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REVIEWS

Histidine Acid Phytases of Microbial Origin

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Abstract—This review is focused on analysis of the biological diversity of phytase-producing microorganisms capable of degrading phytate to inorganic phosphate. General approaches to microbial phytase classification are discussed, with a particular emphasis on histidine acid phytases (HAPs), which catalyze specific cleaving of *myo*-inositol hexakisphosphate. The effect of glycosylation and various effectors on enzyme thermostability and activity of phytases are described. The data on the biosynthesis of histidine acid phytases, their substrate specificity, and on the mechanism of *myo*-inositol hexakisphosphate hydrolysis are considered. A conclusion is made concerning the biotechnological potential of this group of microbial enzymes.

Keywords: histidine acid phytases, microorganisms, *myo*-inositol hexakisphosphate, biochemical properties, substrate specificity, phytate hydrolysis

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Microorganisms—archaea, bacteria, yeasts, and microscopic fungi—inhabit various ecological niches and have a high hydrolytic potential. They can use various hardly accessible compounds even in highly polluted soils. Phosphorus is one of the essential macronutrients necessary for all organisms. On the Earth surface, phosphorus is present in the form of minerals that are components of natural phosphates: apatites and phosphorites, most of which are being used in the production of phosphate fertilizers. While plants take up phosphorus from soil almost solely as phosphate anions, mainly, as HPO_4^{-2} and $H_2PO_4^-$, these forms are characterized by low availability for land plants and high sorption capacity (Secco et al., 2017; Farias et al., 2018). At the same time, natural replenishment of soil with phosphate compounds is rather negligible and the addition of phosphate fertilizers to soil is therefore highly important for increasing crop yield. The major part of phosphorus in soil is often present in the form of phytates, and their low solubility is the cause of limited uptake of this phosphorous compound by plants (Haefner et al., 2005; Singh and Satyanarayana, 2011). Phytate is a *myo*-inositol hexakisphosphate, a strong chelating polyanionic agent capable of forming insoluble complexes with metal cations, proteins, peptides, and amino acids in soil and feeds, which results in decreased bioavailability of phosphorus and other components of the phytate complex. Phytates are the components of grain feeds, but they are not assimilated by monogastric animals and are considered as an antinutritional factor, due to which agricultural producers suffer considerable damage (Bohn et al., 2008). In addition, the limited ability of such animals to assimilate phytate complexes contributes to enhanced excretion of undigested mineral-phytate salts and environmental pollution, which leads to the growth of toxin-producing microorganisms, algae bloom on the water surfaces of lakes and rivers, and nitrogen oxide formation (Roy et al., 2016; Cangussu et al., 2018).

With respect to the chemical structure, inositol is a cyclic derivative of a hexabasic alcohol, and its conformation varies depending on the positions of hydroxyl groups with axial or equatorial spatial orientation. The interaction between hydroxyl groups and six phosphoric acid residues results in the formation of nine stereo isomers of inositol phosphate, the predominant residues being *myo*-inositol hexakisphosphate (phytate, *myo*-$InsP_6$). Each hydroxyl group of phytate binds to only one phosphate residue, and the phosphate group bound with the second carbon atom of inositol (position d-2) is axially oriented in space (Fig. 1) (Kerovuo et al., 2000; Balaban et al., 2016).

The monoester bonds of organic compounds are hydrolyzed by enzymes of a large class of phosphomonoesterases (phosphatases), and their hydrolysis is associated with metabolic regulation and with the regulatory network of cell signal transduction (Shin et al., 2001). In spite of the great diversity and broad distribution of phosphomonoesterases, many enzymes of this class cannot hydrolyze phosphomonoester bonds in inositol phosphate molecules. The enzymes that can hydrolyze phytate and its complexes are distinguished into a separate class of phytases in the vast phosphohydrolase family.

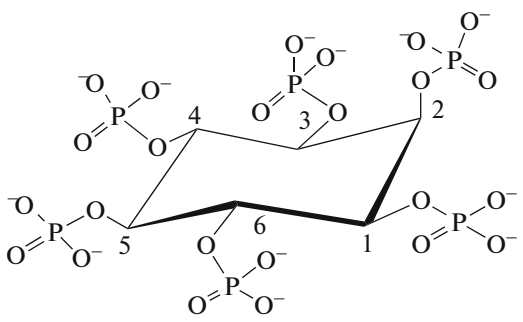

Fig. 1. *myo*-Inositol hexakisphosphate.

Although intracellular phytases with low catalytic activity have been found in plant root cells, they are not secreted into the rhizosphere, and the efficiency of phosphorus production from soil phytate in sufficient quantity is low (Richardson et al., 2001; Singh and Satyanarayana, 2011). Monogastric animals (pigs, birds) do not assimilate phosphorus from plant feeds due to the absence of phytases in the gastrointestinal tract, or their negligible quantities insufficient for phytate utilization (Priyodip and Balaji, 2018). Therefore, exogenous inorganic phosphate is added to the feed as a phosphorus source, which leads to the higher cost of agricultural products (Konietzny and Greiner, 2004; Yao et al., 2011). Microbial phytases are also often added to animals feed, which results in enhanced availability of phosphorus from vegetable feeds and reduced formation of complexes between phytate and minerals (Sommerfeld et al., 2017).

