Cloning of *Bacillus subtilis* phytase gene construct in *Escherichia coli*

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**ABSTRACT**

**Background and Objectives:** Phytase has a hydrolysis function of phytic acid, which yields inorganic phosphate. *Bacillus* species can produce thermostable alkaline phytase. The aim of this study was to isolate and clone a *Phytase* gene (*Phy*) from *Bacillus subtilis* in *Escherichia coli*.

**Materials and Methods:** In this study, the extracellular *PhyC* gene was isolated from *Bacillus subtilis Phytase C*. After purification of the bands, DNA fragment of *Phy* gene was cloned by T/A cloning technique, and the clone was transformed into *Escherichia coli*. Afterward, the pGEM-Phy was transferred into *E. coli* Top-10 strain and the recombinants were plated on LB agar containing 100 µg/ml ampicillin. The colonization of 1171 bp of gene *Phytase C* was confirmed by PCR. The presence of gene-targeting in vector was confirmed with enzymatic digestion by *XhoI* and *XbaI* restriction enzymes.

**Results:** The Phytase gene was successfully cloned in *E. coli*. The result of cloning of 1171 bp *Phytase* gene was confirmed by PCR assay.

**Conclusion:** Our impression of this article is that several methods, such as using along with microbial, plant phytase reproduction, or low-phytic acid corn may be the better way from a single phytase.

**Keywords:** *Bacillus subtilis*; Cloning; *Escherichia coli*; Phytase; Probiotics

**INTRODUCTION**

Phytate (Myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate), is the major source of inositol (1, 2) and the main storage form of phosphorus (P), typically accounting for 60-90% of the total P content of oilseed crops, cereals and legumes (3). Phytate acts as an antinutrient factor since it causes mineral deficiency by chief dietary minerals such as, Fe²⁺, Zn²⁺, Mg²⁺, and Ca²⁺ (4). Making these complexes is basically non absorption from the human gastrointestinal tract (5) and monogastric animals, such as poultry, pig and fish (6). Therefore, this creates problems in the usability of P in their meal (7). Suzuki et al. dis-
covered this enzyme in 1907 (8). Phytases, myo-inositol hexakisphosphate hydrolases, are a specific class of phosphatases (9) which are capable of hydrolysis of Myo-inositol- (1,2,3,4,5,6)-hexakisphosphate and inorganic P (10). Phytase releases at least one phosphate from phytate (11); this is supposing as a principal metabolic process in many microscopic organisms (1). Phytate remains in monogastric-beast-derived dung causing serious P pollution, contributing to accumulate of water resource (1). Today, enhancement of public concern regarding the environmental effect of high P levels in animal excrement has driven phytase usage in animal diet and the biotechnological importance of phytase (12, 13).

Phytases are generally widespread in nature, for example, it discovered in plants, animal (14, 15), and in microorganisms (16, 17). Most of the methodical work has been done on organism’s phytases, mainly on those originating from filamentous fungi such as Aspergillus ficuum (18), A. fumigatus (19), and Cladosporium species (20); yeasts phytases like Schwanniomyces occidentalis (21), Pichia anomala (14); Gram-Positive bacteria such as Bacillus subtilis (22); and gram-negative bacteria such as Escherichia coli (23), Pseudomonas bacteria (24), Klebsiella spp. (25). Overview of bacterial phytase genes by genetic engineering has enhanced the bioavailability of numerous inorganic nutrients (26). Choice dietary enrichment of animal feed and the expulsion problem of P pollution opened up bright prospects for the study on this enzyme.

Now, the main source of organisms important for the production of phytase is bacteria isolated from various sources (27), such as Bacillus phytase. This kind of enzyme has been studied widely, because these kinds of phytase have unique features, and also the feasibility of their mass production for applicability in animal nutrition (28). Bacillus subtilis is a Gram-positive, rod-shaped and spore-forming bacteria (29) that produces many secondary metabolites (30) such as subtilin (31), α-amylase (32), phytase, and nattokinase (33). This organism is not considered pathogenic and (34) the optimal temperature is 25-35°C (11).

