# ISOLATION, SCREENING AND PRODUCTION OF PHYTATE DEGRADING ENZYME (PHYTASE) FROM LOCAL FUNGI ISOLATE

**S. S. Qasim*** **K. A. Shakir** **A. B. Al- Shaibani**
**Assist. Instructor** **Prof.** **Prof.**
**Coll. Of Agric.-Univ. of Baghdad** **Coll. Of Biotech.-Al-Nahrain Univ.**
altaay1977@yahoo.com dr_khalida55@yahoo.com abdulbaqir943@yahoo.com

**ABSTRACT**

**Twenty-six isolates of fungi were isolated from samples of different sources. The isolates were screened for the production of phytase (phytate degrading enzyme) by propagating the isolates on phytase screening medium (PSM) and monitoring the formation of clear zone around their grown colonies. Only thirteen isolates were able to form a clear zone indicating their abilities to produce the phytase enzyme. By subjecting the isolates to the quantitative screening under both solid-state fermentation (SSF) and submerged fermentation (SmF), it was found that fungal isolate ($G_1$) which was identified as *Aspergillus tubingensis* and marked as *SKA* was the most distinguished one among others by giving enzymatic activity in both methods with values reached (49.83 and 26.53 unit/ml) in SSF and SmF respectively. Phytase of fungal isolate *A. tubingensis SKA* was carried out by utilizing different substrates and supplements under SSF and SmF and the results showed the superiority of SSF on SmF for the production of phytase especially when wheat bran was used with an enzymatic activity value of (50.35 and 45.42 unit/ml) respectively.**

**Key words: Phytic acid, *Aspergillus tubingensis SKA*, solid-state fermentation.**
***Part of Ph.D. Thesis of the first author.**



# عزل، غربلة وإنتاج الانزيم المحلل للفايتيت (الفايتيز) من عزلة فطر محلية

سمر صالح قاسم* خالدة عبد الرحمن شاكر عبد الواحد باقر الشيباني
مدرس مساعد أستاذ أستاذ
كلية الزراعة–جامعة بغداد كلية التقانات الاحيائية–جامعة النهرين
altaay1977@yahoo.com dr_khalida55@yahoo.com abdulbaqir943@yahoo.com

**المستخلص**

**تم عزل ست وعشرون عزلة فطرية محلية من مصادر مختلفة وغربلت هذه العزلات للوقوف على كفاءتها في انتاج الفايتيز من خلال تنميتها على وسط غربلة الفايتيز phytase screening medium(PSM) ومراقبة تكون الهالة الشفافة حول المستعمرات الفطرية النامية. وقد أظهرت ثلاث عشرة عزلة هالة شفافة حول مستعمراتها كدلالة على انتاج الانزيم. وأجريت الغربلة الكمية للعزلات بطريقتي تخمرات الحالة الصلبة solid-state fermentations (SSF) وتخمرات الحالة السائلة submerged fermentations (SmF)، وتميزت العزلة ($G_1$) والتي شخصت فيما بعد على انها *Aspergillus tubingensis* ورمز لها بالرمز *SKA* عن مثيلاتها حيث كان عدد الوحدات الانزيمية المحصل عليها من هذه العزلة بطريقتي SSF وSmF (49.83 و26.53 وحدة/مل) على التوالي. أنتج انزيم الفايتيز من العزلة الفطرية *A. tubingensis SKA* باستخدام مدعمات ومضافات مختلفة بطريقتي تخمرات الحالة الصلبة والحالة السائلة واظهرت النتائج تفوق تخمرات الحالة الصلبة على الحالة السائلة في عملية الإنتاج وخاصة مع استخدام نخالة الحنطة إذا كان عدد وحدات الفايتيز المحصل عليها من العزلة (50.35 و45.42 وحدة/مل) على التوالي.**

**الكلمات المفتاحية: حامض الفايتيك *Aspergillus tubingensis SKA*، تخمرات الحالة الصلبة.**
***البحث مستل من أطروحة دكتوراه للباحث الأول**