The attention of researchers is focused on microbial phytases playing the key role in the degradation of phytate and its complexes. Many soil bacteria, fungi, and yeasts produce extracellular phytases with high catalytic activity (Vats and Banerjee, 2004). The introduction of such microorganisms into soil contributes to an efficient increase in the bioavailability of soil phytate. Thus, the commercial preparation Natuphos (BASF, Germany) on the basis of the *Aspergillus niger* NRRL 3135 recombinant strain with high phytase activity leads to substantial degradation of soil phytate. Its application improves the quality of nutrient feeds for poultry and domestic animals and promotes environmental protection when using animal husbandry wastes as fertilizers (Lei and Stahl, 2001; Bohn et al., 2008; Rao et al., 2009; Ushasree et al., 2017).

New interesting data on applications of microbial phytases of probiotic microorganisms in fermented foods have been obtained in recent years. It is known that probiotic bacteria are very important for human health. In particular, phosphorus bioavailability can be increased during the fermentation of probiotic-containing foods. It has been shown that the probiotic bacteria *Bacillus subtilis* and *B. coagulans* present in fermented soy beans, as well as *Lactobacillus acidophilus*, *L. fermentum*, and *L. plantarum* present in fermented milk, cheese, and beet, as well as other probiotics can be more effectively used for phytate utilization than phytases of plant origin, which improves the uptake of minerals and phosphorus by animals (Borgi et al., 2015; Priyodip et al., 2017).

Most extracellular phytases studied so far are of fungal origin. Their producers are *A. niger*, *A. fumigatus*, *A. terreus*, *Emericia nidulans*, etc. The yeasts *Saccharomyces cerevisiae*, *Schwanniomyces castellii*, *Debaryomyces castellii* also secrete extracellular phytases. Bacterial phytases are mainly intracellular enzymes, but gram-positive bacteria of the genus *Bacillus* synthesize extracellular phytases. The phytases of gram-negative bacteria *Aerobacter aerogenes*, *Pseudomonas* sp., *Klebsiella* sp. have an intracellular localization, while the phytase of the bacterium *Escherichia coli* is localized in the periplasmic space (Konietzny and Greiner, 2004; Oh et al., 2004; Akhmetova et al., 2012).

Microbial phytase producers are basic components of the microflora of plant rhizosphere and the microbiota of gastrointestinal tract in animals. Such symbiosis with microorganisms has a favorable effect on plants and animals (Akhmetova et al., 2012). The sequencing of a great number of genomes of microorganisms from different phylogenetic groups made it possible to establish the presence of phytase genes in the overwhelming majority of bacterial genomes. The proteins encoded by these genes belong to different classes of phytases that vary in their specificity and mechanism of action and are active at different pH values. Microbial phytases differ from eukaryotic ones by a comparatively high level of catalytic activity and have a considerable practical potential for agriculture.

Previously we have analyzed the structural properties of alkaline bacillary β-propeller phytases, their enzymatic properties, substrate specificity, as well as the mechanism of catalysis of the calcium-substrate complex Ca^{2+}-$InsP_6$ by the alkaline β-propeller phytase of *B. amyloliquefaciens* DS11 (Balaban et al., 2016). Bacillary phytases have a unique spatial structure of a six-bladed β-propeller (Ha et al., 2000). The nucleotide sequences of phytase genes within this class are identical by 90–98%; however, they have no similarity with any of the groups of acid phytases (Mullaney and Ullah, 2007; Tran et al., 2010). Bacillary β-propeller phytases are characterized by strict substrate specificity to the calcium-phytate complex and unique dependence of catalytic activity on calcium ions and, to a lesser extent, other doubly charged cations (Tran et al., 2011; Lei et al., 2013). The catalytic mechanism is based on successive cleavage in the phytate complex of every second phosphate group in the adjacent position to the first phosphate group, i.e., the bidentate chelation of calcium in *myo*-inositol hexakisphosphate molecule is necessary for the catalysis (Oh et al., 2006; Balaban et al., 2016).

In this work we have analyzed the wide range of microbial phytases–fungal, yeast and bacterial, belonging to the class of histidine acid phytases (HAPs). In particular, we discuss the results of analysis of the biochemical and enzymatic properties of the best known and well-studied HAPs and describe their distinctive features compared to another large class: β-propeller phytases. Moreover, we have performed a comparative analysis of the substrate specificity of HAPs and of the pathways of phytate hydrolysis and described the practical potential of biotechnological applications of phytases of this class.

CLASSIFICATION OF MICROBIAL PHYTASES

The classification of microbial phytases is based on determination of the first site of phosphate group cleavage from a substrate molecule during hydrolysis. With respect to this characteristic, phytases are divided into three groups: 3-phytases (*myo*-inositol hexakisphosphate 3-phosphohydrolases, EC 3.1.3.8) initiating phytate dephosphorylation at the third (d-3) or first (d-1) carbon atom of inositol; 6-phytases (*myo*-inositol hexakisphosphate 6-phosphohydrolases, EC 3.1.3.26) dephosphorylating phytate at the sixth (d-6) or fourth (d-4) carbon atom; and 5-phytases (*myo*-inositol hexakisphosphate 5-phosphohydrolases, EC 3.1.3.72) dephosphorylating phytate at the fifth (d-5) carbon atom of the inositol ring (Roy et al., 2016). The group of 3-phytases includes fungal and bacterial phytases produced by *Aspergillus* sp., *Neurospora crassa*, *Pseudomonas* sp., *Klebsiella* sp., etc. 6-Phytases have been found in seeds of higher plants, as well as in such members of the family of enterobacteria, as *E. coli* and *Pantoea agglomerans* and in protozoa (the infusorian *Paramecium*) (Greiner et al., 2000; Greiner, 2007). 5-Phytase was found, in particular, in the pollen of *Lilium longiflorum* L. (Barrientos et al., 1994).

