PHYTASE GENE ISOLATION FROM BACTERIA OBTAINED FROM HOT SPRINGS OF AJK

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ABSTRACT: Phytases are enzymes capable of hydrolyzing phytic acid to less phosphorylated myo-inositol derivates. Phytases are becoming essential supplement to animal feeds. *Bacillu s*sp. produces a thermostable phytase which renders it suitable for commercial production. A locally isolated strain of *Bacillus subtilis* was used for the amplification of phytase gene. Amplicons of 1300 and 1120bps were obtained by PCR. For the study twenty samples of water were obtained from hot springs found at different places in district Kotli village TattaPani (AJK). Plate streaking method was used for the isolation of pure bacterial colonies. Single bacterial colony was purified after five passages on nutrient agar media. Screening of phytase producing bacteria was done by modified Phytase screening medium. Isolated colonies were spread on this medium which had Calcium phytate as substrate for phytase enzyme. Local isolate of *Bacillus* sp. was employed for amplification and isolation of phytase gene. Dubous salt medium was prepared in order to obtain the growth of *Bacillus subtilis*. Genomic DNA of *Bacillus subtilis* was extracted. Oligonucleotide primers were designed using primer-3 software program. Primers were blast in order to check the relevance and suitability of primers for amplification of desired gene. Consequently, a 1300bp gene, by using phy lit primer (primer set 3) was obtained after optimization of PCR conditions. Band size of desired gene for primer set 3 was 1300bp and 1059bp. For primer set 1 and 2 expected band sizes were 1120bp and 1236bp, respectively and these two primer set were also optimized. Thus it is concluded from the study that desired gene band of 1120bp as well as other non-specific bands of 100bp, 400bp and 800bp were obtained which is a clear indication of presence of phytase gene in hot springs.

Key words: Enzyme, Polymerase Chain Reaction, *Bacillus*, Phytase

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INTRODUCTION

Cereals, legumes and oilseeds are the main ingredients of animal feed. Phosphorus is mainly stored in the form of phytic acid or phytate in the feed obtained from these plant sources (Lei and Porres, 2003; Haefner *et al.*, 2005; Dev *et al*, 2016). Simple stomach animals mainly pig, birds (Ajith *et al.*, 2018a) (including poultry) and fish cannot metabolize phytic acid phosphorus, because of the reason that they have very limited level of phytic acid degrading phytase enzyme activity in their digestive system (Maenz and Classen, 1998; Cao *et al.*, 2007; Secco *et al*., 2017; Ajith *et al.*, 2019). Thus, in order to meet the nutritional requirements in poultry and swine feed inorganic form of phosphate is added frequently in feed (Oh *et al.*, 2004; Demirkan *et al*., 2016). In the feces of these animals, excess amounts of undigested phytate and inorganic phosphate are discharged which may contribute to the eutrophication of water by decreasing oxygen concentration and increasing algal growth in the areas of widespread livestock farming (Cho *et al.*, 2006).

Phytase enzyme when added in feedstuffs increases minerals and phosphorus bioavailability in simple stomach animals and in environment it reduces phosphorus pollution (Lei *et al.*, 1993; Maenz 2001; Singh *et al*., 2013; Kumar *et al*., 2017b). Naturally, Phytases are found in microorganisms, plants and animal tissue (Konietzny and Greiner 2002; Ajith *et al.*, 2018b). Microorganisms are the best source of phytase production that are frequently used and studied for phytase enzyme characterization (Haefner *et al.*, 2005).

Many bacterial species show phytase enzyme activity particularly those which are associated with *Bacillus* and *Enterobacter* genera. *Aerobacter aerogenes* showed phytase enzyme activity as stated by (Greaves *et al.*, 1967; Kumar et al., 2013; Neira *et al.*, 2018), *Escherichia coli* showed phytase enzyme activity as

stated by Greiner *et al.,* (1993). *Pseudomonas* specie showed phytase enzyme activity as stated by Cosgrove *et al.,* (1970) and *Bacillus subtilis* also showed phytase enzyme activity as stated by Powar and Jagannathan (1982) and Shimizu (1992). Due to the reason that the optimum pH of these phytases is near to the poultry crop functional pH, they may be of more useful for poultry as feed additives.