## INTRODUCTION

Oilseeds, cereals and legumes crops are the main ingredients of the food basket of the population especially in developing countries (30); they represent good sources for the essential nutrients especially for the proteins and minerals. In addition whole grain products which are recommended for consumption for their therapeutic role as anti-cardiovascular diseases, diabetes and certain type of cancers (23, 28). However all these products contain phytic acid (*myo*-inositol hexakisphosphate (IP6) the principle storage form of phosphorus, which represents about 60-90% of the total phosphorus in plant tissues (40). Phytic acid is an indigestible constituent for human and nonruminant animals and it has a unique structure with six phosphate groups that makes it highly charged and acts as a strong chelator of cations causing inhibition for the absorption on minerals, as well as its ability to form complexes with proteins reducing their solubility and digestibility (9, 20). Phytases (*myo*-inositol hexakisphosphate phosphohydrolases), have the ability to catalyze the hydrolysis of phytic acid to lower inositol polyphosphates or in some cases free inositol and release inorganic phosphate (Pi), (37). Phytate degrading enzymes (phytases) have potential application in food industries and that is due to their role in the reduction of phyic acid content in food (36). Phytases are present in many plants, animal tissues and microbial sources (bacteria, yeasts and molds), (8, 14). In the case of fungi various species were described as a good producers of phytases specially *Apergillus* (26, 32, 33, 34). Fungi known for their ability to produce extracellular enzymes compared to intracellular enzymes produced by yeast and bacteria, and that was reflected positively on the operation cost, especially for the industrial scale (38). Production of phytase under submerged fermentation (SmF) was promoted as the best technique. Yet, in recent years, production of phytase by solid-state fermentation (SSF) has grown up, due to the advantages of this method of fermentation both in economic and practical perspectives. These included better product recovery, low-technology cultivation equipment, higher product concentration and lower plant operation cost (6, 11, 27, 41). This paper describes the isolation and screening as well as the production of phytase from local fungi isolate (*Aspergillus tubingensis SKA*) by two fermentation techniques, solid-state and submerged fermentations.

## MATERIALS AND METHODS

### INSTRUMENTS AND CHEMICAL

Autoclave (Consolidated sterilizer system / USA), hot plate stirrer (Cimaric), pH meter (Sartorius), water bath and spectrophotometer (Thermo Fisher Scientific), incubator (Memmert / Germany), shaking incubator (Eppendorf / Germany). Crushed corncobs (Beck's Hybrids Seed Company / USA), soybean, sesame, whole-wheat flour and wheat bran (Bob's Red Mill / USA), all other chemicals were purchased from Sigma-Aldrich, Fisher Scientific and *Bio-Rad* (USA).

### ISOLATION AND MAINTENANCE OF FUNGI

Samples of poultry farm soil, cattle shed soil; legume soil, dried legumes, wheat bran, soybean and contaminated bread were collected from different locations of the College of Agriculture/University of Baghdad. Isolation was carried out on malt extract agar, containing (in g/L): malt extract 30.0, mycological peptone 5.0 and agar 15.0, the pH was adjusted to 5.4 by using 1 M HCl before autoclaving at 121°C for 15 min. (16). The isolated fungi were maintained at 4°C on malt extract agar slants that were completely covered with sterilized liquid paraffin, and stored at 4°C.

### QUALTITATIVE SCREENING

Qualitative screening was carried out on modified phytase screening medium (PSM) containing (in g/L): D-glucose 15.0, sodium phytate 3.0, $NH_4NO_3$ 5.0, $MgSO_4.7H_2O$ 0.5, KCl 0.5, $FeSO_4.7H_2O$ 0.01, $MnSO_4.4H2O$ 0.01 and Agar 15.0, the pH was adjusted to 5.5 using 1 M HCl before autoclaving under 121°C for 20 min according to the method of Howson and Davis, (17) and the modified method of Singh and Satyanarayana, (38). All isolates were incubated on 30 °C and monitored for clear zone of sodium phytate hydrolysis. Acid producing fungi have the ability to solubilize sodium phytate causing the formation of clear zone. Thus, the zone forming isolates were subjected to double

staining method according to Bae *et al*., (5).

