Journal of Phytology 2011, 3(9): 20-22
ISSN: 2075-6240
www.scholarjournals.org

JOURNAL OF PHYTOLOGY

www.journal-phytology.com

Molecular Cloning and Expression of a Phytase Gene from the Thermophilic Fungus, *Thermomyces lanuginosus* - RMB

Sowmiya Balasubramanian and Peramachi Palanivelu*

Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai – 625 021, Tamil Nadu, India

Article Info

Article History

Received : 19-06-2011
Revised : 03-08-2011
Accepted : 07-08-2011

*Corresponding Author

Tel : +91-452-2458208
Fax : +91-452-2459105

Email:
PP@mrna.tn.nic.in,
ppmkupp@gmail.com

Summary

Phytases are enzymes which hydrolyse phytate to produce inorganic phosphate and *myo*-inositol and thus they are promising candidates for food and feed industries. *Thermomyces lanuginosus* is known to produce a thermostable phytase. In this work, the *Thermomyces lanuginosus* strain RMB was chosen for cloning and expression of the phytase gene. Intronless primers were designed for phytase gene amplification and expression in *E. coli,* using the phytase gene sequence of *T. lanuginosus* (ATCC 200065). A product of ~1400 bp characteristic of phytase gene was obtained on PCR amplification of the fungal genomic DNA. This gene was cloned into pGEM-T easy vector and the positive clones were confirmed by restriction digestion and sequenced. The cloned gene obtained in this study will have a potential for producing the thermostable enzyme in large amounts, which could find applications in the feed industry to enhance the quality for poultry and piggery by supplementing it in their diets.

Key Words: Thermophilic fungus, *Thermomyces lanuginosus*, Thermostable phytase, Cloning

Introduction

During the last 20 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection, and biotechnological applications. Phytate, *myo*-inositol hexakisphosphate, is the major storage form of phosphorus in food or feeds of plant origin. With six reactive phosphate groups in the molecule it becomes a strong chelating agent and chelates the essential metal ions such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Zn^{2+} [1-3]. Phytases, a specific group of phosphatases, are required to initiate the release of phosphorus from phytate. Monogastric animals such as pig, fishes and and poultry lack phytase activity in their gastrointestinal tracts. Therefore, nearly all of the dietary phytate phosphorus ingested by these species is excreted into the environment resulting in phosphorus pollution in areas of intensive animal production [3-5]. The concept of adding phytases to the feed of monogastric animals to reduce the amount of phosphate excreted in the manure and to circumvent supplementation of the feed with inorganic phosphate has been already described [6].

A major drawback to the wide use of phytases and of feed enzymes in general is the constraint of thermal stability (65° to 95°C) required for these enzymes to withstand inactivation during the feed-pelleting and/or expansion processes. Therefore, the availability of heat-resistant enzymes would be useful to solve the aforementioned problem. Hence in the present study a thermophilic filamentous fungus, *Thermomyces lanuginosus*, was chosen to clone and express the phytase gene in large quantities so that it can be utilized in feed industries.

Materials and Methods

Chemicals:

Phytate (dipotassium salt), Cetyltrimethylammonium bromide, (CTAB) Isopropyl thiogalactoside (IPTG), deoxynucleoside triphosphates (dNTPs) and β-mercaptoethanol were purchased from Sigma Aldrich Chemical Company, U.S.A. Calcium phytate was obtained from Himedia Labs, Mumbai, India. All the other chemicals used were of analytical grade manufactured in India.

Strain and growth media:

Thermomyces lanuginosus - RMB strain was obtained from Dr. Ramesh Maheshwari, Indian Institute of science, Bangalore, India. Mycelial inoculum was prepared by incubating the spores in inoculation medium (growth medium containing 1mg/ml mycological peptone), in gyratory incubator shaker at 50˚C for 24 hrs at 150 rpm. A 3% mycelial inoculum was transferred to the growth medium containing Vogel's stock solution (50N), Vogel's trace element stock [7] and 2% sodium phytate, (pH 6.0) and incubated at 50˚C for 24 h as above.

