on the activity of extracellular phytase from the isolate (C4).

The enzyme has a near neutral pH optimum. This is particularly important since the enzyme should function in the small intestine (pH near neutral), where phosphate absorption takes place in food and feed.

There were many studies said that same bacterial phytase and especially those from *Bacillus* had pH optimum at (6.5-7.5). Besides, many researches reported the optimum pH of phytase from bacteria, fungi and yeast. Most phytase from bacterial strains usually had optimum pH of (4.0-8.0). On the other hand, yeast and fungi phytase typically had optimum pH of (2.0-6.0). [Pandey *et al;* 2001] reviewed partially *Klebsiella oxytoca* MO-3 phytase which was stable at pH (4.0-8.0) with optimum activity in the range of (5.0-6.0). [Yoon *et al;* 1996] studied on *Enterobacter* sp. (4) phytase and found that it had optimum temperature at (7.0-7.5) and was most stable at pH (6.0-8.0). [Kim *et al;* 1998] reported that phytase from *Bacillus* sp. had optimum pH of (7.0-8.0). [Golovan *et al;* 2000] reported two isoforms for and *E. coli* phytase with optimum activity at pH (4.5).

4.4.2. Effect of temperature on phytase activity

Temperature has a profound influence on enzyme activity and, in general, it is highly desirable that enzymatic preparations intended for the use in industrial applications must not only be thermo-stable but must have a high activity at elevated temperature as most of the industrial enzymatic operations are carried out at high temperatures. As the major propose of using phytase in animal feed is the constraint of thermal stability for this enzyme to withstand inactivation during food processing and feed-pelleting process.

Therefore, the effect of temperature on enzyme activity was studied. The enzyme activity of crude phytase from the isolate (C4) was assayed at various temperatures. The temperature profile of the phytase was conducted at the temperatures (30) °C, (35) °C, (37) °C, and (40) °C using the phytase assay method at the given temperature (Figure 4.11). The optimal temperature was found to be (37) °C. The enzyme activity increased considerably when the temperature reaction was raised up to near (37) °C, however the activity decreased suddenly at (40) °C.

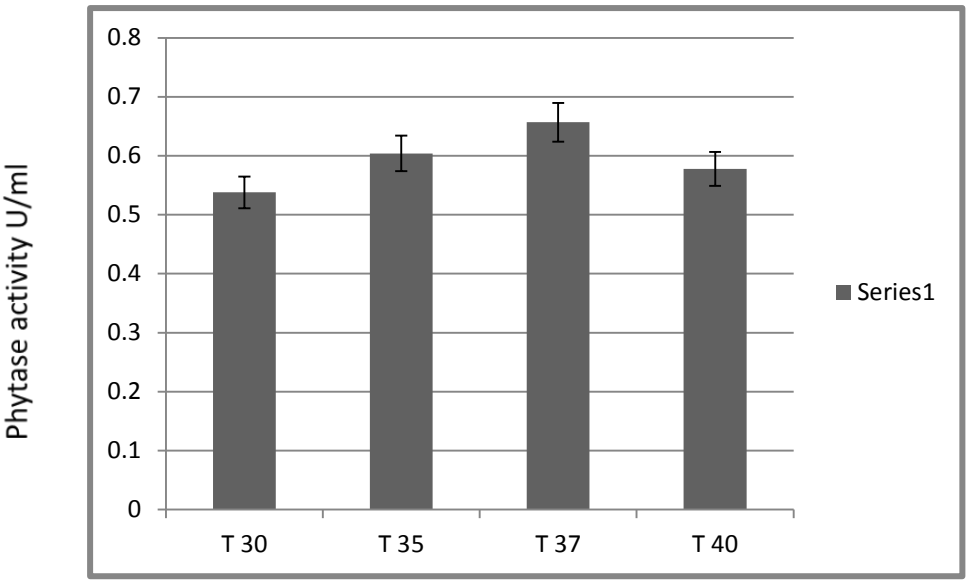


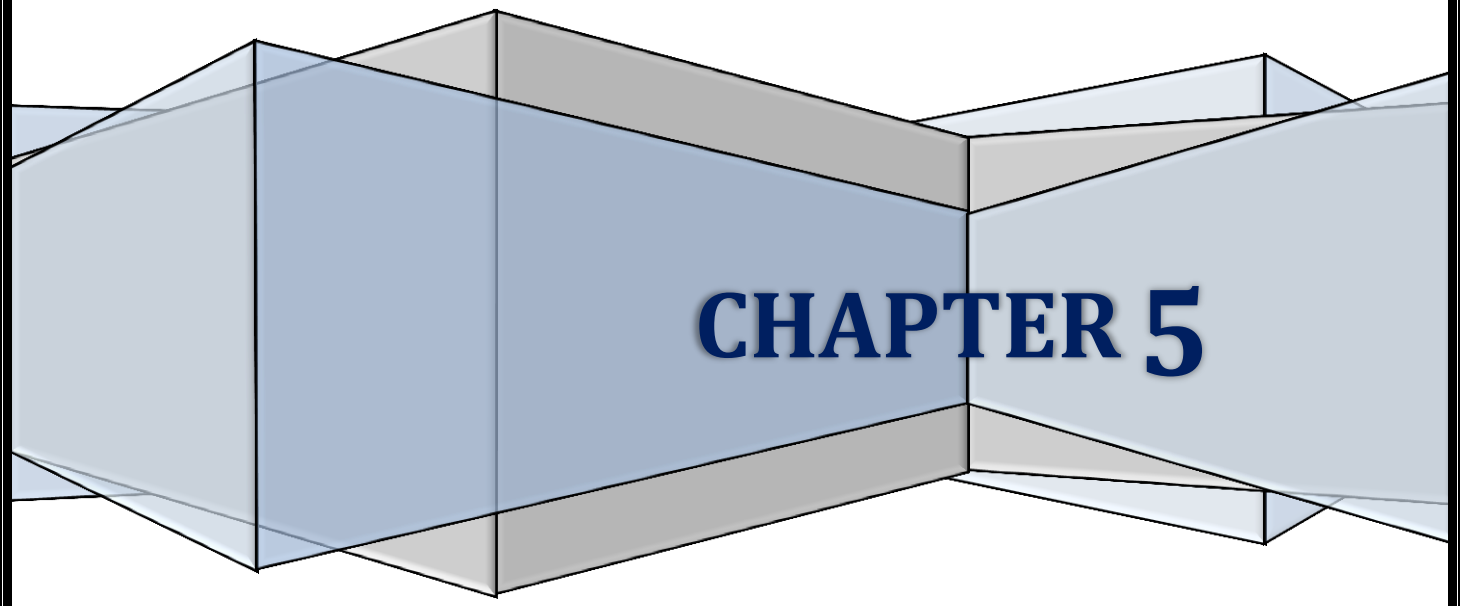
Figure 4.11. Effect of temperature on the activity of extracellular phytase from the isolate (C4).