**QUANTITATIVE SCREENING UNDER SOLID-STATE AND SUBMERGED FEREMNTATIOS**

The isolated fungi that showed clear zone on (PSM) were quantitatively screened for the production of phytase by both solid-state and submerged fermentations. For the (SmF) the isolates were cultivated in 250 ml Erlenmeyer flasks containing 100 ml. phytase screening broth (PSB). An inoculum density of ($10^6$ spore/ml) was used for each isolate and then incubated at 30°C for 5 days and 150 rpm shaking incubator. The fungal biomass was separated from the medium through filtration and cell-free filtrate was used for phytases assay. For the (SSF) the isolates were cultivated in 250 ml Erlenmeyer flasks containing 10 g of wheat bran as the fermentation substrate (support). Each flasks was inoculated with the pre-prepared fungal spores at a density of ($10^6$ spore/g), and then incubated at 30°C for 5 days. Crude enzyme (phytase) was extracted at the end of incubation period by the addition of 50 ml acetate buffer (0.2 M, pH 5.5) in a rotary shaker at 200 rpm for 1 h at room temperature. The mixture was separated from solid biomass by filtration through (Whatman No. 4), then the filtrate was centrifuged at 10,000 rpm for 10 min. The supernatant was used as the source of crude enzyme for phytase assay.

**SELECTION FOR THE MOST EFFICIENT METHOD OF FERMEN-TATION FOR THE PRODUCTION OF PHYASTE**

Phytase production by the isolate *Aspergillus tubingensis SKA* was carried out under both solid-state and submerged fermentations with different treatments to select the most efficient method of production. For the submerged fermentations, isolate *Aspergillus tubingensis SKA* was propagated into 250 ml Erlenmeyer flasks; each flask contains 100 ml of (PSB) with different treatment: (PSB) without any treatment as control, (PSB) with the substitution of glucose and sodium phytate with 5 gm wheat bran; soy flour; sesame flour and whole wheat flour per100 ml of the medium, as a source of carbon and phytate, (PSB) without the addition of sodium phytate. Then incubated at 30°C for 5 days and 150 rpm shaking incubator. For solid-state fermentation, isolate *Aspergillus tubingensis* was propagated into in 250 ml Erlenmeyer flasks; each flask contain 10 g of the fermentation substrate (support). Different substrates were used for that purpose: wheat bran, soybean flour, sesame flour, whole-wheat flour, crushed soybean and crushed corncobs. Then incubated at 30°C for 5 days, enzyme was extracted as mentioned previously.

**PHYTASE ASSAY**

Phytase activity was estimated according to the modified method of Awad *et al*., (3), and the liberated inorganic phosphate was determined according to the method of Fiske and Subbarow, (13). One unit of phytase is defined as the amount of the enzyme that releases one µg inorganic phosphate per ml per min under the assay conditions.

**RESULTS AND DISCUSSION**

After culturing on malt extract agar and propagating at 30°C for 5 days, twenty-six isolates were obtained from the samples of different sources included in this study as listed in Table (1). According to the morphological characteristics and depending on the identification keys of Harigan and McCance, (16), Pitt and Hocking (29), fungal genera mentioned in the table were identified for each source of sample.

**Table 1. Genera of fungi isolated from local sources included in this study.**

| Isolate symbol | Source | Genus |
|---|---|---|
| $F_1$ | Dried legumes | *Fusarium* |
| $F_2$ | Dried legumes | *Fusarium* |
| $F_3$ | Poultry farm soil | *Fusarium* |
| $P_1$ | Poultry farm soil | *Penicillium* |
| $P_2$ | Legume soil | *Penicillium* |
| $A_1$ | Cattle shed soil | *Aspergillus* |
| $A_2$ | Contaminated bread | *Aspergillus* |
| $Al_1$ | Legume soil | *Alternaria* |
| $Al_2$ | Cattle shed soil | *Alternaria* |
| $A_3$ | Wheat bran | *Aspergillus* |
| $P_3$ | Soybean | *Penicillium* |
| $Al_3$ | Contaminated bread | *Alternaria* |
| $A_4$ | Contaminated bread | *Aspergillus* |
| $A_5$ | Poultry farm soil | *Aspergillus* |
| $P_4$ | Dried legumes | *Penicillium* |
| $F_4$ | Contaminated bread | *Fusarium* |
| $A_6$ | Legume soil | *Aspergillus* |
| $G_1$ | Contaminated bread | *Aspergillus* |
| $F_5$ | Cattle shed soil | *Fusarium* |
| $R_1$ | Cattle shed soil | *Rhizopus* |
| $T_1$ | Dep. of Plants Protection/College of Agriculture | *Trichoderma* |
| $Ri_1$ | Dep. of Plants Protection/College of Agriculture | *Rhizoctonia* |
| $P_5$ | Dep. of Plants Protection/College of Agriculture | *Penicillium* |
| $A_7$ | Wheat bran | *Aspergillus* |
| $A_8$ | Legume soil | *Aspergillus* |
| $A_9$ | Cattle shed soil | *Aspergillus* |

