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Comparative Analysis of Peripheral Alkaline Phytase Protein Structures Expressed in *E. coli*

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Abstract

Background: Degradation of phytic acid to inorganic phosphate in domestic animals' diets requires thermostable phytase. Although *Basillus subtilis* phytase shows a potential to be degraded phytate complex in high temperature, the enzyme activities and yields need to be increased to make them possible for industrial application.
Methods: The phytase gene from *Bacillus subtilis* DR8886 was isolated from Dig Rostam hot mineral spring in Iran and cloned into pET21(+) and pET32(+). Expression was induced with 1.5 mM IPTG and the proteins were purified.
Results: The recombinant protein affected by thioredoxin (Trx) from pET32a-PhyC was estimated to constitute about 31% of the total soluble protein in the cells; its concentration was 3.5 µg/ml, and its maximal phytase activity was 15.9 U/ml, whereas the recombinant phytase from pET21a-PhyC was estimated to comprise about 19% of the total soluble protein; its concentration was 2.2 µg/ml, and its maximal phytase activity was 69 U/ml. The molecular masses of recombinant phytase with and without Trx were about 60 kDa and 42 kDa, respectively. Zymography confirmed that the recombinant enzymes were active. Although the concentration of the alkaline phytase expressed by pET32a was approximately 59% greater than that expressed by pET21, its phytase activity was approximately 77% less.
Conclusion: This study showed that the peripheral gene (Trx) encoded by the pET32a (+) vector are the principal reason for the decrease in recombinant phytase enzyme activity.

Keywords: *Bacillus subtilis* DR8806, PhyC Gene, Thioredoxin

Introduction

Phytase enzymes are powerful tools for recycling phosphorus (P) from phytate (myo-inositol hexakisphosphate) (1). Phytic acid, a polyanionic chelating agent, serves as an anti-nutrient by chelating the divalent cations Zn, Fe, Cu, Mg, and Ca, making them unavailable for absorption in the intestines of monogastric animals, which include poultry, swine, and fish (2). Further complementation by inorganic phosphate does not decrease the anti-nutritive effect of phytic acid and leads to increased phosphorus contamination in eutrophication of surface waters by defecation of copious amounts of P in the manure (3). Among the four classes of known phytases, the beta-propy l phytase class (BPP) is widely identified in soil, plants, and bacteria (1). The only group present in aquatic bacteria, BPP, referred to as alkaline phytase, releases P from phytate at alkaline pH (4, 5). BPPs were first identified in the Gram-positive Bacillus species (6). Bacillus spp. and pollens of some plants such as Typha latifolia and Lilium longixorum can produce phytase (7, 8). The X-ray analysis of BPP from Bacillus amyloliquefaciens revealed a six-bladed β propeller structure along with the crucial amino acid residues involved in the conformation of two phosphate- and six calcium-binding sites (9). Therefore, BPP requires Ca2+ for

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catalytic activity and displays strict substrate specificity for the calcium-phytate complex (10). Bacillus spp. are of great biotechnological interest for their thermal stability in feed-pelleting to give the myo-inositol triphosphate isomers Ins(2,4,6)P3 and Ins(1,3,5)P3 (2), which are rare compounds with pharmaceutical importance. B. subtilis has "generally recognized as safe" (GRAS) status, allowing the use of its expressed proteins in pharmaceutical applications. Although B. subtilis phytase offers the potential to degrade phytate complexes, the enzyme's activity and yield need to be increased to make them suitable for industrial applications. For this reason, it is important to isolate a range of microorganisms and their enzymes for phytate degradation (5) and to identify the most efficient expression system. *Escherichia coli* (*E. coli)* are commonly used to produce a variety of recombinant proteins. Trx is a gene fusion expression system that uses thioredoxin as the fusion partner. The Trx system is especially beneficial to enhance production of soluble fusion proteins in the cytoplasm of *E. coli*. In most instances, heterologous proteins produced as thioredoxin fusions are folded properly and demonstrate full biological activity (16). In this study, we outline the cloning, sequencing, and expression of thermostable alkaline phytase from a newly isolated B. subtilis DR8806, which was previously isolated from the Dig Rostam hot mineral spring (Coordinates 32°17′00″N 57°30′00″E) in Iran (17). Furthermore, biochemical properties of recombinant phytases were characterized and phytase activities from the proteins expressed from the pET21 and pET32 vectors were compared to evaluate the influence of Trx on protein activity.