Current intention is to isolate strain of bacteria which can produce effective and functional phytases which are thermostable at high temperature. *Bacillus subtilis* was the first bacterial specie that was first isolated and used to produce thermostable phytase (Kim *et al.,* 1997; Lazali *et al*., 2013; Soni *et al.,* 2018)**.** *Bacillus* strain was used for the equivalent gene replication and screening of two phytases (Kim *et al.,* 1998; Kerovuo*et al.,* 1998). The enzymes which were purified have almost 90% similarity at amino acid level. When compared with *Bacillus* phytases of other type, related biochemical and biophysical properties were confirmed by these two enzymes (Powar, 1982; Shimizu, 1992). In modern era, numerous phytases obtained from *Bacillus* sp. were identified having unique characteristics e.g., heat-stability, substrate specificity, reaction mechanisms and highly neutral pH (Oh *et al.,* 2001; Tye *et al.,* 2002; Castillo *et al.,* 2019). Hence, research should be performed on thermostable phytases that have broad temperature ranges i.e., above 50^{o}C and high specificity as well as neutral pH as compared to fungal phytases to improve the bioavailability of minerals and phosphorous contents bound in the phytic acid or phytate (Pen *et al.,* 1993; Lucca *et al.,* 2002). Present study was an effort for amplification and isolation of phytase gene from bacteria obtained from local resources of TattaPani (Aziz *et al*., 2015).

MATERIALS AND METHODS

Sampling: Twenty samples of water were obtained from hot springs found at different places in district Kotli village TattaPani (AJK). For this purpose, sterile thermal glass bottles were used. The water samples were then brought to Molecular Biochemistry Laboratory (MBL), University of Agriculture Faisalabad for further processing.

Isolation of Thermophilic bacteria: Plate streaking method was used for the isolation of pure bacterial colonies. Single bacterial colony was purified after five passages on nutrient agar media. For checking the thermostability, isolated colonies of bacteria were incubated at different temperature ranges e.g. 50^{o}C, 55^{o}C, 60^{o}C, 65^{o}C and 70 ^{o}C (Balsam *et al*., 2017).

Screening of phytase producing bacteria: Screening of phytase producing bacteria was done by modified Phytase screening medium (Yoon *et al.,* 1996). Isolated colonies were spread on this medium which had Calcium phytate as substrate for phytase enzyme. The positive indication is production of extracellular phytase production by bacteria whereas; the negative indication is absence of extracellular phytase production.

Bacterial strain selection: Locally isolated, *Bacillus* sp. was employed for amplification and isolation of phytase gene. The strain identified as *Bacillus subtilis* was confirmed by Institute of microbiology, University of Agriculture Faisalabad.

Sporulation media preparation: Dubous salt medium was prepared in order to obtain the growth of *Bacillus subtilis*. The plates as well as slants for sporulation of bacteria were kept in incubator at 40°C for 24 hours Van der Salm *et al*., 1994.

Genomic DNA of *Bacillus subtilis* was extracted by using the technique given by Yamada *et al.,* (2002). The DNA pellets were washed with 70% ethanol, air dried, re-suspended in 50 µL TE buffer (Yamada, 2002). Quantification of extracted DNA samples was done by using Nano Drop (Thermo Scientific) 2000c. The absorbance ratio 260/280nm and DNA concentration µg/mL was noted for record.

Oligonucleotide primers were designed using primer-3 software program. Three sets of phytase gene specific oligonucleotide primers were designed according to nucleotide sequence of phytase gene in NCBI. Primers were blast in order to check the relevance and suitability of primers for amplification of desired gene. The detail of primers is given below:

Primers set 1	
BPHYc1120F	**5′-AAG CAT AAG CTG TCC GAT CC-3′**
BPHYc1120R	**5′-TTC TTC ACA TGC AAA AAG CAG-3′**
Primers set 2	
PHYbs1236F	**5′-CTC ACA TTC GGA CAA TCT TCA C-3′**
PHYbs1236R	**5′-GCT TCT GTC GGT CAT TTT TCT C-3′**
Primers set 3	
PhyC F	**5′-CTG TCT GAT CCT TAT CAT TT-3′**
PhyC R	**5′- TCC GCT TCT GTC GGT CA - 3′**

The PCR conditions were optimized for the successful amplification of phytase gene. Two µL of DNA was added in two autoclaved PCR tubes (500 µL) independently. Forward and reverse primers each amount 2 µL was added independently in two tubes having DNA. Concentration of $MgCl_2$ was optimized by adding it in range of 1.5 mM -3.5 mM. *Taq* buffer 2 µL, dNTPs 2 µL and Taq DNA polymerase 0.4 µL were added and the volume was prepared to 20 µL. The PCR tubes were then incubated in thermocycler at different annealing temperatures, ranging from 46to 56°C. At the end, PCR products were run on 1% agarose gel having 1X TAE buffer.