Most of the related studies found that the natural environments are important sources for the isolation of phytase producing fungi. Lata *et al*., (24) stated that obtaining a microorganism possesses the qualities that make it appropriate for this pattern of studies is the main goal for which the researchers seek to isolate from natural environments, in which the basic natural substances affect the metabolism of the microorganism and directs its metabolic pathways to produce the required enzyme. Thyagarajan *et al*., (39) isolated the phytase producing fungus *Hypocrea lixii* from poultry field soil by a screen plate method. Soil is the most widely used source rich in microorganisms, and it had been used by many researchers to achieve their goal in the isolation of phytase producing fungi (19, 21, 22). When the ability of the 26 fungal isolates to produce phytase was tested by propagating on phytase screening medium (PSM), results in Table (2) show that the majority of them (18 isolate) were able to form clear zone around their grown colonies. While the rest 8 isolates were unable to do so due to their lack of possessing such enzyme, so they were excluded from the further experiments. Figure (1) illustrates formation of clear zones by some of the fungal isolates as indication of phytases production. With the presence of 1.5% glucose in the medium, it is very likely for the microorganisms to produce organic acids such as citric acid, malic acid, acetic acids and more others. The formation of these acids decreases the pH of the medium making sodium phytate soluble and that would form a zone of clearness. For that, a simple staining method was conducted according to Bae *et al*., (5) to distinguish the clear zone, which formed because of the production of phytase by consumption of sodium phytate from the one that appeared because of the organic acids' production. Out of the eighteen isolates grown and formed clear zone on phytase screening medium, five of them ($T_1$, $A_7$, $P_4$, $F_2$ and $P_3$) were unable to form such zones by applying of the simple staining method, while the rest thirteen isolates exhibited clear zones by this technique also. For the above findings, those five isolates were excluded from the following experiments regarding the quantitative screening for production of phytase. Qualitative screening is an important step to determine the ability of the isolates in the production of an enzyme; this can be done by propagating microorganisms under selective pressures, by supplementing growth medium with the substrate of the required enzyme and that would induce the production of the enzyme itself. Several studies like (15, 24, 25) were also conducted such technique. Quantitative screening is defined as an additional confirmatory test for the capabilities required in order gaining knowledge about the microorganisms that are isolated from previous steps. It has provided a variety of information necessary to assess the ability of microorganisms in industrial uses (7).

**Table 2. Ability of fungal isolates to grow on phytases screening medium (PSM) and produce phytase enzyme**

| Isolate symbol | Growth on (PSM) | Simple staining method |
|---|---|---|
| $Ri_1$ | - | excluded |
| $P_5$ | - | excluded |
| $F_5$ | - | excluded |
| $F_1$ | - | excluded |
| $Al_2$ | - | excluded |
| $Al_3$ | - | excluded |
| $F_3$ | - | excluded |
| $A_2$ | - | excluded |
| $T_1$ | + | - |
| $A_7$ | + | - |
| $P_4$ | + | - |
| $F_2$ | + | - |
| $P_3$ | + | - |
| $P_1$ | + | + |
| $P_2$ | + | + |
| $A_1$ | + | + |
| $Al_1$ | + | + |
| $A_3$ | + | + |
| $A_4$ | + | + |
| $A_5$ | + | + |
| $F_4$ | + | + |
| $A_6$ | + | + |
| $G_1$ | + | + |
| $A_8$ | + | + |
| $R_1$ | + | + |
| $A_9$ | + | + |

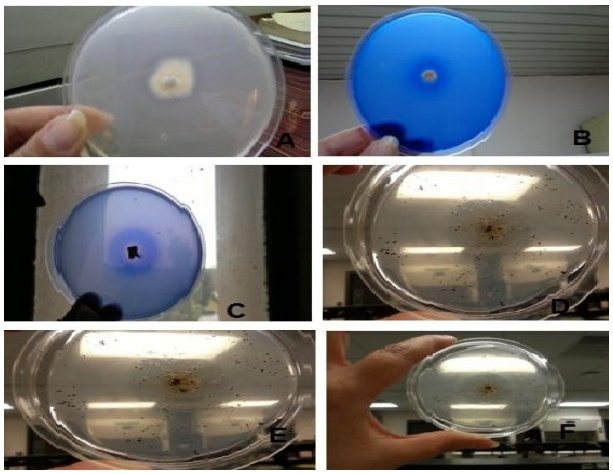


**Figure 1. Clear zone formation of some fungal isolates. (A) isolate $A_3$; (B) isolate $P_3$; (C) isolate $G_1$ on PSM; (D, E and F) clear zone of isolate G1 on a medium consist of sodium phytate + agar**