Materials and Methods

Bacterial strains, plasmids and media

B. subtilis DR8806 (acquisition number: IBRCM10742, Iran), kindly provided by Asoodeh (Ferdowsi University of Mashhad, Iran), was used as the source of the phytase gene (*PhyC*). The isolated strain was identified as Bacillus strain based on the phenotypic characteristics and phylogenetic analysis of the 16SrDNA sequence (17). *E. coli* DH5α and BL21 (DE3) were used as hosts for cloning and expression. Plasmid pTZ57 R/T (Thermo, Maryland, USA) was manipulated as a vector for cloning and nucleotide sequencing, and plasmids pET21a (+) and pET32a (+) (Novagen, USA) were used for overexpression. The *E. coli* and *B. Subtilis* DR8806 were grown in Luria Broth (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl).

Identification of Bacillus subtilis DR8806

Genomic DNA from B. subtilis DR8806 was extracted in accordance with (18). The 16S rRNA gene of the isolate was amplified and sequenced using forward primer F (5' TGGCGGCGTGCCTAATACAT 3') and reverse primer R (5' CGGGTGTTACAAACTCTCGTGGT 3'). The 16S rRNA sequence homology searches in GenBank were performed using the BLAST server.

PCR amplification of the phytase gene

The phytase gene (phyC) was amplified from genomic DNA using forward primer phyCOF (5' GTGCACGTTCATAAAAGGAGGAAG 3') and reverse primer phyCOR (5' TTGCATGTTTATTTTCCGCTTCT 3'), which were designed based on the phyC sequences of the closest organisms identified by a 16S rRNA sequence similarity search. Polymerase chain reaction (PCR) was carried out using 2.5 µl of Pfu DNA polymerase buffer (Thermo Scientific, USA), 1.25 units of Pfu DNA polymerase (Thermo Scientific, USA), 1µg of genomic DNA, 2.5 µl of dNTPs, and 0.25 µM of primers in a total volume of 25 µl. Amplification of the phyC region was accomplished with initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation, annealing, and extension for 1 min at 95 °C, 61 °C, and 72 °C, respectively, and the final extension was carried out at 72 °C for 10 min. Poly-A addition was performed using 1 U of Taq polymerase and dATP for 45 min at 72 °C. The purified poly-A-tailed product was cloned into the InsT/A cloning vector pTZ57 R/T and used to transform *E. coli* DH5α competent cells. After colony PCR screening, recombinant plasmids were purified from the insert-positive clones by the alkaline lysis method and sequenced.

Molecular Gene Cloning

The mature phytase gene (phyC) was amplified by PCR from the recombinant plasmid pTZ57R/T using linker primers phyLF (5'CGGGATCCCGCCTGTCCGATCCTTATCAT TTTACCG3') and phyLR

(5'GCCCAAGCTTGGGTTATTTTCCGCTTCTG TCGGTCAGT3'). The resultant PCR product contained the open reading frame of phyC with a BamHI restriction site at the 5' end and a HindIII restriction site at the 3' end. The purified PCR product was double digested with BamHI and HindIII, and cloned into the expression vectors pET21a(+) and pET32a(+), which were previously digested with the same enzymes. *E. coli* BL21 (DE3) cells were transformed with the recombinant plasmids.