After that, PCR conditions were optimized by running PCR on different annealing temperatures ranges from 46 °C to 56 °C and by changing $MgCl_2$ concentration ranges from 1.5 mM-3.5 mM. The following PCR profile was set on thermocycler: Initial denaturation was carried out at 95°C for 5 minutes, annealing was carried out at 49°C for 1 minute while extension was carried out at 72°C for 1 minute. The PCR products were then run on 1% agarose gel with 1X TAE buffer.

RESULTS

Four isolates were found thermostable as their growth maintained at different temperatures of 50°C, 55°C, 60°C, 65°C and 70°C, respectively.

None of the isolated colonies of bacteria produced translucent region on PSM medium which was clear indication that no one of them was phytase producing. That's why a local isolated strain, which was renowned for its phytase activity, was then employed for further study.

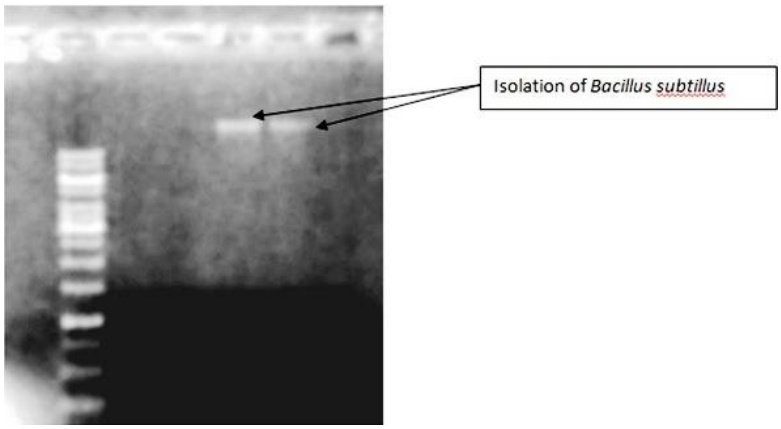

Figure-1. Samples of DNA isolated from *Bacillus subtilis* using phy lit primer

PCR Specific and Non Specific Amplification at 49 °C Annealing Temperature and 3 mM $MgCl_2$ by using primer set 3: Consequently, a 1300 bp gene, by using phy lit primer (primer set 3) was obtained after optimization of PCR conditions. Band size of desired gene for primer set 3 was 1300bp and 1059bp. For primer set 1 and 2 expected band sizes were 1120b and 1236bp, respectively and these two primer set were also optimized later on.

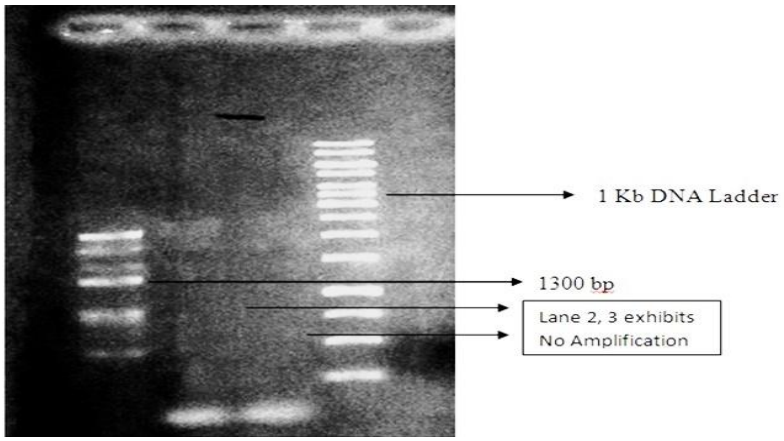

Figure- 2: Specific and Non-specific amplification of 1300 bp Phytase gene together with non-specific bands.

