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Physiological roles of histidine acid phytase from *Pantoea* sp. 3.5.1.

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ABSTRACT

Microbial phytases represent a potential alternative way to produce myo-inositol phosphate isomers with therapeutic properties, whereas the chemical synthesis of these compounds is inefficient and costly. The aim of this work is to study the physiological role of histidine acid phytase in *Pantoea* sp 3.5.1 cells. Promoter region of the phytase gene was studied and potential binding sites for transcription factors RpoD15 and MetJ were identified by the methods of bioinformatic analysis. Genomic locus carrying the phytase gene was characterised and compared with the same genomic loci of other enterobacteria. It was found that the isolated and purified phytase from *Pantoea* sp. 3.5.1 refers to the Agp- group of histidine acid phytases and has dual physiological role in the bacterial cell. As glucose-1-phosphatase it is involved in glycolysis processes. The data obtained by stereospecifity analysis helped to identify that phytase from *Pantoea* sp. 3.5.1 is the enzyme that carries out phytate hydrolysis by the second path and forms D/L-myo-inositol-1,2,4,5,6-pentakisphosphate as the end product.

Keywords: phytase, glucose-1-phosphatase, *Pantoea*, myo-inositol phosphates

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INTRODUCTION

Phytases [E.C. 3.1.3.8, E.C. 3.1.3.26 and E.C. 3.1.3.72] - enzymes able to produce a stepwise dephosphorylation of the phytate (myo-inositol (1,2,3,4,5,6 hexakisiphosphate), which constitutes up to 50% of total organic phosphorus in soils and plays role of a phosphorus reservoir in plant seeds during their germination. Microbial phytases attract the attention of biotechnologists in terms of their practical use in animal husbandry, agriculture and environmental protection. Phytases as a feed additive are present in about 75% of the diets of monogastric animals, and the volume of world phytase market exceeds $350 million per year [1]. Use of phytate-hydrolyzing enzymes in medicine is also promising. Myo-inositol phosphates are important components of signaling systems of living organisms and have pharmacological properties: reduce the severity of the symptoms of cardiovascular disease [2], prevent the formation of kidney stones [3], reduce the risk of colon cancer [4], etc.

However, a great variety and inaccessibility of individual myo-inositol phosphates still precludes from their use. Chemical synthesis of myo-inositol phosphates is a complex multistage process, which is carried out at extreme temperatures and pressure with the formation of a mixture of isomers of myo-inositol pentakisi-, tetrakisi-, tri-, bi- and monophosphates. In general, there are 6 isomers of Ins5P, 15 isomers of Ins4P, 20 isomers of Ins3P, 15 isomers of Ins2P and six isomers of InsP. Purification of the desired isomer from the mixture is a time-consuming and economically unefficient process [5].

The phytate-hydrolyzing enzymes can be used to obtain specific isomers of inositol phosphates [6]. Pharmacological properties of myo-inositol phosphates depend on the number and distribution of the phosphoric acid residues in the inositol ring. Phytases consistently and stereospecifically hydrolyze the myo-inositol hexaphosphates, therefore, production of the required isomer of myo-inositol phosphates with the use of phytases is a potential alternative to chemical synthesis. There are several known types of phytases - 3-phytases, 4-phytases and 6-phytases - depending on the first attacked phosphoric acid residue in the molecule. Therefore, using the phytases of various microorganisms allows producing various isomers of myo-inositol phosphates [7].

Therefore, there is an active search for new phytase producers, investigation of phytases' absolute stereospecificity and their role in the cell, as well as regulation of their biosynthesis at the genetic level and the influence of various factors on the enzyme activity. Previously, we isolated *Pantoea* sp. 3.5.1 strain showing the phytase activity from the soils of the Republic of Tatarstan [8]. We have developed an effective method of phytase purification from the bacterial cell lysate and obtained a homogeneous enzyme preparation. We have identified for the first time the primary structure of the phytase from *Pantoea sp.* 3.5.1, and studied the kinetic characteristics, enzymatic properties and substrate specificity of the enzyme [8]. Objective of this study is the investigation of the physiological role of histidine acid phytase in *Pantoea sp* 3.5.1 strain cells.