Numerous phytase genes have been successfully cloned in several bacterial hosts, transgenic maize, and pigs. E. coli is often selected as a primary host microorganism for the production of this enzyme (35). In 2003, it has been reported that the expression of Bacillus phytase in E. coli is amounted to about 20% of the soluble proteins (24). In the present study, we aimed to clone a novel phytase gene (PhyC) from Bacillus subtilis in E. coli for future extracellular phytase production.

MATERIALS AND METHODS

Bacterial strains and plasmids. B. subtilis was used as a source of chromosomal DNA. Standard strain of B. subtilis bacteria was prepared in the Department of Microbiology Pasteur Institute of Iran and was grown at 37°C in Luria Bertani broth (LB broth) and LB agar plates. Medium was supplemented with ampicillin (100 mg/ml). Grown colonies were biochemically confirmed as B. subtilis positive.

Bacillus subtilis was grown on Luria-Bertani (LB) agar plate at 28°C for 24 h. E. coli strain Top10F', pGEM-T easy vector (Invitrogen, San Diego, CA) was used for TA cloning (using TA cloning kit (Promega, U.S)) and pET32 vector (was used for subcloning (Novagen, Germany)) were cultured in LB broth medium overnight at 37°C.

DNA extraction. Genomic DNA was isolated from bacterial colonies using DNA extraction kit (DNPTM, Cinna Gen, Iran,) according to manufacturer’s instruction. The quality and quantity of final extracted DNA were checked on 1% agarose gel electrophoresis stained with ethidium bromide and Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) at a wavelength of 230, 260 and 280 nm (36).

Gene amplification. In Escherichia coli the ompF gene encodes a major outer membrane porin protein that is differentially regulated by the OmpR protein. OmpR acts as a positive as well as a negative regulator of ompF expression by binding to DNA sequences in the ompF promoter region. Set of primers used for PCR reaction of upstream and downstream regions ompF and PhyC genes of Bacillus subtilis are listed in Table 1. The amplification was done using Thermal Cycler (Mastercycler Gradient, Eppendorf, Germany), in the final reaction volume of 25 µl. The PCR mixture consisted of 1 µg of DNA samples, 1 µM of each primer, 200 µM dNTPs, 200 µM MgCl₂, 1 U of Smart Taq DNA polymerase (Fermentas, Germany) and 2.5 µl of 10× PCR buffer (37). Amplification was performed in a thermal cycler and initiated with a pri-
Table 1. Specific oligonucleotide primers and annealing temperature

| Primer name | Sequence* | TM (°C) |
|-------------|-----------|---------|
| ompF-up     | F: 5'-ATGTCTAGAAGAGATTTTGTGCAGG-3'  
R: 5'-CGTGGTACCTTTTATACCTCCATGG-3' | 61°C |
| PhyC        | F: 5'-TCTAGGTACCAGTATCTCAAAAAACAC-3'  
R: 5'-ATTGAGCTTATTTTCCGCCTGTACGTGTC-3' | 61°C |
| ompF-down   | F: 5'-TGACAGCTCCCTTGGTAGTTGGTG-3'  
R: 5'-ATGCCTCGAGTGTGTTGAAGTAGTAGG-3' | 61°C |

*The underlined sequence are Restriction enzyme sites

mary denaturation step at 95°C for 5 min, followed by 32 cycles of 94°C for 1 min, 61°C for 1 min and 72°C for 1 min. The program was followed by a final extension at 72°C for 5 min.

**Evaluation of PCR products.** The PCR products were run on 1% agarose gel and visualized by ethidium bromide staining. Electrophoresis buffer was TBE [Tris-base 10.8 g, 89 mm, Boric acid 5.5 g, 2 mm and EDTA 4 ml of 0.5 M EDTA (pH 8.0)]. The Constant voltage of 85V for 30 min was used for product separation. After electrophoresis, images were obtained in UVI doc gel documentation systems (UK). The amplified fragments were purified with gel extraction kit (Bioneer Co., Korea), according to the manufacturer’s protocol.

