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# Isolation, Identification and Molecular Characterization of Phytase Producing Thermotolerant Fungus, *Aspergillus Terreus*

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## ABSTRACT

Phytase catalyses dephosphorylation of phytate to myo-inositol and orthophosphate. In plant based animal diet, phytate is the chief storage form of phosphorus. Monogastric animals like poultry, pigs, fishes and humans could not metabolise phytate bound phosphorus due to the lack of phytase enzyme in their guts. In the current study, phytase producing fungi were isolated on PSM with 0.5% ca-phytate, pH 4 and incubation temperature of 45 $^{0}$C. The thermo-tolerant fungus with highest phytase activity was selected. The fungus was identified morphologically as well as microscopically and confirmed by molecular characterization as *Aspergillus terreus*. The fungal isolate exhibited maximum phytase activity (11.80 ± 0.02 Uml$^{-1}$) at pH 4 and 55 $^{0}$C. Phytase from this fungus can have application in animal feeds because acidic pH and high temperature tolerance is desirable for applications in industrial processes.

**KEYWORDS:** *Aspergillus terreus*, phytase, phytate, PSM, fungi

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## INTRODUCTION

Phosphorus is the second most abundant mineral that plays an important role in almost every biochemical pathway in the body. Therefore, it is necessary for the normal growth and development of animals. Phytic acid is the principal storage form of organic phosphorus and inositol which accounts for 60-80% of the total phosphorus stored in the plants seeds (Lott *et al.,* 2000)[1]. The animal feed ingredients mainly comprised of plants seeds therefore the non-ruminant animals could not utilize the phytate bound phosphorus from their diet due to the absence of phytate hydrolysing enzyme in their guts (Singh and Satyanaryana, 2010)[2]. To meet the P requirement additional inorganic phosphorus is added to their diets, which not only increases the feed cost but also causes pollution when excreted phosphorus enters the water bodies via soil run-off causing eutrophication (Schroder *et al.*, 1996)[3].

Phytic acid also act as an anti-nutritional factor because at physiological pH it has six reactive phosphate groups that chelates almost every positive molecule such as cations, proteins, starch and lipids reducing their digestibility (Urbano *et al.,* 2000; Lei *et al*., 2003; Pagano *et al*., 2007)[4,5]. Due to the above mentioned problems, there is a considerable interest in phytate hydrolysing enzymes, phytase.

Phytase (myo-inositol hexakisphosphate phosphohydrolase) catalyzes dephosphorylation of phytate to inositol and orthophosphate. Phytase have a big share in the feed market due to their widespread application as a feed supplement (Greiner and Konietzny, 2006)[6]. The use of phytase is environment friendly because the enzymatic degradation of phytic acid will not produce any toxic by-products (Ciofalo *et al.*, 2003)[7]. Commercial production of phytase as feed additives is mostly focused on fungi and yeasts, as they are the most prolific extracellular producer of phytase enzyme (Farahat *et al.,* 2008)[8]. Therefore, there is an ongoing research for the production of phytase that can meet the prime requisites necessary for application in feed as well as human food consumption, soil remediation and medical purposes. The present study reports isolation, morphological and molecular identification of *Aspergillus terreus*, a promising phytase producing fungus.

## MATERIAL AND METHODS

### *Sample collection*

Various samples (soil, compost, decaying leaves, vegetables, fruits etc.) were collected from different geographical regions in sterile polythene bags. Warm and humid sites were preferred for sample collection.

## *Isolation of phytase producing fungi*

Samples (1g) were suspended in 100 ml of 0.85% saline solution and kept on incubator shaker at 150 rpm for 1 hour. 0.5 ml of soil suspension was inoculated on potato dextrose agar medium. The pH of medium was maintained acidic (pH 4) using sodium citrate buffer. The medium was sterilized by autoclaving at 121 $^{o}$C, 15 psi for 15 min. The plates were incubated at 45 $^{0}$C for 7 days. The fungal isolates obtained were then screened for phytase production on plates using phytase screening medium (Howson and Davis, 1983)[9]. The plates were then observed for zone of clearance around the colony. The isolates showing distinct zone of hydrolysis around the colonies were considered as affirmative isolates and studied further on the basis of size of zone (mm). For elimination of any false results a secondary confirmation was done using counter staining method (Bae *et al.*, 1999)[10].