The thirteen isolates that were obtained from the previous step of qualitative screening were subjected to the quantitative screening, which was carried out under two methods: submerged fermentation (SmF) and solid-state fermentation (SSF) to achieve the most efficient one in production of phytase and to demonstrate the ability of the superior isolate in this regard. As shown in Fig (2), it is obvious that all the thirteen isolates had the ability to consume sodium phytate and produce phytase.

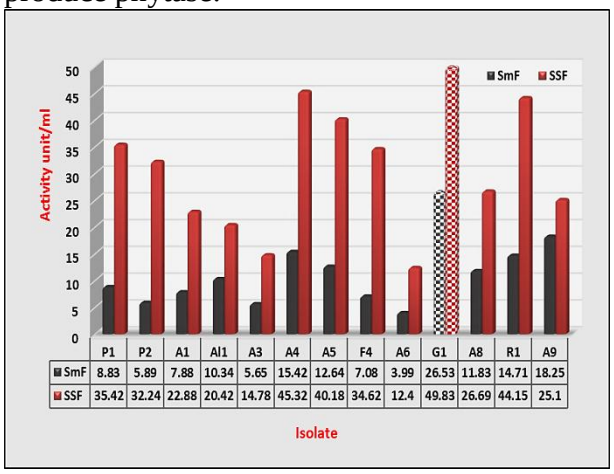


**Figure 2. The quantitative screening of the selected fungal isolates on phytases screening medium.**

Despite that the isolates varied in their capabilities to produce the phytase enzyme, isolate ($G_1$) was the most distinguished one among others by giving the enzymatic activity in both methods with values reached (49.83 and 26.53 unit/ml) in SSF and SmF respectively. Depending on such superior results, this isolate was selected to be used in the rest of the experiments of the research. From the results above, it is obvious that all the isolates recorded their highest enzymatic activity by using the SSF method in comparison with the SmF method. Almost similar findings were achieved by Shivanna and Venkateswaran, (35) who found that phytase activity produced by *Aspergillus ficcuum* SGA 01 was higher by using SSF in about 3-5 folds than that by SmF method. In another study, phytase activity produced by *Aspergillus ficcuum* PTTC was found to be higher when the solid-state bioreactor was used in comparison to the submerged intake bioreactor (4). Aguilar *et al*., (1) confirmed that all products, including enzymes, produced under SSF have high concentrations, which facilitate subsequent purification steps, and Identification of isolate ($G_1$) to the species was conducted at the Fungus Testing Laboratory / University of Texas- Health Science Center/ San Ontario / USA, depending on morphological and molecular identification of three genes (ITS, Tub and CAL) it was found that this isolate is belonged to *Aspergillus tubingensis* and marked as *SKA*. Phytase enzyme was produced from fungal isolate *A. tubingensis SKA* by SSF and SmF fermentation methods. The fermentation have been carried out by utilizing different substrates for SSF, and by adding different supplements to SmF. Results in Figures (3 and 4) illustrate superiority of the SSF on the SmF, especially when using wheat bran as a substrate and as a supplement, respectively.