MATERIALS AND METHODS

Strain

We used the strain *Pantoea sp.* 3.5.1 isolated from forest soil of the Republic of Tatarstan by the maximum phytase activity. Bacterial identification was conducted by sequencing the 16S rRNA-encoding gene, MALDI BioTyper (Bruker Daltonik) and MLSA-analysis.

Phytase purification

Phytase *Pantoea* sp. 3.5.1 was isolated and purified from the cell lysate by FPLC-chromatography using MonoS HR 5/5 and MonoQ HR 5/5 columns (Pharmacia, Germany), followed by gel filtration in 16/60 Sephacryl S-100 HR column (Pharmacia, Germany) as described by Suleimanova [8].

Protein electrophoresis

Electrophoresis was performed in 12.5% polyacrylamide gel (PAAG) in the presence of SDS by Laemmli method [9].

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MALDI TOF mass spectrometry

After the trypsinolysis of the purified phytase preparation, the obtained peptides were identified with a mass spectrometer Vision2000 TOF (ThermoBioanalysis, UK). Data were processed with the programs Peptide Mass Fingerprint http://www.matrixscience.com.

Methods of bioinformatic analysis

The comparative analysis of the primary structure of phytases sequences of genome fragments available on the NCBI server (http://www.ncbi.nlm.nih.gov) were used. Alignment of the sequences was performed with the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast). Analysis of the phytase gene promoter region was performed with the BPROM server (http://www.softberry.com). Alignment of the genomic loci containing phytase genes of *Enterobacteriaceae* was carried out by BioCYC server (http://www.biocyc.org/). Comparative analysis of the GC-content of phytase genes compared to the genomic environment was conducted on ASAP server (https://asap.genetics.wisc.edu/asap). A hypothetical 3D model of phytase from *Pantoea sp.* 3.5.1 was built with the program PyMOL, the identification of metal ion binding sites on a hypothetical protein model was performed by PIONCA software [10]. Ways of bacterial metabolism based on genomic data were reconstructed with KEGG algorithm (http://www.genome.jp/kegg/).

RESULTS AND DISCUSSION

To characterize the properties and stereospecificity of the phytase from *Pantoea sp.* 3.5.1, the protein was purified from the bacterial cell lysates with the use of chromatography in MonoS HR 5/5 and MonoQ HR 5/5 columns, followed by gel filtration in 16/60 Sephacryl S-100 HR column, as described in [8]. As a result of a three-stage purification, a homogeneous phytase preparation was obtained with the activity yield of 14%, and 3641-fold purification. The homogeneity was confirmed by electrophoresis in PAAG under denaturing conditions (Fig. 1).

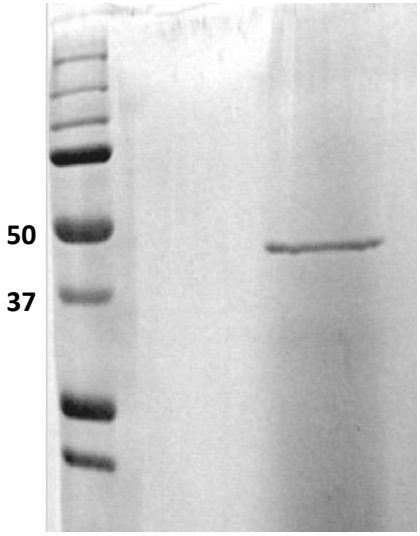

Fig. 1. PAAG SDS electrophoresis of the homogeneous preparation of the phytase from *Pantoea sp*. 3.5.1 after the three-stage purification.

