Cloning and Expression of *Escherichia coli* K13 Phytase Gene (*appA13*) Isolated from Seawater

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A bacterial strain was isolated from seawater to screen for phytase activities. A colony had the highest activity and was identified as an *Escherichia coli* strain. Using primers derived from *E. coli* acid phosphatase *appA* sequence, we cloned a 1,495 bp DNA fragment connected with the pGEM-T vector. It was over-expressed under *lac* promoter combined with its native promoter in *E. coli* DH5α. The expression of the phytase gene occurred during late exponential growth and the intracellular phytase production was 16.9 units/mℓ. The yield of recombinant phytate was 412-fold higher than that of wild type *E. coli* K13.

Key words: *Escherichia coli*, Phytate, Phytase, Expression

Introduction

Phytate (*myo*-inositol 1,2,3,4,5,6-hexakisdihydrogen phosphate) is the principal storage form of phosphorus in plants, such as cereal grains and legumes. It typically represents approximately 75-80% of the total phosphorus found in nature. Phytases (*myo*-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8 for 3-phytase and EC 3.1.3.26 for 6-phytase) catalyze the hydrolysis of phytate, thereby releasing inorganic phosphate. Since the digestive tracts of monogastrics including fish, pig, and poultry lack phytase, phosphate of phytate in their feeds can not be used, and thus inorganic phosphorus has to be added to secure sufficient phosphate supply (Reddy et al., 1982). As a result, phytate and phosphate are excreted in manure, causing environmental problems in areas of intensive livestock production. Furthermore, phytate is viewed as an antinutritional factor because it interacts with essential dietary minerals and proteins, thus limiting the nutritional value of cereals and legumes (Rimbach et al., 1994). So, the enzymatic

hydrolysis of phytate into the less-phosphorylated inositol derivatives in the intestine of monogastics is desirable. Although plant seeds such as wheat, bean, and maize have some phytase activity (Gibsin and Ullah, 1988), microorganisms may be a more feasible source of the enzyme. Phytase genes have been isolated from fungi including *Aspergillus niger* (van Hartingseldt et al., 1993), *Emericella nidulans* and *Talaromyces thermophilus* (Pasamontes et al., 1997), and *Myceliophthora thermophila* (Mitchell et al., 1997). Phytases have also been identified from bacteria such as *Eschericia coli* (Greiner et al., 1993), *Aerobacter aerogenes* (Greaves et al., 1967), *Pseudomonas* sp. (Irving and Cosgrove, 1971), *Bacillus* sp. (Kim et al., 1998a), and *Enterobacter* sp. (Yoon et al., 1996). But among them, the gene was only identified from *E. coli* and *Bacillus* sp. (Dassa et al., 1990, Rodriguez et al., 1999, Kim et al., 1998b).

In the present study, we isolated an *E. coli* strain from seawater that produced relatively high phytase activity. A DNA fragment encoding phytase was amplified by PCR from the strain, cloned and sequenced. Also it was overexpressed in *E. coli* DH5α.

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Materials and Methods

Isolation and identification of phytase-producing bacteria

Seawater taken near Busan was spread onto phytase-screening plates (PSM) containing 1.5% D-glucose, 0.5% calcium phytate, 0.5% NH_4NO_3, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot H_2O$ and 1.5% agar adjusted to pH 7.0 and incubated at 30°C for 24 h. The colonies with clear zones around were selected and grown further at 30°C for 24 h with shaking in a PSM or marine medium. The culture supernatant and cell extract were taken by centrifugation and assayed at 37°C for phytase activity. Morphological and physiological characteristics, and partial 16S rDNA sequences of the isolated colony were determined by standard procedures.

Phytase assay

Phytase activity was assayed by measuring the rate of increase of inorganic orthophosphate (Pi), using the ascorbic acid method of Fiske and Subbarow (1925). A reaction mixture containing 100 $\mu\ell$ enzyme preparation and 400 $\mu\ell$ of 2 mM sodium phytate in 0.1 M sodium-acetate buffer (pH 5.0) was incubated at 37°C for 15 min. The reaction was stopped by adding 500 $\mu\ell$ of 15% trichloroacetic acid. The released inorganic phosphate was measured by incubation with 4 mℓ of reagent A (1:1:1 :2 ratio of 6 N H_2SO_4/ 2.5% ammonium molybdate/ 10% ascorbic acid/ H_2O) at 37°C. After 30 min, the absorbance at 820 nm was measured. One unit of phytase activity was defined as the amount of enzyme required to liberate one μ mole of phosphate per min under the assay condition. Acid phosphatase activity was assayed using p-nitrophenylphosphate as described by Ullah and Gibson (1986) and the release of p-nitrophenol was calculated by measuring the absorbance at 400 nm.