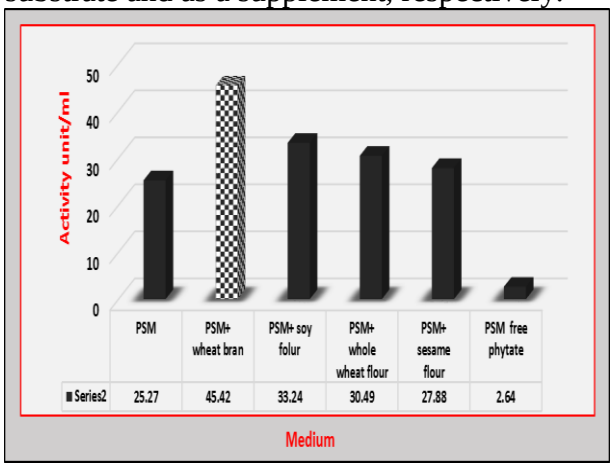


**Figure 3. Production of phytase from fungal isolate *A. tubingensis SKA* by submerged fermentation (SmF) method**

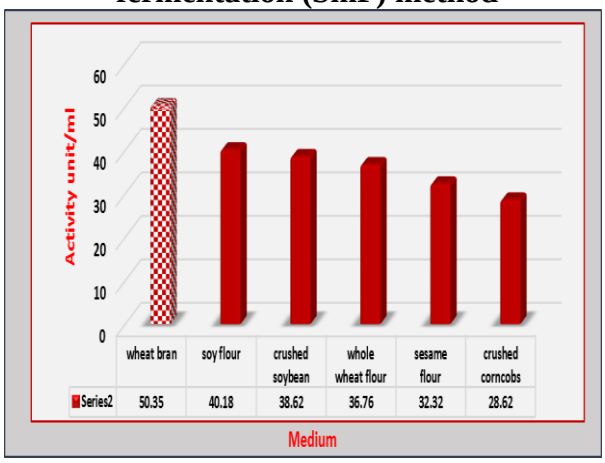


**Figure 4. Production of phytase from fungal isolate *A. tubingensis SKA* by solid-state fermentation (SSF) method.**

Highest enzyme activities was achieved by using wheat bran in SSF and SmF

fermentation methods when they reached (50.35 and 45.42) unit/ml, respectively. In SmF (Fig. 3) it was obvious that supplementing the fermentation medium (PSM) with natural substances such as (wheat bran, soy flour, whole-wheat flour, and sesame flour), increased phytase productivity compared to the original medium (PSM). Such a supplementation is so necessary to upturn the nutritional value of the fermentation medium and reduces the cost of production process compared to using synthetic media, (10, 12). Lowest value of phytase activity (2.64 unit/ml) by using SmF method (Fig. 3) was achieved by using the phytate-free (PSM) medium; such a result suggested that the phytase form isolate *A. tubingensis SKA* is a constitutive enzyme, since it can be produced either in the presence or in absence of phosphate in the growth medium. Same result was reported by Howson and Davis, (17) who found that 12 strain of fungi from different genera (*Aspergillus*, *Rhizopus*, *Mucor* and *Geotrichum*); produced phytases in the (PSM) and potatato dextrose broth, which indicated that their enzymes are constitutive. In a study by Lata *et al*. (24), phytase from the fungus *Aspergillus heteromorphus* MTCC 10685 was found to be an inducible enzyme since the organism was only able to produce the enzyme in the presence of sodium phytate as a sole source of phosphate in the growth medium. Isolate *A. tubingensis SKA* showed unrivaled results in the production of phytase by using the SSF technique (Fig 4). The highest value of enzymatic activity was achieved by using wheat bran, a common substrate in this technique, which is known by its high nutritional value (a rich source of carbon, protein and other nutrients) and low cost. For such characteristics, wheat bran aroused the attention of researchers over years to be highly recommended for using in the production of microbial enzymes (2, 12, 18, 22). The minimum value of phytase activity (28.62 unit/ml) under SSF was obtained by using crushed corncobs, adversely Awad *et al*., (3) gained the highest activity of phytase from *Penicillium purpurogenum* by using corncobs. Such findings can referred to the chemical and nutritional components of corncobs, which may affect the growth of microorganisms. The variation in utilizing various nutrient substrates is due to the differences in reaction of microorganisms to each substrate (38). Superior values of phytase activity under SSF (Fig 4) from the results above encouraged adopting this technique for production of phytase from isolate *Aspergillus tubingensis*. Moreover, the culture medium of SSF is known by its simplicity, low coast, and good concentration of produced enzymes in addition to its suitability to fungi compared to other microorganisms. Fungi can tolerate the decrease in humidity, beside their abilities to grow on the surface of the substrate particles and the ability of their hyphae to penetrate the inter particles spaces and colonized them (1, 31).

**REFRENCES**

1.Aguilar, C. N.; G., Gutierrez-Sanchez; P. A., Rado- Barragan; R., Rodriguez-Herrera; J. L., Martinez-Hernandez and J. C. Contreras-Esquivel 2008. Perspectives of solid-state fermentation for production of food enzymes. American Journal of Biochemistry and Biotechnology, 4(4): 354-366.

2.Awad, G. E. A.; M. M. M., Elnashar and E. N., Danial 2011. Optimization of phytase production by *Penicillium funiculosum* NRC467 under solid-state fermentation by using full factorial design. World Applied Sciences Journal, 15(11): 1635-1644.