Polymerase Chain Reaction at 49 ^{o}C annealing temperature shows Specific and Non-specific amplification. Lane 1 displays specific amplification of 1300 bp Phytase gene together with non-specific bands. Lane 4 shows 1Kb DNA ladder. Lane 2 and 3 displays no amplification. Polymerase Chain Reaction for phytase gene amplification using 3 sets of primers at 47 ^{o}C annealing temperature and 3mM $MgCl_2$ concentration:

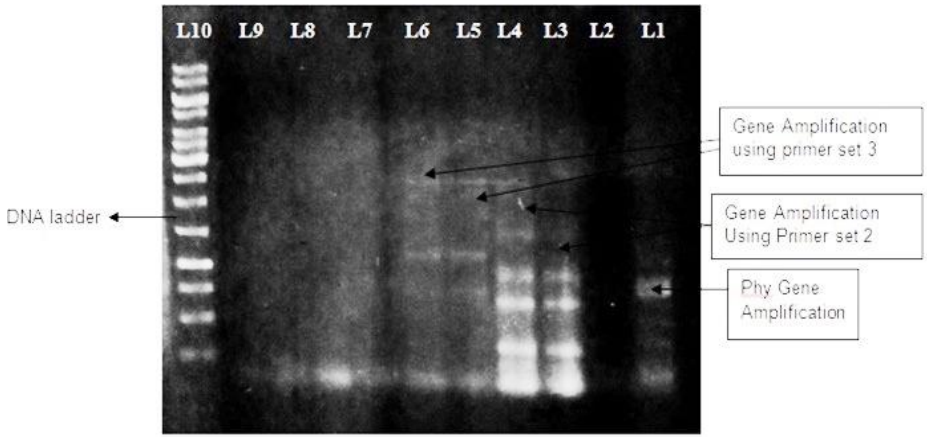

Figure- 3. Phytase gene amplification.

Polymerase chain reaction for phytase gene amplification using three sets of primers. L1 displays phy gene amplification employing primer set 1. Similarly L3 and L4 display phy. gene amplification employing primer set 2. L5 and L6 shows gene amplification by primer set 3. L10 displays 1 Kb DNA marker. Remaining lanes display no amplification. Polymerase Chain Reaction phytase gene amplification results at 49 ^{o}C annealing temperature and 3mM $MgCl_2$ concentration using two sets of primers PCR reaction was performed by keeping all other conditions same. Only annealing temperature was changed as it was kept on 49 ^{o}C and 2.4 µL $MgCl_2$ concentrations was used for 20 µL PCR samples and 6 µL for 50 µL PCR sample.

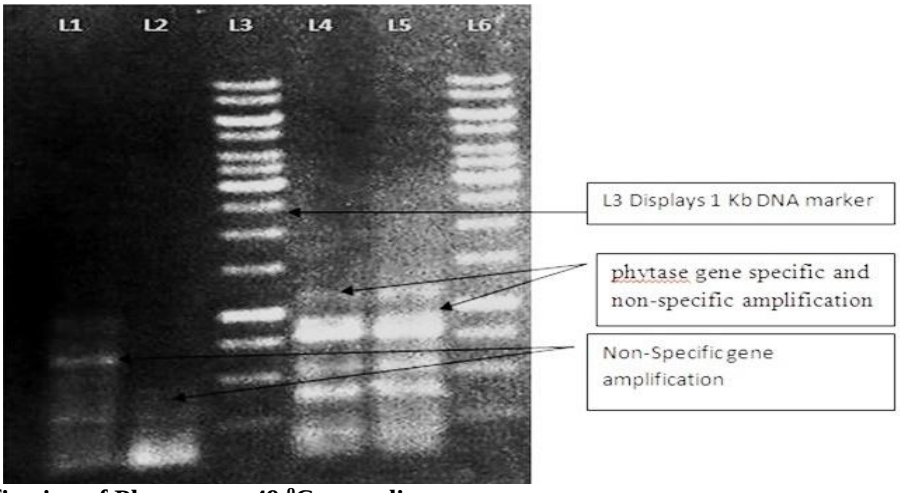

Figure- 4. Amplification of Phy gene at 49 ^{o}C annealing temperature.