[Golovan *et al;* 2000] purified phytase from recombinant *E. coli* and characterized the enzyme to have optimum temperature at (60) °C. [Vats and Banerjee; 2004] reported that general phytase showed high activity in the temperature range of (50) to (70) °C while optimum temperature was mostly between (45) and (60) °C. [Choi *et al;* 2001] studied the effect of temperature on *Bacillus* sp. KHU-10 phytase activity and found that the enzyme had the highest activity at (60) °C in the presence of (10) mM CaCl2. The summarization of phytase from various bacterial sources was shown in (Table 4.5). Most phytases were possible to apply in animal feed industry.

Table (4.5). Optimal temperature of phytases from various bacterial sources.

Source of phytases	Optimum temperature (°C)
Bacillus sp. KHU-10	(60) °C with CaCl2 (40) °C without CaCl2
Pseudomonas putida harbouring E. coli	(55) °C
Bacillus sp. DS11	(75) °C
Klebsiella pneumoniae	(37) and (55) °C
Citrobacter braakii	(50) °C
Malaysian waste water bacterium	(65) °C
E. coli	(60) °C

* * * * *



CHAPTER 5

5.1. Conclusions

The local isolated *Bacillus subtilis* strain X3 produces it's significant amount of phytase during (72th) hour of incubation at (37) °C with the pH of (6), which would considerably make it useful for applications in feed and food.

This study confirmed

- (15) Phytase producing bacterial isolates was isolated from various soil sources of Damascus countryside using PSM media.

- Secondary screening that has been done on the isolates (R5-C2-C4) using LPSM media affirmed that the isolate (C4) has the best phytase activity (0.818 U/ml).

- Microscopic, Biochemical and 16S rDNA sequence studies revealed the isolate (C4) as *Bacillus subtilis* strain X3.

- The isolate (C4) produces significant amount of phytase during (72th) hour of incubation at (37) °C with the pH of (7).

- The crude phytase was characterized and found that the optimum pH value for the enzyme was (6).

- The optimum temperature for the phytase enzyme activity was found to be (37) °C.

5.2. Recommendations

It would be of great importance in the future to pursuit this interesting project through conducting investigations in the following domains:

- Isolation and characterization of phytate degrading lactic acid bacteria and applying them in fermented food process.

- Devoting more researches to discover organisms with suitable characteristics of phytase in nature for increasing the production yield by cloning and expression in a well-controlled host.

- Production of recombinant phytases in a GRAS host would render them more advantageous for food applications [Tran, 2010].

- Further characterization for the obtained phytase, and determine the amino acids sequences of its active site.

- Modifying the enzyme properties to suit application requirements (stability at low pH and at high temperature which allowing the enzyme to survive the feed pelleting process, food processing conditions, and adequate gastric performance) [Singh *et al;* 2011].

- Preparation of specific IPPs (inositol tris-phosphate) can be used as enzyme substrates for metabolic investigation, as enzyme inhibitors and therefore potentially as drugs.

- Further analysis of the relationship between specific residues or other structural features and the stereo-specificity of phytase may lead to the ability to design an enzyme that could dephosphorylate a position on IP6 of our choosing [Madadlou *et al;* 2011].

* * * * *

References

References

- Afinah, S., Yazid, A. M., Anis Shobirin, M. H., and Shuhaimi, M. 2010. Phytase: Application in food industry. *International Food Research Journal, 17:13-21.*

- Agostini, J. S., Nogueira, R. B., and Ida, E. L. 2010. Lowering of phytic acid content by enhancement of phytase and acid phosphatase activities. *BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY, 53:975-980.*

- Ali, M., Shuja, M. N., Zahoor, M., and Qadri, I. 2010. Phytic acid: How far have we come. *African Journal of Biotechnology, 9:1551-1554.*

- Almedia, F. N. 2010. Effects of microbial phytase on the standardized total tract digestibility of phosphorus in soybean meal, corn, and corn co-products. *Master Thesis, University of Illinois, Urbana, Champaign, P4-11, 4-18.*

- Angel, R., Applegate, T. J., Ellestad, L. E., and Dhandu, S. D. 2010. Phytic acid chemistry: How important is it for phosphorus digestibility in poultry. *Multi-State Poultry Meeting, 1:1-22.*

- Applegate, T. J., and Angel, R. 2004. Phytase: Basics of enzyme function. *Farm Animal Management Purdue, 5:1-5.*

- Arjula, R. R., Malireddy, K. R., and Isanaka, R. 2010. Bacterial recombinant phytase. *patent application publication, 1:1-16.*

- Baker, D. H., and Augspurger, N. R. 2003. Efficacy of phytase enzymes for releasing phytate-bound phosphorus: Effects of phytase source, phytase level, and pharmacologic supplementation. *Animal Sciences, 1:95-100.*

- Bharathidhasan, A., Chandrasekaran, D., Natarajan, A., Ravi, R., Viswanathan, K., and Ezhilvalavan, S. 2009. In vitro enzyme assay of commercial feed enzymes. *Tamilnadu J. Veterinary & Animal Sciences, 5:20-24.*