Amplification of phytase gene using PCR:

High molecular weight genomic DNA was isolated according to the method of Basha and Palanivelu [8]. In order to amplify and express the phytase gene in *E. coli*, an upstream primer sequence without the intron and a downstream primer sequence were designed from the *T. lanuginosus* phytase sequence that had been already published (Genozymes Project Public Genomes). The PCR amplification was carried out in 25 µl total reaction volume using a programmable thermal cycler. The PCR reaction mixture in a total volume of 25 µl contained 2.5 µl 10X buffer,

1.0 µl dNTP mix (10 mM), 0.5 µl primers (10 µM) , 0.5 µl template DNA (0.1 µg) and 0.5 µl *Taq* polymerase (3 units/µl). Amplification of the phytase gene region with upstream primer 5' ATGGCATTATTGACGGCCT 3' and downstream primer 5' TCAAAAGCAGCGATCCCA 3' was successfully achieved with initial denaturation at 94˚C for 4 min followed by 30 cycles of denaturation for 30 sec at 95˚C, annealing for 30 sec at 55˚C and extension for 1 min at 72˚C, respectively and the final extension was carried out at 72˚C for 10 min. The PCR product was analyzed on 0.8% (w/v) agarose gel and purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, USA) as per the manufacturer's instructions.

Ligation of phytase gene in cloning vector and transformation:

The ligation of amplified phytase gene was performed in pGEM-T easy vector (Promega) which is a convenient, simple cloning system of PCR amplified DNA fragments. The ligation reaction mix was prepared as follows: 7.5 µl of 2X ligation buffer, 1 µl pGEM-T easy vector, 5 µl of purified PCR product and 1 µl of T_4 DNA ligase enzyme. The final volume was made up to 15 µl with nuclease free water. Ligation was carried out at room temperature followed by incubation at 4˚C for overnight and the ligation mix was subsequently used for transformation. Transformation was carried out by mixing 10 µl of ligation product to 100 µl of freshly prepared *E. coli* DH5α and BL21 (DE3) competent cells. The mixture was kept on ice for 30 min followed by heat shock at 42˚C for 50 sec and then placed immediately on ice for 5 min. Upon transformation, 900 µl of LB medium was added and incubated at 37˚C for 1 hr on a shaker at 180 rpm. The transformed cells were spread on LB agar plates containing ampicillin (100 µg/ml) and incubated at 37˚C for about 18 hrs.

Screening and analysis of recombinant clones:

The colonies grown on LB-ampicillin (100 µg/ml) plates were used to isolate plasmid DNA. The plasmid DNA was isolated according to the method of Basha and Palanivelu [9].The confirmation of positive clones was carried out by restriction digestion of the recombinant plasmid using *Eco*R1 enzyme for the confirmation of the inserted phytase gene.

Expression of phytase gene in E. coli:

To analyze the expression of the thermophilic phytase gene, the transformants from *E. coli* BL21 were used. The transformants were grown on a gyratory shaker for about 14 hrs at 30°C in LB medium containing 100 µg ampicillin and 1 mM IPTG. The cells were pelleted, washed with 0.1M acetate buffer, pH 5.5, and permeabilized with 150 µl permeabilization buffer (0.1 M acetate buffer, pH 5.5, containing 5% CTAB, 0.25% sodium deoxycholate and 10 mM β-mercaptoethanol). The supernatant fluids and the permeabilized cells were used for enzyme assay.

Enzyme assay was done according to Sigma Aldrich protocol using dipotassium phytate as substrate. The reaction mixture in a total volume of 1. 0 ml contained 100 mM acetate buffer (pH 5.5) and 50 mM phytate solution. The reaction was initiated by the addition of the enzyme solutions. The reaction tubes were incubated at 37°C for 30 min followed by the addition of 4 ml of molybdate colour reagent. The absorbency was measured at 420 nm. One unit of phytase activity is defined as release of 1 µmole of inorganic phosphate under the assay conditions.

