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Gene Cloning Studies of a Novel Phytase from *Bacillus megaterium* EBD 9-1 Strain

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Abstract
Phytases are hydrolytic enzymes which release inorganic phosphate by cleaving phosphate groups of phytate and attracted attention because of their usage in many industries providing benefit in eco-friendly way. Therefore, in this study we tried to clone the phytase gene from newly isolated *Bacillus megaterium* EBD 9-1 strain into the *E. coli* DH5α strain. The entire genome sequences of *Bacillus megaterium* DSM319, *Bacillus megaterium* QM B1551 and *Bacillus megaterium* WSH-002 strains are available in the GenBank database. However, a phytase sequence was not found in any of them. Thence, phytase sequences of different *Bacillus* species were aligned, and a degenerate primer pair was prepared. We tried to clone the phytase gene of the *Bacillus megaterium* EBD 9-1 strain using degenerate primers.We were able to amplify, clone and sequence 3 amplification products, but the sequencing results indicated that we could not clone the phytase gene. This study is important for literature because of being the first report on cloning studies of phytase from *Bacillus megaterium*.

Keywords: *Bacillus megaterium*, Phytase, Cloning

INTRODUCTION

Enzymes are significant macromolecules in living systems which play a role in almost every chemical reaction. Phytases are important enzymes which are used as eco-friendly feed additives and also used in food, pharmaceutical, paper industry over the last two decades. The phytase enzyme which was firstly literatured in 1907, is chemically known as myo-inositolhegzaphosphate phosphohydrolase [1]. Phytases release inorganic phosphate and lower phosphoric esters (reduced inositol phosphate esters) by cleaving one or more phosphate (orthophosphate) groups which linked to the inositol core of phytate [2]. Phytic acid, systematically named as myo-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate or myo-inositol hexakisphosphate, is an ester acid which consisting of myo-inositol core and six inorganic phosphates linked to it [3-5]. The salts of phytic acid are called phytate in another saying phytates are anionic forms of phytic acid. Phytin refers to the calcium, magnesium or potassium salts of phytic acid [6]. In plants, phytic acid is found in seed, grain, root, tuber, pollen and spore besides that, organic soils also contain phytic acid. In plants, phytin and phytate are found especially in seeds. Phytate is regarded as a storage form of phosphate in grains and legumes because the phytate comprises more than 80% of the phosphate in plant.

Phytic acid is considered to be both a nutritional and an anti-nutritional factor. For animals such as ruminants (e.g., cow, sheep, goats), which possess phytase producing flora for digesting phytic acid, phytate is a nutritional substance. On the other hand, for animals such as non-ruminants (e.g., pigs, poultry, fishs), which do not own phytase producing flora for digesting phytic acid, phytate is an anti-nutritional substance. Because this is evaluated as the retention of phosphorus in such a way that it can not be used [7]. Due to its chemical structure, the phytic acid contains six phosphate groups and is anionic, therefore at physiological (neutral) or basic pH, it binds to molecules such as cation, protein, amino acid, starch and also enzyme [8]. When phytic acid is negatively charged, it binds to divalent and trivalent cations (Zn^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Mg^{2+}, Mn^{2+}) and it forms insoluble salts or chelate compounds. Since proteins are positively charged at pH values below their isoelectric points, phytic acid binds to proteins with electrostatic attraction and it forms phytate-protein or phytate-mineral-protein complex. Besides that, it binds to proteolytic enzymes such as pepsin, trypsin, and chymotrypsin and decreases the activity of these enzymes [9]. Phytic acid also diminishes the digestibility of nutrients such as amino acid and starch by forming complexes with them and afterwards by preventing the formation of the enzyme-substrate complex. For all this reason, the existence of phytic acid causes a reduction in the availability of these minerals, proteins, nutrients, and enzymes. Also, it is very significant to maximize the utility of proteins and phosphorus in the rations. Because eutrophication is closely related to phosphorus. Using free phosphorus as fertilizer increases the phosphorus level of the rivers and ponds. This increase in organic nutrients in the freshwater disrupt ecological balance.