- Billington, D. C. 1993. The inositol phosphates: Chemical synthesis and biological significance. *Weinheim Verlag Chemic, 1:153.*

- Bohn, L., Meyer, A. S., and Rasmussen, S. K. 2008. Phytate: Impact on environment and human nutrition. A challenge for molecular breeding. *Journal of Zhejiang University SCIENCE B, 9:165–191.*

References

- Bradford, M. M. 1976. Rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem, 72:248-254.*

- Caoa, L., Wang, W., Yang, C., and Yangb, Y. 2007. Application of microbial phytase. *ENZYME AND MICROBIAL TECHNOLOGY, 1:498-505.*

- Choi, Y. M., Suh, H. J., and Kim, J. M. 2001. Purification and Properties of Extracellular Phytase from *Bacillus* sp. KHU-10. *Journal of Protein Chemistry, 20:1-2, 287-292.*

- Chu, H. M., Guo, R. T., Lin, T. W., Chou, C. C ., Shr, H. L., Lai, H. L., Tang, T. Y., Cheng, K. J., Selinger, B. L., and Wang, H. J. 2004. Structures of Selenomonas Ruminantium phytase in complex with per sulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. *Structure, 12:2015-2024.*

- Coulibaly, A., Kouakou, B., and Chen, J. 2011. Phytic acid in cereal grains: Structure, healthy or harmful ways to reduce phytic acid in cereal grains and their effect on nutritional quality. *American Journal of Plant Nutrition and Fertilization Technology, 1:1-22.*

- Dahiya, S., Singh, N., and Rana, J. S. 2009. Optimization of growth parameters of phytase producing fungus using RSM. *Journal of Scientific & Industrial Research, 68:955-959.*

- Dvorakova, J. 1998. Phytase: Sources, preparation and exploitation. *Folia Microbial, 43:323-338.*

- Fitriatin, B. N., Arief, D. H., Simarmata, T., Santosa, D. A., and Joy, B. 2009. Phosphatase-Producing Bacteria Isolated from Sanggabuana Forest. *Poster presented on International Conference on Agriculture at the Crossroad, November, Bandung, Indonesia, 1:1-8.*

- Fitriatin, B. N., Arief, D. H., Simarmata, T., Santosa, D. A., and Joy, B. 2011. Phosphatase-producing bacteria isolated from Sanggabuana forest and their capability to hydrolyze organic phosphate. *Journal of Soil Science and Environmental Management, 2:299-303.*

- Godoy, S., Chicco, C., Meschy, F., and Requena, F. 2005. Phytic phosphorus and phytase activity of feed ingredients. *INTERCIENCIA, 30:24-28.*

- Golovan, S., Wang, G., Zhang, J., and Forsberg, C. W. 2000. Characterization and overproduction of the Escherichia coli appA encoded bifuntional enzyme that exhibits both phytase and acid phosphatase activity. *Can. J. Microbiol, 46:59-71.*

- Greiner, R., Konietzny, U., and Jany, D. K. 1993. Purification and characterization of two phytases from E. coli. *Arch Biochem Biophys, 303:107-13*.

- Greiner, R., and Konietzny, U. 1996. Construction of a bioreactor to produce special breakdown products of phytate. *Biotechnol, 48:153-159.*

- Greiner, R., Konietzny, U., and Jany, D. K. 2006. Phytate - an undesirable constituent of plant-based foods. *Journal für Ernährungsmedizin, 8:18-28.*

- Greiner, R., and Konietzny, U. 2006. Phytase for food application. *Food Technol Biotechnol, 44:125–140.*

- Gulati, H. K., Chadha, B. S., and Saini, H. S. 2007. Production and Characterization of Thermostable Phytase from Bacillus leavolacticus isolated from rhizosphere soil. *J Ind Microbiol Biotechnol, 34:91-98.*

- Ha, N. C., Oh, B. C., Shin, S., Kim, H. J., and Oh, T. K. 2000. Crystal structures of a novel, thermostable phytase in partially and fully calcium-loaded states. *Nat Struct Biol, 7:147-153.*

- Haefner, S., Knietsch, A., Scholten, E., Braun, J., Lohscheidt, M., and Zelder, O. 2005. Biotechnological production and applications of phytases. *Appl Microbial Biotechnol, 68:588–597.*

- Heinonen, J. K., and Lahti, R. J. 1981. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Analytical Biochem, 113:313-317.*

- Hidvegi, M., and Lasztity, R. 2003. Phytic acid content of cereals and legumes and interaction with proteins. *Periodica polytechnic ser.chem.eng, 46:59-64.*

- Hosseinkhani, B., Emtiazi, G., and Nahvi, I. 2007. Analysis of phytase producing bacteria (Pseudomonas sp.) from poultry faeces and optimization of this enzyme production. *African Journal of Biotechnology, 8:4229-4232.*

References

- Jorquera, M. A., and Mora, M. 2010. Bacillus-like phosphobacteria in agronomic volcanic soils from Chile. *World Congress of Soil Science, Soil Solutions for a Changing World, 1:17-19.*

- Kerovuo, J. 2000. A Novel phytase from Bacillus, characterization and production of the enzyme. *Master Thesis, Faculty of Science of the University of Helsinki, Finland, P12-14, 28-29.*

- Khan, A. J., Ali, A., Azam, F. I., and Zeb, A. 2007. Identification and isolation of low phytic acid wheat (triticum aestivum l.) inbred lines / mutants. *Nuclear Institute for Food and Agriculture (NIFA) Tarnab, 39:2051-2058.*

- Kim, Y. O., Kim, H. K., Bae, K. S., Yu, J. H., and Oh, T. K. 1998. Purification and properties of a thermostable phytase from Bacillus sp. DS11. *Enzyme Microb. Technol, 22:2-7.*