**T/A cloning.** The amplified products were cloned in pGEM-T easy vector (Promega Co.) and the resulting recombinant plasmids were used to transform into competent *E. coli* strain Top10F* (Escherichia coli* Top10F competent cells (Stratagene) was employed as the host for gene cloning and transformed cells were grown in LB medium with 100 μg/mL ampicillin) in Luria Bertani (LB) Medium (Merek KGaA, Germany). *E. coli* colonies carrying the recombinant vector were selected on LB medium with ampicillin (100 mg/mL). The presence of amplified *PhyC* and upstream and downstream regions of *OmpF* was confirmed by restriction enzyme analysis. Digestion and transformation procedures were performed according to the manufacturer’s instructions.

**Subcloning of the PhyC, downstream and upstream ompF and construction of expression vector pET32.** The 384 bp fragment of the upstream *OmpF* gene from the recombinant plasmid (pGEM- *OmpF*-up) expression vector was also confirmed by digestion with *XbaI* and *KpnI* enzymes. Also, cloning vector (pET32) were digested with *XbaI* and *KpnI* and then ligated to generate the recombinant plasmid (pET32- *OmpF*-up). After preparation, the plasmid was transformed under heat shock (42°C) and calcium chloride (CaCl₂) for the 90 seconds into *E. coli* strain Top10F*. Extraction and purification of subcloned plasmids were done using 1.5% agarose gel electrophoresis using purification kit (Bioneer, South Korea) according to the manufacturer’s instructions. All steps were performed for the 385 bp fragment of downstream *ompF* and a 1171 bp fragment of *PhyC* genes, that were digested with *SacI/Xhol* and *KpnI/SacI*, respectively. cloning vector (pET32) was introduced with T4 ligase to construct the recombinant plasmid (pET32- *ompF*-up) using a T/A cloning kit (DNA ligation kit mighty mix, TaKaRa). *Escherichia coli* Top10F competent cells (Stratagene) was employed as the host for gene cloning and transformed cells were grown in LB medium with 100 μg/mL ampicillin. The detail protocol of cloning and transformation was followed as described previously (36, 37).

**RESULTS**

**Gene amplification.** Results of PCR-amplified products for *PhyC* and the andupstream and downstream regions of *ompF* are shown in Fig. 1. The results showed that the *B. subtilis* and *E. coli* contained *PhyC* and upstream and downstream regions of *ompF*, respectively.

**T/A cloning.** The recombinant plasmid was transformed into competent cells in LB medium containing ampicillin, then cloned contained *ompF*-up and *ompF*-down regions and *PhyC* gene was digested...
with Xbal/KpnI, SacI/XhoI and KpnI/SacI, respectively (Fig. 2).

**Subcloning of the PhyC gene.** OmpF gene regions and PhyC gene, which has the restriction points of Xbal/ KpnI, SacI/XhoI and KpnI/SacI were inserted in the polyclonal site (PCS) in the pET32 plasmid. TOP10F competent cells were used for transformation and culturing in LB medium containing ampicillin. The results of the pET32 vector contained ompF-up, PhyC and ompF-down genes were digest-ed with Xbal/KpnI, KpnI/SacI and SacI/XhoI, respectively (Fig. 3A). The gene constructs ompF-up-PhyC-ompF-down digested with Xbal/XhoI restriction enzyme, to finally confirm subcloning (Fig. 3B).

**DISCUSSION**

Public knowledge of the environmental impact of organic agriculture has led to legislation that extends the measure of phosphate in the animal excretory product in certain parts of the world, and will likely be increased in other parts of the world in the near future. Under these circumstances, phytase will be extensively used in animal diets to improve phytate-P bioavailability and decrease P expulsion. The global market of phytase as an animal nourish additive is predictable to be 500 million dollars. The important movement has been made in the phytase investigation during the past 20 years. Transgenic mice expressing microbial phytase have been newly developed as a testable sample to work the usefulness of exogenous phytase expression (38).

