

Isolation, characterization and 18S rDNA sequence analysis of phytase producing fungi from Indonesia

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Abstract. Hailu HW, Sajidan, Pangastuti A, Greiner R. 2017. Isolation, characterization and 18S rDNA sequence analysis of phytase producing fungi from Indonesia, Indonesia. *Biodiversitas* 18: 10-14. Phytase breaks down the phytate portion of grains and oil seeds; thereby, releasing phosphorus and minerals for animals. The objectives of this research were to screen phytase-producing novel species of fungi; to conduct cultural and phylogenetic characterization; to perform 18S rDNA sequencing and BLAST analysis. Samples of fungi were collected from different sources, cultured on potato dextrose agar and potato dextrose broth. Phytase assay was conducted based on Vanadate-molybdate method. Three isolates with highest enzyme activity were selected for pH and temperature optimization. DNA was extracted by modifying plant DNA extraction and yeast genomic DNA extraction kits. The 18S rDNA gene was amplified and sequenced. The result indicated that optimum temperatures ranged from 60-75°C and the optimum pH for isolates from papaya (Angr), palm bark (Abark) and sugarcane soil field (Asoil) were 4.5, 3.0 and 4.5, respectively. Based on 18S rDNA sequence analysis the phytase producing fungal isolates Angr, Abark and Asoil were identical to *Aspergillus niger*, *Beauveria felina* and *Nigrosabulum globosum*, respectively.

Keywords: 18S rDNA, BLAST, fungi, phytase, phylogeny

INTRODUCTION

Phosphorus is an essential nutrient for pigs, and poultry animals for bone formation and mineralization (Yao et al. 1988). Its accessibility plays a key role in soil prolificacy, crop production, animal health and nutrition, waste management and water quality (Konietzny and Greiner 2002; Mishra et al. 2013; Reddy et al. 2013). Phytate (*myoinositol* (1,2,3,4,5,6) hexakisphosphate) is the major form of phosphate found in cereals, legumes and oilseeds representing around 75-80% of the total phosphorous in their seeds (Yao et al. 1988; Liu et al. 1998; Hegeman and Grabau 2001; Konietzny and Greiner 2002; Quan et al. 2006; Yu et al. 2012; Mishra et al. 2013).

However, phytate is naturally found chelated with cations like Fe³⁺, Zn²⁺, Ca²⁺, Mg²⁺, Mn²⁺ and K⁺ (Xiong 2006; Greiner et al. 2009; Yu et al. 2012; Mishra et al. 2013). This chelating nature prevents their uptake in the gastro-intestinal tract of monogastric animals such as poultry and pigs or even fish species and therefore, phytate has been considered as an anti-nutritional factor since it always combines with proteins and cations thereby decreasing their bioavailability. The release of phosphate from the phytate molecule depends on phytase activity in the gastro-intestinal tract. Large amount of excreted phosphate contributes to environment pollution. Phytase plays a role of reducing phosphate output in manure by

breaking down phytate thereby making phosphate to be readily available for bio-absorption (Liu et al. 1998; Hegeman and Grabau 2001; Quan et al. 2006; Xiong 2006). Monogastric animals need external phytase to digest phytate. Giving phytase as a feed supplement allows phytate-phosphorus to be utilized by monogastric animals. Supplementation of swine and poultry diets with microbial phytases can improve significantly the bioavailability of phytate-bound phosphorus and reduce P excretion (Maritza et al. 2012; Mishra et al. 2013; Reddy et al. 2013). For example, phytase from different *Aspergillus* species such as *A. niger*, *A. oryzae* and *A. melleus* were reported (Wösten et al. 2007) to have an important role in the breakdown of phytatic acid into phosphate, mono-inositol, and minerals.

This research was aimed to explore novel fungal isolates for potential production of phytase enzyme. It helps explore the huge potential of Indonesia for phytase production from fungal spp.

MATERIALS AND METHODS

Sample collection and isolation of fungi

Different fungal isolates from various sources (soil, tree bark, air conditioners, open air, and fruits such as papaya, orange) were collected in sterile polythene bags, in Surakarta, Central Java, Indonesia. Serial dilutions of 10⁻²,

10^{-3} and 10^{-4} were prepared from 1 gram sample. The serially diluted samples were plated by taking 100 μ L of each sample onto sterilized potato dextrose agar containing 1 g/L of ampicillin (antibiotic was added to inhibit bacterial growth) in Petri dishes and incubated at room temperature for 4-7 days.

