ISSN 0026-2617, Microbiology, 2012, Vol. 81, No. 3, pp. 267–275. © Pleiades Publishing, Ltd., 2012.
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REVIEWS

Microorganisms as Phytase Producers

A. D. Mukhametzyanova, A. I. Akhmetova, and M. R. Sharipova[1]

Kazan State University, Russia

Received July 20, 2011

Abstract—Replenishing of the stores of inorganic phosphate is among the most urgent environmental problems. In soil, phosphorus within inorganic compounds is mostly (over 80%) present as insoluble, phytic acid-based conglomerates. Phytates are strong chelating agents, binding the cations of bivalent metals, as well as peptides and low-molecular metabolites into resilient poorly degradable compounds. Their hydrolysis in nature is carried out by microbial phytases, which may potentially be used for an innovative microbial technology. The review deals with microbial degradation of the derivatives of phytic acid. Bacterial species capable of phytase synthesis for stepwise specific cleaving of phytates and their derivatives are discussed. Information analysis was carried out in order to search for the genes encoding phytases in bacterial genomes. Directional modification of the genes of bacterial phytases in order to develop new biotechnologies for agriculture and forage industry is considered. Application of microbial enzymes in agriculture and medicine is analyzed. Bacteria phytases are concluded to have a high practical potential. Microbiology is capable of providing the theoretical and experimental basis for development of the new biotechnology.

Keywords: microorganisms, phytases, phytate, inositol phosphates, microbial biotechnology.

DOI: 10.1134/S0026261712030095

Phosphorus is among the macroelements of living cells. It is present there as ortho- and pyrophosphoric acids and as a component of nuclear acids, high-energy compounds, phospholipids, coenzymes, enzymes, and hormones. In many agricultural areas, most of the phosphorus introduced with fertilizers (>90%) becomes unavailable to plants and therefore is not utilized. Interaction of the introduced phosphorus with soil components (adsorption and precipitation) results in its transformation into organic conglomerates or insoluble inorganic minerals [1].

Phosphorus of organic compounds (P_{org}) constitutes 30 to 50% of the total soil phosphorus and is abundant in organic-rich soils [2]. Phytic acid and its derivatives (phytates) are the most common forms of soil P_{org} [3]. Phytate content in soils varies significantly and is to some degree determined by the type of soil and ways of its utilization [4].

The goal of this review is to assay the contribution of microorganisms to the degradation of soil phytates.

Phytates, integral components of cereals and cereal crops, were discovered over a century ago [5]. They are reservoirs of phosphorus which may be potentially used by microorganisms, plants, and animals.

Phytic acid is a specific chemical derivative of a six-fold alcohol, inositol, with six molecules of phosphoric acid residues bound to its six hydroxyl groups (Fig. 1). Phosphoric acid residues are chemically active and can bind the ions of metals (calcium, sodium, potassium, zinc, and copper). Phytic acid may also react with amino acid residues, making them unavailable to plants. Thus, phytates, apart from acting as phosphorus reservoirs, bind a significant portion of the microelements, proteins, carbohydrates, and amino acids, transferring them into complex insoluble conglomerates [6].

Phytic acid is accumulated in plant seeds during maturation, together with such storage compounds as starch and lipids. It exists there mostly as salts of mono- and bivalent cations. Phytic acid in plant seeds carries out a variety of functions: it acts as a source of phosphorus, cations, and *myo*-inositol (a precursor of cell wall components), and participates in the processes of seed germination [7]. The content and localization of phytic acid in plant seeds varies depending on the species. In grain crops (wheat and rice), phytic

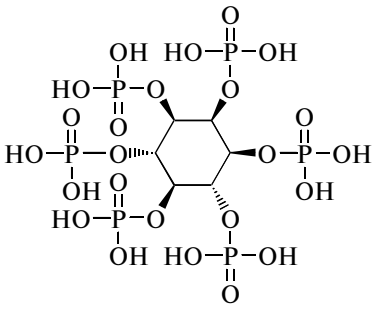

Fig. 1. Structure of phytic acid.