Lane 1 and 2 displays non- specific gene amplification using primer set 2. Lane 3 displays 1 Kb DNA marker. Lane 4 and 5 displays phytase gene specific and non-specific amplification using primer set 1. Desired gene band of 1120bp as well as other non-specific bands of 100bp, 400bp and 800bp was obtained. Lane 6 displays 1 Kb DNA marker.

DISCUSSION

Animal feeds are naturally pelleted in a practice which includes short exposure at temperature above 80°C. Therefore, enzymes use in feed must be thermostable and also functional fully at body temperature. That's why thermostable bacteria were preferred to produce Phytase enzyme. From all the natural fungal phytases, only *Aspergillus fumigatus* is

capable of keeping its enzyme activity at 100 °C for above 20 minutes and at this temperature this enzyme only loses 10% activity (Pasamontes *et al.,* 1997; Balsam *et al*., 2017). While all fungal phytases other than these display structural changes and reduce enzyme activity while exposed to high temperature (Berka *et al.,* 1998; Wyss *et al.,* 1999; Singh *et al*., 2013). In distinction, in comparison, phytase enzyme produced by *TS-phy*in *B. amyloliqueficiens*, maintained 50% enzyme activity when kept at 90 °C for 10 minutes the *phyC*phytase of *Bacillus subtilis* maintained 61% enzymatic activity when kept at 95°C for 15 minutes (Ha *et al.,* 2000; Aziz *et al*., 2015). Therefore, *PhyC*phytase obtained from *Bacillus subtilis* was highly thermostable (Méndez-García *et al*., 2015). Thus, because of its greater thermal stability, *PhyC* phytase was a better candidate for commercial use in feed-pelleting applications.

In modern times, numerous phytases had been identified and purified from *Bacillus* sp. that have distinctive features e.g. substrate specificity, thermostability, reaction process and neutral pH (Oh *et al.,* 2001; Kumar *et al*.,2013; Tye *et al.,* 2002; Lazali *et al*.,2013).

Conclusion: It was concluded from the study that genomic DNA of *Bacillus subtilis* was extracted. Oligonucleotide primers were designed using primer-3 software program. Primers were blast in order to check the relevance and suitability of primers for amplification of desired gene. Thus it was concluded from the study that desired gene band of 1120bp as well as other non-specific bands of 100bp, 400bp and 800bp were obtained which is a clear indication of presence of phytase gene in hot springs.

REFERENCES

Aziz, G., M.A. Nawaz, T.A. Yaqub, J.K. Nazir and A.K. Sami (2015). Isolation and characterization of phytase producing bacterial isolates from soil. J. Anim. Plant Sci. 25: 771-776.

Ajith, S., D. Shet, J. Ghosh, V.B. Awachat, K. Bhat, D. Pal and A.V. Elangovan (2018a). Effect of immobilized fungal phytase on growth performance and bone traits of broilers fed with low dietary calcium and phosphorus. Vet. World. 11(6): 758–764.

Ajith, S., D. Shet, J. Ghosh and A.V. Elangovan (2018b). Immobilised phytase production from Aspergillus foetidus MTCC 11682 using an optimized media. Biotechnol. J. Int. 21(3): 1-13.

Ajith, S.G., S. Jyotirmoy, S.V. Divya, S.B. D. Punith and A.V. Elangovan (2019). Partial purification and characterization of phytase from *Aspergillus foetidus* MTCC 11682. AMB Express. 9. 10.1186/s13568-018-0725-x.

Berka, R.M., M. Michael, M. Kimberly, T. Byun and V.K. Alan (1998). Molecular characterization and expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginosus*. Appl. Environ. Microbiol. 11: 4423-4427.

Balsam, T. M., I.A.D. Hala., J. Atef, A.L. Saleh and K. Christian (2017). Isolation and characterization of thermophilic bacteria from Jordanian hot springs: *Bacillus licheniformis* and *Thermomonas hydrothermalis* isolates as potential producers of thermostable enzymes. Int. J. Microbiol.

Cao, L., W. Wang, C. Yang, Y. Yang, J. Diana, A. Yakupitiyage and Z. Luo (2007). Application of microbial phytase in fish feed. Enzyme Microbiol. Technol. 4: 497-507.