On the other hand, it is also reported that there are some positive effects besides the negative effects of phytic acid. Phytic acid is a strong natural antioxidant because it chelates with free iron, in this way, in the treatment of Alzheimer's and Parkinson disease, it increases the natural immune activity of the cells and reduces the heavy metal toxicity [10]. Because of its antimutagenic properties, it protects against various cancers (e.g., colon, breast, prostate, liver, pancreatic, blood and bone marrow) by affecting signal transduction pathways, cell cycle control genes, oncogenes and tumor suppressor genes [11]. Since it is hypolipidemic,

it inhibits arteriosclerosis and coronary heart disease. It also has the ability to prevent obesity, diabetes, tooth decay, clotting and also the formation of kidney stones [12]. Phytic acid is very important for being the source of myo-inositol phosphate. The main role of myo-inositol phosphate in the animal cell is to ensure the integrity of the cell membrane. İn addition, specific inositol phosphate mixtures have been used for rheumatoid arthritis, respiratory tract diseases (e.g. asthma) and as the analgesic [13]. Besides that, it has been shown that some esters of inositol-triphosphate have an inhibitory effect against retroviral infections, including HIV [14].

Phytase enzymes have been isolated from various bacteria (*Bacillus* sp., *E. coli*, *Pseudomonas* sp., *Citrobacter* sp.), fungus (*Aspergillus* sp., *Penicillium* sp.), yeasts (*Saccharomyces cerevisiae*, *Candida krusei*) and protozoans (*Paramecium* sp.). Nowadays, the most important source of phytase enzymes is *Aspergillus niger*. But, *Bacillus* phytases have been studied comprehensively because of their unique properties and, applicability in animal feed market [15]. The primary step of microbial enzyme production is the selection of proper microorganisms that have high enzyme activity. Another strategy that has gained favor for production of the enzyme, is optimizing the growth conditions. But the level of enzyme activity produced by an organism naturally is mostly unsatisfactory and needs to be boosted more for industrial production. This increase in enzyme production levels is often achieved by using recombinant organisms. There are many studies in the literature that have been made to improve phytase enzyme yield and to facilitate the purification and expression of phytase enzyme. The phytase gene has been isolated from many organisms and has been cloned into various host organisms and expressed [16]. Most genes encoding phytase enzymes have been isolated from *Aspergillus niger*, *Bacillus subtilis, Aspergillus fumigatus, Escherichia coli* and *Schwanniomyces occidentalis*. Mostly, bacteria (*E.coli, Lactobacillus* sp.), various strains of fungus (*Aspergillus* sp.), yeasts (*Pichia pastoris, Saccharomyces cerevisiae*) and, plants (*Nicotina tabacum, Medicago sativa*) have been used as the host organism for this studies [17].

In our previous studies, we screened phytase-producing *Bacillus* species in soil samples of Turkey, and have isolated a good producer. The effects of some physical and nutritional parameters were studied for the optimum production of phytase enzyme [18], afterwards, the enzyme was partially purified and biochemically characterized [19]. In the present paper, we report the cloning studies of phytase produced from a novel new isolate.

MATERIALS AND METHODS

Bacterial strain, vector, host, and culture conditions

Bacillus megaterium EBD 9-1 strain, which was isolated and named in our previous study, was used as novel phytase gene source [18, 19]. *Escherichia coli* strain DH5α was used for bacterial transformation and plasmid propagation. pGEM®-T Easy vector (Promega) was used for cloning PCR products.

Bacillus megaterium EBD 9-1 strain was cultured in Laura-Bertani medium in a shaking incubator (150 rpm) at 37 °C for 24h. Laura-Bertani medium was contained (% w v-1): tryptone 1; yeast extract 0.5; NaCl 1 (pH 7.0) [20]. The growth medium was sterilized by autoclaving at 121℃ for 40 minutes.