Phytase assay

Vanadate-molybdate method was used to investigate the activity of phytase enzyme. The reaction mixture was 100 μ L of phytase, 600 μ L of 0.4% phytate solution at pH 5.0. It was incubated at 37°C for 30 minutes. The reaction was ended by adding three mixtures of solutions (21.67% nitric acid; 0.081 mol/L of ammonium molybdate; 0.02 mol/L ammonium vanadate) at 2: 1: 1 ratio. The phytase activity was measured by Uv-Vis spectrophotometer at 451nm, using 0.1-0.6 mM KH_2PO_4 as standard. It is calculated in terms of phytase unit. One phytase unit is defined as the amount of enzyme required to release 1 μ mol of inorganic phosphate per minute from sodium phytate at 37°C.

Temperature and pH optimization

To study the optimum Temperature of the crude phytase enzyme, the following temperature ranges were used for the standard phytase assay: 28°C, 37°C, 50°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C and 90°C. The pH ranges studied were from pH 2.5-8.0. To study the optimum pH of the crude phytase enzyme, the following buffers were used for the standard phytase assay: pH 3.5-6.0 sodium acetate-HCl; pH 6.0-7.0, 0.1 M Tris-acetic acid; pH 7.0-8.0 Tris-HCl.

Fungal genomic DNA extraction and 18s rRNA amplification

Modified Promega protocol was used to extract *Aspergillus* and other fungal genomic DNA by combining yeast and plant tissue genomic DNA isolation kits. The quality and quantity of DNA extract was analyzed by

biophotometer. Standard PCR Protocol of KAPA2G Fast ReadyMix PCR Kit for 18s rDNA amplification was used. The 18S rDNA was amplified with primers that target highly conserved region of 18s rRNA. Forward Primer (EP3): 5'GGAAGGGRTGTATTTATTAG-3' and Reverse Primer (EP4): 5'TCCTCTAAATGACCAAGTTTG-3' were used (Mishra et al. 2013). The total 25 μ L PCR mix consisted of 8 μ L PCR grade water (ddH₂O), 12.5 μ L of 2x KAPA2G Fast ReadyMix, 1.25 μ L of 10 μ M Forward Primer, 1.25 μ L 10 μ M Reverse Primer and 2 μ L of DNA template. The PCR running conditions were: Initial Denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 15 sec, annealing at 48°C for 15 sec, extension at 72°C for 5 sec and final extension at 72°C for 2 min.

DNA electrophoresis and sequencing

PCR products were separated in 1% agarose gel electrophoresis (Bio-Rad) with voltage, current and time of 85v, 400 m AMP and 60 minutes, respectively. The gel was stained in solution of ethidium bromide for 15 minutes and washed by distilled water for 15 minutes. DNA bands formed visualized under UV illumination GelDoc. Successfully amplified DNA samples were packed with accession number and sent to Singapore 1st base laboratory for determining 18S rDNA sequence of each screened sample. Sequence results were used to perform BLASTn alignment (<http://www.ncbi.nlm.nih.gov/BLAST/>). Phylogenetic relationships were determined using MEGA 6.0 for fungus considered positive for phytase production related species retrieved from GenBank.

RESULTS AND DISCUSSION

Phytase characterization

There are total 12 isolates as shown in Figure 1. All the fungal cultures except isolate from orange (isolate number 5) have positive result for phytase production and the result is shown in Figure 2.