Bacterial strain, plasmid and media

E. coli DH5α was used as host for transformation. Plasmid pGEM-T (Promega, USA) was used as a vector for cloning and nucleotide sequencing. *E. coli* DH5α was cultured in LB broth (1% trypton, 0.5% yeast extract, 0.5% NaCl) or on LB agar plate. When needed, ampicillin was added at a con-

centration of 50 μg/mℓ.

DNA amplification and sequencing

Because the colony that produced the highest phytase activity was identified as an *E. coli* strain, we used primers derived from the DNA sequence of *E. coli* pH 2.5 acid phosphatase gene (*appA,* GeneBank Acession number M58708) to isolate the phytase gene. Primers f1 (forward: 1−22): 5′-TAAGGAGCAGAAACAATGTGG-T-3′, r1 (reverse; 1474−1495): 5′-GGGGTACCTTACAAACTGCACG-3′ were purchased from the Takara company. The whole sequence was amplified using f1 and r1 primers. The PCR reaction was performed by the GeneAmp PCR system 9700 (Perkin Elmer, Norwalk, CT). DNA sequencing was performed with the Big Dye DNA sequencing Kit and ABI PRISM genetic analyzer 310 (Perkin-Elmer Co., USA.) according to the manufacturer's instructions, using universal and internal synthetic primers.

Electrophoretic analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% acrylamide gel. Proteins were stained with Coomassie brilliant blue R-250. Phytase activity bands on SDS-PAGE gel were detected by modifying the method of van Hartingsveldt et al. (1993). Electrophoresis was performed by the standard procedure. Subsequently, the gel was washed two times for 30 min each in 0.1 M sodium acetate buffer (pH 5.0) containing 25% isopropanol, and then two times for 30 min each in the same buffer without isopropanol. The gel was then incubated for 30 min at 37°C in the following solution (0.2% α-naphtylphosphate, 0.1% Fast Garnet GBC Salts, 1 mM Na-phytate, and 0.5 M sodium acetate buffer, pH 5.0). When a dark brown color appeared, the solution was decanted and the gel was washed with water.

Results and Discussion

Isolation and identification of phytase-producing bacteria

Phytase-producing bacterial strains were isolated from seawater samples near Busan using a selective media (PSM). Eight strains showed phytase activity

and finally the highest phytase-producing strain, K 13 (41 U/mℓ) was selected. This strain was a gram negative bacteria with high catalase activity. This strain could grow under unaerobic conditions. Table 1 shows the result of biochemical tests for this strain done as described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Table 1. Biochemical characteristics of strain K13

Characteristics	Result
Gram staining	−
Enzyme activity of	
β-galactosidase	+
Arginine dihydrolase	−
Lysine decarboxylase	+
Ornithin decarboxylase	+
Urease	−
Tryptophan deaminase	−
Cytochrome oxidase	−
Gelatinase	−
Citrate utilization	−
H₂S production	−
Indole production	+
Acetone production (V-P test)	−
Acid form	
Glucose	+
Mannitol	+
Inositol	−
Sorbitol	+
Rhamnose	+
Sucrose	+
Melibiose	+
Amygdalin	−
Arabinose	+

+, positive reaction; −, negative reaction.

On the basis of partial 16S rDNA sequence (data not shown) and biochemical characteristics, strain K13 was identified as *E. coli,* and named as *E. coli* K13.

