Phytase: The Feed Enzyme, an Overview

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17.1 Introduction

Mankind uses enzyme since ancient times. The production of cheese, wine and vinegar, brewing of beer and leavening of bread all include enzymatic processes of prehistoric origin. Enzymes have an advantage that the process in which they are used not only becomes highly specific and fast but also eco-friendly. Enzymes replace chemical reactions which control environmental degradation. With the advancement in the field of biotechnology, new applications of industrial enzymes are emerging, and it is expected they will rule catalysing processes in factories and homes in nearby future. Phytic acid, phytin or phytate have been the substrates for phytase enzymes and discovered in 1903 (Mullaney et al. 2007). Phytate term is the widely used for mixed salt of phytic acid (*myo*-inositol hexaphosphate; IP_6). While phytic acid is the free form of IP_6, deposited complex of IP_6 with magnesium, potassium and calcium is known as phytin that exists in plants.

Phytic acid, also known as myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate, is an organic form of phosphorus and chief component of plant-origin food. In plant seeds that are consumed as animal feed element (such as cereal grains, oilseed meals and legumes), it signifies primary source of inositol and the most important storage form of phosphorus (Bedford 2000; Ravindran et al. 1995). Phytic acid is a significant constituent of plant-derived foods which put in 1–5% by weight of edible cereals, legumes, pollens, oil seeds and nuts. The molecular formula of phytic acid as $C_6H_{18}O_{24}P_6$ with molecular weight of 659.86 was given by Posternak in 1965. Generally, 50–80% of total phosphorus of plant-based food exists as phytate (Harland and Morris 1995). Monogastric animals such as swine and poultry carry little or no phytase in their elementary canal; therefore, phytic acid phosphorus confers anti-nutritive values because of its low metabolizing rate

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(Reddy and Pierson 1994). The solubility and digestibility of starch is affected due to binding of phytic acid with starch molecules (Desphande and Cheryan 1984). Phytic acid present in animal excreta is hydrolysed by microorganisms. The phosphorus released out of microbial enzymatic action is eventually pervaded into river and lake water and may cause eutrophication if accumulated in water bodies (Kuhar et al. 2009) (Fig. 17.1).

When one or more acidic hydrogens of the phosphate groups in phytic acid are substituted by a counter ion, the compound is usually known as phytate salt. Based on the pH and divalent metal cations, phytate salt either exists as a metal-phytate complex or a metal-free phytate. The binding magnitude relies on both pH and divalent metal cations to phytate ratios. In addition, at acidic pH and high cation concentration, a metal-phytate complex is formed due to direct electrostatic interaction. According to ionic radii of the metal cations, they explicitly bind to the phosphate groups of phytate. Preferably, metal cations having large ionic radii give rise to the bidentate metal complex (Oh et al. 2004) (Fig. 17.2).

The phytin was earlier considered as a storage product. It was speculated that mostly phosphorus was stored in the seed which was released to be incorporated into ATP on germination. Fifty to eighty percent of total stored phosphate stored in plant seeds exists as phytic acid (Kumar et al. 2012). The role of inositol phosphate is known to mediate the inward transportation of materials of the cell. Berridge and Irvine (1984) reported their function in signal transduction and in cellular transport as secondary messengers. Phytic acid is, in its less complexed state, highly reactive and easily forms complexes with Zn^{2+}, Ca^{2+}, Mg^{2+} and Fe^{2+}, carbohydrates and proteins (Harland and Oberleas 1977). The complexes are sparingly soluble in the small intestine and, hence, least interactive with phytase (Angel et al. 2002). Researchers thought to devise a technique to amputate phytic acid in a way that is economically reasonable with mineral supplementation by considering its values as a phosphorus source and anti-nutritive properties. Catalysing phytic acid is assumed as an effective way of enhancing the nutritional merit of several plant foodstuffs. Bioavailability of the phytate phosphorus was improved by supplementing the diet with phytase enzyme (Ali et al. 2010).

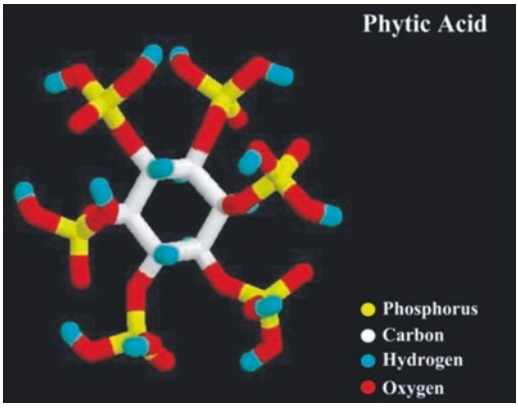

Fig. 17.1 Structure of phytic acid. (Source: W. Schmidt – USDA/ARS)

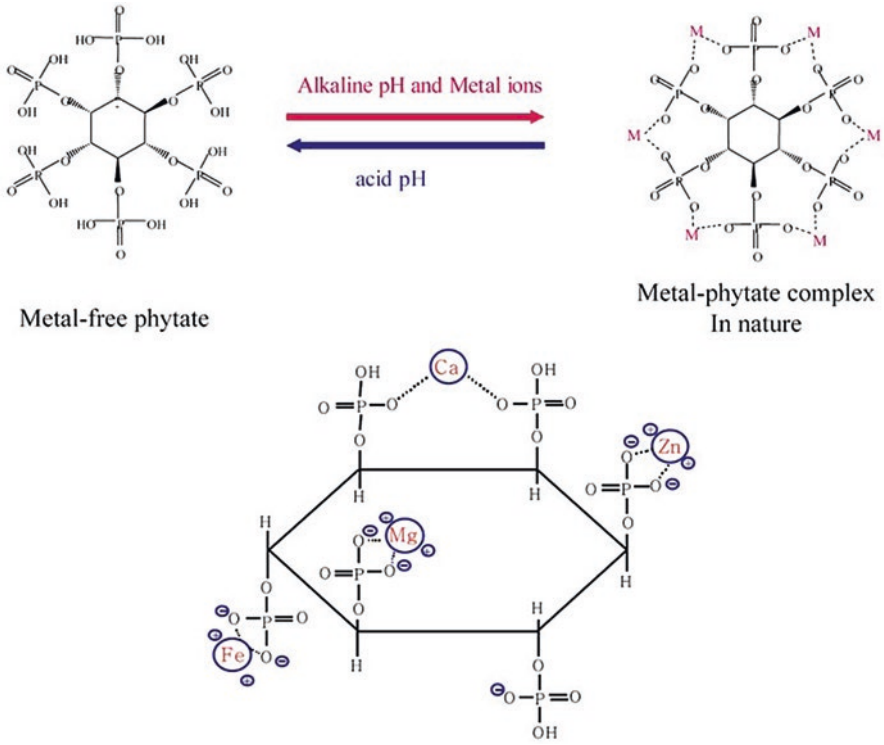

Fig. 17.2 Divalent metal cation complexes. (Source: Oh et al. 2004)

17.2 Phytates in Food Ingredients

Phytates represent 60–90% of the total phosphate and appear during maturation of the seed and its dormancy (Loewus 2002). Hence, phytate is a common component of plant-derived food. The daily intake of phytate may be up to 4500 mg based on the quantity of plant-derived foods in the diet and quality of food processing (Reddy 2002). Normal daily intake of phytate is likely to be 2000–2600 mg in case of herbivores and for the diets of rural people in developing countries and around 150–1400 mg for omnivores (Reddy 2002).

17.2.1 Phytate as an Antinutrient

Research infers that in monogastric animal feed, phytate or phytic acid has a high anti-nutrition effect since monogastric animals are deficient in phytate-degrading enzyme and so unable to utilize the phosphorus part in phytate (Selle and Ravindran 2008). Phytate act in a wide pH range because of having negatively charged ion and a high affinity for food components of cationic nature such as

minerals, proteins and trace elements (Konietzny and Greiner 2003). This association does not only have dietary implications but also affect food ingredients quantitatively as well as qualitatively such as corn steep liquor, starch or plant protein isolates (Desphande and Cheryan 1984; Fredrikson et al. 2001; Kvist et al. 2005). Phytic acid mainly exists as salts of mono- and divalent cations (e.g. calcium-magnesium-potassium salt in soybeans and potassium-magnesium salt in rice) in distinct parts of legumes and cereal grains. Besides other storage matter such as lipids and starch, it accumulates in grains and seeds during maturation. In case of legumes and cereals, it is deposited in the globoid crystals and aleurone particles, respectively (Reddy et al. 1982; Tyagi and Verma 1998). Moreover, non-occurrence of endogenous phytate-degrading enzymes and the limited microflora in the upper part of the digestive tract in human small intestine, only a small fraction of phytate is hydrolysed (Iqbal et al. 1994; Boling et al. 2000). With the decrease in phosphate residues on the myo-inositol ring, the stability and solubility of myo-inositol phosphate-mineral complexes also drop down. Thus, elimination of phosphate residues from phytate leads to lowered uptake of essential dietary minerals in the intestine (Sandberg et al. 1999). The anti-nutritional impact of phytic acid in diets for poultry and pigs was reported by Woyengo and Nyachoti (2013). It reduces mineral digestibility in broilers and pigs.

17.2.2 Phytate-Rich Diets and Health Benefits

Phytate consumption is likely to have positive effect on human health. It was known to slash kidney stone formation (Grases et al. 2001) and to offer defence against coronary heart disease and atherosclerosis (Jariwalla et al. 1990) along with wide type of cancers (Vucenik and Shamsuddin 2003). Dietary deprivation of phytate in the human regulates the levels of phytate and its dephosphorylation products in plasma, urine and other biological fluids (Grases et al. 2001).Therefore, low-phytate diet in developed countries is likely to be responsible for rise in disease incidences in Western societies such as renal lithiasis, diabetes mellitus, coronary heart diseases and atherosclerosis in comparison with the developing countries (Greiner and Konietzny 2006). Phytic acid has been used in diet treatments of rectal and colon cancers (Admassu 2009). Dietary phytate prevents the growth of cancerous cells in the liver and pancreas (Arnarson 2015). It was known to give healthy effects in the gastrointestinal tract and other target tissues through its chelating ability. Phytate is likely to exercise its anticancer impact by influencing cell signalling mechanisms in mammals (Vucenik and Shamsuddin 2003) as a result of occurrence of several *myo*-inositol phosphates, including phytate, as intracellular molecules and the second messenger D-*myo*-inositol (1,4,5)-trisphosphate play diverse pivotal roles in cellular processes such as cell proliferation via mobilizing intracellular Ca^{2+} (Shears 1998). In human erythroleukaemia cells, levels of diverse intracellular *myo*-inositol phosphate esters are affected by extracellular phytate (Shamsuddin et al. 1992). D-*myo*-Inositol (1, 2, 6)-trisphosphate, for example, is known to be related with

impediment of diabetes complications and treatment of cardiovascular diseases as well as chronic inflammations (Claxon et al. 1990), and because of its antitumor and antiangiogenic effects, *myo*-inositol (1, 3, 4, 5, 6)-pentakisphosphate was proposed to offer anticancer therapeutic strategies (Maffucci et al. 2005).

17.2.2.1 Phytate/Protein

Phytate is identified to interact with proteins at both alkaline and acidic pH for complex formation as shown in Fig. 17.3 (Cheryan 1980). Phytic acid is anioinic at acidic, basic and neutral pH (Maenz 2001). Such activity may disrupt protein structure which eventually can lower enzymatic activity, proteolytic digestibility and protein solubility. Conversely, the importance of protein-phytate interaction in nutrition is still under study. Depending upon protein source, phytate-protein interactions depressingly influence protein digestibility in vitro (Cheryan 1980). A harmful effect of phytate on the nutritional value of protein, though, was not evidently established yet in monogastric animals (Sebastian et al. 1998). Few research advocates that phytate does not influence protein digestibility; others have reported an enhanced availability in amino acid along with lower levels of phytate. This variance may arise as a result of use of diverse protein sources. Phytase can inhibit digestive enzymes such as lipase, α-amylase or proteinase including pepsin, chymotrypsin and trypsin (Greiner and Konietzny 2006). The inhibitory activity rises with rising number or levels of phosphate residues in *myo*-inositol molecule. This inhibitory action may be

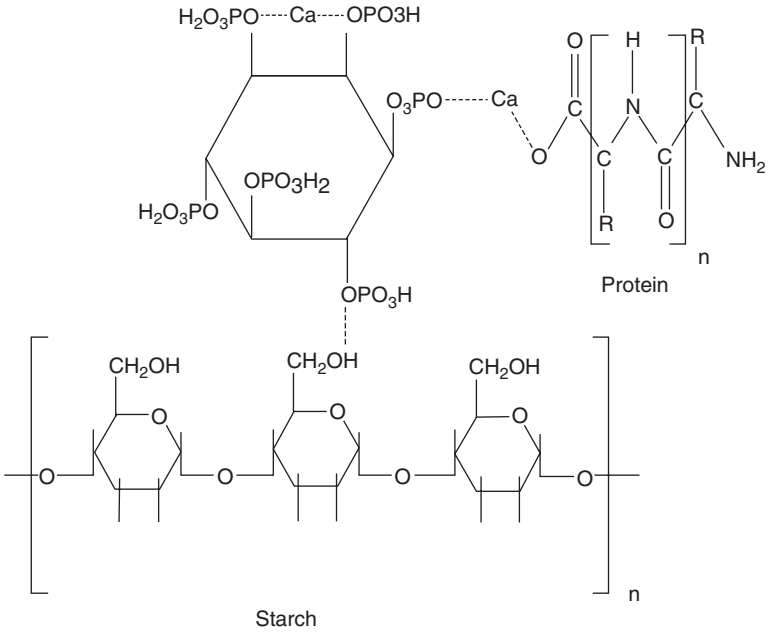

Fig. 17.3 Interaction of phytic acid with metals, proteins and carbohydrate. (Source: Singh et al. 2011)

as a consequence of the non-specific quality of phytate-protein interactions, the chelation of calcium ions which are vital for the activity of trypsin and α-amylase, or due to interaction with substrates of these enzymes. Phytate is known as an inhibitor of α-amylase in vivo in a detrimental relationship between blood glucose response and phytate intake (Jenab and Thompson 2002). Hence, phytate-rich food is assumed to have high nutritional values against diabetes mellitus, which is amongst the universal nutrition-dependent diseases in Western society.

17.2.2.2 Phytate/Mineral

In humans, only isolated form of *myo*-inositol pentakisphosphate can diminish absorption of zinc, iron and calcium, whereas *myo*-inositol tetrakis- and trisphosphates do not affect absorption process of minerals. However, the latter have negative effect of phytate on iron absorption if higher phosphorylated *myo*-inositol phosphates, *myo*-inositol tetrakis- and trisphosphates, exist. Therefore, zinc absorption and the sum of *myo*-inositol tris- through hexakisphosphate from cereal and legume meals exhibit a negative correlation (Sandberg et al. 1999); likewise, it is also true for zinc absorption. Overexpression of phytase at some stage in seed development can lead to lowered phytate quantity in the mature seed (Coello et al. 2001). Upregulated levels of seed phytase may lead to enhancement in mineral absorption by lowering phytate amount in plant-based food at the time of processing and digestion in elementary canal of human. Phytate exerts opposite effect on vitamin absorption; therefore, animals fed with fodder of more phytate content usually show signs of emaciation, off-feed, retarded growth and reproduction failure (Konietzny and Greiner 2003; Lopez et al. 2002; Iqbal et al. 1994). At higher pH than isoelectric point of proteins, it can bind and form protein-mineral-phytate complex that is insoluble, resilient to enzyme hydrolysis and decrease the effectiveness of protein consumption (Kies et al. 2006; Dersjant et al. 2015).

17.3 Phytase

Phytases naturally occurs in plants and microorganisms, particularly in fungi as 3-phytases or 6-phytases. Most of the phytases resides in the family of histidine acid phosphatases. The enzymes hydrolyse phytic acid to phosphoric acid and myo-inositol in a staircase fashion through the formation of myo-inositol phosphate intermediates (Mullaney and Ullah 2003; Yu et al. 2012; Dersjanti et al. 2015). In living systems phosphomonoester hydrolysis is a vital process of metabolic regulation, energy metabolism and signal transduction pathways. Phosphorus is an important element for the growth of all living organisms and also in livestock production. Feed must be augmented with inorganic phosphorus being critical for energy production, forming macromolecular structure and assisting in metabolic regulation; thus, phosphorus in sufficient level is necessary to support the normal growth and development of all living organisms (Carla and Elizabeth 2001).