Cho, J., K. Choi, T. Darden, P.R. Reynolds, J.N. Petitte and S.B. Shears (2006). Avian multiple inositol polyphosphate phosphatase is an active phytase that can be engineered to help ameliorate the planet's phosphate crisis. J. Biotechnol. 2: 248-259.

Cosgrove, D.J., G.C.J. Irving and S.M. Bromceld (1970). Inositol phosphate phosphatases of microbiological origin. The isolation of soil bacteria having inositol phosphate phosphatase activity. Aust. J. Biol. Sci. 23: 339-343.

Dev, S.S., E.A. Nisha and A. Venu (2016). Biochemical and molecular characterization of efficient phytase producing bacterial isolates from soil samples. Int. J. Curr. Microb. App. Sci. 5(5): 218-226.

Demirkan, E., E. Baygın and A. Usta (2014). Screening of phytate hydrolysis Bacillus sp. isolated from soil and optimization of the certain nutritional and physical parameters on the production of phytase. Turk. J. Biochem. 39(2): 206-214.

Greaves, M.P., G.Anderson and D.M. Webley (1967). Hydrolysis of inositol phosphates by *Aerobacter aerogenes*. Biochim. Biophys Acta. 132: 412-418.

Greiner, R., U. Konietzny and K.I.D. Jany (1993). Purification and characterization of two phytase from *Escherichia coli*. Arch. Biochem. Biophys. 303: 107-113.

Ha, N.C., B.C. Oh, S. Shin, H.J. Kim, T.K. Oh, Y.O. Kim, K.Y. Choi and B.H. Oh (2000). Crystal structures of a novel thermostable phytase in partially and fully calcium-loaded states. Nat. Struct. Biol. 7: 147-153.

Haefner, S., A. Knietsch, E. Scholten, J. Braun, M. Lohscheidt and O. Zelder (2005). Biotechnological production and application of phytases. Appl. Microbiol . Biotechnol. 5: 588-597.

Kerovuo, J., M. Lauraeus, P. Nurminen, I. Kalkkinen and J. Apajalahti (1998). Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. Appl. Environ. Microbiol. 6: 2079-2085.

Kim, Y.O., H.K. Kim, K.S. Bae, J.H. Yu, and T.K. Oh (1997). Purification andproperties of a thermostable phytase from *Bacillus* sp. DS11. Enzyme Microb. Technol. 22: 2-7.

Kim, Y.O., J.K. Lee, H.K. Kim, J.H. Yu and T.K. Oh (1998). Cloning of the thermostable phytase gene (phy) from *Bacillus* sp. DS11 and its overexpression in *Escherichia coli*. FEMS Microbiol. Lett. 162: 185-191.

Konietzny, U and R. Greiner (2002). Molecular and catalytic properties of phytate-degrading enzymes (phytases). Int. J. Food Sci. Technol. 37: 791-812.

Kumar, V., A.N. Yadav, P. Verma, P. Sangwan, A. Saxena, K. Kumar and B. Singh (2017). β-Propeller phytases: Diversity, catalytic attributes, current developments and potential biotechnological applications. Int. J. Biol. Macromol. 98: 595–609.

Kumar, V., P. Singh, M. A. Jorquera, P. Sangwan, P. Kumar, A. K. Verma and S. Agrawal. (2013). Isolation of phytase-producing bacteria from himalayan soils and their effect on growth and phosphorus uptake of indian mustard (Brassica juncea). World J. Microbiol. Biotechnol. 29: 1361-1369.

Lei, X.G., K.P. Ku, E.R. Miller, M.T. Yokoyama and D.E. Ullrey (1993). Supplementing corn soybean meal diets with microbial phytase maximizes phytate Pi utilization by weanling pigs. J. Anim. Sci. 71: 3368-3375.

Lei, X.G and J.M. Porres(2003). Phytase enzymology, applications and biotechnology. Biotech. Lett. 25: 1787-1794.

Lucca, P.,R. Hurrell and I. Potrykus(2002). Fighting iron deficiency anemia with iron-rich rice. J. Am. Coll. Nutr. 21: 184-190.

Lazali, M., M. Zaman-Allah, L. Amenc, G. Ounane, J. Abadie and J. J. Drevon (2013). A phytase gene is overexpressed in root nodules cortex of Phaseolus vulgaris-rhizobia symbiosis under phosphorus deficiency. Planta. 238(2): 317-324

Maenz, D.D. and H.L. Classen (1998). Phytase activity in the small intestinal brush border membrane of the chicken. Poult. Sci. 77: 557-563.