Our systematic awareness of phytase has yet to profit a solution to meet its vast nourishing and environmental request. More investigation is needed into discovering novel phytases (39), although phytase as a single source of P has induced this generation. This finding proposes that this generation is induced only when inorganic phosphate is a limiting factor (40). Engineering improved phytases based on three-dimensional structure, and developing more cost-effective expression systems should be continued.

The PCR amplified 1171 base pair DNA fragment corresponding to the PhyC gene from *B. subtilis* was cloned using T/A cloning vector. Successful cloning was achieved in transforming *E. coli* host selected on ampicillin-containing medium. The clones were confirmed using colony PCR and restriction digestion of positive clones with Xbal/XhoI restriction enzyme, to final confirmed subcloning.

Considering catalytic properties, it is extremely desirable to have an operating system for the production of *E. coli* phytase for some reasons. First, the pH optimum is set more in the acid range than that of the frequently used *B. subtilis* phytases. Thus, the high activity of *E. coli* phytase can be used more excellently in the acidic environments of the digestive of simple-stomached animals (38). Another reason,
E. coli phytase has the maximum specific activity of all phytases tested so far. Third, the E. coli phytase in contrast to B. subtilis phytase, is resistant to protolithic degradation in the intestines. This article focused on numerous phytases that had been from multiple sources rather than the searches of the basic factors affecting variability in phytase reaction. An essential material in respect of phytate and phytase is lacking in many parts, which needs to be combined and produced for a more complete conception of this topic. The original phytase feed enzymes were produced generally from fungi. But recent expansions in the production of enzymes in other forms of microorganisms, such as yeast and bacteria, have led to new exogenous phytases (41).

In the current study, the results are in agreement with the results of earlier studies in different nations. In 2008, Rao et al. isolated and cloned a novel Phyc from Bacillus subtilis in E. coli; to recover the active enzyme from inclusion bodies, and to describe the recombinant Phyc (1). However, the kind of Phyc was produced at slower rate levels (42). In 2009 Ozusaglam and Ozcan reported the phytase from B. subtilis VTT E-68013 was not expressed in B. coagulans. Though, this was the first report to our knowledge that Phyc was searched to clone in B. coagulans (43). Tran et al. (2009) reported the cloning of thermostable alkaline Phyc from a newly isolated Bacillus subtilis MD2 in E. coli (35).

Guerrero-Olazarán et al. in 2010 investigated cloning and expression of a Bacillus Phytase C Gene in Pichia pastoris and reported were cloned and inserted. Also, they showed that both recombinant and native phytases were calcium concentration and pH-dependent (42).

pMK3P isolated from E. coli was used to introduce into B. coagulans DSM1 by Electro transformation (44). And Ozusaglam et al. have cloned this gene from B. subtilis VTT E-68013 in L. plantarum strain 755 (43). In 2007 the cloning phytase was used in animal feeding. Moreover, they demonstrated that reduced phosphorus pollution of animal waste and improved phosphorus nutrition (39). In another study, the Phyc gene of P. syringae MOK1 cloned and sequenced, and recombinant expressed the Phytase in E. coli (16). The E. coli pET expression system was able to express Phytases of E. coli and Bacillus subtilis origins (38). The expressed Bacillus subtilis Phytase accounted for 20% of the total soluble proteins in E. coli (24).

The purpose of the present study was to isolate the phyc gene. B. subtilis was selected that produces phytase. The defined aims of the study were isolated via a Phytase-producing Bacillus strain, the
corresponding gene was cloned and developed an efficient and biologically safe production system for the enzyme in the later step. As no single phytase or expression system is likely to be able to meet the various needs for this enzyme, several methods, such as using along with microbial, plant phytase reproduction or low-phytic acid corn, may be the right direction to pursue.

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