The primary structure of the phytase *Pantoea sp.* 3.5.1 was determined by MALDI-TOF mass spectrometry. The conserved sequences **RHN**LR**A**P and **HD** detected in the primary structure of the phytase allowed us to classify the phytase of *Pantoea sp*. 3.5.1 as a hydrolase of the histidine acid phosphatases family. However, the class of histidine acid phytases is also divided into three groups - AppA-phytases, Agp-phytases and PhyK-phytases, which differ in substrate specificity and in the level of specific activity (Table 1). These differences in substrate specificity can be associated with variations in amino acid sequences of the substrate-binding site, while the catalytic nuclei of these enzymes are conserved [11].

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Table 1. Alignment of the amino acid sequences of the active sites of different groups of histidine acid phytases

Phytases	Active center motive
AgpP *Pantoea* sp. 3.5.1 (KJ783401)	39 40 41 42 43 44 45 312 313 **R H** N L **R** A **P H D**
Agp-phytases AgpE *Enterobactercloaceae* (AJ783768.1)	**R H** N L **R** A **P H D**
Glucose-1-phosphatase *Escherichia coli* (M33807.1)	**R H** N L **R** A **P H D**
Glucose-1-phosphatase *Pantoea agglomerans* DQ435815.1	**R H** N L **R** A **P H D**
AppA-phytases AppA *Escherichia coli* (YP_006095380.1)	**R H** G V **R** A **P H D**
6-phytase *Shigella dysenteriae* (YP_008848635.1)	**R H** G V **R** A **P H D**
3-phytase *Aspergillus niger* CBS (XP_001401628.2)	**R H** G E **R** Y **P H D**
PhyK-phytases PhyK *Pantoea vagans* (YP_003930004.1)	**R H** G V **R** P **P H D**
PhyK *Klebsiella pneumonia*(AAL59319.1)	**R H** G I **R** P **P H D**
PhyK *Raoultella terrigena* (CAE01322.1)	**R H** G I **R** P **P H D**

Identification of the gene encoding the phytate-hydrolyzing enzyme of *Pantoea sp.* 3.5.1 involved a full bacterial genome sequencing and identification of two phytase genes belonging to subgroups of Agp and PhyK phytases, however, no genes encoding the enzymes of AppA subgroup were found [12]. Alignment of the identified amino acid sequence of the purified protein with the genomic sequence of *Pantoea sp.* 3.5.1 allowed us to detect a single gene, encoding the isolated enzyme, in the genome of these bacteria. During alignment of the nucleotide sequence of the *Pantoea sp.* 3.5.1 phytase gene with the sequences located in the global databases, we revealed a high degree of homology to the glucose-1-phosphatase of bacteria (Table 2).

Table 2. Homology of nucleotide sequences of the phytase *Pantoea sp.* 3.5.1 gene with the genes of glucose-1-phosphatases (*agp*)

Bacteria	GenBank ID	% of similarity with *Pantoea* sp. 3.5.1 phytase
P. vagans C9-1	YP_003930444.1	84%
P. ananatis LMG 5342	HE617160	81%
P. ananatis PA13	CP003085.1	81%
P. ananatis LMG 20103	CP001875.2	81%
P. ananatis AJ13355	AP012032.1	81%
Pantoea sp. At-9b	CP002433.1	80%
Escherichia coli 042	FN554766.1	72%

Thus, our findings indicate that the purified phytate-hydrolyzing enzyme of *Pantoea sp.* 3.5.1 is a histidine acid phytase and belongs to an evolutionarily conserved subgroup of glucose-1-phosphatases (Agp).

We have conducted a bioinformatic analysis of the promoter region of the *Pantoea sp* 3.5.1 phytase gene sequence, and identified the potential binding sites for the transcription factors RpoD15 and MetJ (Fig. 2). Genes under the control of the RpoD15 transcription factor refer to the household genes and are necessary

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for normal functioning of the bacterial cells [13]. MetJ – is a regulatory protein, which, together with S-adenosylmethionine (SAM), inhibits the expression of the methionine regulon [14].