[1] Corresponding author; e-mail: marsharipova@gmail.com

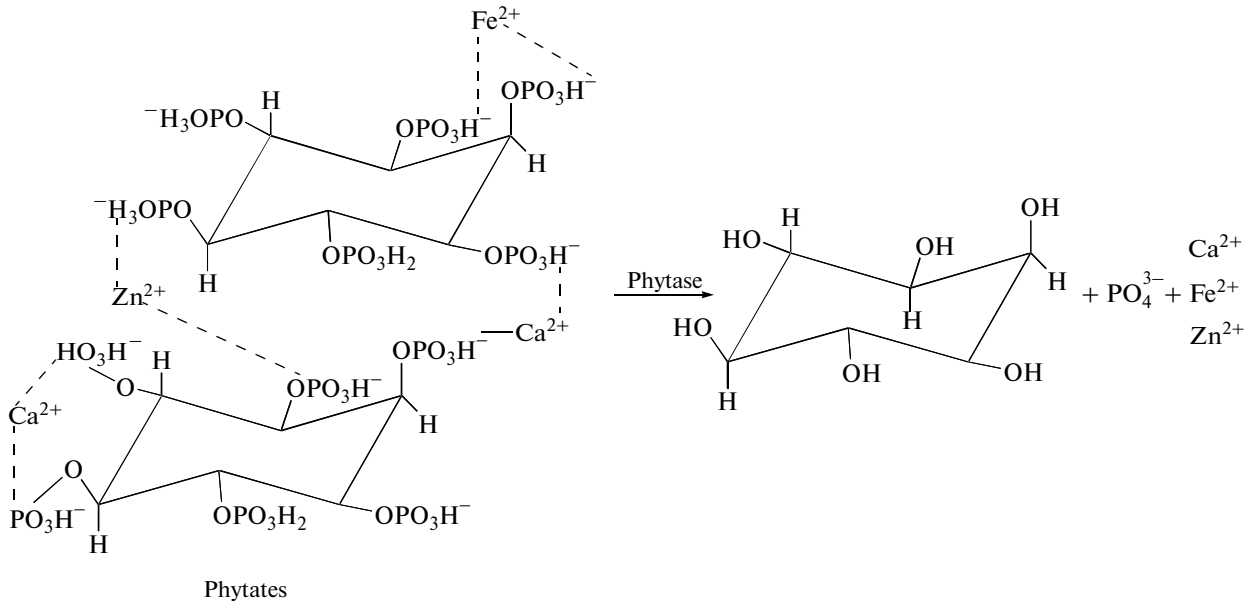

Fig. 2. Mechanism of phytase action. Bacterial phytases cleave phosphate residues from the phytate molecule, thus transferring phosphorus and bound metal ions to an available form.

acid is mainly concentrated in the aleuronic layer and husks, while in oil and pulse plants it is distributed all over the grain. Phytic acid was also found in roots and tubers, vegetables, fruits, nuts, and pollen of various plant species [8].

At the stage of seed germination, plant phytase is activated in order to obtain phosphorus, while phytase activity in mature grain is extremely low [7]. Moreover, plant phytases are highly thermolabile. Phosphorus compounds in the seeds used as feed are therefore not available to animals. Production of phytases on digestive tracts of pork, poultry, and other monogastric animals is almost nonexistent. Under conditions of low or zero phytase activity, phytic phosphorus and associated nutrients pass through the gastrointestinal tract of an animal. Availability of phosphorus from granular feeds is therefore decreased to 15–22% of its content in the feed, while the degree of utilization of associated minerals decreases by 8.7–25.8% [9]. Phytic phosphorus passes unchanged through the digestive tract and is excreted with manure, which may be used as an organic fertilizer. Soil contamination and emergence of insoluble phosphates in groundwater and underground water bodies are associated with high levels of inassimilable phosphorus in the fertilizers [10].

The first phytase capable of phytate hydrolysis was described in 1907 [11]. The pressing environmental issues of the recent two decades resulted in increased attention to phytases due to the possible development of innovative technologies for the application of these enzymes in livestock farming, agriculture, and environmental protection.

Phytases are a specific group of phosphatases, which are capable of phytate hydrolysis with formation of lower phosphorylated inositol derivatives. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) discriminated between two types of phytases, 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). Their classification is based on the position of the first phosphate group cleaved by the enzyme: 3-phytases are mostly of microbial origin, while 6-phytases are plant enzymes [12]. The mechanism of action of all described phytases is based on the enzymatic hydrolysis of the bonds between inositol and phosphoric acid residues. A sixfold alcohol and phosphates are the products of this reaction (Fig. 2).