3.Awad, G. E. A.; M. M. I., Helal; E. N., Danial and M. A., Esawy 2014. Optimization of phytase production by *Penicillium purpu-rogenum* GE1 under solid-state feremntation bu using box-behnken design. Saudi Journal of Biological Sciences, 21: 81-88.

4.Badamchi, M.; Z., Hamidi-Esfahani and S., Abbasi 2013. Comparison of phytase production by *Aspergillus ficuum* under submerged and soil-state feremntation conditions. Focusing on Modern Food Industry, 2(3): 129-137.

5.Bae, H. D.; L. J., Yanke; K. J., Cheng and L. B., Selinger 1999. A novel staining method for detection phytase activity. Journal of Microbiological Methods, 39: 17-22.

6.Becerra, M. and M. I., Gonzalez Siso 1996. Yeas β-galactosidase in solid-state ferment-ation. Enzyme and Microbial Technology, 19: 39-44.

7.Bhargav, S.; B. P., Panda; M., Ali and S., Javed 2008. Solid-state fermentation: an overview. Chem. Biochem. Eng., 22(1): 49-70.
8.Correa, T. L. B.; M. V., Queiroz and E. F., Araujo 2015. Cloning, recombinant expression and characterization of a new phytase from *Penicillium chrysogenum.* Microbiological Research, 170: 205-212.
9.Cowieson, A.; T., Acamovic and M., Bedford 2006. Pytic and phytases: Implications for Protein Utilization by Poultry. Poultry Science, 85: 878-885.
10.Devidas, P. C.; B. H., Pandit and P. S., Vitthalrao 2014. Evaluation of different culture media for improvement in bio-insecticides production by indigenous *Bacillus thuringiensis* and their application against larvae of Aedes aegyptus. The Scientific World Journal, 2014: 1–6.
11.El-Batal, A. I. and H., Abdel Karem 2001. Phytase production and phytic acid reduction in rapeseed meal by *Aspergillus niger* during solid-state fermentation. |Food Research International, 34: 715-720.
12.El-Gindy, A. A.; Z. M., Ibrahim; U. F., Ali and O. M., El-Mahdy 2009. Extracellular phytase production by solid-state cultures of *Malbranchea sulfurea* and *Aspergillus niveus* on cost-effective medium. . Research Journal of Agriculture and Biological Sciences, 5(1): 42-62.
13.Fiske, C. H. and Y., Subbarow, 1925. The colorimetric determination of phosphorus. The Journal of Biological Chemistry, 2: 375-400.
14.Gaind, S. and S., Singh 2015. Production, purification and characterization of neutral phytase from thermotolerant *Aspergillus flavus* ITCC 6720. International Bioreme-diation and Biodegradation, 99:
15-22.
15.Gull, I.; A., Hameed; M. S., Aslam and A., Athar 2013. Optimization of phytase production in solid-state fermentation by different fungi. African Journal of Microbiology Research, 7(46): 5207-5212.
16.Harrigan, W.F. and M. E., McCane 1976. Laboratory Methods in Food and Dairy Microbiology. London, New York. Academic Press.
17.Howson, S. J. and R. P., Davis 1983. Production of phytate-hydrolysing enzyme by some fungi. Enzyme Microb. Technol., 5: 377-382.
18.Jafari-Tapeh, H.; Z., Hamidi-Esfahani and M. H., Azizi 2012. Culture condition improvement for phytase production in solid-state fermentation by *Aspergillus ficuum* using statistical method. ISRN Chemical Engineering, 2012: 1-5.
19.Jena, S. K. and C. C., Rath 2014. Effect of environmental and nutritional conditions on phosphatase activity on *Aspergillus awamori*. Current Research in Environmental and Applied Mycology, 4(1): 45-56.
20.Kies, A. K.; L. H., De Jonge; P. A., Kemme and A. W., Jongbloed 2006. Interactions between protein, phytate and microbial phytase. In vitro studies. Journal of Agricultural and Food Chemistry, 54: 1753-1758.
21.Kumar, A. and A. B., Bahatt 2011. Isolation and screening of phytase producing fungi from soil. Advanced Biotechnology, 10(12): 27-29.
22.Kumar, M. and Sushma 2012. Optimization of production media of novel phytase from *Aspergillus niger* using wheat bran waste. International Journal of Science and Research, 3(6): 949-951.
23.Kumar, Vikas; Amit K. Sinaha; Hrinder P.S., Makkar and Klaus, Becker 2010. Dietary roles of phytate and phytases in human nutrition: A review. Food Chemistry, 120: 945-959.
24.Lata, S.; S., Rostogi; A., Kapoor and M., Imran 2013. Optimization of culture conditions for the production of phytase from *Aspergillus heteromorphous* MTCC 10685. International Journal of Advanced Biotechnology and Research, 4(2): 224-235.
25.Lee, D.; S., Choi and Y., Hwang 2005. Culture conditions and characterization of new phytases-producing fungal isolate *Aspergillus sp.* L117. Mycobiology, 33(4): 223-229.
26.Moreira, K. A.; P. N., Herculano; M. H. C., Maciel; T. S., Porto; M. R., Spier; C. M., Souza-Motta; L. F., Porto and C. R., Soccoi 2014. Optimization of phytase production by *Aspergillus japonicas* Saito URM 5633 using cassava bast as substrate in solid-state fermentation. African Journal of Microbiology Research, 8(9): 929-938.

