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Thermostable phytase from a *Bacillus* sp.

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Heterologous production, mutation, characterization and assay development

Thuy Thi Tran

Doctoral Thesis 2010
Department of Biotechnology
Lund University

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Thermostable phytase from a *Bacillus* sp.

Heterologous production, mutation, characterization and assay development

Thuy Thi Tran
(Trần Thị Thúy)

Department of Biotechnology
Doctoral Thesis
December 2010

Academic thesis which, by due permission of the Faculty of Engineering of Lund University will be publicly defended on Friday, December 17 at 10:30 a.m. in Lecture Hall C, at the Center for Chemistry and Chemical Engineering, Sölvegatan 39, Lund, for the degree of Doctor of Philosophy in Engineering.

Faculty opponent: Professor Peter Stougaard, Institute of Agriculture and Ecology, Denmark

Doctoral thesis, 2010
Department of Biotechnology
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Thermostable phytase from a *Bacillus* sp.: Heterologous production, mutation, characterization and assay development

Abstract

Phytase is an important enzyme in the food/feed industry. It catalyzes the hydrolysis of phytate, an anti-nutrient compound present in cereals and grains, to release orthophosphate and myo-inositol-6-phosphate with lower degrees of phosphorylation. Phytic acid is a strong chelator capable of complexing 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 and the absorption of minerals from food/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.

All the commercially available phytases are histidine acid phytases with optimum activities at low pH and with low thermostabilities. Alkaline phytases (also called β-propeller phytases) are active at neutral or slightly alkaline conditions, calcium-dependent, and are quite thermostable so as to withstand the high temperatures during the pelleting of animal feeds. They can hence exhibit activities in the small intestine of animals as well as during storage of feeds. Alkaline phytases have several other potential applications.

This thesis presents the studies on a phytase from *Bacillus* sp. MD2 isolated in Vietnam. The focus has been directed to (1) developing a new kinetic method to determine the phytase activity, (2) cloning, expression and production of recombinant phytase from *Bacillus* sp. MD2, (3) the metal dependence of the catalytic properties and stability of the recombinant phytase, and (4) site-directed mutagenesis of the recombinant phytase to improve certain properties of the enzyme relevant for food/feed applications.

Key words: Phytase, phytate, kinetic assay, β-propeller phytase, recombinant protein, metallo-enzyme, site-directed mutagenesis, lactose induced production

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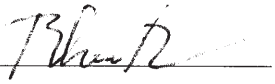
Signature  Date 2010-11-15

Abstract

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A kinetic assay for phytases has been developed based on the turbidity reduction of phytate-protein complexes used as substrates. This method offered a reliable way to measure the enzyme activity of both histidine acid phytases and ß-propeller phytases. The method was found to be simpler, faster, and to measure the activity under conditions closer to those existing in the gastrointestinal tract of animals, thus making it more suitable for evaluating phytases for feed and food applications in comparison with the traditional method based on the release of phosphate from sodium phytate.

The phytase gene from *Bacillus sp*. MD2 was cloned and expressed in *Escherichia coli*. Cultivation of the recombinant bacteria in a minimum medium using a fed-batch strategy combined with the control of the inorganic phosphate concentration resulted in a high level of production of the recombinant phytase. Lactose could be used as an alternative inducer to isopropyl-β-D-thiogalactoside, thus reducing the production cost. A significant amount

of the expressed phytase (90% of the total active enzyme) leaked out into the medium, thereby facilitating the subsequent downstream processing.

A close investigation of the effect of divalent metal ions on the activity and stability of the recombinant phytase was performed. Calcium played a critical role in stabilizing the enzyme and in activating the substrate (phytate) to fulfil the activity of the enzyme. Other metal ions (Ba^{2+}, Mn^{2+}, Mg^{2+} and Sr^{2+}) could replace Ca^{2+} in the active site of the enzyme and recover more than 90% of the enzyme activity with the calcium complex substrate. On the other hand, the presence of Ca^{2+} on the phytate was crucial for an optimal expression of the phytase activity.

The relationship between structure and function of the phytase was probed by site-directed mutagenesis. Single site mutations, S283R and E229V, on the catalytic surface increased the specific activity of the enzyme by 13 and 19%, respectively. Mutation of the catalytically important residue E227 to Ser shifted the optimum pH of the enzyme to the acidic side and simultaneously improved its acid stability. After 3 h of incubation at pH 2.6, the mutant phytase (E227S) retained over 80% of its initial activity while the wild-type phytase displayed only 40% of its original activity. Moreover, the E227S mutant phytase, unlike its wild-type, showed a higher relative activity towards calcium phytate, sodium pyrophosphate and *p*-nitro phenyl phosphate, thus suggesting a broader substrate specificity.

Alkaline phytases have a lower specific activity than their acid counterparts. An increased knowledge of the enzymes for designing mutations to improve their specific activity and catalytic activity at low pH are thus necessary for rendering industrial applications possible.

List of papers

The thesis is based on the following papers, referred to in the text by their Roman numerals. The papers are attached as appendices at the end of the thesis. Reprints are published by kind permission of the editors/publishers concerned.

I. A simple and fast kinetic assay for phytases using phytic acid-protein complex as substrate

Thuy Thi Tran, Rajni Hatti-Kaul, Søren Dalsgaard and Shukun Yu

Accepted for publication in Analytical Biochemistry

II. A thermostable phytase from *Bacillus* sp. MD2: cloning, expression and high-level production in *Escherichia coli*.

Thi Thuy Tran, Gashaw Mamo, Bo Mattiasson and Rajni Hatti-Kaul

Journal of Industrial Microbiology and Biotechnology (2010) 37:279-287

III. Thermostable alkaline phytase from *Bacillus* sp. MD2: effect of divalent metals on activity and stability.

T.T. Tran, S.O. Hashim, Y. Gaber, G. Mamo, B. Mattiasson and R. Hatti-Kaul

Submitted

IV. Altering the activity and specificity of a thermostable alkaline phytase from *Bacillus* sp. MD2 by site directed mutagenesis.

T.T. Tran, G. Mamo, L. Bóxo, N.N. Le, Y. Gaber, B. Mattiasson and R. Hatti-Kaul

Submitted

My contributions to the papers

Paper I. I planned the work with Shukun Yu based on his idea. I performed the experimental work. The results were analyzed with the contributions from all the co-authors. I wrote the first draft of the manuscript.

Paper II. I planned and performed the experimental work, wrote the manuscript with assistance from Rajni Hatti-Kaul. Results were discussed with all the co-authors.

Paper III. I planned and performed the experimental work with the help of some of the co-authors. Results were discussed with all co-authors. I wrote the first draft of the manuscript.

Paper IV. I planned the work with Gashaw Mamo, performed half of the experimental work and wrote the first draft of the manuscript.

Abbreviations

ANFs	Anti-nutritional factors
BPP	ß-propeller phytase
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetracetic acid
GRAS	Generally regarded as safe
GIT	Gastro intestinal tract
HAP	Histidine acid phosphatase phytase
IP_1, IP_2, IP_3,IP_4,IP_5	Inositol mono-, bis-, tris-, tetrakis-, pentakis-phosphate
IP_6	Phytic acid/ *myo*-inositol hexakisphosphate
IPTG	Isopropyl β-D-thiogalactoside
IUB	International Union of Biochemistry
IUPAC	International Union of Pure and Applied Chemistry
NCBI	National Center for Biomedical Informatics
NMR	Nuclear magnetic resonance
PAP	Purple acid phosphatase phytase
PDB	Protein database bank
P_i	Inorganic phosphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Amino acid name	**One-letter code**	**Three-letter code**	**Amino acid name**	**One-letter code**	**Three-letter code**
Alanine	A	Ala	Leucine	L	Leu
Arginine	R	Arg	Lysine	K	Lys
Asparagine	N	Asn	Methionine	M	Met
Aspactic acid	D	Asp	Phenylalanine	F	Phe
Cystein	C	Cys	Proline	P	Pro
Glutamic acid	E	Glu	Serine	S	Ser
Glutamine	Q	Gln	Threonine	T	Thr
Glyceine	G	Gly	Tryptophan	W	Thp
Histidine	H	His	Tyrosine	Y	Tyr
Isoleucine	I	Ile	Valine	V	Val

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1. Introduction

Saving energy and resources has become vital in modern life. One way of doing so is to change our habits in food consumption. In general, there is a need for altering our life style into a more environmentally friendly, safe and saving mode.

The global ecological system consists of numerous complicated food chains. Man is one of the organisms at the top of such a nutrient web. It has been considered that the longer the food chain, the more energy is lost and the more CO_2 as well as pollution is emitted. Plants and other autotrophic organisms are always the first link of a food chain (primary producers), after which different species in the animal kingdom can make many food chain links as consumers. Finally, the microorganisms (decomposers) are the ones that close the food chain. Man is normally a top predator, an intelligent carnivore in a food chain, who can deliberately shorten those in which humans are involved, which leads to energy saving and decreased pollution. The use of energy can act as an indicator for the environmental impact in the production of food/feed, as many environmental problems, like climate change, acidification potential and depletion of non-renewable resources, are related to energy consumption [1, 2].

Since the increase of the mechanization in our society, our physical activity has declined, and our dietary intake needs to be modified in order to provide an adequate supply of micronutrients, but with fewer calories [3]. We are contemporarily encouraged to eat more products from plants (vegetable oil and margarine…) and reduce consumption of meat; we also try to eat more natural fibers (unpolished, wholegrain cereals) as opposed to well-processed food. Furthermore, efforts are dedicated towards raising livestock and poultry in cheaper, safer and less polluting ways. Between 1972 and 1992, poultry feeds have been successfully shifted from fish meal as the main protein source to cheaper plant protein sources such as soybean meal [4]. Also, fish feed is being developed in the same way [4].

A problem related to the consumption of more plant products is the presence of anti-nutritional compounds in vegetarian diets. Some specific nutrients (vitamins, amino acids, minerals…) present in low concentrations or even absent in vegetable products, need to be supplemented to balance the nutrient value of foods and feeds. Some antinutritional factors (ANFs) such as phytate, protease inhibitors, lectins, anti-vitamins, saponins, estrogens, lysinolamine, carbohydrates from soluble fibers, and certain immunogenic proteins need to be inactivated or destroyed [5]. Nutritionists have put much effort into improving the quality of plant meals of poultry feed to reach a quality equal to that of fish meals [6].

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 [7]. 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 [8, 9, 10]. 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 [11, 12, 13].

In addition, most of the phosphorus in phytate is unavailable to monogastric animals as they lack the enzyme to digest phytates [6, 13, 14]. Hence, addition of inorganic phosphorous, a non-renewable and expensive mineral [13], to feeds for monogastric animals is a common practice, which incurs costs and also contributes to water pollution [15, 16]. The price of feed-grade phosphorus has increased more than four-fold in the last few years [17, 18, 19]. Unutilized phytate from vegetable feed is excreted by animals, and is becoming an environmental pollutant in areas of intensive livestock production [13]. 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 [20, 21].

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 [13]. Phytase is an enzyme that catalyses the sequential hydrolysis of phytate (myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate; IP_6) to less phosphorylated myo-inositol derivatives with concomitant release of inorganic phosphate [22].

1.1 Scope of the thesis

The work presented in this thesis is focused on the phytase enzyme as an additive for the food/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. A new kinetic method for assaying phytase activity based on turbidity reduction of IP_6-protein complexes has been developed (**Paper I**). The diversity of phytases, structural information on various phytases and their biophysical and biochemical properties are summarized in chapter 3. *Bacillus* sp. MD2 was isolated as a phytase-producing organism. The gene encoding the phytase was cloned and expressed in a heterologous host. The use of appropriate expression and cultivation strategies resulted in a high-level production of the recombinant phytase (**Paper II)**. The effect of the presence of various divalent metal ions on the enzyme and/or substrate on the phytase activity was investigated and presented in **Paper III**. The phytase gene was then subjected to mutations to determine the effect on the activity of the enzyme under varying conditions and with several substrates (**Paper IV**).

2. Phytate and phytases

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/feed.

2.1 Phytate

The terms phytic acid, phytate and phytin refer to the free acid, salt and calcium/magnesium salt, respectively. In the literature, the terms phytic acid and phytate have been used interchangeably [9]. About 75-85% of the total phosphorus in plant seeds is in the form of phytate [9, 23] 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 [24, 25]. Later on, numerous researchers have isolated phytate in the form of a 'globoid' and confirmed its main chemical content to be C, P and metal ions [7]. The name 'inositol-phosphoric acid' was suggested by Winterstein in 1897 [26], and revised as '*myo*-inositol 1, 2, 3, 4, 5, 6 hexakis (dihydrogen) phosphate' by IUPAC-IUB in 1968 [27].

Structure of phytic acid has been an intensively discussed subject in the chemical society for many years during the twentieth century [7, 9]. The two structures suggested by Neuberg [28, 29] and Anderson [30] 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. 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 [7] and have led to the conclusion that the structure proposed by Anderson is the predominant form found in plant materials.

Several investigations on the phytate structure by NMR [31, 32], crystallography [33] 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, C-6) are equatorial [32, 34]. Blank et al., 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 [35]. By combining ^{13}C NMR, ^{31}P NMR, and Raman spectroscopic analyses, Isbrandt and Oestel concluded that *myo*-insitol 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 causes the inverted conformation, i.e., 5-axial/1-equatorial, to prevail [31].

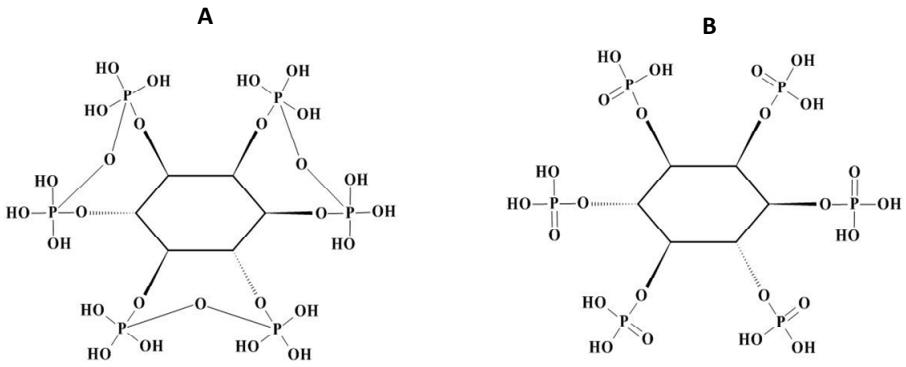

Figure 2.1 Structure of phytic acid proposed by (A) Neuberg, 1908 and (B) Anderson, 1914

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) [7, 9]. This feature makes IP_6 a very strong chelating agent. The negative charges of IP_6 can complex with positively charged moieties of a number of metal ions, peptides, proteins and starch [7, 9, 36, 37]. Several examples of metal ion complexations are given in Figure 2.2. Such complexation leads to turbidity of the solution which is dependent upon the pH as well as on the concentration of IP_6 and of the metal ions (Figure 2.3 and [7, 38]). It has been reported that IP_6-metal ion complexes are formed under neutral or slightly alkaline conditions [39, 40, 41]. On the other hand, the IP_6-protein complexes are formed under acidic conditions where most of the proteins originating from plants are positively charged [41]. IP_6-metal ion-protein complexes have also been suggested to be formed under neutral and slightly alkaline conditions [41, 42]. When phosphate groups are removed from the inositol hexakisphosphate (which leads to fewer phosphate groups than in IP_6), the mineral binding strength becomes progressively lower and the solubility increases [43].

When studying the formation and stability of the IP_6-lysozyme complex (**Paper I**), it has been found to give rise to turbid solutions. This turbidity is dependent on the pH and concentration of IP_6 and lysozyme in the solution (**Paper I**). The maximum complexation occurs at pH 3-3.5 and a IP_6:lysozyme molar ratio > 1.5. IP_6 has been found to be a stronger chelating agent than EDTA [44, 45].

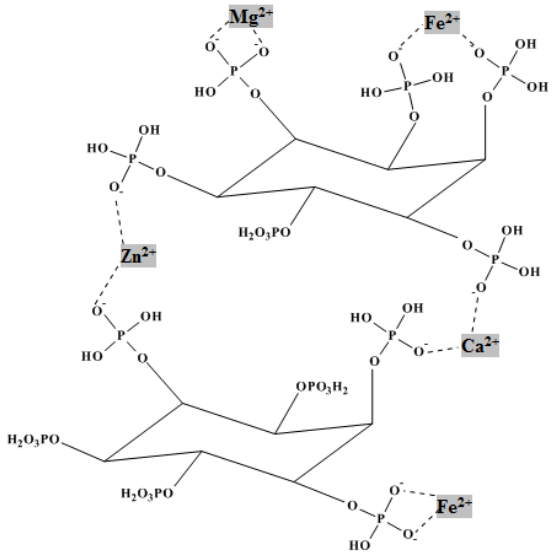

Figure 2.2 Different ways in which metal ions complex with phosphate groups in IP_6 molecules.

Metal ions may interact with $-OH^-$ of the same or another phosphate groups in an IP_6 molecule. They can also interact with $-OH^-$ of the phosphate groups in various IP_6 molecules.

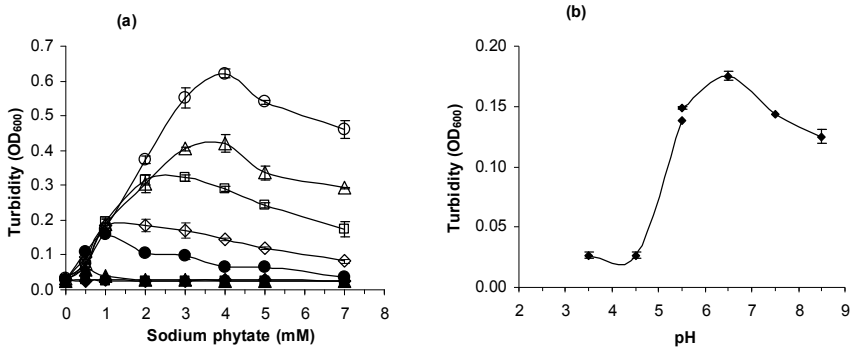

Figure 2.3 Turbidity of the IP_6- Ca complex as a function of (a) the concentration of sodium phytate (IP_6) and Ca^{2+} and (b) the solution pH.

(a) The complex was prepared in 50 mM Tris-maleate buffer (pH 7.5) containing 0-7 mM sodium phytate and $CaCl_2$ at several concentrations: 0 mM (♦), 1 mM (■), 2 mM (▲), 5 mM (●), 7 mM (◊), 10 mM (□), 12 mM (Δ), and 15 mM (○).

(b) The complex was prepared in 50 mM glycine-HCl buffer (pH 2.5-3.5), 50 mM sodium acetate buffer (pH 3.5-5.5) and 50 mM Tris-maleate buffer (pH 5.5-8.5) containing 1 mM sodium phytate and 5 mM $CaCl_2$

2.1.2 Natural distribution and physiological functions

Phytate/phytin is primarily found in seeds [24, 25, 46], however, its location within the seed differs among plants. For instance, 90% of the phytin in corn is in the germ portion of the kernel [47, 48], whereas in wheat and rice most of the phytin is in the aleurone layers of the kernel and the outer bran [47]. Phytin in oilseeds and grain legumes is associated mostly with protein and concentrated within globoids, the subcellular inclusions, which are distributed throughout the kernel. Phytate in soybean seeds, on the other hand, appears to have no specific location [46]. Phytate has also been found in roots and tubers [3, 49, 50], fruits and vegetables, nuts [3, 50], as well as pollen of various plant species [51, 52, 53]. Among several plant feedstuffs, sesame, pumpkin/squash, and flax (linseed) have the highest IP_6 content on a dry weight basis (3.7 to 4.7%) [54]. Other plant feedstuffs comprise between 1 and 3% dry weight of IP_6 [36].

The IP_6 molecule contains 28.2% P [39]. It is normally found as a potassium-magnesium salt in rice [55, 56], wheat [57], broad beans (*Vicia faba*) [47, 58], and sesame seeds [47]; and as a calcium-magnesium-potassium salt in soybeans (*Glycine max*) [58, 59] and Great Northern beans (*Phaseolus vulgaris*) [60]. Phytate rapidly accumulates in grains and seeds during their ripening period and maturation, accompanied by other substances such as starch, proteins, and lipids [61, 62, 63]. Cosgrove and Irving proposed the role of phytate in seeds to be : 1) as a phosphorus reserve, 2) for energy storage, 3) as a competitor for adenosine triphosphate during the rapid biosynthesis of phytin near seed maturity when seed metabolism is inhibited and dormancy is induced, 4) 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 5) as a regulator of the readily available seed Pi level [64]. IP_6 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 [65].

IP_6 has been found in nucleated erythrocytes of birds, fresh water fish and turtles [66, 67, 68], as well as in organic soils [69, 70]. Lower degrees of phosphorylation of inositol (IP_2, IP_3, IP_4, IP_5) and even IP_6 has been recognized as components of cell signaling and phosphate transfer systems present in possibly all animal and plant tissues [71, 72, 73]. Although plants routinely synthesize IP_6, there is evidence that humans also sparsely synthesize IP_6 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 IP_6 per day [43, 74].

2.1.3 Anti-nutritional effect

From a plant nutrient perspective, phytate is important for growing seedlings and promoting good crop yields [75]. In many areas of the world with large populations, the availability in soil of phosphorus to plants is limited [76], causing an increased use of fertilizers which supplement P_i to plant soil. However, from an animal nutrition point of view, phytate is considered as an anti-nutritional compound due to its strong chelating properties [75].

Many essential metal ions (Ca^{2+}, Zn^{2+}, Fe^{2+}…) present in food/feed are bound to IP_6 and form precipitates under neutral or slightly alkaline conditions [38, 41, 77]. The stability of the complexes formed between IP_6 and metal ions is in the order $Zn^{2+} > Cu^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+}$ [78], whereas at pH 7.4 this order is reported to be $Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{2+} > Ca^{2+}$ [79]. 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 [80]. The simultaneous presence of two different cations raises the proportion of the IP_6-metal complex that precipitates [81].

The formation of IP_6-Ca complexes has been reported to be anticalcifying, leading to rickets in puppies [82] and in humans [83, 84]. There are however some reports that human subjects become adapted to a high phytate diet after a short period of time, suffering no ill effects [84, 85]. Nutritional rickets has been reported in the populations of Northern India, Pakistan, Iran, and among the Bedouins, and is related to the high phytate intake in the form of chapatis (unleavened bread) - a major mode of cereal consumption [86, 87, 88]. Phosphate deficiency provides an added factor contributing to the appearance of rickets; a relationship between phytic acid, mineralization, and vitamin D has been reported by Van Den Berg et al. [89].

Magnesium deficiency connected to a high-phytate diet (4% IP_6-soy protein complex) has been reported in chickens [45] and rats [90]. Zinc deficiency symptoms, also caused by a high-phytate diet, have been studied in humans [11, 91], chickens [78, 92], swines [93], and rats [94]. The formation of IP_6-Ca-Zn in the presence of excess calcium even elevates the symptoms of zinc deficiency [95] since IP_6-Ca-Zn complexes are shown to be less soluble than their IP_6-Zn counterparts. The addition of sodium phytate to white bread [96] or milk [97] decreases iron absorption in humans 15-fold. There are many studies reported the negative effects of phytate on bioavailability and absorption of iron in animals [94, 98] although soybean protein (which contains much phytate) was reported to be a good source of dietary iron [99]. Later on, scientists have found that a major iron component from hard wheat bran and soybean is present as monoferric phytate [100, 101] which has a high availability, much like that of dietary inorganic iron or ferrous ammonium sulfate [101, 102]. The addition of 1% phytate to an egg albumin diet significantly reduced the absorption of copper and manganese in rats [94].

IP_6 also interacts with positively charged dietary proteins leading to the formation of IP_6-protein aggregates and precipitates [36, 103, 104], which decreases their accessibility to proteases, thus resulting in inefficient protein digestion [8, 9, 10]. 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 [41, 105]. Complexation with IP_6 has been reported for several proteins from wheat and oat bran [106], the great northern beans [103], corn germs, soybean flakes, and sesame meal [47], soybeans and peanuts as well as cottonseed [107]. There is thus strong evidence that phytic acid-protein interactions negatively affect protein digestibility [43]. The IP_6-metal ion-protein complex formed under neutral and alkaline conditions was suggested to be fairly labile [77] but it could lead to the synergistic effect of mineral deficiency and inefficient protein digestion.

The binding of IP_6 to starch occurs as a result of hydrogen bond formation which might lead to an inefficiency of starch digestion resulting in blood glucose reduction [37]. The binding of IP_6 with protein and starch may also reduce the availability of these nutrients from the diet [39]. Nevertheless, the interaction between IP_6 and starch has not been well characterized and still needs more studies *in vivo*.

By chelating metal ions, IP_6 indirectly impairs the function of some digestive enzymes which need metal ions for their activity and/or stability. A number of metalloenzymes such as carboxypeptidases, leucine aminopeptidase, amylase, alkaline phosphatase, and aminopeptidases [108, 109] containing either zinc or calcium are present in the small intestine and are essential for the degradation of food/feedstuffs. IP_6-Cu has been reported to cause more than 95% decrease in activity of carboxypeptidase A by exchanging Cu^{2+} with Zn^{2+} in enzyme molecules at pH 7.5 [110]. IP_6 demonstrated the same inhibition level as EDTA on the activity of α-amylase [111]. Calcium is known as a contributing factor in autocatalysis of trypsinogen to trypsin, and also as a stabilizer of trypsin. Hence, when IP_6 chelates calcium, the trypsin production and stability are affected [108]. Depletion of intestinal alkaline phosphatase and phytase in the gastrointestinal tract of monogastric animals has been ascribed to high dietary calcium which forms IP_6-Ca complexes [40]. Strong inhibition of the nondigestive enzyme thymidinine kinase is related to zinc deficiency symptoms by high levels of dietary phytate [112].

2.1.4 Environmental perspective

It is well known that plants need inorganic phosphorus (P_i) for their growth and that they accumulate phytate in their seeds, roots, and other tissues during ripening. IP_6 needs to be degraded to return P_i to the soil - thereby closing the phosphorus cycle. However, excess P_i, especially in surface-water, can cause eutrophication [113, 114], 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.

Phytates, which are consumed in food/feed by humans, swine, poultry, and other monogastric animals, often end up in excrements. The manure disposal problems associated with intensive animal farming operations are now widely recognized. It has been shown that humans and monogastric animals do not possess any intestinal phytase [115, 116, 117], and that the hydrolysis of phytate occurring in the gastrointestinal tract is due to a non-specific action of intestinal phosphatases or of gut bacteria [3]. Hence, P_i supplementation in feeds to fulfill the nutritional requirements of animals is common practice, incurring an extra expense to the feed and increasing IP_6 and P_i excreted to litter. Microorganisms present in soil and water which possess a wide range of phytate hydrolytic enzymes [118, 119] have contributed to the phytate hydrolysis in livestock litter [120]. When IP_6 and P_i enter into rivers, they give rise to cyanobacterial blooms, hypoxia and death of water-borne organisms [121, 122]. 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 as discussed above, phytic acid and inositol intermediates have been implicated in the treatment of several diseases. Some reviews have raised the following question: “Phytate: a bad or good food component?” [43, 74].

Phytate has been reported to block the formation of free radicals (•OH), suppress iron-catalyzed oxidative reactions, hence providing antioxidant [123] and anticarcinogenic [124, 125] benefits. Other advantages of phytate have been reported, such as improving the glucose response [126], decreasing plasma cholesterol and triglycerides [127], detoxification of heavy metals such as cadmium and lead [128], as well as therapy for calcium urolithiasis [129] and against dental caries [130, 131]. It has moreover been shown to inhibit *Vibrio vulnificus* on septicemia-induced mice [44, 132].

2.2 Phytases and their applications

Phytases (*myo*-inositol hexakisphosphate hydrolases) are a special class of phosphatase enzymes that are able to catalyze the sequential hydrolysis of phosphate ester bonds of phytate (IP_6) [23, 39, 133]. This yields P_i and myo-inositol or a series of its lower phosphoric esters [43, 134, 135].

2.2.1 Phytase sources

Phytases are widely distributed in plants, animals, and microorganisms. Nys et al. found a large variation of phytase activity in the seeds of several varieties of plants [136]; high

levels 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 [137, 138]. Many bacteria, yeasts and fungi produce phytases and have thus been frequently reviewed in the literature [118, 120, 139]. Phytase-producing microorganisms have been isolated from a wide range of sources including soil, fermented food/feed, water and also from the gastrointestinal fluid of ruminants. Phytase in calf liver and blood was the first report on animal phytases [140], however no other mammalian blood phytase has been reported. Bitar and Reinhold purified phytases from rat, chicken, calf, and human intestines [141] and reported that the activity of human intestine phytase was 30 times lower than that of rats. Moreover, it did not seem to play a significant role in phytate digestion in the gastrointestinal tract [141, 142].

2.2.2 Determination of phytase activity

Most of the phytase assays developed so far are end point measurement of the color formed between IP_6 or P_i and the color reagents after certain reaction times. Methods to quantify IP_6 are quite complicated, comprising numerous steps of extraction, precipitation with $FeCl_3$, centrifugation, and washing [143, 144]. They are also expensive since they require the use of HPLC analysis [145] or infrared spectroscopy (FT-IR) [146]. Hence, a more convenient phytase assay involves measurements of P_i release after stopping the enzymatic reaction. This method is based on the formation of a colored complex between P_i and ammonium molybdate [147, 148, 149, 150, 151].

The end point measurement of P_i has been widely used to determine phytase activity of several commercial phytases. However, drawbacks include it being time-consuming and based on the use of a toxic color reagent. Moreover, the accuracy is very dependent on choosing a suitable dose of enzyme and an accurate time for stopping the enzymatic reaction.