Cloning and sequencing of the K13 phytase

PCR was performed with designed primers by using genomic DNA of the isolated *E. coli* K13 as the template. A 1,495 bp amplified fragment was cloned into pGEM-T vector and sequenced. This fragment designated as *appA13,* encoded a protein of 432 amino acids (Fig. 1). The upstream region of *appA13* contains sequences of hexanucleotides which, although not typical, resemble −35 and −10 promoter sequences (TTAGCA and TATAAT, res-

pectively). A Shine Dalgarno-like sequence (AA-GCGG) could be found at a reasonable distance from the ATG initiation codon of *appA13.* The deduced peptide contained both the N-terminal motif (RHGXRXP, position: 38−44) and C-terminal motif (HD, position; 325−326), which is characteristic for histidine acid phosphatases (Wodzinski and Ullah, 1996). Many fungal phytases and *E. coli* acid phosphatase belong to histidine acid phosphatase (Mitchell et al., 1997). Substrate specificity and the kinetic parameters, however, showed that the *E. coli* acid phosphatase should be designated as a phytase (Greiner et al., 1993). To date, two *E. coli* phytase genes, *appA* and *appA2,* have been reported by Dassa et al. (1990) and Rodriguez et al. (1999), respectively, with 95% of sequence homology. Among the available sequence databases, *appA13* shared some homology with *E. coli appA* and *Bacillus* sp. DS11 phytase gene (Kim et al., 1998b) (98% and 47% in nucleotide sequence, respectively). The only difference between *appA* and *appA13* is an exchange of several amino acids: two within the signal peptide and two in the coding region. Compared to *A. niger* phytase *phyA* gene (van Hartingseldt et al., 1993), the deduced amino acid sequence of *appA13* had only a 19% homology.

Expression of *appA13* in *E. coli*

The 1,495 bp PCR fragment encoding phytase was cloned into a pGEM-T vector, and transformed it in *E. coli* DH5α. It was observed that the transformants on the plate incubated at 37℃ showed different colony morphology. To clarify this reason, we prepared the plasmids and analyzed them. When the phytase gene and its promoter were in the opposite direction of *lac* promoter, the transformants grew well and their colony morphology was normal but the phytase was less expressed. In contrast, when a recombinant plasmid pAP13 was constructed, which consists of the phytase gene and its promoter under *lac* promoter, the transformants appeared ill-formed and pale as though collapsed by phage infection. These features seem to be due to high production of phytase encoded by a foreign gene in *E. coli.* The expression of the recombinant phytase was attained by decreasing the growth temperature. That means, the transformants grew well

(f1)

TAAGGAGCAGAAACAATGTGGTATTTACTTTGGTTCGTTGGCATTTTGTTGATGTGTTCGCTCTCCACCCTTGTG 75

TTGGTATGGCTGGACCCGCGATTGAAAAGTTAACGAACGTAAGTCTGATGTGGCGCATTAGCGTCGCATCAGGCA 150
                                                          -35