Expression and purification of Recombinant Phytases

E. coli cells carrying recombinant pET21a (+) and pET32a (+) were cultivated overnight in LB broth medium at 37 ◦C with shaking at 180 rpm. Then, 200 ml of overnight culture was inoculated into 4L of LB broth containing 100 µg/ml ampicillin and grown at 37°C until the optical density (OD) at 600 nm was reached. To induce expression, isopropyl β-D-thiogalactoside (IPTG) was added to the culture broth to a final concentration of 1.5 mM and the culture was incubated 5 h at 37 ◦C. The induced cells were collected by centrifugation at 12,000 × g and 4 °C for 20 min and buffer A (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole and 1 mM PMSF, pH: 8.0) was added, followed by 6 cycles of intermittent sonication at 25 W for 30 s. The cell lysate was centrifuged at 12,000 × g and 4 °C for 20 min and the supernatant, containing the soluble recombinant protein, was collected. Both the soluble and insoluble recombinant proteins were assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The clear supernatant was loaded onto a pre-equilibrated Ni2+ -NTA affinity column (Bio-Rad, CA, USA) and washed with buffer B (50 mM NaH2PO4, 300 mM NaCl, and 20 mM imidazole, pH 8.0). Target proteins were eluted with buffer C (50 mM NaH2PO4, 300 mM NaCl, and 250 mM imidazole, pH 8.0). All purification steps were performed at 4 ◦C according to the instructions provided by Novagen, USA.

Phytase activity assay

Enzyme activity assays were performed on cytoplasmic fractions according to the Novagen pET system manual. The elution buffer samples obtained from the Ni2+-NTA affinity column were assayed for phytase activity by incubating 150 µl of the concentrated protein with 600 µl of the substrate solution (0.2% w/v sodium phytate (Sigma, St Louis, MO, USA) in 0.1 M sodium acetate buffer, pH 5.0), for 30 min at 39 ◦C (19). A 750 µl aliquot of 5% (w/v) trichloroacetic acid solution was added to stop the reaction. 6 ml of color reagent (1.5% (w/v) ammonium molybdate, 5.5% (v/v) sulfuric acid solution, and 2.7% (w/v) ferrous sulfate) were added to 1.5 ml of the sample solution. The production of phosphomolybdate was measured spectrophotometrically at 700 nm. Results were compared to a standard curve prepared with inorganic phosphate (KH2PO4). One unit of phytase activity was defined as the amount of the enzyme that hydrolyzed 1 mol of phosphate per minute under the assay conditions (5).

Electrophoresis and zymogram analysis

The purity and molecular masses of the recombinant phytases were analyzed by SDS-PAGE. One hundred µl samples were mixed with 20 µl of sample loading buffer (62.5 mM Tris buffer, pH 6.8 containing 0.05% bromphenol blue, 0.72 M 2-mercaptoethanol, 10% glycerol, and 2% sodium dodecyl sulfate) according to (20). Samples were heated at 100 °C for 10 min. The denatured samples were electrophoresed by SDS-PAGE on 12% separating and 4% stacking gels (20). The zymogram was performed in accordance with (21). Briefly, gels were shaken in 1% Triton X-100 for 1 h at room temperature and then in 0.1 M sodium acetate buffer pH 5.0 for 1 h at 37 ºC. Phytase activity was detected by incubating the gels for 16 h in 0.1 M sodium acetate buffer pH 5.0 containing 0.4% (w/v) sodium phytate. Gels were stained with 2% (w/v) aqueous cobalt chloride for 5 min at room temperature, the cobalt chloride solution was decanted, and a freshly-prepared solution containing equal volumes of 6.25% (w/v) aqueous ammonium molybdate and 0.42% (w/v) ammonium vanadate was added. Phytase activity was visualized as zones of clearing in an opaque background.

Results

Isolation and cloning of phyC

The 1,407 nucleotide 16s rRNA gene of the isolate displayed 99% similarity to sequences from B. subtilis, B. amyloliquefaciens, and B. velezensis. The 1188-bp phyC PCR product was ligated into a pTZ57 R/T vector and its nucleotide sequence was analyzed. The presence of phyC was confirmed by colony PCR, and the linker primer from a recombinant pTZ57 R/T