Microbial phytases are actively secreted into soil, where they participate both in decomposition of fresh plant debris and in the liberation of phosphorus from soil organic compounds. Thus, microbial phytases are the key enzymes of the P_{org} cycling in the soil (Fig. 3) [13]. Importantly, weak phytase activity was found in plant roots. This enzyme is not secreted into the rhizosphere, so that plants are unable to assimilate the bound phosphorus from soil phytates. Microorganisms play a unique role in the degradation of these compounds in soil [14]. Micromycetes are the most active producers of microbial extracellular phytases. Among 2000 phytate-hydrolyzing microbial isolates from soil, the majority produced intracellular phytase [15]. Extracellular phytase activity was found in 30 isolates, which belonged to mycelial fungi: 28 members of the genus *Aspergillus*, one *Penicillium*, and one *Mucor* species [16].

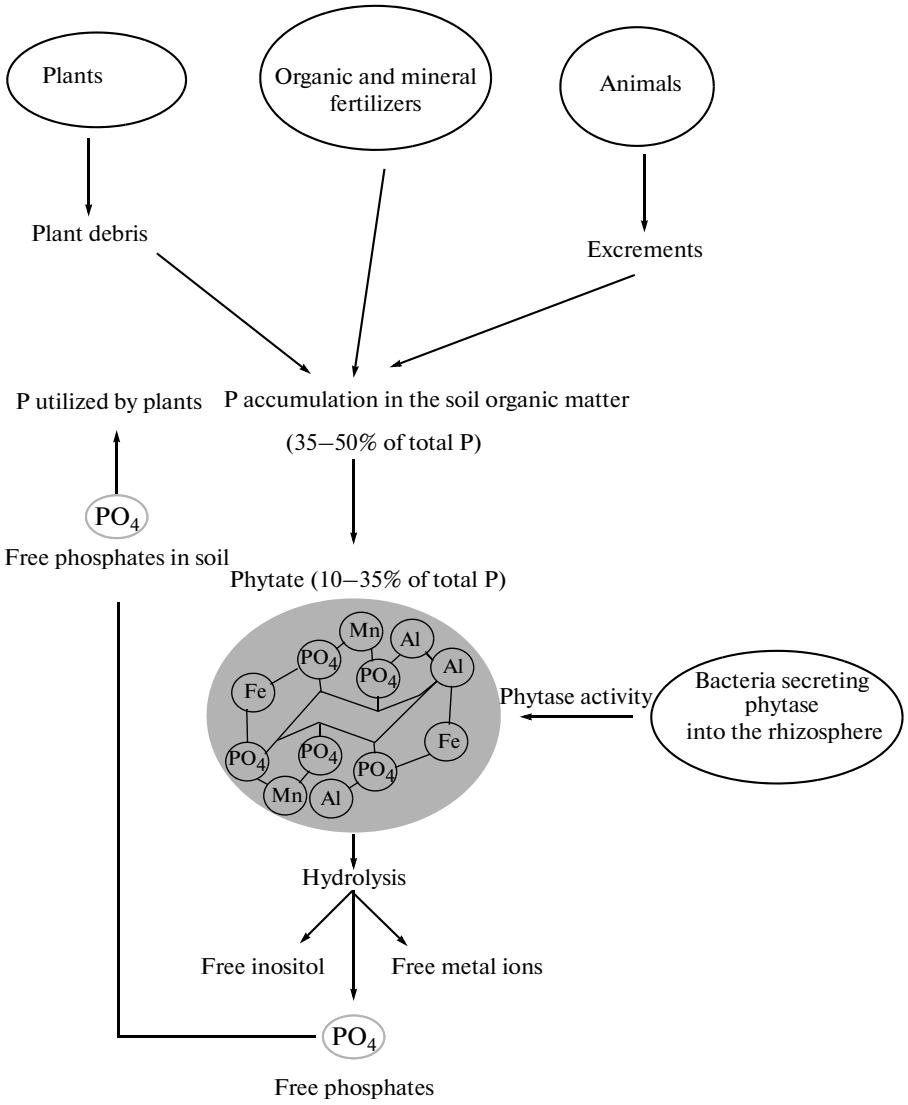

Fig. 3. Phosphorus mobilization by microorganisms: phytase-synthesizing bacteria of the rhizosphere provide for sequential utilization of phosphorus from insoluble soil phytates and elimination of chelating complexes with release of metal ions, amino acids, and vitamins.