In case of nutrition, two features of phytic acid are vital: (1) monogastric animals including poultry and pigs cannot consume phytic acid phosphorus, since they have

low phytase activity in their elementary tracts and phytic acid cannot be resorbed. Hence, pigs and poultry feed is augmented either with phytase-enriched feed or inorganic phosphate. (2) Phytic acid is anti-nutritive factor; it develops insoluble complexes with dietary essential metals, viz. zinc, calcium, magnesium and iron, reducing their bioavailability; thus, the enzymatic hydrolysis of phytic acid into less phosphorylated myo-inositol derivatives is desired (Vohra and Satyanarayana 2003). Generally the litter is useful to land as fertilizer. This waste encloses phosphorus that is consumed by diverse plants, though excess phosphorus can surfeit land and come into streams and lakes triggering serious health, environmental and economic problems.

Rigorous animal-based farming led to rise in environmental pollution because of high load of phosphorus excretion by the monogastric animals, due to their inherent inability to digest plant phytate (Vohra et al. 2006; Kuhar et al. 2009). Application of phytase in poultry and pig supplements increases the feed quality, lowers the phytate phosphorus excretion and assists in sustaining the environmental balance of the related areas. Consequently, phytase enzyme can be employed for regulating environmental pollution which arises due to eutrophication and constant chelation of nutrient factors from soil. The research on phytase spans from more than ten decades from its discovery by Suzuki et al. (1907).

All the desirable properties do not exist within single phytase; thus, on the basis of series of the available phytases, an accord phytase could be engineered (Lehmann et al. 2000). Recombinant DNA technology including site-directed mutagenesis could be used for additional remodelling of the features. The approaches employed for the engineering and developing of an ideal phytase are depicted in Fig. 17.4.

17.3.1 Molecular Classification of Phytases

Several alkaline phytases and histidine acid phosphatase (HAP) genes from diverse species were cloned and sequenced. Phylogenetic investigation of the protein sequences of various phytases evidently indicates two major classes, which finely correlate with the classification of phytases related to their electrostatic surface potential and biochemical and catalytic properties (Figs. 17.5 and 17.6; Table 17.1).

17.3.1.1 Histidine Acid Phytases

One of the major classes of phytases (Class I) is the family of HAPs sharing a highly conserved RHGXRXP motif (Van Etten et al. 1991).

The HAP class of phytases can be further subcategorized into three distinct groups (*PhyA*-*PhyC*), on the basis of biochemical properties, such as optimal pH and the position specificity of phytate hydrolysis and amino acid sequence

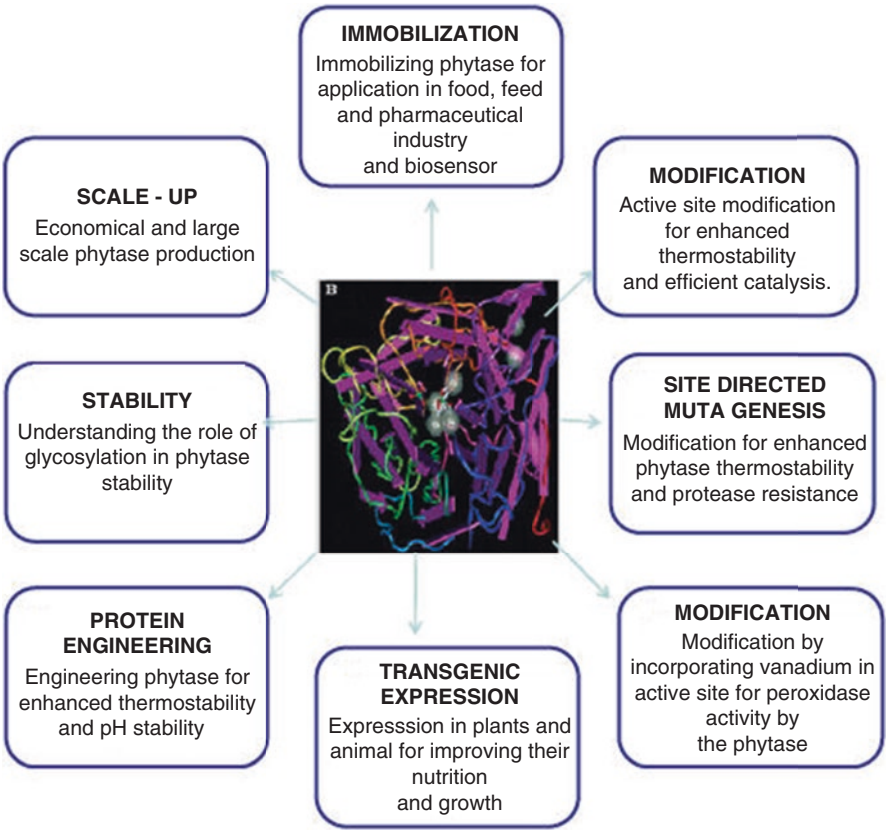

Fig. 17.4 Designing an ideal phytase for biotechnological applications. (Source: Singh et al. 2011)

homology. At acidic pH, HAPs are capable to hydrolyse five phosphate groups from phytate to give rise to myo-inositol monophosphate as the end product. Free from their origin, the acid phytate-degrading enzymes investigated to date in regard with phytate degradation (*A. terreus*, *Emericella nidulans*, *A. niger*, *Myceliophthora thermophila*, *Pseudomonas*, *S. cerevisiae*, *E. coli*, rye, rice, barley P1, barley P2, oat) secrete five amongst the six phosphate groups of phytate, and the end product was known as Ins(2)P (Cosgrove 1970; Wyss et al. 1999; Nakano et al. 2000). This signifies that phytate-degrading enzymes have an intense fondness for equatorial phosphate groups, but they are virtually not capable to slice the axial phosphate group. Rarely, traces of unbound myo-inositol were perceived.

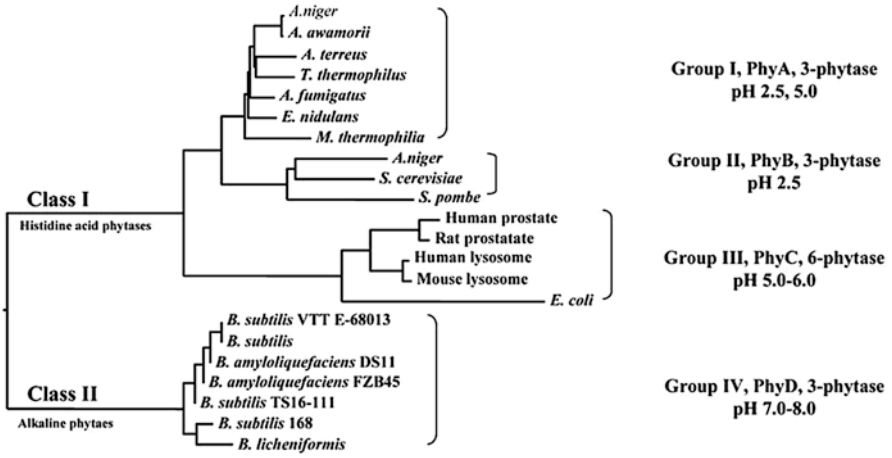

Fig. 17.5 Phylogenetic analyses of various HAPs and alkaline phytases. (Source: Oh et al. 2004)

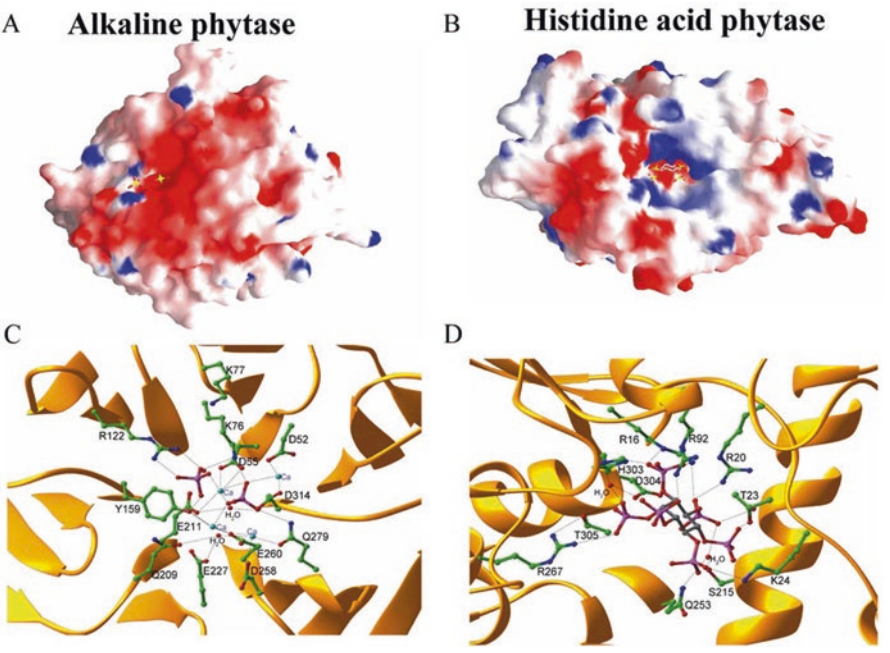

Fig. 17.6 Electrostatic surface potential of alkaline phytase and HAP in the active-site region. (Source: Oh et al. 2004)

Table 17.1 Molecular and biochemical characteristics of HAPs and alkaline phytases

	Histidine acid phosphatases (HAPs)			Alkaline phytase
Characteristics	PhyA	PhyB	PhyC	PhyD
Active site	(+) charged amino acids	(+) charged amino acids	(+) charged amino acids	(−) charged amino acids
Crystal structure	A large α/ß and a small α domain	A large α/ß and a small α domain	A large α/ß and a small α domain	Six-bladed ß propeller
Effect of Ca ions	Inhibition	Inhibition	Inhibition	Stimulation
Effect of EDTA	Stimulation	Stimulation	Stimulation	Inhibition
Final product	IP+5Pi	IP+5Pi	IP+5Pi	IP+3Pi
Glycosylation	Yes	Yes	No	No
Molecular mass (KDa)	62–128	270	42–45	38–45
Nature of phytate	Metal-free phytate	Metal-free phytate	Metal-free phytate	Calcium phytate
Optimum pH	2.5–5.0	2.5–5.0	5.0–6.0	7.0–8.0
Optimum temp.	55–60 °C	55–60 °C	40–60 °C	55–70 °C
Position specificity	D-3 position of phytate	D-3 position of phytate	D-6 position of phytate	D-3 position of phytate
Substrate specificity	Broad	Broad	Broad	Specific
Thermostability	Low (60°)	Low (60°)	Low (60°)	High 85–95 °C

Source: Oh et al. (2004)

17.3.1.2 Alkaline Phytases

Another main class (Class II) includes alkaline phytases that vary from HAPs in several features, such as molecular mass, optimal pH, tertiary structure, calcium ion requirement for enzymatic catalysis and substrate specificity. Based on such biochemical variations and phylogenetic data, alkaline phytases from *Bacillus* sp. and some plant seeds can be categorized as another group: *PhyD* (Oh et al. 2004). These phytases, referred as alkaline phytases, catalyse phytate existing as a metal-phytate complex in plants which have been identified from *Bacillus* sp. (Choi et al. 2001; Gulati et al. 2007a; Kerovuo et al. 1998, 2000) and pollen of certain plants such as *Lilium longiflorum* (Scott and Loewus 1986) and *Typha latifolia* (Hara et al. 1985). Such phytases can likely be employed for remedy of the animal feed before feeding (i.e. during feed mixing, pelleting and storage). In contrast to acid phytases, the alkaline phytate-degrading enzymes from lily pollen (Barrientos et al. 1994), cattail (Hara et al. 1985) and *B. subtilis* (Kerovuo et al. 2000) are incapable of taking a myo-inositol phosphate with three or lesser phosphate residues as a substrate.

Therefore, a myo-inositol trisphosphate isomer is the final product of phytate catalysis by alkaline phytate-degrading enzymes. The end product of phytate catalysis by the lily enzyme was found to be Ins(1,2,3)P_3 (Barrientos et al. 1994), but the *B. subtilis* enzyme was found to produce Ins(1,3,5)P_3 and Ins(2,4,6)P_3 (Kerovuo et al. 2000). Hence, the enzyme from *B. subtilis* is the first phytate-degrading enzyme competent of removing the axial phosphate group at the C-2 position of the myo-inositol ring (Kerovuo et al. 2000).

The surfaces of the substrate-binding sites of *Bacillus amyloliquefaciens* (A) and *Escherichia coli* phytase (B) are coloured according to their local electrostatic potentials ranging from −7 kt/e in red to +7 kt/e in blue, using GRASPP (Honig and Nicholls 1995). Stick models of two phosphates (A, C) and phytate (B, D) are shown in the substrate-binding site.

17.3.2 Structure and Types of Phytases

The basic characters of many phytate-degrading enzymes have been recognized as representatives of formerly identified classes of phosphate (Mullaney and Ullah 2003; Chu et al. 2004; Yao et al. 2012; Dersjant et al. 2015). The International Union of Biochemistry and Molecular Biology (IUBMB) in consultation with the IUPAC-IUB, Joint Commission on Biochemical Nomenclature (JCBN) (1975), on the basis of the position-specific phosphate ester group on the phytate molecule on which hydrolysis is started, listed three types of phytases:

17.3.2.1 3-Phytase (EC 3.1.3.8)

Recommended name, a 3-phytase; systematic name, *myo*-inositol hexakisphosphate-3-phosphohydrolase; hydrolyses the ester bond at the third position of *myo*-inositol hexakisphosphate to d-*myo*-Ins-1,2,4,5,6-pentakisphosphate and orthophosphate (Sajidan et al. 2004).

17.3.2.2 5-Phytase (EC 3.1.3.72)

Recommended name, a 5-phytase; systematic name, *myo*-inositol hexakisphosphate-3-phosphohydrolase; hydrolyses the ester bond at the third position of *myo*-inositol hexakisphosphate to d-*myo*-Ins-1,2,3,4,6-pentakisphosphate and orthophosphate (Chu et al. 2004).

17.3.2.3 6-Phytase (EC 3.1.3.26)

Recommended name, a 6-phytase; systematic name, *myo*-inositol hexakisphosphate-6-phosphohydrolase; hydrolyses the ester bond at the 6th position of *myo*-inositol hexakisphosphate to d-*myo*-Ins-1,2,3,4,5-pentakisphosphate and orthophosphate. Subsequent ester bonds in the substrate were hydrolysed at variable rates (Lassen et al. 2001; Chu et al. 2004).