vector was used to amplify the mature sequence of the phytase gene. Bioinformatics analysis from the nucleotide sequence revealed an 1149-bp open reading frame (ORF), starting with an ATG initiation codon at base 90 and extending to base 1242 (Fig. 1). The primary amino acid sequence deduced from the nucleotide sequence of phyC revealed a fragment of 383 amino acids. According to (4), the putative signal peptide cleavage site is located between residues 26 and 27. It is believed that residues 27 to 29 might be analogous to a pro-peptide, but three amino acids would be very short for a pro-peptide. Kim et al. suggested that the phytase gene contains a 30-amino acid signal sequence and a cleavage site between Leu-30 and Ser-31 (22). Signal software predicted a 29-amino acid signal peptide. The mature peptide sequence lacked RHGXRXP, a conserved motif at the catalytic site of acidic phytases, similar to our previous report (23). The sequence of phyC, its deduced amino acid sequence, putative -35 and -10 sequences, a ribosomal binding site, and a transcription terminator are shown in Fig. 1.

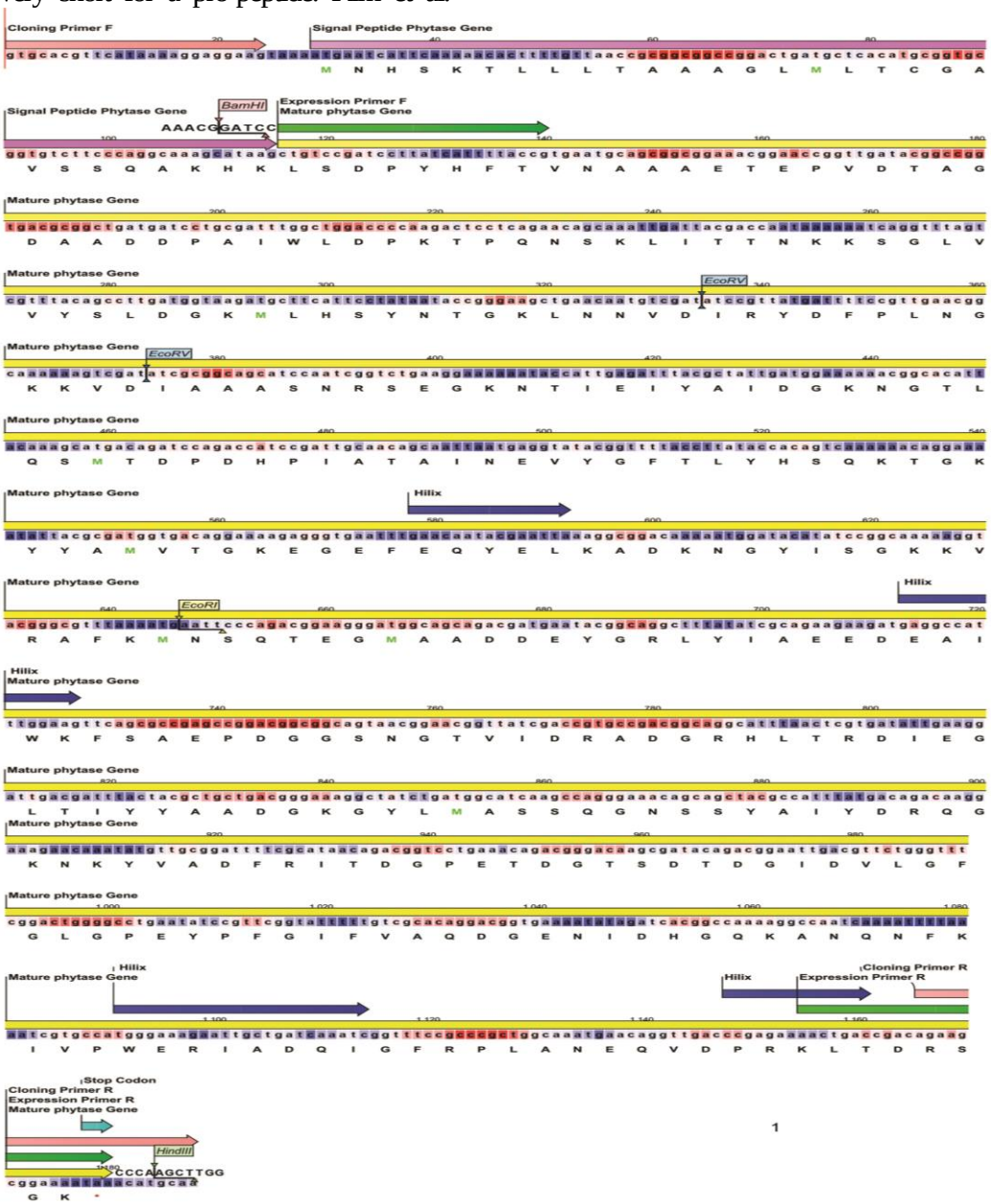

Fig. 1. Phytase gene characterization and primer binding sites in *Bacillus subtilis* DR8806. The violet arrow shows the native signal peptide. The deduced amino acid sequence of phytase was determined by bioinformatics analysis.

Overexpression of phyC

pET32a(+) was used because solubility can also be influenced by the vector, cloning sites, or host cells (15). A mature phytase gene containing BamHI and HindIII restriction sites was double digested and inserted between the BamHI and HindIII restriction sites at the multiple cloning site of pET21a(+) and pET32a(+).The structures of pET21-phyC and pET32-phyC are shown in Fig. 2. PhyC was under the control of the T7 promoter and fused downstream of Trx in pET32a(+). The ligation mixtures were used to transform *E. coli* BL21 (DE3) cells and the resulting library was screened by colony PCR and digested with BamHI and HindIII (Fig. 2).