Méndez-García,C., A.I. Peláez, V. Mesa, J. Sánchez, O.V. Golyshinaand M. Ferrer (2015). Microbial diversity and metabolic networks in acid mine drainage habitats. Front. Microbiol. 6: 475.

Maenz, D.D. (2001). Enzymatic characteristics of phytases as they relate to their use in animal feeds. Enzyme Farm Anim. Nutr. 61-84.

Neira, V.A.A., C.N. Aguilara, A. Ilyinab, E.J.C. Contreras, C.M.G. Carneiro-da, A. Michelena and J.L. Martinez (2018). Purifcation and biochemical characterization of an Aspergillus niger phytase produced by solid-state fermentation using triticale residues as substrate. Biotechnol. Report. 17: 49–54.

Oh, B.C., B.S. Chang, K.S. Park, N.C. Ha, H.K. Kim and B.H. Oh (2001). Calcium-dependent catalytic activity of a novel phytase from *Bacillus amyloliquefaciens* DS11. J. Biochem. 40: 9669-9676.

Oh, B.C., W.C. Choi, S. Park, Y.O. Kim, and T.K. Oh (2004). Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Appl. Microbiol. Biotechnol. 63: 362-372.

Pasamontes, L., M. Haiker, M.Wyss and M. Tessier (1997). Gene cloning, purification, and characterization of a heat stable phytase from the fungus *Aspergillus fumigatus*. Appl. Environ. Microbiol. 63: 1696-1700.

Powar, V.K and V. Jagannathan (1982). Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. J. Bacteriol. 151: 1102-1108.

Pen, J., T.C. Verwoerd, P.A. Paridon, R.F. Beudeker, P.J. Elzen, K. Geerse, J.D. Klis, H. Aersteegh, A.J. Ooyen and A. Hoekema (1993). Phytase-containing transgenic seeds as a novel feed additive for improved phosphorus utilization. Biotechnol. 11: 811-814.

Shimizu, M (1992). Purifcation and characterization of phytase from *Bacillus subtilis* (Natto) n-77. Biosci. Biotech. Biochem. 56: 1266-1269.

Secco, D., N. Bouain, A. Rouached, C. Promuthai, M. Hanin, A.K. Pandey, H. Rouached (2017). Phosphate, phytate and phytases in plants: from fundamental knowledge gained in *Arabidopsis* to potential biotechnological applications in wheat. Crit. Rev. Biotechnol. 12: 1–13.

Singh, N.K., D.K. Joshi and R.K. Gupta (2013). Isolation of phytase producing bacteria and optimization of phytase production parameters. Jundish. J. Microbiol. 6(5): 1-7.

Tye, A.J., F.K.Y. Siu, T.Y.C. Leung and B.L. Lim (2002). Molecular cloning and the biochemical characterization of two novel phytases from *B. subtilis* 168 and B. licheniformis. Appl. Microbiol. Biotechnol. 59: 190-197.

Van-der-Salm, T.P.M., C.J.G. Van der Toorn, C.H. Hänisch ten Cate, L.A. M. Dubois, D.P. De Vries and H.J.M. Dons (1994). Importance of the iron chelate formula for micropropagation of

Rosa hybrida L. 'Moneyway'. Plant Cell Tissue Organ Cult. 37(1): 73-77.

Wyss, M. R., A. Brugger, R. Kronenberger, R. Remy, G. Fimbel, M. Oesterhelt and A.P. Gvan-Loon (1999). Appl. Environ. Microbiol. 65: 367-373.

Yamada, Y.M., M. Koiche, O. Hossan, N. Kumito, Y. Yayoi and O. Masako (2002). Comparison of two different methods for extraction of mitochondrial DNA from human pathogen yeast. Jap. J. Infect. Dis. 55: 122-125.

Yoon, S.J., Y.J. Choi, H.K. Min, K.K. Cho, J.W. Kim, S.C. Lee, and Y.H. Jung (1996). Isolation and identification of phytase producing bacterium, *Enterobacter sp*. 4, and enzymatic properties of phytase enzyme. Enzyme Microb. Technol. 18: 449-454.