17.4 Sources of Phytase

17.4.1 Plant Sources

Phytase occurs in seeds of various crops, viz. wheat, rice, maize, soybeans, lettuces, mung beans, faba beans, barley, pea, white mustard, potato, radish, spinach grass, rye and other legumes or oil seeds (Gibson and Ullah 1990). First, Suzuki et al. (1907) have made a preparation of phytase and detected phytase activity in wheat and rice bran. They isolated inositol as a reaction product of phytase. Phytase has been isolated in pure form and characterized from soybean (Gibson and Ullah 1990). Laboure et al. (1993) isolated the phytase in pure form from maize seedlings to characterized it and lateron, cDNA clone was also synthesized for phytase (Maugenest et al. 1997). Roots of several plant species have also been reported for phytases activity (Hayes et al. 2000; Richardson et al. 2000), although the cloning of phytase genes and their characterization in plants are fewer. Primarily, low abundances of phytase genes at transcription and translation levels were reported in the plant species, viz. maize (Maugenest et al. 1997, 1999) and *M. truncatula* (Xiao et al. 2005).

cDNA library was created from the germinated cotyledons of soybean by employing a fragment of *M. truncatula* phytase gene as the probe and used to clone a new phytase gene *Sphy1*. Phytic acids and its derivatives stored in seeds at the time of the seed germination and the early growth of seedlings could be hydrolysed by the activity of *Sphy1*. The activity of phytase rises swiftly during germination which finally decomposed and used in the form of phosphate and inositol; however, seeds confer activities of both constitutive and germination-inducible phytases. Certain feedstuffs hold significant phytase activity (wheat, rye, wheat bran, barley), while others show little or no phytase activity (corn, sorghum, oats and oilseeds) (Eeckhout and de Paepe 1994). Crushed grain diets in both pigs and broilers show a very high correlation with overall phosphorus retention (Barrier-Guillot et al. 1996). The phytase activity was reported to be highly variable (915–1581 U/Kg) in the wheat samples (Eeckhout and de Paepe 1994). Mostly, this deviation can be assumed due to cultivar differences (Barrier-Guillot et al. 1996) and probably due to grain storage time and conditions. It cannot be considered as a consistent source in most commercial swine and poultry operations as a result of this high variability of phytase activity in feedstuffs. Forty-five to 60 °C is the optimal temperature range of plant phytases (Wodzinski and Ullah 1996) which, however, may be partially or totally inactivated due to high steam pelleting temperatures or overheating (Ravindran et al. 1995). Wheat phytase lose significant activity if incubated a proteolytic digestive enzyme, pepsin (Phillippy 1999).

17.4.2 Animal Sources

McCollum and Hart (1908) firstly reported animal phytase in calf liver and blood. It was also detected in the blood of lower vertebrates such as birds, fishes, reptiles and

sea turtle (Rapoport et al. 1941) and in the intestine of pig, cow and sheep (Spitzer and Philips 1972). Phytase was partially purified from rat, calf, chicken and human intestines (Bitar and Reinhold 1972). First phytate hydrolysis was observed by Patwardhan (1937) in the rat intestine. Human intestine shows about 30 times lower phytase activity than rat. Maximum phytase activity was found in the duodenum and minimum in the ileum, but humans have limited capacity to digest undergraded phytases (Iqbal et al. 1994). Microbial flora-based phytase activity was reported in the ruminants. A rat hepatic multiple inositol polyphosphate phosphatase (MIPP) gene showing phytase activity was cloned and expressed by Craxton et al. (1997). The MIPP mRNA was found highly active in the kidney and liver than other tissues. *Paramecium* also contains a phytase-like enzyme activity (Freund et al. 1992).

17.4.3 Microbial Sources

Many bacteria, fungi and yeast show phytase activity; however, it is most frequently detected in fungi. Richardson et al. (2009) reported microbial phytase activity in different types of soil. Microbial phytases are generally cell associated, and they produce only intracellular enzyme, with the exception of *Lactobacillus amylovorus*, *Bacillus subtilis* and *Enterobacter* sp.4. More than 200 fungal isolates of the genera *Aspergillus*, *Mucor*, *Penicillium* and *Rhizopus* have been examined for phytase production. An active extracellular phytase is secreted by all isolates. *Aspergillus niger* was known as the highest active extracellular fungal phytase producer with three different varieties, out of which two are mesoinositol-hexaphosphate phosphohydrolase (E.C. 3.1.3.8.) *PhytA* have two pH optima 2.5 and 5.0 (Shieh et al. 1969; Ullah and Gibson 1987) and a non-specific phosphomonoesterase (E.C. 3.1.3.2.). *PhytB* with a pH optimum of 2.0 (Shieh et al. 1969) to 2.5 (Ullah and Cummins 1987) hydrolyse phytin (Shieh et al. 1969; Ehrlich et al. 1993). A non-specific phosphomonoesterase (Pase) is produced by *A. niger* with a pH optimum of 6.0, which does not hydrolyse phytate (Ullah and Cummins 1988). Neither Pase nor *PhytB* hydrolyses phytate at pH 5.0 (Ullah and Cummins 1987, 1988). *PhytA* is 38% less active at pH 2.5 instead of its Michaelis constant (K_m) at both pH 2.5 and 5.0 is same (Ullah 1988). Both *PhytA* and *PhytB* are repressed by orthophosphate in partially purified form, although the *PhytA* is repressed to a lesser extent (Shieh et al. 1969). A slow-releasing organic phosphate supply such as Hylon starch phosphate can repress *PhytA* production (Gibson 1987).

Amongst yeast, extracellular phytase has been reported in *Schwanniomyces castellii* (Lambrechts et al. 1993), *Arxula adeninivorans* (Sano et al. 1999) and *Pichia anomala* (Vohra and Satyanarayana 2003) and characterized. Phytases from *Schwanniomyces castellii* show the molecular weight of 490,000 Dalton. It is similar to that reported for the phytases from *A. ficuum* being a glycoprotein with an approximate glycosylation rate of 31%. The phytase enzyme bears wide specificity with phytate as its favourite substrate. *Arxula adeninivorans* was found to be one of the rare yeasts competent of synthesizing phytase as a sole source of carbon and phosphate. Nakamura et al. (2000) examined several yeast species for extracellular

phytase activity. *Pichia spartinae* and *Pichia rhodanensis* harboured the greatest levels of phytase activity. An extracellular HAP phytase-producing thermophilic mould which enhances the growth of wheat seedlings was reported by Singh and Satyanarayana (2010).

Microorganisms such as *Klebsiella aerogenes* (Greaves et al. 1967), *Bacillus* sp. (Shimizu 1992; Kim et al. 1998a, b; Kerovuo et al. 2000; Gulati et al. 2007a, b), *Escherichia coli* (Greiner et al. 1993), *Klebsiella ohmeri* BG3 (Li et al. 2009), ruminal bacteria (Yanke et al. 1998), fungal species *A. fumigatus* (Pasamontes et al. 1997) and plants (Houde et al. 1990) were screened for obtaining better phytases. The *E. coli* phytase harbours the greatest specific activity at about eightfold greater than that of the *A. niger* enzyme (Wyss et al. 1999) which is now used as a commercial feed additive. Cost of cell splintering and the separation of cell debris would be too high for industrial enzymes including phytase; therefore, extracellular production is required. Periplasmic proteins are released into the extracellular space through the Kil protein (e.g. *E. coli*). It has been found that the expression level of recombinant proteins can be radically increased by using such system. A significant production of enzyme can be achieved by extracellular system where several heterologous enzymes are naturally expressed at only a very low level (Miksch et al. 2002).

17.5 Production of Phytase

17.5.1 Production Techniques

Phytase production techniques involve submerged fermentation (SmF) and solid-state fermentation (SSF) (Table 17.2). Main factors influencing yield of phytase production taken into consideration for selecting a particular production technique are types of strain, the culture conditions, availability of nutrients and nature of substrate. Phytase production from *Hansenula polymorpha* under oxygen-limited conditions in SmF has been reported by Stockmann et al. (2003).The culture parameters for phytase-producing fungus NSF-7 under SmF conditions and assay conditions using RSM approach for phytase enzyme production under SmF by *Bacillus cereus* MTCC 10072 were optimized (Dahiya et al. 2009, 2010).

The pre-culturing of phytase-producing strains in dextrose medium under limited oxygen supply leads to 25% enhanced production and eradicates 20-h-long lag phase which was practically observed without such limitation. Three different cultivation protocols, namely, submerged fermentations (Ullah and Gibson 1987), solid state (Ebune et al. 1995) and semi-solid (Han and Gallagher 1987), are employed for the production of phytase from *Aspergillus ficuum* NRRL 3135. Culture conditions, especially media composition (wheat bran and full-fat soybean flour), inoculum age and period of SSF affect yield of the phytase production by *A. niger* (Krishna and Nokes 2001). Phytase production by culturing *Rhizopus oligosporus* NRRL 5905, *Mucor racemosus* NRRL1994 and *A. ficuum* NRRL3135 on canola meal, cracked corn, soybean meal and wheat bran in SSF was extensively studied and achieved (Bogar et al. 2003a, b). An enhanced phytase production was

Table 17.2 The biochemical properties of phytases from various organisms

Microbial strain	pH_{opt}	T_{opt}	Fermentation	Carbon source	Nitrogen source	References
Filamentous fungi						
A. fumigatus SRRC 322	5.0	37	SmF	Hylon starch	$NaNO_3$	Mullaney et al. (2000)
Aspergillus niger	5.5	30	SmF	Glucose starch	–	Vats and Banerjee (2005)
Aspergillus ficuum	5.0	30	SmF	Corn starch, glucose	$NaNO_3$	Shieh and Ware (1968)
Aspergillus oryzae	6.4	37	SmF	Glucose	$(NH_4)_2SO_4$	Shimizu (1993)
Rhizopus oligosporus	5.5	27	SmF	Corn starch, glucose	$NaNO_3$	Casey and Walsh (2004)
Rhizopus oryzae	5.5	30	SSF	Glucose	NH_4NO_3	Ramachandaran et al. (2005)
Mucor racemosus	5.5	30	SSF	Starch	$NaNO_3$	Roopesh et al. (2005)
Peniophora lycii	5.5	26	SmF	Maltose, dextrin, soya flour	Peptone	Lassen et al. (2001)
Thermoascus aurantiacus	5.5	45	SmF	Starch, glucose, wheat bran	Peptone	Nampoothiri et al. (2004)
Rhizomucor pusillus	8.0	50	SSF	Wheat bran	Asparagine	Chadha et al. (2004)
Myceliophthora thermophila	5.5	45	SmF	Glucose	$NaNO_3$	Mitchell et al. (1997)
Sporotrichum thermophile	5.0	45	SmF	Starch, glucose	Peptone	Singh and Satyanarayana (2008)
Sporotrichum thermophile	5.0	45	SSF	Sesame oil cake, glucose	$(NH_4)_2SO_4$	Singh and Satyanarayana (2006)
Yeasts						
Pichia anomala	6.0	25	SmF	Glucose	Beef extract	Vohra and Satyanarayana (2001)
Schwanniomyces castellii	4.4	77	SmF	Galactose	$(NH_4)_2SO_4$	Segueilha et al. (1992)
Arxula adeninivorans	5.5	28	SmF	Galactose	Yeast extract	Sano et al. (1999)
Pichia rhodanensis	4.5	70	SmF	Glucose	–	Nakamura et al. (2000)
Pichia spartinae	4.5	75	SmF	Glucose	–	Nakamura et al. (2000)

(continued)

Table 17.2 (continued)

Microbial strain	pH_{opt}	T_{opt}	Fermentation	Carbon source	Nitrogen source	References
Candida krusei	4.6	40	SmF	Glucose	Polypeptone	Quan et al. (2001)
Bacteria						
Bacillus subtilis	7.0	37	SmF	Glucose	NH_4NO_3	Kerovuo et al. (1998)
Bacillus amyloliquefaciens	6.8	37	SmF	Glucose	Casein, peptone	Idriss et al. (2002)
Escherichia coli	7.0	37	SmF	–	Tryptone	Sunita et al. (2000)
Klebsiella aerogenes	7.0	30	SmF	Sodium phytate	Yeast extract	Tambe et al. (1994)
Lactobacillus sanfranciscensis	5.5	37	SmF	Maltose, glucose	Yeast extract	Angelis et al. (2003)
Lactobacillus fructivorans	5.5	37	SmF	Maltose, glucose	Yeast extract	Angelis et al. (2003)
Lactobacillus lactis subsp. *lactis*	5.5	37	SmF	Maltose, glucose	Yeast extract	Angelis et al. (2003)
Lactobacillus rhamnosus	6.5	37	SmF	Glucose	Yeast extract	Raghavendra and Halami (2009)
Lactobacillus amylovorus	6.5	37	SmF	Glucose	Yeast extract	Raghavendra and Halami (2009)
Pediococcus pentosaceus	6.5	37	SmF	Glucose	Yeast extract	Raghavendra and Halami (2009)

Source: Singh et al. (2011)

optimized on wheat bran supplemented with starch and ammonium sulphate by using Plackett-Burman and central composite designs. Canola oil cake kept on specific fermentation conditions (pH 5.3, 30 °C, 54.5% moisture content) in SSF by using *R. oligosporus* can be used for phytase production without additional nutrients (Sabu et al. 2002). Cultivation of *E. coli* in fed-batch fermentation under constant glucose concentration and low oxygen level in the medium show rapid glucose uptake rate at constant low level of oxygen (5–10%) and lead to high extracellular phytase activity (120 U/ml) in shorter cultivation time (14 h) (Kleist et al. 2003). A thermostable, extracellular phytase can be produced in submerged fermentation (SmF) by using *Bacillus* sp. DS11 in the wheat bran and casein hydrolysate medium at 37 °C (Kim et al. 1998a). Moreover, the phytase gene cloned in *B. subtilis* produces phytase on 100 times enhanced rate in modified Luria broth medium (Kim et al. 1999b). Similarly, cultivation of *Aspergillus* sp. 5990 (higher optimum temperature for catalytic activity than the commercial Natuphos from *A. ficuum* NRRL 3135) by employing SmF at 37 °C, pH 7.0, gives fivefold higher activity of phytase in liquid culture (Kim et al. 1999a). Other researchers also found three to five times

higher phytase activity in SSF than SmF by culturing *Aspergillus niger* CFR 335 and *Aspergillus ficuum* SGA 01 (Shivanna and Venkateshwaran 2014). A qualitative correlation was established amongst medium composition, morphology and phytase production by using *A. niger* (Papagianni et al. 2000). Culturing a new bacterial species from the rumen of cattle, namely, *Mitsuokella jalaludinii*, for phytase production in batch fermentation exhibits glucose suppression in a medium consisted of rice bran-soybean milk (2:1). The finest phytase production requires no surfactant at pH 7.0, 39 °C (Lan et al. 2002). High yield and purity of the enzyme in a relatively low-cost system is likely to be a competitive production. Very low quantity of phytase is obtained from wild-type organisms even after tedious and cost-intensive purification which make them unfit for large-scale/industrial production of the enzyme. Consequently, recombinant DNA technology emerges out to be cost-effective and efficient processes for phytase production.

17.5.2 Physical Parameters

17.5.2.1 Temperature

Rising temperature up to a maximal temperature is accompanied with an enhancement in phytase activity. Rise in temperature beyond limit brings about heat-induced denaturation of the enzymes. Optimal temperature for phytate hydrolysis depends upon the source of enzyme and generally varies from 35 to 80 °C (Table 17.2) (Singh and Satyanarayana 2008; Li et al. 2008, 2009; Tran et al. 2010; Dahiya et al. 2010). Usually, plant-based phytases confer highest activity at lower temperatures than their microbial counterparts (Jog et al. 2005). Application of phytase in food industries finds high suitability of microbial phytase as compared to plant counterpart because of the higher pH and thermal stability of the former. Specific activity has an effect on the economy of the anticipated use; therefore, it is one key factor for commercial exploitation of an enzyme. Phytases characterized from lily pollen, soybean, mung bean, maize, and *Penicillium simplicissimum* exhibit specific activity at <10 U/mg (Kim et al. 2003; Lassen et al. 2001; Ullah et al. 2003), whereas *Citrobacter braakii*, *Candida krusei*, and *Peniophora lycii* exhibit specific activity at >1000 U/mg (Tseng et al. 2000; Hegeman and Grabau 2001; Jog et al. 2005) at 37 °C. Additionally, most plant-based phytases are permanently inactivated at higher temperatures than 70 °C within minutes, but most of the microbial counterparts retain considerable activity even after longer incubation period. Similar treatment of these enzymes at 70 °C for 10 min did not bring about loss of activity, and enzyme isolated from *Pichia anomala* can even endure a prolonged treatment at 70 °C with no loss of activity (Vohra and Satyanarayana 2002).