Phytases have also been found in bacteria of various taxa (*Aerobacter aerogenes* [17], *Pseudomonas* sp. [18], *Bacillus subtilis* [19], *Klebsiella* sp. [20], *Escherichia coli* [21], *Enterobacter* sp. 4 [22], *B. amyloliquefaciens* [23]) and yeasts (*Saccharomyces cerevisiae, Candida tropicalis*, etc.) 24, 25]. Although most strains of lactic acid bacteria do not possess phytase activity, it was demonstrated for some strains. *Lactobacillus sanfranciscensis* was found to be the best phytase producer among the lactic acid bacteria isolated from leaven [26].

During microbial degradation of phytate, the phosphoric acid residues are liberated in different order and with different rate (Fig. 4). After dephosphorylation of the first phosphate group, the histidine acidic phytases catalyze the subsequent liberation of the phosphates adjacent to the free hydroxyl group. Most plant phytases differ in the first stage of formation of the intermediate product, *myo*-inositol pentaphosphate. Microbial 6-phytases produce another set of intermediates at the first stage of hydrolysis (Fig. 4). Acidic phosphatases with phytate-hydrolyzing activity identified in *Enterobacteriaceae* preferably hydrolyze glucose-1-phosphate and cleave the phosphate residue in D-3 position in the phytate molecule [27]. Alkaline phosphatases of macereed, lily pollen, and *B. subtilis* produce *myo*-inositol triphosphates as the end products of the reaction (Fig. 4) [12].

Bacterial phytases are usually intracellular enzymes, although *Bacillus* and *Enterobacter* species produce extracellular phytases, and *E. coli* phytase is a periplasmic enzyme [27].

Four classes of enzymes with phytase activity are presently considered: histidine acidic phosphatases