Depending on the pH optimum of activity, phytases are divided into acid and alkaline enzymes. Acid phytases have been found in yeasts and fungi (*Saccharomyces cerevisiae*, *Candida tropicalis*, *Aspergillus* sp.), in microorganisms of the family *Enterobacteriaceae* (*E. coli*, *P. agglomerans*, *P. vagans*, *Shigella* sp.), and in lactic acid bacteria *Lactobacillus sanfrancicensis* (Mukhametzyanova et al., 2012). The alkaline group of phytases is represented by β-propeller phytases (BPPs) isolated from the cells of gram-positive spore-forming bacteria of the genus *Bacillus* (Yao et al., 2011).

Acid phytases are subdivided into several classes of enzymes: histidine acid phytases (HAPs), purple acid phytases (PAPs), cysteine acid phytases (CPs), and tyrosine phosphatases with a phytase activity (PTP-like inositol polyphosphatases), including the recently discovered phytase PhyAme (inositol polyphosphatase, IPPase) of the anaerobic bacterium *Megasphaera elsdenii* (Puhl et al., 2009; Yao et al., 2011). HAPs are the largest group of acid phytases.

The representatives of different classes of microbial phytases have characteristic structural features, different enzymatic properties, substrate specificity and catalytic mechanisms promoting efficient hydrolysis of natural *myo*-inositol hexakisphosphate at different pH and temperature values, resulting in the formation of such final products, as free phosphate, metal cations, free inositol, or less phosphorylated phytate (Kerovuo et al., 2000; Mullaney and Ullah, 2007). All phytases are stereospecific, with a strict preference for equatorially located phosphate groups in the *myo*-inositol hexakisphosphate molecule, and do not hydrolyze axial phosphate groups (Greiner et al., 2000; Lei and Porres, 2003; Bohn et al., 2008).

Histidine Acid Phytases of the HAP Class

The well-studied acid phytases of the class of HAPs include many fungal, yeast, and some bacterial enzymes from *A. niger*, *A. fumigatus*, *A. ficuum*, *Sch. castellii*, *Pichia rhodanensis*, *E. coli*, *K. oxytoca*, *K. terrigina*, *Pseudomonas* sp., etc. The common feature of HAPs is the presence in their active center of the conservative N-terminal motif RHGXRXP, the C-terminal motif HD, and the cysteine motif associated with the formation of disulfide bridges (Nakamura et al., 2000; Mullaney and Ullah, 2005). The pH optimum of activity of histidine acid phytases is within the region of 2.5–6.5; the temperature optimum is mainly determined at 50–60°C, although there are phytases with a higher temperature optimum (*A. terreus*, *Sch. castellii*) (Tables 1 and 2). It was proposed to divide HAPs into three groups of the basis of their molecular and biochemical characteristics: PhyA, PhyB, and PhyC (Oh et al., 2004).

Biochemical Properties of Microbial Phytases of the Class of HAPs

Group PhyA. This group includes the phytases produced by *A. niger*, *A. ficuum*, *A. fumigatus*, *A. terreus*, *Emericella nidulans*, *A. nidulans,* etc. (Vohra and Satyanarayana, 2003). The phytases of this group are monomeric proteins, and some of them have two optima of pH activity. For instance, the *A. niger* NRRL 3135 phytase PhyA has two pH optima (2.0–2.5 and 5.0–5.5) and a high specific activity of phytate hydrolysis (102 U/mg protein) (Table 1) (Mullaney and Ullah, 2003). In contrast to this phytase, the *A. fumigates* ATCC 34625 phytase has two pH optima of 4.0 and 5.0–6.0 and a 4 times lower specific activity (Wyss et al., 1999; Konietzny and Greiner, 2002; Mullaney and Ullah, 2003). The fungal phytases PhyA of *A. terreus* 9A1 and *E. nidulans* (GenBank no. U59803) have one optimum of pH activity in the range of pH 5.0–6.5 (Table 1), and the activity of these phytases drastically decreases at a pH below 3.0 or above

Table 1. Biochemical properties of group PhyA phytases (Wyss et al., 1998, 1999a, 1999b)

Phytase producers	Molecular mass, kDa	pH optimum	Temperature optimum, °C	Thermostability		Specific activity by phytate hydrolysis, U/mg
				preincubation time, min	°C	
PhyB *A. niger* NRRL 3135	48–50	2.0–2.5 and 5.0–6.0	50–58	20	30–50	102.5
A. fumigates 34625	48.3–48.9	4.0 and 5.0–6.5	58–60	20	90	23–28
A. terreus 9A1	48–49	5.0–5.5	70	20	50	142–196
E. nidulans (GenBank no. U59803)	49	6.0–6.5	55	–	60	29–33

Table 2. Biochemical properties of group PhyB phytases (Sequeilha et al., 1992)

Phytase producers	Molecular mass, kDa	pH optimum	Temperature optimum, °C	Thermostability		Specific activity by phytate hydrolysis, U/mg
				preincubation time, min	°C	
PhyB *A. niger* NRRL 3135	50.8	2.0–2.5	55–65	20	30–70	126
Sch. castellii CBS 2863	One subunit 125, three subunits by 70	4.4	77	60	74	522
D. castellii CBS 2923	51–53	4.0–4.5	60	60	60–66	157

7.5 (Mullaney and Ullah, 2003; Oh et al., 2004; Mullaney and Ullah, 2007). The pH optimum of activity for most of the studied yeast phytases (*Candida* sp., *Pichia* sp., *Saccharomyces* sp., etc.) at the optimal temperature of 60°C is in the pH range of 4.0–5.0 but, when the temperature decreases to 37°C, the pH optimum is determined at lower values of 3.0–4.0 (Nakamura et al., 2000).