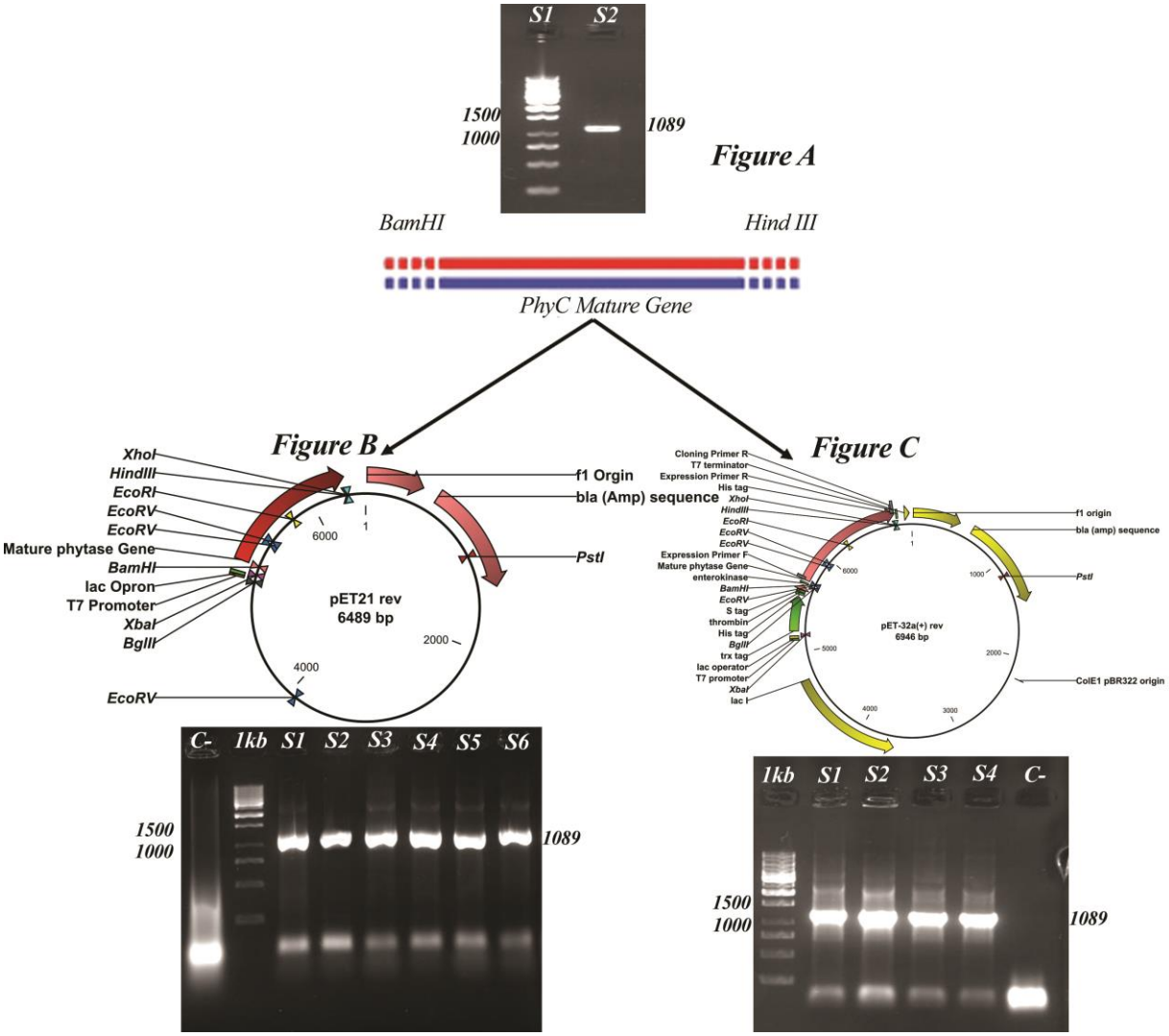

Fig. 2. Isolation and cloning of *phyC* gene pathways. A: Electrophoresis of *phyC* PCR product on 1% agarose gel, S2: *phyC* PCR product. B: Cloning of phyC in pET21 (+), S1-S6: Colony PCR of *phyC*. C: Cloning of phyC in pET32 (+), S1-S4: Colony PCR of *phyC* PCR product. B: Cloning of phyC in pET21 (+), S1-S6: Colony PCR of phyC. C: Cloning of phyC in pET32 (+), S1-S4: Colony PCR of phyC.

Recombinant phytase Properties

The pET32a-*PhyC* construction facilitated the expression of phytase with the Trx leader peptide at the N-terminus, which is expected to be responsible for facilitating the export of the target protein to the periplasmic space. The recombinant constructs without the leader peptide (pET21-*phyC*) resulted in most of the phytase being insoluble, but activity was detected in the culture. The concentrations of the recombinant proteins extracted from pET32a-*phyC* and pET21a-*phyC* constructs were 3.5 and 2.2 µg/ml, respectively. The maximal phytase activities for phytase with and without Trx of 15.9 and 69 U/ml were both obtained

about 5 h after induction at pH 7. The reduced activity seems to be due to the fusion protein's complete release into the medium, although some degradation by intracellular proteases cannot be ruled out. According to (12), there was a lag phase of 4 h in the induction using lactose during fed-batch cultivation followed by an increase in the phytase activity to 71 U/ml during the same period as IPTG-induced production. For the pET32a-*phyC* construction, the recombinant phytase was estimated to constitute about 31% of the total soluble protein in the cells, while the recombinant phytase from the pET21a-*phyC* construction was estimated to be about 19% of the total soluble protein. The apparent molecular masses of phytase with and without Trx were 59 and 42 kDa, respectively. The calculated molecular mass of phytase based on the deduced amino acid sequence is 39 kDa (Fig. 2).

Phytase zymogram

Phytase-active protein bands may be identified using non-specific phosphatase chromogenic substrates (25); however, this process is limited due to poor substrate specificity. Phytase proteins extracted from recombinant *E. coli* were resolved on standard gels, renatured, and incubated with sodium phytate. After exposure of the phytate-infused gels to the counterstaining reagents, insoluble phytate complexes formed where phytate was present. Zones of clearing were observed where active phytase bands were present (Fig. 4). This counterstaining method has proven applicable for phytase screening on polyacrylamide gels.