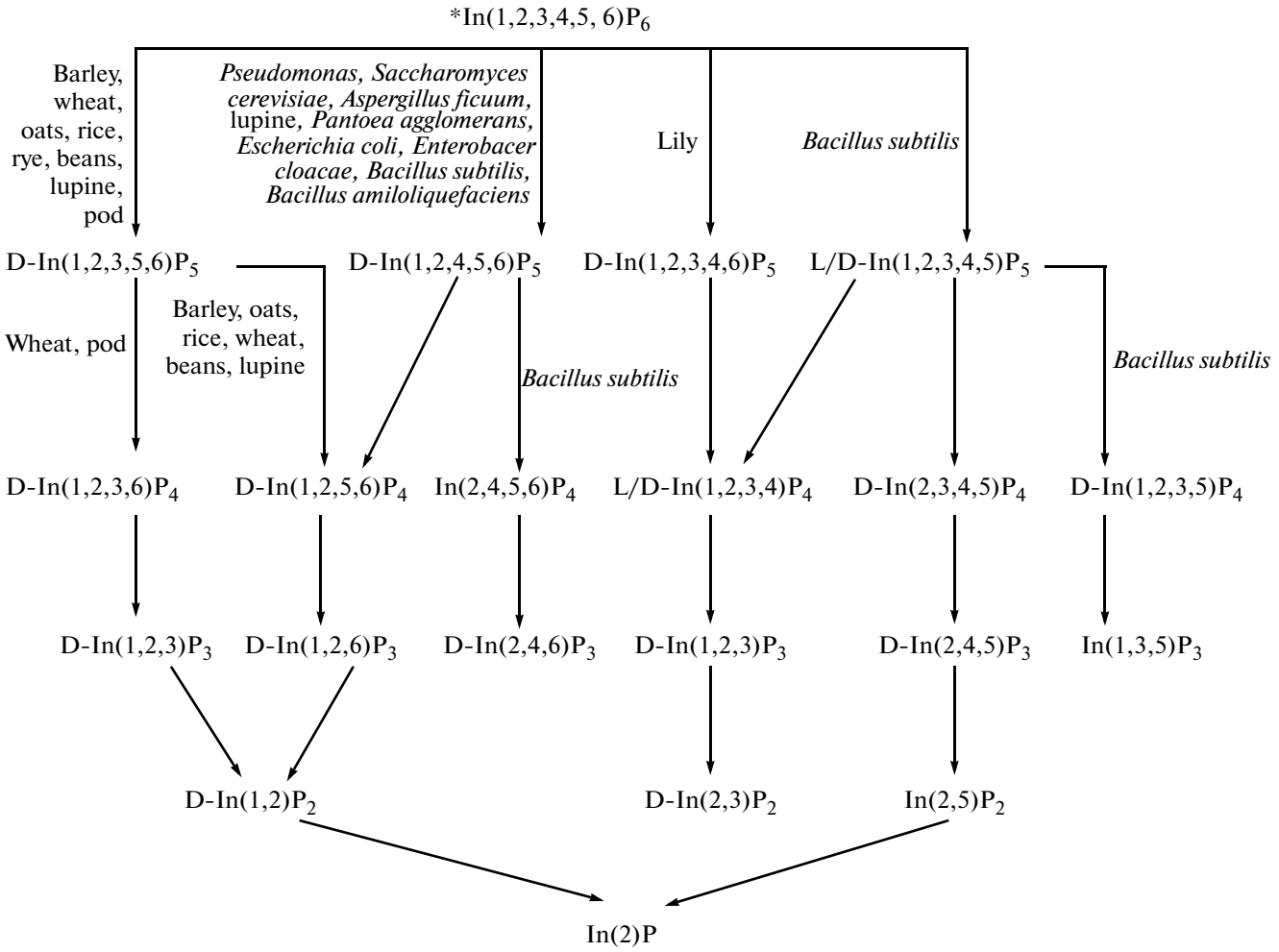

Fig. 4. Pathways of sequential phytate hydrolysis by acidic (plants and microorganisms) and alkaline phytases (bacilli and lilies). Phosphate residues in the phytate molecule are released by phytate-hydrolyzing enzymes at different rates and in a different order. Independent on their origin, at acidic pH most phytases liberate five out of six phosphate residues, with *myo*-inositol-2-phosphate as the terminal product of dephosphorylation. After liberation of the first phosphate, the histidine acidic phytases carry out hydrolysis of the neighboring hydroxyl groups, while the β-propeller phytases preferably hydrolyze every second phosphate, with *myo*-inositol triphosphates as terminal products. D-In(1,2,3,4,5,6)P_6 stands for D-inositol-(1,2,3,4,5,6) hexaphosphate—(*).

(HAP), β-propeller phytases (BPP), cysteine phosphatases (CP), and purple acidic phosphatases (PAP). Members of each class possess different catalytic mechanisms and the individual properties which make possible the efficient utilization of *myo-inositol* hexaphosphates at different pH values [28].

Histidine acidic phosphatases (HAP) is a large class of enzymes which are widely spread among microorganisms and occur in plants and animals. They share the same catalytic mechanism. The amino acid sequence of the active center contains conservative N- and C-terminal motifs, RHGXRXP and HD, respectively. During folding, these sequences become closely located and form the catalytic center responsible for two-stage hydrolysis of the substrate [29]. The *E. coli phytase* is the best studied enzyme of this class [30].

β-*Propeller phytases (BPP)* form a separate class. Unlike histidine acidic phytases, they have no homology with any known group of phosphatases [28]. This class was named for the molecular structure of the protein, which consists mostly of β-structural shields and resembles a six-blade propeller. The β-propeller phytases were found in *Bacillus* species. Unlike histidine acidic phosphatases, the bacillar enzymes lack the RHGXRXP and HD conservative motifs of the active center. β-Propeller phytases contain six calcium-binding sites in the molecule. The binding of six calcium ions by the highly specific sites of the protein globule results in its increased thermostability. The binding of three additional calcium ions with the nonspecific calcium-binding sites at the surface of the molecule provides the catalytic activity of the enzyme by transition of one conformer into another, which is preferable for binding with phytate [31].

Cysteine phytases were found in anaerobic bacteria *Selenomonas ruminantium* from the rumen. Unlike the

monogastric animals, ruminants are able to assimilate phytate due to the presence of phytase-producing microorganisms in the rumen. Among the anaerobic bacteria of the rumen, *S. ruminantium* was found to possess phytase activity, which was inhibited by iron ions and some other metals [32]. The gene encoding the *S. ruminantium* phytase was cloned and its product is presently under investigation [33]. Investigation of the crystal structure of the protein showed that it did not belong to the histidine or β-propeller phytases. Its structure and proposed catalytic mechanism suggest that it is a member of the superfamily of cysteine phosphatases containing the conservative HCXXGXXR(T/S) motive in their active center [28].