The pH stability of fungal phytases varies within a broad range of pH values: 2.5–7.5. In the *E. parum* BCC 17694 phytase, pH stability is in the range of pH values of 2.5–7.0; in the *A. fumigates* ATCC 34625 phytase, this range is less (pH 4.0–7.5) (Pasamontes et al., 1997; Fugthong et al., 2010).

The temperature optimum of yeast phytases is above 60°C, while fungal phytases have a temperature optimum mainly within the range of 45–60°C, though the *A. terreus* 9A1 phytase has a temperature optimum of 70°C (Table 1). The phytases of group PhyA have low thermostability (Pasamontes et al., 1997; Han et al., 1999; Nakamura et al., 2000; Rodriquez et al., 2000; Lei and Stahl, 2001). Thus, the *A. niger* phytase PhyA remains active after 20-min preincubation in the temperature range of 30–50°C, but its activity drastically falls at a 10-min increase in preincubation temperature to 60°C and more. The *A. terreus* 9A1 phytase loses 50% of the activity after 20-min heating at 50°C (Table 1) (Pasamontes et al., 1997; Wyss et al., 1998; Han et al., 1999). Against the background of low thermostability of this group of phytases, the thermostability of the *A. fumigates* ATCC 34625 phytase is unique. The enzyme retains 90% of its activity after 20-min heating at 90°C and higher; after 2-h heating at 90°C, the residual activity is 70%. The authors believe that resistance to high temperatures is related to the capacity of the *A. fumigates* ATCC 34625 phytase for correct refolding after thermal denaturation (Pasamontes et al., 1997; Rao et al., 2009). Thermostability can be influenced by the nature of the buffer. Experiments with the *A. fumigatus* recombinant phytase expressed in *P. pastoris* have shown that the protein retains 70% of its initial activity when heated at 90°C for 20 min in Na-acetate buffer, while in Na-citrate buffer its activity falls to 60% (Rodriquez et al., 2000). In contrast to the high thermostability of the *A. fumigates* ATCC 34625 phytase, the phytases from other four strains of *A. fumigates* were not resistant to heating. It is supposed that the differences in thermostability of phytases from different *A. fumigates* strains are associated with posttranslational protein modification (Mullaney et al., 2000).

Table 3. Biochemical properties of group PhyC phytases (Greiner et al., 1993; Kim et al., 2003; Ray et al., 2012)

Phytase producers	Molecular mass, kDa	pH optimum	Temperature optimum, °C	Thermostability		Specific activity by phytate hydrolysis, U/mg
				perincubation time, min	°C	
AppA *E. coli* K-12 ATCC 33965	42	4.5	55	60	30–50	1800
Shigella sp. CD2	45	5.5	60	30	15–80	780
C. braakii YH-15	47	4.0	50	30	25–50	3457

Group PhyB. This group includes the PhyB phytase isolated from the same producer *A. niger* NRRL 3135 as the PhyA phytase, as well as yeast phytases from *Sch. castellii* and *D. castellii* (the authors have denoted the second one as PhyDc). The phytases have one pH optimum in the range of 2.5–4.5 and the temperature optimum of 50–60°C; however, the temperature optimum of the *Sch. castellii* yeast phytase was 77°C (Table 2) (Sequelha et al., 1992; Konietzny and Greiner, 2002; Ragon et al., 2008). Originally, the PhyB phytase from *A. niger* NRRL 3135 was described as an acid phosphatase with the optimum of activity at pH 5.0. Further study revealed the enzyme to show its maximum activity of phytate hydrolysis at pH 2.5. This phytase differs from the *A. niger* NRRL 3135 PhyA phytase not only in the presence of a single pH optimum but also in the structure with a tetramer as an active form. The tetrameric structure of PhyB phytase contributes to the increase in thermostability of the enzyme, although the phytase is incapable of refolding after denaturation (Kostrewa et al., 1999; Mullaney and Ullah, 2003). The tetrameric structure was found in the phytases from *Sch. castellii* CBS 2863 and *D. castellii* CBS 2923 with the high temperature optima: 77 and 60°C, respectively (Table 2). The *Sch. castellii* phytase has a high thermostability: it does not lose activity after 60-min preincubation at 74°C, while the native phytase PhyDc shows a 70% loss of activity under the same conditions (Sequelha et al., 1992; Konietzny and Greiner, 2002; Vohra and Satyanarayana, 2003).

Group PhyC. This group of enzymes includes bacterial histidine acid phytases AppA from *E. coli*, *Shigella* sp. CD2 and *Citrobacter braakii* YH-15, as well as the rat and human acid phytases with crystalline structures similar to the structure of *E. coli* phytase (Lim et al., 2000; Oh et al., 2004). Two microbial phytases (P1 and P2) were originally isolated in 1993. Homogeneous protein preparations were obtained from the gram-negative bacterium *E. coli* K12 (ATCC 33965), and their biochemical properties were studied (Greiner et al., 1993). These are periplasmic monomeric unglycosylated proteins with the pH optimum of 4.5 and the temperature optimum of 55°C. The pH stability of the enzymes at 4°C is determined within a broad pH range of 3.0–9.0 for 14 days. The *E. coli* phytases are not thermostable: the activity drastically dropped after 60-min preincubation at 60°C and disappeared completely at 70°C (Table 3).