17.5.2.2 pH

Depending upon pH optimization, phytases can be categorized into acid phytases having pH 3.5–6.0 and alkaline phytases having pH 7.0–8.0 as optimum pH range. Most phytases characterized so far reveal highest phytate-degrading activity under the acidic pH range (Table 17.2). *Bacillus* species (Kerovuo et al. 1998; Shimizu 1992;

Kim et al. 1998a, b), the rat intestine (Yang et al. 1991) and lily pollen (Jog et al. 2005) produce alkaline phytases. Most of the phytases characterized so far bear a single precise pH optimum with the only exception the phytase from *Aspergillus fumigatus*, which shows wide pH optimum with minimum 80% of the maximal activity at pH values from pH 4.0 to 7.3 (Wyss et al. 1999). Majority of microbial enzymes are stable at pH over 8.0 and under 3.0; on the other hand, plant counterparts are least stable under such pH conditions. For instance, an *E. coli* phytase remains active at pH 2.0 and pH 10.0 even exposed to 4 °C for 2 h (Greiner et al. 1993). In addition to high-temperature stability and high specific activity, low pH resistance of the phytase is also essential if it is consumed as feed additives because of occurrence of such acidic conditions and high protease concentration of gastric passages in animals. Two new phytase genes from *Yersinia rohdei* and *Y. pestis* have been cloned to express in *Pichia pastoris* (Huang et al. 2008). Both the recombinant phytases show maximum activity at pH 1.5–6.0 (optimum pH 4.5) at 55 °C. A comparative study of commercial phytases advocates the *Y. rohdei* phytase to be more resilient to pepsin, persist higher activity under gastric conditions and produce two- to tenfold higher inorganic phosphorus from soybean meal. These advanced characters advise that the *Y. rohdei* phytase is suitable supplement to animal feed. Certain new phytase genes harbouring great tolerance against low pH and high temperature have been isolated and cloned by several laboratories (Farhat et al. 2008; Rao et al. 2008). The highest activity of *E. coli* phytases under a pH range of 2–5.5 as compared to *A. niger and P. lycii* makes it more desirable for industrial application (Tran et al. 2011).

17.5.3 Nutritional Parameters

17.5.3.1 Carbon Source

Growth and metabolism of microorganisms depends upon readily available form of carbon sources (Gautam et al. 2002). Selection of carbon source relies on the fundamental structure and digestibility of the organism. Even though fungus possesses same number of carbons as that of sucrose and glucose (Vats and Banerjee 2005), mostly, they do not produce phytase because of absence of cellular mechanisms to convert disaccharides such as cellobiose, lactose and maltose into simpler glucose molecules (Takanobu 2002). A disaccharide like trehalose is highly resistant to acid hydrolysis and quiet stable in solutions under acidic conditions and high temperatures (Takanobu 2002). A trisaccharide like raffinose has higher moles of carbon than other sugars; however, it cannot raise enzyme activity due to repression control mechanism of the enzyme (Vohra and Satyanarayana 2003). *Arxula adeninivorans* produce more phytase molecules on availability of galactose than glucose (Sano et al. 1999). *Aspergillus niger* produce high levels of enzyme on starch followed by a mixture of glucose and starch (Vats and Banerjee 2002). Fructose forms large pellets or clumps which do not trigger phytase production and release of extracellular enzyme (Ullah and Dischinger 1993). *Bacillus licheniformis* LH1 produce higher phytase on beef extract than strain LF1 (Roy et al. 2013). Amongst all sugars,

glucose is all-time favourite carbon source for fungi, yeast and bacteria so far (Vohra and Satyanarayana 2001; Ramachandaran et al. 2005; Kuhar et al. 2009) (Table 17.2).

17.5.3.2 Nitrogen Source

Selection of nitrogen source depends on the type of microorganism employed. In *Aerobacter aerogenes* and *Klebsiella aerogenes*, organic forms of nitrogen such as 1% of each peptone and yeast extract give better results for the production of phytase (Jareonkitmongkol et al. 1997). Potassium nitrate and urea as nitrogen source can lower about 50–70% enzyme activity of *A. niger* in both SmF and SSF (Vats and Banerjee 2004). Comparable inhibition results were obtained when various organic and inorganic nitrogen sources were used for phytase production in *Rhizopus oligosporus* and *Aspergillus ficuum* (Pinky et al. 2002). Generally, ammonium nitrate (Ramachandaran et al. 2005; Kerovuo et al. 1998) acts as sole source of nitrogen followed by yeast extract (Angelis et al. 2003; Raghavendra and Halami 2009). Ammonium sulphate is also efficient nitrogen source for phytase production in *Bacillus licheniformis* LF1 than in LH1 (Roy et al. 2013).

17.5.3.3 Phosphorus Source

In addition to regulatory function, phosphorus (P) is amongst the main constituents in biosynthesis of nucleic acids and cell membranes as well as energy metabolism (Singh and Satyanarayana 2011). Phosphate plays pivotal function in phytase production, and its dose in medium substantially regulates enzyme production rate (Gargova and Sariyska 2003). Strain-dependent phytase repression in the presence of excess orthophosphate can be reversed by supplying a slow-releasing organic phosphate source (Gibson 1987). Inorganic phosphorus in a complex medium usually exerts insignificant suppression rate of phytase synthesis (Fredrikson et al. 2002). A similar phenomenon of phosphate repression on phytase synthesis was also reported in many yeast strains (Nakamura et al. 2000).

Apt dose of inorganic phosphates significantly influences the process of phytase production. High phosphate conditions are repressing, although restrictive phosphate conditions result in increased phytase expression. A comparative study of phytase-producing microorganisms reported that under very low range of inorganic phosphate of about 0.0001–0.005%, A. *ficuum* produced highest amount of phytase (Wodzinski and Ullah 1996). Gibson (1987) also verified the impact of phosphorus dose in the medium and studied the production from many sources and speculated variable cleavage behaviour of phosphoester linkage in some starch sources leads to slow but steady supply of phosphorus. A sudden decline in phytase synthesis by *A. niger* even at 0.05% phosphorus in the medium with complete inhibition of production at 0.1% and above advocates the end product inhibition concept in phytase synthesis (Vats and Banerjee 2002). In phosphorus-exhausted medium, the strain produced 184 nkat/ml phytase with a specific activity of 21,367 nkat/mg protein. Similarly, *A. ficuum* on 10 mg *P*/100 g semi-solid substrate supplemented with soybean meal in the growth medium lead to higher phytase activity of 82.5 U/g substrate instead of 8.0 U/g substrate in control without phosphate (Han and Gallagher 1987). UV irradiation of *A. niger* NRRL 3135 repressed *phyA* production in the

mutant up to 60% under the *Pi* dose of 0.006% (w/v), but phytase production in wild-type strain was not affected considerably by higher concentration of phosphorus (0.006–0.015%, w/v) (Chelius and Wodzinski 1994). 20 mg/dm^3 phosphorus supports maximum secretion of phytase from *Aspergillus* sp. 307 at dual pH optima (pH 5.0 and 2.5), beyond which a regression in production was observed (Gargova et al. 1997). The extracellular phytase production by using *Aspergillus* sp. 5990 was highest at lower phosphate concentration (50 mg/l), whereas by increasing concentration up to 100 mg/l, enzyme production was highly repressed (Kim et al. 1999a). On the other hand, phosphate supplementation in the range from 0.05% to 0.5% to the rice bran-soybean milk medium did not influence phytase production by *M. jalaludinii* (Lan et al. 2002). The repression of phytase synthesis by inorganic phosphorus was insignificant in a medium of increased composition complexity (Fredrikson et al. 2002).

17.5.3.4 Effect of Surfactants on Production

The inclusion of surfactants triggers the development of minor pellets in the broath which lead to enhanced yield of phytase (Boling et al. 2000; Mallin 2000). These are surface active agents who lower surface and interfacial tensions and have wetting and penetration actions, detergency, emulsification, gelling, flocculating actions and microbial growth enhancement, etc. (Elliott et al. 1986). Surfactants enticed researchers to be employed for pellet formation in broath cultures. Pelletization process enhances the extracellular enzyme secretion in submerged fermentation (Kerovuo et al. 1998). Tween-20 is amongst the effective surfactant that causes flocculation and formation of finer pellets for improved yield of the enzyme in submerged cultures. Inclusion of sodium oleate or Tween-80 during the culture of *A. carbonarius* increases phytase production and reduces phytic acid content in canola meal during SSF (Maenz and Classen 1998). Tween-20 triggered phytase synthesis by culturing *A. niger* CFR 335 as a result of increase in cell permeability in SmF. Ethylenediaminetetraacetic acid (EDTA), also known by several other names, which is a chemical used as chelating agent was found to readily inactivate *Bacillus* phytases (Kim et al. 1998a, b), although it promptly stimulates *A. fumigatus* phytase activity (Kim et al. 1999a). EDTA, sometimes, binds to form strong complexes with Mn^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, etc., inhibiting the fungal growth and lowering phytase production (Van Etten et al. 1991). Likewise, *A. niger* CFR 335 phytase was repressed by EDTA because of its antifungal activity. Similar antifungal activities of EDTA against *Candida albicans* and in human salivary mucin have already been reported (Kleist et al. 2003). Increased yields of phytase in *A. niger* NCIM 563 with 0.5% Triton X-100 and in *A. niger* NRRL 3135 with 0.5% Triton X-100, sodium oleate and Tween-80 have been shown (Mullaney and Ullah 2003; Wyss et al. 1998).

17.6 Regulation of Phytase Synthesis

17.6.1 Microbial Phytase Regulation

So far, *E. coli* (Greiner et al. 1993) and *Raoultella terrigena* (Zamudio et al. 2002) have been extensively studied amongst bacteria for phytase production. In bacterial system, phytase is an inducible enzyme whose expression is via complex regulation; however, phytase production is not regulated consistently in different bacteria (Liu et al. 1998). There is a robust control inhibition by orthophosphate levels in the culture medium for production of extracellular fungal phytate-degrading enzymes.

17.6.1.1 Factors Affecting Regulation

Media

The production of both the *E. coli* (Greiner et al. 1993) and *Raoultella terrigena* (Zamudio et al. 2002) phytase was switched off in exponentially growing bacteria and turned on as soon as the cultures come into the stationary phase in non-limiting media. Since the formation of the enzymes began with the decreasing growth rate, it was recommended that either energy or a nutrient limitation, known to arise in the stationary phase, could be at the beginning of its induction. Only carbon starvation amongst the nutrient limitation tested was able to trigger an instant synthesis of the *Raoultella terrigena* phytase (Greiner et al. 1997), although in *E. coli*, phytase synthesis was provoked upon starvation of bacteria for inorganic phosphate, while carbon, nitrogen, and sulphur were ineffective factors (Touati and Danchin 1987). The main response to the control of a specific nutrient in *E. coli* was instigation of a certain set of genes which facilitates an improved uptake of the scarce nutrient or the utilization of homologous nutrients belonging to same class. These nutrient-specific systems involve the *NtrB/NtrC/*σ^{54} regulon that is triggered under nitrogen limitation, the cyclic AMP (cAMP) and its receptor the catabolite activator protein (CAP) for the use of substitute carbon sources, and the *PhoB/PhoR* regulon that is triggered under phosphorus limitation (Hengge-Aronis 1996). If the medium is completely exhausted for an essential nutrient, the cells transfer into the stationary phase. The synthesis of many proteins is provoked during transition into stationary phase, and a core set of proteins is triggered irrespective of class of nutrient under depletion. As stated earlier, phytase synthesis in *E. coli* is stimulated in non-limiting media entering into the stationary phase and under anaerobic environment (Greiner et al. 1993). The phytase-encoding gene *appA* expression solely relies on the *rpoS*-encoded sigma factor σ^S, which is known as key regulator for several stationary phase-responsive genes. Very often σ^S-dependent genes are controlled by many promoters, and only one of them is regulated by σ^S. Therefore, all genes are not known to be σ^S controlled for expression. Starvation for phosphate in minimal medium instead for glucose or ammonia leads to strong activation of *rpoS* expression followed by a rise in phytase activity.

Level of Inorganic Phosphate

A strong regulatory inhibition of phytase synthesis by inorganic phosphate dose usually occurs in all microbial phytase producers including moulds, bacteria and yeast, except rumen bacteria (Greiner et al. 1997; Yanke et al. 1998). The downregulation of phytase formation by inorganic phosphate was insignificant with higher medium composition intricacies. The components in the complex media accounting for the lowered repression rate are not known. The proficient activation or repression of phytase synthesis by phosphate starvation in most bacteria (Greiner et al. 1993; Konietzny and Greiner 2002) doubts its putative function in supplying the cell with phosphate hydrolysed from substances such as phytate. This assumption is advocated by the detection of a phytase in the stalk of *Caulobacter crescentus*, an oligotroph gram-negative alpha purple proteobacterium that thrives in aquatic environments with low nutrient availability (Ireland et al. 2002). Phosphate is the regulatory factor in the climate in which *Caulobacte*r exists, and the proposed role of the stalk is phosphate uptake. Stalks get longer for increasing surface area for phosphate uptake when phosphate is limiting as well as the occurrence of a phytase facilitates the uptake of the organic phosphate by the stalk.

Nature of Carbon Source

Besides this, phytase synthesis relies on the nature of the carbon source supplied for growth. Glucose, a catabolite repressor, has been broadly used to increase phytase production. In *E. coli*, the cAMP-CAP instead of the carbon source itself is clearly regulatory in nature (Liu et al. 1998). Phytase production in both *E. coli* and *Raoultella terrigena* was negatively controlled by cAMP (Touati and Danchin 1987; Zamudio et al. 2002), which is assumed to be a part of amphibolic metabolism of glucose and galactose as well as directly or indirectly in regulating the expression of an essential stationary growth regulator.

Substrate Concentration

In *Mitsuokella jalaludinii* substrate, induction mechanism was found (Lan et al. 2002), although phytate had no influence on the synthesis of phytase (Greiner et al. 1993). Therefore, to optimize culture conditions for the synthesis of phytate-degrading enzymes by employing microorganisms is not a simple job; the control of production may vary from microorganism to microorganism. Hence, inability to find phytate-degrading activity does not essentially entail that the experimental microorganism is not a phytate-degrading enzyme producer by any means, but that the growing conditions are not suitable for enzyme production. Substrate dose beyond 300 μM shows inhibitory action for production of the phytase-like enzyme from *Paramecium* (Freund et al. 1992). The *Rhizopus oligosporus* and *Klebsiella* sp. phytases were repressed by the substrate (Sutardi and Buckle 1988; Shah and Parekh 1990), but only under higher substrate concentrations. Fungal phytase activity was also repressed by substrate dose beyond 1 mM (Ullah 1988). Soybean and maize root phytases were inhibited at 20 mM and 300 μM substrate, respectively

(Sutardi and Buckle 1988; Hubel and Beck 1996). Under higher substrate dose, the charge residing over phosphate groups may interfere the local surroundings of the catalytic domain of the enzyme. This might hinder transformation of the enzyme-substrate complex to enzyme and product (Ullah and Phillippy 1994), although repression due to the formation of sparingly soluble protein-phytate complex cannot be affected.

17.6.2 Phytase Regulation in Plants

Cereals, oilseeds, legumes and nuts of higher plants confer phytate-degrading enzymes, although an insignificant phytase activity is also confined to the root part of the plants. The phytate-degrading activity of grains, pollen and seeds mediates phytate degradation during germination to produce phosphate, myo-inositol and minerals for availability during plant growth and development (Reddy and Pierson 1994). Root phytase has been illustrated as mechanisms of plants to increase use of soil phosphate. Organic phosphate usually represents half of the total soil phosphate in which a major constituent ensues in the form of inositol penta- and hexakisphosphate (Richardson et al. 2001). In low phytase activity in roots and the lack of ability of phytase secretion into the rhizosphere, phytate is scantily utilized by plants (Hayes et al. 2000). Therefore, it is advisable that *Bacillus*- and *Enterobacter* sp.-like soil microorganisms colonizing the plant rhizosphere act as plant growth-promoting rhizobacteria (PGPR) by making phytate phosphate absorbable to the plant because of extracellular phytase activity (Idriss et al. 2002). Enabling the plants to utilize phytate phosphate either via activity of extracellular secreted *Aspergillus* phytase in the plant root or via inclusion of purified phytase as well as soil microorganisms secreting extracellular phytase into the rooting medium shows significance of phosphate availability from soil phytate for plant nutrition under phosphate scarcity (Richardson et al. 2001).