Results and Discussion

Cloning and confirmation of thermophilic fungal phytase gene in pGEM-T easy vector:

Figure 1 shows the high molecular weight genomic DNA isolated from the thermophilic fungus, *Thermomyces lanuginosus*. The RNA contamination was cleared by digesting with DNase free RNase.

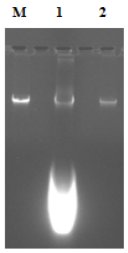

Figure 1. Agarose gel electrophoresis of genomic DNA from *Thermomyces lanuginosus*
M- Lambda DNA marker, Lane 1- Genomic DNA before RNase treatment, Lane 2- Genomic DNA after RNase treatment.

The amplified PCR product with phytase gene specific primers and the eluted PCR product are shown in Figs. 2 A & 2B. The PCR reaction amplified an amplicon size of ~1.4 kb which covers the entire gene without the first and the only intron and signal peptide sequences. The purified PCR product was ligated in pGEM-T easy vector and transformed into *E. coli* DH5α and BL-21.

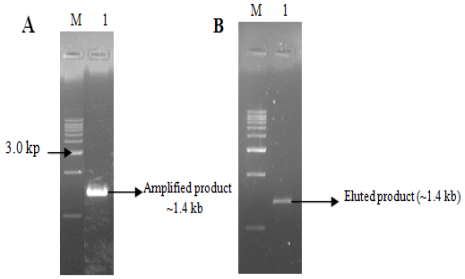

Figure 2. Agarose gel electrophoresis of the amplified PCR product with phytase gene specific primers
Fig. 2A: M-1kb DNA ladder, Lane 1-Amplified product of phytase gene (~1.4 kb)
Fig. 2B: M-1kb DNA ladder, Lane 1- Eluted product of phytase gene (~1.4 kb)

Transformants were screened again on LB-amp plates and the positive transformants were selected for plasmid isolation. Fig. 3 shows the recombinant plasmid isolated from the positive clones, before and after digestion with *Eco*R1. It released the cloned fragment of ~1.4 kb in size.

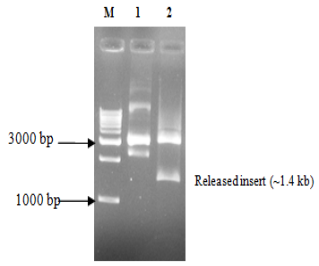

Figure 3. Agarose gel electrophoresis of recombinant plasmid before and after digestion with *Eco*R1
M-1kb DNA ladder, Lane 1- Undigested recombinant plasmid, Lane 2- Digested recombinant plasmid and released insert of ~1.4 kb.

After confirmation of the insert, the plasmid was sequenced by automated sequencing method. The sequences were subjected to BLASTn. It showed 95% and 96% identity with forward and reverse primers, respectively confirming that it is the phytase clone (data not shown). Successful cloning of phytase genes from *Kodamaea ohmeri* in pMD19-T [10], *Bacillus subtilis* [11] *A. aculeatus* RCEF 4894 and *Aspergillus ficuum* in pMD18-T [12, 13] have also been reported. Furthermore expression of phytase gene in eukaryotic system like *P. pastoris* [14] and *S. cerevisiae* [15] followed by purification and molecular characterization has also been achieved [16].

Expression of the phytase gene in E. coli

The phytase gene containing *E. coli* transformants in BL21 (DE3) were induced with IPTG and the permeabilized cells and the culture supernatant fluids were used for enzyme assays. About 4 fold increase in activity was observed in the induced cells as compared to the controls (data not shown). No significant activity was observed in the supernatant fluids. Attempts are being made to express the gene in eukaryotic hosts like *P. pastoris* and *S. cerevisiae* with strong inducible promoters.