- Kim, T., Mullaney, E. J., Porres, J. M., Roneker, K. R., Crowe, S., Rice, S., KO, T., Ullah, AH. J., Daly, C. B., Welch, R., and Lei, X. G. 2006. Shifting the PH profile of PhyA phytase to match the stomach PH enhances its effectiveness as an animal feed additive. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 72:4397–4403.*

- Konietzny, U., and Greiner, R. 2002. Molecular and catalytic properties of phytate-degrading enzymes (phytases). *International Journal of Food Science and Technology, 37:791–812.*

- Konietzny, U., and Greiner, R. 2004. Bacterial phytase: potential application, *in vivo* function and regulation of its synthesis. *Brazilian Journal of Microbiology, 35:11-18.*

- Krieg, N. R., and Holt, J. G. 1984. Bergey's manual of systematic bacteriology. *Williams & Wilkins Co., Baltimore, 1:1-8.*

- Lassen, S. F., Breinholt, J., Ostergaard, P. R., Brugger, R., Bischoff, A., Wyss, M., and Fuglsang, C. C. 2001. Expression, gene cloning, and characterization of five novel phytases from four basidiomycete fungi: Peniophora lycii, Agrocybe Pediades, a Ceriporia sp., and Trametes Pubescens. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 76:4701–4707.*

- Lei, X. G., and Porres, J. M. 2003. Phytase enzymology, applications, and biotechnology. *Biotechnology Letters, 25:1787–1794.*

References

- Lei, X. G., Porres, J. M., Mullaney, E. J., and Pedersen, H. B. 2007. Phytase: Sources, structure and application. *J. Polaina and A.P. MacCabe Industrial Enzymes, 1:505–529.*

- Mardigan, M. T., Martinko, J. M., and Parker, J. 2000. Brock biology of microorganisms. *Prentice-Hall, Inc., Upper Saddle River, N.J, 9:447-448.*

- Marlida, Y., Delfita, R., Adnadi, P., and Ciptaan, G. 2010. Isolation, characterization and production of phytase and its application for feed. *Pakistan Journal of Nutrition, 9:471 474.*

- Meor Hussin, A. S., Farouk, A., and Greiner, R. 2009. Potential phytate-degrading enzyme producing bacteria isolated from Malaysian maize plantation. *African Journal of Biotechnology, 8:3540-3546.*

- Meor Hussin, A. S., Farouk, A., and Salleb, M. H. 2010. Phytate-degrading enzyme and its potential biotechnological application: A review. *J.Agrobiotech, 1:1-16.*

- Minihane, A. M., and Rimbach, G. 2002. Iron absorption and the iron binding and anti-oxidant properties of phytic acid. *International Journal of Food Science and Technology, 37:741–748.*

- Mullaney, E. J., and Ullah, AH. J. 2003. The term phytase comprises several different classes of enzymes. *Biochemical and Biophysical Research Communications, 312:179–184.*

- Mullaney, E. J., and UIlah, AH. J. 2007. Phytases: Attributes, catalytic mechanisms and applications. *CAB International, 1:97-109.*

- Musapuor, A., Afsharmanesh, M., and Shahrbabak, H. M. 2006. Use of microbial phytase for decrease of pollutant due to environmental poultry excreta phosphorus. *International Journal of Agriculture&Biology, 8:35-37.*

- Nielsen, M. M., Damstrup, M. L., Thomsen, A. D., Kjarsg, S., and Hansen, R. A. 2007. Phytase activity and degradation of phytic acid during rye bread making. *Eur Food Res Technol, 225:173–181.*

- Noorbatcha, I. A., Samsudin, N., and Salleh, H. M. 2009. Modification and characterization of phytases. *INTERNATIONAL CONFERENCE ON CHEMICAL & BIOPROCESS ENGINEERING, 1:34-35.*

References

- Oh, N. S., and In, M. J. 2009. Phytate degradation by Leuconostoc Mesenteroides KC51 cultivation in soymilk. *African Journal of Biotechnology, 8:3023-3026.*

- Olempska-Beer, Z. S., Merker, R. I., Ditto, M. D., and Dinovi, M. J. 2006. Food-processing enzymes from recombinant microorganisms. *Regulatory Toxicology and Pharmacology, 45:144–158.*

- Ozusaglam, M. A. 2009. Cloning of phytase gene in probiotic bacterium Bacillus coagulans. *Advanced Studies in Biology, 1:15-24.*

- Pandey, A., Szakacs, G., Soccol, C. R., Rodriguez-Leon, J. A., and Soccol, V. T. 2001. Production, purification and properties of microbial phytases. *Bioresource Technology, 77:203-214.*

- Phillippy, B. Q., Johnston, M. R., Tao, S. H., and Fox, M. 1998. Inositol phosphates in processed foods. *Journal of Food Science, 53:496-499.*

- Phillippy, B. Q., and Wyatt, C. J. 2001. Degradation of phytate in foods by phytases in fruit and vegetable extracts. *JOURNAL OF FOOD SCIENCE, 66:535-539.*

- Raboy, V. 2009. Approaches and challenges to engineering seed phytate and total phosphorus. *Plant Science, 177:281–296.*

- Reddy, N. R. 2002. Occurrence, distribution, content, and dietary intake of phytate. *Journal of Food Science, 1:1-27.*

- Rodríguez, H., and Fraga, R. 2000. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances, 17:319–339.*
- Sandberg, A. S., and Svanberg, U. 2006. Phytate hydrolysis by phytase in cereals; Effects on in vitro estimation of iron availability. *Journal of Food Science, 56:1330-1333.*

- Sanz-Penella, J. A., Tamayo-Ramos, J. A., Sanz, Y., and Haros, M. 2009. Phytate reduction in bran-enriched bread by phytase-producing Bifidobacteria. *J.Agric.Food Chem, 1:1.*

- Shamna, K. S., Rajamanikandan, KC. P., Mukesh Kumar, D. J., Balakumaran, M. D., and Kalaichelvan, P. T. 2012. Extracellular production of Phytases by a Native Bacillus subtilis Strain. *Annals of Biological Research, 3:979-987.*

References

- Singh, B., and Satyanarayana, T. 2010. Application of phytase. *Journal of scientific & Industrial Research, 69:411-414.*

- Singh, B., Kunze, G., and Satyanarayana, T. 2011. Developments in biochemical aspects and biotechnological applications of microbial phytases. *Biotechnology and Molecular Biology Review, 6:69-87.*

- Singh, N. K., Joshi, D. K., and Gupta, R. K. 2013. Isolation of Phytase Producing Bacteria and Optimization of Phytase Production Parameters. *Jundishapur Journal of Microbiology, 6:6419.*

- Singh, P., Kumar, V., and Agrawa, S. 2014. Evaluation of Phytase Producing Bacteria for Their Plant Growth Promoting Activities. *International Journal of Microbiology, 1:1-7.*

- Siren, M. 1986. New Myo-inositol triphosphoric acid isomer. *Patent: SW052950*.