Isolation of genomic DNA

The overnight cultures that have 0.8-1.0 Optical Density at 600 nm wavelength were centrifuged (13000 rpm, 1 min.), and the pellets were used for isolation of genomic DNA. The genomic DNA of *Bacillus megaterium* EBD 9-1 was isolated using the G-spin™ Genomic DNA Extraction Kit [for bacteria] (Intron Biotechnology) as recommended by the manufacturer. The concentration and purity of isolated chromosomal DNA was measured using Nanodrop 2000 spectrophotometer. The absorbance values at 230, 260 and 280 nm wavelengths were monitored and the A260/280 and A260/230 ratios were calculated. Elution buffer in the genomic DNA extraction kit was used as blank. Integrity and size of chromosomal DNA were confirmed by electrophoresis on 0.8% (w v-1) agarose gel. The gel was run at 100V for 50 - 60 min. in 1X TBE buffer and visualized by ethidium bromide staining.

Phytase gene amplification by the PCR (Polymerase Chain Reaction)

Forward and reverse primers were tried to design based on the phytase gene sequence of *Bacillus megaterium* in the NCBI database. The entire genome sequence of *Bacillus megaterium* DSM319, *Bacillus megaterium* QM B1551 and *Bacillus megaterium* WSH-002 strains are available in the NCBI database, while, there was no sequence of *Bacillus megaterium* phytase gene. For this reason, phytase sequences of five different *Bacillus* species (*Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus siamensis* and *Bacillus atrophaeus*) were aligned, and a degenerate primer pairs were designed. Each PCR tube contained 320 ng genomic DNA as a template, 10X PCR buffer solution (75 mM Tris-HCl 25ºC, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01 % (v v-1) Tween20), 200 µM dNTP mixture, 1.5 mM $MgCl_2$, 1.25 Unit Taq DNA polymerase enzyme, 0.4 µmol forward primer and 0.4 µmol reverse primer. Reaction mixture final volume was completed to 25 µL using DNase/RNase-free deionized sterile water in each PCR tube, also the annealing temperature (Tm) was calculated for degenerate primer pair. The degenerate forward primer 5' - TAY CAC AGY CAR AAA ACM GG-3' and reverse primer 5' - ACR ATT TTR AAR TTY TGA TT- 3' were used in the following cycling conditions: 94 ºC for 5 min for 1 cycle, 94 ºC for 1 min, 40 ºC for 1 min, and 72ºC for 1 min for 34 cycles, and 72ºC for 5 min for 1 cycle. To control the PCR products' integrity and size, the PCR amplicons were separated on 1.5 % agarose (w v-1) gel at 100V for 120 min in 1X TBE buffer and visualized by ethidium bromide staining. Afterwards, the PCR amplicons were extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen). For determining of the concentration and purity of PCR amplicons, optical density values were measured at wavelengths of 230, 260 and 280 nm using Nanodrop 2000 spectrophotometer, and A260/280 and A260/230 ratios were calculated. Elution buffer in the genomic DNA extraction kit was used as blank.

Cloning of the phytase gene, and plasmids' isolation from transformants

After purification, the amplified DNA fragments were transferred into pGEM®-T Easy vector using ligation. Ligation was carried out using 3 µL PCR product (100 ng µL-1), 1 µL pGEM®-T Easy vector (50 ng µL-1) (Promega), 5 µL 2X ligase buffer solution (Promega) and 1µL T4 DNA ligase enzyme (3 U µL-1) (Promega). The ligation mixture was incubated overnight at 4°C. *E. coli* DH5α strain was used as hosts for transformation, for this purpose, competent cells have been prepared in accordance with the procedure

of Mandel and Higa [21]. Afterwards, *E. coli* DH5α competent cells were transformed with the ligated products by the heat shock method. Following transformation, phytase positive transformants were detected using blue/white screening technique. Transformed *E. coli* DH5α cells carrying recombinant plasmids were selected on LB broth agar plate ((% w v-1): tryptone 1; yeast extract 0.5; NaCl 1; agar 1.5 (pH 7.0)) which was containing also isopropyl-β-D-thiogalactopyranoside (IPTG) (24 µg mL-1), X-Gal (40 µg mL-1) and ampicillin (10 µg mL-1). The transformation was controlled by the growth of cells in ampicillin-containing medium and by the formation of blue colonies due to the using of X-Gal as substrate (activated β-Galactosidase activity by IPTG). After detection of positive transformants, white colored positive clones were selected and stocked.