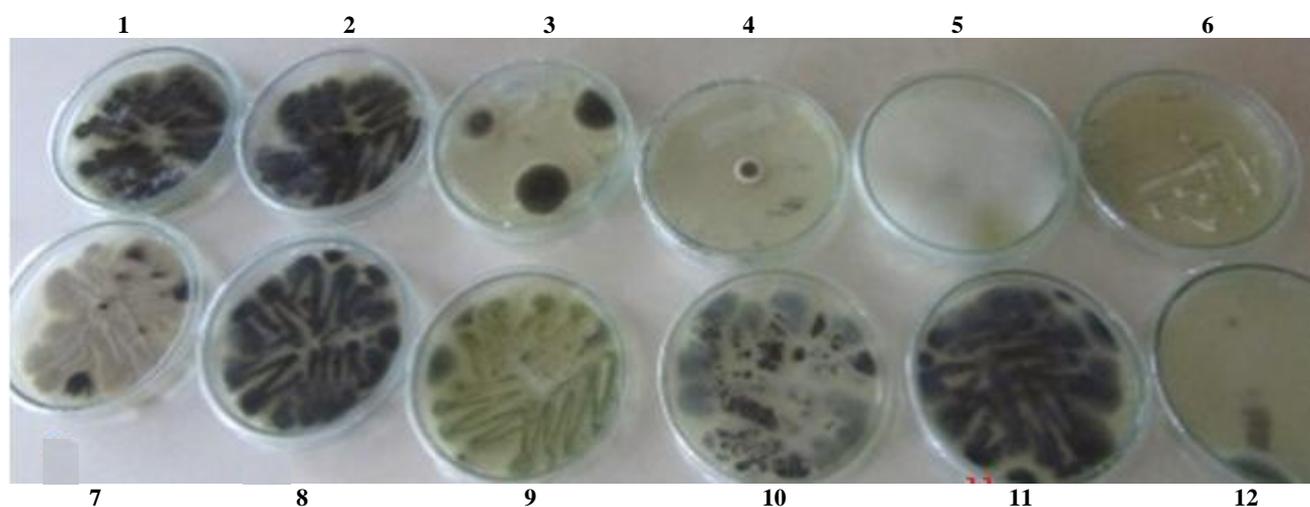


Figure 1. Fungal isolates for phytase production. 1-4. *Aspergillus niger* (as comparison), 5. Isolate from orange, 6. Isolate from rice field, 7. Isolate from palm bark, 8-9. Isolate from papaya fruit (Angr), 10-12. Isolate from sugarcane soil field

As shown in Figure 2, the isolates from papaya (Angr), *A. niger*, palm bark (Abark) and sugarcane field (Asoil) have the highest phytase activities of 1199.8, 1114.5, 1149.2 and 1175.2 mU/mL, respectively. The three fungal species identified are promising for the production of phytase for further application and commercialization. Three isolates (Angr, Abark and Asoil) with relatively higher phytase activities were selected for further optimization of temperature and pH, and molecular analysis of the isolates. Similarly, phytase activities of 2.20 ± 0.1 U/mL/min for the fungi *Sporotrichum thermophile*, 0.04 ± 0.01 U/mL/min for *Aspergillus fumigatus*, 0.22 ± 0.02 U/mL/min for *Humicola insolens*, and 0.53 ± 0.02 for *Thermomyces lanuginosus* were reported (Javed et al. 2010). High Phytase activity makes the enzyme highly desired for applications in many areas such as animal nutrition, aquaculture, human nutrition and health, and agriculture (Sarsa 2013).

Optimum temperature

Different enzymes have various optimum temperature and pH at which the enzymes function maximally. The results of phytase activity at different temperatures are presented in Figure 2.

The activity of phytase enzyme from the isolates was determined at different temperatures and the enzymatic activity was determined according to the reaction of the phytase with sodium phytate substrate. Isolates Angr (A) exhibited optimal activity at 60°C, isolate Asoil (B) exhibited maximum phytase activity at 70°C, whereas for isolate Abark (C) maximum phytase activity was at 75°C. This indicated that these enzymes are thermostable which makes it ideal for industrial enzyme application. Enzymes used for animal feed supplements should withstand temperatures of 60 to 90°C, during feed pelleting process (Markus et al. 1998).