- Tahir, A., Mateen, B., Saeed, S., and Uslu, H. 2010. Studies on the production of commercially important phytase from decaying organic soil. *Micrologia Aplicada International, 22:51-57.*

- Tran, T. T. 2010. Thermostable phytase from a *Bacillus* sp. heterologous production, mutation, characterization and assay development. *Doctoral Thesis, Department of Biotechnology Lund University, Sweden, P17.*

- Turner, B. L., Richardson, A. E., and Mullaney, E. J. 2005. Inositol phosphates in animal nutrition: Dietary manipulation and phosphorus excretion by animals. Inositol phosphates in the soil–plant–animal system: Linking agriculture and environment. *Soil Science Society of America, 1:25-26.*

- Tuyet, T. T., Long, N. D., and Khanh, H. Q. 2004. Production of phytase by *Aspergillus niger* NRRL 363. *Journal of Agricultural Sciences and Technology, 4:28-32.*

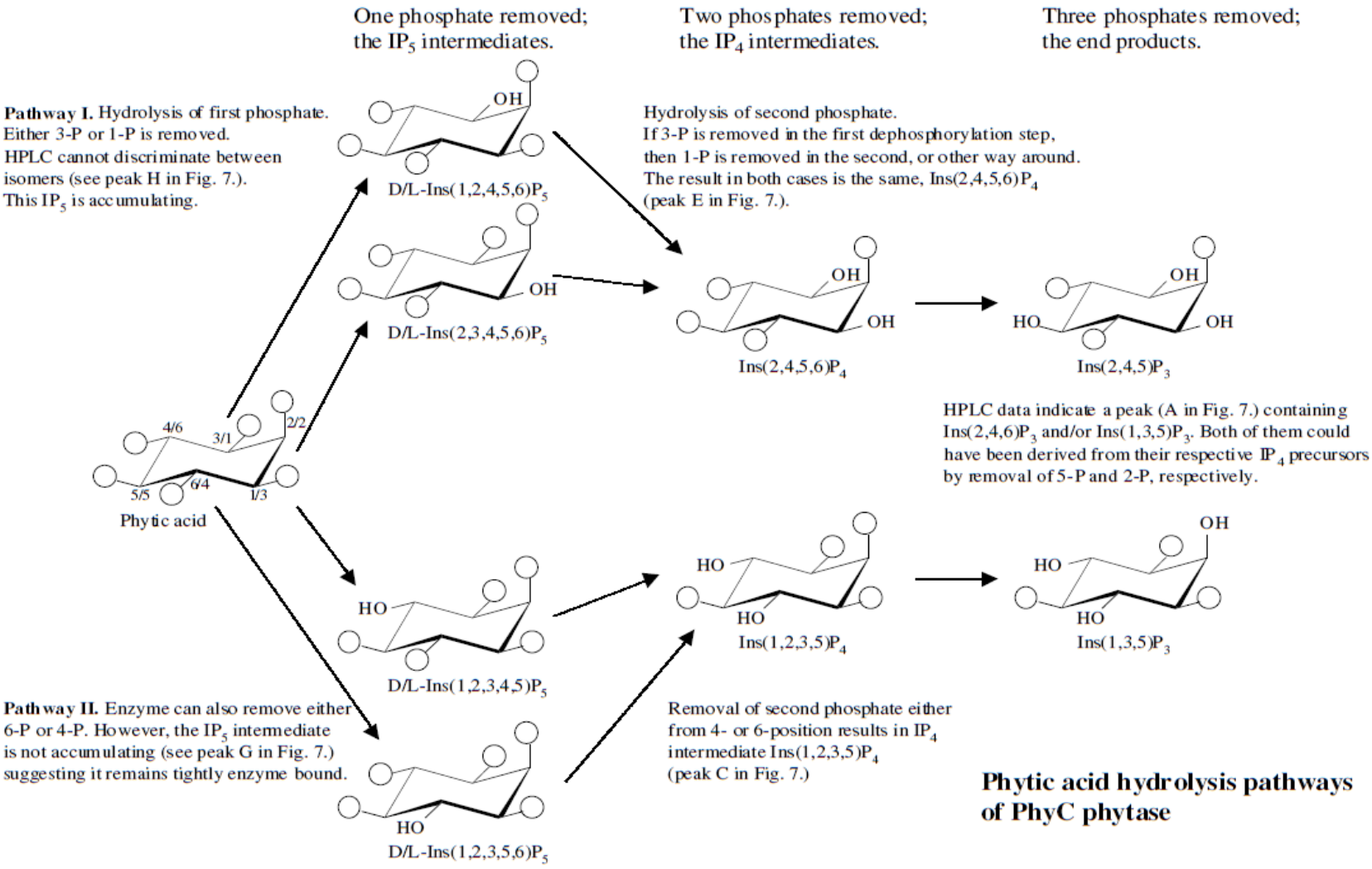
- Vats, P., and Banerjee, U. C. 2004. Production studies and catalytic properties of phytases (Myo-inositol hexakisphosphate phosphohydrolases): an overview. *Enzyme and Microbial Technology, 35:3-14.*