Recombinant plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen) as recommended by the manufacturer. The concentration and purity of the recombinant plasmids were measured using Nanodrop 2000 spectrophotometer. The absorbance values at 230, 260 and 280 nm wavelengths were monitored and the A260/280 and A260/230 ratios were calculated. Elution buffer in the QIAprep Spin Miniprep Kit (Qiagen) was used as blank.

Screening of isolated recombinant plasmids and sequencing

To confirm the cloning, calculations were made according to the concentration of the isolated recombinant plasmid DNA, and recombinant plasmids were digested with EcoRI restriction endonuclease enzyme (New England Biolabs). 25 µL reaction mixture containing 1000 ng plasmid DNA, 1X cutsmart buffer (New England Biolabs), 10 Units EcoRI restriction enzyme (New England Biolabs). The reaction mixture was completed to 25 µl using DNase/RNase-free deionized sterile water and incubated at 37°C for 120 minutes and afterwards at 65°C for 20 minutes. In order to check the presence of expected lengths DNA fragments, digested plasmids were separated on 1.5% agarose (w v-1) gel at 100V for 120 min in 1X TBE buffer and visualized by ethidium bromide staining. The isolated plasmids were sequenced using the T7 primer and ABI PRISM-310 Genetic Analyzer (Applied Biosystems, CA, USA) apparatus.

RESULTS AND DISCUSSION

In our study, we attempted to clone the phytase gene of the new isolate *Bacillus megaterium* EBD 9-1 strain. The whole genome sequences of the *Bacillus megaterium* DSM319, *Bacillus megaterium* QM B1551 and *Bacillus megaterium* WSH-002 strains are available at the NCBI databank. However, no similar phytase sequence was found in any of the published sequences. To amplify the phytase gene of *Bacillus megaterium* EBD 9-1, we have aligned the phytase sequences of several *Bacillus* species (*Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus siamensis* and *Bacillus atrophaeus*) that are present in GenBank. Genomic DNA from strain was isolated according to the protocols outlined above. The concentration of the extracted genomic DNA was 160 ng µL-1 and was sufficiently pure. DNA extraction procedures for these methods were carried out in duplicate. Extracted chromosomal DNA was analyzed by electrophoretic methods and Figure 1(a) shows the completeness of DNA. The phytase gene was tried to be amplified by PCR using degenerate primer pairs. After the PCR product was electrophoresed on a 1.5% agarose gel, a band approximately 900bp and two bands approximately 500 bp were observed (Figure 1 (b)). The three bands eluted from the agarose gel showed the adequate purity based on the A260/230 and A260/280 ratios, and concentration of each three bands were measured approximately 98 ng µL-1.

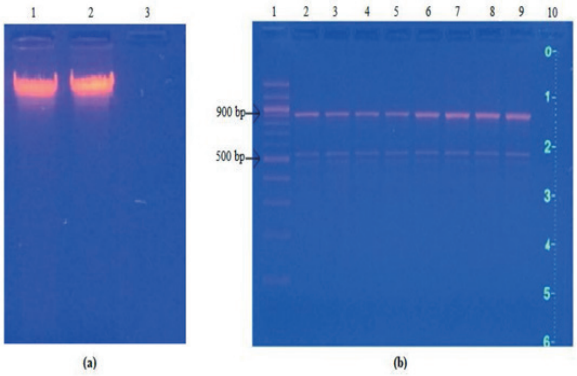


Figure 1. Results in agarose gel. (a) Extracted Genomic DNA from *Bacillus megaterium* EBD 9-1 strain. Lane 1 – 2: Extracted genomic DNA, Lane 3: No template control. (b) Amplification of phytase gene by PCR. Lane 1: 100 bp DNA Ladder, Lane 2 – 9: Phytase gene amplicons, Lane 10: No template control.

Three DNA fragments were ligated with pGEM®-T Easy vector digested with the same restriction endonuclease enzyme (EcoRI). The ligation mixture was used to transform competent *E.coli* DH5α strain cells. As a result, positive clones, which were colored white because of losing the ability to produce beta-galactosidase enzyme, were selected. To confirm the presence of the recombinant plasmid in the transformed cells, the plasmid DNA was extracted from the competent *E.coli* DH5α strain cells, and concentration and purity of samples were found to be appropriate. The presence of the insert in the recombinant plasmid was confirmed by restriction-digestion of the recombinant plasmid with EcoRI restriction endonuclease enzyme, and subsequently by observing the bands of the expected size on agarose gel (Figure 2).