Several isoforms of variable controlled phytases are known in a certain plant. Grains, pollen and seeds enclose both germination-inducible and constitutive phytases (Lin et al. 1987; Greiner et al. 2000). The biochemical mechanism undertaking phytase activity is not appropriately identified; however, certain instances are referred, for example, in pollen, it was advised that phytase enzymes activated during germination may be formed from pre-existing, long-lived messenger RNA (mRNA) (Jackson and Linskens 1982; Lin et al. 1987). Rise in phytate-degrading activity during germination in cereals and legumes is also doubtful. Some report favours de novo synthesis (Bianchetti and Sartirana 1967), while others merely propose activation of pre-existing enzymes as consequence of the rise in phytate-degrading activity (Eastwood and Laidman 1971). Phytate regulation process is linked to the presence of gibberellic acid which is assumed to enhance the secretion of phytases to give it access to phytate but does not induce their synthesis (Gabard and Jones 1986); however, few reports suggest that gibberellic acid can induce phytase activity (Srivastava 1964). Two main mechanisms are likely to occur in the

control of phytase activity by phosphate. Acid phytate-degrading enzymes are robustly repressed by phosphate; consequently the enzyme activity itself may be regulated by phosphate. Phosphate added in due course in the germination sequence can suppress the rise in phytate-degrading activity at the transcription level (Sartirana and Bianchetti 1967).

17.6.3 Phytase Regulation in Animals

Animal phytases are poorly studied than the microbial phytases. Though dietary phytate has hostile nutritional effect for animals including man, the occurrence of phytase activity in the gastrointestinal tract of diverse animals was studied. Instead, phytase activity of the mammalian intestine has been endorsed to the action of imprecise intestinal alkaline phosphatases (Davies and Motzok 1972; Davies and Flett 1978); moreover, intestinal alkaline phosphatase and intestinal phytase enzymes are distinctive proteins (Bitar and Reinhold 1972).

17.7 Purification of Phytase

General biochemical techniques such as acetone precipitation, ammonium sulphate fractionation, gel filtration, affinity chromatography, ion-exchange chromatography and hydrophobic interaction are employed for purification of phytase enzymes. Main difficulty during purification of phytases from plant source is the problem in separation of phytases from tainting non-specific acid phosphatases (Konietzny et al. 1995). Besides this, purification of the enzymes from plants requires prolonged germination period. The low stability rate as that of microbial enzymes further makes purification of phytases from plant sources more complicated. Microorganism-based extracellular phytases have been easily achieved from the culture filtrate in rather high amount. A three-step approach has been proposed to purify the phytase from *A. niger* NRRL 3135, including ion-exchange chromatography and chromatofocussing. The phytase was purified about 22-fold with a retrieval of 58% (Ullah and Gibson 1987). Two discrete new extracellular phytases from *A. niger* were first purified by employing phenyl-Sepharose column chromatography, Rotavapor concentration, and Sephacryl S-200 gel filtration (Soni and Khire 2007). *Cladosporium* sp. (Gulati et al. 2007) and *Thermomyces lanuginosus* that yield high amount of phytase were separated from air, and phytase was concentrated to electrophoretic homogeneity by gel filtration and ion-exchange chromatography (Quan et al. 2004). *Rhizopus oligosporus* (DSMZ1964), which is commonly used in tempeh formation, was cultured in rice flour suspension, and intracellular phytases RO1 and RO2 were extracted and purified in a five-step method to give 1.3% (RO1) and 1.6% (RO2) in relation to phytase activity in crude extract. *E. coli*-based two periplasmic phytases have been purified over 16,000-folds with a reclamation of 7% and 18%, respectively, employing a five-step purification method, including ammonium sulphate fractionation, hydrophobic interaction

and ion-exchange chromatography (Greiner et al. 1993). The purification of a phytase from faba beans (Greiner 2001) and oat (Greiner and Larsson Alminger 1999) was attained by employing a seven-step purification process, including acetone precipitation and ammonium sulphate fractionation. The enzyme from faba beans was purified 2190-fold with a recovery of 6% and that of oat 5380-fold with a recovery of 23% and from *A. niger* was purified 51-fold with recovery of 20.3%; *R. oryzae* results in 20.7-fold purification and specific activity of 141.83 U/mg of protein with 26% phytase recovery. The purification of the intestinal phytase of rat can be achieved by a six-step method, involving ethanol precipitation, butanol extraction, ion-exchange chromatography and gel filtration to give 1136-fold purification with a recovery of 19% (Yang et al. 1991). Certain microbial phytate-degrading enzymes have been available by cloning and heterologously expressing the corresponding genes instead of extraction and purification of the enzymes from the wild-type organisms. The recombinant phytases from *Emericella nidulans* (Wyss et al. 1999), *A. fumigatus* (Wyss et al. 1998, 1999), *A. terreus* (Wyss et al. 1999) as well as from the thermophilic fungi *Thermomyces lanuginosus* (Berka et al. 1998) have been characterized biochemically. Measuring the orthophosphate released by the enzymatic action employing a method based upon colorimetric measurements of phosphomolybdate can be used to determine phytase activity (Heinonen and Lahti 1981). The phytate preparation used for revealing phytase activity must devoid of lower myo-inositol phosphates or other phosphorylated compounds. The release of orthophosphate has to be restricted in enzymatic hydrolysis of phytate being a stepwise process in which each lower myo-inositol phosphate may become a substrate for further hydrolysis. The release of orthophosphate is restricted to a maximum 4% of the entirely available phytate phosphate in the assay (Konietzny et al. 1995) by excluding dephosphorylation of lower myo-inositol phosphates in the assay mixtures by the phytase enzyme under study. The separation and quantitative determination of phytate and lower myo-inositol phosphates can be done by reverse-phase C18 high-performance liquid chromatography (HPLC) (Skoglund et al. 1997). This approach is able to detect precisely the decline in phytate during enzymatic hydrolysis, but in regard with revealing phytase activity, the process is too long and considerable extent of lower myo-inositol phosphates in the assay mixtures has been produced until the decline in phytate could be quantified with sufficient precision.

17.8 Biochemical and Molecular Characterization of Enzyme

17.8.1 Effect of Temperature

The activity of purified phytases rises with the temperature increase as evident for most of the cases and was found maximum at its optimum temperature which further declines sharply at temperatures beyond its optimum value. The optimum temperature of phytase activity from *Arxula adeninivorans* is 75 °C at pH range between 4.5 and 5.0 (Sano et al. 1999), while several yeast strains (*Pichia*, *Torulaspora*, *Candida*, *Schwanniomyces* sp., *Kluyveromyces*) and fungus *Thermomyces lanuginosus* are having optimum temperatures in the range of 60–80 °C (Nakamura et al.

Table 17.3 Physico-chemical and kinetic properties of some of the purified phytases

Sources	Mol. Wt. KDa	Isoelectric point pI	Optimum		Substrate specificity	*Km* (mM)	References
			pH	Temp. (°)			
Bacteria							
Bacillus sp. DS11	44	5.3	7.0	70	Specific	0.55	Kim et al. (1998a)
Bacillus amyloliquefaciens							Kim et al. (1999b)
Bacillus subtilis	36–38	6.25	6.0–6.5	60	Specific	0.50	Kerovuo et al. (1998, 2000)
Bacillus licheniformis(168 *phy*A, *phyL*)	44, 47	5.0, 5.1	4.5–6.0	55, 65	Specific		Tye et al. (2002)
Enterobacter sp.			7.0–7.5	50		0.70	Yoon et al. (1996)
Escherichia coli	42	6.3–6.5	4.5	60	Specific	0.13	Greiner et al. (1993)
Klebsiella terrigena	40		5.0	58	Specific		Greiner et al. (1997)
Mitsuokella multiacidus					Specific		Yanke et al. (1998)
Prevotella ruminicola							Yanke et al. (1998) and Cheng et al. (1999)
Pseudomonas syringae	45		5.5	40	Specific	0.38	Cho et al. (2003)
Lactobacillus sanfranciscensis	50		4.0	45			De Angelis et al. (2003)
Citrobacter braakii	47		4.0	50		0.46	Kim et al. (2003)
Yeasts							
Arxula *adeninivorans*			4.5	75	Specific	0.23	Sano et al. (1999)
Pichia pastoris	95		2.5,5.5	60			Han and lei (1999)
Schwanniomyces castellii	490			77			Segueilha et al. (1992)
S. occidentalis					Specific	0.038	Nakamura et al. (2000)
Saccharomyces cerevisiae	120		2.0–2.5	55–60			Han et al. (1999)
Fungi							
Aspergillus ficuum (phy A)	85	4.5	2.5, 5.0	58	Specific	0.027	Ullah and Gibson (1988)
Aspergillus ficuum	68	4.0	2.5	63	Broad	.103	Ehrlich et al. (1993) and Ullah (1987)

(continued)

Table 17.3 (continued)

Sources	Mol. Wt. KDa	Isoelectric point pI	Optimum pH	Optimum Temp. (°)	Substrate specificity	*Km* (mM)	References
Aspergillus oryzae	120	4.15	5.5	50	Broad	.33	Shimizu (1993)
Aspergillus niger SK	57		2.5,5.5	50	Specific	.0187	Nagashima et al. (1999)
Aspergillus Niger ATCC 9142	84		5.0	65	Broad	0.10	Casey and Walsh (2003)
Peniophora lycii (*phyA*)	72	3.61	4–4.5	50–55	Specific		Lassen et al. (2001)

Source: Vats and Banerjee (2004)

2000). *B. laevolacticus*-based phytase production has optimal temperature of 50 °C (Sano et al. 1999). The other bacteria such as *E. coli, Lactobacillus amylovorus*, *M. jalaludinii*, and *Bacillus* sp. yield optimally in mesophilic temperature range of 37–39 °C (Lan et al. 2002; Sreeramulu et al. 1996; Sunitha et al. 1999) optimally at 40 °C. Generally, phytase exhibits high activity in the temperature range of 50–70 °C, whereas optimum temperature is mainly between 45 and 60 °C (Gulati et al. 2007a, b; Singh and Satyanarayana 2008; Dahiya et al. 2010) (Table 17.3). Phytase (PhyC) isolated from *B. subtilis* VTTE-68013 has an optimum temperature at 55 °C (Kerovuo et al. 1998). *Pseudomonas syringae* MOK1-based phytase gives its optimal activity at 40 °C (Cho et al. 2003). *Bacillus* sp. MD2 phytase has optimal temperature for activity between 67 and 73 °C (Tran et al. 2010) and phytase from *B. megaterium*, *B. coagulans*, *B. pumilus* and *B. licheniformis* shows activity at temperature up to 80 °C and also exhibits thermostability and 50% activity at 70 °C (Dechavez et al. 2011). The optimum temperature of enzyme from *Bacillus cereus* isolate MTCC 10072 was 60 °C that is comparable to *A. ficuum* NTG-23 phytase (60 °C) and *A. niger* ATCC 9142 phytase (65 °C) (Casey and Walsh 2003).

Phytase from *A. niger* NW205 (63 °C) (Kostrewa et al. 1999) and *Kodamaea ohmeri* (65 °C) (Li et al. 2008) shows higher optimal temperature than that of *A. niger* van Teighem phytase (52–55 °C) (Vats and Banerjee 2005), *A. niger* NRRL 3135 *phyA* (58 °C) (Kostrewa et al. 1999) and *Bacillus* sp. KHU-10 phytase (40 °C) (Choi et al. 2001).

The *A. fumigatus*-based phytate-degrading enzymes are most resilient to high temperatures as incubation of the enzyme at 90 °C for 20 min resulted in only 10% loss of the initial activity (Pasamontes et al. 1997). It was found that this enzyme is not thermostable, but had a unique property to refold entirely into native-like, fully active conformation by giving heat treatment (Wyss et al. 1998). Practically, the crude extract of phytate-degrading enzymes are highly stable than purified enzyme for a technical application in feed and food processing.

17.8.2 Effect of pH

The histidine acid phosphatases and alkaline phytases are the two broad categories of phytase on the basis of pH of its activity. Acidic phytases are more favourite because of their wide application in animal feeds and broader substrate specificity than those of alkaline phytases (Kaur and Satyanarayana 2010). The potential of a phytase to catalyse phytate in the elementary canal is established by its enzymatic properties. As stomach is the core site of action for the augmented phytase, high tolerance to pepsin and acidic pH is undoubtedly enviable (Selle and Ravindran 2008). The purified phytase of *A. niger* showed significant activity below pH 3.0 in contrast to the *P. lycii* enzyme. Beside this, the purified phytase have high tolerance level to pepsin and acidic pH values than the industrially available *P. lycii* and *A. niger* enzymes. Its pepsin tolerance and resistance of acidic pH values is similar to that of the *E. coli* enzyme which is extraordinarily high under acidic conditions. Moreover, exposure at extremely low pH of 2.0 for longer duration did not cause in a substantial loss of activity (Greiner et al. 1993). A wide pH range of activity has been reported for thermophilic fungi, *Myceliophthora thermophile* and *A. fumigatus* (Wyss et al. 1999). *Rhizopus oryzae* phytases have dual pH optima at 1.5 and 5.5. Phytate purification from *Bacillus subtilis* can be achieved at a pH optimum between 7.0 and 7.5 (Powar and Jagannathan 1982). *B. subtilis* (natto) phytase was prepared to homogeneity and has shown a pH optimum between pH 6.0 and 6.5 (Shimizu 1992). Phytase from *B. subtilis* VTTE-68013 was isolated, purified and characterized at optimum pH 7.0 (Kerovuo et al. 1998). An extracellular phytase from *Bacillus* sp. KHU-10 was isolated, and the optimum pH was determined to be pH 6.5–8.5 at 40 °C without 10 mM $CaCl_2$ whereas pH 6.0–9.5 at 60 °C with 10 mM $CaCl_2$ (Choi et al. 2001). The enzyme was quiet stable from pH 6.5 to 10.0. Gulati et al. (2007a, b) illustrated that the partly purified phytase from *B. laevolacticus* was finest active at 70 °C and pH 7.0–8.0.

pH profile of phytases is also tailored by protein engineering. The pH range for enzyme activity of the *E. coli* phytase (Rodriguez et al. 2000a, b) or the *A. niger* phytase (Mullaney et al. 2002) was widened at acidic pH by mutagenesis. Moreover, phytases with diverse pH optima, ranging from 2.5 (*A. niger PhyB*) to 7.5 (several *Bacillus* sp.), are reported in literature (Oh et al. 2004). Although various potential sites of action such as the crop of poultry with a nearly neutral pH or strong acidic stomach make the focus to an ideal pH profile, the activity and stability of phytases are quite challenging (Lei and Stahl 2001). Successful trials of feeding were carried out with acidic phytase. The phytase having neutral pH optimum exhibits related biological activity. The prospective of these enzymes for industrial applications is yet to be scrutinized for neutral and alkaline phytases.

17.8.3 Catalytic Characterization

Complete degradation of phytic acid by phytases releases free inositol and orthophosphate via several intermediate products, viz. the mono-, di-, tri-, tetra- and

penta-esters of inositol. So, the release of orthophosphate or lower InsPs can determine phytase activity. Typically, inorganic phosphate is estimated by colorimetric technique where phosphomolybdate produced was isolated in organic solvent. Separation and quantitative estimation of phytic acid and lower InsPs is done by reverse-phase C18 HPLC (Burbano et al. 1995). Phytases are discriminated from acid phosphatase that is vulnerable of degrading phytate by its specificity for phytic acid under analysis (Konietzny et al. 1995). Cloning and overexpression of *phy* genes from six new *A. fumigatus* isolates help to examine the innate variability in amino acid sequences and their effect on catalytic activity of phytase (Brugger et al. 2003). Amongst all the proteins, phytase from *Sartorya fumigata* (anamorph of *A. fumigatus*) was highly variable (86% amino acid sequence identity) with more specific activity and discrete variation in catalytic properties.