Fig. 2. Phytase gene promoter sequence. A potential site for binding with the protein RpoD15 is marked with purple box, and with protein MetJ - with orange box. Regulatory -10 and -35 regions are in bold, the start codon is in red. A potential site for translation initiation is represented by ATG codon.

The characterization of the gene encoding a phytate-hydrolyzing protein of *Pantoea sp.* 3.5.1 involved a comparative analysis of the genomic locus containing this gene. The sequence of 20,000 bp genomic locus of *Pantoea sp.* 3.5.1 has 84% homology to the same locus of the strain *P. vagans* C9-1, which genome is presented on BioCYC server (http://www.biocyc.org/). Therefore, the analysis of the genomic locus was carried out on the basis of the related strain (*P. vagans* C9-1). The organization of the genomic locus is highly conservative within the genus *Pantoea*: an *agp* gene has the same length and the same neighboring genes on 3'- and 5'- flanking ends (Fig. 3). The glucose-1-phosphatase in the same chain is superseded by two genes, encoding the serine tRNA, the acylphosphatase gene, the gene encoding the Coenzyme A-binding protein (*yccU*) and helicase IV gene (*helD*); in the other chain - genes of transport protein YccA, sulfate-reductase YccK, methyltransferase YccW, heat shock protein HspQ, methylglyoxal synthase MgsA, and the protein of inner membrane YccF (Fig. 3).

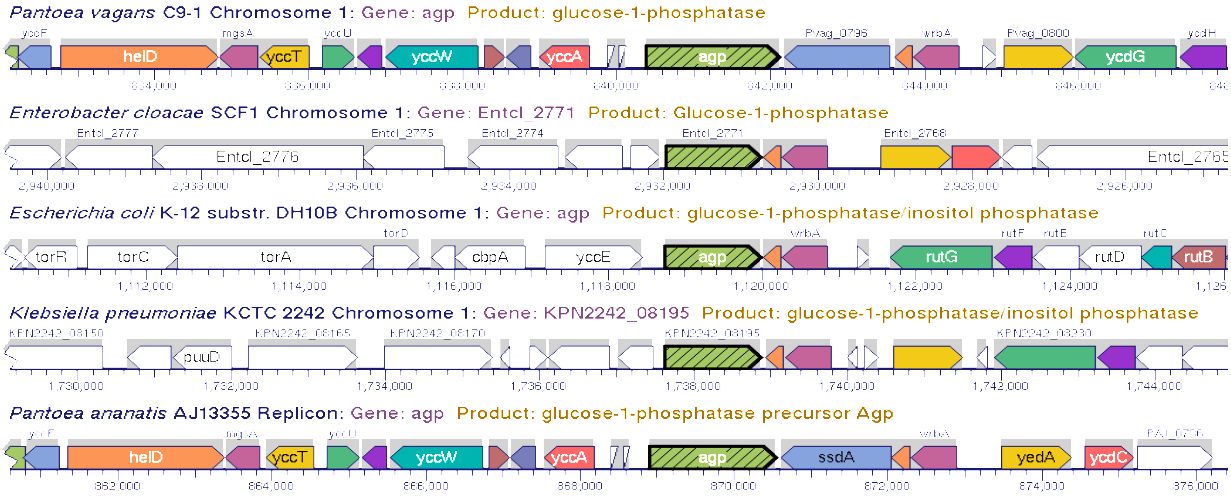

Fig. 3. Structural organization of a genomic locus containing genes of enterobacterial glucose-1-phosphatase. The orthologous genes are marked with the same color; gene keys are given in the text.