Since interactions of IP_6 with proteins and metal ions form turbid complexes [7, 41, 77, 107, 152], the reduction in turbidity of the solution by the action of phytase can be used as an indirect parameter to measure phytase activity. The turbidity of IP_6-metal ion complexes has also been used for screening microorganisms producing phytases on agarose plates [119, 153, 154] and for observing the phytase band on SDS-PAGE with the zymogram method [153].

A kinetic assay for phytases using the IP_6-lysozyme complex as a substrate has been developed (**Paper I**). The IP_6-lysozyme complex has a significantly higher turbidity as compared to other complexes and is quite stable during storage. For this reason, it was chosen as a complex substrate for the phytase assay. Figure 2.4 shows a scheme of the IP_6-lysozyme complex substrate preparation and the phytase assay using this complex. The turbidity (OD_{600}) was monitored kinetically and the phytase activity was measured in

OD/min. Figure 2.5 presents a good correlation between the turbidity reduction (OD_{600}) and the P_i release (OD/min) by the action of *A. niger* phytase.

Compared to the conventional end-point method based on P_i measurements, the kinetic assay described in **Paper I** was shown to be simpler, faster, and more closely related to the *in vivo* physiological conditions. It was thus more suitable for evaluating phytases for feed and food applications. A drawback was however that the linear range for turbidity at OD_{600} (0.1 - 0.9) was quite narrow compared to the linear range for the colorimetric assay of P_i release. In addition, the reaction mixture needed to be well mixed before and during each measurement to avoid precipitation of the complex. The method has been shown to be reproducible and reliable for both alkaline ß-propeller phytase and HAP phytases (**Paper I**).

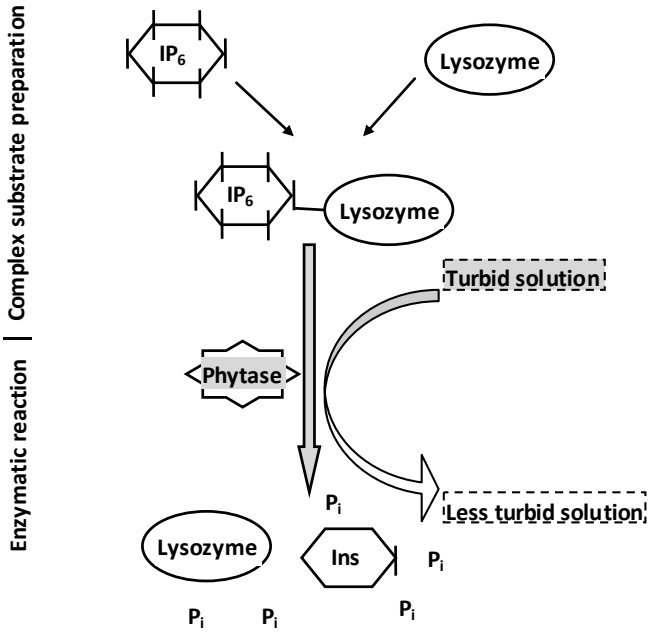

Figure 2.4 Scheme of the kinetic assay of phytase activity.
IP_6: *myo*-inositol phosphate; P_i: inorganic phosphate; Ins: inositol

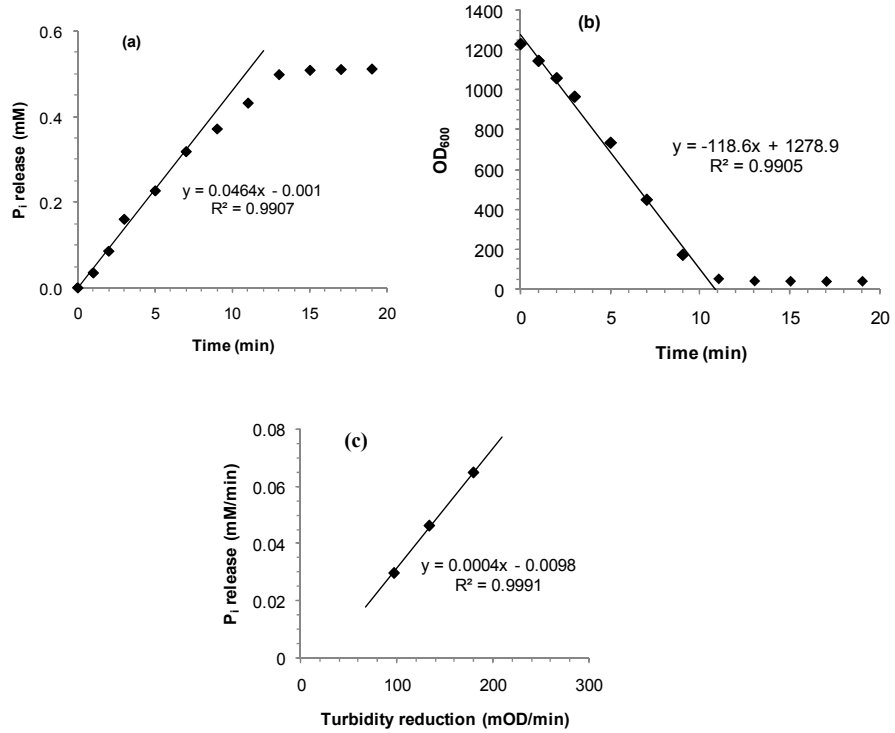

Figure 2.5 Phytase activity measured by (a) P_i release and (b) turbidity reduction; and (c) the relationship between turbidity reduction and phosphate release during reactions with varying concentrations of *A. niger* phytase.

2.2.3 Application as feed additives

Until now, phytase has mainly, if not solely, been used as a feed supplement in diets for swine and poultry, and to some extent for fish [13, 155]. As discussed above, phytate is considered to be an anti-nutritional factor and its hydrolysis by phytases increases the bioavailability of various nutrients. This leads to a reduction of the costs of adding dicalcium 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. Despite that there are several reports on the presence of phytase in the gastrointestinal tract of poultry and livestock [141, 156, 157], phytate phosphorus is still poorly available to humans and monogastric animals (poultry, fish, pigs…) [3, 39, 43] since their intestinal enzymes are depressed by high levels of dietary IP_6 [158], calcium [159, 160], magnesium [161] and P_i [158, 161] in the feed. Thus, additions of phytase instead of dicalcium phosphate to the feed will probably increase.

Vats and Banerjee estimated that 250 g of phytase could replace 10 kg of dicalcium phosphate in animal feeds, and that the actual demand of phytase enzyme for livestock was approximately 200 tonnes per annum [118]. It was suggested from laboratory experiments and field trials that 500-1000 phytase units could replace approximately 1 g of inorganic phosphorus supplementation and reduce total phosphorus excretion by 30-50% [162, 163].

The addition of microbial phytases to feeds has led to increase phytate phosphorus utilization in turkeys [163, 164] and chickens [164, 165, 166]. Adding Finase, a commercial phytase from Alko Co. (Finland), to a corn-soybean pig diet converted one third of the unavailable phosphorus to an available form [167]. A similar phytate phosphorus conversion in chicken and pig diets was reported for Allzyme from Altech (USA) and Natuphos from BASF (USA) [163, 168]. Nelson et al. found an increase in bone ash of 1-day-old chickens fed with a corn-soya diet with phytase from *A. niger* together with the increase of available phosphorus (60%) and the decrease of phosphorus in droppings (50%) [169]

In addition, adding phytase to feed also increases the absorption of minerals, the growth performance and the feed conversion ratio for the animals. Reports from Sebastien et al. showed an increase in body weight (13.2% for male and 5.8% for female) of 180-day-old broiler chickens fed with low-phosphorus diets containing Natuphos 1000 [170]. This increase in body weight was accompanied by a raise of the relative retention of total P, Ca, Cu and Zn by 12.5, 12.2, 19.3, and 62.3%, respectively, as well as a reduction of excretion of phosphorus by 42-51% [170]. The feed conversion ratio and growth rate of growing pigs fed with low-P diets containing microbial phytase were comparable to, or even better than, those obtained on control diets [165].

Phytase in feeds can be inactivated by temperature during feed processing [14], by the low pH or pepsin in the upper part of the gastrointestinal tract of an animal. Phytase for feed applications should therefore be heat tolerant [6], pepsin- and pancreatin- resistant [13], have a tolerance to low pH, be cheap, and work well in a broad pH range in both the upper and lower part of an animal's gastrointestinal tract and at the animal's body temperature [13]. The neutral and slightly alkaline conditions in the lower part of the gastrointestinal tract have been reported to decrease the solubility of IP_6-metal complexes thus lowering the efficacy of phytase [40].

Another problem when employing phytase in the feed industry is that it is an occupational allergen that can cause specific IgE immune responses. The granulated Bio-Feed® Ronozyme phytase from Novo Nordisk is a dust-free product which offers advantages over the powdered enzyme [6]. Feed phytase could also be used in the form of phytase-expressing transgenic plants [171] or ready in the saliva of transgenic animals [172].

2.2.4 Application in foods

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 [173].

Adults consume about 1 g of inositol per day from both animal and plant sources [43]. 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 [155]. An increased dietary consumption of cereal fibers, legumes and soy protein isolates leads to a higher intake of phytate [174]. 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 [175].

Although phytate has been reported to be an antioxidant with anticancer activity, its negative effect related to mineral deficiency [86, 87, 88] is much more severe in undeveloped and developing countries. Different strategies have been developed [176] 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 [176]. Since only a very low phytate-degrading activity occurs in the human intestines [177], supplemental phytases to degrade phytate during food processing and in the gastrointestinal tracts has been suggested [173]. The phytase for food applications would need to be thermostable 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.

Simell *et al.* used Finase phytase for the preparation of phytate-free soy protein [175]. The phytase was used either as a powder [178] or in the immobilized form [179] to prepare phytate-free soybean milk. Sandberg et al. reported that supplement of *A. niger* phytase to flour containing wheat bran would help to increase the iron absorption in humans [180]. Some suggestions for reducing the phytate in foods have been given by Greiner and Konietzny, include producing low phytate mutants in maize, barley, rice and soy bean, enhancing levels of seed phytase by gene transfer, eating more geminating 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) [155]. Addition of phytase during bread-making not only lowers the phytate level, but also releases calcium from the IP_6-Ca complex for α-amylase, hence providing an indirect improvement of the bread quality [155]. 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 humans [155].

In order to improve mineral availability, degradation of inositol hexakis- and pentakisphosphates is essential [181]. 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 since animals live for a relatively short period of time and do not normally receive high levels of dietary iron [182, 183]. 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 [184]. The presence of undigested phytate in the colon may provide protection against the development of colonic carcinoma [142].

2.2.5 Preparation of myo-inositol phosphate

Some lower *myo*-inositol phosphate derivatives are of pharmaceutical interest. Hawkins et al., Phillipy and Graf reported on antioxidant properties of *myo*-inositol 1,2,3 tris phosphate and *myo*-inositol 1,2,3,6 tetrakis phosphate [185, 186]. Many other tri phosphate *myo*-inositols are related to the transmembrane signaling and mobilization of calcium from intracellular space [187]. Siren described a method to prepare *D-myo*-inositol 1,2,6-trisphosphate, *D-myo*-inositol 1,2,5-trisphosphate, L-*myo*-inositol 1,3,4-trisphosphate and *myo*-inositol 1,2,3-trisphosphate using *Saccharomyces cerevisiae* phytase [188]. Starting with immobilized phytase from *Escherichia coli*, Greiner and Konietzny prepared inositol 1,2,3,4,5-pentakisphosphate, inositol 2,3,4,5-tetrakisphosphate, inositol 2,4,5-trisphosphate and inositol 2,5-bisphosphate [189]. Advantages of hydrolysis by phytase include its stereo-specificity and mild reaction conditions [174].

3. Diversity of phytases

Since the first phytase was found by Suzuki et al. in 1907 [190], many different phytases from a variety of sources have been discovered and described.

3.1 Classification of phytases

The International Union of Biochemists [191] currently distinguishes between two classes of phytase enzymes: a 3-phytase (EC 3.1.3.8) and a 6-phytase (EC 3.1.3.26), depending on the position (3 or 6) on the inositol ring where the dephosphorylation is initiated. The 3-phytases yield 1,2,4,5,6-pentakisphosphate, while the 6-phytases give 1,2,3,4,5-pentakisphosphate as the first product along with P_i. The 3-phytases do not always completely dephosphorylate IP_6, whereas the 6-phytases do [14]. The 3-phytases are normally produced by microorganisms and 6-phytases are present in plants [7, 134]. However, there are some exceptions: soybean phytase is a 3-phytase [192] and *Escherichia coli* phytase is a 6-phytase [193].

Based on their active site geometry and catalytic mechanism, phytases are classified into histidine acid phosphatase (HAP) phytases, ß-propeller phytase (BPP) and purple acid phosphatase (PAP) phytases [194]. Based on the pH optimum for catalysis, phytases can be designated as acid, neutral or alkaline [194]. Oh and coworkers have used the phytases´ amino acid sequences and their biochemical properties to divide them into two major groups (HAPs and alkaline phytases) with four sub groups (PhyA, B, C, D: Figure 3.1) [22]; however plant phytases were not included in their classification.

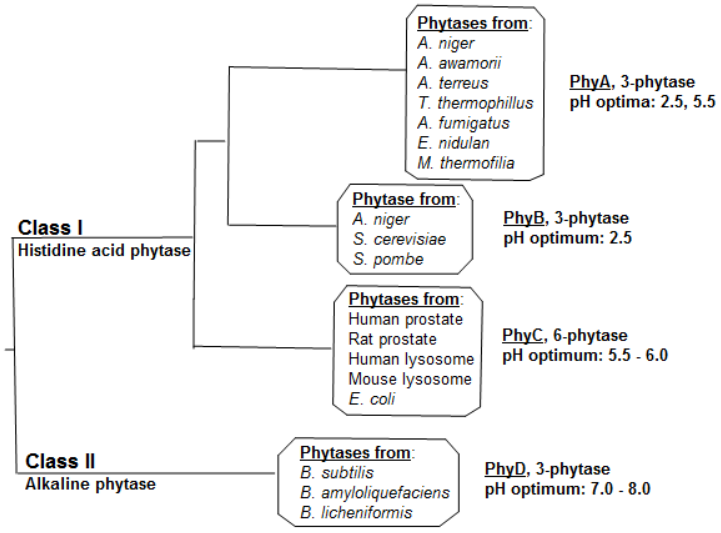

Figure 3.1 Classification of phytases based on their sequence analysis and biochemical properties [22].

3.2 Phytase genes and protein structures

Most phytases that have been found so far are monomers, with the exception of phyB from *Aspergillus niger* and PL phytase from *Penophora lycii* that are tetrameric and dimeric proteins, respectively [22, 195, 196].

3.2.1 Histidine acid phytases

The histidine acid phytase (HAP) group has been the most extensively studied. Members of this group are found among prokaryotes (appA phytase from *Escherichia 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 [197, 198]. This allows a two-step mechanism in the hydrolysis of phosphomonoesters [194]. The phyA and phyB from *A. niger* and appA from *E. coli* are the representatives which have been the most characterized. Some of the distinguished characteristics of the DNA sequence, protein structure and properties of the HAP phytases are summarized in Table 3.1.

Table 3.1 Some sequence and structure characteristics of HAP phytases.

	phyA	**PL phytase**	**phyB**	**appA**
Microorganism	*A.niger*, *A. awamori*	*Penophora lycii*	*A.niger*, *A. awamori*	*E. coli*
DNA sequence homologous to *phyA*	100%	38%	23.1%	15.1%
Active site	RHGXRXP	RHGXRXP	RHGXRXP	RHGVRAP
Substrate biding site	2 acidic and 4 basic residues	-	2 acidic residues	-
Protein structure	monomer	dimer	tetramer	monomer
Glycosylation	9-10 Asp relates to glycosylation	11 Asp relates to glycosylation	glycosylated	Non glycosylated
Disulfide bridge	5 disulfide bonds	4-5 disulfide bonds	5 disulfide bonds	-
Classification	3- phytase	6-phytase	3-phytase	6-phytase
pH optimum	2.5 and 5.0	5.5	2.0	2.5
References	[197, 198, 199, 200, 201]	[195]	[196, 202, 203]	[193, 204, 205, 206, 207, 208]

Three-dimensional structures of phyA from *A. niger* NRRL3135 and appA from *E. coli* are available in the protein data base (PDB). The proteins contain a large α/ß-domain and a smaller α-domain with a relatively large and positively charged active site at the interface of these two domains [201, 208, 217]. Six residues related to substrate-specific sites of phyA phytase were found to encircle the active site cavity [200, 201, 208]. On the other hand, four subunits of phyB from *A. niger* are comprised of two identical dimers, where each monomer consists of a large α/ß-domain and a smaller α-domain, forming an active site like the phyA molecule [196]. 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. 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 [196]. A dynamic light scattering study of the structure of PL phytase from *Penophora lycii* indicated the enzyme to be a dimer [195]. However, the crystal structure of the PL phytase has not yet been determined to confirm its dimeric conformation.

DNA sequence analyses of yeast [209, 210] and maize phytases [211, 212] has revealed that they also belong to the HAP phytase group with both RHGXRXP and HD conservative motifs.

3.2.2 β-propeller phytases

Alkaline ß-propeller phytases (BPP) include phytases from *Bacillus* spp. [213, 214], pollen of *Thypha lattifolia* [215], and *Lilium longiflorum* [52, 216]. Gene sequences have been reported for many *Bacillus phytases* but only the protein structure of the phytase from *Bacillus amyloliquefaciens* DS11 has been solved [218]. This group of phytases does not possess RHGXRXP and HD motifs as HAP phytases do. Rather, they are metallo-enzymes with two separate calcium binding sites (three calciums at high-affinity binding site and the other three at low-affinity calcium binding site [218]). This suggests a difference in the mode of phytate hydrolysis [219].

In spite of the diverse sequences of β-propeller phytases (65-99% similarity) [213, 220, 221], they are conserved at these two calcium binding sites [221]. Tye et al. reported that only the residue Val60 out of 25 residues related to calcium binding sites was non-conserved in phytase from *B. subtilis* 168 [221]. In addition to the substitution of L60V at the center of the enzyme molecule, the phytase from *B. licheniformis* exhibited two more non-conserved residues (T339, E340) near the C-terminus [221] at the periphery of the enzyme molecule. These three residues are coordinated at high affinity calcium binding sites [221], which impart stability to a β-propeller phytase.

Unlike the structure of HAP phytase, β-propeller phytase contains mostly ß sheets in 6 blades arranged like a propeller (Figure 3.2) with a narrow and highly negatively charged

active cleft on the top of the enzyme molecule [218]. Each blade of the β-propeller phytase molecule consists of four to five anti-parallel ß strands; the fourth strand of each blade is connected to the first strand of the next blade across the top of the enzyme molecule. The sixth blade is laid along the central shaft of the propeller, constituting a central channel filled with many well-bound water molecules [218]. This sixth blade consists of 1 ß strand from the N-terminal end and 3 ß strands from the C-terminal end of the protein molecule, joining the two ends of the enzyme molecule, and contributing to the stability of the enzyme. An extra ß strand at the N-terminal end of the protein molecule connects the 6th and the 5th blade, which also supposedly contributes to the stability of the enzyme [218]. This so-called "double clasp" structure together with the three calcium ions at the high-affinity calcium binding sites are responsible for the elevated thermostability of the β-propeller phytase from *Bacillus amyloliquefaciens* DS11 [218, 222]. Three low-affinity binding calcium ions were found to coordinate inside the active site of TS-phy from *Bacillus amyloliquefaciens* DS11 phytase. Together with the side chains of surrounding amino acids, they have a key catalytic function.

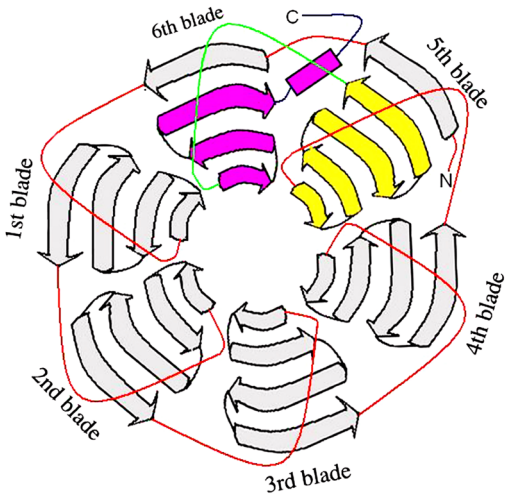

Figure 3.2: Scheme of the structure of β-propeller phytase (redrawn according to Ha et al. [218])

3.2.3 Purple acid phosphatase phytase

Phytase from the cotyledons of germinating soybeans (*Glycine max*) called GmPhy [223] has the active site motif of a purple acid phosphatase (PAP) with binuclear Fe(III)-Zn(II) at the active site [224]. Although the structure of this phytase has not yet been reported, it is the only PAP-like phytase presently known to demonstrate a significant activity towards phytate [194].

Phytase from *Klebsiella terrigena* [225] and *K. aerogenes* [226] have not yet been characterized. Nevertheless, they are reported to be monomeric 3-phytases [225], possessing two inducible forms. The larger form is 700 kDa and the smaller one is 10-13 kDa, with separate pH optima (5.2 and 4.5, respectively) [226]. This is the smallest known protein fraction exhibiting phytase activity [6].

3.3 Substrate specificities and mechanisms of phytate hydrolysis by phytases

HAP phytases show broad substrate specificities with the highest affinity for phytate [174]. PhyB is a special case of the HAP phytases which demonstrates a high activity to many other substrates (*p*-nitrophenyl phosphate, fructose 1-6-bisphosphate, fructose 6-phosphate, glucose 6-phosphate, ribose 5-phosphate, etc) [22, 133]. ß-propeller phytases are known to be very specific to phytate and show very little or no activity to other phosphate esters [213, 214]. Oh et al. reported that the preferential substrate for *Bacillus amyloliquefaciens* DS11 is a bidentate (P-Ca^{2+}-P) formed between Ca^{2+} and two adjacent phosphate groups of its natural substrate phytate (IP_6) [227].

The K_m values of reported phytases are very different, ranging from 0.02 mM for phytase from *Typha latifolia* pollen [215] to 0.7 mM for phytase from *Enterobacter* sp. [228]. K_m values of phytase from plants and fungi seem to be lower than those from bacteria. For instance, the K_m of phyA and phyB from *A. niger* are 0.027 [229] and 0.103 mM [230], respectively, whereas the K_m of appA phytase from *E. coli* is 0.13 mM [193], and that of phytases from *Bacillus* is 0.5-0.55 [213, 214].

Figure 3.3 shows the difference in the electrostatic surface and the active site pocket of HAP phytase from *E. coli* and ß-propeller phytase from *Bacillus* sp. MD2. It is clear that the surface around the active site of HAP phytase is much more positively charged than that of ß-propeller phytase, which makes the enzyme more suitable for hydrolyzing metal-free IP_6 under acidic conditions. On the other hand, the negatively charged active site of BPP phytase with 3 calcium ions embedded inside is more favorable for hydrolyzing IP_6-metal ion complexes under neutral and alkaline conditions.

The histidine residue in the conserved motif, i.e., RHGXRXP, of HAP phytase serves as a nucleophile in the formation of a covalent phosphohistidine intermediate [205, 231] whereas the aspartic acid residue of the C-terminal conserved HD motif serves as a proton donor to the oxygen atom of the scissile phosphomonoester bond [231]. HAP phytase has been seen to initiate the IP_6 hydrolysis at the 3rd P position as in the case of phyA or phyB or at the 6th P position as in the case of appA phytase from *E. coli* and PL phytase from *Penophora lycii* [6]. The end product of HAP phytases is P_i and inositol monophosphate.

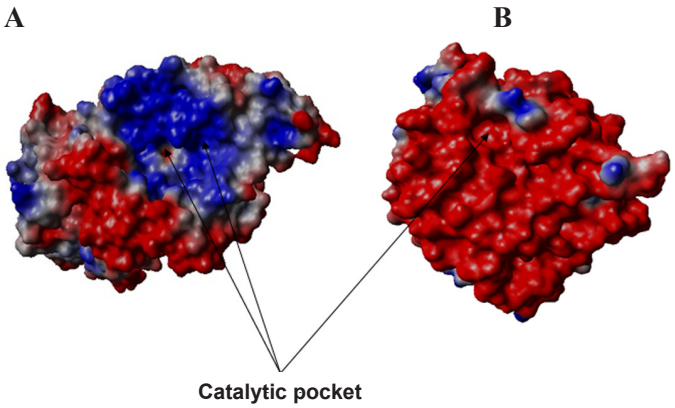

Figure 3.3 Surface representations of (A) histidine acid phytase from *E. coli* (PDB 1DKL) and (B) alkaline phytase model from *Bacillus* sp. MD2 (**Paper IV**). Negatively and positively charged surfaces are colored in red and blue, respectively.

In the case of ß-propeller phytase, the hydroxide ion (Wat1) bridging two calcium ions (Ca5 and Ca6) acts as a nucleophile, directly attacking the phosphorous atom of phytate occupying the cleavage site (Pho1) of the active site [232]. The role of the general acid (-B:H^+) in the catalysis, which concerns donating a proton to the leaving group, can be served by either Wat2, Wat8, or Lys76 [232]. Shin et al. proposed a catalytic mechanism for TS-phy related to two phosphate binding sites in the active site of ß-propeller phytase: one is a cleavage site (Pho1) responsible for the hydrolysis of a phosphomonoester group in IP_6, and the other is an affinity site (Pho2) that increases the binding affinity for a vicinal phospho monoester group in IP_6 [232]. The proposed mechanism is depicted in Figure 3.4. Shin et al. also provided evidence that BPP phytase could initiate the hydrolysis of IP_6 at any position of phosphomonoester in the substrate, IP_6, and could catalyze the hydrolysis of *myo*-inositol 2-monophosphate but with very low speed (159 times lower than that with IP_6) [232]. Consequently, ß-propeller phytase cannot be classified as 3- or 6-phytase. Later on, Oh et al. reported the preferred substrate for TS-Phy to be a bidentate (P-Ca^{2+}-P) formed between Ca^{2+} and two adjacent phosphate groups of its natural substrate phytate (IP_6) [227], and the 2-4-6 inositol trisphosphate is often the final product. This explains the preferential pathway of ß-propeller phytase in hydrolysis of alternate phosphate groups in the IP_6 molecule.

Kerovuo and coworkers analyzed several isomers of inositol phosphate generated from phytate hydrolysis by Phy C from *Bacillus subtilis* VTTE 68013 with HPLC and suggested two alternative pathways for the hydrolysis of phytic acid (Figure 3.5). These two pathways resulted in two *myo*-inositol trisphosphate end products: inositol(2,4,6)P_3 and inositol(1,3,5)P_3 [233]. Computer-modeling analyses of enzyme-substrate complexes

revealed the different binding of the substrate (IP_6) to the active site of Phy C. An intended cleavable phosphate group of IP_6 is directed to the active site by its two neighboring phosphate groups which also bind to the enzyme [233]. Although the mechanism proposed by Kerovuo et al. did not point out the role of calcium in the catalytic mechanism, it provided a good explanation of the preference of ß-propeller phytase in the hydrolysis of alternate phosphate groups in an IP_6 molecule.

From the analysis of various isomers of inositol phosphate generated from phytate acid hydrolysis by *Bacillus subtilis* 168, *Bacillus amyloliquefaciens* ATCC 15841, and *Bacillus amyloliquefaciens* 45, Greiner et al. concluded that the sequential removal of phosphate groups from IP_6 of ß-propeller phytase occurs via two independent routes that proceed either via D-inositol(1,2,4,5,6)P_5 through inositol(2,4,5,6)P_4 to inositol(2,4,6)P_3, or D-inositol(2,5,6)P_3, or via D-inositol(1,2,4,5,6)P_5 through D-inositol(1,2,5,6)P_4 to D-inositol(1,2,6)P_3 [234]. D-inositol(2)P is the final product of both routes in the presence of excess of enzyme and for a prolonged reaction time [234]. In this case, ß-propeller phytase could be classified as 3-phytase.

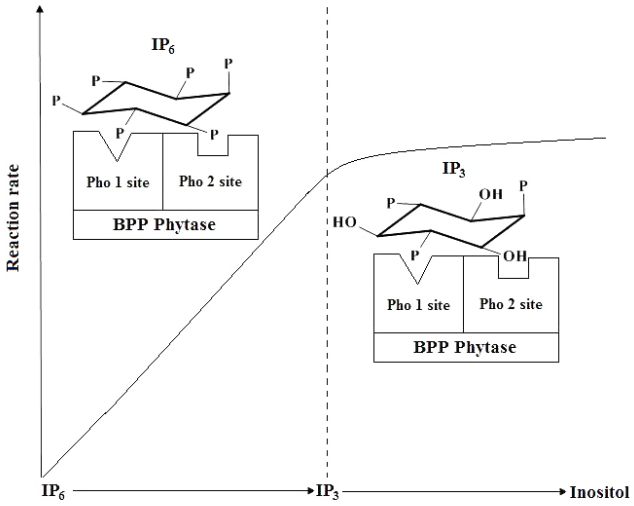

Figure 3.4 Scheme of phytate hydrolysis by TS-Phy (redrawn according to Shin et al. [232]). The reaction is fast when two adjacent phosphate groups of the substrate simultaneously occupy both the cleavage (Pho1) and the affinity (Pho2) sites. The reaction is very slow when only one phosphate group occupies the cleavage site (Pho1), and the affinity site (Pho2) is empty. P stands for $H_2PO_4^-$.

