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Molecular Cloning of Phytase Gene from *Bacillus subtilis* NCIM-2712

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Summary

Phytases are enzymes which hydrolyze phytate. *Bacillus* species are known to produce a thermostable phytase. The *Bacillus subtilis* strain NCIM-2712 was chosen for cloning of *phy* gene. Primers were designed for *phy* gene amplification using the *phy* gene sequence of *B. subtilis* (AF298179). A sequence of 1059 bp characteristic of *phy* gene was obtained on PCR amplification. This gene was cloned into InsT/A cloning vector and the positive clones were confirmed by colony PCR with gene specific primers and restriction digestion. Phytase is a promising candidate for feed applications. The cloned gene obtained in this study will have potential for producing recombinant enzyme, which would enhance the feed quality for poultry and piggery by supplementing it in their diets.

Key Words: *Bacillus subtilis*, phy gene, Cloning, Phytase

Introduction

Phytase is widely present in nature occurring in microorganisms, plants as well as in some animal tissues. In bacteria, phytase activity has been found abundantly in *Bacillus* species like *Bacillus sp.* DS 11, *B. amyloliquefaciens* [1], *B. subtilis* VTTE-68013 [2], *B. subtilis* 168 *and B. licheniformis* [3]. The development of the *B. subtilis* cloning system has been mainly hindered by the absence of suitable vector replicons. Several plasmids can be introduced and maintained in the highly transformable *B. subtilis* strain 168 [4, 5] however, they lacked genetic markers for selection and DNA cloning. Bacterial phytase has been cloned and characterized from *Escherichia coli* [6]. Cloning of novel phytase gene from *B. subtilis* VTTE-68013 has also been reported [2]. *Bacillus* phytases differ from well characterized fungal phytase and other bacterial phytase possessing a six-bladed-propeller folding architecture and belong to β-propeller group [1, 3]. Unlike, other β-propeller structures, it does not show any conserved sequence repeat in the β-strands [7]. They have strict substrate specificity, thermostablity and exhibit a pH optimum in the range from 6.0 to 8.0 [8]. Metal dependency for structural integrity is another unique feature of β-propeller group of phytases. Although phytases from several species of bacteria, yeast, and fungi have been characterized, commercial production of these enzymes as feed additives currently focuses on *Aspergilli* and yeasts, as they are the most prolific extracellular producers of these enzymes [9, 10]. The role of phytase supplementation has been well proven and documented in poultry [11]. But, it is important to realize that any single phytase may never be able to meet the diverse needs for all commercial and environmental applications. Therefore, there is ongoing interest in screening microorganism for novel and efficient phytase. However, on the basis of strict substrate specificity, pH profile, resistance to proteolysis as well as high thermostability, bacterial phytases are a real alternative to fungal enzymes [12]. Particularly, *Bacillus* species have been reported to produce extracellular phytases [1, 2, 13, 14]. Hence, in the present investigation, the *Bacillus subtilis* NCIM-2712 strain was chosen to isolate and clone *phy* gene with a view to its over-expression in future endeavors.

Materials and Methods

Strains and growth media:

Bacillus subtilis NCIM-2712 strain was procured from NCL, Pune, India. It was grown on the nutrient agar media and incubated at 37˚C for 16 h. A single colony was inoculated in the nutrient broth medium and incubated at 37˚C for 16 h at 180 rpm.

Amplification of phy gene using PCR:

Genomic DNA extraction from bacterial strain was carried out according to the standard protocol [15]. A set of forward and reverse primer to amplify the *phy* gene was designed by primer express software using the published *phy* gene sequence (AF298179). The primers were dissolved in TE to make a final working concentration of 25 pmol. The PCR amplification was carried out in 25 µl of total reaction volume using programmable thermal cycler (ABI, USA). PCR was optimized by testing various components of the reaction mixture in different concentrations *viz.,* $MgCl_2$ (1-3 mM), forward and reverse primers PhyF (5'CTGTCTGATCCTTATCATTT3') and PhyR (5' TCCGCTTCTGTCGGTCA 3') (5 to 40 pmols) and annealing temperature (40-55˚C). Amplification of the *phy* gene region was successfully achieved with initial denaturation at 95˚C for 5 min followed by 35 cycles of denaturation, annealing and extension for 1 min at 95˚C, 52˚C and 72˚C, respectively and the final extension was carried out at 72˚C for 10 min. The PCR product was visualized on 1.0% (w/v) agarose gel and purified using Zymoclean™ gel DNA recovery kit (Zymo research, USA) following manufacturer's instructions.

Ligation of phy gene in cloning vector and transformation:

The ligation of amplified *phy* gene was carried out in InsT/A cloning vector (pTZ57R/T) (Fermentas) which is a

convenient system for direct one step cloning of PCR-amplified DNA fragment. The ligation reaction mix was prepared as follows: 10.0 µl of purified PCR product, 1.5 µl of InsT/A cloning vector, 2.5 µl of ligase buffer, 1.0 µl of T_4 ligase enzyme and 2.5 µl of PEG. The final volume was made to 25 µl with nuclease free water. Ligation reaction was carried out at 22˚C for 12 h in thermomixer (Eppendorf, Hamburg, Germany) and the ligation mix was subsequently used for transformation. Transformation was carried out by mixing 10 µl of ligation product to 200 µl of the *E. coli* DH5α competent cells. The mixture was kept on ice for 1 h. Heat-shock was given for 45 s at 42˚C and rapidly chilled on ice for 10 min. Upon transformation, 1 ml SOB, 20 µl glucose and 10 µl $MgCl_2$ were added in to it and incubated at 37˚C for 2 h with 180 rpm shaking. The transformed cells were spread on ampicillin (75 µg/ml) containing LB agar plate and incubated at 37˚C for 18 h.