The histidine acid phytase isolated from the cells of *Shigella* sp. CD2 and designated by the authors as phytase AppAs is localized in the periplasmic space and its nucleotide sequence is identical by 98% to the sequence of the *E. coli* AppA phytase (Roy et al., 2016). The AppAs phytase, in contrast to the *E. coli* AppA phytase, is thermostable and retains 100% of its activity after 30-min preincubation of the enzyme at 70°C. The AppAs phytase, similarly to the fungal phytase from *A. fumigatus* ATCC 34625, can restore the active conformation of the protein molecule after heat-induced denaturation, with retaining its activity (Roy et al., 2012).

The PhyC group of enzymes also includes the intracellular phytase from *C. braakii* YH-15, with the nucleotide sequence identical by 62% to the sequence of the *Shigella* sp. CD2 phytase. The maximum activity is determined at the optimal pH 4.0 and the temperature of 50°C; the specific activity is almost 2 times higher than that of the *E. coli* AppA phytase. The phytase has a low thermostability: only 40% of the activity is retained after 30-min preincubation at 55°C (Table 3) (Kim et al., 2003). However, the recombinant phytase expressed in *S. cerevisiae* shows a high thermostability: the residual activity is 66% after 30-min preincubation at 70°C (Kim et al., 2006).

Altogether the enzymes of groups PhyA and PhyB are 3-phytases and the enzymes of group PhyC are 6-phytases.

INFLUENCE OF GLYCOSYLATION AND EFFECTORS ON PHYTASE THERMOSTABILITY, MOLECULAR MASS, AND ACTIVITY

Glycosylation promotes correct protein folding and increases the thermostability and molecular mass of the enzymes. Fungal phytases of the class of HAPs are glycosylated proteins with variable molecular masses depending on the degree of glycosylation of N-glycosylated sites of the enzymes by carbohydrate fragments, but glycosylation does not affect the optimal

pH and temperature values (Oh et al., 2004; Mullaney and Ullah, 2007). Thus, the molecular mass of the *A. fumigatus* phytase containing up to seven glycosylation sites is 60–100 kDa, while the molecular mass of the unglycosylated enzyme is 48.27 kDa (Pasamontes et al., 1997; Lei and Stahl, 2001). The molecular mass of the *A. niger* NRRL 3135 unglycosylated phytase PhyA with ten glycosylation sites is 48–50 kDa, but the glycosylated phytase expressed in *S. cerevisiae* has a varying molecular mass (within a broad range of 62–128 kDa) and a high thermostability: 75% of activity is retained after 15-min heating at 80°C (Ha et al., 1999; Oh et al., 2004). The *C. braakii* bacterial phytase expressed in *S. cerevisiae* cells has five glycosylation sites and shows a high thermostability (Kim, 2006). The N-glycosylated phytases from *Yersinia kristensentii* and *Y. rohdei* expressed in *P. pastoris* improved thermostability by 5°C and retained their activity up to 80% at a pH decrease to 1.5 (Niu et al., 2016). Phytase thermostability may be associated not only with glycosylation but also with the buffer systems used for determination of enzyme activities and the degree of purity of the enzyme preparations, and may also depend on the interaction between proteins and the organisms in which protein expression takes place (Rao et al., 2009).

Glycosylation can influence not only thermostability but also other properties of phytases: catalytic properties and pI of the enzyme that can alter the behavior of proteins during purification; glycosylation may also reduce the level of expression of heterologous phytases (Wyss et al., 1999a). It has been shown that deglycosylation by the Endo-H endoglycosidaseof the recombinant phytase PhyA from *E. parum* BCC17604 expressed in *P. pastoris* results in the complete loss of its activity, i.e., glycosylation is crucially important for the maintenance of recombinant enzyme activity (Fugthong et al., 2010).

Effectors. The activity of histidine acid microbial phytases is not significantly influenced by divalent metal cations at low concentrations (0.1–1.0 mM), but the increase in concentration to 2 mM leads to inhibition of the activity of phytases from *A. fumigatus*, *A. terreus* CBS, *E. nidulans*, *Shigella* CD2, and *C. braakii* YH-15 (Sequelha et al., 1992; Greiner et al., 1993; Kim et al., 2003; Rao et al., 2009; Roy et al., 2012). At the same time, Cu(II), Zn(II), and Fe(II) cations inhibit the activity of all groups of histidine acid phytases even at low concentrations (1 mM). The inhibitory effect seems to be accounted for by the formation of insoluble complexes between phytate and metal cations, which leads to lower substrate availability (Konietzny and Greiner, 2002).

