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Heterologous Expression of B*acillus ginsengihumi* Phytase Gene.

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ABSTRACT

Phytate is the most abundant reservoir of phosphorus, which can potentially be used by microorganisms, plants and animals. Microbial phytases, capable of hydrolysing insoluble phytates, can be used as animal feed additives. In the present study, we isolated new phytase-producing bacteria - *Bacillus ginsengihumi* M2.11, which was identified using Microbial Identification System and 16S rRNA sequencing. We first cloned and sequenced the 1149 bp open reading frame encoding *Bacillus ginsengihumi* M2.11 phytase into the expression vector pET-46 Ek/LIC by the LIC-cloning method (cloning without ligase). The recombinant *E.coli* strain expressing *Bacillus ginsengihumi* M2.11 phytase was obtained. The molecular weight of phytase was 41 kDa.

Keywords: phytase, cloning, Bacillus, expression.

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INTRODUCTION

Wheat, barley, rye and other grains that have not only nutritious, but also negative effects provide the substantial share of the monogastric animals' diets [1]. Cereals, legumes and oilseeds contain phytic acid, which binds phosphorus and makes it for 70-80% unavailable to digest. This limits the usage of these crops in feeding, and especially in the development of high - concentrated rations for the intensive breeding of livestock and poultry [2]. 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. 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 [3]. 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 [4].

Bacteria hydrolyze phytates by releasing the bounded phosphates via enzyme - phytase [5]. Phytases are a specific group of phosphomonoesterase capable of hydrolyzing phytate on less phosphorylated compounds. The production of phytases in the digestive tracts of pigs, poultry and other monogastric animals is very limited [6]. Typically, under the conditions of low or no phytase activity phytic acid phosphorus and associated with it beneficial nutrients pass through the gastrointestinal tract without assimilation [7].

Nowadays, there is an urgent need to develop new science-intensive biotechnologies, based on bacterial enzymes that can serve as feed additives for phytate utilization. Therefore, issues related to the environmental screening of phytate-degrading microorganisms, isolation and purification of phytate-hydrolyzing enzymes and creation of the new biotechnologies are relevant and will help to resolve the problem.

Here we report screening, isolation and identification of a novel phytate-degrading bacteria *Bacillus ginsengihumi* M2.11 and cloning of its phytase gene into the expression vector.

MATERIALS AND METHODS

Screening and isolation of phytate-degrading bacteria

Soil samples were collected from several agricultural enterprises and natural eco-niches of the Republic of Tatarstan, Russian Federation in September 2009. In order to isolate phytate-degrading bacteria, soil samples were serially diluted and plated on PSM agar medium (2% Glucose, 0.4% Sodium phytate, 0.2% $CaCl_2$, 0.5% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4 \times 7H_2O$, 0.001% $FeSO_4 \times 7H_2O$, 0.001% $MnSO_4 \times H_2O$, 3% Agar, pH 7.0) [8]. Plates were incubated at 28°C, and examined for zones of clearance after 24h of incubation up to 5 days. Morphologically distinct colonies showing halo zones on repeated subculture on PSM medium were selected for further studies.

Identification of Isolated Bacteria

Genera of isolated microorganisms were determined using microbial identification system based on the gas chromatography analysis of fatty acid methyl esters of bacterial cell membranes. Bacterial cultures after 20 h of growth on LB agar plates were harvested and placed into a test-tube. Ester fraction was isolated as described in the protocol (MIS Operating Manual, Version 6.2, 2012). Fractions were placed in a chromatographic column and analysed (Agilent GC 78900 Series).