Screening of recombinant (InsT/A) clones having phy gene:

The colonies growing on LB plates containing ampicillin (75 µg/ml) were used to isolate plasmid DNA. The plasmid DNA was extracted adopting miniprep plasmid isolation method [15]. The confirmation of positive recombinant clones was carried out by colony PCR and restriction endonuclease digestion of the plasmids. A double digestion reaction was carried out for InsT/A-phy plasmid for the confirmation of the inserted *phy* gene using *Sac*-I and *Hind*-III enzymes.

Results and Discussion

Cloning of phy gene in InsT/A cloning vector:

PCR amplified 1059 bp phytase gene was ligated in InsT/A cloning vector as shown in Fig. 1. Construct was designated as InsT/A-phy. In the present study, transformation was done by using heat-shock method. Transformed colonies growing on LB agar- ampicillin plate are shown in Fig. 2. Initial confirmation of the insertion of amplified *phy* gene into the cloning vector was done by colony PCR. The colony PCR with *phy* gene specific primers revealed a PCR product of 1059 bp indicating successful transformation (Fig. 3). For further confirmation of positive clones, a double restriction digestion with *Sac*-I and *Hind*-III enzymes was carried out and the cloned fragment of 1059 bp was observed (Fig. 4).

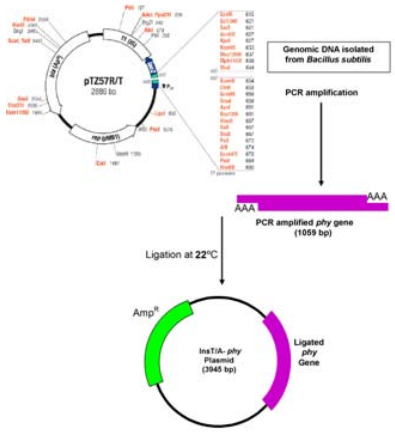

Figure 1. Cloning strategy for inserting PCR amplified *phy* gene in InsT/A cloning vector [Source: www.fermentas.com]

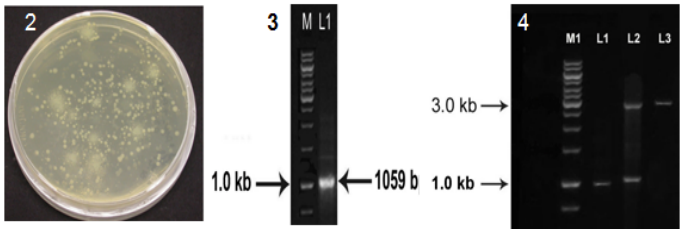

Figure 2. LB-agar ampicillin plate showing transformed *E. coli* DH5α colonies having InsT/A- phy construct.
Figure 3. 1% agarose gel electrophoresis showing colony PCR amplified 1059 bp *phy* gene from clone; M–1Kb DNA Molecular weight marker, L1–Colony PCR amplified product of *phy* gene.
Figure 4. 1% agarose gel electrophoresis showing restriction digestion analysis of InsT/A-phy clone containing 1059 bp gene insert by *Sac*-I and *Hind*-III enzymes; M1–1Kb DNA Molecular weight marker, L1–PCR amplified product of *phy* gene, L2–RE digested clone, L3–Lineralized InsT/A vector.

The first bacterial phytase gene cloning was reported for *app*A gene, from *E. coli* which encodes periplasmic phosphoanhydride phosphohydrolase [16]. Molecular gene cloning of phytase gene has also been reported from *B. amyloliquifaciens* FZB45 [17], *Pseudomonas syringae* MOK1 into *E. coli* using pGEMT–easy vector [18], *B. subtilis* US417

[14], partial *phy* gene of 989 bp from *B. subtilis* VTTE-68013 [2]. The recovery of active phytase enzyme from inclusion bodies and characterization of the recombinant phytase produced from cloning and expression of a novel phytase gene from *Bacillus sp.* in *E. coli* was also achieved [19].

Conclusion

Use of plant-based feed in poultry and piggery is inevitable in the near future too. Phytate-rich plant ingredients restrict the bioavailability of phosphorus along with other minerals, thereby increasing discharge of phosphorus into water bodies leading to eutrophication. However, it is evident that phytase supplementation of feed improves the bioavailability of the phosphorus and nitrogen. The increased bioavailability of nitrogen and phosphorus in the diet leads to reductions in feed costs through curtailing the addition of inorganic phosphorus. The role of phytase supplementation has been well proven and documented in poultry. The identified and confirmed InsT/A-phy clone will be helpful for further over-expression of this gene for large scale production of cost effective phytase enzyme through fermentation and their utilization in animal feed.

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