Similar optimum temperatures were reported for *Aspergillus* species at 55°C for *A. niger* (Natuphos) and *A. Niger* NRRL3135, 50°C for *A. niger* SK-57, 60°C (*A. niger* 113 and *A. fumigatus*), 49°C for *A. terreus* A1, 45°C for *A. terreus* CBS. Additionally, for yeasts the thermo-stability of phytase activities were observed at temperatures of 60°C for *Candida intermedia*, 60-65°C for *Kluyveromyces thermotolerans*, 65°C for *Candida tropicalis* (Xiong et al. 2004), 60°C for *Pichia anomala* (Sano et al. 1999), 70-75°C for *P. rhodanensis* (Xiong et al. 2004), 75°C for *Arxula adenivorans* (Nakamura et al. 2000), 75-80°C for *P. spartinae* (Xiong et al. 2004), 77°C for *Schwanniomyces castellii* (Vohra and Satyanarayana 2002).

pH optimum

The optimum pH for isolates Angr was pH 4.5, Asoil was pH 4.5 and Abark was pH 3.0. This indicates that each isolates have different optimum pH values at which the fungal species have maximum phytase activity at those particular pH ranges or values. Optimum phytase activities were reported at pH 4.5 for *Candida intermedia*, *Candida tropicalis* and *Arxula adenivorans* (Nakamura et al. 2000; Sano et al. 1999), pH 4.0-5.0 for *Kluyveromyces*

thermotolerans (Nakamura et al. 2000), pH 4.0 for *Pichia anomala* (Vohra and Satyanarayana 2002), 4.0-4.5 for *P. rhodanensis* (Nakamura et al. 2000), pH 4.5-5.5 for *P. spartinae* (Nakamura et al. 2000), pH 4.4 for *Schwanniomyces castellii* (Segueilha et al. 1992).

The active site structure of enzymes plays an important role for its specificity and change in pH has an effect on the structure. For example, the pH-dependent catalytic site alkaline pH possibility providing alternative activity of *A. fumigatus* phytase was reported to be linked to three water molecules that may prevent the substrate from binding thus block the nucleophilic attack of the catalytic imidazole nitrogen (Liu et al. 1998).

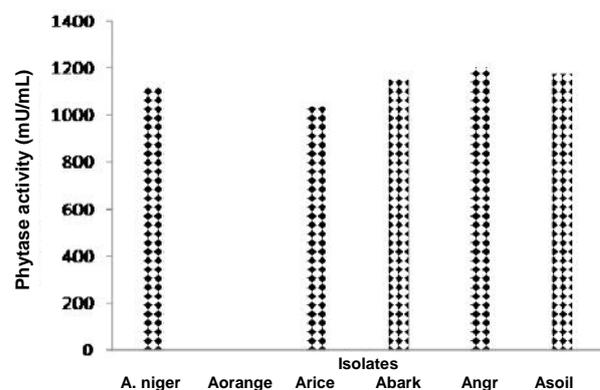


Figure 2. Phytase activity (mU/mL) of the fungal isolates *A. niger* (as comparison), isolate orange fruit (Aorange), rice soil field (Arice), Palm bark (Abark), papaya fruit (Angr) and sugarcane soil (Asoil)

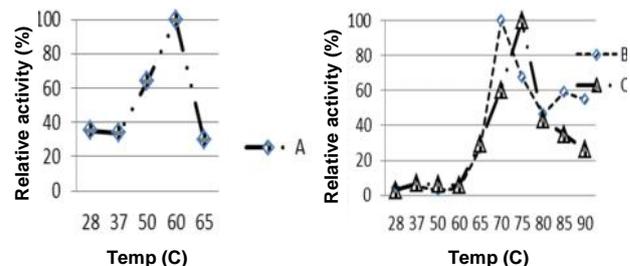


Figure 2 Optimum temperatures for phytase enzyme activity of the isolates. A. Angr, B. Asoil, C. Abark

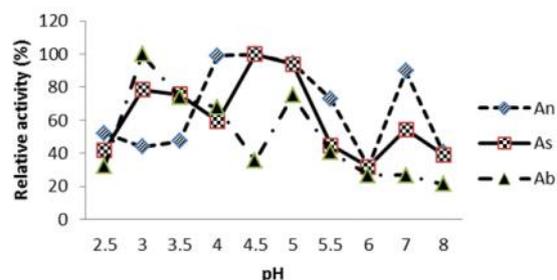


Figure 3. Phytase activity optimum pH for isolates: An = Angr, As = Asoil, Ab = Abark

Molecular characterization and bioinformatics

Isolates with highest enzyme activity were selected for PCR amplification. Three of the isolates were successfully amplified. The 18S rDNA PCR product sizes were around 1500 bp as shown in Figure 4.

