Iranian Journal of Microbiology
Volume 13 Number 5 (October 2021) 664-670

ORIGINAL ARTICLE

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

Mahdiyar Iravani Saadi[1], Abbas Doosti[2], Heeva Jalali[3*], Ehsan Nabi Abdolyousefi[1], Mansooreh Hooshiyar[4], Reza Tabrizi[5], Esmat Noshadi[1,2*]

[1]Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Biotechnology Research Center, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran
[3]Department of Animal Science, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran
[4]Department of Microbiology and Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
[5]Non-Communicable Diseases Research Center, Fasa University of Medical Sciences, Fasa, Iran

Received: January 2021**, Accepted:** August 2021

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, Fe^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+} (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-

*Corresponding author: Heeva Jalali, MSc, Department of Animal Science, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran. Tel: +98-918-9786698 Fax: +98-871-33620553 Email: heeva.jalali@uok.ac.ir

*Corresponding author: Esmat Noshadi, Ph.D, Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran; Biotechnology Research Center, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran. Tel: +98-36122263 Fax: +98-71-36122261 Email: e.noshadi@yahoo.com

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), a-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 esxtraction. 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 $MgCl_2$, 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'-ATGTCTAGAAGAAGATTTTGTGCCAGG -3'	61°C
	R: 5'-CGTGGTACCTATTTATTACCCTCATGG -3'	
PhyC	F: 5'- TTAGGTACCATGAATCATTCAAAAACAC -3'	61°C
	R: 5'- ATTGAGCTCTTATTTTCCGCTTCTGTCAGTC -3'	
ompF-down	F: 5'- GTAGAGCTCGCTTTGGTATCGTTGGTG -3'	61°C
	R: 5'- GTGCTCGAGTTTTTGTTGAAGTAGTAGG -3'	

*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 1min. 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 (Bineer 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 (Merck 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) *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 ($CaCl_2$) 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*/*XhoI* 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 *XbaI/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 *XbaI/ 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

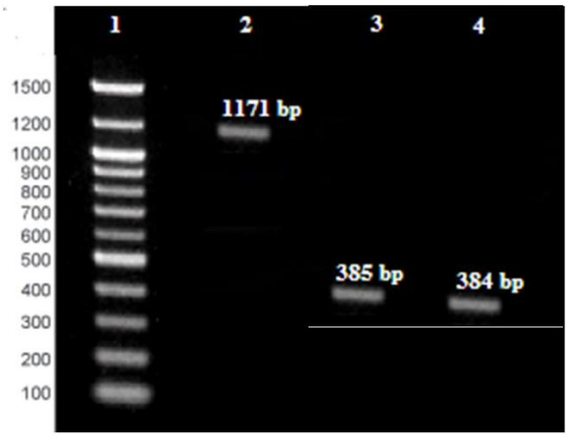

Fig. 1. Analysis of PCR amplified *PhyC* and *OmpF* gene products by agarose gel electrophoresis. Lane 1: 100 bps DNA marker (Fermentas, Germany), lanes 2: 1171 bp *PhyC* gene fragment, lane 3: 385 bp *ompF*-down and lane 4: 384 bp *ompF*-up fragments.

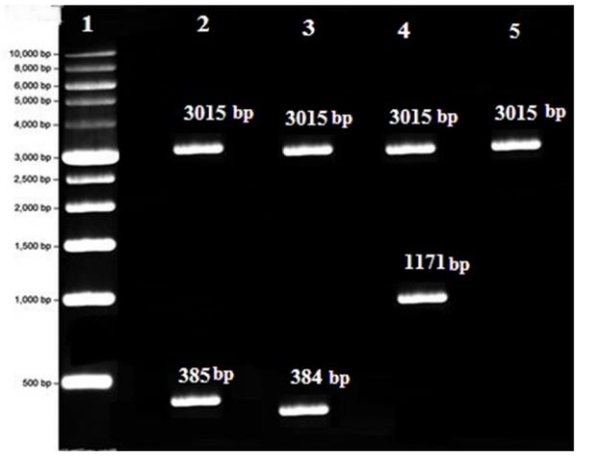

Fig. 2. Analysis of digested pGEM-*PhyC* plasmid by restriction enzymes. Lane 1: 1Kb DNA marker (Fermentas, Germany), lanes 2-4: pGEM- *ompF*- down, pGEM- *ompF*- up and pGEM-*PhyC* were digested with *SacI/XhoI, XbaI/ KpnI* and *KpnI/SacI* respectively. Lane 5: 3015 bp pGEM-T easy vector.