- Westers, L., Westers, H., and Quax, W. J. 2004. Bacillus subtilis as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. *Biochimica et Biophysica Acta, 1694:299-310.*

- Xuan, N. T., Hang, M. T., and Thanh, V. N. 2009. Cloning and over expression of phytase gene (PhyA). *World Academy of Science, Engineering and Technology, 56:750-753.*

- Yoon, S. J., Choi, Y. J., et al. 1996. Isolation and identification of phytase producing bacterium, *Enterobacterium* sp. 4, and enzymatic properties of phytase enzyme. *Enzyme Microb Technol, 18:449-454.*

Appendix


One phosphate removed;
the IP5 intermediates.
Two phosphates removed;
the IP4 intermediates.
Three phosphates removed;
the end products.
Pathway I. Hydrolysis of first phosphate.
Either 3-P or 1-P is removed.
HPLC cannot discriminate between
isomers (see peak H in Fig. 7.).
This IP5 is accumulating.
D/L-Ins(1,2,4,5,6)P5
D/L-Ins(2,3,4,5,6)P5
Hydrolysis of second phosphate.
If 3-P is removed in the first dephosphorylation step,
then 1-P is removed in the second, or other way around.
The result in both cases is the same, Ins(2,4,5,6)P4
(peak E in Fig. 7.).
Ins(2,4,5,6)P4
Ins(2,4,5)P3
HPLC data indicate a peak (A in Fig. 7.) containing
Ins(2,4,6)P3 and/or Ins(1,3,5)P3. Both of them could
have been derived from their respective IP4 precursors
by removal of 5-P and 2-P, respectively.
4/6
3/1
2/2
5/5
6/4
1/3
Phytic acid
D/L-Ins(1,2,3,4,5)P5
Ins(1,2,3,5)P4
Ins(1,3,5)P3
Pathway II. Enzyme can also remove either
6-P or 4-P. However, the IP5 intermediate
is not accumulating (see peak G in Fig. 7.)
suggesting it remains tightly enzyme bound.
Removal of second phosphate either
from 4- or 6-position results in IP4
intermediate Ins(1,2,3,5)P4
(peak C in Fig. 7.)
D/L-Ins(1,2,3,5,6)P5
Phytic acid hydrolysis pathways
of PhyC phytase

Arabic Summary

المقدمة:

تعتبر الأحياء الدقيقة مصدرا" هاما" للعديد من المنتجات المفيدة كالإنزيمات والأحماض الأمينية والمضادات الحيوية وغيرها من الجزيئات الفعالة حيويا"، واليوم التفتت التقانات الحيوية للبكتريا كمصدر للإنزيمات باعتبار الإنزيمات من مصدر بكتيري أقل كلفة واستهلاكا" للوقت من المصادر الأخرى. منذ اكتشاف إنزيم الفايتاز لأول مرة عام 1907 تم اكتشاف وتوصيف عديد من أنزيمات الفايتاز ومن مصادر متنوعة، حيث نالت إنزيمات الفايتاز خلال العشرين سنة الماضية اهتماما" كبيرا" من قبل العلماء والمهتمين في مجالات الأغذية وحماية البيئة والتقانة الحيوية [Singh *et al;* 2011] لأهميتها البالغة في هذه المجالات، من خلال التفاعل الذي يقوده إنزيم الفايتاز حيث يقوم بتفكيك حمض فايتيك إلى فوسفات أحادية لا عضوية و أينوسيتول بوجود الماء [Applegate and Angel, 2004]. واهتمت الأبحاث مؤخرا" بإنزيم الفايتاز من أصل ميكروبي وخاصة المستخلص من بكتريا التربة نظرا" لكونه الأفضل في تطبيقات الإنزيم في مجال التقانة الحيوية.

مبررات البحث:

نظرا" لأهمية التطبيقات الحالية لإنزيم الفايتاز في الصناعات الغذائية وصناعة الأعلاف وتطبيقاته الكامنة [Haefner *et al;* 2005] في الفوائد الصيدلانية لمشتقات الأينوسيتول الناتجة عن التفاعل الذي يقوده الإنزيم، فإن الحصول على عزلات بكترية محلية قادرة على إنتاج إنزيم الفايتاز يعد هدفا"، لتعزيز القيمة الغذائية للأغذية النباتية المستهلكة بكثرة في سورية (الحبوب والبقول) وزيادة مردود الأعلاف للحيوانات من جهة، ولتعزيز إنتاجه على المستوى التجاري محلياً مما يغني عن استيراده بتكلفة مرتفعة من جهة أخرى. ولأن إنزيم الفايتاز الذي يبدي فعالية مرغوبة في مجال PH واسع، وثباتية حرارية جيدة، يعد واعدا" في المجالات التطبيقية للإنزيم، هدفت هذه الدراسة لتوصيف إنزيم الفايتاز المستخلص من عزلة بكترية محلية وذلك من خلال قياس تأثير درجة الحرارة و ال PH على فعاليته.

أهداف البحث:

- عزل بكتريا منتجة لإنزيم الفايتاز من التربة السورية.
- تحديد هوية العزلة البكترية المنتجة لإنزيم الفايتاز الأعلى فعالية.
- الإختبارات الإنزيمية وتوصيف إنزيم الفايتاز.

الدراسة المرجعية:

الفايتاز إنزيم واسع الإنتشار في الطبيعة، حيث يوجد في الأحياء الدقيقة كالبكتريا والفطور والخميرة والأوليات [Pandey *et al;* 2001]، كما يوجد في الخلايا النباتية للبذور وحبوب الطلع وبنسب أقل في خلايا الجذور [Konietzny and Greiner, 2002] لتأمين الفوسفور والأينوسيتول والمعادن الضرورية لنمو النبات وتطوره، أما في النسج الحيوانية فالمجترات تفرز إنزيم الفايتاز في حين لا تفرزه أحاديات المعدة كالدواجن، الخنزير والأسماك [Pandey *et al;* 2001]، كما لا يفرزه الإنسان أيضا".