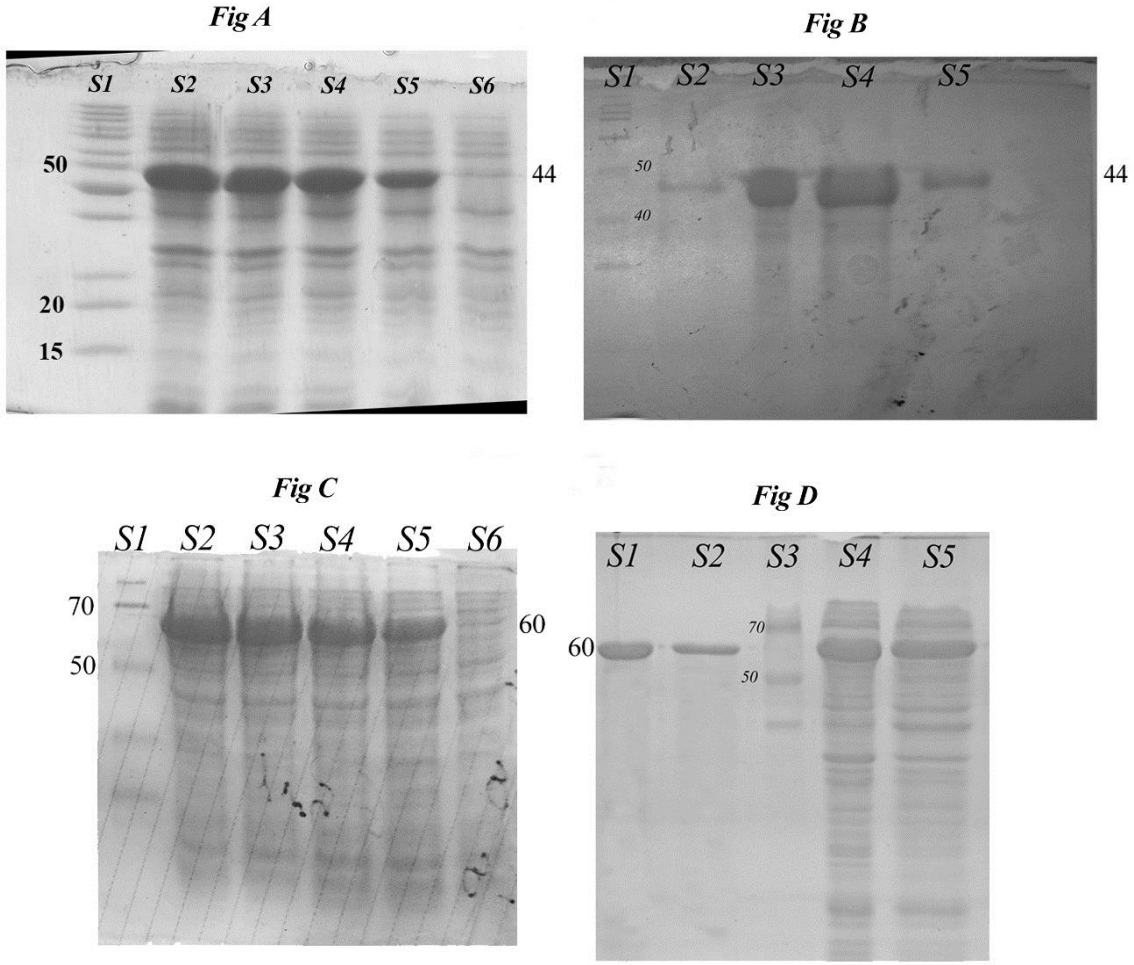

Fig. 3. SDS-polyacrylamide gel electrophoresis analysis of the recombinant phytase. Fig A: Lane S1: molecular mass markers, lane S2-S6 cell extracts from *E. coli* BL21 (DE3) contained recombinant pET21a(+) cultured for 4, 3,2,1,0 h after 1.5 mM IPTG induction. Fig B: Lane S1: molecular mass markers, lane S2-S5: purified fraction of recombinant protein expressed in pET21a (+) following affinity purification. Fig C: Lane S1: molecular mass markers, lane S2-S6 cell extracts from *E. coli* BL21 (DE3) contained recombinant pET32 a(+) cultured for 4, 3,2,1,0 h after 1.5 mM IPTG induction. Fig D: Lane S1-S2 purified fraction of recombinant protein expressed in pET21a (+) following affinity purification, S3: molecular mass markers, lane S4-S5: cell extracts from lysis solution from purified fraction.