The region below the gene of glucose-1-phosphatase in *P. vagans* is conservative within the genus: the same chain always includes a transporter protein gene (yedA), the opposite chain includes a gene of NAD-dependent succinate-aldehyde dehydrogenase (ssdA) in the same operon with genes of cytoplasmic protein YccJ and flavoprotein WrbA, the pyrimidine permease gene present in the genomes of other representatives of the family *Enterobacteriaceae* (*Escherichia coli, Klebsiella pneumonia, Shigella flexneri*), but absent in *Pantoea ananatis* (Fig. 3).The *agp* genes of other enterobacteria (*Escherichia coli, Klebsiella pneumonia, Shigella flexneri, Enterobacter cloacae)* differ in size – they are on average 500 base pairs shorter than the same genes in the

Pantoea genus. Genomic locus from the 3'-end of glucose-1-phosphatase gene has a heterogeneous structure, and shows no homology between the geni.

The degree of conservation of these loci can be judged by the content of GC-pairs in the individual genes or operons. For example, GC-composition of the genomic DNA of *Pantoea* sp. 3.5.1 is 55.77%, and GC-composition of *agpP* gene is 54.8%. Evolutionary conservation of the gene indicates its important functional role in cellular metabolism of these bacteria.

It is known that metal ions affect the activity of the phytate-hydrolyzing enzymes. We have found that Ca^{2+}, Mn^{2+} and Mg^{2+} ions at a concentration of 1 mM increase the activity of the *Pantoea* sp. 3.5.1 phytase twice. The activity level of phytase from *Pantoea sp.* 3.5.1 was decreased by Fe^{2+}, Cu^{2+} and Zn^{2+} ions, which inhibited the enzyme activity on 10%, 16% and 42%, respectively [8].

The ability of Ca^{2+}, Zn^{2+} and Mg^{2+} ions to affect the phytase activity was confirmed by *in silico* data. Based on data of the primary structure we have built a 3D-model of the *Pantoea sp.* 3.5.1 phytase. Using a hypothetical model and PIONCA software (Protein Ion Calculator) [10] we identified the ion binding sites in the active site of the enzyme (Fig. 4). Thus, the analysis showed that the obtained enzyme can be activated under the effect of Ca^{2+} and Mg^{2+}, and inhibited in the presence of Zn^{2+} ions.

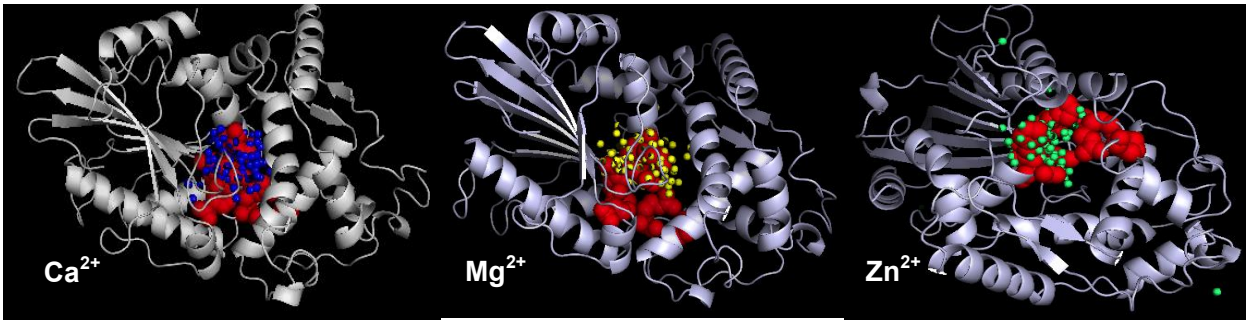

Fig. 4. Hypothetical 3D-model of the *Pantoea sp.* 3.5.1 phytase. Amino acid residues that form the active site of the enzyme are marked with red. Color points mean the metal ion binding sites: blue - calcium ions, yellow - magnesium ions, green - zinc ions.

In the process of the phytate microbial degradation, the phosphorous acid residues are released in different order. After the hydrolysis of the first phosphate group from phytate, the histidine acid phytases catalyze the subsequent release of phosphates, adjacent to a free hydroxyl group. Based on the analysis of the mechanism of hexaphosphate hydrolysis with the *Pantoea* sp. 3.5.1 phytase, we have found that D/L-myo-inositol 1,2,4,5,6-pentakisiphosphate is the only end-product of the phytate hydrolysis with the phytase from *Pantoea sp.* 3.5.1. This allowed us to classify the selected enzyme as 3-phytase by the first phosphate release from a third carbon in the inositol ring [8].