ompF-up, *PhyC* and *ompF*-down genes were digested with *XbaI/KpnI, KpnI/SacI* and *SacI/XhoI*, respectively (Fig. 3A). The gene constructs *ompF*-up-*PhyC*-*ompF*-down digested with *XbaI/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 phytate 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 *XbaI/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. subtillis* 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,

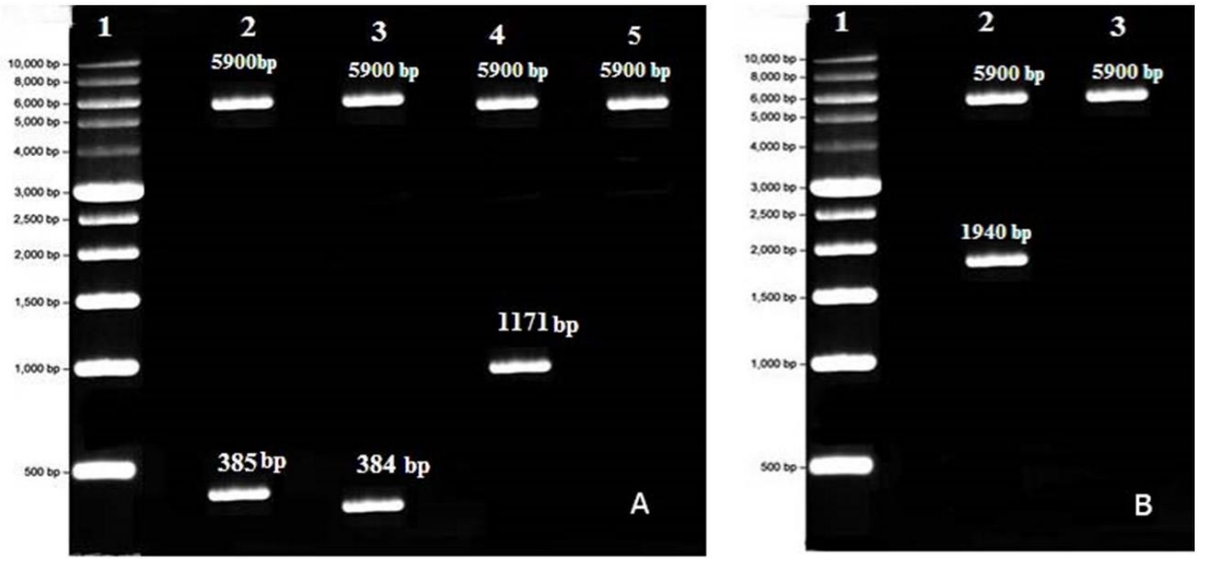

Fig. 3. Analysis of digested pET32-*PhyC* plasmid of restriction enzymes. Lane 1: 1Kb DNA marker (Fermentas, Germany), lanes 2-4: pET32- *ompF*- down, pET32- *ompF*- up and pET32-*PhyC* were digested with *SacI/XhoI, XbaI/KpnI* and *KpnI/SacI* respectively. Lane 5: 5900 bp pET32 vector. B. Final confirmed gene construct. Lane 1: 1 Kb DNA marker (Fermentas, Germany), lane 2: gene constructs digested, that 1940 bp belong to *ompF*- up -*PhyC*- *ompF*- down construct and 5900 bp pET32 vector, lane 3: pET32 supercoil vector.

E. coli phytase has the maximum specific activity of all phytases tested so far. Third, the *E. coli* phytase in contrast to *B. subtillis* 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 Ozusagla 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.

ACKNOWLEDGEMENTS

The authors would like to express their deep sense of gratitude and sincere thanks to the staff of the Biotechnology Research Center of the Islamic Azad University of Shahrekord Branch in Iran.