الركيزة التي يعمل عليها إنزيم الفايتاز هي الفايتين، وهو ملح الصوديوم لحمض الفايتيك وهذا الحمض يعد مضادا" غذائيا" (Antinutrient) لقدرته على تشكيل معقدات مع الشوارد المعدنية والعناصر النادرة مانعا" إياها من الإمتصاص في الجهاز الهضمي للإنسان والحيوانات أحاديات المعدة. كما أن حمض فايتيك قادر على تشكيل معقدات مع كثير من الإنزيمات التي تقوم بعملية الهضم مثل الببسين، التربسين، ألفا أميلاز و البيتا غالاكتوزيداز مؤديا" لخفض فعاليتها، ومع النشاء أيضا". ويذكر أن حمض فايتيك يخزن في الحبوب والبقول والبذور الزيتية وهو المصدر الأولي للفوسفور والأينوستول في تلك الأغذية [Greiner *et al;* 2006]. يقوم إنزيم الفايتاز بتفكيك حمض فايتيك إلى فوسفات أحادية لا عضوية و أينوسيتول مؤديا" لتحطيم المعقد الغذائي بحيث تصبح مكوناته من المعادن والعناصر النادرة والبروتينات والنشاء [Mullaney *et al;* 2003] متاحة للإمتصاص الهضمي في أحاديات المعدة والإنسان، هذا ويذكر أن حلقة الأينوسيتول المتحررة من التفاعل السابق تتصف بعديد من التطبيقات الصيدلانية فيمكن أن تستخدم كمسكنات للألم وكعلاج للعديد من الأمراض كالتهاب المفاصل والربو [Kerovuo, 2000].

ولإنزيم الفايتاز تطبيقات مهمة في عديد من المجالات الصحية والإنتاجية ففي المجال الإنتاجي تفيد إضافة إنزيم الفايتاز إلى علف أحاديات المعدة في تعزيز إتاحة الفوسفور والمعادن النادرة مثل (Cu, Mn, Zn, Fe) مما يؤدي إلى دعم النمو الطبيعي للحيوانات و يضبط كمية الفوسفور المطروحة في المخلفات مما يساهم في التخفيف من تلوث البيئة باعتبار الفوسفور هو العامل الأكثر تلويثا" للبيئة [Musapuor *et al;* 2006]. أما تطبيقات إنزيم الفايتاز في مجال تغذية الإنسان فتؤدي إضافة هذا الإنزيم لغذاء الإنسان لرفع القيمة الغذائية للأغذية الآتية من مصدر نباتي (الحبوب والبقول) حيث تؤدي

إضافتة لهذه الأغذية لتفكيك الفايتين فيها إما خلال عمليات الهضم في الجهاز الهضمي أو خلال عمليات التصنيع الغذائي [Meor Hussin *et al;* 2009]. ونذكر أخيرا" أن أحد المجالات الصناعية المهمة لاستخدام إنزيم الفايتاز هي استعماله كعامل حيوي في تفكيك حمض الفايتيك خلال عمليات تصنيع الورق [Kerovuo, 2000]، حيث يعد أحد عوامل التكنولوجيا النظيفة في إنتاج الورق بدلا" من استخدام الحموض المعدنية القاسية المؤدية لإنتاج مواد تصنيع وسيطة مسرطنة وسامة.

خطوات العمل:

- **عزل بكتريا منتجة لإنزيم الفايتاز من التربة السورية**

عزلت البكتريا المنتجة لإنزيم الفايتاز في هذه الدراسة من ترب متنوعة (تربة محيطة بجذر البقوليات، تربة حظائر المجترات، تربة المداجن) في ريف دمشق باستخدام طريقة الطبق الصلب PSM. فأظهرت (15) عزلة بكتيرية القدرة على إنتاج إنزيم الفايتاز حيث أعطت هالات واضحة على الطبق والتي كان منها (9) عزلات من التربة المزروعة بالبقوليات وأشير لها بالرمز (R1–R9) و(4) عزلات من تربة حظائر المجترات وأشير لها بالرمز (C1–C4) وعزلتان من تربة المداجن وأشير لها بالرمز (P1–P2) وحفظت العزلات النقية في وسط مغذي يحتوي % (40) غليسيرول في الدرجة (20–) °س. وبعد أن أعيدت زراعة كافة العزلات بطريقة الطبق الصلب من جديد تم قياس قطر المستعمرة (C) وقطر الهالة (Z) لحساب كفاءة الحلمهة لها بدقة فتراوحت بين % (50– 5)، حيث أبدت العزلات (R5–C2–C4) أفضل كفاءة واختيرت بالتالي ليجرى عليها الغربلة الثانوية في الوسط السائل لإنتاج الإنزيم LPSM في شروط ثابتة من درجة الحرارة (37) °س ورقم pH (7.0) وزمن تحضين (72) ساعة. فأبدت العزلة (C4) أعلى فعالية إنزيمية (0.818) وحدة إنزيمية/مل، واختيرت بالتالي لإجراء اختبارات تحديد الهوية بهدف الدراسة اللاحقة لخصائص الإنزيم الذي تفرزه هذه العزلة.

- **تحديد هوية العزلة البكترية المنتخبة**

تم تحديد هوية العزلة البكترية المنتخبة (C4) بداية بواسطة الفحص المجهري (صبغ غرام) والإختبارات الحيوية الكيميائية (API 50 CH system) لتحديد جنس ونوع العزلة ثم أجري التشخيص الجزيئي (16S rDNA sequence analysis) لتحديد السلالة. وبينت النتائج اعتمادا" على تصنيف Bergey أن العزلة المدروسة من الجنس والنوع *Bacillus subtilis* في حين بين التشخيص الجزيئي أنها السلالة *Bacillus subtilis* Strain X3.