## *Phytase enzyme activity assay*

The fungal isolates exhibiting distinct zone of hydrolysis were screened quantitatively for enzyme production in phytase screening broth containing calcium phytate. A spore suspension of $1x10^{7}$ spores/ml was prepared using heamatocytometer and used as inoculums to inoculate Erlenmeyer flasks (250 ml) containing 50 ml production medium and incubated at 45 $^{0}$C in an incubator shaker at 150 rpm for 7 days. 2 ml culture was withdrawn aseptically from every flask periodically. Extracellular crude phytase was extracted by filtration using Whatman No. 1 filter paper. The cell free supernatant obtained after centrifugation at 10,000 rpm for 10 minute was used as crude phytase enzyme for activity assay. Phytase assay was done by measuring the amount of phosphate released using sodium phytate as substrate. The reaction mixture comprised 0.5 ml buffer, 0.5 ml substrate and 1 ml enzyme. The reaction was carried out at 40 $^{0}$C for 10 min. and stopped by adding 1 ml 5% TCA. The phosphate release was estimated using spectrophotometer according to method described by Fiske and Subbarow (1925)[11]. Standard curve was prepared using potassium dihyrodgen phosphate in range of 15-150 µg/ml. 'One unit of enzyme activity was defined as the amount of enzyme used to liberate one µmole inorganic phosphate/ml/minute under the assay conditions'.

## *pH and temperature optima of phytase enzyme*

The pH optima of enzyme were determined by incubating reaction mixture on different pH ranging from pH 2.0-9.0 [0.2 M glycine/HCl buffer (pH 2.0 to 3.0), 0.2 M acetic acid/sodium acetate buffer (pH 4.0 to 6.0), 0.2 M Tris/HCl (pH 7.0 to 8.0)]. The temperature optima of enzyme were

determined by incubating the reaction mixture at different temperature from 30 $^{o}$C -70 $^{o}$C with an interval of 10 $^{o}$C and the phytase activity was assayed.

### *Morphological and microscopic characterization*

The isolates were identified on the basis of morphological characters such as overall color, color of conidia, reverse color, texture, zonation and sporulation on three different types of media, *viz.* czapek dox agar (CDA), czapek yeast agar (CYA) and malt yeast agar (MYA) (Samson *et al.*, 2007; Gontia-Mishra *et al.*, 2013). The fungal isolates were also analyzed microscopically by slide culture technique (Riddell, 1950) using lactophenol cotton blue.

### *Molecular characterization*

Fungal isolate was cultured on potato dextrose agar medium. The plates were incubated at 45 $^{0}$C for 4-5 days. The DNA was isolated from fungal mycelium using fungal DNA isolation kit (HIMEDIA) following the manufacturer's protocol. PCR amplification was carried out using universal fungal primers [ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3')] (White and Bruns, 1990) in 100 µl reaction mixture containing 4 µl dNTPs (10mM), 10 µl assay buffer, 1 µl Taq DNA polymerase enzyme, 400 ng each forward and reverse primers and 1 µl genomic DNA. The reaction conditions were as follows: initial denaturation at 95 $^{0}$C for 5 min, 35 amplification cycles of denaturation at 94 $^{0}$C for 30 sec, annealing at 52 $^{0}$C for 30 sec and primer extension at 72 $^{0}$C for 45 sec; followed by a final extension at 72 $^{0}$C for 7 min. The PCR product was run on 0.8% agarose gel and visualized under UV transilluminator. The nucleotide sequence of the PCR product was determined by PCR-direct sequencing done by Chromous Biotech Pvt. Ltd., Bangalore, India to identify the culture and its closest neighbor.

## RESULTS AND DISCUSSION

### *Isolation of phytase producing fungi*

Twenty nine fungal isolates, which were able to form colonies at 45 $^{0}$C and pH 4, were obtained out of all samples. Further on qualitative screening; nineteen fungal isolates were found to produce a distinct zone of clearance around the colony on PSM containing ca-phytate. The zone of clearance is formed due to the enzymatic hydrolysis of phytate-phosphorus. Thus the fungal isolates with distinct clearance zone were considered to exhibit phytase activity (as depicted in Figure 1.). However, the clear zone may also be formed due to the production of various acids by the fungi which lower the pH of medium near colonies and increase solubility of calcium. Thus, the

counterstaining method was employed to eliminate any false positives. All the isolates were found to be positive to counterstaining method.