AATCAATAATGTCGGATATGAAAAGCGGAAACATATCG ATGAAAGCGATCTTGCTCCCATTTTTATCTCTTCTG 224
       -10   SD   M K A I L L P F L S L L   12
ATTCCGTTAACCCCGCAATCTACATTCGCTCAGAGTGAGCCGGAACTGAAGCTGGAAAGTGTGGTGATTGTCAGT 299
I P L T P Q S T F A Q S E P E L K L E S V V I V S   37
CGTCATGGTGTGCGTGCACCAACCAAGGCCACGCAACTGATGCAGGATGTCACCCCAGACGCATGGCCAACCTGG 374
F H G V R A P T K A T Q L M Q D V T P D A W P T W   62
CCGGTAAAACTGGGTTGGCTGACACCGCGCGGTGGTGAGCTAATCGCCTATCTCGGACATTACCAACGCCAGCGT 449
F V K L G W L T P R G G E L I A Y L G H Y Q R Q R   87
CTGGTAGCCGACGGATTGCTGGCGAAAAAGGGCTGCCCGCAGTCTGGTCAGGTCGCGATTATTGCTGATGTCGAC 524
I V A D G L L A K K G C P Q S G Q V A I I A D V D   112
GAGCGTACCCGTAAAACAGGCGAAGCCTTCGCCGCCGGGCTGGCACCTGACTGTGCAATAACCGTACATACCCAG 599
E R T R K T G E A F A A G L A P D C A I T V H T Q   137
CCAGATACGTCCAGTCCCGATCCGTTATTTAATCCTCTAAAAACTGGCGTTTGCCAACTGGATAACGCGAACGTG 674
A D T S S P D P L F N P L K T G V C Q L D N A N V   162
ACTGACGCGATCCTCAGCAGGGCAGGAGGGTCAATTGCTGACTTTACCGGGCATCGGCAAACGGCGTTTCGCGAA 749
T D A I L S R A G G S I A D F T G H R Q T A F R E   187
CTGGAACGGGTGCTTAATTTTTCCGCAATCAAACTTGTGCCTTAACCGTGAGAAACAGGACGAAAGCTGTTCATTA 824
L E R V L N F P Q S N L C L N R E K Q D E S C S L   212
ACGCAGGCATTACCATCGGAACTCAAGGTGAGCGCCGACAATGTCTCATTAACCGGTGCGGTAAGCCTCGCATCA 899
T Q A L P S E L K V S A D N V S L T G A V S L A S   237
ATGCTGACGGAAATATTTCTCCTGCAACAAGCACAGGGAATGCCGGAGCCGGGGTGGGGAAGGATCACTGATTCA 974
M L T E I F L L Q Q A Q G M P E P G W G R I T D S   262
CACCAGTGGAACACGTTGCTAAGTTTGCATAACGCGCAATTTTTATTTACTACAACGCACGCCAGAGGTTGCCCGC 1049
H Q W N T L L S L H N A Q F Y L L Q R T P E V A R   287
AGCCGCGCCACCCCGTTGTTGGATTTGATCATGACAGCGTTGACGCCCCATCCACCGCAAAAACAGGCGTATGGT 1124
S R A T P L L D L I M T A L T P H P P Q K Q A Y G   312
CTGACATTACCCACTTCAGTGCTGTTTATCGCCGGACACGATACTAATCTGGCAAATCTCGGCGGCGCACTGGAG 1199
V T L P T S V L F I A G H D T N L A N L G G A L E   337
CTCAACTGGACGCTTCCAGGTCAGCCGGATAACACGCCGCCAGGTGGTGAACTGGTGTTTGAACGCTGGCGTCGG 1274
L N W T L P G Q P D N T P P G G E L V F E R W R R   362
CTAAGCGATAACAGCCAGTGGATTCAGGTTTCGCTGGTCTTCCAGACTTTACAGCAGATGCGTGATAAAACGCCG 1349
L S D N S Q W I Q V S L V F Q T L Q Q M R D K T P   387
CTGTCATTAAATACGCCGCCCGGAGAGGTGAAACTGACCCTGGCAGGATGTGAAGAGCGAAATGCGCAGGGCATG 1424
L S L N T P P G E V K L T L A G C E E R N A Q G M   412
TGTTCGTTGGCCGGTTTTACGCAAATCGTGAATGAAGCACGCATACCGGCGTGCAGTTTGTAAGGTACCCC 1495
C S L A G F T Q I V N E A R I P A C S L * (r1)   433

Fig. 1. Nucleotide sequence of the *appA13* gene and its deduced amino acid sequence. The underlined sequences are the primers used to amplify K13 phytase gene. Putative Shine-Dalgarno sequences are indicated (SD), as are −35 and −10 promoter-like sequences for *appA13*. Putative signal sequence is underlined. The conserved regions related to the enzymatic activity sites are boxed.

at 30℃ and their colony morphology was normal. In addition, the phytase gene was high-expressed. The expression of recombinant phytase and cell growth was observed throughout the culture time in LB medium with 50 μg/mℓ ampicillin (Fig. 2). The intracellular phytase production reached the highest
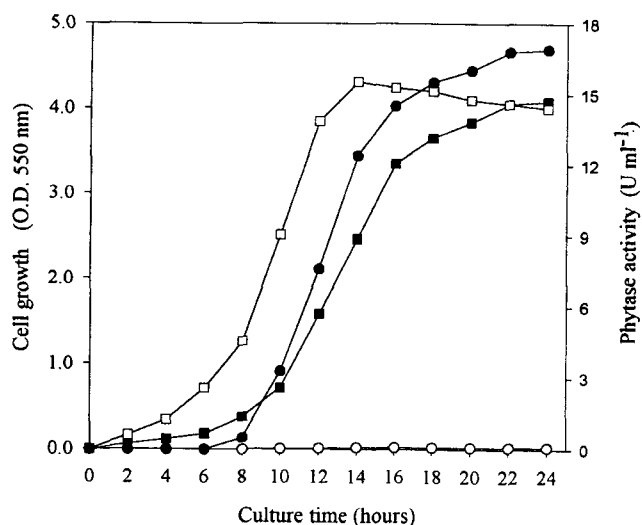
Fig. 2. Cell growth and phytase expression of *E. coli* DH5α (pAP13) in LB medium. The strain was grown at 30℃ in LB medium with 50 μg/mℓ ampicillin and its growth together with intercellular enzyme activity were measured periodically. Growth (■) and enzyme production (●) by *E. coli* DH5α (pAP13); growth (□) and enzyme production (○) by *E. coli* DH5α as a control.