Genomic DNA from the selected strains was isolated using the Fermentas Genomic DNA Purification Kit (Thermo Scientific, Lithuania). The 16S rRNA genes of selected isolates were amplified using polymerase chain reaction (PCR) by standard 27F forward primer (5' GAGTTTGATCCTGGCTCAG 3') and 1492R reverse primer (5' TACCTTGTTACGACTT 3'). The PCR reaction mixtures contained 2.5 µl of 2 mM dNTPs mixture, 1 µl of each primer (10 pmol), 2.5 µl of 10xTaq-buffer, 1 µl of DNA, 1 µl of Taq-polymerase and adjusted with the sterile MiliQ water to the final volume of 25 µl. Reaction mixtures were incubated for 4 min at 95°C, followed by 30 cycles of denaturation for 45 s at 95°C, annealing for 45 s at 57°C, and extension for 100 s at 72°C, followed by a final extension for 3 min at 72°C. The PCR-products were purified using the Fermentas PCR purification kit (Thermo Scientific, Lithuania) and sequenced at Syntol (Moscow, Russia). The DNA sequences

were compared with the sequences in GenBank database using the BLAST Internet tool (http://www.ncbi.nlm.nih.gov).

Phytase Assay

Phytase activity was determined by measuring the amount of inorganic phosphate liberating from the sodium phytate during the reaction. The enzyme solution (10-50 µl) was added to 350 µl of 100 mM sodium acetate buffer, pH 4.5, containing 10 mM sodium phytate (Aldrich, Germany) as a substrate and incubated at 37°C for 30 min. The reaction was stopped by adding 1.5 ml of freshly prepared AAM-solution acetone : 5 N H_2SO_4 : 10 mM ammonium molybdate (2:1:1), allowed to proceed for 2 min, then 100 µl of 1 M citric acid was added to conserve the result. The released inorganic phosphorous was quantified at 355 nm using the modified ammonium molybdate method [9]. Blanks were prepared by adding AAM-solution before the enzyme was added. To calculate the enzyme activity a calibration curve was built using the concentrations of inorganic phosphate from 5 to 600 nmol. One unit of phytase activity (U) was defined as the amount of enzyme that produces 1 µmol of inorganic phosphorous per min at 37°C.

Cloning of phytase gene

Amplification of phytase gene was performed using primers (NCBI-dir/NCBI-rev 5'gctaagcactgccgcgggtt3' / 5'gccgtcagaacggtctttcagctt3' and ORF-dir/ORF-rev 5'atgaaggttccaaaaacaatgctg3' / 5'ctagccgtcagaacggtctttca3') containing complementary nucleotides for annealing, start and stop-codons and sequences, coding His-tag at the N-terminus. PCR-product of phytase gene was cloned into the pET-46LIC vector and then transformed into competent *E.coli* DH5α cells. Obtained recombinant plasmids were analysed using restriction enzymes (BamHI and NotI) and PCR reaction. Plasmid pET-LIC/Phy, which contains phytase gene as an insert, was transformed into a commercial recipient strain *E.coli* Rosseta 2 (DE3) T1R. Transformants were grown on the selective growth medium TB (Overnight Express Instant TB Medium, Novagen) [10] supplemented with chloramphenicol and carbenicillin (100 mg / ml).

Immunoblotting

Recombinant strain cells were disrupted and lysates were separated by SDS-PAGE as described by Sambrook J., 2001. Western blot was carried out as described in protocol [11].

RESULTS AND DISCUSSION

Isolation and identification of phytate-degrading bacteria

Six rhizosphere soil samples were screened for the presence of phytate-degrading enzyme producing bacteria on PSM solid medium, which contains calcium phytate as a sole source of phosphorus. About one hundred thirty strains were initially isolated: 29 × 10^3 colony forming units (CFU) per gram of soil from the large farm complex GUP "Mayskiy" soil, > 100 × 10^3 CFU per gram of soil from the forest soil (near village Agerze, Aznakaevo district), 7 × 10^3 CFU per gram of soil from Kazan city streets soil and 0.4 × 10^3 CFU per gram of soil from the large farm complex Kazan Greenhouse Sovkhoz soil. However, after several screening steps only 12 isolates were selected for further studies based on their ability to form the clear zones around the colonies on PSM solid medium after 5 days of incubation at 37°C (Figure 1).