**Figure 1. Zone of hydrolysis shown by *A. terreus* on PSM**

## *Phytase enzyme activity assay and its optimization*

Phytase activity was estimated by measuring the amount of liberated inorganic phosphate and its reaction with color reagent. The fungal isolates that were producing a clear zone of hydrolysis were screened for phytase production. Fungal isolate CSA4 was selected having highest enzyme activity of 11.80 ± 0.02 $Uml^{-1}$ of culture filtrate and a zone diameter of 27.5 mm after 5 days of incubation. The optimum temperature and pH values for maximum phytase activity by this isolate were determined. The pH and temperature at which the isolate exhibit maximum phytase activity was found to be pH 4.5 and 55 $^{o}$C, respectively (Figure 3a and 3b). Mostly fungi showed an optimum pH ranging from 4.0-6.0 (Marlida *et al*., 2010) and temperature ranging from 50 $^{o}$C -60 $^{o}$C.

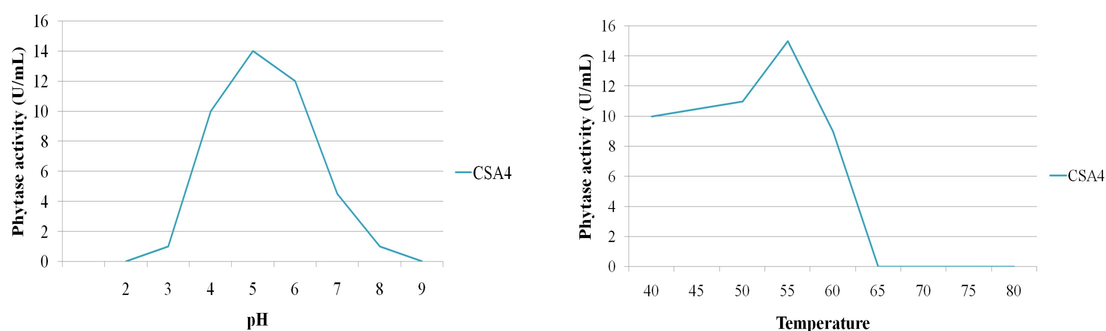


**Figure3**. Phytase activity of bacterial isolate at different pH (a) and temperature (b)

### *Morphological and microscopic characterization*

The selected isolate, CSA4, was studied for various colony morphology characteristics as shown in Table 1, (Figure 2a). According to observed characters, the fungal isolate was suggested to be *A. terreus* (Zain *et al.*, 2009). This fungus is readily distinguished from the other species of *Aspergillus* by its cinnamon-brown colony colouration. The microscopic analysis for the affirmative conformation of fungal isolate was done. The hyphae were wide and septate with long conidiophores. The vesicle was elliptical and biserate (Figure 2b). Similar findings were also reported by Balajee (2009). Thus the isolate CSA4 was confirmed as *A. terreus.*