17.8.4 Effect of Metal Ions

The need for metal ions for enzyme activity depends upon phytases. Metal ions can modify phytate-degrading activity, but it is hard to reveal whether the inhibitory influence of different metal ions is consequence of binding to the enzyme or the appearance of sparingly soluble metal ion-phytate complexes. The formation of a precipitate during addition of Fe^{2+} or Fe^{3+} to the analyte mixtures advises a fall in the active substrate concentration by the appearance of sparingly soluble iron-phytate (Konietzny et al. 1995). Cu^{2+} and Zn^{2+} can highly inhibit most phytate-degrading enzymes. Generally, ethylenediaminetetraacetic acid (EDTA) does not have major effect on the acid phytate-degrading enzymes except the phytases from *A. fumigatus*. The enzyme of *A. fumigatus* was activated up to 50% by EDTA treatment (Wyss et al. 1999), although alkaline phytate-degrading enzymes were highly inhibited by EDTA (Kerovuo et al. 2000) which is evident that a metal ion is required for optimal activity. Ca^{2+}-dependent and EDTA-activated enzymes are produced by *B. amyloliquefaciens* (Kim et al. 1998a), *B. subtilis* (Shimizu 1992; Kerovuo et al. 2000), cattail pollen (Hara et al. 1985) and lily pollen (Scott and Loewus 1986). Reducing agents including reduced glutathione, dithiothreitol and 2-mercaptoethanol do not show key influence on the enzymatic activity of phytase enzymes possibly because of having no any free and accessible sulphydryl group or that free sulphydryl groups play an insignificant role in enzymatic structure and activity. This conclusion is verified by the fact that most mature microbial phytases have an even number of cysteine residues which might be occupied in disulfide bridges as illustrated for the phytases from *E. coli* (Lim et al. 2000). The role of disulphide bonds in the phytate-degrading enzyme from *A. niger* is essential for the structure and activity of the enzyme which contribute in the folding of the protein (Ullah and Mullaney 1996). 2-Mercaptoethanol and dithiothreitol can activate phytases from *Cladosporium* sp. FP-1 which can be inhibited by Pb^{2+}, Ba^{2+}, phenylmethyl sulfonyl fluoride, iodoacetate and P-chloromercuribenzoate (Quan et al. 2004). *Rhizopus oryzae*-derived phytases are resilient to heavy metal ions and proteolysis. Cysteine residues do not appear in the phytases from *B. amyloliquefaciens* (Kim et al. 1998a, b)

and *B. subtilis* (Kerovuo et al. 1998). Fluoride is a robust competitive inhibitor of many acid bacterial, fungal and plant phytates. The inhibition constants range varies from 0.1 to 0.5 mm. However, fluoride cannot inhibit the alkaline phytases from lily pollen (Baldi et al. 1988), *B. subtilis* (Powar and Jagannathan 1982) and *B. amyloliquefaciens* (Kim et al. 1998a, b). Transition metals such as molybdate and vanadate are also recognized as inhibitor of phytases as their oxoanions exert their inhibitory effects by making complexes which mimic the trigonal bipyramidal geometry of the transition state (Zhang et al. 2001). In addition to the hydrolysis product phosphate, the substrate phytate was also known to inhibit various phytases. The modest phytate concentration essential to inhibit phytase activity ranges from 20 mm for the soybean enzyme (Gibson and Ullah 1988) and up to 300 mm for the maize root enzyme (Hubel and Beck 1996). The resident environment of the active site of the enzyme may be affected by the net charge as a result of the phosphate groups present in high substrate concentrations. This might hinder change of the enzyme-substrate complex to enzyme and product along with the formation of sparingly soluble protein-phytate complexes.

17.8.5 Effect of Different Substrates

The efficiency and restrictions of phytase supplementation may also rely on substrate specificity. The phytases with wide substrate specificity voluntarily disintegrate phytate to myo-inositol monophosphate with no main deposition of intermediates, though phytases with restricted substrate specificity lead to myo-inositol tris- and bisphosphate accumulation when phytate degradation linked with a gradual phosphate release rate (Wyss et al. 1999). To date, only the phytases from *Bacillus amyloliquefaciens* (Kim et al. 1998a, b) and *Bacillus subtilis* (Powar and Jagannathan 1982; Shimizu 1992) were known as substrate specific for polyphosphate compounds such as ATP, sodium phytate and sodium tripolyphosphate. The end product of phytate dephosphorylation was recognized as a myo-inositol trisphosphate (Greiner et al. 2002; Kerovuo et al. 2000). The pure phytase though releases five phosphate residues per phytate molecule which insinuate that myo-inositol monophosphate is the end product of enzymatic phytate degradation. Characterization investigation illustrated that the purified enzyme is best suitable for potential commercial interest as an animal feed additive. A wide substrate specificity range is an enviable property in phytase which has earlier been found in *M. thermophila* and *Emericella nidulans* previously (Pasamontes et al. 1997). *Rhizopus oligosporus*-derived phytases show wide affinity for different phosphorylated compounds. Phytases generally exhibit wide substrate specificity. Frequently hydrolysed substrates include guanosine monophosphate (GMP), guanosine trisphosphate (GTP), nicotinamide adenine dinucleotide phosphate (NADP), adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), *p*-nitrophenyl phosphate, 1-naphthyl phosphate, phenyl phosphate, 2-naphthylphosphate, galactose 1-phosphate, fructose 1,6-diphosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, pyridoxal phosphate, α-glycerophosphate,

ß-glycerophosphate, o-phospho-l-serine and pyrophosphate. Merely a small number of phytases have been illustrated as very specific for phytate, viz. the alkaline phytase enzymes from *B. amyloliquefaciens* (Kim et al. 1998a) and *B. subtilis* (Powar and Jagannathan 1982; Shimizu 1992). The acid phytase enzymes from *A. niger*, *A. terreus* (Wyss et al. 1999) and *E. coli* (Greiner et al. 1993) have also been found to be specific for phytate. All phytase enzymes studied so far adhere to Michaelis-Menten kinetics except phytase enzymes from *Myceliophthora thermophila* and *Emericella nidulans* (Pasamontes et al. 1997). Usually, phytases from microbial sources show the highest turnover number, although the related plant enzymes carry out the maximum relative rates of hydrolysis with ATP and pyrophosphate, even though many phytases characterized so far conferred the maximum affinity to phytate among all phosphorylated compounds studied. The kinetic efficiency of an enzyme is confirmed via the k_{cat}/K_m values for a given substrate. The *E. coli* phytases have a highest value of a k_{cat}/K_m value of 1.34×10^7 M $^{-1}$ $s^{-1}min^{-1}$ reported so far (Golovan et al. 2000). The previously accounted turnover number of 6209 s^{-1} and k_{cat}/K_m value of 4.78×10^7 M^{-1} s^{-1} for the phytase enzyme from *E. coli* (Greiner et al. 1993) was hyped due to a low estimation of enzyme concentration by the Bradford assay employed for protein estimation (Golovan et al. 2000). However, the relative rates of hydrolysis of certain phosphorylated compounds such as pyrophosphate and ATP by plant-based phytases are considerably higher than those of phytate; such phytases gave the highest k_{cat}/K_m values with phytate. Hence, the kinetic parameters illustrate phytate to be the probable substrate for the phytase enzyme from plants under certain physiological circumstances. Microbial phytase-based investigations deduced that enzymes with a wide substrate specificity voluntarily degrade phytate to myo-inositol monophosphate without accumulation of intermediates, but enzymes with restricted substrate specificity produce myo-inositol tris- and bisphosphate accumulation during phytate degradation simultaneous with a growing rate of phosphate release, which conclude that lower myo-inositol phosphates are poorer substrates than phytate (Wyss et al. 1999). Moreover, the variation in the substrate specificity of the two classes of phytate-degrading enzymes reveals a selective difference in the specific activities with phytate to a substantial extent, because phytase enzymes with wide substrate specificity innately had lesser specific activities with phytate as substrate, whereas the phytases with restricted substrate specificity had greater specific activities. Phytase from plants, *B. subtilis* and *B. amyloliquefaciens*, do not adhere to this classification. The plant enzymes have wide substrate specificity and greater specific activity; but the *Bacillus* enzymes seem to be highly specific for phytate instead of apparently low specific activity. Phytate degradation by plant-based phytases is accompanied with a growing rate of phosphate release along with an accumulation of myo-inositol tetrakis-, tris- and bisphosphates (Greiner 2001).

17.8.6 Effect of Calcium Ions

Bacillus phytases show metal ion dependency by requiring calcium for their activity and stability (Kim et al. 1998a, b; Powar and Jagannathan 1982; Shimizu 1992; Oh et al. 2001). Ca^{2+} attaches two oxyanions of the phosphate groups in phytate to produce a precise positively charged calcium-phytate complex. Ca^{2+} works as an important activator to diminish the negative charge around the active site cleft due to the occurrence of three Ca^{2+} binding sites at the active site cleft, whereas excessive quantity of Ca^{2+} acts as a competitive inhibitor (Oh et al. 2001). Additionally, Ca^{2+} exert an essential influence on the stability against temperature and pH (Choi et al. 2001). Removal of metal ions from the enzyme by EDTA leads to absolute inactivation in *B. subtilis* phytase (Kerovuo et al. 2000). The conformational change may lead to the loss of enzymatic activity confer, as the circular dichroism spectra of holoenzyme and metal-depleted enzyme were unrelated. Metal-depleted enzyme was partially capable of restoring the active conformation if incubated by application of calcium. Slighter reactivation was noticed with other divalent metal ions and their combinations. Intake of phytase (and phytate) influences digestibility of calcium along with carbohydrates and amino acids.

17.8.7 Thermostability of Enzyme

Recent focus on the isolation, characterization and engineering of enzyme along with the determinants of thermostability is due to the increasing commercial significance of thermostable enzymes. Thermostability is essential for the profitable application of enzymes in animal feed which are exposed to 60–90 °C during pelleting process. Thus designing phytases for enhancing their catalytic properties under diverse conditions is of much interest. The pH activity profile of phytases (*A. fumigatus* and consensus phytases) was engineered by rational mutagenesis for the probable commercial applications of phytase in animal feed (Tomschy et al. 2002). New consensus method can be used for improving the intrinsic thermostability of fungal phytases (Lehmann et al. 2000). Sequence comparison investigations of 13 phytases from six distinct fungi and selection of highly conserved location of each residue led to the de novo simulation of consensus phytase with improved thermostability. Later, the consensus amino acid sequences of six additional phytase sequences were included and studied 38 amino acid replacements by site-directed mutagenesis. The inclusion of stabilizing amino acids in consensus phytase sequence enhanced the unfolding temperature of phytase 1 and 10, thereby sustaining the general validity of consensus approach for improving thermostability by engineering of proteins (Lehmann et al. 2000). Improvement of thermal resistance and enhancement in specific activity are two significant issues not only for animal feed but also for applications of phytases in food processing. Diverse strategies have been employed to prepare an enzyme capable of tolerating higher temperatures. *E. coli* phytases expressing in the yeast *Pichia pastoris* after introduction of three glycosylation sites into the amino acid sequence by site-directed mutagenesis shift temperature

optimum from 55 to 65 °C and show considerable improvement in its thermal stability at 80 and 90 °C (Rodriguez et al. 2000b). Thermostability is mainly a significant factor since feed pelleting is usually carried out at temperatures between 65 and 95 °C, even though the process is carried out by employing after-spray equipment for pelleted diets and/or by passing heat destruction by chemical coating of phytase to tolerate high temperatures will certainly be superior candidates for feed supplements. Contrast to the commercially available phytases from *P. lycii* and *A. niger*, the pure enzyme shows a greater thermal stability. *E. coli*-based commercially available phytase was found to confer a higher thermal stability than the purified enzyme (Garrett et al. 2004). The purified phytase retained half of the initial activity if exposed to 70 °C for 10 min, although the enzymes from *A. niger* lose 60% activity and the phytase from *P. lycii* was completely inactivated at 70 °C after 15 s (Ullah and Sethumadhavan 2003). The *E. coli* enzyme did not lose any activity after heating at 62 °C for 1 h even 27% residual activity was retained at 85 °C after 10 min (Garrett et al. 2004). The pure phytase lose 88% of the initial activity at 80 °C after 10 min. The *Pichia anomala* and *Schwanniomyces castellii* phytases are highly tolerable to high temperatures reported so far (Segueilha et al. 1992; Vohra and Satyanarayana 2002). Exposure of these enzymes at 70 °C for 10 min did not cause any considerable loss of activity, and even the *Pichia anomala* phytase was reported to tolerate a 70 °C temperature after 30 h treatment without any significant loss of activity (Vohra and Satyanarayana 2002). So far, no phytase was used for commercial application. The pelleting of feed facilitates animals to take a balanced diet and helps preservation of enzyme-containing food and feed, though enzyme is exposed to temperatures around 80–85 °C for 2 min during the pelleting process (Wyss et al. 1998).

17.8.8 Proteolysis Resistance

An effectual phytase must harbour a sharp tolerance to hydrolytic breakdown by proteinases occurring in the elementary canal. Fungal and bacterial phytases exhibit various perception to pepsin and trypsin (Kerovuo et al. 1998; Rodriguez et al. 1999). Bacterial phytases appear to bear more tolerance than fungal counterpart (Igbasan et al. 2000). However phytase from *S. thermophile* is unaffected by trypsin and pepsin and also stay insensitive to EDTA Singh and Satyanarayana (2010) Site-directed mutagenesis promise to engineer the protease- susceptible positions of phytases by blocking or modifying them which generally reside in the exposed loops at the surface of the molecules (Wyss et al. 1999).

17.8.9 Immobilization of Phytase

Phytases perform serial action on *myo*-inositol hexakisphosphate to release numerous lower isomers. Therefore, a proficient immobilized bioreactor can generate several isomers of phytic acid also rendering the molecule non-chelator of metal ions,

proteins, etc. *Candida krusei* cells were immobilized in Ca^{2+}-alginate gel beads for the formation of various InsPs where the pure isomers were separated by ion-exchange chromatography (Quan et al. 2002). Even single isomer of each inositol phosphate was produced by NMR analysis except *myo*-inositol pentakisphosphate. Extracellular phytase were produced by *R. oligosporus* and *A. ficuum* in SSF pH 6.0, 30 °C, 58.3% RH by with polystyrene as inert solid support (Gautam et al. 2002). Ullah and Cummins (1987) designed a packed-bed bioreactor for culturing *A. niger* NRRL 3135 phyA by covalently immobilizing on Fractogel TSK HW-75F. Although no swing in pH optima was found, temperature optima swinged from 58 to 65 °C with a rise of K_m for phytate, and there was increased release of phosphorus up to 50%. HPLC analysis of products only detected $InsP_2$ and InsP in the eluate subsequent to repeated hydrolysis of phytate in bioreactor. Immobilization by using protein backbone can drop the phytase activity and bioreactor's output. Immobilization approach can enhance thermostability of phytases (Pandey et al. 2001). Simple alginate immobilization of *Sporotrichum thermophile* can sustainably produce phytase up to five repeated cycles (Singh et al. 2011). It may be because of the distortion of functioning centre of phytase by widespread cross-linking of enzyme to the protein matrix. Preclusion of extensive cross-linking is tough due to heavily glycosylated native phytase. It should be feasible to amputate glycosyl residues by site-directed mutagenesis to give an enzyme that can be immobilized by a few carbohydrate moieties along with retaining a high level of phytase activity (Dischinger and Ullah 1992). The covalently immobilizing of *E. coli* phytase on NHS-activated Sepharose® can enhance heat resistance of the enzyme (Greiner and Konietzny 1996). Liu et al. (1999) shifted the temperature optima of *A. ficuum* phytase from 50 to 58 °C by immobilizing it in gelatin gels and further hardening with formaldehyde. Noticeably K_m has enhanced to 3.28 mM (K_m = 2.34 mM for free enzyme) and the residual enzyme activity was 34.6% only.