We have shown that the phytase *Pantoea* sp. 3.5.1 refers to glucose-1-phosphatase group, which primary function is receiving glucose from glucose-1-phosphate, an important intermediate of the biosynthesis and catabolism of galactose and glycogen metabolism [15]. This enzyme function *in vivo* explains a constitutive synthesis of phytase in *Pantoea sp.* 3.5.1.

KEGG program algorithm (Kyoto Encyclopedia of Genes and Genomes) allows reconstructing the ways of bacterial metabolism on the basis of the genome. Our analysis allowed us to estimate the contribution of Agp protein (phytase) in the metabolism of *Pantoea vagans* C9-1 - the closest phylogenetic relative of *Pantoea sp.* 3.5.1 (Fig. 5).

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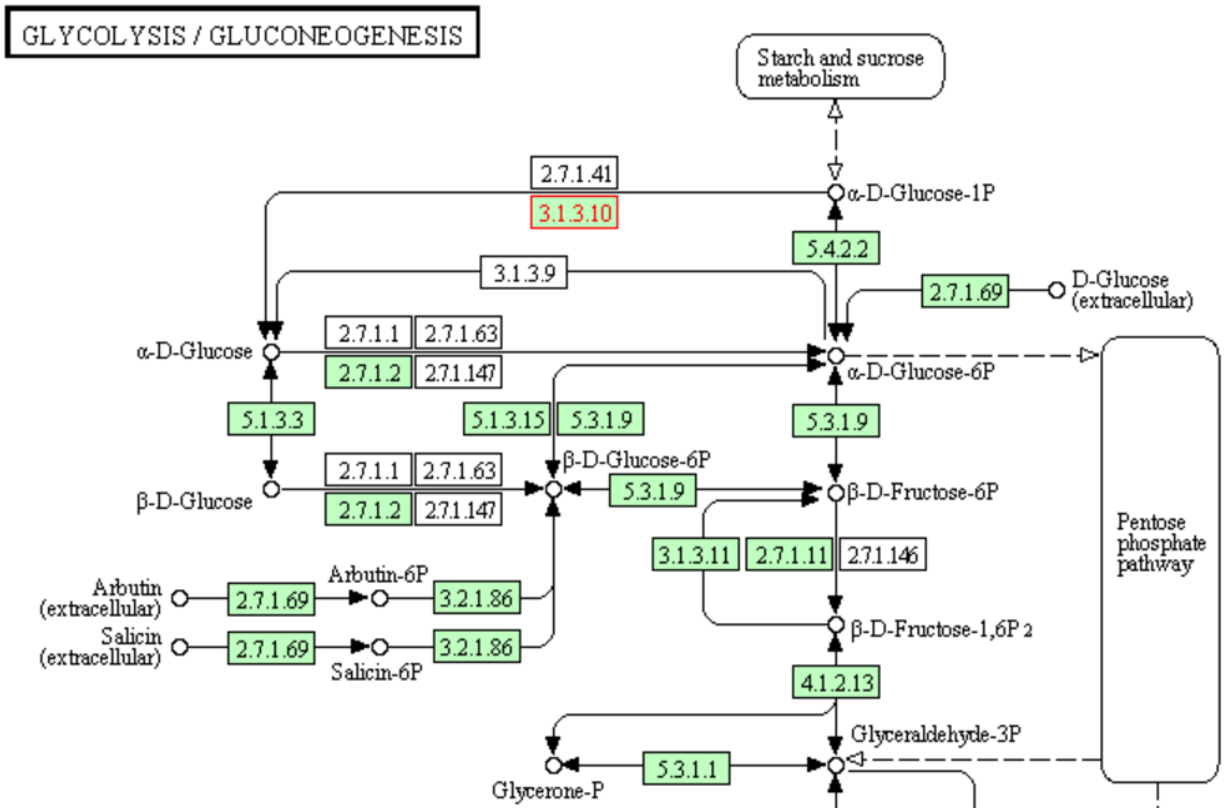

Fig. 5. Reconstruction of the metabolic pathways involving the product of *agpP* gene of *P. vagans* C9-1 based on KEGG database. Enzymes which genes are identified in the bacterial genome are indicated in green color, the colorless frame shows non-identified proteins, red circle - AgpP protein.