Investigation of the effects of chemical reagents on the activities of microbial histidine acid phytases has shown that EDTA does not influence the activity of enzymes such as PhyA from *A. niger*, *Shigella* sp. CD2, *E. coli*, *S. castellii* (Sequelha et al., 1992; Wyss et al., 1999a; Konietzny and Greiner, 2002). *para*-Chloromercuribenzoate (*p*-CMB) had no substantial effect on the activities of phytases from *A. fumigatus*, *A. niger*, *A. terreus* CBS, and *E. nidulans,* which decreased in its presence by no more than 11%. It seems that there are no free SH groups in the structure of these phytases, or that sulfhydryl groups play an insignificant role in manifestation of their activities (Wyss et al., 1999a). It has been shown that *p*-CMB at a concentration of 1 mM inhibits the activity of the *S. castellii* phytase by 95%, suggesting that SH groups are associated with the catalytic center of the enzyme (Sequeilha et al., 1992). The detergent sodium dodecyl sulfate (SDS-Na) completely inhibits the activity of HAPs, probably due to the interaction between the negative charge of the detergent and the positive charge of the phytase active center, resulting in substrate unavailability for the active center of the enzyme (Fugthong et al., 2010).

The phytase of the pathogenic bacterium *Shigella* sp. CD2 and the recombinant phytase of *C. braakii* expressed in the cells of *S. cerevisiae* are stable in the presence of pepsin and trypsin. Papain, elastase, and pancreatin insignificantly inhibit the activity of the *C. braakii* phytase (residual phytase activity during the incubation with these enzymes is 85, 80 and 70%, respectively) (Kim et al., 2003).

Substrate Specificity of Histidine Acid Phytases

Since the phytate complex with metal cations is positively charged, the microbial histidine acid phytases with positively charged groups in the active center region cannot hydrolyze the phosphomonoester bonds of the substrate due to electrostatic repulsion. At a lower pH value, phytases of the class of HAPs hydrolyze only the phytate free from metal cations (Lim et al., 2000; Oh et al., 2004).

It is known that alkaline phytases are characterized by narrow substrate specificity, hydrolyze the calcium-phytate complex at pH 7.0–8.0, but do not cleave phosphomonoester bonds in other phosphorylated substrates (Oh et al., 2004). Phytases of the class of HAPs, in contrast to β-propeller phytases, can hydrolyze not only *myo*-inositol hexakisphosphate but also other phosphate esters. Histidine acid phytases with broad substrate specificity but low specific activity, such as those from *A. fumigatus*, *E. nidulans*, and PhyB *A. niger*, have been described. Other microbial phytases show high specific activity for phytate hydrolysis and narrow substrate specificity for the hydrolysis of other phosphorylated substrates, e.g., the *A. niger*, *A. terreus* and *E. coli* PhyA phytases (Table 4) (Wyss et al., 1999b; Bohn et al., 2008). Such phytases also include the phytases from *Sch. castellii* CBS 2863 and *D. castellii* CBS 2923. The commonly used commercial preparation Phyzyme XP (Danisco, Denmark) was developed on the basis of the *E. coli* phytase AppA with a specific activity by an order of magnitude higher

Table 4. Substrate specificity of phytases by phosphorylated substrate hydrolysis (Wyss et al., 1998, 1999a, 1999b)

Phytase producers	Specific activity by substrate hydrolysis, U/mg:					
	phytate	pNPP	fructose-6-phosphate	glucose-6-phosphate	phenyl phosphate	ATP
PhyA *A. niger* NRRL 3135	102.5	18	0	5	15	18
A. terreus 9A1	148	15	2	45	10	48
A. fumigatus 34625	26.5	108	5	30	110	20
E. nidulans (GenBank no. U59803)	28.0	34	10	12	48	15
PhyB *A. niger* NRRL 3135	120	900	250	900	900	700
AppA *E. coli* K-12	812	25	0	0	0	3

compared to the *A. niger* phytase PhyA (Youn et al., 2011). The preferred specificity for phytate is demonstrated by the *Shigella* sp. CD and *C. braakii* YH-15 phytases vs. low activity on other phosphorylated substrates, such as ATP, ADP, glucose-6-phosphate, fructose-6-phosphate, and *p*-nitrophenyl phosphate (pNPP). It would be interesting to note that the *C. braakii* YH-15 phytase has a unique ability to hydrolyze all six phosphate groups of *myo*-inositol hexakisphosphate to free inositol, including the phosphate group at position d-2, which is axially bound to the inositol molecule (Kim et al., 2003; Roy et al., 2012). The high specific activity, thermostability and resistance to proteolytic degradation make microbial phytases promising for the production of feed additives for animals.

Mechanism of Myo-Inositol Hexakisphosphate Hydrolysis by the AppA E. coli Phytase

The mechanism of hydrolysis by microbial phytases of the class of HAPs is based on successive cleavage of phosphoric acid residues from the *myo*-inositol hexakisphosphate molecule; the final products of such hydrolysis are a molecule of *myo*-inositol monophosphate and five molecules of inorganic phosphate. The formed low-phosphorylated intermediate products are released in different order and at different rates (Wyss et al., 1999b). Phytate hydrolysis proceeds via a two-step mechanism. The first step is the nucleophilic attack by the histidine residue of the conservative motif RHGXRP at the phosphorus atom with formation of a covalent intermediate. The second step is hydrolysis of the formed phosphohistidine bond of the intermediate by the aspartate residue of the C-terminal motif HD, which is a proton donor for the oxygen atom (Oh et al., 2004; Yao et al., 2011). Thus, the reaction of *myo*-inositol hexakisphosphate Ins(1,2,3,4,5,6)P_6 hydrolysis by the *E. coli* phytase AppA begins with the phosphate group cleavage at position d-6 of the phytate with the formation of the intermediate Ins(1,2,3,4,5)P_5 which, as it has been shown experimentally, does not accumulate and is rapidly hydrolyzed to *myo*-inositol tetrakisphosphates. It has been shown experimentally that the phosphate group is released from *myo*-inositol pentakisphosphate Ins(1,2,3,4,5)P_5 mainly by the major pathway of hydrolysis with the formation of intermediate Ins(2,3,4,5)P_4, while two other intermediates, Ins(1,2,3,4)P_4 and Ins(1,2,4,5)P_4, are formed in small quantities (Greiner et al., 1993). *myo*-Inositol tetrakisphosphate Ins(2,3,4,5)P_4 accumulates, because the rate of hydrolysis decreases due to inhibition by the released inorganic phosphate. Further hydrolysis to less phosphorylated products proceeds at a low rate with formation of *myo*-inositolmonophosphate Ins(2)P_1 as a final product, its phosphate residue forming an axial bond with inositol molecule at position d-2 (Greiner, 2007). In the hydrolysis of *myo*-inositol hexakisphosphate by 3-phytases of the class of HAPs, the final product is also *myo*-inositol monophosphate, but the hydrolysis begins at position d-3 of *myo*-inositol hexakisphosphate (Fig. 2).