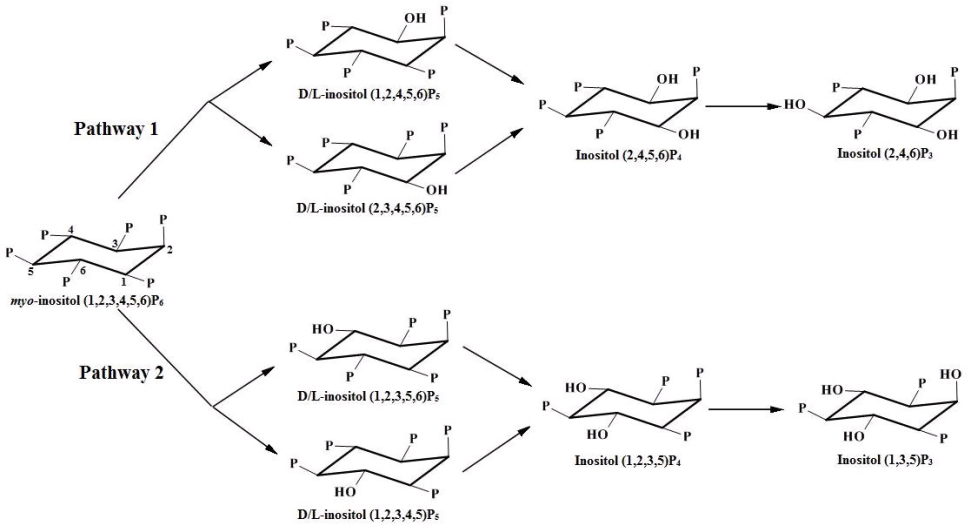

Figure 3.5 Scheme of the IP_6 hydrolysis pathways of Phy C from *Bacillus subtilis* VTTE 68013 (redrawn according to Kerovuo et al., [232]). P stands for $H_2PO_4^-$.

3.4 Biophysical and biochemical properties of phytases

Biophysical and biochemical properties of both HAP and ß-propeller phytases have been summarized in numerous reviews [13, 22, 118, 120, 139, 235].

The molecular weight of phytases from bacteria normally range from 35 to 50 kDa, except the one from *Klebsiella aerogenes* which has two inducible molecular forms as described above [226]. Phytases from eukaryotic organisms (yeasts, fungi, plants and animals) are often glycosylated and have higher molecular weights: 85-150 kDa for fungal phytases, around 500 kDa for yeast phytases, and 50-150 kDa for phytases from plants and animal tissues.

Most of the known phytases have an optimum temperature in the range of 45-80 °C. The highest optimum temperature found for phytase from *Schwanniomyces castellii* is 77 °C [236], from *Bacillus* sp. MD2 it is 73 °C (**Paper II**), and from *Bacillus amyloliquefaciens* DS11 it is 70 °C [214, 222]. These relatively high temperature optima preclude phytases from their full activity at the stomach temperatures of swine or poultry (37-40 ◦C), and cause an even poorer performance of phytases in fish [13]. The thermostability of phytases is correlated to their optimum temperatures. Most phytases are inactivated at temperatures of 40-65 °C [22] including the phytase from *S. castellii*. Phytase from *A. fumingatus* has been reported to lose only 10% of its activity after 10 min

of exposure to 100 °C [237], but inactivation begins at 60 °C [238]. Since commercial feeds are often pelletized at high temperature (60-80 °C) [13], thermostable phytases such as ß-propeller phytase from *Bacillus amyloliquefaciens* DS11 (with a melting temperature around 80 °C), from *Bacillus* sp. MD2 (**Paper III**) and from *A. fumingatus*, present a true advantage.

Most HAP phytases have pH-optima in the range of 4.5-6.0, except for phyB from *Aspegillus niger* that is optimally active at 2.5 [13, 22, 118]. Phy A from *Aspegillus niger* has two pH optima at 2.5 and 5.5, and the ß-propeller phytases have pH optima between pH 6 and 7.5 ([213, 214, 239] and **Paper III**). Hence, HAP phytases can work in the upper part of an animal's intestinal tract where acidic conditions prevail, whereas ß-propeller phytases can work in the lower parts, such as the small intestine, where neutral and alkaline conditions are predominant. The ability to withstand the low pH in the animal stomach is important. HAP phytases are stable under acidic conditions, whereas ß-propeller phytases cannot work at low pH but show remarkable stability at low pH (**Paper II, III**, [214]).

Resistance to proteolysis is of great interest for food/feed applications. A higher resistance has been reported for *E. coli* phytase as compared to *A. niger* phytase [240, 241, 242]. *Bacillus* phytase is known to be extremely resistant towards papain, pancreatin and trypsin but susceptible to action of pepsin (Paper II, [243]. During incubation of different phytases with supernatants of the digestive fluid from various segments of the digestive tract of hens, Simon et al. [241] found the most resistant phytases to be from *E. coli, Bacillus* sp. and Consensus phytase (Table 3.2).

Table 3.2 Residual phytase activities (%) after 60 min of incubation at 40 ºC in digesta supernatants from various segments of the digestive tract of hens [240].

Phytase	Crop	Stomach	Duodenum	Jejunum	Ileum
Aspergiluss A	98.5	60.4	93.6	60.2	54.5
Peniophora	96.8	59.2	94.8	91.1	84.8
Escherichia coli	96.9	92.8	96.8	86.7	80.4
Bacillus	93.5	70.8	95.3	91.5	97.3
Consensus	91.5	86.5	88.3	78.0	85.5

The activity and stability of ß-propeller phytases are reported to be strongly dependent on calcium [151, 222, 243], and EDTA is a competitive inhibitor to the enzyme activity [222]. On the other hand, the activity of HAP phytases often increases slightly in the presence of EDTA. NaCl (500 mM) was found to diminish the dip between two pH optima (pH 2.5 and 5.5) in the pH profile of *Aspergillus niger* phytase; as well as to shift the pH optimum of *E. coli* phytase to pH 2.0 from 5.5 [244].

3.5 *Bacillus* sp. MD2 and its thermostable phytase

The strain MD2 was isolated in Hanoi (Vietnam) from a sample of infant excrement. It showed positive phytase activity (0.2 units/ml) after 3 days of incubation at 37 °C in a medium of meat pepton agar (MPA) containing 1% sodium phytate. Morphological studies displayed the strain MD2 to be a Gram positive bacteria, rod-shaped with round ends, 0.5 x 1.5µm, and motile by flagella (Figure 3.6). On agar medium, its colony was circular (0.2-0.3 mm) with rough edges and a dense point at the center. The 1407 nucleotide long 16S rRNA gene sequence (GenBank accession number GU143091) of the isolate showed a high similarity (99%) to *Bacillus subtilis* and *B. amyloliquefaciens* sequences. Figure 3.6 presents *Bacillus* sp. MD2 on the phylogenic tree with other closely related microorganisms.

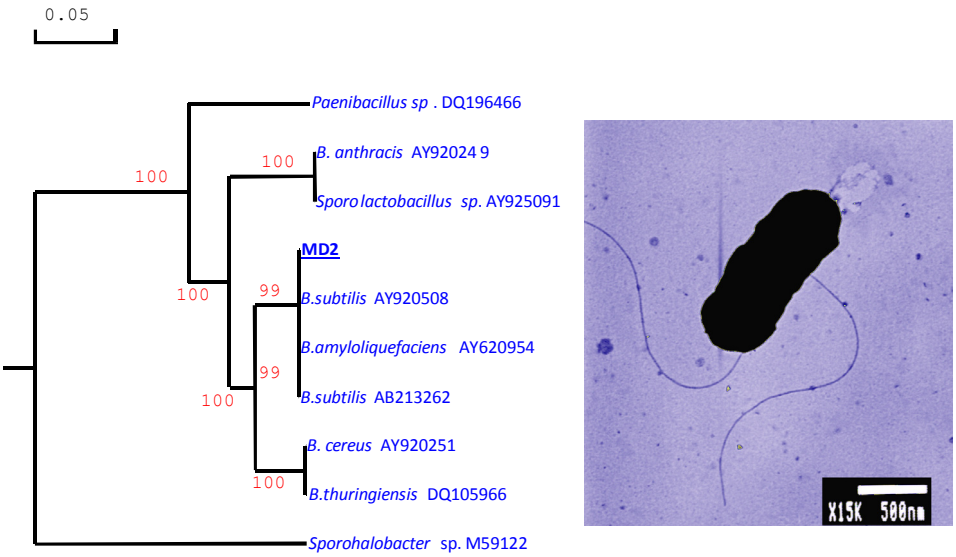

Figure 3.6 Phylogenic tree depicting the classification of MD2 isolate (left); and the morphology of the MD2 isolate obtained with scanning electron microscopy at a magnification of 15000 x (right).

The phytase encoding gene (GenBank accession number GU143090) of *Bacillus* sp. MD2 was amplified and sequenced in order to deduce the amino acid sequence. Just as the other phytases of *Bacillus* origin, the mature peptide of 354 amino acids does not possess the HAP phytase conserved RHGXRXP motif. The sequence is highly similar to that of TS-phy from *Bacillus amyloliquefaciens* DS11 [220]. There are two residues at positions 81 and 148 where Ala and Asn in TS-phy are replaced by Val and Asp, respectively. The

sequence also closely resembled that of *phyC* from *Bacillus subtilis* VTTE68013 [213], differing only by 8 residues (**Paper II**).

The recombinant *Bacillus* sp. MD2 phytase had a specific activity of 39 U/mg, a molecular weight of 47.5 kDa and an optimal activity at pH 6-7 and 67-73 ºC (**Paper II** Figure 3.7a). It was highly stable in the pH range of 3.6-9.6 at room temperature, and exhibited a remarkable thermostability in the presence of 5 mM $CaCl_2$, retaining about 40 % of the activity at pH 7 after 10 min at 100 ºC (Figure 3.7b).

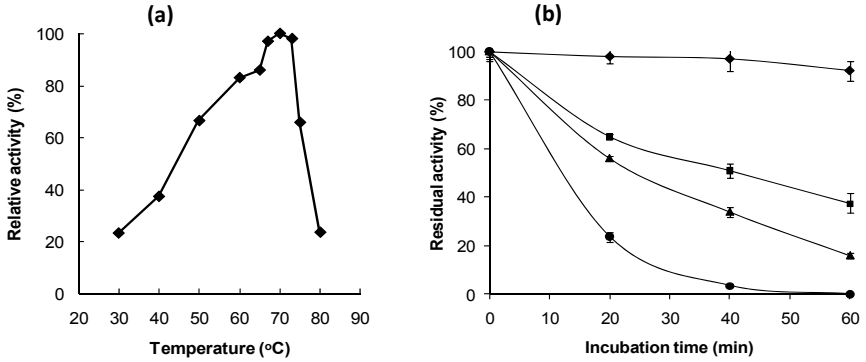

Figure 3.7 Effect of temperature on (a) the activity and (b) the stability of the recombinant *Bacillus* sp. MD2 phytase: (a) The enzyme activity was determined using sodium phytate dissolved in buffer of pH 7 containing 5 mM $CaCl_2$. (b) The enzyme was incubated in Tris-HCl buffer, pH 7 containing 5 mM $CaCl_2$ at 70 °C (♦), 80 °C (■), 90 °C (▲) and 100 °C (●), and then cooled prior to activity measurements at 70 °C (**Paper II and III**).

A distinguishing feature of the MD2 phytase with respect to the other alkaline phytases possessing a strict specificity for phytate substrate [3, 27] was that the enzyme - although being most active with phytate - also displayed a certain degree of activity with other phosphate substrates such as ADP, ATP, α-fructose-1,6-diphosphate, *p*-nitrophenyl phosphate and sodium pyrophosphate. The K_m and V_{max} values of the enzyme determined at 70 °C using sodium phytate as the substrate were 0.9 mM and 41 μmol of P_i per min per mg of protein, respectively.

The enzyme showed the highest activity at 70 °C when adding 2-4 mM $CaCl_2$ to the assay buffer, but the activity was reduced at a $CaCl_2$ concentration higher than 4 mM. At 37 °C, the enzyme demonstrated a lower activity as opposed to at 70 °C, and the effect of the $CaCl_2$ concentration was less than at 70 °C (**Paper III**).

Thermostability of the enzyme in the presence/absence of various divalent metal ions was determined by differential scanning calorimetry (DSC) (**Paper III**). In the presence of 5 mM $CaCl_2$, the T_m value (the melting temperature; midpoint of the temperature

denaturation curve) of the enzyme increased by about 12 °C. The optimum temperature for enzyme activity was shifted from 40 °C (without added calcium) to 70 °C when 1-5 mM of $CaCl_2$ was added to the reaction mixture; moreover, a more than 3-fold increase in enzyme activity was achieved (Figure 3.8). The shift in optimum temperature of the enzyme in the presence of calcium was ascribed to the role of the latter with regard to the thermostability of the enzyme. The optimum pH of MD2 phytase was also shifted toward the acidic region (0.5-1 pH-unit) at both 37 and 70 °C in the presence of 1-5 mM $CaCl_2$ (Figure 3.9). This was believed to be due to a combined effect of a more favorable binding of calcium-phytate (in contrast to phytic acid) and facilitated release of the leaving group (inorganic ortho-phosphate) after the cleavage of the phosphomonoester bond caused by the relative abundance of H^+ at lower pH.

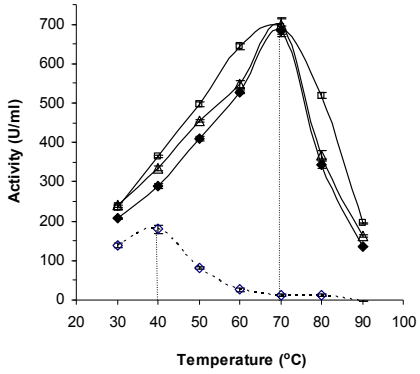

Figure 3.8 Effects of calcium and temperature on the activity of the recombinant *Bacillus* sp. MD2 phytase (**Paper III**). The enzyme activity was determined by assaying the calcium-loaded phytase at different temperatures using sodium phytate dissolved in buffer at pH 7 containing 0 (◊), 1 (□), 3 (Δ), 5 mM $CaCl_2$ (♦). The dotted lines show the optimal temperature for the enzyme activity.

Removal of calcium from the MD2 phytase molecule by intensive dialysis against 15 mM EDTA resulted in an average of 0.79 calcium ions retained per enzyme molecule. This form of metal-depleted enzyme demonstrated less than 2% of the original activity, which was fully recovered when the enzyme was re-charged with 1 or 5 mM $CaCl_2$. Moreover, 96-99% of the activity was obtained when supplemented with Sr^{2+}. Even with Ba^{2+}, Mn^{2+}, and Mg^{2+} salts, the activity recovery was as high as 90% at 37 °C, and even higher at 70 °C. This indicated that these metal ions were able to bind to low-affinity triadic calcium sites and the catalytically important water molecules in the active site, hence fulfilling the activation role of Ca^{2+} (Paper III). However, the thermal stability (described by T_m) of the phytase was not improved as much as in the presence of calcium. This suggests that the presence of calcium at the high-affinity sites was important for the thermal stability of the enzyme.

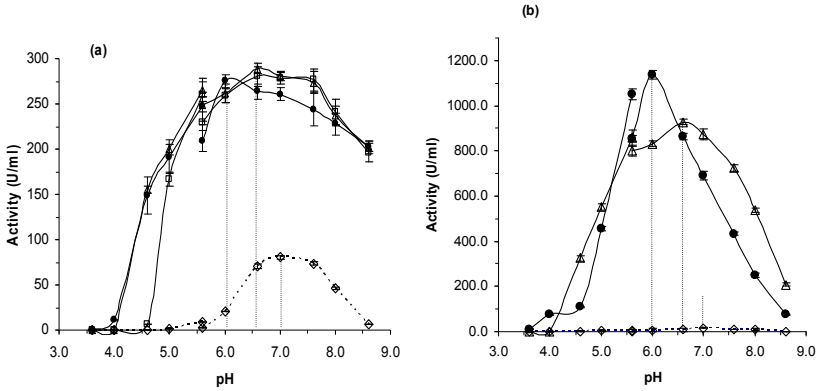

Figure 3.9 Effect of calcium on the pH profile of *Bacillus* sp. MD2 phytase at (a) 37 °C and (b) 70 °C (**Paper III**). The enzyme activity was determined in 0.1 M acetate buffer (pH 3.6-5.6) and Tris-maleate buffer (pH 5.6-8.6) containing 0 (◊), 1 (□), 3 (Δ), 5 mM $CaCl_2$ (●) and 1.5 mM sodium phytate. The dotted lines display the optimal pH for the enzyme activity.

MD2 phytase exhibited a high specificity towards the IP_6-Ca complex substrate. When replacing Ca^{2+} with other metal ions in the IP_6-metal ion complex substrate, no dephosphorylation took place. The enzyme showed a limited activity to IP_6-Mg and IP_6-Sr complex substrates (28 and 25% relative activity, respectively) at 37 °C but not at 70 °C. When the calcium-depleted enzyme was recharged with various metal ions, it demonstrated no activity with the corresponding IP_6-metal ion complex substrates, except with the IP_6-Ca complex (100% relative activity) at both 37 and 70 °C; with IP_6-Mg complex (23% relative activity) and IP_6-Sr complex (21% relative activity) at 37 °C but not at 70 °C (**Paper III**).

4. Phytase production and -engineering

At the close of the 20th century, annual sales of phytase as an animal feed additive were estimated to US$ 500 million and are continuing to rise [19]. This growth of the market for phosphate additives to animal feed has fostered a critical step in the commercial development of phytases [119].

4.1 Production of phytase

Although phytases are widely distributed in nature, the production in wild-type organisms is far from an economically viable level. Of all the studied microorganisms, the production of phytase is the highest in the fungi of the genus *Aspergillus*, with the most elevated levels of extracellular phytase produced by wild-type *Aspergillus niger* of around 7 U/ml (recalculated from the raw data based on the standard definition of a phytase unit) [119]. Hence, cloning and expression of phytase genes in suitable host organisms is necessary in order to reach higher productivities. The commercialization of a food/feed enzyme is based on access to suitable delivery systems and the ability to produce the enzyme in an economical fashion [14].

The critical factors affecting the yield include the culture conditions, type of organism, nature of the substrate and availability of nutrients and these parameters should be taken into account when selecting a particular production technique [118]. Recombinant phytase from *Aspergillus* sp. has been achieved by three cultivation methods: solid state [245, 246], semi-solid [247] and submerged fermentation [154, 229]. The technical control and the possibility of scaling up to an industrial level have limited the application of the solid state fermentation despite it having shown some advantages regarding use of agricultural by-products, as well as saving water and energy.

Krishna et al. reported high product titer and a productivity for phytase production by solid-state fermentation of *Aspegillus niger* (844±121 U/g substrate after 144 h cultivation) comparable to that achieved with submerged fermentation [246]. A commercial phytase named Natuphos™ from Gist-Brocades is the first recombinant phytase expressed by *A. niger* NRRL 3135 *phy*A gene cloned in multiple copies in a PluGBug® system and produced in an *A. niger* host by submerged fermentation [6, 248]. Bio-feed phytase Ronozyme™P from Novo Nordisk is also a recombinant phytase originating from *Peniophora lycii* - a basidiomycete from where the phytase gene was cloned, overexpressed and produced in an *A. niger* system by submerged fermentation [6]. Other commercial phytases such as Phyzyme XP (Danisco, Brabrand, Denmark) and Optiphos (JBS United, Indiana, USA) are recombinant types expressed from the *E. coli* phytase gene in *Bacillus* host systems.

So far, submerged fermentation is the best choice for phytase production both in prokaryote (*E. coli, Bacillus*) and eukaryote (*A. niger*) host systems. Almost all phytase

genes that have been cloned to date originate from mesophiles and are expressed in mesophilic host systems. Hence, the optimum temperature for the production of phytases from a majority of microorganisms lies in the range of 25-37 °C and the optimum pH values of most bacterial and fungal phytase productions range from 5.0 to 7.0 [120].

The choice of a carbon source is of importance for all phytase production systems. Glucose at 1-2% concentration has been used for the recombinant *Bacillus subtilis* system [213, 249], wheat bran (6%) was a good carbon source for phytase production by *Bacillus* sp. DS11 [214], while for fungi, malt yeast extract broth was utilized [119]. Simple sugars such as glucose and sucrose when used as the sole sources of carbon for phytase production in *A. niger* NRRL 3135 gave rise to mycelia pellets and low yields of phytase were observed [119]. The source of nitrogen seems to be a critical component in the production medium of recombinant *Bacillus subtilis* in order to obtain high yields of phytase [249, 250], however, *E. coli* cultivation needs a simple nitrogen source such as $(NH_4)_2SO_4$ (**Paper II** and [251]). Inorganic phosphate (P_i) was reported to inhibit the phytase production of yeast and mold [119, 154, 229, 247]; P_i became critical for phytase production if a phosphate-depleted promoter was used in a recombinant system to trigger the expression [154, 229, 247, 249, 250]. Due to the formation of mycelia, surfactant [119, 247], the size and age of the inocula [14] were found to have a strong effect on the phytase production in a fungal system.

The phytase gene has been cloned and expressed in plants (maize, soy bean and rice) in order to grow crops with low phytate levels [252, 253, 254]. Plants have also been used as hosts or “bioreactors” to produce phytase in alfalfa leaves [255], and tobacco leaves [256, 257] in order for most of its product to be present in the juice. The equipment investment for this biofarming is minimal and potentially turns a byproduct into a source of additional income for the farmer [6].

Golovan et al., have successfully developed a transgenic pig carrying the *E. coli appA* phytase gene which expressed phytase in saliva. This allows the pigs to dephosphorylate the phytate in the pig diet [172]. The offsprings of the transgenic pig expressed an average of 2000-3000 U/ml of phytase in their saliva, providing an essentially complete digestion of dietary phytate phosphorus, thereby relieving the requirement for inorganic phosphate supplements, and reducing fecal phosphorus output by up to 75% [172].

4.1.1 *Escherichia coli* as a recombinant host and high cell density cultivations

Escherichia coli is often the primary choice as a host microorganism for laboratory-scale production of recombinant proteins since it is a well known system of high productivity. Recombinant proteins produced in an *E. coli* host normally accumulate inside the cells (intracellular protein) or in the periplasmic space (periplasmic protein). However, in certain cases, the stress on the outer membrane due to periplasmic accumulation of

proteins results in a loss of membrane integrity or in the triggering of an autolytic response caused by changes in the cultivation conditions [258, 259, 260]. Leakage of the periplasmic enzymes by increasing the growth temperature is likely to be caused by induction of a heat-shock response and an activation of the phospholipase activity in the outer membrane [260].

A phytase gene amplified from genomic DNA of *Bacillus* sp. MD2 was inserted between the restriction sites for *Hind*III and *Xho*I at the multiple cloning site of pET-22b(+) to construct the recombinant pE10C2. This construction rendered it possible to express the phytase with a *pel* B leader peptide at the N-terminal end, which was expected to facilitate the export of the target protein to the periplasmic space. The recombinant constructs without the leader peptide resulted in most of the phytase being insoluble, and very little activity was detected in the culture. Supplementation of 10 mM $CaCl_2$ to the medium during induction gave rise to the expression of a higher phytase activity; a maximum activity of 3.6 U/ml was obtained with 10 mM $CaCl_2$ in the medium during shake flask cultivations. More than 90% of the expressed recombinant phytase leaked out of the cells into the medium (Figure 4.1 and **Paper II**). Such a high level of leakage into the extracellular medium is an advantage for downstream processing of the phytase as there is no need for cell disruption and separation of cell debris and the amount of host cell proteins is much lower in the extracellular medium. Indeed the specific activity of recombinant phytase in the cell-free fraction of the fed-batch cultivations was about 35 U/mg total protein, constituting more than 90% of the total protein.

Many strategies have been devoted to the establishment of high cell density processes to increase the level of production of recombinant target proteins by *E. coli*. The idea behind most of them has been to control the substrate concentration at certain levels in order to avoid an overflow metabolism and the resultant acetate accumulation under glucose excess condition [251, 261, 262]. Åkesson and coworkers [262] have previously proposed a feeding strategy that is automatically controlled by the dissolved oxygen level such that aerobic conditions are maintained in spite of the limitations in oxygen transfer, i.e., the feeding rate is decreased when a maximum oxygen transfer capacity is reached in the bioreactor. By employing this strategy in the cultivation of recombinant *E. coli* harboring pE10C2 and controlling a suitable P_i level for phytase induction, a high phytase production (327 U/ml) could be achieved after 15 hours of fed-batch cultivation including 5 hours of induction by IPTG (**Paper II**). Induction by lactose (20 mM) led to a higher cell density (57 g cell dry weight/l as compared to 33.7 g cell dry weight/l in the case of IPTG induction). However, the phytase production was delayed, and the total phytase activity was 71 U/ml at the end of the cultivation. This is the highest phytase productivity in submerged fermentation of a prokaryote host reported so far (Table 4.1). It seems possible to achieve higher productivities with prolonged induction times.

Table 4.1 Comparison of the phytase production in batch and fed-batch cultivations of *E. coli* with recombinant *Bacillus* sp. MD2 phytase with other systems.

Cultivation type, inducer	Host strain	Total phytase production (U/ml)	Reference
Batch, IPTG	Recombinant *E. coli*	10.3	Paper II
Batch, lactose	Recombinant *E. coli*	2.25	[263]
Fed-batch, IPTG	Recombinant *E. coli*	327	Paper II
Fed-batch, lactose	Recombinant *E. coli*	71	Paper II
Batch, IPTG	Recombinant *B. subtilis*	2	[250]
Fed-batch, IPTG	Recombinant *B. subtilis*	28.7	[264]
Fed-batch, IPTG	Recombinant *B. subtilis*	48	[249]
Fed-batch, IPTG	Recombinant *E. coli*	120	[251, 265]

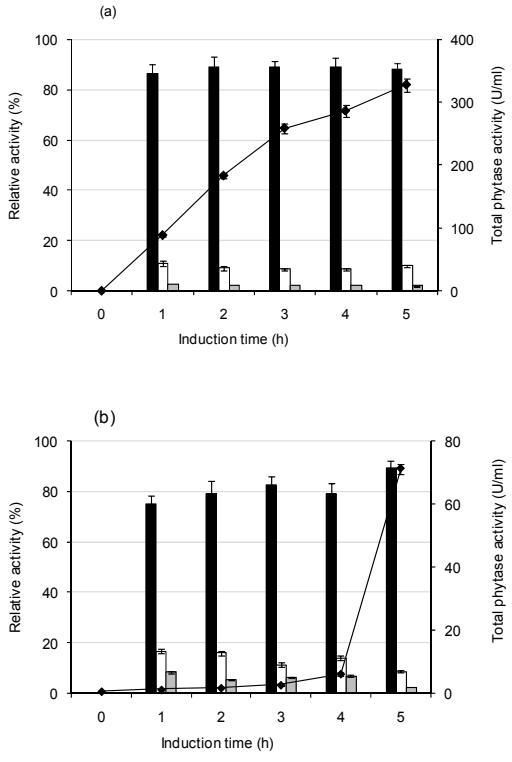

Figure 4.1 Distribution of recombinant phytase as a function of time during fed-batch cultivation in 2.5 liters of culture medium induced by (a) 1 mM IPTG and (b) 10 mM lactose: relative activity in extracellular (■), periplasmic (□), and cytoplasmic (▣) fractions, and total phytase activity (♦) (**Paper I**).

4.2 Genetic engineering of phytases

Although the properties of phytases vary, there is no single wild-type enzyme that is perfect or ideal for the field application [13]. Each specific application defines its own demands of the enzyme properties. For the feed application, which is presently a major application of phytase, an "ideal" phytase should be catalytically efficient, proteolysis-resistant, thermostable and cheap [13]. Genetic engineering has been a useful tool to modify and improve properties of phytases. The consensus-approach has commonly been used to search for a way to combine/increase specific properties from various phytases with similar protein structures. Three-dimensional structure studies also represent a good tool when searching for a specific point in the enzyme structure for mutation design. In almost all cases of phytase engineering, site-directed mutagenesis is the selected method [266, 267, 268, 269, 270] however, random mutagenesis has also been utilized [271].

The catalytic efficiency of an enzyme is its specific activity at the applied condition. The higher the specific activity at the temperature and pH prevailing in the gastrointestinal tract of the animal or during storage, the better is the phytase for feed application. By employing site-directed mutagenesis, Rodriguez et al. were able to improve the catalytic efficiency and thermostability of acid phosphatase/phytase (pH 2.5) from *E. coli* expressed in *P. pastoris* [268]. Tomschy et al. found an active site residue 297 of *A. niger* related to its specific activity; and later on by using site-directed mutagenesis to create a mutant phytase with a 7-fold improvement of the specific activity of a heat-stable *A. fumigatus* phytase [269, 270]. When probing the structure and function of MD2 phytase (**Paper IV**), two mutant variants of phytase (E229V and S283R) carrying a single mutation site to reduce the negative charge and increase the positive charge of the catalytic surface of MD2 phytase were found to increase the specific activity by about 19 and 13%, respectively, compared to the wild-type MD2 phytase.

Thermostability is another desired property for feed phytase since the enzyme may be inactivated by elevated temperatures (65-95 °C) during feed processing [6, 14]. If an additional processing step is required after pelletization in order to add the phytase, the commodity cost will increase. A phytase that combines the desirable traits with high heat tolerance would therefore be a superior enzyme for a majority of current animal feed applications [6]. Some phytase genes from thermophilic fungi (*Myceliophthora thermophila, Talaromyces thermophilis, Thermomyces lanuginosus*) have been cloned [272, 273, 274], and the analysis of the gene sequence of these thermostable phytases by means of the consensus-approach has led to the design of a new phytase with a higher stability and with a high specific activity at ambient temperature. Lehmann et al. performed a sequence comparison study of 13 phytases from six different fungi and utilized the consensus-approach to select the most conserved position of each residue to *de novo* construct a consensus phytase with improved thermostability [266]. Later, they included consensus amino acid sequences of six more phytase sequences and studied 38

amino acid substitutions by site-directed mutagenesis to stabilize amino acids in a consensus phytase sequence and further increase the unfolding temperature [267]. By employing error-prone PCR, Kim and Lei proposed two substitutions of K46E and K65E, capable of introducing additional hydrogen bonds with adjacent residues, thereby improving the thermostability of *E. coli* AppA2 phytase through a stabilization of local interactions. Their two variants (K46E and K65E/K97M/S209G) showed over 20% improvement in thermostability during incubation at 80°C for 10 min, and a 6-7 °C increase in melting temperature (T_m) [271].