As shown in Figure 5, all the three phytase producing isolates were successfully amplified. This indicates that the primer used to amplify 18S rDNA was specific so that it targets the required conserved DNA region. BLAST analysis of the partial 18S rRNA gene sequences for the three fungal isolates with the highest phytase activity are presented in Table 1.

Based on the BLAST analysis on similarity, the result of isolates of 18S rRNA sequence with gene bank it was found that the fungal isolate designated as Angr has 97% similarity with *Aspergillus niger* strain CEPC 11, which has 1723 bp 18S ribosomal RNA gene length, isolate Asoil was identified as *Nigrosabulum globosum* with 94 % (novel) similarity and 18S ribosomal RNA gene length of 1638 bp, whereas Isolate Abark was 98% similar to *Beauveria felina* strain CBS 173.71 with 18S ribosomal RNA gene length of 2285 bp.

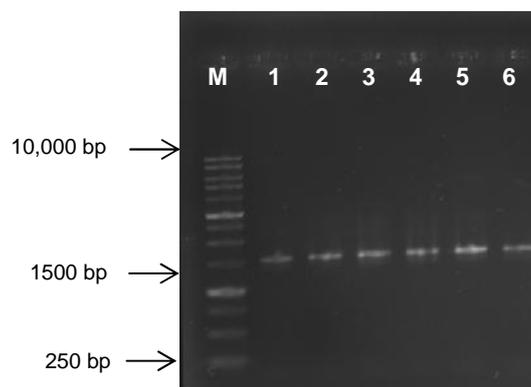


Figure 4. Electrophoresis of 18s rRNA PCR product bands observed for Isolate from Angr (1 and 2), Asoil (3 and 4) and Abark (5 and 6) using 1 Kb DNA marker (M)

Table 1. Identification result of the three phytase producing fungal isolates

| Sample | Similarity | Spp size (bp) | Query cover | Identity | Accession |
|--------|---|---------------|-------------|----------|------------|
| Angr | <i>Aspergillus niger</i> strain CEPC 11 18S ribosomal RNA gene, partial sequence | 1723 | 83% | 97% | KM516789.1 |
| Asoil | <i>Nigrosabulum globosum</i> strain ATCC 22102 18S ribosomal RNA gene, partial sequence | 1638 | 80% | 94% | AF096180.1 |
| Abark | <i>Beauveria felina</i> strain CBS 173.71 18S ribosomal RNA gene, complete sequence | 2285 | 90% | 98% | AY261368.1 |