Purple acidic phosphatases (PAP) are common among bacteria, fungi, plants, and mammals [28]. Similar to other metal-containing enzymes, these phosphatases require metal ions for their catalytic activity [34].

Bacterial phytases of *Bacillus* and *Enterobacter* species generally have pH optimum in the range from 6.0 to 8.0 and temperature optimum from 45 to 77°C [14]. The *A. niger* phytase with optimal pH at 5.0–5.5 is not thermostable and is incapable of refolding after thermal denaturation, unlike another *A. niger* phytase with pH optimum at 2.2, for which an insignificant decrease in activity at 80°C was reported [16]. Expression of the gene of this *A. niger* phytase in *S. cerevisiae* resulted in a significant decrease in its thermostability, probably due to deglycosylation of the molecule. Attempts to increase the thermostability by increasing glycosylation in the experiments with several phytases/phosphatases showed that glycosylation had no effect on the thermostability of these enzymes per se. High thermostability of fungal phytases was observed in the *A. fumigatus* recombinant enzyme expressed in *Pichia pastoris*: the protein lost ~10% of initial activity after boiling for 10 min [35].

Microbial phytases from *A. niger* and *E. coli* were more resistant to proteolytic degradation with trypsin than wheat phytases [36]. Phytases of *Bacillus* species were resistant to papain, pancreatine, and trypsin, but were highly sensitive to pepsin [37]. The sensitivity of bacillar phytases to pepsin is thought to result from the conformational modification during protein denaturation at low pH.

Comparative analysis of the genes encoding phytase was carried out for the genomes of *Bacillus* strains from the NCBI database. Aligning of the sequences of bacterial phytase genes available in the world databases revealed high homology (Fig. 5). The structure of the *phyC* phytase gene from *B. subtilis* (AN AJ277890) was completely homologous to the sequence of the phytase gene from *B. amyloliquefaciens* strain BAP (AN AY836773) (*phyC*). Homology of the structure of the *phyC* gene of *B. subtilis* with the gene encoding the phytase precursor in *B. licheniformis* (AN AF469936) (*phyL*) was 73% (352 nucleotide out of 482).

Comparative analysis of the structure of the *phyC* gene of *B. subtilis* and the phytase genes of fungi (*Aspergillus* spp.) and gram-negative bacteria (*Pseudomonas* spp, *Klebsiella* spp., *E. coli*) did not reveal any homology. Homology of the phytase genes was, however, revealed within each of these microbial genera. For example, the structure of the *phyA* gene of *A. niger* (AN AY7457391) exhibited 92% homology to the sequences of phytase genes from *A. ficuum* (AN AF537344.1) (*phyA*) and *A. awamori* (AN DQ192035.1).

Biosynthesis of phytate-hydrolyzing enzymes in most microorganisms is induced by phosphate starvation, indicating the role of phytases in providing phosphorus supply for the cells. This was confirmed by identification of phytate-hydrolyzing enzymes in stalked aquatic bacteria (prosthecate bacteria) *Caulobacter crescentus*, inhabitants of oligotrophic environments where phosphates are limiting microbial growth [38]. Phosphate adsorption is one of the hypothetical functions of the *C. crescentus* stalk; it becomes longer in bacteria under phosphate stress. Increase of the stalk area and the phytase content there facilitates more efficient consumption of P_{org} from the environment. Growth of cyanobacteria of the family *Rivulariaceae* on phytate was shown to improve when thin, hairlike appendages were formed [39]. Among lactic acid bacteria, only bacteria of the leaven are capable of phytate hydrolysis. Since lactic acid bacteria are adapted to favorable growth conditions and the growth media enriched with nutrients and energy sources, requirements for phytate-hydrolyzing enzymes have been eliminated by natural selection. The *S. cerevisiae* phytases are proteins of the *pho* regulon, which are involved in the processes of specific phosphate adsorption from the environment [40]. Importantly, expression of the phytase genes in *E. coli* is not regulated by the *pho* regulon [12]. Their main function is probably not associated with phosphate assimilation, although phytases affect the overall phosphorus content in the periplasm [28].