Conclusion

As there are potential uses for novel and modified phytases in food and feed industries an attempt has been made to clone the thermostable phytase from the thermophilic fungus*, Thermomyces lanuginosus*. The intronless gene has been cloned in *E. coli,* sequenced and expressed. The intronless cloned phytase gene could be used further for expression studies and for large scale production in eukaryotic expression systems for cost effective production of thermostable phytase for supplementation in animal feed.

Acknowledgements

The authors wish to thank Miss. T.S Anitha for suggestions on the manuscript.

References

[1] Grinna, L. S. and J. F. Tschopp. 1989. Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast, *Pichia pastoris*. Yeast 5 (2):107-115.

[2] Reddy, N. R., S. K. Sathe and D. K. Salunkhe. 1982. Phytates in legumes and cereals. Adv. Food Res. 28:1-92.

[3] Vohra, A and T. Satyanarayana. 2003. Phytases: Microbial sources, production, purification, and potential biotechnological applications. Crit. Rev. Biotechnol. 23: 29-60.

[4] Casey, A and G. Walsh. 2004. Identification and characterization of a phytase of potential commercial interest. J. Biotechnol. 110: 313-322.

[5] Ullah, A., E. Mullaney and H. Dischinger. 1994. Advances in phytase research. Biochem. Biophys. Res. Comm. 203:182–189.

[6] Nelson, T. S., T. R. Shieh, R. J. Wodzinsky and J. H. Ware. 1971. Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. J. Nutr. 101:1289–1294.

[7] Palanivelu P. 2009. Analytical Biochemistry and Separation techniques, IV edition, Twenty first century publications, Madurai,

[8] Basha, S.Y. and P. Palanivelu. 1995. Two simple non-enzymatic procedures to isolate high molecular weight DNA from fungi. Curr. Sci. 68: 587-588.

[9] Basha, S. Y. and P. Palanivelu. 1994. A modified alkaline lysis procedure for isolation of plasmids from *E. coli*. Curr. Sci. 66: 335-336.

[10] Xiaoyu, L.I., L.I.U. Zhiqiang., C.H.I Zhenming., L.I. Jing and W. Xanghong. 2009. Molecular cloning, characterization, and expression of the phytase gene from marine yeast *Kodamaea ohmeri* BG3. Mycological Res. 13: 24-32.

[11] Sunil Yadav, Keerti Tantwai, Lalit Prasad Singh Rajput, Megha Kadam Bedekar, Sunil Kumar, Iti Gontia and Sharad Tiwari. 2011. Molecular Cloning of Phytase Gene from *Bacillus subtilis* NCIM-2712. J. Phytol. 3(7): 35-37.

[12] Zhong-You Ma, Shun-Chang Pu, Jing-Jing Jiang, Bo Huang, Mei-Zhen Fan and Zeng-Zhi Li. 2011. A novel thermostable phytase from the fungus *Aspergillus aculeatus* RCEF 4894: gene cloning and expression in *Pichia pastoris*. World J. Microbiol. Biotechnol. 27: 679–686.

[13] Li, G.L., J.H. Zhu., J. Sun., Z.W. Wu., J. Chen., J. Yan., L.F. Wang., G. Chen., H.Z. Jiang and M.G. Li. 2003. Cloning of the phytase gene *phyA* from *Aspergillus ficuum* 3.4322 and its expression in yeast. Fungal Diversity 13: 85-93.

[14] Ngo Thanh Xuan, Mai Thi Hang and Vu Nguyen Thanh. 2009. Cloning and over Expression of an *Aspergillus niger* XP Phytase Gene (*phyA*) in *Pichia pastoris.* World Acad. Sci. Engg. and Technol. 56.

[15] Yanming, H., D. B. Wilson and X.G. Lei. 1999. Expression of an *Aspergillus niger* phytase gene (*phyA*) in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 65: 1915-1918.

[16] Sariyska1, M.V., S.A. Gargova1., L.A. Koleva and A.I. Angelov. 2005. *Aspergillus Niger* Phytase: Purification and Characterization. Biotechnol. Biotec eq.19: 98-105.