Phytase in feed ingredients can also be inactivated by the low pH of the upper portion of the gastrointestinal tract [275], as well as by the action of pepsin and pancreatin from gastric secretions [275]. By site-directed mutagenesis of the *phy*A gene, which leads to the creation of a single mutation, i.e., K300E, Mullaney et al. increased the phytase activity of *A. niger* NRRL 3135 phytase (phyA) at intermediate pH levels (3.0-5.0) and improved phytic acid hydrolysis at 37 °C by 6 and 19% at pH 4.0 and 5.0, respectively [276]. Later on, based on the crystal structure of PhyA, they prepared 21 single or multiple mutants, among which 17 mutants lost one pH optimum (at pH 2.5) or shifted the pH optimum from pH 5.5 to more acidic values. The mutant E228K exhibited the best overall changes, with a shift of the pH optimum to 3.8 and 266% greater ($P < 0.05$) hydrolysis of soy phytate at pH 3.5 as opposed to the wild-type phyA enzyme [277]. By rational mutagenesis, Tomschy et al. successfully engineered the pH activity profiles of phytases (*A. fumigatus* and consensus phytases) for the possible industrial applications of phytase in animal feed [278]. Jermutus *et al.* was successful in applying designed structure-based chimeric enzymes to improve the thermostability, specific activity, and pH optimum of phytase [279]. The consensus-approach has also been applied to β-propeller phytase to synthesize a phytase gene expressing a broader pH profile and a higher thermostability at pH 7.5 [280].

As described in **Paper IV**, site-directed mutagenesis at a single site, E227S, resulted in a shift in the pH optimum of the enzyme to the acidic side (Figure 4.2), as well as in an improvement of the stability under acidic conditions. This mutant phytase, displayed over 80% of its initial activity after 3 h of incubation at pH 2.6 as compared to the wild-type phytase which retained only 40% of its original activity. Moreover, the mutant phytase demonstrated a higher activity as opposed to its wild-type counterpart on calcium phytate, sodium pyrophosphate and *p*-nitro phenyl phosphate (**Paper IV**).

The protease-sensitive sites of phytases, normally in the exposed loops at the surface of the molecules, may be blocked or modified using site-directed mutagenesis [281].

To obtain cheaper enzymes for food/feed applications, most commercial phytases are now produced in recombinant systems. Genetic engineering has been applied to improve enzyme specificity also in addition to the production yield. By employing UV radiation, Chelius and Wodzinski isolated a mutant of *A. niger* NRRL 3135, producing 3.3-fold higher phytase (phyA) as opposed to the wild-type strain [282]. Attempts have also been

made at creating a chimeric protein by fusing different enzyme molecules which showed both endoglucanase and phytase activities across broad ranges of pH (i.e., 4.0-8.0) and temperature (i.e., 25-75 °C) [283].

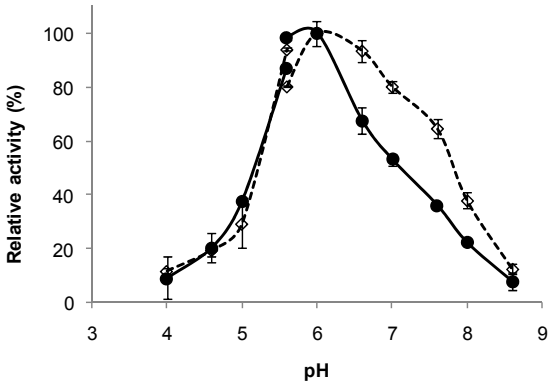

Figure 4.2 Effect of pH on the activity of native *Bacillus* sp. MD2 phytase (◊) and mutant E227S (●) (**Paper IV**). The enzyme activity was determined in 0.1 M buffers of different pH values (acetate buffer pH 3.6-5.6; Tris-maleate buffer pH 5.6-8.6) containing 5 mM $CaCl_2$ and 1.5 mM sodium phytate at 70 ºC for 20 min.

5. Concluding remarks and future perspectives

Phytases have been of interest for over a hundred years, owing to their usefulness from a nutritional as well as environmental perspective. Research has been devoted to discovering organisms with suitable characteristics of phytase in nature, increasing the production yield by cloning and expression in a well-controlled host, and modifying the enzyme properties to suit application requirements.

So far, the main application of phytases has been as a feed additive. In this area, only histidine acid phytases are commercialized. They are active under acidic conditions such as in the stomach of animals; however, they are not active under high concentrations of Ca^{2+} under neutral and slightly alkaline conditions such as in the intestines of animals. Moreover, they are not stable enough to withstand the high temperatures of feed pelletizing processes. Great interest has been addressed on mutation studies in order to increase the thermostability and modify the characteristics of histidine acid phytases. Alkaline ß-propeller phytases, although not active under acidic conditions, show a high thermostability and activity in the presence of calcium. The combination of histidine acid phytase and ß-propeller phytase in feed would give a synergistic effect in increasing the bioavailability of phosphorus in the digestion tract of animals. This has been demonstrated in studies with chickens, and needs to be confirmed for other poultry and livestock.

Phytases are not yet employed in food processing applications, however, there have been many studies focused on improving food quality for people in developing countries. ß-propeller phytase could find potential in food applications since it is thermostable enough to withstand Pasteurization and active during long-term storage of foods at room temperature. The benefit of the lower phosphorylation of inositol phosphate (e.g., IP_4, IP_3, IP_2) for health would make ß-propeller phytases more advantageous than histidine acid phytases since the end product is inositol trisphosphate, and not inositol monophosphate. Furthermore, the production of recombinant ß-propeller phytases in a GRAS host would render them more advantageous for food applications.

Specific inositol phosphate derivatives are of pharmaceutical interest and require synthesis. For this, phytases represent a good choice compared to chemical synthesis thanks to their specific activity during synthesis of enantiomer-pure products as well as their environmental friendliness.

This thesis has been devoted to *Bacillus* sp. MD2 phytase, a ß-propeller phytase. In a first study, the phytase gene was subjected to cloning and expression, and production of the enzyme in a heterologous host (**Paper II**). With further optimization of the production scale, time and use of lactose as an alternative inducer, the production would be both higher and cheaper. The characterization of this thermostable alkaline phytase, with regard to the effect of divalent metal ions (**Paper III**), demonstrated that it is an interesting model of a metallo-enzyme which needs Ca^{2+} for activity; not only in the enzyme active site but

also on its substrate. The Ca^{2+} on the enzyme could partially be replaced by some other metal ions to render the enzyme active. Promising results were obtained when using thermostable alkaline MD2 phytase for hydrolyzing such IP_6-metal ions complexes in feed. Moreover, crystallization of the enzyme in the presence of different metal ions may help to fully understand the role of the latter on the activation/inhibition of the enzyme as well as on enzyme stability.

Some mutants of phytase from *Bacillus* sp. MD2 showed promising improvements with regard to the specific activity and properties of the enzyme (**Paper IV**). Further investigations to improve these mutant variants by gene shuffling, saturated mutagenesis, crystallization, and X-ray studies of the enzyme structure could contribute to a better understanding of the structure-function relationships of the enzyme.

Finally, a new kinetic method for assaying phytase activity based on turbidity reduction of the IP_6-lysozyme complex substrate has shown great potential for assaying the phytase activity faster and safer in a high-throughput manner (**Paper I**). This approach can also be used for assaying the enzyme variants generated in mutagenesis trials.

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Paper I

A simple and fast kinetic assay for phytases using phytic acid-protein complex as substrate

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Abstract

Phytase (EC 3.1.3.-) hydrolyzes phytate (IP_6) present in cereals and grains to release inorganic phosphate (Pi), thereby making it bioavailable. The most commonly used method to assay phytase, developed nearly a century ago, measures the P_i liberated from IP_6. This traditional end point assay is time consuming and well known for its cumbersomeness in addition to requiring extra caution for handling the toxic regents used. This paper reports a simple, fast and nontoxic kinetic method adaptable for high through-put for assaying phytase using IP_6-lysozyme as a substrate. The assay is based on the principle that IP_6 forms stable turbid complexes with positively charged lysozyme in a wide pH range and hydrolysis of the IP6 in the complex is accompanied with a decrease in turbidity monitored at 600 nm. The turbidity decrease correlates well to the released P_i from IP_6. This kinetic method was found useful in assaying histidine acid phytases, including 3- and 6-phytases, a class representing all commercial phytases, and alkaline *β*-propeller phytase from *Bacillus* sp. The influences of temperature, pH, phosphate and other salts on the kinetic assay were examined. Salts including NaCl, $CaCl_2$, and phosphate all showed a concentration-dependent interference.

Keywords: Phytate-protein interaction; microbial phytases; kinetic assay for phytases

Abbreviations: FTU, phytase activity unit; IP_6, phytic acid, sodium phytate; IP_x, *myo*-inositol phosphate esters, where x denotes the number of phosphate ester bonds; P_i, inorganic phosphate.

Introduction

Histidine acid phytases constitute an important group of enzymes for feed and grain processing industries due to their high specific activity and wide pH optima for activity [1]. Phytase catalyzes the sequential hydrolysis of phytate (*myo*-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate; IP_6), a principal storage form of phosphorous in cereals and legumes, to less phosphorylated *myo*-inositol derivatives with concomitant release of inorganic phosphate (P_i) [1]. Hydrolysis of phytate overcomes a number of its negative effects on human and animal nutrition [2-7] and on the environment [8, 9]. It is well established that phytic acid binds positively charged metal ions [10] and bio-molecules [11] making them unavailable as nutrients. In grain processing it is important to add phytase in order to make Ca^{2+} available for α-amylase [12]. Interaction with positively charged dietary proteins leads to the formation of phytate-protein aggregates and precipitates,

which decreases their accessibility to proteases, thus resulting in inefficient protein digestion [13-18]. The decrease in protein solubility results from masking of positive charges on the protein molecules by phytic acid, which changes the isoelectric points of the proteins [16, 11].

In vitro studies with phytase reported in literature have been performed with IP_6 as the substrate and either the degradation of IP_6 or the amount of Pi released has been followed. This method for assaying phytase may not provide a true picture of the enzyme activity since the phytic acid *in vivo* may not exist in its free acid or sodium salt form. Furthermore, quantitative determination of the IP_6 degradation is tedious and time consuming [19-21]. It requires the use of chromatographic techniques with multiple devices as neither IP_6, IP_{1-5} nor Pi can be detected directly by absorbance or fluorescence, which put limitations on developing this technique for a high through-put screening [21-23]. Measurement of P_i released from IP_6 by phytase is an end point assay that provides color development proportional to the amount of Pi released, but it requires a suitable choice of enzyme dose and assay time beside the tediousness and carefulness in handling the toxic vanadate and molybdate reagents [24-27]. The color development may vary with the reaction conditions such as pH. Attempts have been made to assay phytase kinetically using *p*-nitrophenyl phosphate or *p*-nitrophenyl pyrophosphate as substrate. However, it is difficult to differentiate whether the yellow color produced upon substrate hydrolysis is due to phytase or the action of phosphatases that occur widely in biological materials [28-29]. Moreover, not all phytases show good activity toward these two artificial substrates [30-32].

Due to the importance in animal nutrition, phytase becomes one of the most assayed enzymes in feed research and feed industrial analysis laboratories as well as in feed mills. Research laboratories need to screen protein engineered phytase variants with improved performances, especially heat-stability and pepsin resistance while breeders need a phytase assay in order to be sure if the phytase added to their feed is still active so that they can save the addition of calcium phosphate. However, due to the toxic chemicals or the specific equipments needed for the phytase assay, routine phytase assay has not been done outside a well-equipped laboratory. Thus it is clear that there is a need for a simple and faster alternative method for assaying phytases. Our preliminary tests have shown that phytases catalyze the hydrolysis of the protein/peptide-phytate complexes and, to a less extent, complexes with calcium ion, releasing P_i and simultaneously reducing the turbidity of the substrate solution. In the present study, the use of IP_6-lysozyme complex as substrate to determine phytase activity kinetically by monitoring the turbidity reduction was established and compared with that of the traditional method. The developed method was used for the determination of activities of different bacterial and fungal phytases over a wide range of pH.

Materials and methods

Enzymes and chemicals

The bacterial phytases (EC 3.1.3.26) used in this study included: *E. coli* phytase at 10 000 FTU/g (Phyzyme XP, Danisco, Brabrand, Denmark), which is here referred as *E. coli* phytase variant 1, *E. coli* phytase at 2000 FTU/g (Optiphos, JBS United, Indiana, USA),

which is here referred to as *E. coli* phytase variant 2, and *Bacillus* sp. MD2 phytase at 163.5 FTU/ml, which was cloned and expressed in our laboratory [33]. The fungal phytases used in this study included: *Aspergillus niger* phytase (EC 3.1.3.8) at 5000 FTU/g (Natuphos, BASF Animal Nutrition, Germany) and *Peniophora lycii* phytase (EC 3.1.3.26) at 10 000 FTU/g (Ronozyme P-(CT), DSM Nutritional Products Europe Ltd, Switzerland). Sodium phytate (IP_6) and chicken egg white lysozyme (EC 3.2.1.17) were purchased from Sigma-Aldrich and other chemicals of analytical grade were obtained from Merck.

Phytase extraction and activity determination

All the four commercial phytases were obtained in granulated form and were extracted as described below except the *Peniophora* phytase, which was first ground in a mortar-pestle to improve the extraction. The granulated enzymes or the ground powder of *Peniophora* phytase (200-500 mg) were dispersed in 50 ml of MilliQ water in 100 ml beakers and stirred using a magnetic stirrer at room temperature (22°C) for 30 min. The suspensions were then left standing overnight (16-18 h) at 5°C to allow the particles to settle down. The supernatant was then withdrawn gently using Pasteur pipettes, and diluted with MilliQ water to a final enzyme concentration of 20 FTU/ml based on the FTU values provided by the manufacturers. The standard phytase solutions were stored as stock enzyme solutions at -20°C for subsequent experiments. Phytase from animal feed (12.5 g) was extracted with MilliQ water (37.5 ml), supernatant was obtained by centrifugation and assayed for phytase activity by using IP_6 and IP_6-lysozyme complex as substrate, respectively.

The activity of the different commercial bacterial and fungal phytases was determined based on the amount of P_i released from IP_6 in 0.25 M acetate buffer pH 5.5 at 37°C [24, 25]. The activity of *Bacillus* sp. MD2 phytase was assayed in 0.1 M Tris-HCl (pH 7.0). The enzyme stock solutions were diluted to approximately 0.03 FTU/ml in 0.25 M acetate buffer (pH 5.5) for histidine acid phytases or in 0.1 M Tris-HCl buffer (pH 7.0) containing 1 mM $CaCl_2$ and 0.01% (v/v) Tween 20 for *Bacillus* sp. MD2 phytase. Two milliliters of 7.5 mM IP_6 in 0.25 M acetate buffer (pH 5.5) was added to 1 ml of the enzyme solution pre-incubated at 37°C for 5 min. The reaction was performed for 60 min followed by the addition of 2 ml of freshly prepared stop reagent containing 1.5:1.5:1 mixture of 10% ammonium heptamolybdate, 0.24% ammonium vanadate and 65% nitric acid. Subsequently, the reaction mixtures were centrifuged at 8000 g at room temperature for 10 min before the absorbance of the supernatant at 415 nm was recorded. A P_i calibration curve was made by treating standard P_i solutions of 0– 4.0 mM KH_2PO_4 without added phytase under the same conditions as described above. All samples were assayed in triplicates. The phytase activity was calculated from the calibration curve of absorbance (OD_{415}) versus P_i concentration (mM). One unit of phytase activity (FTU) was defined as the amount of phytase that releases 1 µmole of P_i per minute under the assay conditions. The activity values obtained for the five phytases were used to design all subsequent enzymatic reactions.

Preparation of phytate substrate complex

Phytate-protein substrate complexes were prepared in 96-well flat-bottomed plates in a total volume of 120 µl. Sixty microliter of a specific buffer, 12 µl of 25 mg/ml lysozyme, 12 µl of 3 mM IP_6 and 36 µl of MilliQ water were added to each well, and mixed at 1400 rpm on an Eppendorf thermomixer (model: Comfort MTP) for 2 min at room temperature to form a homogeneous IP_6 -lysozyme complex.

Other substrate complexes (IP_6-soy protein, IP_6-lysine and IP_6-Ca complex, respectively) were prepared in the same way as IP_6-lysozyme by varying the final concentration of IP_6 and the corresponding ligands (soy protein, lysine or calcium) to get the desired turbidity.

Kinetic measurement of the turbidity reduction of the substrate complex catalyzed by phytases

The substrate complexes prepared as described above were pre-heated at 37°C with vigorous shaking (1400 rpm) for 2 min prior to the addition of 1-12 µl phytase to reach the desired final enzyme concentration in FTU. The reaction was carried out at 37°C for 20-60 min using a microplate reader ($PowerWave_x$, BioTek Instruments, Vermont, USA). The reaction was monitored at 600 nm and data were collected every 30 seconds. Before each reading the microplate was shaken at full scale for 5 seconds. Reactions with MilliQ water instead of phytase samples were used as controls for each enzymatic reaction. All samples were assayed in triplicates. Activity of each phytase sample was calculated as the rate of turbidity reduction of the substrate complex in milli optical density at 600 nm (mOD_{600}) per minute.

Inorganic phosphate analysis by Konelab

In order to measure Pi released from the substrate complex in the reactions described above, the reactions were stopped at different time intervals by the addition of 30 µl of 2.5 N HCl. Microplates having the reactants were centrifuged at 8000 g for 10 min, and 100 µl of the clear supernatants were transferred manually to 0.5 ml sample cups for Pi analysis by Konelab™ Analyser (Thermo Scientific, Ulm, Germany) according to the instructions from the manufacturer. All samples were measured in triplicates.

HPLC analysis of inositol phosphate esters

The substrate IP_6 and its degradation products (IP_{1-5}) from the phytase catalyzed reactions were analyzed on a Dionex DX-500 ion chromatograph system (Sunnyvale, CA, USA) with a Dionex CarboPac PA-100 column (4 x 250 mm) and a Dionex knitted coil (75 µl) for post column reaction. The reactions were stopped by the addition of 30 µl of 2.5 N HCl at a specific time and the solutions were filtered through 96-well filter membranes (Pall life sciences, Deland, Florida, USA) with 0.45 µm pore size before being injected into the HPLC column with an injection volume of 100 µl. Separation was performed by a linear gradient of 1-92% 1 N HCl at a flow rate of 1 ml/min. The eluant was mixed in the post column mixing chamber with 0.1% $Fe(NO_3)_3.9H_2O$ in 2% $HClO_4$ [23] for detection of the inositol phosphate esters. Samples were analyzed in triplicates.

Results and Discussion

Effect of phytic acid and lysozyme concentration on turbidity of phytic acid-lysozyme complex

Most proteins of plant origin, such as those derived from soybean, peanut, cottonseed and rapeseed, have their isoelectric points (p*I)* in the acidic range (around pH 4). Hence the solution of the IP_6 complex with these proteins develops turbidity when the pH is lower than their p*I* [34, 35]. For example, the solution of IP_6-soy protein complex is turbid only when the pH of the solution is decreased below pH 4. On the other hand, chicken egg white lysozyme has a p*I* of around 11 [36] and is considerably stable in the acidic pH range. IP_6-lysozyme complex was found to show turbidity and stability in a wide pH range from pH 2.5 to pH 8.5.

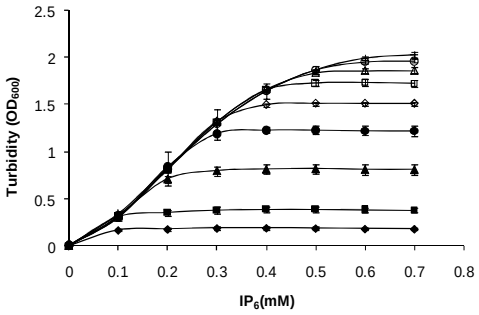

Fig. 1. Effect of IP_6 and lysozyme concentration on the turbidity of IP_6-lysozyme complex solution. The complex was prepared in 50 mM sodium acetate buffer pH 4 containing 0-0.7 mM IP_6 and lysozyme at different concentrations: 30 μM (♦), 58 μM (■), 116 μM (▲), 174 μM (●), 231 μM (◊), 289 μM (□), 347 μM (Δ), 405 μM (○), and 463 μM (+) in a total volume of 120 μl. Experimental details are described in the Materials and Methods section.

Figure 1 shows that the turbidity of the IP_6-lysozyme complex increases with increase in the concentrations of IP_6 and lysozyme at pH 4.0. At around 0.5 mM IP_6 and 0.35 mM of lysozyme, the turbidity levels off. Highest turbidity of the solution is seen at an IP_6: lysozyme molar ratio >1.5: 1. The ratio of 0.3 mM IP_6: 0.23 mM lysozyme (approximately 2.5 mg lysozyme per ml) was subsequently chosen to prepare an IP_6-lysozyme complex for use as substrate for various phytase assays since it showed high turbidity (OD_{600}>1) and the reduction in turbidity of the complex was linearly related to the hydrolysis of the IP_6 in the complex. Changes in pH, temperature, and salt concentration were subsequently examined for their impact on the turbidity and stability of IP_6-lysozyme complex as shown below.

Factors affecting turbidity and stability of phytic acid-lysozyme complex

Table 1 shows the half life of the IP_6-lysozyme complex based on its turbidity at different temperatures and pH. Between pH 2.5 and 5.5, the complex was stable for more than 10 days at room temperature (22-25°C) and for two months at 5-8°C without any significant reduction in turbidity. However, at higher temperatures (>30°C), the stability of the complex was dramatically reduced, particularly at pH 5.5 and above due to the instability of lysozyme under these conditions. It was observed that denatured proteins including lysozyme did not form stable complexes with IP_6 partly because the proteins themselves precipitate upon denaturation.

Table 1: Stability of the IP_6-lysozyme complex at various pH and temperatures.

Temperature	**2.5 ≤ pH < 3.5**	**3.5 ≤ pH < 5.5**	**≥ pH 5.5**
45°C	10 min	> 50 min	6 – 12 min
37°C	75 min	110 – 170 min	10 – 70 min
30°C	> 1.5 h	Stable for 60 min	> 90 min
Room temperature (20 - 25°C)	16 days	40 – 45 days	12 – 13 days
Refrigeration (5 - 8°C)	84 days	No change in 2 months	> 2 months

The IP_6-lysozyme complex (0.3 mM:0.23 mM) was prepared in a total volume of 120 µl in 50 mM glycine-HCl (pH 2.5-3.5), 50 mM sodium acetate (pH 3.5-5.5) and 50 mM Tris-maleate (pH 5.5-8.5) containing 0.3 mM IP_6 and 0.23 mM lysozyme. The IP_6-lysozyme complexes at different pH values were incubated at different temperatures and their turbidity was followed. Data were reported as half life ($t_{1/2}$) of the turbidity reduction of the IP_6-lysozyme complex.

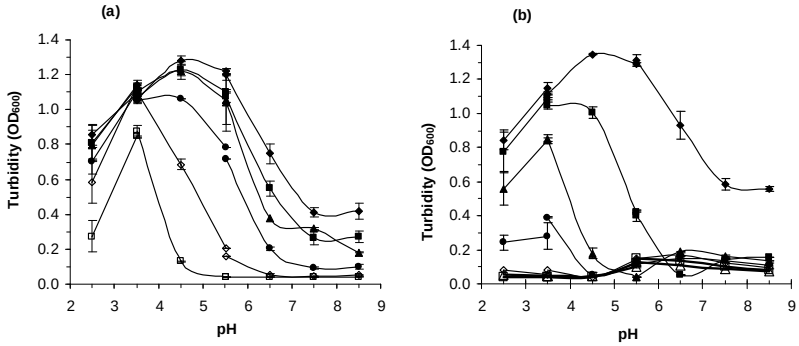

Fig. 2. Effect of pH and concentration of (a) NaCl and (b) $CaCl_2$, respectively, on the turbidity of IP_6-lysozyme complex (in a ratio of 0.3 mM:0.23 mM) in 50 mM glycine-HCl buffer (pH 2.5-3.5), 50 mM sodium acetate buffer (pH 3.5-5.5), and 50 mM Tris-maleate buffer (pH 5.5-8.5) containing 0.3 mM IP_6, 0.23 mM (2.5mg/ml) lysozyme and (a) NaCl at different concentrations: 0 mM (♦), 5 mM (■), 10 mM (▲), 15 mM (●), 30 mM (◊), and 45 mM (□), or (b) $CaCl_2$ at different concentrations: 0 mM (♦), 1 mM (■), 3 mM (▲), 5 mM (●), 7 mM (◊), 10 mM (□) and 15 mM (Δ) in a total volume of 120 µl. Experimental details are described in the Materials and Methods section.

The pH dependence of the turbidity of IP_6-lysozyme complex is further shown in Fig. 2. As expected, the turbidity was high (OD_{600} of 1-1.2) in the acidic region (pH 3.5-5.5), and started to decrease around neutral pH and became constant at the lowest level between pH 7.0-8.5, corresponding to about 30% of the highest turbidity value.

From Fig. 2 one can further see the effect of some common salts, NaCl and $CaCl_2$, which may be present in assay buffers and bio-material extracts, on the turbidity of the IP_6-lysozyme complex. It can be seen that the turbidity of IP_6-lysozyme complex was quite stable at NaCl concentrations below 15 mM in the acidic region (pH 3-5.5) (Fig. 2a). Increase in NaCl concentration at 30 and 45

mM resulted in significant decrease in turbidity except at pH 3.5. The solution of IP_6-lysozyme complex became clear in the presence of 100 mM of NaCl at all pH values in the range of 3.5-8.5.

Investigation of the effect of increasing concentrations of calcium salt at varying pH showed that the turbidity of IP_6-lysozyme complex was stable at pH 3-4 at <3 mM $CaCl_2$, but it decreased dramatically at higher $CaCl_2$ concentration, especially at pH >4.5 (Fig. 2b). It is well known that divalent and trivalent metal ions such as Zn^{2+}, Ca^{2+}, Fe^{3+} have strong interactions with IP_6 [10]. Figure 3 shows that EDTA (in the form of sodium salt), the commonly used chelator in buffers, was not able to restore the high turbidity in the presence of Ca^{2+} and at concentrations higher than 10 mM, EDTA itself also contributed to reducing the turbidity of the solution. Due to the higher affinity to calcium or iron, IP_6 has been used as an alternative chelating agent in therapy for calcium urolithiasis [37] and for inhibiting *Vibrio vulnificus* on septicemia-induced mice [38, 39].

Inorganic phosphate is the main product of the phytase-catalyzed reaction, and it can potentially compete with phytate to bind to the positive charged lysozyme. In the standard kinetic assay of phytase having phytate at a concentration of 0.3 mM, the final Pi can be 1.5 mM when all phytate molecules are converted to Pi and *myo*-inositol monophosphate. It was found that at pH 3.5 in 50 mM glycine-HCl the turbidity of IP_6-lysozyme complex was not affected at a P_i concentration up to 50 mM and the turbidity disappeared at 80 mM P_i (Fig. 4). At pH 3.5 to 8.5, P_i up to 5 mM did not interfere with the turbidity of IP_6-lysozyme complex (data not shown).

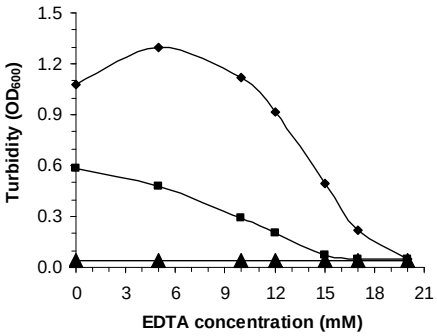

Fig. 3. Effect of EDTA and $CaCl_2$ concentrations on the turbidity of IP_6-lysozyme complex (0.3 mM:0.23 mM) in 50 mM glycine-HCl pH 3.5 containing 0.3 mM IP_6, 0.23 mM lysozyme and $CaCl_2$ at different concentrations: 0 mM (♦), 5 mM (■), and 10 mM (▲) in a total volume of 120 µl.

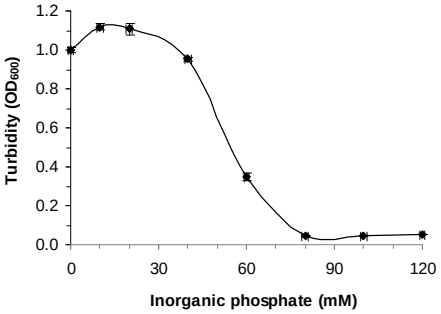

Fig. 4. Effect of phosphate concentration on the turbidity of IP_6-lysozyme complex (0.3 mM:0.23 mM) in 50 mM glycine-HCl pH 3.5 containing 0.3 mM IP_6, 0.23 mM lysozyme and different concentrations of KH_2PO_4 in a total volume of 120 µl.

The above results suggest that in spite of the variation in the turbidity with varying testing conditions, the IP_6-lysozyme complex can be an ideal substrate for determining phytase activity by following the decrease in turbidity provided that negative controls

(without phytase or with denatured phytase) for each enzymatic reaction are run simultaneously. Under the assay conditions neither the bacterial nor the fungal phytases themselves in the range of 0.1 to 2.8 FTU/ml reaction mixture caused detectable turbidity with IP_6.