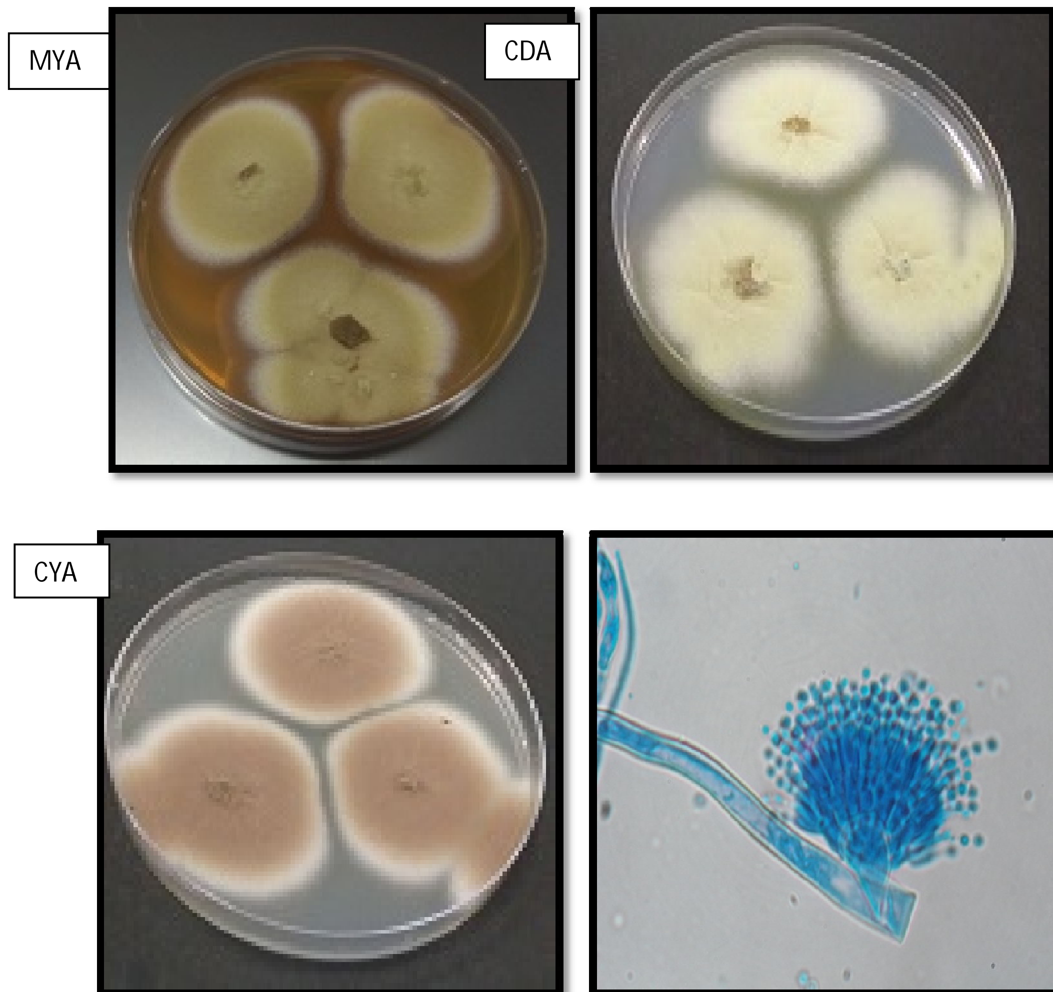


**Figure 2. Colony morphology on different media (MYA, CDA and CYA) and microscopic analysis of fungal isolate**

**Table1- Morphological features of fungal isolate on various culture media.**

| Media | Colony Diameter (mm) | Colony texture | | | Sporulation |
|---|---|---|---|---|---|
| | | Texture | Surface colour | Reverse colour | |
| CDA | 37 ±1.23 | Powdery | Yellow with brown periphery | Yellow | Moderate |
| CYA | 40 ±1.09 | Powdery | Brown with white periphery | Orange | Moderate |
| MYA | 45 ±1.17 | Powdery | Green with white periphery | Peach | Moderate |

## *Molecular identification and phylogenetic analysis*

The isolate CSA4 was subjected to molecular identification using ITS region. The ITS region is the most widely sequenced DNA region in molecular ecology of fungi (Peay *et al.,* 2008) and has been recommended as the universal fungal barcode sequence (Schoch *et al.,* 2012). It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). The PCR product was estimated to be approximately ≈600bp and its sequence was obtained. Phylogenetic analysis was done based on the alignment of this sequence with the corresponding sequence of various fungal species in the database using the system software aligner. The sequence alignment revealed a close of fungal isolate (CSA4) relation to *Aspergillus* sp. The phylogenetic tree was created using weighbour method with alphabet size 4 and length size 1000 (William *et al.*, 2000). The distance matrix was generated using Jukes-contor correction model and sampling distribution was estimated using bootstrap. The fungal isolate showed maximum sequence similarity of 99% with *Aspergillus terreus.* The sequence of this isolate was submitted to NCBI's GenBank under the following accession number: MF447153. Phylogenetic relationship of the fungal isolate with other fungi is shown in Figure 4.