BIOSYNTHESIS OF MICROBIAL PHYTASES AND ITS REGULATION

In most microorganisms, phytase is an inducible enzyme and its expression undergoes complex regulation; however, phytase biosynthesis is differently controlled in different microorganisms. The regulation of phytase production has been studied in detail only in *E. coli*, *Raoultella terrigena* (*Klebsiella terrigena*) and *S. cerevisiae* (Touati et al., 1987; Greiner et al., 1993; Greiner et al., 1997; Zamudio et al., 2001; Andlid et al., 2004). In micromycetes, phytase biosynthesis is associated with growth; the enzyme activity increases from the beginning of cell growth to the beginning of the stationary phase. However, under non-limiting conditions, the formation of many bacterial phytases begins when the cultures enter the stationary phase of growth. Thus, the nutrient or energy limitation occurring in the stationary phase can induce phytase production.

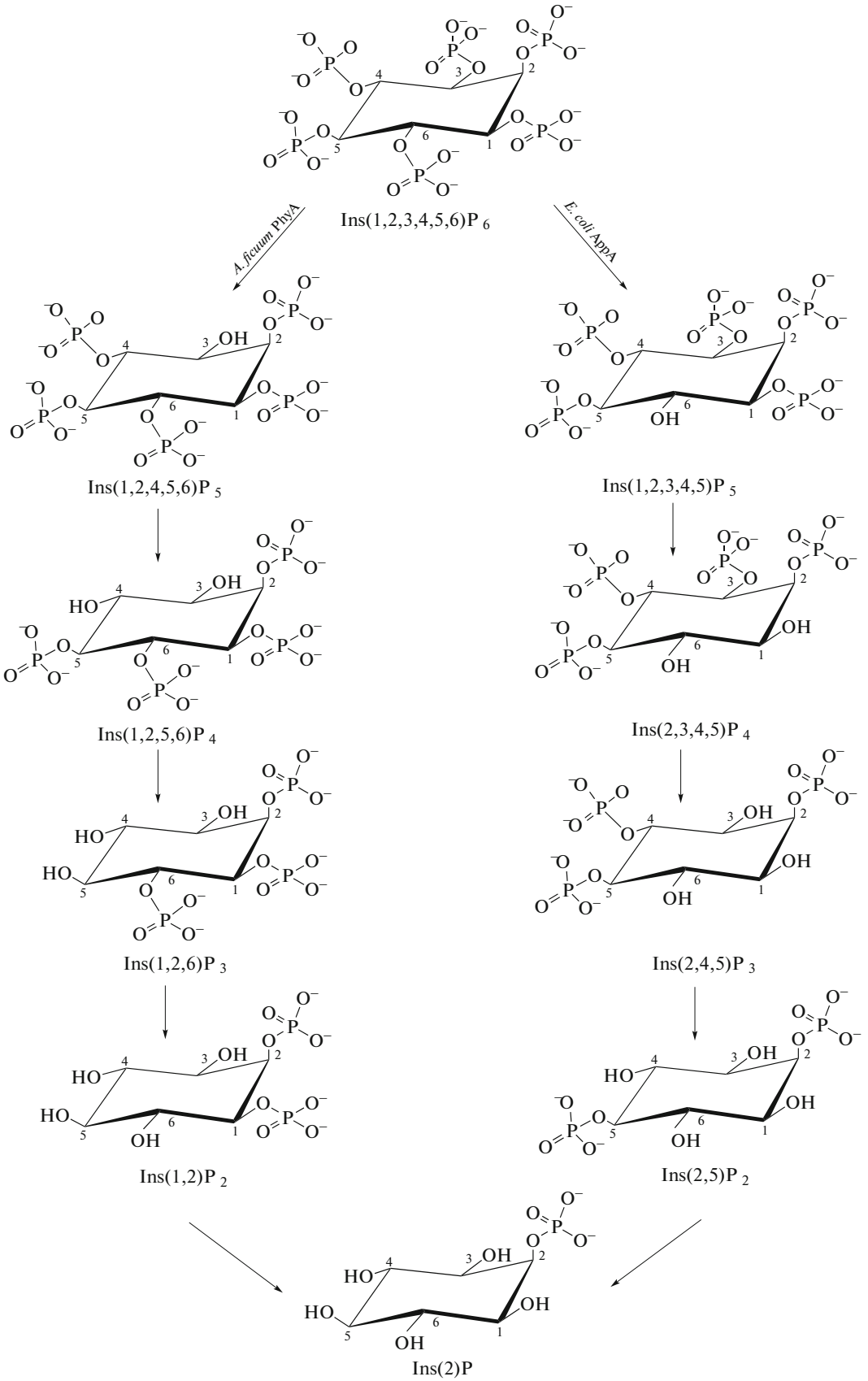

Fig. 2. The pathways of *myo*-inositol hexakisphosphate Ins(1,2,3,4,5,6)P_6 hydrolysis by the *E. coli* AppA and *A. ficuum* PhyA phytases.