A comparison of 5 microbial phytases for their activity toward IP_6 and various IP_6-ligand complexes

Table 2 shows that all the five phytases of bacterial or fungal origin were able to hydrolyze the IP_6 in the IP_6 -protein complexes as indicated by P_i release determined by Konelab analysis. It is noted that the *E. coli* phytases (*E. coli* phytase variant 1 and 2) hydrolyzed IP_6 as well as IP_6-lysozyme and IP_6-soy protein several fold faster than the two fungal phytases (Table 2), which is in line with literature data showing that *E. coli* phytase has higher activity at pH 3 than its fungal counterparts [40]. The activity of a phytase at pH around 3 is an important criterion for its efficiency as a feed enzyme for animal nutrition. Phytases from *Peniophora lycii* and *Bacillus* sp. MD2 showed the least hydrolysis of the three substrates under these assay conditions. In contrast to the *A. niger* phytase, all the four phytases showed 1.3 to 2.3 fold higher activity toward IP_6-lysozyme and IP_6-soy protein than IP_6 based on the Pi released. The difference in the substrate preference of these five phytases could not be related to the ester bonds that are first hydrolyzed since phytases from *E. coli* and *Peniophora lycii* initiate their hydrolysis reaction by attacking the ester bond at the 6^{th} position in the phytate molecule [41, 42], while *A. niger* phytase hydrolyzes initially at position 3 [43]. *Bacillus* phytase is a hybrid 3/6-phytase, which initially attacks the phosphate groups at 3^{rd} and/or 6^{th} position [43] or any position of phosphate [44] in phytate molecules.

Table 2: Activity of different microbial phytases on IP_6-lysozyme and IP_6-soy protein complex as compared to IP_6 as substrate.

Phytases	**Relative activity (%)**		
	IP_6-soy protein	**IP_6-lysozyme**	**IP_6-Na**
Escherichia coli phytase variant 1	164.3	229.0	100.0
Escherichia coli phytase variant 2	137.8	151.8	102.7
Aspergillus niger phytase	31.8	23.1	37.0
Peniophora lycii phytase	24.5	13.0	9.8
Bacillus sp. MD2 phytase	5.6	9.5	4.3

The assay was carried out in a total volume of 120 µl in 50 mM glycine-HCl pH 3.0 at 37°C for the 5 different phytases added at a dose of 0.1 FTU/ml. The reaction rates in terms of P_i release (µmol P_i/ml/min) was measured by stopping the reaction at different time intervals and analyzing P_i on Konelab. Activity of *E. coli* phytase variant 1 (0.096 µmol P_i/ml/min) on IP_6 was set as 100%. Activities of the phytases on the other substrates are reported relative to the activity of *E. coli* phytase variant 1 on IP_6.

The observations with the *E. coli* phytase shown in Table 2 were further supported by analysis of the reaction products by HPLC which separated IP_6 and its degradation

products IP_5, IP_4, IP_3, and IP_2 [22]. It was confirmed that the *E. coli* phytases showed higher activity in degrading IP_6 in the IP_6-lysozyme complex than IP_6 in the form of sodium phytate (data not shown). The exact reason why IP_6-protein may be a much better substrate for the *E. coli* phytases remains an enigma and needs to be examined by ^{31}P-NMR. On the other hand, as IP_6 can be bound with seed proteins in plant seeds and with food and feed proteins in the upper parts of the digestive tracts of monogastric animals having an acidic environment, the efficient hydrolysis of IP_6 in the IP_6-protein complex under conditions close to *in vivo* is a prerequisite for a good feed phytase.

The *E. coli* phytase variant 1 and *A. niger* phytase as representatives of bacterial and fungal, 6-, and 3-phytases, respectively, were chosen to further examine their activity toward IP_6-lysine complex. Lysine as a positively charged amino acid and a food and feed additive was chosen as it can potentially complex with IP_6 *in vivo* in the digestive tract. IP_6-lysine complex, unlike IP_6-lysozyme complex, was not turbid at all pH values between 3.5 and 8.5. Hence, its role as a substrate complex with IP_6 for phytase had to be evaluated by the release of Pi.

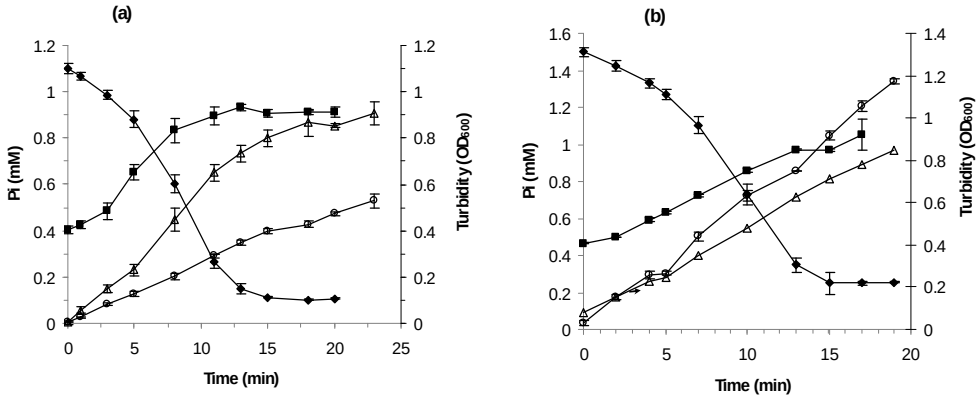

Fig. 5. Activity of (a) *E. coli* phytase variant 1 (0.1 FTU/ml) and (b) *A. niger* phytase (0.5 FTU/ml) with IP_6-lysozyme substrate based on turbidity reduction (♦) and P_i released (■); with IP_6 based on P_i released (Δ); and with IP_6-lysine complex based on P_i released (○). The three substrates were prepared in 50 mM glycine-HCl (pH 3.5) containing 0.3 mM IP_6 (for IP_6 substrate) and either lysozyme 0.23 mM (for IP_6-lysozyme substrate) or lysine 23 mM (for IP_6-lysine substrate) in a total volume of 120 µl. Reactions were performed at 37°C for 25 min with continuous mixing. Turbidity of the reactions was determined every 30 sec. For Pi determination, the reactions were stopped by adding 30 µl of 2.5 M HCl, centrifuged and the supernatants were analyzed for P_i on Konelab.

Figures 5a shows that while hydrolysis of IP_6-lysozyme complex by *E. coli phytase* variant 1 was faster compared to IP_6, in line with the results shown in Table 2, hydrolysis of IP_6-lysine complex was slower. In contrast, *A. niger* phytase did not show much difference in its initial reaction rates with the two substrates IP_6-lysine complex and IP_6 (Fig. 5b). It should be noted in Fig. 5a and 5b that the *A. niger* phytase was dosed 5 times

higher than the *E. coli* phytase considering its lower activity at pH 3.5.

Beside IP_6-protein complexes, IP_6-Ca^{2+} complex can also develop turbidity and can therefore be used for the assay of phytases by following the decrease in turbidity. However, the turbidity of IP_6-Ca^{2+} complex was low (OD_{600}<0.2), especially at pH values lower than pH 5.5. IP_6-Ca^{2+} complex could, however, be used as an alternative substrate to IP_6-lysozyme for the assay of neutral and alkaline phytases (data not shown).

The relationships between turbidity decrease and phosphate release

If the phytase activity assay based on the turbidity reduction of IP_6-lysozyme complex catalyzed by 3- and 6-phytases can reflect the hydrolysis of IP_6, it is essential that it is validated by the well established method measuring Pi release. Figure 6 shows that turbidity reduction (OD_{600}) of IP_6-lysozyme in the kinetic assay developed here correlated well to P_i released from the same enzymatic reaction using both *E. coli* (Fig. 6a and 6b) and *A. niger* phytases (Fig. 6d and 6e). Phytase activity in these assays could thus be measured as mOD/min, which is equal to the slope of turbidity reduction in the linear range (OD_{600}= 0.1-0.9). The reaction rate in mOD/min could be converted to the common unit of phytase activity (FTU/ml) by correlating it to the P_i released (Fig. 6c and 6f). In Fig. 6c and 6f, it is estimated that every 1 μmole Pi released per min based on the results from Konelab analysis is related to a 3.03±0.27 unit OD decrease at 600nm per min.

Effect of substrate and phytase concentration

The optimal substrate concentration was found to be 0.3 mM IP_6 and 0.23 mM of lysozyme, which was used in all assays in this study. With lower substrate concentration, the turbidity was low which made the linear range of the reaction shorter while with high substrate concentration, the turbidity decrease was not linear with the substrate concentration. It should be noted that the kinetic assay of phytase developed here is based on the monitoring of substrate hydrolysis instead of product formation as in most enzyme assays. This is also one of the reasons one can not use high substrate concentration. Due to these this kinetic method was found not useful for the estimation of K_m and V_{max} of phytases just like the conventional colorimetic assay of phytases. The suitable method of estimation of K_m and V_{max} is by HPLC [22-23].

At an assay of pH 3 to 3.5, assay temperature of 30 ^oC and a reaction time of 20 to 60 min, the suitable phytase concentration for *E. coli* and *A. niger* phytases was in the range of 0.1 to 0.8 FTU/ml reaction mixture. The phytase activity of some cereals, such as rye, triticale, wheat and barley, is in the range of 500 to 5000 FTU/kg [45], while microbes of *Aspergilli, E. coli* and *Bacillus* sp. are reported to have a phytase activity of 0.1 to 1.8 FTU/ml broth [33, 41, 46]. In the current study, feed samples containing corn and soy flour and *E. coli* phytase variant 1 at 400 to 2300 FTU/kg could be assayed by the kinetic method, whereas with phytase level at 200 FTU/kg or lower in the feed the kinetic method was not suitable as the incubation had been overnight long.

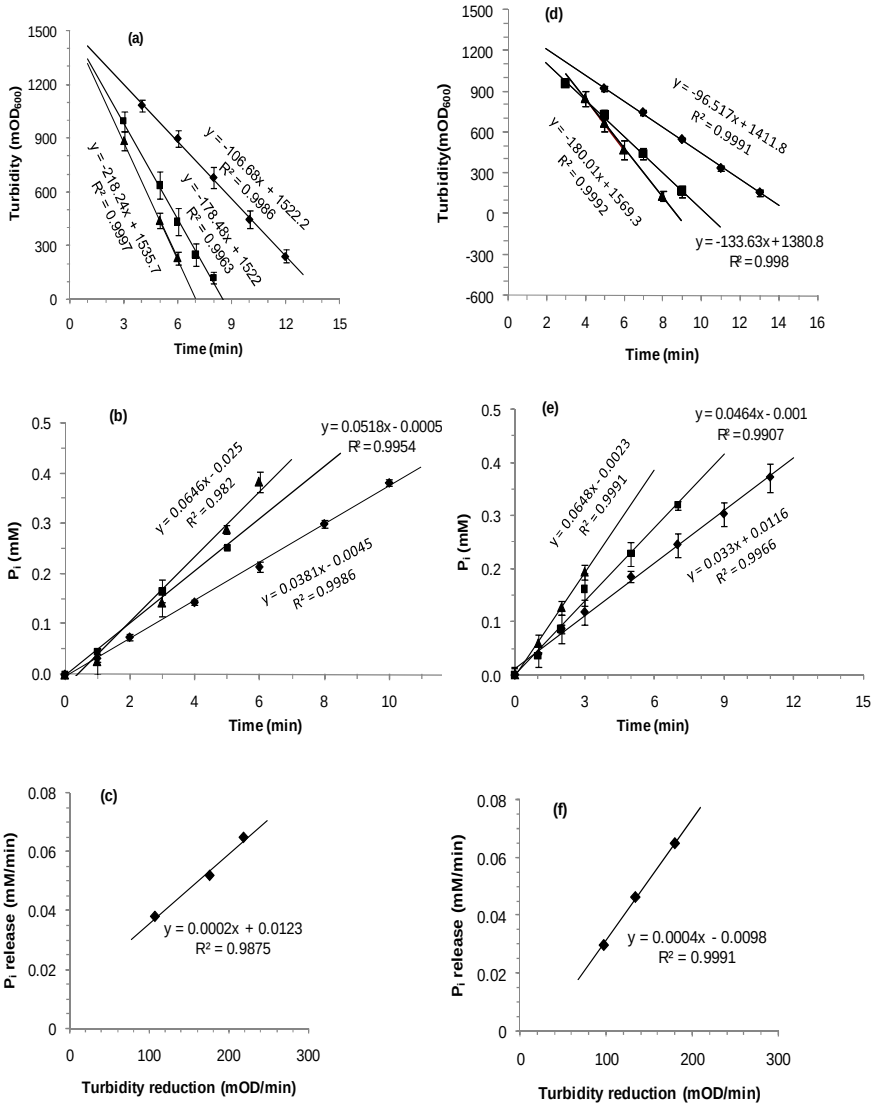

Fig. 6. Relationship between turbidity reduction and phosphate release during reaction with different concentrations of phytases: (a, b, c) *E. coli* phytase variant 1, 0.1 FTU/ml (♦), 0.2 FTU/ml (■), and 0.3 FTU/ml (▲), and (d, e, f) *A. niger* phytase, 0.1 FTU/ml (♦), 0.15 FTU/ml (■), and 0.2 FTU/ml (▲), in 50 mM acetate buffer (pH 5.5 at 37°C) with a total reaction volume of 120 µl. Turbidity measurements are shown in Fig. 6a, 6d; P_i released in Fig. 6b and 6e; and relationship between turbidity reduction (mOD/min) and P_i release (mM/min) in 6c and 6f.

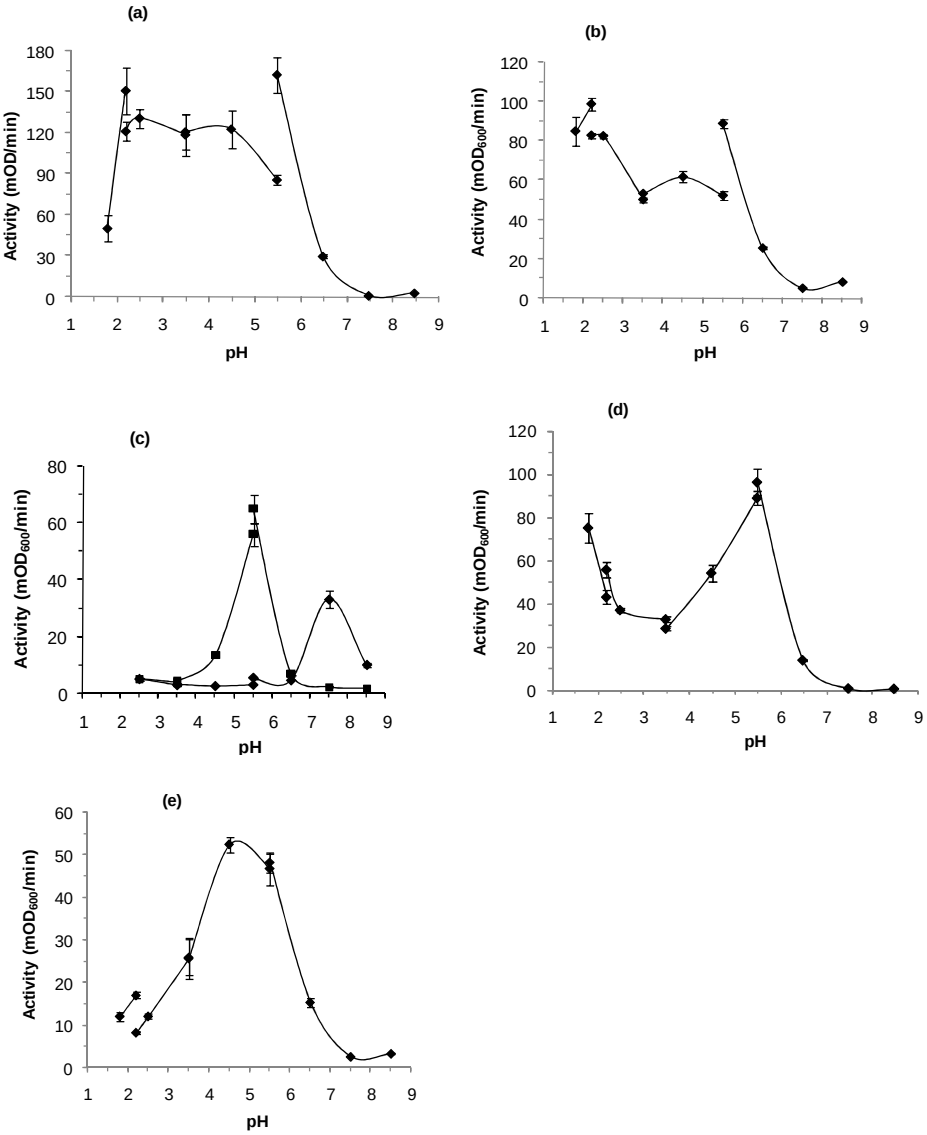

Fig. 7. pH profiles of (a) *E. coli* phytase variant 1, (b) *E. coli* phytase variant 2, (c) *Bacillus* phytase, (d) *A. niger* phytase, and (e) *Peniophora lycii* phytase on IP_6-lysozyme substrate complex. Reactions were carried out in 50 mM potasium-HCl (pH 1.5-2.5), 50 mM glycine-HCl (pH 2.5-3.5), 50 mM sodium acetate (pH 3.5-5.5) and 50 mM Tris-maleate (pH 5.5-8.5), respectively, containing 0.3 mM IP_6 and 0.23 mM lysozyme in a total volume of 120 µl at 37°C. The enzyme dose for each reaction was 0.1 FTU/ml based on P_i released from IP_6 in conventional phytase activity assay. In Fig. 7c, enzymatic reactions were performed without $CaCl_2$ (♦) and with 1 mM $CaCl_2$ (■).

The pH profiles of five microbial phytases using phytic acid-lysozyme complex as substrate

In order to evaluate the usefulness of the kinetic method developed, it is also important to test this method in a wide pH range that is relevant for phytases. Figure 7 shows the activities of the five different microbial phytases as a function of pH in the range of pH 2-8.5 using IP_6-lysozyme complex as the substrate. In general the pH profiles obtained by the kinetic method (Fig. 7a-7e) agree well with those reported in the literature using IP_6 as substrate [31, 32, 37, 41, 42, 40, 47]. From Fig. 7a and 7b, one can see that the two *E. coli* phytases showed similar pH profiles with IP_6- lysozyme complex as with IP_6 alone [41, 47] except for an extended pH optimum to the acidic region (pH 2-5.5). This could mean that IP_6-lysozyme complex as a substrate makes the phytase more stable and therefore active in the acidic region. In Fig. 7a and 7b, it can be noted that even at the same pH the activity measured varied greatly due to the different buffers used that have different ionic strengths which in turn affect the turbidity of the system. Using IP_6-lysozyme complex as substrate, it was observed that *Bacillus* sp. MD2 phytase, a calcium dependent enzyme [31, 32], showed a shift of 2 pH units in its pH optimum toward the acidic region in the presence of 1 mM $CaCl_2$ (Fig. 7c). *Aspergillus niger* phytase showed two optimal peaks of activity at pH 5.5 and pH 2 (Fig. 7d) as with IP_6 except that the second pH optimal peak shifted 0.5 unit toward the acidic region (pH 2 instead of pH 2.5) compared to IP_6 alone as substrate [37]. The pH profile of *Peniophora* phytase showed one optimum around pH 4–5, which is in accordance with that reported earlier for the enzyme with IP_6 [42]. The broadening of pH optimum in the case of *E. coli* phytases and the *A. niger* phytase, and also the shift of pH optimum in the case of *Bacillus* phytase are obviously advantageous considering the pH of the digestive tract in monogastric animals which is generally in the range of pH 2.5-6.0.

Conclusions

IP_6 and lysozyme complex at a concentration of 0.3 and 0.23 mM, respectively, forms a turbid solution and can be used for the kinetic assay of the activity of phytases by monitoring the decrease in absorbance. The decease in turbidity correlates well with the release of Pi. The method is useful for assaying histidine acid phytases, represented by all commercial phytases, and β-propeller phytase tested in this study. Other IP_6-ligand complexes including IP_6 -soy protein, IP_6-lysine and IP_6-Ca^{2+} were also investigated as substrates, but they were less suitable than the IP_6-lysozyme complex. Compared to the conventional end-point colorimetric method based on P_i measurement, the kinetic assay described here is simple, fast, safe, adaptable to high through-put screening, and also more close to the *in vivo* physiological conditions, making it more suitable for use in phytase protein engineering, in feed mills and industrial analysis laboratories to estimate the phytase activity before use in feed and food applications.

The usefulness of this method has been further demonstrated in studying the pH profiles of five different phytases. The main limitation of this method is that the

enzymatic reaction needs to be mixed well before measurements to avoid precipitation of the IP_6-lysozyme complex, which can lead to greater deviation in measurement. The linear range for turbidity at OD_{600} (0.1–0.9) is quite narrow compared to the linear range for the colorimetric assay of P_i release. As this kinetic method is based on the monitoring of substrate consumption instead of product formation as in most enzyme assay, it is not suitable for the estimation of K_m and V_{max} of phytases.

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Paper II

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ORIGINAL PAPER

A thermostable phytase from *Bacillus* sp. MD2: cloning, expression and high-level production in *Escherichia coli*

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Abstract Phytase is used as a feed additive for degradation of antinutritional phytate, and the enzyme is desired to be highly thermostable for it to withstand feed formulation conditions. A *Bacillus* sp. MD2 showing phytase activity was isolated, and the phytase encoding gene was cloned and expressed in *Escherichia coli.* The recombinant phytase exhibited high stability at temperatures up to 100°C. A higher enzyme activity was obtained when the gene expression was done in the presence of calcium chloride. Production of the enzyme by batch- and fed-batch cultivation in a bioreactor was studied. In batch cultivation, maintaining dissolved oxygen at 20–30% saturation and depleting inorganic phosphate below 1 mM prior to induction by IPTG resulted in over 10 U/ml phytase activity. For fed–batch cultivation, glucose concentration was maintained at 2–3 g/l, and the phytase expression was increased to 327 U/ml. Induction using lactose during fed-batch cultivation showed a lag phase of 4 h prior to an increase in the phytase activity to 71 U/ml during the same period as IPTG-induced production. Up to 90% of the total amount of expressed phytase leaked out from the *E. coli* cells in both IPTG- and lactose-induced fed-batch cultivations.

Keywords Alkaline phytase · *Bacillus* sp. · Fed-batch cultivation · Protein secretion

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Introduction

Phytase is an important enzyme used as an additive in animal feed. The enzyme catalyses the sequential hydrolysis of phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate; IP6) present in plant material to less phosphorylated myo-inositol derivatives with concomitant release of inorganic phosphate [28]. The phosphate is made available to monogastric animals, such as pigs, chickens and fish, that do not produce phytase and also their microflora cannot degrade phytate. This eliminates the need for external addition of phosphorous to the feed, which incurs costs and also contributes to environmental pollution [3, 23, 43]. Removal of phytate from the feed also has other advantages in terms of overcoming its negative nutritional effects such as decrease in the bioavailability of vital minerals [20, 21, 27, 30], impairing the function of digestive enzymes or decrease in the digestibility of feed protein [2, 3, 17]. For the phytase to be effective, it needs to be thermostable enough to withstand the high temperatures used during feed preparation.

A wide variety of phytases are known from different organisms; however, the focus has been on the enzymes of fungal origin, such as *Aspergillus* spp. [36, 38], which are significantly active at low pH as in stomach where phytate exists in a metal-free form but has low thermostability [19, 22, 28, 45]. A few phytases, known as alkaline phytases, which degrade phytate that is present as a metal-phytate complex in plants, have been reported from *Bacillus* spp. [3, 7, 12–14, 37] and pollen of some plants such as *Typha latifolia* [8] and *Lilium longiflorum* [35]. Such phytases can potentially be used for treatment of the animal feed prior to feeding (i.e., during feed mixing, pelleting and storage).

Several phytase genes have been successfully cloned and expressed in different microbial hosts, corn kernels of

transgenic maize and saliva of transgenic pigs [5, 12, 13, 17, 19, 39]. *Escherichia coli* is often the primary choice as host microorganism for production of recombinant proteins. Some of the earlier attempts with expression of *Bacillus* phytases in *E. coli* have resulted in production of inactive enzyme in the form of inclusion bodies [12, 33]. This entails additional steps for recovery of the active protein [33]. Kim et al. [15] have reported production of *Bacillus* sp. DS11 phytase to 20% content of the total soluble protein in *E. coli* BL21(DE3) using the pET-22b(+) vector with the inducible T7 promoter.

Many strategies have been devoted to the establishment of high cell density processes to increase the level of production of recombinant target proteins by *E. coli*. Most of them are related to controlling the substrate concentration at certain levels in order to avoid overflow metabolism and the resultant acetate accumulation under glucose excess conditions [1, 10, 18, 31]. Åkesson and coworkers [1] have previously proposed a feeding strategy controlled automatically by the dissolved oxygen level such that aerobic conditions are maintained in spite of the limitations in oxygen transfer, i.e., the feeding rate is decreased when maximum oxygen transfer capacity is reached in the bioreactor.

In this study, we report cloning and expression of a thermostable alkaline phytase from a newly isolated *Bacillus* sp. MD2 in recombinant *Escherichia coli*, and its production by high cell density cultivation using a strategy based on dissolved oxygen level to control stirrer speed and to regulate feeding to avoid anaerobic conditions and acetate accumulation.

Materials and methods

Bacterial strains, plasmids and media

Isolate MD2 was obtained from an excrement sample in Hanoi (Vietnam) on meat peptone agar (MPA) medium containing 1% sodium phytate. *Escherichia coli* strains DH5α and BL21(DE3) were purchased from Invitrogen (Carlsbad, CA) and Novagen (Madison, WI), respectively. The cloning vector pJET1.2 and expression vector pET-22b(+) were purchased from Fermentas (MBI Fermentas, Germany) and Novagen, respectively. pE10C2 plasmid was constructed in this study from pET-22b(+) and the phytase gene from *Bacillus* sp. MD2. The recombinant *E. coli* strain was cultivated either in LB medium supplemented with 100 μg/ml of ampicillin or synthetic glucose-minimal medium composed of (per liter): 10 g glucose, 2 g $(NH_4)_2SO_4$, 4.807 g K_2HPO_4 and 1.067 g $NaH_2PO_4.H_2O$, 0.5 g $(NH_4)_2HC_6H_5O_7$, 2 mM $MgSO_4$, 2 ml trace element solution (composed of 0.5 g $CaCl_2$, 16.7 g $FeCl_3$, 0.18 g $ZnSO_4.7H_2O$, 0.16 g $CuSO_4.5H_2O$, 0.15 g $MnSO_4.H_2O$, 0.18 g $CoCl_2.6H_2O$, and 20.1 g sodium EDTA per liter) [9], and 100 mg ampicillin.

Identification of *Bacillus* sp. MD2

Genomic DNA was extracted from the isolate MD2 according to Sambrook et al. [34]. The 16S rRNA gene of isolate was amplified and sequenced using a forward primer 8–27f (AGAGTTTGATCCTGGCTCAG) and a reverse primer 1492R (GGTTACCTTGTTACGACTT). The 16S rDNA sequence was compared with sequences available in public databases. Sequencing was done using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocols. Sequence reactions were electrophoresed using the ABI PRISM® 3100 DNA sequencer.

Cloning and expression of a phytase gene from the isolate MD2

Genomic DNA was extracted from the isolate MD2 following the method of Sambrook et al. [34]. The phytase encoding gene was amplified from the genomic DNA using a forward primer Phyf (TATAAAGCTTCTGTCTGATC CTTATCATTTTACCG) and a reverse primer Phyr (TCTCTCGAGTTTTCCGCTTCTGTCGGTCAG), which were designed based on the phytase gene sequence of closest organisms identified by 16S rDNA sequence similarity search. The gene amplification was performed by polymerase chain reaction (PCR) using 5 μl of *Pfu* DNA polymerase buffer (Fermentas), 1.25 units of *Pfu* DNA polymerase (Fermentas), 1.25 units of *Vent* DNA polymerase (New England BioLabs), 1 μg genomic DNA and 0.25 μM primers in a total volume of 50 μl. The reaction was run for 15 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C. Finally, an additional post-cycle extension was carried out for 7 min at 72°C. The amplified fragments were purified and cloned into blunt cloning vector pJET1.2 and transformed to *E. coli* DH5α competent cells. After colony PCR screening, plasmids were purified from the insert positive clones by alkaline lysis method and then digested with *Hind*III/*Xho*I. The released inserts were re-cloned into expression vector pET-22b(+) to construct pE10C2 that was transformed into *E. coli* BL21(DE3) cells. All the cloning steps were done according to Sambrook et al. [34].

Shake flask cultivation of the recombinant *E. coli*

The *E. coli* BL21 (DE3) cells harboring the recombinant vector pE10C2 were grown on a LB-agar plate containing 100 μg/ml of ampicillin at 30°C. A single colony was picked from the plate and cultured for 5 h in 5 ml LB broth containing 100 μg/ml of ampicillin at 30°C and 200 rpm.

This culture was used to inoculate 100 ml of the same medium, which was incubated under similar conditions in a gyratory shaker incubator. When the optical density (OD) of the culture at 620 nm was about 0.7, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. Culture samples (3 ml) were withdrawn at different time intervals for analysis of enzyme activity in extracellular, cytoplasmic and periplasmic fractions according to Novagen pET System manual (http://www.novagen.com). The extracellular activity was determined in the cell-free supernatant obtained after centrifugation of the sample at 8,000 g for 10 min at 4°C. The cell pellet was washed with physiological saline and resuspended in 30 mM Tris–HCl buffer pH 8 containing 20% (w/v) sucrose and 5 mM $CaCl_2$, and then EDTA was added to a final concentration of 1 mM. After gentle shaking at room temperature, the cells were harvested by centrifugation and subjected to osmotic shock by re-suspending in ice-cold 5 mM $MgSO_4$, and shaken for 10 min on ice. After centrifugation, periplasmic activity was determined in the supernatant, while the remaining cell pellet was re-suspended in 0.1 M Tris–HCl buffer, pH 7, containing 5 mM $CaCl_2$, and the cytoplasmic content was released by four rounds of sonication. All the assays were performed in triplicates.