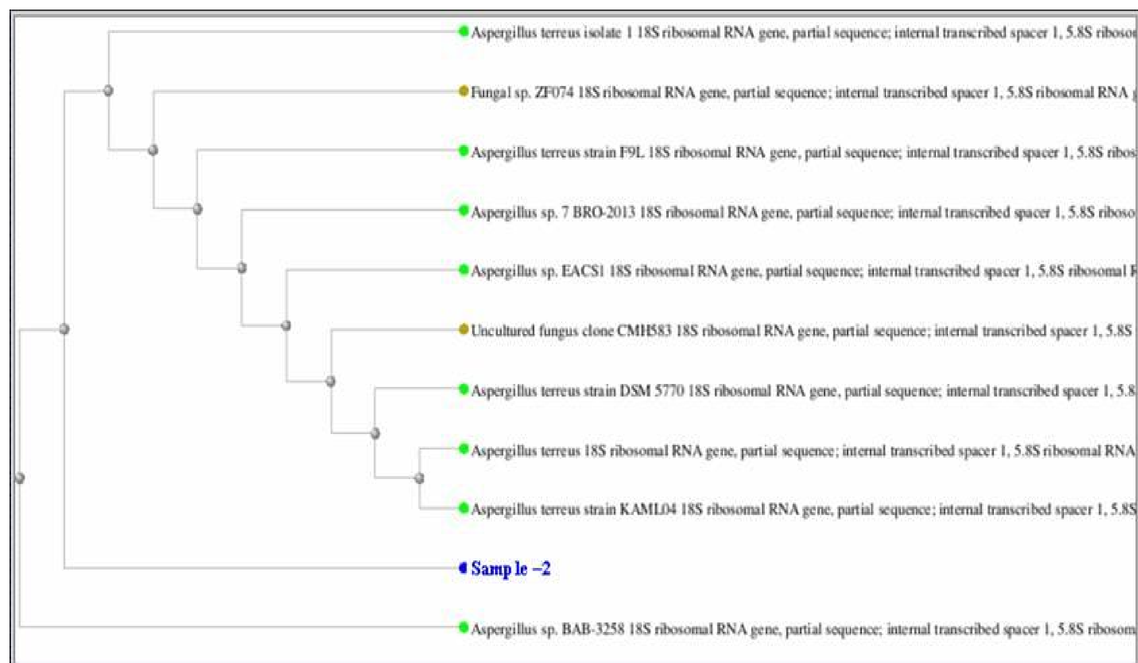


**Figure 4. Phylogenetic tree showing the relationship of isolated fungus (CSA4) with closely related fungi**.

*Aspergillus terreus*, also known as *Aspergillus terrestris*, is a fungus found worldwide in soil. This saprotrophic fungus is prevalent in warmer climate. *A. terreus* is a thermotolerant species since it has optimal growth in temperatures between 35–40 °C (95–104 °F), and maximum growth within 45–48 °C (113–118 °F) (Anderson *et al.,* 1980). High temperature tolerance is desirable for pelleting of phytase enzyme. Yamada *et al.* (1968) reported phytase from *A. terreus* with optimal temperature and pH values of 4.5 and 70 $^{0}$C, respectively. Shieh and Ware (1968) also studied *A. terreus* for phytase production. In the present study, the isolated *A. terreus* also showed maximum growth at 45 $^{0}$C with highest phytase activity at pH 4.5 and temperature 55 $^{0}$C, thus phytase from this fungus could find applications in animal feeds.

## CONCLUSION

It can be concluded that phytase from *Aspergillus terreus* have significant values which can be exploited for industrial production of enzyme. Moreover, this enzyme could be used in the animal feeds for improving nutritional status of animal feeds.

### Conflict of interest

The authors declare that they have no conflict of interest.