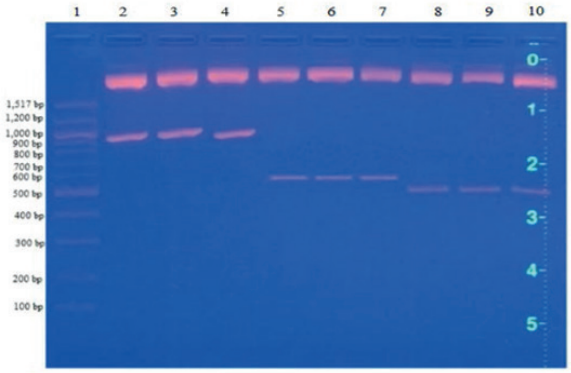


Figure 2. Electrophoretic analysis of EcoRI-cleaved plasmids. Lane 1: 100 bp DNA Ladder, Lane 2 – 4: pGEM®-T Easy/900 bp DNA fragment, Lane 5-7: pGEM®-T Easy/550 bp DNA fragment, Lane 8-10: pGEM®-T Easy/500 bp DNA fragment.

The three amplification products were sequenced to verify the identity of the clones' plasmids, and the sequences of the amplified DNA fragment's were compared with the GenBank database (NCBI) using BLAST (Basic Local Alignment Search Tool). Sequenced DNA fragments did not show any similarity to the phytase sequences deposited in GenBank database. But, 758 base pairs long cloned DNA

fragment showed similarity to the reported carbon starvation protein gene (cstA) of *Bacillus megaterium* QM B1551, *Bacillus megaterium* DSM319 and *Bacillus megaterium* WSH-002 strains. The other two cloned DNA fragments, which were 473 base pairs long, showed similarity to the reported *Bacillus megaterium* arabinose operon regulatory protein (araC) gene sequence and also, certain gas vesicle protein gene (gvpT) sequences of *Bacillus megaterium* QM B1551, *Bacillus megaterium* DSM319, *Bacillus megaterium* WSH-002 strains. In short, although there was phytase activity in our new isolate, we could not clone the phytase gene.

The gene that encodes the phytase enzyme has been isolated primarily from *Aspergillus niger*, *Bacillus subtilis*, *Aspergillus fumigatus*, *Escherichia coli* and *Schwanniomyces occidentalis* [16]. There are many organisms, which are recombinant phytase producers and also hosts for recombinant phytase production [17]. Phytase derived from *Bacillus* sp. was cloned into and expressed in *E. coli* [22, 23], *Lactobacillus plantarum* [24] and *B. subtilis* [22]. The phytase gene from *Aspergillus* sp. was cloned into and expressed in *E. coli* [25], *Pichia pastoris* [26], *Saccharomyces cerevisiae* [27], *Nicotina tabacum* [28] and *Alfalfa* [29]. The phytase gene isolated from *Schwanniomyces occidentalis* was cloned into and expressed in *Candida boidinii* [30]. The phytase genes from *Peniophora lycii*, *Agrocybe pediades*, *Cf. Ceriporia* sp. and *Trametes pubescens* were cloned into and expressed in *Aspergillus oryzae* [31]. Animals can also be used as the host for expression of the phytase gene. For example, the isolated *E. coli* phytase gene was cloned into and expressed in the mouse and pig [32, 33]. Additionally, plants have been sources for cloning studies for example, the phytase gene from *Zea mays* was cloned into and expressed in *E. coli* [34]. And also an *E. coli* acid phosphatase gene with phytase activity was cloned into pBBRT plasmid and transformed into *Pseudomonas putida* [35].

In this paper, using the designed degenerate primers, we were able to amplify, clone and sequence three amplification products, but the sequencing results showed that we could not clone the phytase gene from our new isolate *Bacillus megaterium* EBD 9-1 strain. Based on our knowledge, there is no study to achieve to cloning of the phytase gene from *Bacillus megaterium*, and there is no similar phytase sequence was found in any of the published sequences. For this reason, this study is important for literature.

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