17.8.10 Molecular Characteristics

17.8.10.1 Molecular Weight

Mostly phytases characterized so far act as monomeric proteins with molecular weight between 40 and 70 KDa (Table 17.2), while certain phytases seem to contain multiple protein subunits. The phytase from *Schwanniomyces castellii* was known for its tetrameric protein with three identical small subunits of 70 KDa and one large subunit of 125 KDa (Segueilha et al. 1992), and rat intestine phytase was found to be a heterodimer consisting of 70 and 90 KDa subunit where both subunits were encoded differentially during the development of the rat (Yang et al. 1991), these subunits may signify two separate enzymes. The phytases from maize roots (Hubel and Beck 1996), tomato roots (Li et al. 1997), germinating maize seeds (Laboure et al. 1993), *A. oryzae* (Shimizu 1993) and soybean seeds (Hegeman and Grabau 2001) were found to be homodimeric proteins, but a homohexameric structure was assumed for the *A. terreus* enzyme (Yamamoto et al. 1972). In other reports there was no suggestion of an oligomeric structure of the phytases from soybeans (Gibson

and Ullah 1988) and *A. terreus* (Wyss et al. 1999). This contradictory assessment was advised to be the consequence of using gel filtration or native PAGE (polyacrylamide gel electrophoresis) for determination of molecular mass. These techniques can misjudge the molecular mass, and the inaccuracy intensifies with the level of glycosylation (Wyss et al. 1999). Thus, the assumption of the existence of oligomeric phytate-degrading enzymes should be considered with prudence in support of more credible confirmation that oligomeric forms exist. Two different forms of phytases have been found in *K. aerogenes* (Tambe et al. 1994). One is probably the native enzyme having a remarkably large size (700 KDa), while the other, possibly a fraction of the native enzyme, shows an extremely small molecular mass (10–13 KDa) but a full complement of activity. Phytase from *Sporotrichum thermophile* was reported to be homopentameric glycoprotein with molecular mass 456 KDa (Singh et al. 2011). Fungal and several plant phytases have been noticed to be glycosylated. The galactose and N-linked mannose of native enzyme from *A. niger* NRRL 3135 represent 27.3% of its molecular mass (Ullah 1988). The catalytic properties, isoelectric point and the stability of an enzyme are affected by glycosylation. Amazingly, diverse level of glycosylation does not influence the thermostability and refolding properties of the phytases from *A. niger* and *A. fumigatus*.

17.8.10.2 Amino Acid Sequence and Structural Properties

The amino acid sequences of numerous fungal phytases have been established in recent years. The primary sequences of the phytases from *A. terreus* (Mitchell et al. 1997), *A. niger var. awamori* (Piddington et al. 1993), *Talaromyces thermophilus* (Pasamontes et al. 1997), *A. fumigatus* (Pasamontes et al. 1997), *Emericella nidulans*, *Thermomyces lanuginosus* (Berka et al. 1998) and *Myceliophthora thermophila* (Mitchell et al. 1997) exhibited 60, 97, 61, 66, 48 and 47% homology to the related enzyme from *A. niger* NRRL 3135, while the bacterial phytases from *E. coli* did not exhibit clear sequence similarity (Rodriguez et al. 2000a), although all phytases enjoy the highly conserved sequence motif RHGXRXP known for phosphate acceptor site near the N-terminus. Besides this, they carry a conserved HD motif near the C-end where the aspartate is assumed to be the proton donor for the substrate detaching group. The amino acid sequence of the phytases from maize seedling seems to be entirely distinctive from one of the related enzymes from *A. niger*, but a homologous region of 33 amino acids having the RHGXRXP motif was known (Maugenest et al. 1997). All these phytases come under the subfamily of histidine acid phosphatases (Mitchell et al. 1997). The primary sequence of the phytases from *B. subtilis* (Kerovuo et al. 1998) and *B. amyloliquefaciens* (Kim et al. 1998a) does not have homology to the online databases; moreover, the presumed active-site motifs RHGXRXP and HD found in histidine acid phosphatases are missing. Hence, the *Bacillus* phytase enzymes signify a novel class of phosphatases. Later on, it was found that a phytase enzyme from soybeans was also deficient in the RHGXRXP motif (Hegeman and Grabau 2001). Instead, this enzyme includes characteristic motif of a large group of phosphoesterases, involving purple acid phosphatases. Purple acid phosphatases include binuclear Fe (III)-Me (II) centres where Me may be Fe, Mn or Zn. A phytase (*PhyAsr*) coming under the protein tyrosine phosphatase

(PTP) superfamily has been differentiated from the anaerobic, ruminal bacterium *Selenomonas ruminantium* (Aaron 2006). Investigation reveals that *PhyAsr* has a conserved PTP-like active-site signature sequence (C(X)5R) and a PTP-like fold which assists a standard PTP mechanism of dephosphorylation (Chu et al. 2004). Other thermostable phytase gene of 1404 bp size, expressing for presumed phytases of 468 amino acid residues, has been recognized in *Aspergillus japonicas* BCC18313 (TR86) and BCC18081 (TR170) except for a new phytase gene from *Aspergillus niger* (Promdonkoy et al. 2009). A novel phytase gene, *appA*, of 1302 bp encoding 433 amino acid residues with 27 residues of a putative signal peptide has been cloned by degenerate PCR in *P. wasabiae* (Shao et al. 2008). A phytase gene coming under the β-propeller phytase family and approximately 28.5% identical with *Bacillus subtilis* phytase has been known in *Pedobacter nyackensis* MJ11 CGMCC 2503 (Huang et al. 2009). An extracellular phytase (PHY US417) of 41 KDa molecular weight is extracted to pure form and characterized in *Bacillus subtilis* US417 (Farhat et al. 2008). Besides this, other phytase genes, as a promising ideal feed additive for increasing the phytate phosphorus digestibility in monogastric animals, were cloned and characterized in other species, such as in *Escherichia coli* (*appA*) (Hong et al. 2004), ruminal bacterium *Selenomonas ruminantium*(*SrPf6*) and *Peniophora lycii* (Xiong et al. 2006). HP-HAP-like domain and 24 amino acid signal peptide at N-terminal are present in OSPHY2 gene from rice that produces phytase targeted to cytoplasm.

17.8.10.3 Molecular Biology and Gene Expression

The phytases of the fungi *Talaromyces thermophilus* and *Emericella nidulans* were cloned which encoded 463 and 466 amino acids with a molecular weight of 51,450 and 51,785, respectively (Pasamontes et al. 1997). Both putative amino acid sequences were highly identical up to 48–67% to known phytases. OSPHY2, a histidine acid phosphatase from rice (*Oryza sativa L.*), is 2060-bp-long sequence which codes for polypeptide of 519 amino acids (Li et al. 2011). Lehmann et al. (2000) tried to make consensus phytases by using amino acid sequence comparisons due to non-availability of natural phytases having thermostability for application in animal feeding. An accorded enzyme based on 13 fungal phytase sequences had normal catalytic properties, but exhibited an unpredicted 15–22 °C enhancement in unfolding temperature than parental counterparts. For the first time, the crystal structure of consensus phytase was deduced to compare with that of *A. niger* phytase which helps to gain insight into the molecular basis of increased heat resistance. *A. niger* phytase unfolded at very low temperatures. There is a direct link existing between protein sequence conservation and protein stability for fungal phytases. The expression profile of an *A. niger* phytase gene (*phyA*) was studied in *S. cerevisiae* for determining the effects of glycosylation on the activity of phytase and its thermostability (Han et al. 1999). A 1.4 kb DNA segment harbouring the coding region of the *phyA* gene was incorporated into the expression vector *pYES2* to express in *S. cerevisiae* as an active extracellular phytase. The level of total extracellular phytase activity was influenced by the medium composition and the signal peptide. The expressed phytase had a molecular size of about 120 KDa, a

temperature optimum between 55 and 60 °C and two pH optima at 2.0–2.5 and 5.0–5.5. A 9% loss of activity and 40% of thermostability were reported by deglycosylation of the phytase. The gene (*phyA*) isolated from *A. niger* with optima at pH 5.5 and 2.2 was cloned and expressed in *E. coli* under regulation of T_7lac promoter (Phillipy and Mullaney 1997). One hundred sixty-eight *PhyA* phytase genes from *B. subtilis* along with Phy L gene from *B. licheniformis* were cloned to overexpress in *B. subtilis* in ϕ105M0331 prophage vector system (Tye et al. 2002). Han and Lei (1999) investigated the expression profile of a phytase gene (*phyA*) isolated from *A. niger* in *Pichia pastoris*, and Guerrero-Olazarán et al. (2010) expressed *B. subtilis* phytase gene in *Pichia pastoris*. Both host strains yielded high amounts of active phytase (25–65 units/ml of medium) which was mostly released into the medium. Yao et al. (1998) tailored the phytase gene *phyA2* by removing signal peptide encoding sequence and intron sequence. *Yersinia kristensenii* appA gene with 1326-bp-long ORF expressing protein of 441 amino acid includes 24 amino acid signal peptide which was cloned and heterologously expressed in *P. pastoris* to extract a PH-resistant and thermostable phytase (Huang et al. 2008). The *Thermomyces* phytase maintained its activity at 75 °C and exhibited greater catalytic properties than any fungal phytase at 65 °C as optimum temperature (Berka et al. 1998). A large-scale expression of phytase was achieved in *B. subtilis* (Kim et al. 1999b). Kim et al. (1998a) cloned *Bacillus* sp. DS11 phytase gene into *E. coli* which encoded a 2.2 kb fragment.

17.8.10.4 Isoelectric Point (pI)

All phytates from bacteria, plant and fungi have acidic isoelectric point values except *A. fumigatus* enzyme, which has a pI of about 8.6 (Wyss et al. 1999), *B. licheniformis* (168 *phyA*, *phyL*) has a pI of 5.0, 5.1(Tye et al. 2002), *K. terrigena* has a pI of 5.5 (Greiner et al. 1997) and *B. subtilis* has a pI in between 6.3 and 6.5 (Kerovuo et al. 1998). Golovan et al. (2000) purified phytase of 45 kDa size by chromatofocussing from *E. coli* and separated it into two isoforms of identical size with pI of 6.5 and 6.3. The enzyme from *Bacillus* sp. KHU10 was found to have pI value of 6.8 (Choi et al. 2001). The enzyme from other *Bacillus* sp. exhibited an pI value at 6.25 (Shimizu 1992).

17.9 Application of Phytase Enzyme

17.9.1 Animal Feed

Phytases can be included into commercial swine, poultry and fish diets and have broader applications in animal nutrition due to its property to reduce phosphorus excretion of monogastric animals by substituting inorganic phosphates in the animal diet, add considerably for environmental protection, and make increased availability of minerals, amino acids, trace elements and energy (Vats and Banerjee 2004; Haefner et al. 2005). The phosphorus accessibility can be enhanced by using phytase-rich cereal diet or by incorporating microbial phytase to the feed (Nelson

1967). The enzyme diminishes the requirement for augmentation with inorganic phosphorus because of enhancement in the use of organic phosphorus in poultry and hence noticeably dropping the excretion of phosphorus in fertilizer (Kuhar et al. 2009). The inclusion of phytase as a feed supplement is costly approach due to its inactivation by heating required for pelleting. Thermotolerant phytase is desired for animal feed applications (Mullaney et al. 2000). This problem is conquered by producing phytase endogenously in poultry and swine (Selle and Ravindran 2008). This heat resistance feature of phytases led to its cloning from thermophilic fungi such as *T. thermophilus* and *M. thermophila*. Introduction of disulphide bonds and usage of certain compounds like polyols and salts, sorghum liquor wastes (Chen et al. 2001) and calcium (Kim et al. 1998b) can also improve thermostability of enzyme. For commercial applications of phytase as animal feed, it must be optimally active in the natural pH range of digestive tract of animals. Supplementation of fungal phytase in diets for swine and poultry helps in substantial enhancement in phosphorus retention. Inclusion of fungal phytase up to 1000 U/kg in corn/SBM-based diets of pigs and broilers can improve phosphorus retention from 52% to 64% (Kornegay et al. 1999) and from 50% to 60%, respectively (Simons et al. 1990; Kornegay et al. 1996). Augmentation of broiler chickens with industrial phytase has enhanced concentration of NPP, P, albumin and total protein and reduced serum Mg; however, Ca remain unaffected. It can raise activity of aspartate amino transferases and decrease activity of alkaline phosphatase and alanine amino transferase, but did not influence haematological and biochemical parameters of chickens. Industrial phytases are usually tailored by recombinant DNA technology approach. For instance, a bacterial phytase gene has been successfully introduced into yeast genome for its industrial production. Modern genetic engineering has highly improved functional use of phytases by increasing their pH specificity, thermostability and resistance to proteases in the digestive tract of animal (Li et al. 2009).

Efficiency of phytase augmentation, although, relies on the form of the enzyme (coated, size of the particle, etc.), microbial source, pH and temperature optima of the enzyme, diet manufacturing methodology, diet mineral level (Ca, Mg, Fe, Cu and Zn), constituents used in the diet, form of the diet (pelleted, mash or liquid), type and level of vitamin D metabolites, location of addition of phytase (postpelleting or mixer), disease condition of the animal and other factors (Ravindran et al. 1995). Zeng et al. (2001) reported that *Bacillus* phytase augmentation of 300 U/Kg furnishes the same effect as 1000 U/Kg acidic or neutral phytase augmentation.

Microbial phytases encouragingly influenced the pigs' health recital and their daily gain; moreover, the feed conversion ratios were restructured by organic acids (Kim et al. 2005; Pomar et al. 2008; Akinmusire and Adeola 2009; Hill et al. 2009). Dietary modulations to assist the action of exogenous phytases should be taken into account and implemented suitably. *Lactobacillus* and phytase boost up nutrient accessibility by looking after gut microflora and impede digestive diseases which eventually help in pig production and feed (Veum and Ellersieck 2008). Genetically modified *Lactobacillus* for phytase enzyme is advantageous for the gastrointestinal tract and animal health by improving *Lactobacillus* growth and hindering *E. coli* proliferation in pig gut which is related with low diarrhoea rate. Minimal dietary

concentration of calcium and phosphorus and limited Ca/P ratio seem beneficial. The concurrent addition of phytase and xylanase in wheat-based diets can give synergistic gain in digestibility of certain amino acids (Ravindran et al. 1999; Selle et al. 2003). Phytate is accumulated in the aleurone coating of wheat (Ravindran et al. 1995), and xylanase assists contact of phytase to its substrate in the aleurone-like proteolytic enzymes (Parkkonen et al. 1997). The harmonizing use of 3-phytase, 6-phytase and acid phosphatase in broilers may also enhance phytate degradation rates in poultry (Zyla et al. 2004). Amalgamation of phytase enzyme and carbohydrase improves nutrient availability for digestion and eventually feeding value of wheat soybean meal diets having full-fat rapeseed (canola) for poultry. Usually for phytase, the 'nutrient release or equivalency' values are legitimately unaffected from raw material employed. The original phytase feed is produced mostly from fungi. But recent developments in genetic engineering in other forms of microorganisms, such as bacteria and yeast, produced new exogenous phytases. It is evident that such bacterial phytases may be more effectual in broiler chickens. For instance, the *E. coli* phytase released more P in broilers than two recombinant fungal phytases based on improvement in tibia ash compared to inorganic P supplementation (Augspurger et al. 2003). Higher body weight, low mortality, better feed utilization and low phosphorus content in faeces were found in broiler fed with test diets with lowered phosphorus level and phytase. *E. coli* phytase is reported to be more tolerant to pepsin activity than fungal counterpart (Rodriguez et al. 2000a; Igbasan et al. 2000), which may account for the enhanced release of phytate-bound phosphorus. Nonetheless, 'second-generation' phytase feed enzymes with a naturally higher potential to hydrolyse dietary phytate which can lower phosphorus excretion and produce higher amino acid and energy responses will likely be developed in the future. The ideal enzyme would have good thermostability during feed processing, great specific catalytic activity (per unit of protein), increased activity under broad ranges of gut pH, resistance to proteolysis and better stability under ambient temperatures.

17.9.2 Food Industry

A phytate-rich diet significantly decreases absorption of dietary minerals (Konietzny and Greiner 2002), and the dephosphorylation of phytate at some point in food processing leads to the production of only incompletely phosphorylated myo-inositol phosphate esters with a lower potential to impair with the intestinal uptake of dietary minerals (Sandberg et al. 1999). Occurrence of phytate in plant foodstuffs results in mineral scarcity because of chelation of metal ions. Phytic acid present in rapeseed brings about Mg, Zn and Ca deficiency in chickens. Inclusion of phytase to high phytate holding diet increases assimilation and consumption of phosphorus. Many significant physiological functions in human beings are carried out by individual myo-inositol phosphate esters (Shears 1998). Thus, phytases may obtain application in food processing to make functional foods (Greiner et al. 2002), if such biochemically effective myo-inositol phosphate esters is produced by phytases and

absorbed in the digestive tract of humans. Phytases isolated from *A. niger* decrease IP6 content without distressing quality of brown rice bread during preparation. Mineral uptake is improved by phytase augmentation in food (Kuhar et al. 2009). In addition to increased mineral and trace element bioavailability, inclusion of phytase during food processing can influence cost of the manufacture process with yield and quality of the end products. Practical improvements by including phytase during food processing can be achieved for bread making (Haros et al. 2001), corn wet milling (Antrim et al. 1997), manufacturing of plant protein isolates (Fredrikson et al. 2001) and the fractionation of cereal bran (Kvist et al. 2005).