Batch- and fed-batch cultivations of recombinant *E. coli* in a bioreactor

A 3 liter bioreactor (Chemoferm FCL-B-3, Hägersten, Sweden) containing 2.5 l of minimal medium was autoclaved for 40 min at 121°C. After cooling, the sterilized medium was supplemented with sterilized solutions of glucose, trace elements, $MgSO_4$ and ampicillin, respectively, to the desired concentration. The medium was inoculated with 100 ml culture of *E. coli* BL21(DE3) harboring pE10C2 grown in the same medium for 16 h at 30°C with shaking at 200 rpm. For batch cultivations, phosphate buffer in the medium was replaced by 50 mM Tris-maleate buffer pH 7.0.

Cultivations in the bioreactor were performed at 30°C with stirring at an initial speed of 200 rpm. The dissolved oxygen (DO) level was monitored using a DO probe, and when reduced to 30% saturation, the stirrer speed was increased automatically, and the DO was thereafter controlled at 20–30% saturation. The pH of the culture was maintained by addition of 3.57 M ammonia solution, which was controlled by a signal from the pH probe in the bioreactor. The volume of the ammonia solution consumed during the cultivation was recorded. The concentration of glucose was monitored off line in duplicates every 30 min by Accucheck glucose test strips. Inorganic phosphate (P_i) in the fermentation broth was monitored in triplicates at regular time intervals according to the method of Shimizu [37] (see ahead under phytase activity assay). For induction of the phytase production, the IPTG or lactose was added to the bioreactor to a desired concentration when the concentration of inorganic phosphate had decreased to less than 1 mM.

Fed-batch cultivation was started using a continuous feed of a solution containing 50% (w/v) of glucose, 50 ml/l of 1 M $MgSO_4$ and 10 ml/l of trace element solution in order to maintain the glucose concentration at 2–3 g/l in the bioreactor.

Phytase activity assay

The phytase activity was assayed according to the method described by Shimizu [37]. Unless otherwise mentioned, a mixture of appropriately diluted enzyme sample and sodium phytate (1.5 mM) dissolved in 100 mM Tris–HCl buffer, pH 7, containing 5 mM $CaCl_2$ was incubated at 70°C for 20 min. The reaction was stopped by addition of an equal volume of 15% trichloroacetic acid (TCA) solution. The precipitate obtained was removed by centrifugation, and 500 µl of the clear supernatant was transferred to an Eppendorf tube to which was then added 500 µl ammonium molybdate reagent (containing 1:4 mixture of 2.7% $FeSO_4$ and 1.5% ammonium molybdate in 5.5% H_2SO_4). After 5 min incubation at room temperature, absorbance at 700 nm was read, which was used to calculate the concentration of P_i from a standard curve made using sodium dihydrogen phosphate solution in the concentration range of 0–600 µmol/l P_i. All the samples were assayed in triplicates. One unit of phytase activity was defined as the amount of enzyme that releases 1 µmole of P_i per minute under the assay conditions.

Protein analysis

The purity of the phytase samples prepared from the cultivation broth was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12.5% (w/v) polyacrylamide.

Protein concentration was measured by bicinchoninic acid method using bovine serum albumin as standard. The recorded values were an average of measurements of triplicate samples.

Results and discussion

Isolation and identification of phytase positive strain

The isolate MD2 showing positive phytase activity (0.2 units/ml) was obtained from a sample of infant excreta after 3 days of incubation at 37°C in MPA medium containing

1% sodium phytate. The 1,407-nucleotide-long 16S rRNA gene sequence (GenBank accession number GU143091) of the isolate showed the highest similarity (99%) to *Bacillus subtilis*, *B. amyloliquefaciens* and *B. velezensis* sequences.

Sequence analysis of *Bacillus* sp. MD2 phytase

The phytase encoding gene (GenBank accession number GU143090) of *Bacillus* sp. MD2 was amplified and sequenced so that the amino acid sequence could be deduced. The sequence comprised a mature peptide of 354 amino acids, and like other phytases of *Bacillus* origin, it lacked RHGXRXP, a conserved motif at catalytic site of acidic phytases [28]. The sequence is highly similar to that of *ts-phy* from *Bacillus amyloliquefaciens* DS11 [15], the only difference being at positions 81 and 148 where Ala and Asn in *ts-phy* are replaced by Val and Asp, respectively. Close similarity is also seen with *phyC* from *Bacillus subtilis* VTTE68013 [12], the sequences differing by eight residues.

Expression and localization of recombinant *Bacillus* sp. MD2 phytase

A plasmid construct (pE10C2) was prepared in which the phytase gene was inserted between the restriction sites for *Hind*III and *Xho*I at the multiple cloning site of pET-22b(+). This construction allows expression of the phytase with *pel*B leader peptide at the N-terminal end, which is expected to facilitate the export of the target protein to the periplasmic space. The recombinant constructs without the leader peptide resulted in most of the phytase being insoluble, and there was very little activity detected in the culture. *E. coli* BL21(DE3) cells, transformed with the recombinant plasmid pE10C2 with the leader peptide, were grown in LB medium supplemented with ampicillin in a shake flask at 30°C and induced for expression of the enzyme activity by addition of 1 mM IPTG. Maximal phytase activity of 1.34 U/ml was obtained at about 4 h after induction. The activity in the cytoplasm and periplasm fractions was seen to decrease after 4 h and was eventually lost while the extracellular activity increased steadily (Figure 1a). The loss of cell-associated activity seems to be due to its complete release into the medium, although some degradation by intracellular proteases cannot be ruled out.

Supplementation of the medium with $CaCl_2$ during induction resulted in expression of higher phytase activity, with maximum activity of 3.6 U/ml being obtained at 10 mM $CaCl_2$. The activity was detected in all three fractions, although the extracellular activity was the most predominant. The periplasmic phytase activity started to decrease after 10 h of induction while the activity in the extracellular fraction increased to about 50% of the total

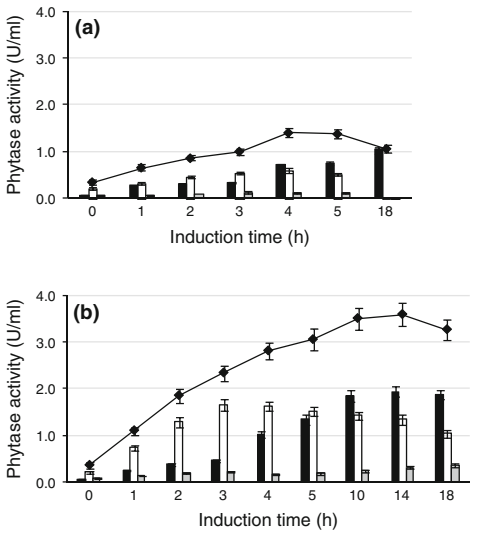

Fig. 1 Distribution of the expressed recombinant phytase in shake flask cultivation of *E. coli* BL21(DE3) at 30°C. Cells grown **a** without $CaCl_2$ and **b** with 10 mM $CaCl_2$ during enzyme induction. Symbols: Total phytase activity (*filled diamond*), and activity in cell free fraction (*filled bar*), periplasmic fraction (*open bar*) and cytoplasmic fraction (*gray bar*)

activity (Fig. 1b). The recombinant phytase was estimated to constitute about 19% of the total soluble protein present in the cells. The higher activity obtained on addition of $CaCl_2$ seems to be due to stimulation of the activity and increased stability of the phytase in the presence of the salt. Increasing the cultivation temperature to 37°C decreased the level of expression, but increased the extent of secretion of the recombinant enzyme into the culture medium (data not shown).

The possibility to secrete the target protein into the extracellular medium provides several advantages for enzyme production including a better folding environment free of cell-associated proteolytic degradation and easier recovery without the need for cell disruption and separation of cell debris [26]. There are several reports on the recombinant proteins directed to the periplasm being exported out of the cells [26, 29, 41]. This has been attributed to the stress on the outer membrane due to periplasmic accumulation of proteins resulting in loss of membrane integrity or triggering of autolytic response caused by change in cultivation conditions. Leakage of the periplasmic enzymes by increasing the growth temperature is likely to be caused by induction of heat-shock response and activation of the phospholipase activity in the outer membrane [41]. There have also been many efforts to engineer *E. coli* to excrete

proteins extracellularly [26]. Recently, secretion of *Bacillus* hydrolytic enzymes using their native signal peptides or that of *E. coli* outer membrane protein, OmpA has been achieved [46]. Miksch et al. [24] have used the coexpression of Kil protein to secrete a recombinant *E. coli* phytase.

Properties of the recombinant *Bacillus* sp. MD2 phytase

In order to characterize the enzyme, the recombinant *Bacillus* sp. MD2 phytase was purified to homogeneity from the crude cell homogenate by heat treatment, immobilized metal affinity chromatography and gel filtration to yield an enzyme with a specific activity of 39 U/mg. The pure enzyme had a molecular weight of 47.5 kDa and was optimally active at pH 6–7 and 67–73°C. The presence of 2–5 mM Ca^{2+} was essential for the enzyme to be active at 70°C. It was highly stable in the pH range of 3.6–9.6 at room temperature, and exhibited remarkable thermostability in the presence of 5 mM $CaCl_2$, retaining about 40% of the activity at pH 7 after 10 min at 100°C (data not shown). This is a desirable feature for the enzymes for animal feed applications in order to tolerate pelleting temperature of 80–85°C. Indeed, evaluation of the *ts*-phytase from *B. amyloliquefaciens*, to which the MD2 phytase is related, has shown promise for application in feed treatment [16].

A distinguishing feature of the MD2 phytase with respect to the other alkaline phytases that possess strict specificity for phytate substrate [4, 28] was that the enzyme, although being most active with phytate, displayed even some degree of activity with other phosphate substrates, such as ADP, ATP, α-fructose-1,6-diphosphate, *p*-nitrophenyl phosphate and sodium pyrophosphate.

Production of the recombinant phytase by batch cultivation in a bioreactor

Since LB medium does not have a good buffering capacity and is also expensive, a minimal medium, with glucose as carbon source and ammonium sulfate and citrate as the inorganic source of nitrogen, was tested for cultivations. Preliminary investigations with phosphate buffer in the medium led to precipitation of calcium phosphate when the cells were induced in the presence of $CaCl_2$. It was thus replaced by Tris-maleate (50 mM), pH 7, for buffering the medium. Cultivations in shake flasks in this medium resulted in very slow cell growth taking about 5–7 h to reach an OD_{620} of 0.7 prior to induction of the cells. During cultivation in a bioreactor at an initial stirrer speed of 200 rpm, the cells started to grow exponentially after 4 h (Fig. 2) at a maximum specific growth rate μ_{max} of 0.49 h^{-1}. The dissolved oxygen started to decrease after 3 h of cultivation, and when it reached 30% saturation the stirrer speed was increased (around 6 h), and the DO level was

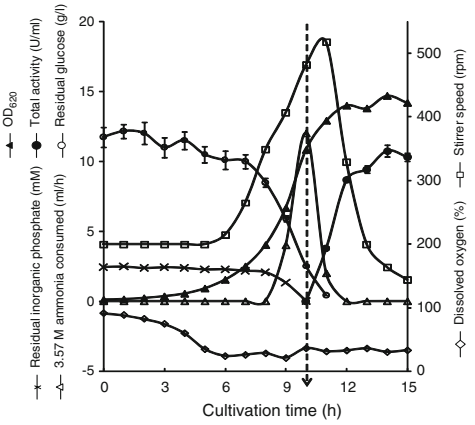

Fig. 2 Batch cultivation of recombinant *E. coli* in 2.5 l of minimal medium with 50 mM Tris-maleate buffer pH 7. Dissolved oxygen, stirrer speed and ammonia consumed are log data from the bioreactor, inorganic phosphate, OD_{620} and total activity were off line measurements in triplicates, while residual glucose was measured off line in duplicates. The arrow shows the induction time

thereafter maintained at 20–30% saturation. Under such conditions, the cells reached an OD_{620} of over 10.8 (cell dry weight of about 3.73 g/l) at 10 h (Fig. 2). At this time, 1 mM IPTG was added as an inducer of phytase production since the level of inorganic phosphate (detected in low concentration in the medium) had dropped to below 1 mM, hence avoiding the risk of calcium phosphate precipitation. Phosphate starvation has previously been used as a strategy for induction of recombinant phytase in *B. subtilis* using a phosphate-starvation inducible *pst*-promoter [13, 44].

As seen in Fig. 2, growth became limited with time due to depletion of glucose that led to an increase in dissolved oxygen and decrease in the stirrer speed. The cell growth decreased (μ_{max} of 0.13 h^{-1}) and stopped after 12 h of cultivation (2 h after induction by IPTG) (Fig. 2), and the maximal phytase activity of 10.3 U/ml was obtained after 4 h of induction. The amount of phytase activity released into the medium remained constant over time, and much of the expressed phytase was accumulated in the cytoplasm. As a result, after 4 h of induction, the relative fraction of the extracellular activity was reduced to about 25%, and the cytoplasmic activity increased to about 42% of the total recombinant phytase (Fig. 3). This change in the distribution behavior of the recombinant phytase could be due to cultivation of cells under low phosphate conditions, which leads to induction of the synthesis of periplasmic phosphate-binding proteins that occupy most of the export sites available in *E. coli* [29]. As a result, the export of the

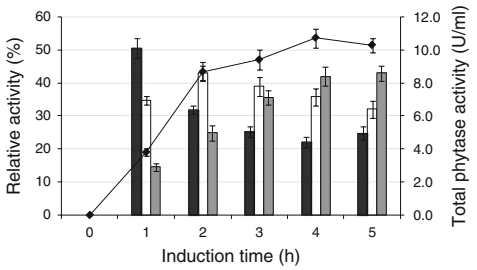

Fig. 3 Distribution of recombinant phytase in 2.5-l batch cultivation: relative activity in the extracellular (*filled bar*), periplasmic (*open bar*) and cytoplasmic (*gray bar*) fraction, and total phytase activity (*filled diamond*)

recombinant protein out of the cells gets limited, and it starts to accumulate inside the cells.

Production of the recombinant phytase by fed-batch cultivation and induction by IPTG

As batch cultivation has its own drawback of nutrient limitation leading to very low cell density, fed-batch cultivation was designed using the minimal medium to prolong the exponential phase of *E. coli* and to get high cell density. It was possible to use phosphate buffer (50 mM, pH 7) for buffering the medium since the larger number of cells obtained during fed-batch should be able to consume the larger amount of the phosphate as nutrient. Cultivation conditions were initially similar to those used for batch cultivation described above. The stirrer speed started to increase at about 7 h of cultivation to compensate for the decrease in dissolved oxygen and reached a maximum of 1,000 rpm after 14 h cultivation time. Glucose concentration was reduced to <3 g/l after 8.5 h, and thereafter a continuous feed of glucose was applied to the reactor according to a programmed feeding profile to maintain the glucose concentration at 2–3 g/l [1, 18, 31]. This resulted in an extension of the exponential phase of the cultivation, μ_{max} of 0.47 h^{-1} between 4–16 h of the cultivation, and increase in cell density to an OD_{620} of 117.6 (cell dry weight of 33.7 g/l) at 16 h (Fig. 4). This corresponded with a decrease in P_i concentration to below 1 mM, and the culture was induced by the addition of 1 mM IPTG and 10 mM $CaCl_2$. The cells continued to grow at a significantly lower growth rate (μ_{max} of 0.1 h^{-1}) after induction to reach an OD_{620} of 188 (49.6 g cell dry weight/l).

A total phytase activity of 327 U/ml (6,600 U/g cell dry weight) was obtained after 5 h of induction, which is about 32 times higher than the production during batch cultivation (Fig. 5a), and is the highest reported so far for recombinant phytase production in a bacterial system (Table 1).

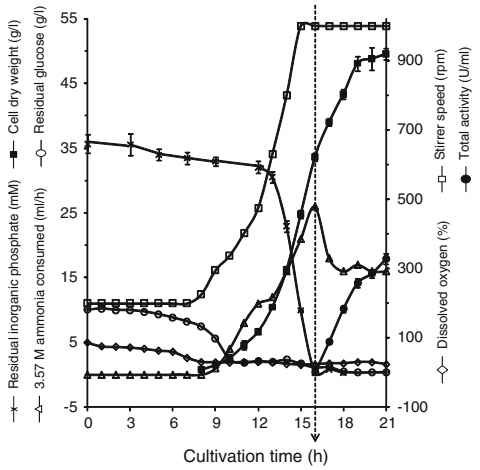

Fig. 4 Fed-batch cultivation of recombinant *E. coli* in 2.5 l synthetic glucose-mineral medium with 50 mM phosphate buffer, pH 7. Dissolved oxygen, stirrer speed and ammonia consumed are log data from the bioreactor; inorganic phosphate, OD_{620}, cell dry weight and total activity were measured off line in triplicates; residual glucose was measured off line in duplicates. The *arrow* shows the induction time

About 85–90% of the expressed recombinant phytase activity was found in the extracellular medium, and the relative enzyme activity in the different cell fractions remained almost constant with time (Fig. 5a). A high level of leakage into the extracellular medium is an advantage for downstream processing of the phytase as there is no need for cell disruption and separation of cell debris, and the amount of host cell proteins is much lower in the extracellular medium. Indeed, the specific activity of recombinant phytase in the cell free fraction of this fed-batch cultivation was 35.3 U/mg total protein, constituting more than 90% of the total protein.

Lactose-induced production of the recombinant phytase during fed-batch cultivation

Induction using lactose has previously been reported to give comparable yields of enzyme activity as with IPTG [6, 42]. Lactose was tested as the inducer of phytase activity in the present system. Preliminary experiments in shake flasks showed that in contrast to induction by 1 mM IPTG, no phytase activity was observed after 1 h of induction with 5–25 mM lactose; however, at 3 h the activity levels were quite comparable with that obtained with IPTG (Table 2). The observed delay in production with lactose as inducer is in accordance with an earlier report [15]. The highest level of production was obtained after

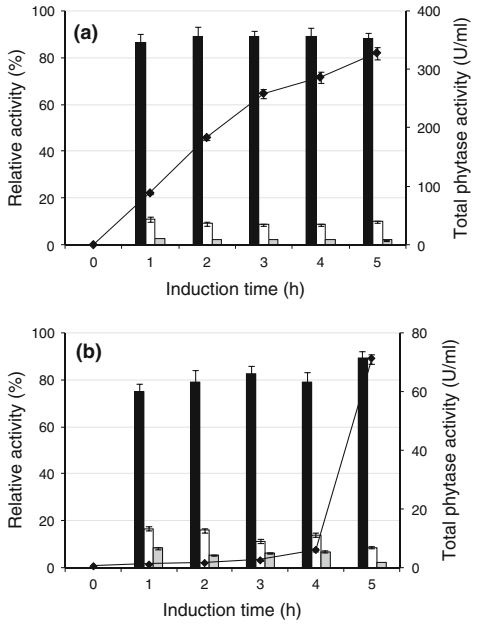

Fig. 5 Distribution of recombinant phytase with time during fed-batch cultivation in 2.5 l culture medium induced by **a** 1 mM IPTG and **b** 20 mM lactose: relative activity in extracellular (*filled bar*), periplasmic and (*open bar*) cytoplasmic (*gray bar*) fractions, and total phytase activity (*filled diamond*)

5 h of induction and was nearly the same at lactose concentrations of 15 mM and above. After 18 h of induction, more than 50% of the phytase activity was found in the extracellular medium.

Production of the recombinant phytase was then studied by fed-batch cultivation with 20 mM lactose as the inducer. The higher concentration of lactose was used assuming that some of it could be consumed by the *E. coli* cells at a low glucose concentration (2–3 g/l) [6]. All the parameters were controlled in the same way as described above for IPTG-induced cultivations. After induction, cells continued growing for 4 h at a maximal specific growth rate of 0.49; however, phytase production was very low in contrast to similar cultivation conditions using 1 mM IPTG as the inducer. Phytase production increased dramatically after 4 h of induction. Delay in enzyme production on lactose induction during fed-batch cultivation of *E. coli* BL21(DE3) cells has been reported earlier [32, 42] and could be explained by the time needed for lactose to be processed to allolactose, an inducer for lac UV5 in *E. coli* chromosome and lac o in pET-22b(+) [11, 25].

Table 1 Comparison of phytase production in batch and fed-batch cultivations in this study with those reported in literature

Cultivation type, inducer	Host strain	Total phytase production (U/ml)	Reference
Batch, IPTG	Recombinant *E. coli*	10.3	This study
Batch, lactose	Recombinant *E. coli*	2.25	[40]
Fed-batch, IPTG	Recombinant *E. coli*	327	This study
Fed-batch, lactose	Recombinant *E. coli*	71	This study
Batch, IPTG	Recombinant *B. subtilis*	2	[17]
Fed-batch, IPTG	Recombinant *B. subtilis*	28.7	[13]
Fed-batch, IPTG	Recombinant *B. subtilis*	48	[44]
Fed-batch, IPTG	Recombinant *E. coli*	120	[18, 24]

Table 2 Expression of phytase activity by recombinant *E. coli* cells induced by different concentrations of lactose

Inducer concentration (mM)	Total activity (U/ml) at different hours after induction				
	0 h	1 h	3 h	5 h	18 h
IPTG, 1 mM	0	1.5	2.50	2.78	2.46
Lactose, 5 mM	0	0	2.05	2.60	2.40
Lactose, 10 mM	0	0	2.12	2.65	2.36
Lactose, 15 mM	0	0	2.33	2.79	2.41
Lactose, 20 mM	0	0	2.28	2.85	2.40
Lactose, 25 mM	0	0	2.31	2.77	2.39

Total activity (U/ml) was the sum of extracellular, periplasmic and cytoplasmic fractions. The enzyme activity was measured in triplicate, and the data show the average value of total phytase activity

Higher cell density (57 g cell dry weight/l) was reached at the end of cultivation due to longer exponential growth phase. However, the total phytase activity after 5 h induction was only 71 U/ml (1.25 U/g cell dry weight), which is approximately 22% of that obtained on induction by IPTG (Fig. 5). This indicated that the conditions used for induction by lactose during high cell density cultivation were not optimal. It has previously been suggested that the residual lactose concentration plays an important role in the induction process; too high concentrations may lead to inhibition, while limitation occurs at concentrations below a critical value [6]. It is thus likely that optimization of the amount of lactose and mode of addition could further increase the phytase activity levels. It is possible that

prolonged incubation (beyond 5 h) could lead to higher enzyme activity in case of both IPTG and lactose-induced fed-batch cultivations but bioreactor capacity posed a limitation.

Distribution of the recombinant phytase during the fed-batch cultivation induced by lactose is shown in Fig. 5b. As in the IPTG-induced cultivation, up to 90% of total phytase activity had leaked out of the cells into the medium. The possibility to use lactose as an inducer for recombinant enzyme production would substantially lower the production cost, especially when added in the form of whey or whey permeate [42].

Concluding remarks

Bacillus sp. MD2 phytase is a highly thermostable enzyme and has good potential as a supplement to feed for monogastric animals. In this study, selection of a suitable expression host and fermentation conditions have resulted in a drastic increase in the production levels of the enzyme. Although large-scale production is normally done using fungal or other microbial hosts that are able to secrete high enzyme amounts, *E. coli* serves as a convenient host for laboratory-scale studies allowing structure-function studies and also evaluation of the product in the desired application. Moreover, extracellular expression is beneficial for avoiding the formation of inclusion bodies or degradation by host proteases, and also lowers the risk of bacterial endotoxins entering the enzyme product.

Currently, the enzyme is being evaluated with regard to feed utilization and animal growth rates. Studies are also on-going at the molecular level to investigate the possibility of increasing the activity of the enzyme in the acidic range.

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Paper III

Thermostable alkaline phytase from *Bacillus* sp. MD2: effect of divalent metals on activity and stability

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Abstract

Phytate, the major source of phosphorus in seeds, exists as a complex with different metal ions. Alkaline phytases are known to dephosphorylate phytate complexed with calcium ions in contrast to acid phytases that act only on phytic acid. A recombinant alkaline phytase from *Bacillus* sp. MD2 has been purified and characterized with respect to the effect of divalent metal ions on the enzyme activity and stability. The presence of Ca^{2+} on both the enzyme and the substrate are required for optimal activity and stability. Replacing Ca^{2+} with Ba^{2+}, Mn^{2+}, Mg^{2+} and Sr^{2+} in the phytase resulted in expression of >90% of the maximal activity with calcium-phytate as the substrate, while Fe^{2+} and Zn^{2+} rendered the enzyme inactive. On the other hand, the calcium loaded phytase showed significant activity (60 %) with sodium phytate and lower activity (17-20%) with phytate complexed with only Mg^{2+}, Sn^{2+} and Sr^{2+}, respectively. On replacing Ca^{2+} on both the enzyme and the substrate with other metal ions, about 20% of the maximal phytase activity was obtained only with Mg^{2+} and Sr^{2+}, respectively. Only Ca^{2+} resulted in a marked increase in the melting temperature (T_m) of the enzyme by 12-21°C, while Ba^{2+}, Mn^{2+}, Sr^{2+} or Cu^{2+}resulted in a modest (2-3.5 °C) increase in T_m. In the presence of 1-5 mM Ca^{2+}, the optimum temperature of the phytase activity was increased from 40 °C to 70 °C, while optimum pH of the enzyme shifted by 0.4-1 pH unit towards the acidic region.

Keywords: alkaline phytase; phytate; divalent metal ions; differential scanning calorimetry.

1. Introduction

Phytate (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate, IP_6) accounts for approx. 50-80% of the phosphorus in seed feedstuffs [1]. This highly reactive acidic compound easily chelates nutritionally important cations, such as Ca^{2+}, Zn^{2+} and Fe^{2+}, needed for the control of cellular processes and released during germination upon the action of intrinsic plant phytases [1, 2]. However, with respect to its presence in the feeds, phytate is most commonly regarded as an anti-

nutritional compound, as it chelates metal cations and some important proteins. The bound cations as well as the phosphorus are partially or completely unavailable to the monogastric animals and need to be supplemented [3]. Hydrolysis of phytate in the feeds is thus of nutritional importance, and even has environmental significance since supplementation of feed with inorganic phosphate becomes unnecessary and consequently phosphorus pollution in the livestock rich regions is reduced. This has led to interest in development and application of phytases.

The phytases hydrolyse the phytate to less phosphorylated *myo*-inositol phosphates and free orthophosphate [4]. Several phytases of fungal, bacterial and plant origin belong to histidine acid phosphatase family; they share a common active site motif and are generally active under acidic conditions. Detailed studies with *E. coli* phytase have shown that the active site region contains several positively charged groups that provide favorable interactions for binding only the metal free form of phytate [5]. On the other hand, phytases from *Bacillus* species and pollen of some plants, called as alkaline or β-propeller phytases, are optimally active under neutral and alkaline conditions [6, 7], and hydrolyse the metal bound phytate [8]. Phytases from *Bacillus amyloliquefaciens* and *Bacillus subtilis* are the most well studied, which share 93% sequence similarity but are considerably different from the histidine acid phosphatases [6, 7]. Moreover, they are highly thermostable and are dependent on calcium ions for activity and thermostability [8, 9]. Investigation of the crystal structure of the thermostable *B. amyloliquefaciens* phytase (TS-Phy) in complex with bound phosphate revealed a different catalytic mechanism for phytate hydrolysis wherein a calcium-bound water molecule in the active site directly attacks a phosphorus atom of the substrate and the pentavalent transition state is stabilized by the calcium ions [10]. In contrast to the acid phytases, an alkaline phytase hydrolyses alternate phosphate bonds on the phytate yielding *myo*-inositol triphosphate as product due to the presence of non-equivalent phosphate binding sites on the enzyme, one meant for cleavage and the other for increasing the binding affinity for the substrate [10].

Owing to the nature of the phytate in the feed and processing of the feed at high temperatures, alkaline phytases are more attractive for use as feed enzymes [11]. We have recently reported on cloning, expression and production of an alkaline phytase from *Bacillus* sp. MD2 [12]. Its amino acid sequence (GenBank accession number GU143090) was highly similar to that of TS-Phy (GenBank accession number U85968). In this paper, we have replaced the calcium on the enzyme and/or substrate with a number of other divalent cations to study their effect on the activity and stability of the purified recombinant *Bacillus* sp. MD2 phytase.

2. Experimental

2.1. Materials

Sodium phytate (P3168) were purchased from Sigma-Aldrich. Other chemicals of analytical grade were obtained from Merck. Ultra pure water obtained from Millipore-MilliQ system was used throughout this study. *Bacillus* sp. MD2 was isolated in Hanoi (Vietnam) from an infant excrement sample

on meat peptone agar (MPA) medium [12]. *Escherichia coli* BL21(DE3) was purchased from Novagen (Novagen Madison, WI, USA) and cultivated using Luria-Bertani (LB) broth or agar supplemented with ampicillin (100 µg/ml). The recombinant plasmid pE10C2, containing the gene encoding *Bacillus* sp MD2 phytase was constructed from pET-22b(+) and expressed in *E. coli* as described in a previous report [12].

2.2. Production and purification of recombinant Bacillus sp. MD2 phytase

E. coli BL21(DE3) cells harbouring the recombinant vector pE10C2 were cultivated in shake flasks using Luria-Bertani (LB) medium supplemented with 100 µg/ml of ampicillin at 30 °C and 200 rpm as described earlier [12]. When the optical density (OD) of the culture at 600 nm was about 0.7, expression was induced by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG). After 5 hours of cultivation, the *E. coli* cells harbouring the recombinant protein were harvested by centrifugation at 8 000 x g for 15 min. The cells were resuspended in 20 mM Tris-HCl buffer pH 7 containing 5 mM $CaCl_2$, and sonicated (four treatments of 60s, with 60s intervals between the cycles) to release the recombinant protein.