level of 16.9 units/mℓ when the cell growth reached A_{550} nm = 4.08, which was 412-fold higher than that of the wild type strain *E. coli* K13. Also, phytase production was very stable even after 24 h incubation and remained constant up to 36 h. When the plasmids from transformants in both 30℃ and 37℃ were analyzed, they were not modified and remained intact (data not shown).

On SDS-PAGE and zymogram, a molecular mass of the expressed phytase was shown to be 43 kDa (Fig. 3), well corresponding to the sizes of other *E. coli* phytases ranging from 42 – 45 kDa (Greiner et al., 1993, Rodriguez et al., 1999). It was found that proteolytic processing of the cloned enzyme within the *E. coli* cell did not occur since only a single band appeared in the activity staining gel.

Some enzymatic properties of the recombinant phytase

To investigate the substrate specificity of the recombinant enzyme, we tested various phosphorylated compounds including *p*-nitrophenyl phosphate, a general substrate for acid phosphatase. The en-
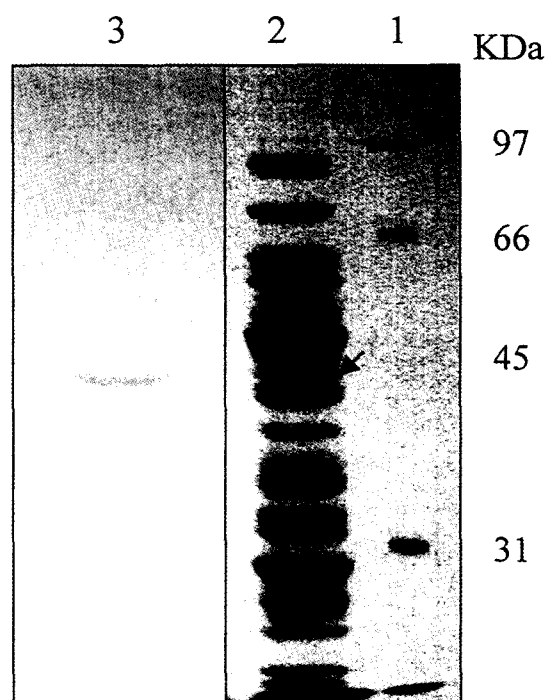

Fig. 3. SDS-PAGE analysis and activity staining of the recombinant phytase from *E. coli* DH5 α (pAP13). The *E. coli* cells were grown for 16 h at 30℃ in LB medium with 50 μg/mℓ ampicillin. Lane 1, molecular weight marker; Lane 2, cell extract from *E. coli* DH5α (pAP13); Lane 3, activity staining of SDS-PAGE.

Table 2. Substrate specifity of *E. coli* K13 phytase

Substrates (2 mM)	Relative activity (%)
Phytate	100
p-nitrophenylphosphate	11.2
Tetrasodium pyrophosphate	5.4
ATP	0.5
ADP	0.4
Glycerophosphate	1.9
Glucose-1-phosphate	0.3
Glucose-6-phosphate	0.2
Fructose-6-phosphate	0
Mannose-6-phosphate	0