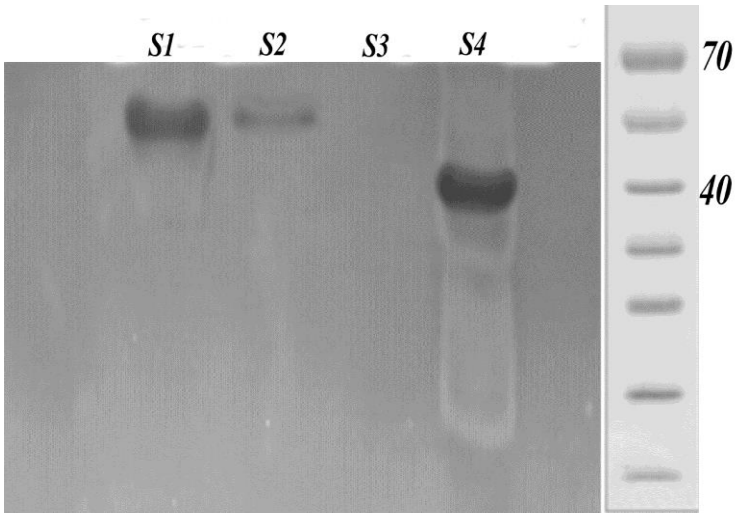

Fig. 4. Zymogram of recombinant proteins. S1 and S2: purified fraction of recombinant protein expressed in pET32 a (+) following affinity purification. S3: Negative control, S4: purified fraction of recombinant protein expressed in pET21a (+) following affinity purification. Zones of clearing show phytase activity.

Discussion

To date, all Bacillus sp. tested have been shown to possess thermostable enzymes (4, 22). The enzymes of B. subtilis found in soil were active at temperatures between 30 and 90◦ C, with maximum activity at 37◦ C (12). In this study, we report for the first time the production of recombinant thermostable phytase from B. subtilis isolated from Dig Rostam hot mineral spring. The pH of the spring water samples was 9.7 ± 0.15 and their temperature was 88 ± 1.5 ◦C (25). For industrial application, the enzyme activities, thermostability, and yields need to be increased; therefore, it is desirable to isolate the phytase gene from species of B. subtilis that are able to grow at 88 ◦C. It is expected that the produced recombinant protein will be active at temperatures above 90 °C. Pelleting and animal feeding studies are now planned to establish that the enzyme is more suited to in-feed application than current commercial products.

Recombinant gene expression in *E. coli* is convenient and relatively inexpensive. However, production of recombinant proteins in bacterial hosts, especially *E. coli*, can be problematic due to insolubility, purification difficulties, degradation, and misfolding. Proteins expressed in the pET32a system contain almost 22 kDa of extra amino acids encoded by the expression vector to increase solubility and prevent protein toxicity. The additional sequence includes the Trx-tag of 109 amino acids, which increases the solubility of the expressed protein, the S- and His 6-tags, used for purification, and a linker sequence. The recombinant phytase was found to be catalytically active despite the presence of these additional amino acids in pET32a(+) (16). In another report, it was shown that the enzyme activity of expressed phytase with the native signal peptide and Trx was 7.7 U/ml (24). In our study, while the addition of Trx led to an increase in gene expression, activity of the fusion protein was less than that of recombinant phytase alone. The protein produced in pET21 was approximately 4.5-fold more active than the protein produced in pET32a(+), suggesting that the presence of Trx inhibited enzyme activity. It has been shown that reducing disulfide bonds in Trx fusion proteins can increase solubility; however, the reduced protein might be inactive (15). Our study demonstrated that peripheral proteins were not required for alkaline phytase production in *E. coli*, and *B. subtilis* from a hot mineral spring was a good choice for the target enzyme.

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