Unlike other microorganisms, anaerobic bacteria of the rumen are adapted to high ambient phosphate concentrations, which do not inhibit phytase synthesis. This unique property enables them to hydrolyze phytates in the rumen efficiently, even at high phosphate concentration, and is an indication of conservative regulation of expression of phytase genes in rumen bacteria [28].

In order to supply phosphates for a microbial cell, phytate-hydrolyzing enzymes require access to the phytates in the environment. Phytate-hydrolyzing enzymes of *E. coli* are periplasmic proteins. Since phytate is readily degraded by both intact and disrupted *E. coli* cells [12], these enzymes probably have access to phytates in vivo. Phytate is possibly transported into bacterial cells [41]. In *Klebsiella pneumoniae* the phytase-encoding gene is co-transcribed with polycistronic messenger RNA encoding the inositol

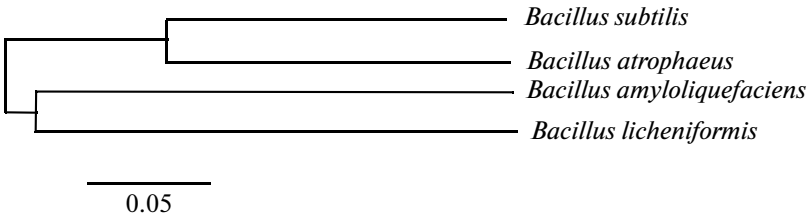

Fig. 5. Homology of phytase genes in *Bacilli*. The tree was constructed using the Mega5 software package (http://www.megasoftware.net). Scale bar, 0.05% divergence.

phosphate transporter [42]. Phytate-hydrolyzing activity of *S. ruminantium* and *Bacteroides multiacidus* is associated with the outer membrane [33]. On the other hand, the mechanisms responsible for growth of bacteria devoid of extracellular phytase activity (e.g., some *Pseudomonas* strains) in the absence of available phosphorus sources remains unknown [41].

The role of phytases is not limited to the degradation of the phosphorus source required for bacterial metabolism. Evidence exists of involvement of the *myo*-inositol phosphate phosphatase activity in signal transduction, cell division, and microbial pathogenesis [43]. Association of the *myo*-inositol flow with pathogenesis is of interest in both basic and practical medical aspects. These compounds probably participate in phospholipid signaling and play an important role in a number of physiological processes responsible for the parasite–host relationship, including survival of the pathogen's cells and the regulation of the transport functions of the membrane [44]. Some pathogens are known to actively secrete *myo*-inositol phosphatases, which are associated with various mechanisms of pathogenesis.

For the biotechnologies for production of modified microbial phytases with required properties, different strategies may be applied. More thermostable phytases are obtained by screening phytases among thermophilic microorganisms and by mutagenesis of the relevant genes in order to increase their thermostability and change their substrate specificity. Protein engineering techniques used for modification of fungal phytases from *Aspergillus* strains resulted in the isolation of highly thermostable enzymes [45].

A significant increase in thermostability was obtained for the "consensus" phytase. This protein is encoded by a synthetic gene constructed from 13 phytase genes with the known sequences. The temperature optimum of the resulting "consensus" phytase increased to 71°C. Further changes in the consensus gene showed the possibility of modification of the catalytic properties of this phytase [46].

The possible expression of microbial phytases in plants which may be used as animal feed is of special interest. Although efficient expression of these enzymes in transgenic plants is technically feasible, scarce data exist on the properties of microbial phytases under these conditions. The gene of a fungal (*A. ficuum* phytase) *phyA* was expressed in tobacco leaves and exhibited the same catalytic properties as the *A. ficuum* phytase. Expression of phytases in agricultural feed plants is considered a promising direction of research [47].