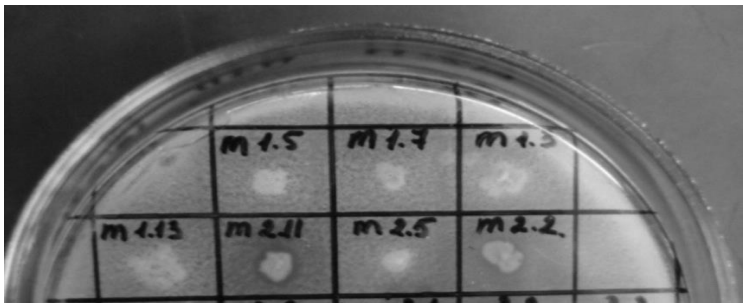

Figure 1: Phytate hydrolysis assay for microbial colonies on PSM medium.

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On the next step, five bacterial strains were isolated according to the largest halo zones, four of which were Gram-negative rods (0.6x6 µm). The fifth isolate M2.11 from the soil sample of the large farm complex GUP "Mayskiy" was identified as Gram-positive bacteria. The identification of selected isolates was carried out by the Microbial Identification System (MIS), based on the cytoplasmic membrane lipid composition analysis. According to the results of MIS-analysis, isolated bacterial strain M2.11 with phytate-hydrolyzing activity belongs to the *Bacillus* genus - the identity of the cell membrane of the isolate to the cell membrane of bacilli was 97.3%. 16S rRNA gene sequencing allowed to identify the high homology to the *Bacillus ginsengihumi* ANA12 (HQ219843.1) strain: the identity of the genes was 97%.

Thus, from the soil samples of the Republic of Tatarstan we isolated gram-positive bacterial strain *B.ginsengihumi* M2.11.

Characterization of *B.ginsengihumi* M2.11 growth and phytase production

One-day culture cells (4-8x0.8µm) of *B.ginsengihumi* M2.11 were rod-shaped, connected in short chains and formed round non-pigmented colonies with a smooth surface and smooth edges. Oval spores did not exceed the diameter of the cells. They appeared on the 24th hour of growth (less than 5%) and reached the maximum value (80%) on 42nd hour of cultivation (Figure 2).

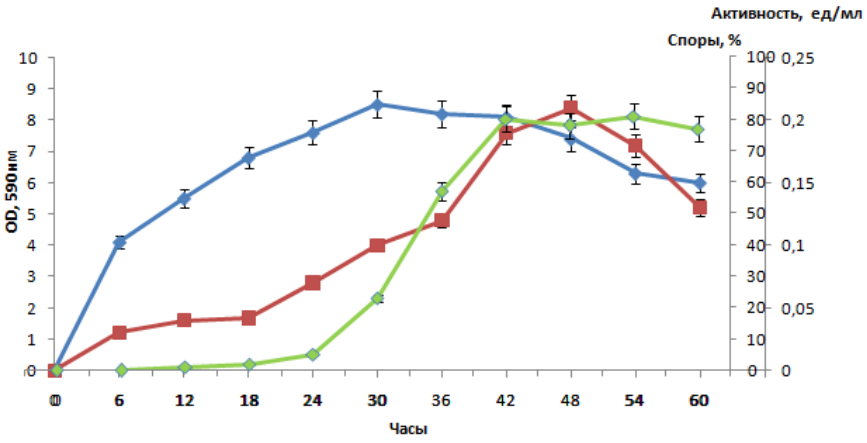

Figure 2: Growth (1), sporulation (2) and extracellular phytase activity (3) in the liquid culture of *B.ginsengihumi* M2.11.

Growth curve and extracellular phytase activity of *B.ginsengihumi* M2.11 on the liquid medium was studied (Figure 2). Phytase production was observed already on the 6th hour of growth and lasted during all growth phases.