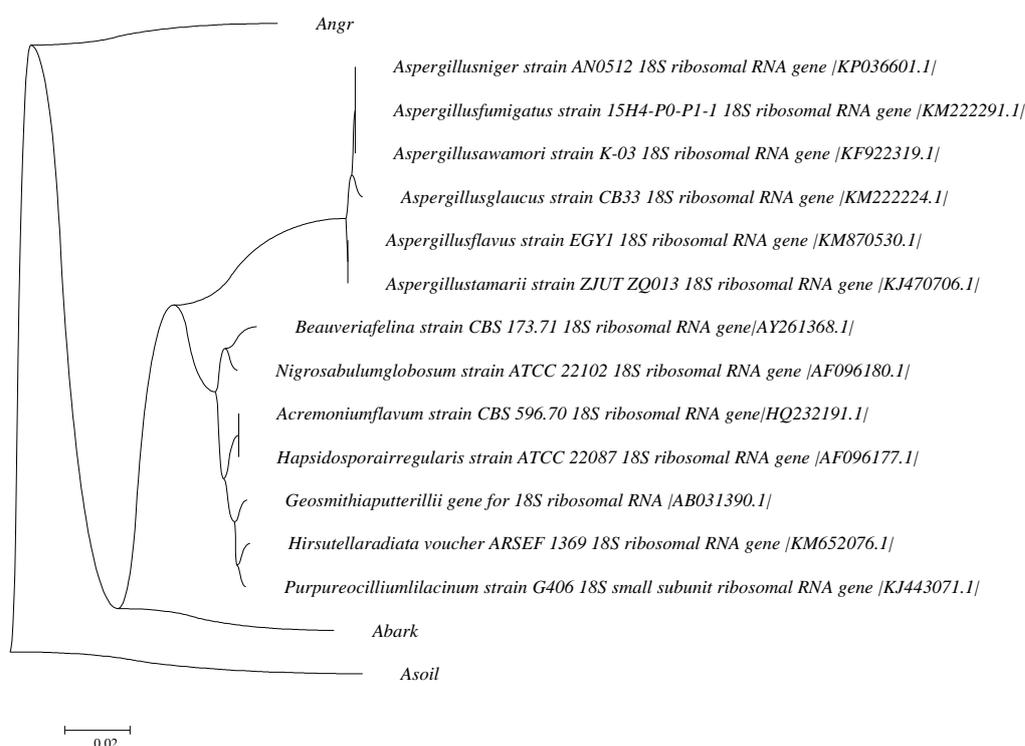


Figure 4. Evolutionary relationships of taxa: The phylogenetic analysis involved 16 nucleotide sequences. The Neighbor-Joining Method available in MEGA6 was used to infer the evolutionary history with the sum branch length of 0.40975987. The Maximum Composite Likelihood method was used to compute the evolutionary distances (Tamura et al. 2013).

The 16S/18S rRNA sequence-based identification of microbes focuses on key parts of the genetic makeup of bacterial, fungal and yeast cells called ribosomal RNA, commonly referred to as 16S rRNA in prokaryotes (e.g. bacteria) or 18S rRNA in eukaryotes (e.g. yeast, *Aspergillus* and fungi). The 18S rRNA gene-based approach was used as a tool for identification unknown fungal species which are able to produce phytase enzyme. Sequence nucleotide analysis of conserved 18S rRNA region helps to find out the sequence similarity of different eukaryotic species based on either pairwise or multiple sequence analysis (Sugita and Nishikawa 2003).

As shown in figure 4. The nucleotide sequences of the genes encoding the 18S rRNA of *Aspergillus niger*, *Nigrosabulum globosum*, *Beauveria felina* and other fungal sequences were elucidated and aligned to the sequences of isolates Angr, Abark and Asoil. The phylogenetic analysis and comparison showed that the isolate Angr is closely related to *Aspergillus*. It is clearly shown that the genus *Aspergillus* is clustered together in top part of the phylogenetic tree showing the phylogenetic relatedness of species. The 18S rDNA is a good candidate for finding consensus conserved regions suitable for genus or higher taxonomic level detections because of its relatively slow rate of molecular evolution (Wu et al. 2003).

To conclude, phytase from isolate Angr (97% similar with *Aspergillus niger* strain CEPC 11) has an optimum temperature of 60°C, and the pH of 4.5 and 7.0. The phytase activity for isolate Asoil (94% similar to *Nigrosabulum globosum* strain ATCC 22102) has optimum temperature of 70°C and peak in pH of 3.0, 4.5 and 7.0, whereas, Isolate Abark (97% similar with *Beauveria felina* strain CBS 173.71) has optimum temperature activity of 75°C and peak in pH of 3.0 and 5.0. This research comes out with additional two novel fungal species (*Beauveria felina* strain CBS 173.71, *Nigrosabulum globosum* strain ATCC 22102) which were never reported so far.