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## REFERENCE

1. Anderson KH and Gams W. Compendium of soil fungi (2nd print ed.). London, UK: Academic press. 1980. ISBN 9780122204029.
2. Bae HD, Yanke LJ and Selinger LB. A novel staining method for detecting phytase activity. J. Microbiol Methods. 1999; 39: 17-22.
3. Balajee SA. *Aspergillus terreus* complex. Med Mycol. 2009; 47: S42–S46.
4. Ciofalo V, Barton N, Kretz K, Baird J and Cook M. Safety evaluation of a phytase, expressed in schizosaccharomyces pombe, intended for use in animal feed. Regulatory Toxicolology and Pharmacology. 2003; 37: 286-292
5. Farahat A, Chouayekh H, Farhat MB, Bouchaala K and Bejar S. Gene cloning and characterization off a thermostable phytase from Bacillus subtilis UC417 and assessment of its potencial as a feed additive in comparison with a commercial enzyme. Mol Biotechnol. 2008; 40: 127-135.
6. Fiske CH and Subbarow Y. The colorimetric determination of phosphorus. J. Biol. Chem. 1925 ; 66: 375.
7. Gontia-Mishra I, Deshmukh D, Tripathi N, Bardiya-Bhurat K, Tantwai R and Tiwari S..Isolation, morphological and molecular characterization of phtate hydrolysing fungi by 18S rDNA sequence analysis. Brz J Microbial. 2013; 44: 317-323.
8. Greiner R and Konietzny U. Phytase for food application. Food Technology and Biotechnology. 2006 ; 44: 125-140
9. Howson SJ and Davis RP. Production of phytate hydrolyzing enzymes by some fungi. Enz. Microb. Technol. 1983; 5: 377-382.
10. Lei XG and Porres JM. Phytase enzymology, application and biotechnology. Biotechnol Lett. 2003; 25: 1787-1794.
11. Lott JNA, Ockenden I, Raboy V, Batten GD. Phytic acid and phosphorus in crop seeds and fruits: a global estimate. Seed Sci Res. 2000; 10: 11–33.
12. Marlida Y, Delfita R, Adnadi P and Ciptaan G. Isolation, characterization and production of phytase from endophytic fungus its application for feed. Pak J Nutr. 2010; 9: 471–474.

13. Pagano AR, Yasuda K, Roneker KR, Crenshaw TD and Lei XG. Supplemental Escherichia coli phytase and strontium enhance bone strength of young pigs from a phosphorus adequate diet. J Nutr. 2007; 137: 1696-1801
14. Peay KG, Kennedy PG and Bruns TD. Fungal community ecology: a hybrid beast with a molecular master. BioScience. 2008; 58: 799–810.
15. Ronald WR. Simple methods of slide culturing. Micologia. 1950; 42: 265-270.
16. Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J. Diagnostic tools to identify black aspergilli. Stud Mycol. 2007; 59: 129-145.
17. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Bolchacova E, Voigt K and Crous PW. Nuclear Ribosomal Internal Transcribed Spacer (ITS) Region as a Universal DNA Barcode Marker for Fungi. 2012; PNAS 109 (16): 6241–6246.
18. Schroder B, Breves G and Rodrhutscord M. Mechanisms of intestinal phosphorus absorption and bioavailability of dietry phosphorus in pigs. Dtsch Tierarztl Wochenschr. 1996 ; 103: 209-214.
19. Shieh TR and Ware JH. Survey of microorganisms for the production of extracellular phytase. Appl microbial. 1968; 16: 1348-135.
20. Singh B and Satyanarayana T. Application of phytase of thermophilic mould Sporotrichum thrmophile: A review. J sci Ind Res. 2010; 69. 411-414.
21. Urbano G, Lopez-Jurado M, Aranda P, Vidal-Valverde C, Tenorio E and Porres J. The role of phytic acid in legumes: Antinutrient or beneficial function? J Physiol Biochem. 2000; 56: 283–294.
22. William JB, Nicholas DS and Aaron LH. Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction. Mol. Biol. Evol. 2000; 17(1): 189-197.
23. Wiley EO, Brooks DR, Siegel-Causey D and Funk VA. *The Compleat Cladist: A Primer of Phylogenetic Procedures.* 1991.
24. Yamada K, minoda Y and Yamamoto S. Phytase from aspergillus terreus. Part 1. Production, purification and some general properties of the enzyme. Agric Biol Chem. 1968; 2: 1275-1282.
25. Zain ME, Razak AA, El-Sheikh HH, Soliman HG and Khalil AM. Influence of growth medium on diagnostic characters of *Aspergillus* and *Penicillium* species. Afr J Microbiol Res. 2009; 3: 280–286