The crude cell lysate obtained after sonication was heated twice at 60 °C for 20 min and cooled on ice. The precipitated proteins and cell debris were removed by centrifugation (10 000 x g, 15 min) and 10 ml of clear supernatant was loaded on a 10 ml IMAC column loaded with nickel ions (Ni-IMAC) pre-equilibrated with binding buffer (20 mM Tris-HCl pH 7 containing 5 mM $CaCl_2$, 300 mM NaCl and 1% glycerol). The column was then thoroughly washed with the binding buffer to remove loosely bound proteins. Bound protein was eluted using a gradient of 0-300 mM imidazole in the same buffer. Fractions with phytase activity were pooled, concentrated to 1 ml and further fractionated by size exclusion chromatography on a 30 ml Sephadex-G75 column at a flow rate of 0.1 ml/min. Eluted fractions were analysed by SDS-PAGE and fractions containing pure enzyme were pooled and concentrated.

2.3. Assay for phytase activity

The phytase activity was measured spectrophotometrically based on the release of inorganic phosphate (P_i) from phytate during 10 min reaction as described previously [12, 13]. One unit of phytase activity was defined as the amount of enzyme that releases 1 µmole of P_i per minute under the assay conditions. All the samples were assayed in triplicates.

2.4. Protein analysis

The molecular weight and homogeneity of the purified phytase were determined by SDS–PAGE, using 12.5 % (w/v) polyacrylamide as described by Laemmli [14]. Protein bands were visualized by staining the gel with Coomassie Brilliant Blue. The total protein concentration of the samples was measured in triplicates using bicinchoninic acid method and bovine serum albumin as standard [15].

The isoelectric point (*p*I) of the enzyme was determined by Ettan IPG phor^{TM} II, IEF system from Pharmacia using immobilized pH gradient IPG gel strips (ReadyStrip IPG Strip, pH 3 to 9 (Bio-Rad 163-2000).

Rehydration of the strips and sample application were carried out following the manufacturer's instructions (Bio-Rad). Focusing was performed at 29 800 V per hour.

2.5. Removal of Ca^{2+} from the recombinant Bacillus sp. MD2 phytase

Purified recombinant phytase from *Bacillus* sp. MD2 was extensively dialyzed against 10 mM Tris-HCl buffer pH 7 containing 15 mM EDTA for three days followed by dialysis against 50 mM acetate buffer, pH 4 or 10 mM Tris-HCl buffer, pH 7 without any chelating agent for one day (with changes every 6 hours by fresh buffer). The calcium content of the protein sample was determined before and after dialysis by atomic absorption spectrometry.

The Ca^{2+} content of phytase samples prepared as described above was determined using Zeenit700 graphite atomic absorption spectrometer (Analytikjena, Germany). The protein samples were appropriately diluted in Milli-Q quality water, and analysis was performed in graphite wall tubes at a wavelength of 422.7 nm. Conditions used for the analysis were: prolonged pyrolysis (25 seconds), lowered temperatures for the steps of pyrolysis (1000 ºC) and atomization (2300 ºC), and combined with an additional gas flow during the atomization step. The concentration of Ca^{2+} in the enzyme samples was calculated by comparing atomic absorbance of samples to those of a standard curve constructed from serial dilutions of a 50 µg/L solution of $CaCl_2$ prepared in Milli-Q water.

2.6. Differential scanning calorimetric (DSC) analysis

Thermal stability of the recombinant enzyme was determined using the Microcal VP-DSC (Northampton, MA) having a cell volume of 0.5072 ml with 2 atm pressure applied over the cells. Prior to scanning, the protein samples were dialysed extensively against the appropriate buffers and degassed by stirring under vacuum for 10 min. DSC scans of 25 µM metal depleted- and metal-activated samples of recombinant phytase in 50 mM acetate buffer, pH 4 were performed by increasing the temperature from 20 °C to 100 °C at a scan rate of 1 °C/min, unless otherwise stated. Buffer scans were subtracted from sample scans prior to the determination of molar excess heat capacities (Cp), by normalizing the experimental thermograms with enzyme concentration and the volume of the calorimeter cell. The apparent denaturation temperature, T_m, was determined as the temperature corresponding to maximum C_p (C_{pmax}).

2.7. Metal docking studies

AutoDock integrated in Yasara software [16, 17] was used to explore the effect of Ca^{2+} and Zn^{2+} on IP_6 binding. A macro command from the YASARA package called "dock_run.mcr" applying Lamarckian genetic algorithm was used to perform the simulations where all options were set to program defaults. The ligand (IP_6) coordinates were extracted from the PDB entry 1DKP. As the *Bacillus* sp. MD2 phytase differs from the *B. amyloliquefaciens* TS-Phy only by two amino acids [12], PDB entries of the latter enzyme 1H6L, TS-Phy co-crystallized with bound phosphate and calcium at the low affinity binding site, and 1CVM, TS-Phy crystallized with Cd^{2+}, were used in the docking experiments. Preparation of the files

for docking was as follow: the two phosphate molecules were removed from 1H6L file; all cadmium atoms of 1CVM file were replaced by Zn atoms.

3. Results

3.1. Purification and properties of recombinant Bacillus sp. MD2 phytase

Bacillus sp. MD2 phytase, expressed in a soluble and active form in *E. coli* BL21(DE3) cells, was purified to homogeneity using a three-step procedure involving heat treatment, immobilized metal ion affinity chromatography and size exclusion chromatography (Table 1, Fig. 1). The pure recombinant enzyme had a specific activity of 39 U/mg, molecular weight of about 47.5 kDa, and isoelectric point of 5.5. In the presence of 5 mM $CaCl_2$ in reaction buffer, the phytase was optimally active at pH 6 and 67-73 °C.

Table 1 Purification of recombinant *Bacillus* sp. MD phytase expressed in *E. coli*.

Sample	Specific activity (U mg^{-1})	Recovery (%)	Purification factor (fold)
Cell lysate	0.20	100	1.0
1st heat treatment	0.46	98	2.3
2nd heat treatment	1.02	97	5.1
Eluate from IMAC*column	37.30	84	184.4
Gel filtration** eluate	39.10	13	193.3

*IMAC: Immobilized metal affinity chromatography

**Gel filtration: Sephadex G-75 column

3.2 Effect of divalent metal ions on phytase activity

Extensive dialysis of the phytase against 15 mM EDTA resulted in a preparation with 0.79 µmole Ca^{2+} per µmole of protein, i.e. 79% of the protein molecules retained one bound calcium.

The Ca^{2+}-stripped enzyme was charged with different divalent metal ions (at 1 or 5 mM concentration) overnight and its activity was determined at 37 °C and 70 °C, respectively, in the reaction mixture

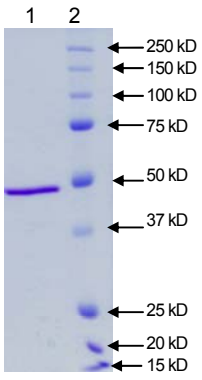

Fig. 1 SDS-PAGE of *Bacillus* sp. MD2 phytase. Purified phytase (lane 1) and protein molecular weight markers (Precision plus protein all blue standards from BioRad) (lane 2).

containing 1.5 mM sodium phytate supplemented with 5 mM $CaCl_2$ (Table 2). The metal depleted phytase exhibited less than 2% of the activity of the original activity, but the activity was fully recovered when the enzyme was re-charged with 1 or 5 mM $CaCl_2$, and 96-99% activity was obtained with Sr^{2+}. Even with Ba^{2+}, Mn^{2+}, and Mg^{2+} salts, activity recovery was as high as 90% at 37 °C, and even higher at 70 °C. As seen in Table 2, only a fraction of activity was recovered with Co^{2+}, Cu^{2+} and Sn^{2+} (for the latter no activity was observed at 70 °C), while the treatment with 5 mM Fe^{2+} and Zn^{2+} resulted in complete loss of activity.

Owing to the similarity of Zn^{2+} with Cd^{2+} and the availability of crystal structure of TS-Phy with Cd^{2+} [18,19], AutoDock was used to predict the orientation of IP_6 in the presence of Zn^{2+} ions instead of Ca^{2+} in enzyme active site to understand the inhibitory effect of Zn^{2+}. As seen in Figure 7, Zn^{2+} (no. 8) pulls away one of the phosphate groups resulting in disorientation of IP_6 (Figure 7b), so the cleavage site is not fully occupied with the phosphate group as in case of Ca-phytase.

Table 2: Effect of divalent metal ions on the activity of metal depleted *Bacillus* MD2 phytase.

	Relative activity (%)		
Assay temperature	37 °C	70 °C	
Metal ion	5 mM	1 mM	5 mM
$CaCl_2$	100.0 ± 1.6	100.0 ± 6.1	100.0 ± 3.1
$BaCl_2$	90.9 ± 3.7	93.2 ± 1.2	98.5 ± 6.7
$CoCl_2$	31.7 ± 0.5	17.6 ± 2.3	19.1 ± 2.9
$CuCl_2$	14.9 ± 0.2	ND	16.8 ± 4.8
$MnCl_2$	91.5 ± 3.1	97.7 ± 0.9	99.5 ± 3.9
$MgCl_2$	88.7 ± 1.2	88.6 ± 5.8	101.0 ± 3.1
$FeCl_2$	ND	ND	ND
$SnCl_2$	12.7 ± 0.6	ND	ND
$SrCl_2$	98.3 ± 1.5	96.6 ± 3.7	98.8 ± 4.1
$ZnCl_2$	ND	ND	ND

Metal depleted enzyme (5µM) was incubated with different metal ions (1 and 5 mM, respectively) overnight at 4 °C. The enzyme samples were then diluted 100 times in 10 mM Tris-HCl buffer pH 7 prior to determination of activity in 0.1 M Tris-HCl pH7 containing 5 mM $CaCl_2$ and 1.5 mM sodium phytate at 37 °C and 70 °C, respectively. Activity of calcium activated enzyme (289.7 U/ml at 37 °C and 5mM $CaCl_2$; 692.7 U/ml at 70 °C and 1mM $CaCl_2$; 685.2 U/ml at 70 °C and 5mM $CaCl_2$) was considered as 100%. Activity of the control sample (metal depleted enzyme without any treatment) showed 0.7 ± 0.1% relative activity at 37 °C and 2.0 ± 0.9% relative activity at 70 °C. ND-not detected.

Subsequently, the calcium bound phytase was used to hydrolyze phytate complexed with different metal ions; no additional calcium was added to the buffer. As expected, maximum activity was obtained with calcium phytate; at 70 °C the activity was more than twice of that at 37 °C. The activity with sodium phytate (without any added metal salt) at 37 °C was about 60% of the maximum, and the only other phytate complexes that led to significant phytase activity (15-30%) were with Mg^{2+}, Sn^{2+} and Sr^{2+} (Table 3). On the other hand at 70 °C, the phytase showed only 1% activity with sodium phytate and 6.5% with Sn-phytate, and no activity with the other metal complexes.

In a separate experiment, the Ca^{2+}-depleted phytase was treated by incubation with different metal ions, and assayed for activity using the phytate complex containing the same metal ion used for the enzyme activation. As shown in Table 4, only Mg^{2+}- and Sr^{2+} provided significant activity (20 % of activity with calcium) at 37 °C.

3.3 Effect of different metal ions on stability of Bacillus sp. MD2 phytase

The effect of the divalent metal salts on *Bacillus* sp. MD2 phytase stability was then investigated using differential scanning calorimetry. Thermal denaturation of the phytase in presence of calcium at pH 7 resulted in melting temperature (T_m) of 75.4 °C (Fig. 2b), which is in accordance with that reported for *B. amyloliquefaciens* DS11 phytase [19]. However, at pH 7, with the various buffers tested (HEPES, MES, Tris-HCl, BR universal buffer), the enzyme tended to aggregate after unfolding, thereby distorting the thermograms (data not shown).

Table 3: Effect of different metal-phytate substrate complexes on the catalytic activity of calcium loaded *Bacillus* sp. MD2 phytase.

Metal ion-phytate	Relative activity (%)	
	37 °C	70 °C
Ca-IP_6	100 ± 1.1	100 ± 2.9
Ba-IP_6	ND	ND
Co-IP_6	ND	ND
Cu-IP_6	1.07 ± 0.03	ND
Mn-IP_6	0.77 ± 0.03	ND
Mg-IP_6	28.3 ± 0.5	ND
Fe-IP_6	2.76 ± 0.04	ND
Sn-IP_6	17.3 ± 1.3	6.5±1.1
Sr-IP_6	25.3±1.1	ND
Zn-IP_6	ND	ND
Na-IP_6	60.9±1.6	1.1 ± 0.2

The enzyme (5µM) was incubated overnight in 10 mM Tris-HCl buffer pH 7 containing 1 mM $CaCl_2$ at 4 °C. The calcium loaded enzyme was diluted 100 times in 10 mM Tris-HCl buffer pH 7 prior to determination of activity in 0.1 M Tris-HCl buffer pH 7 containing 1.5 mM sodium phytate and 4.5 mM different metal ions for 10 min at 37 °C and 70 °C, respectively. The activity of calcium activated enzyme on Ca-IP_6 substrate (289.7 U/ml at 37 °C; 685.2 U/ml at 70 °C) was considered as 100 %.ND: not detected.

Addition of 150 mM NaCl in Tris-HCl buffer at pH 7 prevented the aggregation to some extent (Fig. 2b). But since this study was focused on studying the effect of different metal ions on the activity and stability of the enzyme, the use of NaCl was undesirable. Furthermore, it is well established that Tris-HCl has poor buffering capacity at high

temperatures and is not usually recommended for DSC studies.

Table 4: Effect of different metal ions on the activity of metal depleted *Bacillus* sp. MD2 phytase using the corresponding metal ion-phytate complexes as substrates.

Metal ion-phytate	Relative activity (%)	
	37 °C	70 °C
Ca-IP_6	100 ± 1.3	100 ± 2.9
Ba-IP_6	ND	1.6 ± 1.1
Co-IP_6	ND	ND
Cu-IP_6	0.53 ± 0.02	ND
Mn-IP_6	0.18 ± 0.01	ND
Mg-IP_6	22.87 ± 0.32	ND
Fe-IP_6	1.77 ± 0.03	ND
Sn-IP_6	0.18 ± 0.01	6.5 ± 1.1
Sr-IP_6	20.74 ± 0.54	ND
Zn-IP_6	ND	ND

The enzyme samples (5µM) were incubated overnight in 10 mM Tris-HCl buffer pH 7 containing 5 mM different metal ions at 4 °C after which the samples were diluted 100 times in 10 mM Tris-HCl buffer pH 7 before determining activity in 0.1 M Tris-HCl buffer pH 7 containing 1.5 mM sodium phytate and 4.5 mM different metal ions for 10 min at 37 °C and 70 °C, respectively. The activity of calcium activated enzyme on Ca-IP_6 substrate (289.7 U/ml at 37 °C; 685.2 U/ml at 70 °C) was considered as 100%. ND: not detected.

Subsequently, the DSC analysis was performed in 20 mM acetate buffer at pH 4, where no aggregation was noted. In absence of Ca^{2+}, a T_m value of 42.4 °C was obtained, while in presence of 5 mM calcium, the T_m value increased by 11.9 degrees (Fig. 2a, Table 5). Replacing Ca^{2+} with other metals, Ba^{2+}, Mn^{2+}, Sr^{2+}, and Cu^{2+} in the enzyme resulted in an increase in T_m of about 2-3 °C while the presence of Mg^{2+} and Co^{2+} led to a decrease in T_m by about 1.4 °C.

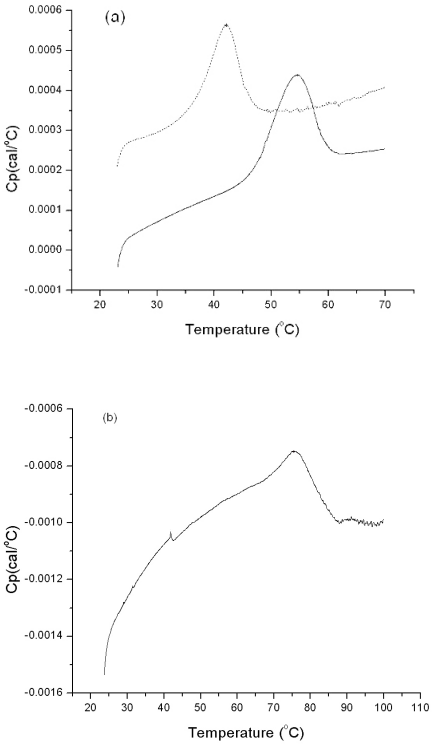

Fig. 2 Raw DSC thermograms of calcium depleted (dotted line) and calcium loaded (solid line) *Bacillus* sp. MD2 phytase in (a) 20 mM acetate buffer pH 4 and (b) calcium loaded MD2 phytase in 20 mM Tris-HCl buffer pH 7 with 150 mM NaCl.

3.4 Effect of calcium ions on MD2 phytase activity and stability

Thermal stability of the MD2 phytase in the presence of 5 mM $CaCl_2$ at pH 7 was also determined in terms of its residual activity

after incubation at a particular temperature. The enzyme retained over 90% of its activity after 3 h incubation at 30-40 °C, 80 min at 50-60 °C (data not shown), or 1 h at 70 °C (Fig. 3). At 80 °C and 90 °C, the half life ($t_{1/2}$) of the enzyme was about 42 and 22 min, respectively. The enzyme retained about 40% of the activity after 10 min incubation at 100 °C (Fig. 3).

Table 5: Thermostability of *Bacillus* sp. MD2 phytase activated by different metal ions.

Enzyme samples	**T_m (°C)**
Non activated enzyme	42.4
Enzyme activated by $CaCl_2$	54.3
Enzyme activated by $BaCl_2$	44.4
Enzyme activated by $MnCl_2$	45.7
Enzyme activated by $MgCl_2$	41
Enzyme activated by $SrCl_2$	45.8
Enzyme activated by $CoCl_2$	41
Enzyme activated by $CuCl_2$	45.7

The enzyme (25µM) was activated in 20 mM acetate buffer pH 4 containing 5 mM of different metal ions at 4 °C for 4 hours before DSC analysis of the T_m value. T_m values were reported as mean values of duplicate analysis of the temperature at which the activated enzyme was completely denatured.

Fig. 4 shows the influence of calcium concentration (0-5 mM) in the reaction on temperature profile of the (calcium loaded) enzyme. The optimum temperature for enzyme activity was shifted from 40 °C (without added calcium) to 70 °C when 1-5 mM of calcium was added to the reaction mixture and over a 3-fold increase in enzyme activity was achieved. The effect of Ca^{2+} on activity of the phytase was further determined at 37 °C and 70 °C (Fig. 5). In the absence of Ca^{2+} very little activity was detected at 70 °C and upon addition of the metal ion at a concentration of 1 mM, a drastic increase in activity was observed. When the Ca^{2+} concentration was higher than 4 mM , inhibition of the enzyme activity was noted and no activity was detected at 10 mM Ca^{2+}. On addition of EDTA to the reaction containing 5 mM $CaCl_2$, the enzyme activity was found to be stimulated and became optimal at 4 mM EDTA. With further increase in EDTA concentration, the enzyme activity dropped sharply and was undetectable in presence of 6 mM EDTA (Fig. 5). At 37 °C, the influence of the calcium concentration was less significant.

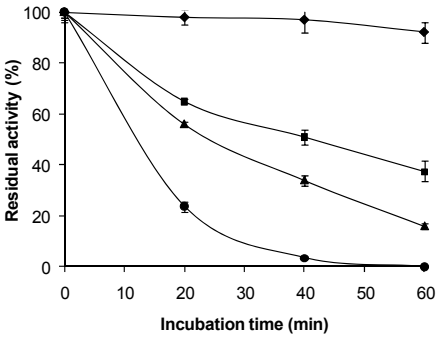

Fig. 3 Effect of temperature on thermal stability of *Bacilus* sp. MD2 phytase at pH 7 at 70 °C (♦), 80 °C (■), 90 °C (▲) and 100 °C (●), respectively. The enzyme was incubated in 0.1 M Tris-HCl buffer, pH 7 containing 5 mM $CaCl_2$ at the respective temperatures and samples were withdrawn at defined time intervals, cooled and residual activity determined under the standard assay conditions (70 °C for 20 min in 0.1 M Tris-HCl buffer, pH 7 containing 5 mM $CaCl_2$ and 1.5 mM sodium phytate).

Effect of calcium chloride (0-5 mM) on MD2 phytase activity at different pH values was studied at 37 °C and 70 °C (Fig. 6a, b). In the absence of Ca^{2+} in the buffer, the enzyme showed optimal activity at pH 7-7.5 and no activity at pH 5 and pH 8.5. In the presence of 1-5 mM $CaCl_2$, a dramatic increase in activity above pH 4 was observed over a broad pH range, resulting in a broader pH profile of MD2 phytase at 37 °C. There was also a shift in optimum pH of the enzyme towards the acidic region with increasing calcium concentration; at 5 mM $CaCl_2$ the optimum pH of the phytase was 6. At 70 °C, pH profile of the enzyme became narrower with increase in $CaCl_2$ concentration as compared to that at 37 °C. The MD2 phytase also showed remarkable stability during storage at room temperature in the pH range of 3.6-9.6 with 5 mM Ca^{2+} (data not shown). Retention of about 40% of the original activity was observed after 1h incubation at pH 2.6.

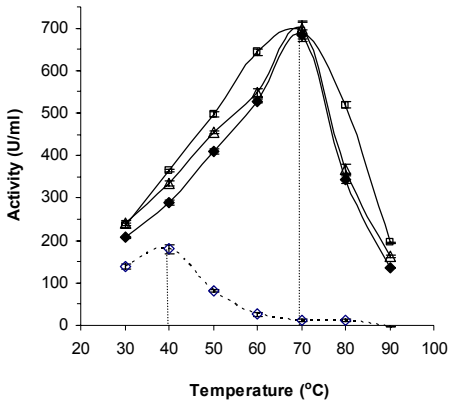

Fig. 4 Effect of calcium and temperature on the activity of the recombinant *Bacillus* sp. MD2 phytase. The enzyme activity was determined by assaying the calcium-loaded phytase at different temperatures using sodium phytate dissolved in buffer pH 7 containing 0 (◇), 1 (□), 3 (△), 5mM $CaCl_2$ (◆). The dotted lines show the optimal temperature for enzyme activity.

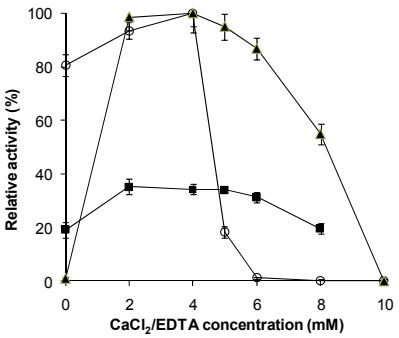

Fig. 5 Effect of $CaCl_2$ and EDTA concentration on the activity of recombinant *Bacillus* sp. MD2 phytase. The assays were performed at pH 7, 37 °C (■) and at 70 °C (▲) with different concentration of $CaCl_2$. The effect of EDTA (○) concentration was investigated at 70 °C.

4. Discussion

Alkaline phytases are widely available in nature [20], however there is only limited knowledge available on their biochemical and catalytic properties [4]. *Bacillus* species constitute an important source of alkaline phytases, which are highly homologous to each other. The phytase from *Bacillus* sp. MD2 shares a high sequence similarity with other *Bacillus* phytases [12]. The enzyme was expressed at a high level in *E. coli* and purified using a simple procedure exploiting its thermostability and affinity to metal ions via the histidine tag. As would be expected, the pure enzyme was found to have features

close to those of other *Bacillus* phytases. The recombinant phytase has molecular weight of 47.5 kDa, specific activity of 39 U/mg, and optimum pH of 6.0 and temperature of 67-73 °C for activity in the presence of 5 mM Ca^{2+} fall within the range known for *Bacillus* phytases [4, 6, 7, 13, 21, 22].

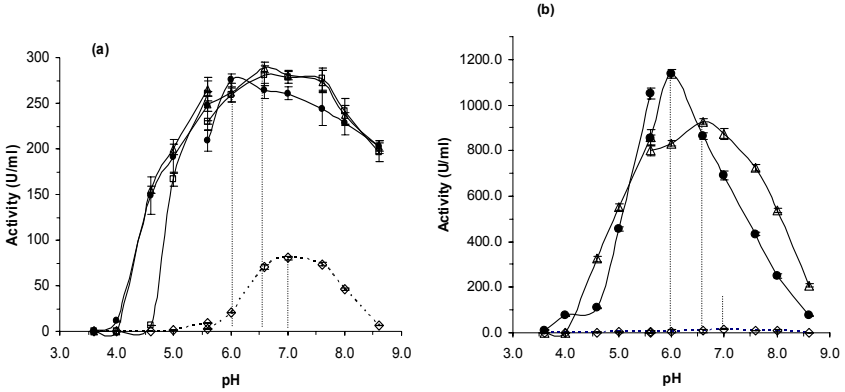

Fig. 6 Effect of calcium on pH profile of *Bacillus* sp. MD2 phytase activity at 37 °C (a) and 70 °C (b). The enzyme activity was determined in 0.1 M acetate buffer (pH 3.6–5.6) and Tris-maleate buffer (pH 5.6–8.6) containing 0 (◇), 1 (□), 3 (△), 5mM $CaCl_2$ (●) and 1.5 mM sodium phytate. The dotted lines show the optimal pH for enzyme activity.

Dependence on the presence of calcium in the enzyme as well as substrate for the activity of alkaline phytase has been reported earlier [8, 9]. Structural analysis of *B. amyloliquefaciens* phytase has shown the presence of bound calcium ions at three high affinity sites; two at the periphery of the molecule (Ca1 and Ca2) and one at the central channel (Ca3), which are important for stabilizing the enzyme structure [5]. Additionally, a low affinity three calcium binding site (Ca4, Ca5 and Ca6) is present in a shallow cleft at the top of the molecule, which forms the active site. The calcium occupied cleft provides a favorable environment for binding of phytate. Binding of yet another calcium ion (Ca7) is induced by phosphate ions by providing a coordination arm [10]. Removal of calcium from *Bacillus* sp. MD2 phytase by extensive dialysis against EDTA resulted in major fraction of the enzyme retaining one calcium atom per protein molecule. According to earlier studies with DS11 phytase, the calcium embedded in the central channel would not be accessible to the chelating agents [8, 19]. The calcium depleted enzyme did show up to 2% of the maximum activity, which could probably be due to some interaction of Ca^{2+} in the buffer with the low affinity calcium binding sites on the enzyme during the assay. Recharging the phytase with Ca^{2+} resulted in 100% recovery of its activity.

The enzyme was also charged with other metal ions, most of which (except tin) belonged either to the same group (Group II, alkaline earth metals) or the same period (transition metals) as calcium. Mg^{2+}, Sr^{2+},

Ba^{2+} (the Group II elements) and Mn^{2+}, respectively, led to almost complete recovery of the activity (Table 2), which confirms that these metal ions are able to bind to low affinity triadic calcium sites and the catalytically important water molecules in the active site, and hence fulfill the activation role of Ca^{2+}. The other metal ions in Table 2 leading to no activity (Fe^{2+} and Zn^{2+}) or limited phytase activity (Co^{2+}, Cu^{2+} and Sn^{2+}) most likely since they also bind to the low affinity calcium binding sites but their binding may further involve different number of bound metals or other preferred donors for these metal ions [23] on the enzyme molecule that may result in either deactivation or interference with binding of the substrate. Our observations differ from the earlier reports that show partial activation of TS-Phy phytase with Ba^{2+}, Mg^{2+} and Sr^{2+}, and no activation with other metal ions (Al^{2+}, Co^{2+}, Cs^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Ni^{2+}) [8, 19], and partial recovery of *B. subtilis PhyC* activity on reactivation by Ca^{2+} and limited recovery by Zn^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}[9]. It may partly be due to different protocols used for treatment of the enzyme with the metal salts.

Ha et al. [19] have earlier shown that Cd^{2+} ions bind to five different sites on top of the TS-Phy molecule - three to the calcium sites and other two located close by that interfere with substrate binding. This would explain also the activity loss observed with Zn^{2+} (Table 2), which belongs to the same group as Cd^{2+} in the periodic table and has similar features in terms of coordination numbers, geometries, preference of donors, etc. [18]. Investigating the effect of Zn^{2+} on phytase effect would be of inerest since it is the second most abundant metal in enzymes and the most encountered metal ion in EC3 (hydrolytic) enzyme class [23]. Contrary to Zn^{2+}, Fe^{2+} has versatile redox properties which enables it to act as a redox center, and is unsuitable for the role of a redox inert metal required in the hydrolytic activity, which can increase the electrophilicity of reacting species (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}) [23].

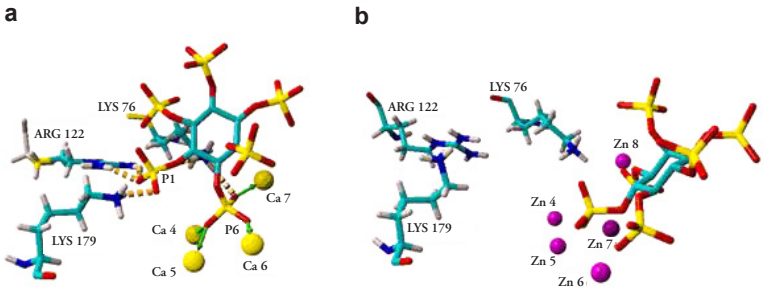

Fig. 7 a) Top view of IP_6 docked into calcium loaded phytase; dashed lines are hydrogen bonds; green arrows indicate the interaction of the 4 Ca^{2+} ions (Ca4-Ca7) and the phosphate group (P6) at the cleavage site, b) Side view of effect of zinc binding to phytase: IP_6 is disoriented in the cleavage site and the phosphate group (P6) lies outside the four Zn atoms. Adaptation of Lys 179 side chain to form hydrogen bond is noticed in case of Ca-phytase. The molecular graphics were created with YASARA (www.yasara.org) and PovRay (www.PovRay.org).