Amplification of the phytase gene and optimization of PCR conditions

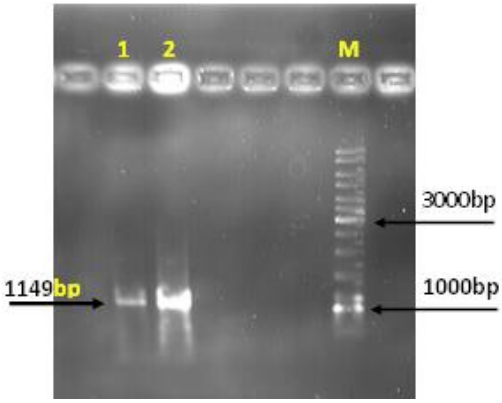

Figure 3: PCR amplification of the phytase gene from genomic DNA of *B.ginsengihumi* M2.11. 1 - The PCR product obtained using primers NCBI-dir / NCBI-rev, 2 - The PCR product obtained using primers ORF-dir / ORF-rev, M – marker DNA.

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Phytase genes of *Bacillus* genus are characterized with high sequence homology. Therefore, primers for *B.ginsengihumi* M2.11 phytase gene cloning were designed on the basis of *B.subtilis* 168 phytase gene (AN NC000964.3) sequence. Two primer pairs (NCBI-dir/NCBI-rev and ORF-dir/ORF-rev) were used to amplify the phytase gene from the genomic DNA of *B.ginsengihum*i M2.11. The size of PCR-products from both primer pairs was 1149 bp and represented the full phytase gene of *B.ginsengihumi* M2.11. The optimization of PCR conditions revealed that annealing temperatures of 58ºC and primer pair ORF-dir/ORF-rev showed the highest specificity of annealing (Figure 3) and were used in further experiments.

Amplification product of the phytase gene was purified and sequenced. Nucleotide sequence of the *B.ginsengihumi* M2.11 phytase gene was established – open reading frame started with the start ATG codon and ended with the stop-codon TAG. Sequence alignment with the phytase gene sequence of *B.subtilis* 168 showed 100% homology of the genes and confirmed the high degree of nucleotide conservation within the phytase genes of *Bacillus* genus.

Thus, we first determined the nucleotide sequence of the *B. ginsengihumi* M2.11 phytase gene, which showed high homology with the *B.subtilis* 168 phytase sequence.

Construction of recombinant expression vector with *B. ginsengihumi* M2.11 phytase gene

The PCR-product of *B. ginsengihumi* M2.11 phytase gene was cloned into the expression vector pET-46 Ek/LIC by the LIC-cloning method (cloning without ligase) (Figure 4). The LIC method uses the 3'→5' exonuclease activity of T4 DNA Polymerase to create specific single-stranded overhangs in the Ek/LIC vector. PCR products with complementary overhangs are created by building appropriate 5' extensions into the primers. Specific complementary DNA fragments were obtained by mixing the vector and the insert in the absence of ligase.

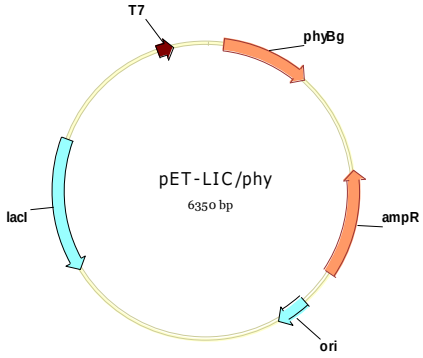

Figure 4: *pET-LIC/Phy* expression vector.

After the transformation of pET-LIC/Phy vector into the recipient *E.coli* DH5α strain the recombinant plasmids were isolated and analysed for the presence of the phytase gene insert (Figure 5). PCR-analysis revealed the presence of *B.ginsengihumi* M2.11 phytase gene insertion into the expression vector. Sequencing of the insert also confirmed the presence of the phytase gene in the vector.