REFERENCES

1. Rao DE, Rao KV, Reddy VD. Cloning and expression of Bacillus phytase gene (phy) in *Escherichia coli* and recovery of active enzyme from the inclusion bodies. *J Appl Microbiol* 2008;105:1128-1137.
2. Singh N, Kuhar S, Priya K, Jaryal R, Yadav R (2018). Phytase: The feed enzyme, an overview. *Advances in Animal Biotechnology and Its Applications.* pp: 269-327.
3. Farhat-Khemakhem A, Farhat MB, Boukhris I, Bejar W, Bouchaala K, Kammoun R, et al. Heterologous expression and optimization using experimental designs allowed highly efficient production of the PHY US417 phytase in *Bacillus subtilis* 168. *AMB Express* 2012;2:10.
4. Bhavsar K, Gujar P, Shah P, Kumar VR, Khire JM. Combinatorial approach of statistical optimization and mutagenesis for improved production of acidic phytase by *Aspergillus niger* NCIM 563 under submerged fermentation condition. *Appl Microbiol Biotechnol* 2013;97:673-679.
5. Nielsen AV, Tetens I, Meyer AS. Potential of phytase-mediated iron release from cereal-based foods: a quantitative view. *Nutrients* 2013;5:3074-3098.
6. Dersjant-Li Y, Awati A, Schulze H, Partridge G. Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *J Sci Food Agric* 2015;95:878-896.
7. Morgan NK, Walk CL, Bedford MR, Scholey DV, Burton EJ. Effect of feeding broilers diets differing in susceptible phytate content. *Anim Nutr* 2016;2:33-39.
8. Boehm Jr JD, Walker FR, Bhandari HS, Kopsell D, Pantalone VR. Seed inorganic phosphorus stability and agronomic performance of two low-phytate soybean lines evaluated across six southeastern US environments. *Crop Sci* 2017;57:2555-2563.
9. Haros M, Bielecka M, Honke J, Sanz Y. Myo-inositol hexakisphosphate degradation by *Bifidobacterium infantis* ATCC 15697. *Int J Food Microbiol* 2007;117:76-84.
10. Lan GQ, Abdullah N, Jalaludin S, Ho YW. Culture conditions influencing phytase production of *Mitsuokella jalaludinii*, a new bacterial species from the rumen of cattle. *J Appl Microbiol* 2002;93:668-674.
11. Haefner S, Knietsch A, Scholten E, Braun J, Lohscheidt M, Zelder O. Biotechnological production and applications of phytases. *Appl Microbiol Biotechnol* 2005;68:588-597.
12. Wang Q, Fu SJ, Sun JY, Weng XY. Characterization of a thermostable alkaline phytase from *Bacillus licheniformis* ZJ-6 in Pichia pastoris. *World J Microbiol Biotechnol* 2011;27:1247-1253.
13. Ries EF, Alves Macedo G. Improvement of phytase activity by a new Saccharomyces cerevisiae strain using statistical optimization. *Enzyme Res* 2011;2011: 796394.
14. Vohra A, Satyanarayana T. Phytase production by the yeast, Pichia anomala. *Biotechnol Lett* 2001;23:551-554.
15. Azeem M, Riaz A, Chaudhary AN, Hayat R, Hussain Q, Tahir MI, et al. Microbial phytase activity and their role in organic P mineralization. *Arch Agron Soil Sci* 2015;61:751-766.
16. Nakamura Y, Fukuhara H, Sano K. Secreted phytase activities of yeasts. *Biosci Biotechnol Biochem* 2000;64:841-844.
17. Huang H, Luo H, Yang P, Meng K, Wang Y, Yuan T, et al. A novel phytase with preferable characteristics from *Yersinia intermedia*. *Biochem Biophys Res Commun* 2006;350:884-889.
18. Bekalu ZE, Madsen CK, Dionisio G, Brinch-Pedersen H. Aspergillus ficuum phytase activity is inhibited by cereal grain components. *PLoS One* 2017;12(5):e0176838.
19. Han N, Miao H, Yu T, Xu B, Yang Y, Wu Q, et al. Enhancing thermal tolerance of *Aspergillus niger* PhyA phytase directed by structural comparison and computational simulation. *BMC Biotechnol* 2018;18:36.
20. Quan CS, Tian WJ, Fan SD, Kikuchi J. Purification and properties of a low-molecular-weight phytase from *Cladosporium* sp. FP-1. *J Biosci Bioeng* 2004;97:260-266.
21. Hamada A, Yamaguchi K, Ohnishi N, Harada M, Nikumaru S, Honda H. High-level production of yeast (*Schwanniomyces occidentalis*) phytase in transgenic rice plants by a combination of signal sequence and co-