17.9.3 Preparation of Myo-inositol Phosphates for Other Health Benefits

Metabolic impacts of some myo-inositol phosphates are known such as diabetes mellitus, coronary heart disease, atherosclerosis, HIV and heavy metatoxicity (Kuhar et al. 2009; Claxon et al. 1990; Ruf et al. 1991; Siren et al. 1991; Carrington et al. 1993), improvements in heart disease by regulating atherosclerosis and hypercholesterolaemia (Jariwalla et al. 1990), lowered risk of colon cancer (Yang and Shamsuddin 1995) and deterrence of kidney stone formation (Ohkawa et al. 1984). Besides this, much interest has been paid on lower myo-inositol phosphates, in particular the intracellular second messengers d-myo-inositol (1,3,4,5)-tetrakisphosphate and d-myo-inositol (1,4,5)-trisphosphate which influence cellular metabolism and secretion by activating intracellular secretion of calcium (Greiner and Konietzny 2006). Various phytates may produce several positional isomers of the lower myo-inositol phosphates and hence in diverse physiological impacts (Singh et al. 2008). Phytase intake endorses against several cancers through antioxidation properties, cell cycle inhibition and interruption of cellular signal transduction and enhancement of NK cell activity (Kuhar et al. 2009). By knowing health benefits of the phytases, they may also find use in food processing to supplement foods with enhanced nutritional qualities, health benefits and maintained sensory feature (functional foods) (Singh et al. 2008, 2011).

17.9.4 Potential in Aquaculture

In aquaculture, 70% of total fish production expenses are spent on feed (Rumsey 1993). Attempts were made to utilize soybean meal or other plant meals in aquaculture to replace a more expensive protein source such as menhaden fish meal from low-cost plant protein for considerable reduction of cost (Mullaney et al. 2000). The anti-nutritive impact of phytic acid is highly challenging in fish because of their small gastrointestinal tracts (Richardson et al. 1985), which consecutively hamper the application of plant-origin protein in fish feed. Therefore, phytase has been assessed as a method to support the application of inexpensive plant meals in the aquaculture industry and to sustain tolerable phosphorus levels in water. Phytase

from *Klebsiella sp*. and *Bacillus sp*. due to its optimum pH and excellent thermostability is appropriate for universal carp feed. Phytases may offer the extra benefit in freshwater and marine aquaculture because of their probiotic function. Attempts have also been made on appliance of cell-bound phytase of *Pichia anomala* (Vohra and Satyanarayana 2003) in increasing the growth of the freshwater fish rohu (*Labeo rohita*), the marine seabass (*Lates calcarifer*) and the magur (*Clarias batrachus*) by modifying phosphate absorption. Phytase incorporated yeast biomass were supplied as food to the fish which were monitored for growth, phosphorus absorption, and phosphorus excretion. Thus, phytase has been assessed as a method to both enhance the application of inexpensive plant meals in the aquaculture industry and to sustain suitable phosphorus levels in the water.

17.9.5 Pulp and Paper Industry

Yellowing of papers is one of the major difficulties faced by the pulp and paper industries. A thermo-resistant phytase performs as a new biological agent who decomposes phytic acid at some stage in pulp and paper processing (Vohra and Satyanarayana 2003). The enzymatic decay of phytic acid would not consequence in the formation of toxic and mutagenic by-product, and simultaneously ageing of paper can be precluded. Accordingly, the application of phytases in pulp and paper industry could be recyclable and would assist in the development of hygienic technologies (Liu et al. 1998).

17.9.6 As a Soil Amendment

Phytic acid and its products may signify about 50% of the total organic phosphorus in the soil in the several locations (Dalal 1997). Findenegg and Nelemans (1993) investigated the impact of phytase on the accessibility of phosphorus in the soil from phytic acid for maize plants. An improved rate of phytin degradation was found upon inclusion of phytase to the soil which enhanced growth stimulus Singh and Satyanarayana (2010). This report also advocates that the appearance of phytase in the roots of genetically modified plants may enhance the availability of phosphorus to plant roots (Day 1996).

17.9.7 Semi-synthesis of Peroxidase

Peroxidases are universal enzymes that catalyse various selective oxidations with hydrogen peroxide as the primary oxidant (Correia et al. 2008). The active site of vanadium chloroperoxidase from *Curvularia inaequalis* strongly bears a resemblance to that of the acid phosphatases, and the apoenzyme of vanadium chloroperoxidase confers phosphatase-like activity (Hemrika et al. 1997). The permutation of phytase with vanadate formed an efficient semi-synthetic peroxidase. The influence of pH on the vanadate

phytase-mediated oxidation of thioanisole found that the pH optimum corresponded with that of phytase. Optimization resulted into the highest enantiomeric excess (ee) of 68% attained in formate buffer at 4 °C. The vanadium-integrated phytase was durable for more than 3 days with only an insignificant decline in activity. A cross-linked comprehensive enzyme of 3-phytase was converted into peroxidase by integration of vanadate (Correia et al. 2008). The cross-linked comprehensive phytase exhibited comparable efficacy and uneven activation as the free enzyme. Furthermore, the cross-linked comprehensive phytase can be reclaimed as a minimum three times with no considerable loss of activity. Certain other acids, hydrolases and phosphatases were tested for peroxidase activity upon integration with vanadate ion. Phytases from *A. fumigatus*, *A. ficuum* and *A. nidulans*, phospholipase D from cabbage and sulfatase from *Helix pomatia* mediated the enantioselective oxygen transfer processes by integrating vanadium.

17.10 Market Trends and Manufacture

The phytase enzymes have come into view as big feed augment. Feed enzymes (protease, phytase, xylanase, amylase, lipase, cellulase, β-glucanase) are recently increasing in the animal nutrition market with rapid growth which is estimated to attain $14.1 billion by 2019. Currently, approximately 6% of available animal feeds carry enzymes, about 80–90% for vitamins, which is known as greatest animal nutrition class (Anons 1998). Numerous major animal nutrition industries are emerging in this area very dynamically, and diverse products under discrete trade names are already offered to the marketplace. For instance, 'Cenzyme' is a product from Cenzone, which is an exclusive combination of intense digestive enzymes with phytase from a fungal source which is used as animal feed (Cenzone 1999). The biggest fraction of market (~40%) in feed enzymes is covered by Finnfeeds International, a unit of Finland's Cultor, followed by BASF (Simon and Igbasan 2002; Misset 2003). The latter, which has marketing contract with Dutch-based enzyme producer company Gist-Brocades, is the global leader marketer of phytase. Finnfeeds has currently manufactured a phytase. Novo industry is also marketing a phytase in Europe since 1998. Alltech has founded a production facility in Mexico for the manufacturing of phytase in 1999 (Table 17.4).

Table 17.4 Phytase preparations authorized in the EU as feed additives

Company	Trademark	Phytase source	Production strain	References
BASF	Natuphos	*Aspergillus niger var. ficuum*	*Aspergillus niger*	Simon and Igbasan (2002), Misset (2003), and European Union (2004a)
AB enzymes	Finase	*Aspergillus awamori*	*Trichoderma reesei*	Simon and Igbasan (2002), Misset (2003), and European Union (2004b)
Novozymes	Bio-Feed phytase	*Peniophora lycii*	*Aspergillus oryzae*	Simon and Igbasan (2002), Misset (2003), and European Union (2004c, d)

17.11 Recent Status of Research and Development in the Area

17.11.1 World Scenario

Phytase was isolated from the rice bran (Suzuki et al. 1907), and its production was initiated by *Aspergillus* sp. (Dox and Golden 1911). The first substantial attempt to make phytase an industrial product was begun which dates back to 1962 at the International Minerals and Chemical Corp., Skokie, IL. Shieh and Ware (1968) extracted *Aspergillus ficuum* phytase, which gave the highest production of phytase, and deposited it as NRRL 3135. Shieh et al. (1969) developed the strain *A. niger* NRRL 3135 for production of phytase with two pH optima, i.e. 5.5 and 2.5.

17.11.1.1 US Studies

The technology developed at IMC was transmitted to the Agricultural Research Service (ARS), US Department of Agriculture (USDA), and Southern Regional Research Centre (SRRC), and the researchers at SRRC, over a period of 16 years, studied phytases (*phy A* and *phy B*) and acid phosphatases produced by *A. niger* NRRL 3135. *phy A* gene from *A. niger* NRRL 3135 was cloned to *gt11* expression vector by the N- terminal and intersequences of *A. ficuum* phytases. The entire gene was consequently subcloned and deposited at GenBank. Simultaneously, the *phy A* gene from *A. niger* NRRL 3135 due to the expression of its multiple copies produced up to tenfold enhanced phytase activities than the wild-type strain (Van Hartingsveldt 1993). Another gene, *phy B*, was cloned and expressed to encode amino acid sequence which was validated by the chemically inferred protein sequence (Ehrlich et al. 1993). Phytase is marketed as food additive in the United States since January 1996 as Natuphos receiving approval from many countries and the Food and Drug Administration (FDA) as GRAS (generally recognized as safe) for use in food.

17.11.1.2 Dutch Studies

The scientists at Gist-Brocades in 1994 analysed and overexpressed *phy A* from *A. niger* NRRL 3135 under the control of amyloglucosidase promoter which produced 52-fold enhancement of phytase yield. About 1400-fold enhancement of phytase yield in one of the wild-type nonproducers was obtained by cloning the enzyme along with amyloglucosidase promoter and *A. niger* CBS 513.88.

17.11.1.3 Pan Labs Studies

Another phytase gene *phy B* has been cloned and overexpressed for enhance production of phytases by researchers at Aiko Ltd., Finland, jointly with Pan Labs. The genes for phytase from *A. niger* var. *awamori* were cloned. Miettinen-Oinonen et al. (1997) overexpressed pH 2.5 acid phosphatase in the *Trichoderma reesei* expression

system under the control of main cellobiohydrolase I (cbh1) promoter, and the enzyme is now available in the market as Finase-F.

17.11.1.4 Novo Nordisk Studies

Expression of phytase from a basidiomycete, *Peniophora lycii*, in *A. oryzae* IFO 41779 (WO 9828409 and US 606298) has been reported (Lassen et al. 2001). The preliminary investigation proves its potential of releasing phosphate from phytic acid at a high initial rate joined with high specific activity. Now, the enzyme is available in the marketplace as Bio-Feed phytase by Novo Nordisk.

17.11.2 Current Status in India

The manufacturing and extraction of phytase-specific phosphate were first reported in India from *B. subtilis* (Powar and Jagannathan 1982). A thermostable fungus, *A. niger* NICM 564, for the production of phytase has been isolated (Mandviwala and Khire 2000). The enzyme was found optimally active at 50 °C, pH 5.0, and retained 75% activity at 55 °C after 1 h incubation. Phytase yield from *Pichia anomala* was enhanced from dried flower buds that have a high activity (68 U/g dry biomass) intracellular enzyme at 20 °C (Vohra and Satyanarayana 2003). An overproducing strain of *A. niger* was isolated from rotten wood logs (Vats and Banerjee 2004). The Indian industries are currently applying dicalcium phosphate (DCP) in animal feeds, and it was found that phytase augmentation can substitute 50–60% dicalcium phosphate. About 10 kg DCP can be substituted by 250 g phytase enzyme; hence, perceptive that 50–60% of DCP can be substituted by phytase, the prospective requirement for phytase in cattle and poultry feed will be around 400 tonnes per annum.

17.11.3 Issues Related to Phytase

Regardless of all benefits of phytase, there are problems over its use that necessitates more research. Phytate being a strong chelator of iron and zinc, in plant foods, in fact can function as an antioxidant to decrease free radical formation interceded by these metals. As food-producing animals survive for a comparatively brief period and do not usually obtain high levels of feeding phytase, dietary iron or low phytic acid (Veum et al. 2001; Sands et al. 2003) constituents do not expect to cause any health problem, although low phytic acid grain may have a possible harmful impact on human health, especially in those with high iron stores attributable to high dietary intakes of highly accessible iron from animal foodstuffs or high dietary intakes of fruits that highly improve the absorption of non-heme iron (Fleming et al. 2002). Hence, sufficient carefulness should be taken in encouraging that low phytic acid grain approach from the animal production points to a wide application. The other problem is whether the rate of catalysis of augmented phytases for phytate-phosphorus form is higher than the rate of assimilation in animals which eventually

secrete more free phosphorus to the environment than the control without phytase (Dao 2003). Indeed, total soluble phosphorus excretion is significantly lowered in animals fed phytase, but their comparative percentage of soluble phosphorus in the total excreta phosphorus is marginally enhanced (Xavier et al. 2003). Meanwhile, suitable phytase ample may be titrated with phosphorus needs and dietary conditions (Kemme et al. 1997) of animals. For workforce handling phytase, breathing contact may cause immune reactions such as work-related asthmatic and other respiratory symptoms (Doekes et al. 1999). The hypersensitivity symptoms can be evaded by enhancing local exhaust systems and wearing of all shielding clothing and masks with P_2 filter (Baur et al. 2002).

17.11.4 Environmental Impression

In livestock, phase feeding of phytase can decrease environmental pollution without any impact on production. Certain genetically modified plants like low-phytate corn or soybeans have revealed 1.4% rises in P availability. Transgenic animals also enhance livestock effectiveness in applying P. Hence a combination of lowered P, precision feeding, phase feeding and enzyme can led to sustainable development in livestock production method and environmental protection simultaneously (Kebreab et al. 2012). Phytic acid occurring in the manure of monogastric animals is enzymatically sliced by soil- and water-borne microorganisms which may cause in eutrophication because of extreme algal growth (Bali and Satyanarayana 2001).

Genetically modified plants that produce microbial phytase can be employed in spite of inorganic phosphorus (P) fertilizers to maintain sustainability in agriculture Singh and Satyanarayana (2010). Phytase expressions in genetically modified plants neither get influence by occurrence of rhizosphere microorganisms nor it influence microbial community in the vicinity of the plant. Rising demand of animal products in upcoming years, harmonizing animal productivity with nutrient output, will entail an intensive attempt amongst producers, waste management specialists and nutritionists to lower the menace associated with animal wastes.

17.12 Conclusion

Recombinant DNA technology promises to produce well-customized commercial phytases. Modern genetic engineering technology has significantly enhanced functional use of phytases by making them thermotolerant, pH specific and withstand to protease activity in the digestive tract of animals. Biochemically, phytase is proteinaceous in nature, thus sensitive to denaturation due to excessive temperature, such as during pelleting. Spraying liquid phytase onto the frozen pellets can maintain the thermostability of the enzyme. Besides this, heat-stable phytases are existing. Storage under high-temperature and moisture conditions may also degrade the phytase (Selle et al. 2003). Therefore, appropriate storage measures and frequent replacement of stuffs containing phytase must be practised routinely. Phytase

containing stuffs must be stored only in dark, cool and dry areas. The manufacturer's advices must be precisely be followed, particularly when phytase is incorporated in vitamins and trace mineral premixes.

Knowledge and technology related to the phytase enzyme have emerged to a new exciting field due to increasingly global use of enzyme. Evidently, augmented phytases enhance dietary phytate phosphorus consumption by food-producing animals and lower environmental pollution of phosphorus from animal excreta in region of intensive animal production. Capability of phytase towards enhancing human nutrition and health and in growing specific phytic acid or inositol-derived products is given more attention and will be broaden as a new trend of phytase. Biotechnology will sustainably provide efficient means for developing and improving phytase enzymes and their delivery systems.

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