Exhaustion of the deposits of phosphate fertilizers, which may lead to a "phosphate crisis" in the future, indicates a promise for the application of microbial phytases as a replacement for inorganic phosphorus fertilizers. Since utilization of phosphorus from soil phytates by plants is insignificant due to the low phytase activity in the rhizosphere, the importance of such technologies is beyond doubt [48]. Research in this direction is, however, hindered by numerous problems. For example, while two phytase genes from maize plants were cloned, the enzymes encoded by these genes were not secreted [4]. Extracellular phytase activity was shown to be required for the plants to hydrolyze the phytates of soil. Transgenic *Arabidopsis* plants expressing the extracellular phytase of *Aspergillus* sp. were able to satisfy their phosphorus requirements by direct assimilation of phytates from soil [49]. Similar experiments were carried with other plants. Genetically modified *Trifolium subterraneum* L. plants secreted the *A. niger* phytase (PhyA) directly from their root cells [4].

The role of microbial phytases in plant nutrition is significant. Introduction of bacterial phytases into the rhizosphere was found to enhance the utilization of phosphorus from soil phytates, indicating the role of these enzymes in plant nutrition [50]. Moreover, active functioning of bacterial phytases in the rhizosphere prevents formation and elimination of chelated phytates, which bind metals and trace elements, making them unavailable to plants. The phytase from the culture liquid of rhizobacteria *B. amyloliquefaciens* FZB45 was shown to promote seed germination and growth of maize under phosphate starvation when phytate was present in the medium [51]. Using the culture liquid of soil bacteria as biofertilizers may be an economic and environmentally friendly way to substitute for the mineral phosphorus fertilizers and limit their application [52]. However, commercial preparations of microbial phytases for agricultural application have not been developed as yet.

Development of feeds for poultry and agricultural animals is another aspect of phytase-based biotech-

nologies. As was stated above, animals decompose phytates by means of the phytases of the ruminal microflora. Phosphorus released during phytate hydrolysis is used by both the microflora and the host organism. Such animals as pigs, chicken, and fish, are unable to metabolize phytic acid due to the absence of relevant enzymes in their gastrointestinal tracts. Phosphates added to the fodder to supply phosphorus to these animals increase the cost of the fodder and promote phosphate contamination of the environment. Addition of phytases to the feed will make it possible to utilize phosphorus compounds directly from the fodder ingredients, resulting in lower levels of unassimilated phosphates in the manure. To use phytase-producing bacteria as food supplements, their enzymes should meet certain requirements. The enzymes should be thermostable, since the fodder for pigs and chicken is usually granulated at temperatures of up to 90°C [53].

myo-Inositol phosphates, the products of microbial phytate decomposition, were found in human organisms, where they may be involved in such cell functions as membrane transport, division, cell differentiation, and cell death. Presently, *myo*-inositol phosphates attract attention of biotechnologists for their application as medical preparations. Some *myo*-inositol phosphates were found to be efficient in protection against the complications caused by diabetes, as well as for treatment of chronic inflammations and cardiovascular diseases. They also were found to possess antitumor properties [54].

Industrial production of these compounds therefore becomes an urgent problem. Nonenzymatic production of *myo*-inositol phosphate isomers is economically inefficient and yields toxic by-products [55]. Directional production of desired isomers by microbial phytases is a new biotechnological approach. The application of microbial phytate-hydrolyzing enzymes and development of efficient phytase-producing strains may become the basis for new microbial technologies for production of specific isomers of inositol phosphates [56]. The pharmacological properties of *myo*-inositol phosphates depend on the number and distribution of phosphate residues in the ring. Microbial phytases hydrolyze inositol hexaphosphates in a sequential and stereospecific manner, and production of both precursors of *myo*-inositol phosphates and free *myo*-inositol by bacterial phytases is therefore a potential alternative to chemical synthesis. Stereospecificity, mild reaction conditions, and economic gain are the major advantages of the enzymatic hydrolysis involving microbial technologies [57].

Thus, the ecological state of the environment, phosphate requirement of all living organisms, and the irreversible deficiency of available phosphorus compounds determine the urgency of the search for the ways of phytate utilization. This necessitates the isolation of new microbial phytase producers and construction of the modified strains synthesizing the enzymes with high catalytic activity and improved characteristics. The natural and modified strains of phytase producers are the basis for innovative biotechnologies in agriculture, bioengineering, pharmacy, and medicine. These technologies will provide for development of available, environmentally friendly biofertilizers, feed supplements, and prophylactic and curative medical preparations.

ACKNOWLEDGMENTS

The work was supported by the Scientific and Science-Educational Personnel of Innovative Russia target program for 2009–2013 and by the Tatarstan grants for young scientists nos. 13-20/G-2010 and 13-24/2011.

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