don modification of the phytase gene. *Plant Biotechnol J* 2005;3:43-55.
22. Tye AJ, Siu FK, Leung T, Lim B. Molecular cloning and the biochemical characterization of two novel phytases from *B. subtilis* 168 and *B. licheniformis. Appl Microbiol Biotechnol* 2002;59:190-197.
23. Priyodip P, Prakash PY, Balaji S. Phytases of probiotic bacteria: characteristics and beneficial aspects. *Indian J Microbiol* 2017;57:148-154.
24. Singh S, Singh S, Sharma PK, Sharma D. Isolation, identification and molecular characterization of phytase producing bacteria, *Pseudomonas* sp. aazad. *J Pure Appl Microbiol* 2017;11:1845-1850.
25. Roy MP, Mazumdar D, Dutta S, Saha SP, Ghosh S. Cloning and expression of phytase appA gene from Shigella sp. CD2 in Pichia pastoris and comparison of properties with recombinant enzyme expressed in *E. coli*. *PLoS One* 2016;11(1):e0145745.
26. Hong CY, Cheng KJ, Tseng TH, Wang CS, Liu LF, Yu SM. Production of two highly active bacterial phytases with broad pH optima in germinated transgenic rice seeds. *Transgenic Res* 2004;13:29-39.
27. Shamna KS, Rajamanikandan KCP, Mukesh Kumar DJ, Balakumaran MD, Kalaichelvan PT. Extracellular production of phytases by a native *Bacillus subtilis* strain. *Ann Biol Res* 2012;3:979-987.
28. Gulati HK, Chadha BS, Saini HS. Production and characterization of thermostable alkaline phytase from *Bacillus laevolacticus* isolated from rhizosphere soil. *J Ind Microbiol Biotechnol* 2007;34:91-98.
29. Barbe V, Cruveiller S, Kunst F, Lenoble P, Meurice G, Sekowska A, et al. From a consortium sequence to a unified sequence: the *Bacillus subtilis* 168 reference genome a decade later. *Microbiology (Reading)* 2009;155:1758-1775.
30. Liu J, Fang C, Jiang Y, Yan R. Characterization of a hemolysin gene ytjA from *Bacillus subtilis. Curr Microbiol* 2009;58:642-647.
31. Heinzmann S, Entian KD, Stein T. Engineering *Bacillus subtilis* ATCC 6633 for improved production of the lantibiotic subtilin. *Appl Microbiol Biotechnol* 2006;69:532-536.
32. Swain M, Ray R. Alpha-amylase production by Bacillus subtilis CM3 in solid state fermentation using cassava fibrous residue. *J Basic Microbiol* 2007;47:417-425.
33. Chang CT, Fan MH, Kuo FC, Sung HY. Potent Fibrinolytic Enzyme from a Mutant of Bacillus s ubtilis IMR-NK1. *J Agric Food Chem* 2000;48:3210-3216.
34. Earl AM, Losick R, Kolter R. Ecology and genomics of *Bacillus subtilis. Trends Microbiol* 2008;16:269-275.
35. Tran TT, Mamo G, Mattiasson B, Hatti-Kaul R. A thermostable phytase from *Bacillus* sp. MD2: cloning, expression and high-level production in *Escherichia coli*. *J Ind Microbiol Biotechno*l 2010;37:279-287.
36. Sambrook J, Fritsch EF, Maniatis T (1989). *Molecular cloning: a laboratory manual* 2nd ed. Cold spring harbor laboratory press.
37. Zimmerman SA, Tomb JF, Ferry JG. Characterization of CamH from *Methanosarcina thermophila*, founding member of a subclass of the {gamma} class of carbonic anhydrases. *J Bacteriol* 2010;192:1353-1360.
38. Golovan S, Wang G, Zhang J, Forsberg CW. Characterization and overproduction of the Escherichia coli appA encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. *Can J Microbiol* 2000;46:59-71.
39. Lei XG, Porres JM. Phytase enzymology, applications, and biotechnology. *Biotechnol Lett* 2003;25:1787-1794.
40. Bikker P, Spek JW, Van Emous RA, Van Krimpen MM. Precaecal phosphorus digestibility of inorganic phosphate sources in male broilers. *Br Poult Sci* 2016;57:810-817.
41. Selle PH, Ravindran V. Microbial phytase in poultry nutrition. *Anim Feed Sci Technol* 2007;135:1-41.
42. Kumar V, Yadav AN, Verma P, Sangwan P, Saxena A, Kumar K, et al. β-Propeller phytases: diversity, catalytic attributes, current developments and potential biotechnological applications. *Int J Biol Macromol* 2017;98:595-609.
43. Ozusaglam MA, Ozcan N. Cloning of phytase gene in probiotic bacterium *Bacillus coagulans. Adv Studies Biol* 2009;1:15-24.
44. Bawane R, Tantwai K, Rajput LPS, Kadam-Bedekar M, Kumar S, Gontia I, et al. Molecular analysis of phytase gene cloned from *Bacillus subtilis. Adv Stud Biol* 2011;3:103-110.