zyme had high activity for phytate but a little or no activity on other substrates (Table 2). The effects of pH and temperature on the activity of recombinant phytase were examined using sodium phytate as a substrate. The enzyme had an optimum pH of 4.5 and was fairly stable at pH values ranging from 3.5 to 8.0 (Fig. 4). This was very similar to *E. coli* phytase (Greiner et al., 1993) and *A. niger* phytase
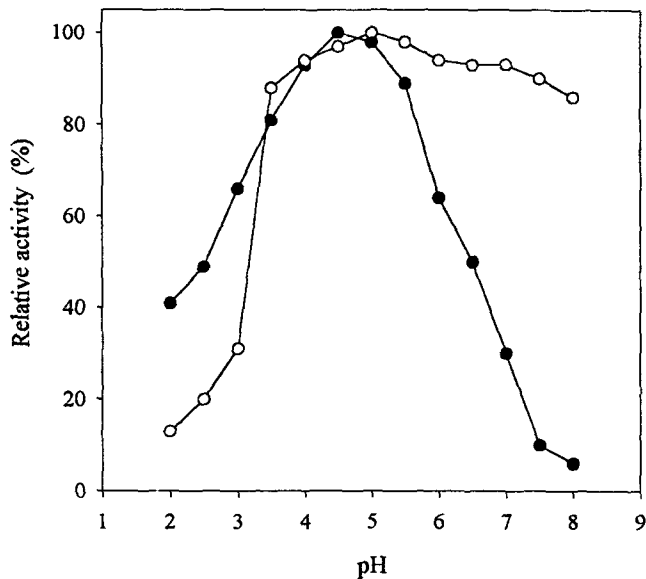
Fig. 4. Effects of pH on phytase activity and stability. The enzyme was assayed at various pHs (●). For the pH stability, the enzyme was preincubated at various pH buffers for 2 h and the remaining activity was measured as standard assay (○). The buffers used as follows: glycine-HCl (pH 2.0—3.5), sodium acetate-acetic acid (pH 3.5—6.0), Tris-acetic acid (pH 6.0—7.0) and Tris-HCl (pH 7.0—9.0). All buffers were used at 0.1 M for enzyme assay.

Fig. 5. Effects of temperature on phytase activi-ty and stability. The enzyme was assayed at various temperatures in 0.1 M sodium acetate buffer (pH 5.0) (●). For the temperature stability, the enzyme was preincubated at various temperatures for 30 min and the remaining activity was measured as standard assay (○).

(van Hartingseldt et al., 1993). The enzyme activity was measured at various temperatures ranging from 30℃ to 70℃. Its optimum temperature was 60℃ and a sharp decrease in activity was observed at 70℃ (Fig. 5). The reported optimum temperatures were 50—60℃ for *Enterobacter* sp. 4 phytase (Yoon et al., 1996), 58℃ for fungal phytase (Yamada et al., 1968) and 60℃ fo: *Bacillus subtilis*(*natto*) N-77 (Shimizu, 1992). The enzyme was stable up to 50℃ for 30 min and it retained 60% of its activity at 55℃.

The properties of *E. coli* phytase including the low pH optimum, high substrate specificity for phytate and high specific activity have been demonstrated that this enzyme is a good candidate for industrial production as a feed additive enzyme. Thus, overexpression of the enzyme has been attempted by various expression systems. However, when this enzyme was produced by *E. coli* expression system such as pET system, recombinant enzyme accumulated as insoluble inactive aggregates known as inclusion bodies. In the present study, we
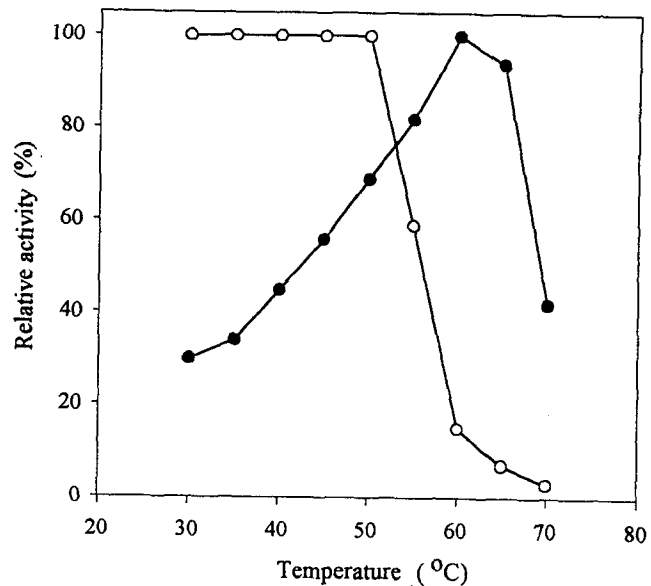
overexprssed the soluble active *E. coli* K13 phytase under *lac* promoter without addition of IPTG, which is *lac* promoter inducer, into *E. coli* DH5α. This may be due to an efficient transciption by the *lac* promoter combined with its native promoter. Phytase production was increased to over 16.9 units/mℓ and the yield was 412-fold higher than that of wild type *E. coli* K13.

Therefore, the recombinant *E. coli* strain transformed by this plasmid, pAP13, seems to be a promising candidate for industrial applications.

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