- **إنتاج إنزيم الفايتاز في الوسط السائل LPSM**

لُقّح معلق بكتيري (10^6) خلية/مل من العزلة المختارة (C4) في دورق سعة (100) مل يحتوي (30) مل من الوسط الانتقائي السائل لإنتاج إنزيم الفايتاز LPSM (نفس تركيب الوسط الصلب دون إضافة آغار) وحضن الدورق في حاضنة متحركة بسرعة (200) دورة/دقيقة بدرجة حرارة (37) °س و لمدة (72) ساعة. تم الحصول على الطافي الحاوي على الإنزيم بتثفيل الوسط بسرعة (4000) دورة/دقيقة لمدة (15) دقيقة بدرجة حرارة (4) °س، وعُدّت مستخلصا" للإنزيم. ولقياس فعالية إنزيم الفايتاز اعتمدت طريقة [Heinonen and Lahti, 1981] من خلال التقدير اللوني لكمية الفوسفور الناتجة عن تفكيك الإنزيم للركيزة عند طول الموجة (700) nm باستخدام مولبيدات الأمونيوم كاشفا" لونيا". وعرفت وحدة الفعالية لإنزيم الفايتاز بكمية الإنزيم القادرة على تحرير (1unit.mol) من الفوسفات اللاعضوية في الدقيقة في (1) مل في شروط التجربة [درجة الحرارة (37) °س، رقم الpH (7.0)]. وأُستخدمت طريقة [Bradford, 1976] في تحديد تركيز البروتين، والتي يعتمد مبدؤها على استخدام كاشف برادفورد الذي يتألف بشكل رئيسي من أزرق الكوماسي Coomassie Blue G250 وهو ملون يرتبط بالبروتينات بروابط كهربائية ساكنة كارهة للماء معطياً معقداً يمتص الضوء بطول الموجة (595) nm، تكون شدة الامتصاص على علاقة طردية مع تركيز البروتين في العينة.

ولأن زمن التحضين هو العامل الأهم عند أمثلة شروط إنتاج الإنزيم ولاختيار الزمن الأنسب لفعالية الإنزيم تمت دراسة تأثير استمرار التحضين لمدة (96) ساعة بنفس الشروط السابقة على العزلة المختارة (C4) فوجد بأنها تحقق أعلى فعالية إنزيمية لها في زمن التحضين (72) ساعة.

- **توصيف إنزيم الفايتاز**

بدراسة تأثير درجة الpH على فعالية إنزيم الفايتاز المنتج من العزلة (C4) في مجال من درجات الpH يتراوح بين (7–4)، كانت درجة الpH المثالية لعمل هذا الإنزيم هي (pH 6.0) حيث كانت أخفض فعالية لإنزيم الفايتاز عند درجة (pH 4.0) وازدادت بشكل واضح حتى وصلت الى درجة (pH 6.0) والتي كانت الدرجة المثلى لعمل الإنزيم ثم تراجعت الفعالية مع استمرار ارتفاع درجة الpH بعد هذه الدرجة. أظهرت نتائج دراسة تأثير درجة الحرارة على فعالية إنزيم الفايتاز المنتج من العزلة (C4) والتي أجريت في مجال من درجات الحرارة بين (40–30)°س وعند درجة (pH 6.0)، أن فعالية الإنزيم ازدادت بارتفاع درجة الحرارة حتى وصلت إلى أعلى قيمة عند درجة الحرارة (37) °س ثم انخفضت مع استمرار ارتفاع درجة الحرارة حتى وصلت إلى أخفض فعالية عند درجة الحرارة (40) °س.

الاستنتاجات:

- تم عزل (15) عزلة بكتيرية منتجة لإنزيم الفايتاز من (30) عينة تربة سورية.
- اختيرت العزلات (R5-C2-C4) ذات أفضل كفاءة حلمهة على الطبق الصلب PSM ليجرى عليها الغربلة الثانوية في الوسط السائل لإنتاج الإنزيم LPSM فأبدت العزلة (C4) أعلى فعالية إنزيمية (0.818) وحدة إنزيمية/مل.
- تم تحديد هوية العزلة (C4) الأكثر كفاءة في إنتاج الإنزيم في الوسط الصلب PSM وفي الوسط السائل LPSM بواسطة الإختبارات المورفولوجية والحيوية الكيميائية والجزيئية على أنها *Bacillus subtilis* Strain X3.
- درس تأثير زمن التحضين على فعالية إنزيم الفايتاز الخام ووجد أن زمن التحضين (72) ساعة على درجة الحرارة (37) °س ورقم الpH (7.0) هو الأفضل لعمل الإنزيم.
- تم توصيف إنزيم الفايتاز الخام ووجد أن رقم الpH المثالي لفعالية الإنزيم هو (6.0) في حين درجة الحرارة المثالية لفعالية الإنزيم كانت (37) °س.

التوصيات:

- عزل بكتريا حمض اللبن القادرة على إنتاج إنزيم الفايتاز من مصادر محلية لأهميتها الاستثنائية في مجال التغذية الوظيفية لإنسان.
- البحث عن مصادر ميكروبية منتجة لإنزيم فايتاز مثالي المواصفات التطبيقية، وزيادة مردود إنتاجه بواسطة التنسيل الجزيئي.
- إنتاج إنزيم الفايتاز المؤشب في خلايا بكتيرية آمنة غذائيا".
- تنقية وتوصيف أوسع لإنزيم الفايتاز المستحصل في هذه الدراسة، ودراسة موقعه الفعال.
- تحوير خصائص الإنزيم للوصول لإنزيم فايتاز مثالي الأداء متحمل للحرارة المطبقة في الصناعات الغذائية ومتحمل للحموضة لأداء فعال في القناة الهضمية لأحاديات المعدة.
- فصل حلقة الأينوستول ثلاثية الفوسفات الناتجة عن التفاعل الذي يقوده إنزيم الفايتاز وتنقيتها لأهميتها الصيدلانية.
- دراسة التفاعل إنزيم _ ركيزة بهدف تصميم إنزيم فايتاز قادر على إنتاج مركبات الأينوسيتول عديدة الفوسفات المرغوبة للتطبيقات الصيدلانية.

Damascus, Syria - 2015