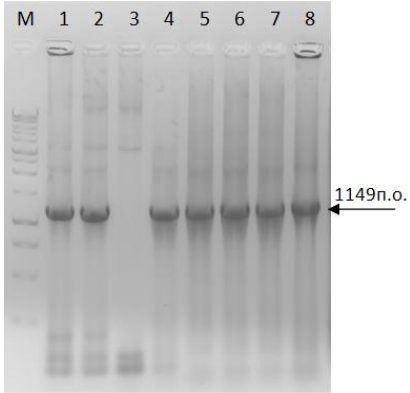

Figure 5: PCR-analysis of the recombinant plasmids. M – marker DNA; 1-8 – PCR-products from the plasmids using phy Phy-LIC fw/rev primer pair.

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pET-LIC/Phy vector was transformed into a commercial protease-free *Escherichia coli* Rosseta 2 (DE3) T1R strain carrying the chloramphenicol resistance gene. Transformants were grown on the specialized enriched growth medium TB supplemented with chloramphenicol and carbenicillin. After 24 hours of growth cells were harvested, disrupted with French-press and analyzed on SDS-PAGE-electrophoresis. Immunoblotting with His-tag antibodies was used to detect expression products (Figure 6).

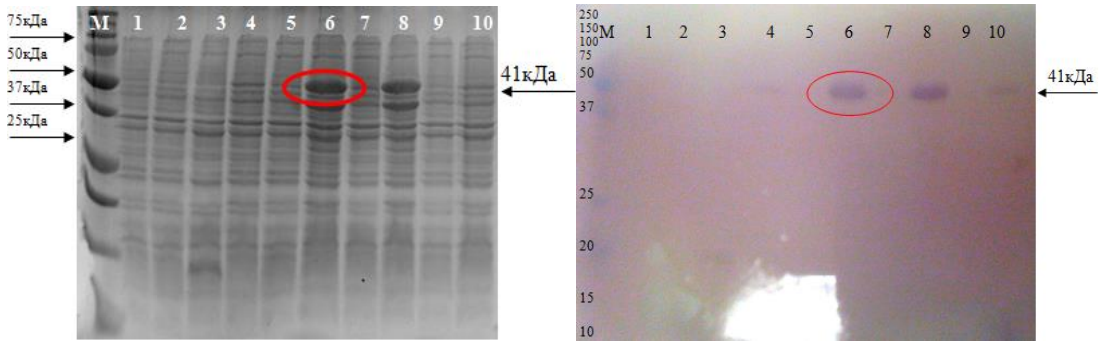

Figure 6: SDS-PAGE (A) and immunoblotting (B) of cell lysates of recombinant strain *E.coli* Rosseta *pET-LIC/Phy* on the 24th hour of cultivation. M – markers, 1–10 – protein products.

Among the products of expression we identified a protein with the molecular weight of 41 kDa (Figure 6). Results of immunoblotting also confirmed the presence of the protein with His-tag. Phytate-hydrolysing activity of recombinant cells lysate was 0.2 U/mg of biomass.

Thus, as a result of successful LIC-cloning of *B.ginsengihumi* M2.11 phytase gene efficient recombinant strain *E.coli* Rosseta pET-LIC/Phy6 producing phytate-hydrolyzing enzyme of bacilli, was obtained. This expression level will allow us to identify and study the phytate-hydrolyzing enzyme of isolated strain.

CONCLUSION

The original phytase feed enzymes used in livestock were produced mainly from fungi but recent developments in molecular biology and gene technology have resulted in bacterial phytases entering the market and there is suggestive evidence that bacterial phytases are more efficacious. Identification of the new phytase-producing bacteria *B.ginsengihumi* M2.11 and heterologous expression of its phytase gene in *E.coli* will allow to produce the enzyme on commercial scale with the potential application as a feed additive.

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