Potential role of AgpP protein involved in glycolysis processes - the enzyme catalyzes the cleavage of the phosphate group from the α-D-glucose-1-phosphate with formation of α-D-glucose.

Based on the obtained data on the broad substrate specificity of the isolated enzyme [8], we analyzed the inositol phosphates metabolism of the bacteria. KEGG-analysis of metabolism of inositol phosphates by the strain *P. vagans* C9-1 has revealed that these bacteria perform the hydrolysis of myo-inositol hexakisiphosphate (phytate) in two ways - with the formation of inositol-1,2,3,4,5-pentakisiphosphate (1) and inositol-1,2,4,5,6-pentakisiphosphate (2) (Fig. 6). The enzyme, cleaving the phosphate from the sixth carbon of the inositol ring, has been previously identified in the genome of *P. vagans* C9-1 as PhyK. We have also identified this gene in the genome of *Pantoea* sp. 3.5.1.

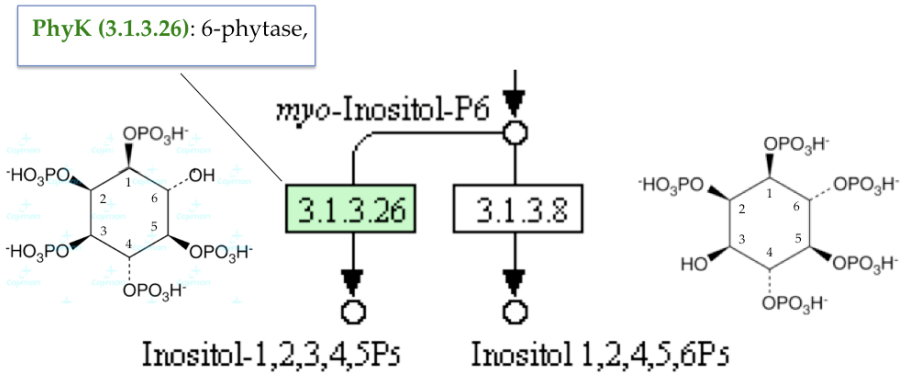

Fig. 6. The analysis of the metabolic ways of myo-inositol hexaphosphate hydrolysis identified in the *P. vagans* C9-1 genome with KEGG database. Enzymes which genes are identified in the bacterial genome are indicated in green color, the colorless frame shows non-identified proteins.

The enzyme with second-waypath hydrolysis has not been identified to date in *P. vagans* genome. The obtained data on the stereospecificity of the phytase isolated from *Pantoea sp.* 3.5.1 suggest that this is the enzyme that performs the second-waypath hydrolysis of phytate with the formation of a D/L-myo-inositol 1,2,4,5,6-pentakisiphosphate. Thus, the obtained enzyme is involved not only in the process of glycolysis, but in the alternative pathway of phytate cleavage.

SUMMARY

Thus, it was found that the phytase *Pantoea sp.* 3.5.1 refers to the conservative group of Agp-phytases from histidine acid phosphatases. We have also shown that phytase from *Pantoea sp.* 3.5.1 has a dual role in the metabolism of bacteria: as glucose-1-phosphatase, it catalyzes the glucose production from glucose-1-phosphate, and the data on stereospecifity of phytase suggest that this enzyme cleaves the phytate with the formation of D/L-*myo*-inositol-1,2,4,5,6-pentakisiphosphate.

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