Zn^{2+} ions were assumed to bind the same Cd^{2+} loci based on the high degree of similarity between Cd^{2+} and Zn^{2+} and the observation of Ha and coworkers, where different metals were found to bind the same loci in the low affinity site [18, 19]. The effect of Zn^{2+} on the binding of IP_6 was explored using AutoDock, which successfully predicts the binding of IP_6 to the cleavage site and binding site in the presence of Ca^{2+} (Figure 7a) in line with the findings of Shin et al. [10]. Interestingly, on binding of Zn^{+2} the phosphate group is not properly placed in the cleavage site of the phytase (Figure 7b). The disoriented position of IP_6 does not allow the stabilization of the pentavalent transitional state, hence explaining the loss of activity in case of Zn^{+2}.

When calcium loaded enzyme was assayed using phytate complexed with other metal ions, the phytase activity was lower and restricted to only a few of these substrates. Sodium phytate was included as a substrate since it is commonly used for assaying phytase activity, and gave about 60% of the maximal activity (obtained with calcium phytate) at 37 °C. Some activity was also observed with Mg^{2+}-, Sn^{2+}- and Sr^{2+}-phytate complexes, but was extremely low with Cu^{2+}, Mn^{2+} and Fe^{2+} complexes and undetectable with Ba^{2+}, Co^{2+} and Zn^{2+} complexes. The decrease in activity of the phytase may be related to the stability of the metal-phytate complexes [1] and the ionic radius of the metal ions. Stronger affinity of IP_6 to various metal ions including Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, and Fe^{2+} than to Ca^{2+} has been established earlier [24, 10]. Higher stability of the metal phytate complex can possibly hamper the phytate degradation. Sodium and calcium share the same coordination number and coordination polyhedron which is cubic to octahedral. Moreover, the atomic radii of sodium (1.02 Å) and calcium (0.99 Å) are very close. This may explain the relatively high phytase activity obtained with Na-phytate. Ca, Mg and Sr (Group II elements) have similar physical and chemical properties. The respective phytate complexes of these metals may also have similar properties and hence the enzyme exhibited a reasonable degree of activity with Mg- and Sr-phytate complexes.

Replacing calcium on both the enzyme and phytate with other cations revealed about 20-23% activity with Mg^{2+} and Sr^{2+} at 37 °C but not at 70 °C. Oh et al. (2001) have earlier shown partial activation of *Bacillus* DS11 phytase with Sr^{2+} but not with any other cation [8]. These results indicate the importance of Ca^{2+} on the substrate for providing optimal activity. While Mg^{2+} and Sr^{2+} could replace the Ca^{2+} on the *Bacillus* MD2 phytase as long as phytate complexed with calcium was used, it seems that the binding of the (seventh) calcium ion (Ca7) brought about by the presence of the substrate phosphate ion in the active site is crucial for stabilization of the enzyme-substrate transition state and resultant activity. This may be ascribed to the coordinative flexibility of the metal ion for interaction at the active site and optimal stability of the calcium-phytate complex. Lack of phytase activity at higher temperature could be attributed to low stability of the enzyme in the absence of calcium.

Stabilization of MD2 phytase by calcium was confirmed by DSC experiments. Thermal

unfolding of the enzyme at pH 7 resulted in protein aggregation after unfolding. This problem led us to run the DSC measurements at a sub-optimal pH for calcium binding and phytase activity. Despite this, an increase in T_m of the phytase by 12 °C was observed in presence of Ca^{2+} (Fig. 2a). The other cations (Ba^{2+}, Mn^{2+}, Sr^{2+} and Cu^{2+}) provided a minor stabilizing effect, while Mg^{2+} and Co^{2+} lowered the enzyme stability (Table 5). This suggests that presence of calcium at the high affinity sites is important for thermal stability of the enzyme, however the reason underlying the effect on stability of the other metal ions, i.e. if the minor stabilization provided by some is due to their interaction with the high affinity sites or to their presence on the low affinity sites, is not clear.

The stability provided by calcium was also seen in the increase in optimum temperature of phytase activity from 40 °C (without Ca^{2+}) to 70 °C (Figure 4) and the significant residual activity after incubation at temperatures up to 100 °C (Figure 3). A drastic activation of the MD2 phytase at 70 °C was observed in the presence of calcium but the effect was not significant at 37 °C (Figure 5). The influence of calcium on MD2 phytase activity showed a similar trend as that reported for TS-Phy phytase [8]. Optimal activity was observed at 1-4 mM Ca^{2+}; higher concentrations resulted in inhibition of the activity which has earlier been ascribed to the action of calcium as a weak competitive inhibitor at high concentration [8]. Chelation of excess Ca^{2+} by EDTA relieved the inhibition, but excess EDTA led to inhibition owing to chelation of the metal essential for activity. Besides increasing the MD2 phytase activity and stability over a wide range of pH, the presence of calcium also led to a shift in the optimum pH of the activity towards the acidic side (Figures 6 and 7). This could be due to a combined effect of a more favorable binding of calcium-phytate (in contrast to phytic acid) and facilitated release of the leaving group (inorganic ortho-phosphate) after the cleavage of the phosphomonoester bond due to the relative abundance of H^+ at lower pH.

Concluding remarks

Alkaline phytases are an interesting group of metalloenzymes whose activity is regulated by the metal ions present on the enzyme as well as the substrate. This study shows that the alkaline phytase of *Bacillus* sp. MD2 can utilize different metal ions besides calcium for its catalytic activity, presumably through binding to the known low affinity calcium binding sites. The presence of calcium ions on the substrate is however a pre-requisite for expression of optimal activity, indicating an important role of Ca7 in the catalytic mechanism [10]. Calcium is also unique in providing the high thermostability to the enzyme as has also been shown earlier [8, 9]. The calcium loaded MD2 phytase was able to degrade phytates complexed with Na^+ and Mg^{2+}, which are among the common salt forms of phytic acid in nature [24, 25], at 37 °C but not at 70 °C. This suggests that such metal phytates can be degraded either during feed storage or in the animal gut but not during pelleting. Further studies will involve crystallization of the MD2 phytase in the presence of different metal ions for fully understanding the interactions and the effects thereof.

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Paper IV

Altering the activity and specificity of a thermostable alkaline phytase from *Bacillus* sp. MD2 by site directed mutagenesis

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ABSTRACT

Site-directed mutagenesis of a thermostable alkaline phytase from *Bacillus* sp. MD2 was performed with an aim to increase its specific activity and activity and stability in an acidic environment. The mutation sites are distributed on the catalytic surface of the enzyme (P257R, E180N, E229V and S283R) and at the active-site (K77R, K179R and E227S). Selection of the residues was based on the idea that acid active phytases are more positively charged around their catalytic surfaces. Thus, a decrease in the content of negatively charged residues or an increase in the positive charges in the catalytic region of an alkaline phytase was assumed to have effect on the enzyme activity and stability at low pH. Moreover, widening of the substrate-binding pocket is expected to improve the hydrolysis of substrates that are not efficiently hydrolysed by wild type alkaline phytase. Analysis of the phytase variants revealed that E229V and S283R mutants increased the specific activity by about 19 and 13%, respectively. Mutation of the active site residues K77R and K179R led to severe reduction in the specific activity of the enzyme. On the other hand, the double mutant (K77R-K179R) phytase showed higher stability at acidic pH (pH 2.6 - 3). E227S variant exhibited a shift in the pH optimum of the enzyme to the acidic side and improved stability in acidic condition. This mutant phytase, displayed over 80% of its initial activity after 3 h incubation at pH 2.6 while the wild type phytase retained only about 40% of its original activity. Moreover, the relative activity of this mutant phytase on calcium phytate, sodium pyrophosphate and *p*-nitro phenyl phosphate was higher than that of the wild type phytase.

Key words: Phytase, site-directed mutagenesis, *Bacillus* sp., substrate specificity.

INTRODUCTION

Phytases, enzymes that catalyze the sequential hydrolysis of phosphate ester bonds of phytate (myo-inositol 1,2,3,4,5,6-hexakisphosphate) to less phosphorylated myo-inositol derivatives with concomitant release of inorganic phosphate [1], are among

the most common animal feed additives [2, 3]. The action of phytase in feeds contributes to the removal of phytate, an anti-nutritional factor, and thereby increases the bioavailability of not only phosphate but also minerals [4-7], protein [8-10] and starch [11]. Consequently, the supplementation of inorganic phosphate to the feeds is reduced/avoided [2], leading to reduced feed costs as well as pollution caused by phosphorus run-off from the manure in intensive livestock breeding areas [12, 13].

Phytases are diverse and are grouped as histidine acid phytases and alkaline phytases (beta-propeller phytases) based on their structure and catalytic properties [1]. Fungal and *Escherichia coli* phytases are histidine acid phytases, which degrade phytate at low pH as in stomach where phytate exists in a metal free form and results in myo-inositol monophosphate [1, 14-17]. Alkaline phytases exhibit Ca^{2+}-dependent catalytic activity and stability, optimal activity around neutral pH, and degrade metal-phytate complex to release myo-inositol triphosphate as end product. Alkaline phytases have been reported from *Bacillus* sp. [9, 18-22] and pollen of some plants such as *Typha latifolia* [23] and *Lilium longiflorum* [24]. Although high thermostablity and resistance to calcium and phosphate inhibition exhibited by this group of phytases is desirable for their use as feed ingredient [25], they have low specific activity, and poor activity and stability in acidic environment encountered in stomach of animals, which pose limitations for their application. Thus, improving these properties of alkaline phytases is of great interest.

Phytases used in animal feed formulations should be preferably active and stable in wide range of pH, exhibit high specific activity, stability both during and after pelleting, and degrade different forms of phytate [2, 26]. However, there is no phytase that fulfills all the application requirements [2]. Hence mutagenesis has often been used to improve single or multiple traits of phytases. Site-directed mutagenesis has been used to improve the specific activity or thermostability of phytases from *Aspergillus fumigatus* [27] and *E. coli* [28], and to change the pH profile of *Aspergillus niger* phytase [29]. Based on sequence comparison of 13 phytases from six different fungi, Lehmann et al., [30, 31] have used consensus-approach and site directed mutagenesis to de novo construct a consensus phytase with markedly improved thermostability. Consensus-approach was also used for alkaline phytase from *Bacillus* species to design an alkaline β-propeller phytase with increased activity in an acidic environment and higher thermostability at pH 7.5 [32].

In the present study, site directed mutagenesis was applied on a thermostable alkaline phytase from *Bacillus* sp. MD2 - with an aim to obtain phytase mutant(s) with higher specific activity and -activity and stability at lower pH.

MATERIALS AND METHODS

Bacterial strains, media and plasmids

Escherichia coli strains (Novablue and BL21(DE3)) were purchased from Novagen (Novagen Madison, WI, USA) and cultivated using Luria-Bertani (LB) broth or LB-agar supplemented with ampicillin (100 μg ml-1) when needed. The expression vector pET-22b(+) plasmids were purchased from

Novagen. The plasmid, pE10C2 was constructed, in our previous study, from pET-22b(+) and phytase gene from *Bacillus* sp. MD2 [33].

Mutagenesis of the phytase gene

The recombinant plasmid pE10C2 was extracted from *E. coli* Novablue cells following the method of Sambrook et al. [34] and used as template for mutagenesis. Seven pairs of mutagenic primers were designed to make the mutations (Table 1), using QuickChange®II site directed mutagenesis kit from Stratagene. The PCR mixture contained about 25 ng of the template plasmid, 62.5 ng of each forward and reverse mutagenic primer, 1 µl of dNTP mix, 2.5 µl of reaction buffer and 1.25 units of PfuUltra HF DNA polymerase (Stratagene) in a total reaction volume of 25 µl. The reaction was run for 16 cycles of 30 s at 95 °C, 1 min at 55 °C and 7 min at 68 °C. The amplified plasmids were digested by DpnI to remove parental methylated plasmids and then 1 µl of the reaction mixture was transformed to competent *E. coli* Novablue cells. Mutant plasmids were then propagated in *E. coli* Novablue cells in LB broth supplemented with ampicillin (100 µg ml^{-1}), extracted by alkaline lysis method [35] and sequenced. Plasmids containing the target mutation were transformed to the expression host *E. coli* BL21(DE3) cells.

Table 1. Mutagenic primers used to construct different phytase variants (Nucleotides in bold are mutated sites).

Primers	Sequence
P257R-F	GGCAGGCATTTAACCC**G**TGATATTGAAGGACTGACG
P257R-R	CGTCAGTCCTTCAATATCA**C**GGGTTAAATGCCTGCC
E180N-F	GCGATGGTGACAGGAAAAA**AC**GGCGAATTTGAACAATACG
E180N-R	CGTATTGTTCAAATTCGCC**GT**TTTTTCCTGTCACCATCGC
E229V-F	CGCAGAAGAAGATG**T**GGCCATCTGGAAGTTC
E229V-R	GAACTTCCAGATGGCC**A**CATCTTCTTCTGCG
S283R-F	GCCAGGGTAACAGC**C**GCTATGCGATTTATGAAAGACAG
S283R-R	CTGTCTTTCATAAATCGCATAGC**G**GCTGTTACCCTGGC
K77R-F	GCAAATTGATCACAACCAATAAAA**G**ATCAGGCTTAGTCGTG
K77R-R	CACGACTAAGCCTGAT**C**TTTTATTGGTTGTGATCAATTTGC
K179R-F	CGCGATGGTGACAGGAA**G**AGAAGGCGAATTTGAACAATAC
K179R-R	GTATTGTTCAAATTCGCCTTCT**C**TTCCTGTCACCATCGCG
E227S-F	GGCAGTCTTTATATCGCAGAA**TC**AGATGAGGCCATCTGG
E227S-R	CCAGATGGCCTCATCT**GA**TTCTGCGATATAAAGACTGCC

Expression of mutant phytases

A single colony of *E. coli* BL21(DE3) cells harbouring the mutant plasmid was picked from LB agar plate containing 100 µg ml^{-1} of ampicillin and cultured for 5 h in 5 ml LB broth containing 100 µg ml-1 of ampicillin at 30 ^{o}C and 200 rpm. This culture was used to inoculate 100 ml of the same medium, which was cultivated under the same conditions. When the optical density (OD) of the culture at 620 nm was about 0.7, isopropyl

β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. After 14 hours of induction, the culture broth was centrifuged at 8000 g for 10 min. The cell pellet was washed with physiological saline solution and re-suspended in 0.1 M Tris - HCl buffer, pH 7 containing 5 mM CaCl2, and subjected for 4 rounds of sonication to release cell associated phytase. The phytase activity in the cell free culture supernatants and cell lysate fractions was determined.

Purification of mutant phytases

Each mutant phytase with a tag of 6 His residues was purified from the cell free culture supernatant using immobilized metal affinity chromatography (IMAC). The culture supernatant was filtered using a 0.45 µm pore size cellulose acetate membrane and 20 ml of the filtrate was loaded on a 10 ml Cu^{2+} bound column pre-equilibrated with binding buffer (20 mM Tris-HCl pH 7 containing 5mM $CaCl_2$, 300 mM NaCl and 1% glycerol). The column was then thoroughly washed with the binding buffer to remove loosely bound proteins. The bound protein was eluted using a gradient of 0-300 mM imidazole dissolved in the same buffer. Fractions exhibiting phytase activity were checked on SDS-PAGE and homogenous fractions were pooled, concentrated using Macro®column (10 KDa cut off, Pall Scientific Corporation) and then equilibrated with 0.1 M Tris-HCl buffer pH 7 containing 5 mM $CaCl_2$.

Phytase activity assay

The enzyme activity was assayed according to the method described by Shimizu [18], involving spectrophotometric measurement of the inorganic phosphate (P_i) released from sodium phytate. Unless otherwise mentioned, a mixture of appropriately diluted enzyme and sodium phytate (1.5 mM) dissolved in 100 mM Tris-HCl buffer, pH 7 containing 5 mM $CaCl_2$ was incubated at 70 °C for 20 min. The reaction was stopped by addition of 15% trichloroacetic acid (TCA) solution. The precipitate obtained was removed by centrifugation and 500 µl of the clear supernatant was transferred to an eppendorf tube to which was then added 500 µl ammonium molybdate reagent (containing 1:4 mixture of 2.7% $FeSO_4$ and 1.5% ammonium molybdate in 5.5% H_2SO_4). After 5 min incubation at room temperature, absorbance at 700 nm was read, which was used to calculate the concentration of P_i from a standard curve made using sodium dihydrogen phosphate solution in the concentration range of 0-600 nmol ml^{-1} P_i. All the samples were assayed in triplicates. One unit of phytase activity was defined as the amount of enzyme that releases 1 µmol of P_i per minute under the assay conditions.

Determination of pH profile and pH stability of mutant phytases

The pH profiles of the mutant and wild type phytases were determined using different buffers (0.1 mM sodium acetate pH 3.6-5.6 and Tris-maleate pH 5.6-8.6, respectively) containing 5 mM $CaCl_2$ and 1.5 mM sodium phytate. The effect of pH on stability of enzymes was studied by treatment of the enzyme in buffers (with 5 mM $CaCl_2$) mentioned above for 3 hours, then diluting the enzyme 10 times in 0.1 mM Tris-HCl buffer, pH 7 containing 5 mM $CaCl_2$ prior to measuring the enzyme activity at 70 ºC for 20 min in 0.1 mM Tris-HCl buffer, pH 7

containing 5 mM $CaCl_2$ and 1.5 mM sodium phytate.

Protein analysis

The molecular weight and homogeneity of the purified phytase were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 12.5% (w/v) polyacrylamide gel. The total protein concentration of the samples was measured by bicinchoninic acid method using bovine serum albumin as standard [35].

Structure analysis

The structures of a histidine acid phytase from *E. coli* (1DKQ) and an alkaline phytase (beta-propeller phytase) from *Bacillus amyloliquficens* (2POO) were downloaded from PDB database. The phytase of *Bacillus* sp. MD2 differs from the *B. amyloliquefaciens* phytase only by two amino acids [33] and hence 2POO was used to generate a model structure for *Bacillus* sp. MD2 phytase. The surface electrostatic potential of the proteins were generated using YASARA software. The same software was used to dock sodium pyrophosphate into the active site of the wild type and mutated *Bacillus* sp. MD2 phytase.

RESULTS AND DISCUSSION

Mutation strategy

A comparison was made between an acidic phytase from *E. coli* (PDB 1DKQ) and an alkaline phytase from *B. amyloliquficens* phytase (PDB 2POO), which is almost identical to the enzyme from *Bacillus* sp. MD2 [33]. The results are summarized in Table 2.

Table 2. Comparison of the active site regions of a hisdine acid phytase from *E. coli* and an alkaline (beta-propeller) phytase from *Bacillus* sp. MD2 which is almost identical to *B. amyloliquficens* phytase (PDB 2POO).

Acid phytase	Alkaline phytase
Eleven residues 'involved'	Fourteen residues 'involved'
Six charged residues	Eleven charged residues
Five positively charged residues (four Arg and a Lys)	Four positively charged residues (an Arg and three Lys)
Big cavity	Small cavity
No catalytically important metal ion	Three catalytically important Ca^{2+} and one Ca^{2+} from the substrate

One of the major differences between these two groups is the number of charged residues in the active site region. The surface electrostatic potential of the catalytic region of phytases is very important for substrate binding and subsequent degradation. The *E. coli* phytase is more positively charged than the *Bacillus* phytase (Fig. 1). This property is expected to facilitate the binding of the negatively charged phytic acid to the positively charged active site and allows the enzyme to be active in the acidic region [1]. On the other hand, the *Bacillus* phytase has more negative charges on its surface, which possibly repel the negatively charged phytic acid and hence do not degrade it. The *E. coli* phytase and the *Bacillus* phytase have five and four positively charged residues, respectively, in their active sites (Table 2). However, four out of five are Arg in *E. coli*

while it is only one out of four in the *Bacillus* phytase. Since Arg has an extra positive charge than Lys, the positive charge density is higher in *E. coli* than in *Bacillus* phytase. Thus, substituting the alkaline phytase catalytic region Lys with Arg or decreasing the number of acidic residues at or around the active site is expected to improve some properties of the alkaline phytase for the low pH environment. Histidine acid phytases are also more promiscuous with respect to substrates than alkaline phytases, which is attributed to their wider catalytic cavity [1]. Thus, widening the catalytic cavity of the alkaline phytases may increase the substrate promiscuity of alkaline phytases.

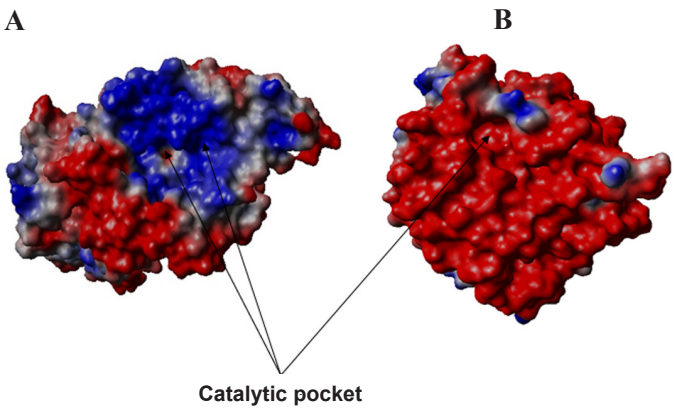

Fig. 1. Surface representation of (A) a histidine acid phytase from *E. coli* (PDB 1DKL) and (B) an alkaline phytase model from *Bacillus* sp. MD2. Negatively and positively charged surfaces are colored in red and blue, respectively.

In this study, a number of sites were chosen, mutated and the mutant enzymes were analysed. Mutation of the catalytically important K77 and K179 to arginines was done based on the assumption that the extra positive charge of Arg might improve the activity of the enzyme in acidic condition. Some amino acids close to the active site residues were also semi-randomly selected for mutation. The *B. licheniformis* phytase sequence (GenBank Acc.No. AAM74021.1), which is reported to be significantly active at lower pH [36] than *Bacillus* sp. MD2 phytase, was used to identify residues that decrease the negative charge (E180N & E229V) or increase the positive charge density (P257R & S283R). Mutating the active site Glu (E227) to Ser would decrease the negative charge density and widen the cavity. Table 3 lists all the mutated phytase variants prepared in this study.

Characterization of mutant phytases

As revealed from the band intensity on the SDS-PAGE (data not shown), there was no detectable difference in the expression level and localization of the recombinant proteins of the mutant and wild type phytases. All the phytase variants were purified under similar conditions and characterized.

Table 3 lists the specific activities of the mutants produced. The specific activity of the purified mutant E180N was similar to that of the native phytase, whereas mutants E229V and S283R showed about 19 and 13% increase in specific activity, respectively. It is possible that further mutation in the vicinity of the active site surface may lead to a significantly higher specific activity. The mutant P257R exhibited lower specific activity than the wild type phytase. The cyclic side chain of Pro gives conformational rigidity to proteins, and hence the mutation P257R which is adjacent to the active site residue D258 may result in loss of catalytically important rigidity. The pH profile and pH stability of these four mutants was similar to the wild type phytase (data not shown). This could be partly due to the insufficient change in the electrostatic potential imparted by the single site mutations. Thus, it would be interesting to test the effect of multiple site mutations that changes the electrostatic potential of the region.

On the other hand, the mutations K77R and K179R led to significant loss of specific activity of the enzyme (Table 3) although they are not directly involved in the catalysis [37, 38]. The pH profiles of K77R and K179R mutant phytase were similar to that of wild type phytase (data not shown). A double mutant (K77R-K179R) was also constructed and its specific activity was almost similar to the specific activity of the single site mutations (Table 3). Although the wild type phytase retained about 19% of its peak activity at pH 4.6, the double mutant phytase was not detectably active at pH 4.6 (data not shown) but was more stable than the wild type phytase at lower pH (Fig. 2).

Table 3. Specific activity of native and mutant phytases of *Bacillus* sp. MD2. Sodium phytate was used as substrate and the assay was done at 70 °C, pH 7

Mutants	Specific activity (U/ mg protein)
Native	32.2±0.6
E180N	31.2±1.02
E229V	37.8±1.98
P257R	18.5±1.9
S283R	35.7±1.95
K77R	7.3±0.24
K179R	5.7±0.3
K77R- K179R	7.5±0.23
E227S	1.3±0.03

Mutating E227 by Ser was expected to decrease the negative charge and widen the catalytic cavity as the Ser side chain is shorter by a carbon atom. E227 interacts with a water molecule that interacts with Ca^{2+} [1] or even directly with Ca^{2+} [30]. Hence, the mutant phytase exhibited very low specific activity as in most of the other mutants shown in Table 3. However, the change in the properties of this particular mutant was interesting. Among all the mutants, only E227S showed a pH optimum shift (by 0.5 units) to the acidic side (Fig. 3). Moreover, the stability of mutant E227S phytase at lower pH was significantly higher. It retained over 80% of its initial activity while the wild type phytase displayed only 40% of its original activity after 3 h of treatment at pH 2.6 (Fig.2).

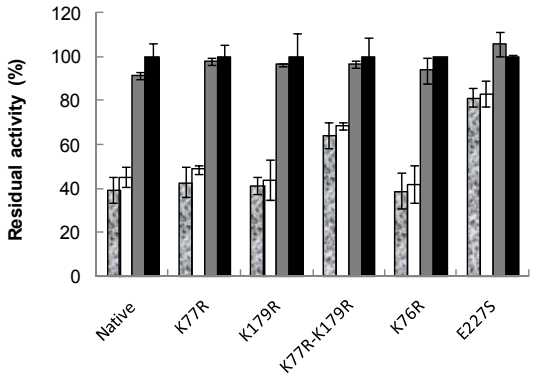

Fig. 2. pH stability of native and some mutant phytases from *Bacillus* sp. MD2 . The enzyme was incubated in buffers of different pH for 3 hours: pH 2.6 (dotted bar), pH 3 (open bar), pH 3.6 (grey filled bar), then diluted 10 times in 0.1 M Tris-HCl buffer pH 7 containing 5 mM $CaCl_2$ activity assays were performed by standard procedure at 70 °C. Control sample of each mutant (no treatment) shows in black filled bar.

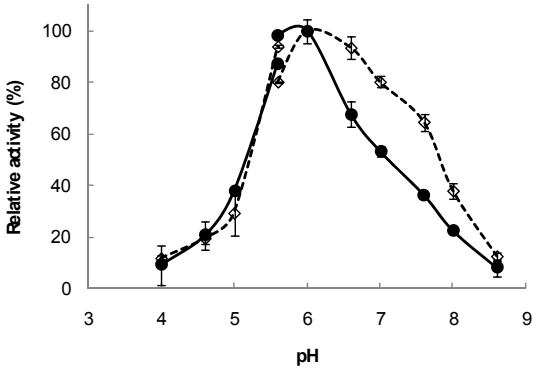

Fig. 3. Effect of pH on the activity of native *Bacillus* sp. MD2 phytase (◊) and mutant E227S (●). The enzyme activity was determined in 0.1 M buffers of different pH values (acetate buffer pH 3.6 – 5.6; Tris-maleate buffer pH 5.6–8.6) containing 5 mM $CaCl_2$ and 1.5 mM sodium phytate at 70 ^oC for 20 min.

Alkaline phytases from *Bacillus* spp. are known to be very specific to phytate [9, 19, 20], which is attributed to a narrower catalytic pocket which accommodates only two phosphate groups of the Ca^{2+}-phytate complex [1]. The mutant E227S acquires a slightly wider cavity as shown in Fig. 4 and showed relatively lower substrate specificity (Table 4). The relative activity of mutant phytase on sodium pyrophosphate was significantly higher than that with the wild type phytase, which was also confirmed by a better fitting of the pyrophosphate to the active site of the mutant phytase in contrast to the wild type phytase (Fig. 4). Moreover, the possible charge repulsion between the negatively

charged residue, E227 and the pyrophosphate is avoided in the mutant phytase.

Table 4: Substrate specificity of *Bacillus* sp. MD2 wild type and mutant phytases

Substrates	Relative activity (%)					
	Native	K77R	K179R	K77R-K179R	K76R	E227S
Sodium phytate	100	100	100	100	100	100
Calcium phytate	69.1	69.5	68.8	66.9	66.2	95.9
Sodium pyrophosphate	9.7	16.5	21.8	8.3	19.3	66.5
p-nitro phenyl phosphate	4.9	9.8	21.2	11.0	14.1	10.2

Reactions were done at 70 °C, pH 7 for 20 min. Different substrates at concentration of 1.5 mM were disolved in 0.1 M Tris-HCl buffer, pH 7 containing 5 mM $CaCl_2$. All enzymatic reactions were in triplicate and standards deviations (SD) of the relative activity were less than 10%.

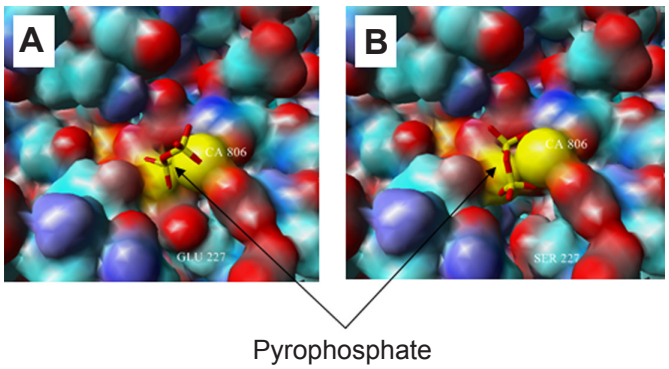

Fig. 4. The docking of sodium pyrophosphate to the *Bacillus* sp. MD2 wild type (A) and E227S mutant (B) phytases.

CONCLUSION

This study has shown the possibility of increasing the enzyme specific activity, acid activity and stability and substrate promiscuity of thermostable alkaline phytase from *Bacillus* sp. MD2. Residues on the enzyme catalytic surface could be interesting mutational targets to improve the specific activity of alkaline phytases. Mutations of catalytically involved amino acids can improve the acid activity/stability and the range of substrates that can be efficiently hydrolyzed. However, a secondary mutation which at least restores the level of the wild type enzyme specific activity is necessary.

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