REFERENCES

- Greiner R, Gomes da Silva L, Couri S. 2009. Purification and characterization of an extracellular phytase from *Aspergillus niger* 11T53A9. *Braz J Microbiol* 40: 795-807.
- Hegeman CE, Grabau EA. 2001. A novel phytase with sequence similarity to purple acid phosphatases is expressed in cotyledons of germinating soybean seedlings. *Plant Physiol* 126: 1598-1608.
- Javed MM, Ahmed W, Zahoor S, Ikram-Ul-Haq. 2010. Solid state culturing of thermophilic fungi for phytase production. *Pak J Bot* 42 (5): 3605-3611.
- Konietzny U, Greiner R. 2002. Molecular and catalytic properties of phytate-degrading enzymes (phytases). *Intl J Food Sci Tech* 37: 791-812.
- Liu BL, Rafiq A, Tzeng YM, Rob A. 1998. The induction and characterization of phytase and beyond: Review. *Enzy Microbiol Tech* 22: 415-424.
- Maritza OB, Cervantes P, Fernanda L, Mauricio MM, Mauricio SY, Sánchez G, Andrés P. 2012. Isolation and characterization of potential phytase-producing fungi from environmental samples of Antioquia (Colombia). *Rev Fac Nal Agr Medellín* 65 (1): 6291-6303.
- Markus W, Pasamontes L, Kuszniir E, Reamy R, Gadiant M, Kohler J, Müller F, Van Loon APGM. 1998. Comparison of the thermostability properties of three acid phosphatases from molds: *Aspergillus fumigatus* phytase, *A. niger* phytase, and *A. niger* pH 2.5 acid phosphatase. *Appl Environ Microbiol* 64 (11): 4446-4451.
- Mishra IG, Deshmukh D, Tripathi N, Bhurat, KB, Tantwai K, Tiwari S. 2013. Isolation, morphological and molecular characterization of phytate-hydrolyzing fungi by 18S rDNA sequence analysis. *Braz J Microbiol* 44 (1): 317-323.
- Nakamura, Y, Fukuhara, H., Sano, K. 2000. Secreted phytase activities of yeasts. *Biosci Biotechnol Biochem* 64 (4): 841-844.
- Quan LQ, Feng LQ, Li J, Da-jiang Z, Mei WH, Hong YQ. 2006. Transgenic expression of the recombinant phytase in rice (*Oryza sativa*). *Rice Sci* 13 (2): 79-84.
- Reddy CS, Vijaylakshmi M, Vani K, Chandra P, Reddy O, Pandey S, Kaul T, Gupta G. 2013. Manipulating microbial phytases for heterologous expression in crops for sustainable nutrition. *Ann Plant Sci* 2 (10): 436-454.
- Sano K, Fukuhara H, Nakamura Y. 1999. Phytase of the yeast *Arxula adenivorans*. *Biotechnol Lett* 21: 33-38.
- Sarsan S. 2013. A review on role of microbial phytases in agriculture. *Intl Interd Res J* 3 (4): 91-99.
- Segueilha L, Lambrechts C, Boze H, Moulin G, Galzy P. 1992. Purification and properties of the phytase from *Schwanniomyces castellii*. *J Ferment Bioeng* 74 (1): 7-11.
- Sugita T, Nishikawa A. 2003. Fungal identification method based on dna sequence analysis: Reassessment of the methods of Pharmaceutical Society of Japan and the Japanese Pharmacopoeia. *J Health Sci* 49 (6): 531-533.
- Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30: 2725-2729.
- Vohra A, Satyanarayana T. 2002. Purification and characterization of the thermostable and acid stable phytase from *Pichia anomala*. *World J Microbiol Biotechnol* 18: 687-691.
- Wösten HAB, Scholtmeijer K, de Vries RP. 2007. Hyper production of enzymes by fungi. In: Samson RA, Dijksterhuis J (eds.) *New Challenges in Food Mycology*. Marcel Dekker Inc., New York.
- Wu ZH, Tsumura Y, Blomquist G, Wang XR. 2003. 18S rRNA gene variation among common airborne fungi, and development of specific oligonucleotide probes for the detection of fungal isolates. *Appl Environ Microbiol* 69 (9): 5389-5397.
- Xiong AS, Yao QH, Peng RH, Li X, Fan HQ, Guo MJ, Zhang SL. 2004. Isolation, characterization, and molecular cloning of the cDNA encoding a novel phytase from *Aspergillus niger* 113 and high expression in *Pichia pastoris*. *J Biochem Mol Biol* 37 (3): 282-291.
- Xiong AS, Zhang Z, Chen JM, Yao QH, Xu F, Liu JG, Peng RH, Han PL. 2006. High level expression of a synthetic gene encoding *Peniophoralycii* phytase in methylotrophic yeast *Pichia pastoris*. *Appl Microbiol Biotechnol* 72: 1039-1047.
- Yu S, Cowieson A, Gilbert C, Plumstead P, Dalsgaard S. 2012. Interactions of phytate and myo-inositol phosphate esters (IP1-5) including IP5 isomers with dietary protein and iron and inhibition of pepsin. *J Anim Sci* 90: 1824-1832.