The *Bacillus* spp. and *E. coli* cells initiated phytase synthesis upon inorganic phosphate depletion, while the carbon, nitrogen and sulfur limitations had no such effect (Touati et al., 1987). The regulatory inhibition of phytase biosynthesis by phosphate was observed in all microbial phytase producers including micromycetes, yeasts and bacteria, except for *R. terrigena* and the bacteria isolated from the rumen (Greiner et al., 1997).

Phytase expression also depends on ambient pH and the carbon source used for cultivation (Konietzny and Greiner, 2004). Repression of phytase synthesis was observed in the presence of simple sugars (glucose) in the medium. It has been shown that cAMP-CAP, rather than the carbon source, is directly involved in the regulation of phytase synthesis in *E. coli*. Phytase production both in *E. coli* and in *R. terrigena* is negatively regulated by cAMP (Touati et al., 1987; Zamudio et al., 2002). For several strains of *Raoultella* sp. it was shown that the presence of phytate in the medium was a necessary condition for phytase biosynthesis (Greiner et al., 1997). Substrate induction was also found in the anaerobe *Mitsuokella jalaludinii*, while the presence of phytate in the medium had no effect at all on phytase formation in *E. coli* (Greiner et al., 1993; Lan et al., 2002). Effective induction or repression of phytase biosynthesis during phosphate starvation in most microorganisms poses the question about the important functional role of phytase in the supply of microbial cells with phosphate during phytate hydrolysis.

PRACTICAL APPLICATIONS OF MICROBIAL PHYTASES

Soil microorganisms have a favorable effect on plant growth and development, increasing the availability of phosphorus from poorly soluble phytates and other important but poorly available nutrients such as nitrogen, group B vitamins, and amino acids. Microorganisms, as phytase producers, are the perfect candidates for making biofertilizers. It has been shown that pasture plants can utilize phytate phosphorus in the presence of soil microorganisms of the genera *Pseudomonas*, *Enterobacter*, and *Pantoea*, which are widespread in the rhizosphere of various plant species (Richardson et al., 2001b; Jorquera et al., 2008). Microbial phytases also promote the release of trace elements from phytate complexes, which are then assimilated by plants. The innovative microbial biotechnologies for the development of biofertilizers based on phytase producers will lead to the economically profitable and environmentally friendly method of increasing the bioavailability of soil phosphorus, which will be an alternative to the application of inorganic phosphorous fertilizers. The fundamental knowledge of microbial phytases and diversity of their producers is necessary for the development of novel technological products.

There are several factors important for microbial phytases to be used for enhancing phosphorus bioavailability in animal nutrition. Phytases must have high thermostability and specific activity, because nutrient additives are produced at high temperatures (70–80°C) when non-thermostable enzymes are quickly inactivated (Oh et al., 2004). For bioadditives to be digested, it is important that phytases were active in a broad pH range and highly resistant to proteolytic enzyme degradation and inhibitors of animal origin. The new phytases obtained from *Enterobacter cloacae* PSB-45 (GenBank Accession no. KR133282) and *Serratia* sp. PSB-15 (GenBank Accession no. KR133277) are characterized by thermostability at 50–70°C and pH stability within a broad range (pH 3.0–8.0), which opens certain prospects for their practical application in agriculture (Kalsi et al., 2016). Microbial phytases with pH optimum in the range of low values and with resistance to pepsin are the preferred agents for increasing the nutritional value of vegetable feeds (Lei and Stahl, 2001). The *E. coli* recombinant phytase AppA with the temperature optimum of 60°C and pH optimum of 4.5 was obtained in order to increase the amount of inorganic phosphorus in poultry feed additives; it was designated by the authors as Xlanase and expressed in seaweed cells. With transgenic Xlanase being used in the nutrition for broiler chickens, the amount of free inorganic phosphorus increased by 41% and the fecal excretion of phytates decreased by 43% compared to those for the chickens receiving conventional poultry feed (Yoon et al., 2011). Due to the low cost, feasibility of cultivation and safety of bacterial enzymes, and owing to the positive effects of hydrolyzed products on the metabolism of animal organisms, the new biotechnologies will allow microbial phytases to be widely used as feed additives for poultry and domestic animals (Konietzny and Greiner, 2004). At present, in addition to the known commercial phytase preparations Natuphos and Xlanase, the industry produces Ronozyme TM (DSM, *Peniophora lycii*), Phyzyme TM (Diversa/DaniscoA/S, *Schizosaccharomyces pombe*), and Quantum TM (Diversa/Syngenta, *E. coli*) (Kalsi et al., 2016). It should also be noted that the products of specific hydrolysis of *myo*-inositol hexakisphosphate by bacterial phytases have a high potential of practical applications as drugs, in contrast to the products of multistage and expensive chemical synthesis (Shamsuddin and Vucenik, 2005; Balaban et al., 2014).

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. This article does not contain any studies involving animals performed by any of the authors.

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