27.Muniswaran, P.K.A.; P., Selvakumar and N. C .L. N., Charyulu 1994. Production of cellulases from coconut coir pith in solid-state fermentation. A comparative study. Journal of Chemical Technology and Biotechnology, 60: 147-151.

28.Nuobariene, L.; A. S., Hansen; L., Jespersen and N., Arneborg 2011. Phytase-active yeasts from grain-based food and beer. Journal of Applied Microbiology, 110: 1370-1380.

29.Pitt J.I. and A. D., Hocking 1997. Fungi and Food Spoilage. pp. 366-368. Blackie Academic professional.

30.Rostami, Hassan and Archana, Giri 2013. An overview on microbial phytases and its biotechnological application. International Journal of Advanced Biotechnology and Research, 4: 62-71.

31.Santos, M. M.; A. S., Rosa; S., Dal'boit; D. A., Mitchell and A., Kriger 2004. Thermal denaturation: is solid-state fermentation really a good technology for the production of enzymes. Bioresour. Technol., 99: 261–268.

32.Sariyska, M. V.; S. A., Gargova; L. A., Koleva and A. I., Angelov 2005. *Aspergillus niger* phytase: purification and characterization. Biotechnol. and Biotechnol. Eq., 19: 98-105.

33.Shah, K. B. and R., Trivedi 2012. Purification and characterization of an extracellular phytase from *Aspergillus tamari*. International Journal of Pharma and Biosciences, 3(2): 775-783.

34.Shieh, T. R. and J. H., Ware 1968. Survey of microorganisms for production of extracellular phytase. Applied Microbiology, 16(9): 1348–1351.

35.Shivanna, G. B. and G., Venkateswaran 2014. Phytase production by *Aspergillus niger* CFR 335 and *Aspergillus ficuum* SGA 01 through submerged and solid-stat fermentation. The Scientific World Journal, 2014: 1-6.

36.Singh, B. and T., Satyanaryana, 2012. Production of phytate-hydrolyzing enzymes by theromphiloic moulds. African Journal of Biotechnology, 11(59): 12314-12324.

37.Soni, Sarvese Kumar. 2009. Phytase from *Aspergillus niger* NCIM 563: Isolation, Purification, Characterization and its Applic-ations. Doctoral thesis. NCIM RESOURCE CENTRE, NATIONAL CHEMICAL

38.Subramaniyam, R. and R., Vimala 2012. Solid state and submereged fermentation for the production of bioactive substances: A comparative study. International Journal of Science and Nature, 3: 480-486.

39.Thyagarajan, R.; S. K. R., Namasivayam and G. N., Kumar 2014. Evaluation of phytase production by Hypocrea *lixii* SURT01 in submerged and solid-state fermentation. International jJournal of Pharmacy and Pharmaceutical Sciences, 6(10): 352-355.

40.Vats, P. and U. C., Banerjee 2004. Production studies and catalytic properties of phytases (*myo*-inositol hexakisphosphate hosphohydrolases): an overview. Enzyme and Microbial Technology, 35: 3-14.

41.Zhu, Y.; J. P., Smit; W., Knol and J., Bol 1994. A novel solid-state fermentation system using polyurethane foam as inter carrier. Biotechnology Letters, 16: 643-648.