Purification and Characterization of Phytase from Fruit Bodies of Local mushroom *Pleorotus ostreatus* Grown by Solid State Fermentation

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Abstract

Phytase produced from an edible local mushroom *P.ostreatus*11L was purified in three steps, in precipitation with ammonium Sulfate (saturation ratio70%), the specific activity of phytase increased from 0.38u/mg protein in crude extract to 0.77 u/mg protein with purification of 2.03 fold and the enzyme yield was 92.05%, in ion exchange chromatography(DEAE-cellulose) step, specific activity was 3.6 u/mg protein with purification of 9.47 fold with a yield of 71. 82%. In the last step, with gel filtration chromatography using Sephadex G75 which gave the highest specific activity reached 7.5 U/mg with the purification folds arrived to 19.74 and enzyme yield was 42.61%. The results showed that there is only one band appeared in SDS-PAGE technique indicating a high purity of phytase enzyme , the purified phytase behaved as a monomeric protein with a molecular mass of about 25.12 kDa . The phytase was active over a broad range of incubation temperature (20 - 50ºC), maximal activity was 0.83 unit/ml when phytase incubated at 30ºC while the enzyme has thermal stability over a broad range of temperatures (20-60ºC) for one h since more than 50 % of the relative enzyme activity was retained after incubation. Phytase was active over a broad range of pH (4-8), maximal activity was 0.81unit/ml when phytase incubated at pH 6 followed by 0.79 unit/ml when phytase incubated at pH 5 without significant differences between them. In addition , the enzyme was full stable at pH 5 and 6 for 1 h of incubation at 37^{o}C. phytase activity didn't reach 70% at the other pH values. Hence it is inferred that the phytase was active over a broad range of pH 4-8. Among various metal ions, only $MgSO_4$ has a syregenic effect with phytase activity in which activity increased as residual activity was 117.36% , while CuSO4 and ZnCl were the most inhibitory agents for *P.ostreatus* phytase.

Key words : purificationphytase, *P.ostreatus*, endophytase, phytase characterization.

Introduction

Phytase (*myo* - inositol hexaphosphate phosphohydrolase, EC 3.1.3.8 or EC 3.1.3.2.6) hydrolyzes phytate, thereby releasing inorganic phosphate [1, 2]. Supplemental microbial phytase in meal diets for swine, poultry and fish effectively improves phytate phosphorus utilization by these animals, The phytases enhances the bioavailibility of minerals, protein and phosphorus in monogastric animals decreasing minerals excretion pollution [3,4,5]. In addition to animal feed, phytase was used in many industrial applications such as, food industry, preparation of myo-inositol phosphate, detoxified agent, Paper industry, soil improvement and the elimination of environmental pollution[6]. Phytase as exo-enzyme was purified from many filamentous fungi including especially *Aspergillus niger*, *A. terreus*, *A. oryzae*, *Neurospora crassa*, *Emericella nidulans* and *thermomyces lanuginosus*. In addition from several yeast species such as *saccharomyces cerviceia*, *Candida tropicalis*, *Kluveromyces fragilis*, *Torulopsis candida*, *Debaryomyces castelli and Schwanniomyces castelli*, but the first commercial phytase was produced from *Aspergillus niger* [7,8,9]. All these organisms produced phytase with submerged cultures using high cost fermenters, while other researches focused on the type of fungi called Mushrooms for production of phytase as endo-enzyme due to low cost- mushrooms cultivation technology. Phytase was purified and characterized from a few types of mushrooms, these mushrooms were edible and cultured, included *Agrocybe pediades*, *Cenporia sp.*, *Peniophoralycii*, *Trametes pubenscence* and *Agaricus bisporus* [10,11], *Lentenus edodes*[12], *Volvariella volvacea* [13] and *Flammulina velotipes*[14]. Whereas there was no any study in the country for the production, purification and characterization of phytase from fungi. In addition low cost mushrooms cultivation, edible mushrooms were a safe food source that didn't contain any toxin. For these reasons, the present study was achieved which aimed to Purify and characterize of phytase.

Materials and Methods

Purification of phytase

Phytase was purified from *P.ostreatus* extraction according to the following steps :

Ammonium sulfate precipitation

Phytase enzyme was precipitated by ammonium sulfate (70%) in ice bath. The mixture was stir up continuously for 30-40 min. Then put in cold centrifuge at 4C, 4000 G for 30 min. The precipitation was dissolved in amount of the Ammonium carbonate mono hydrate buffer. Then, Dialyzed 3 times against D.W for 24 hours. At the end of this step phytase activity and optical density were measured at 250 nm.

Ion exchange chromatography

DEAE-Cellulose Preparation:

DEAE-Cellulose column (20×2.5cm) was prepared according to Whitaker and Bernard (1972) by dissolving 20 g of DEAE-Cellulose resin in 1 liter of distilled water. Then, left to settle down and washed

several times with distilled water until it becomes clear. After that, the resin was resuspended in 0.25 M sodium hydroxide. Finally, the resin was filtered and washed several times with 0.25 M hydrochloric acid solution by distilled water before it was equilibrated with Ammonium carbonate monohydrate buffer (pH 9.0).

Sample loading

After column equilibration, concentrated phytase was transferred and poured gently on to the surface of the DEAE cellulose resin, then washed by ammonium carbonate monohydrate buffer to displace unbinding proteins (wash). The column was then washed with 100 ml of the same buffer at the flow rat of ml/min. The column was eluted with a linear NaCl gradient from (0-0.5)M in the same buffer. Fractions were eluted at a flow rate of 3ml/fraction then, the phytase activity and optical density at 280 nm for each fraction were measured .Fractions represent phytase activity were pooled and kept at 4°C for the next step of purification.

Gel filtration chromatography

Preparation of Sephadex G-75 column

Sephadex G-75 Column of (50×1.5) cm was used. A suitable part of wool was put in the lower of the column and the Sephadex G-75 (pharmacia Fine chemicals) was prepared by suspending 25g of this gel in 500ml of the phosphate buffer then put in boiled water bath for 5 min for sowelling and degasing . the gel was allowed to stele for 18 hours. The column waswashed by phosphate buffer 50 mM and pH 7.0 twice of its volume then, it was ready to be used.

Sample loading

After column equilibrium, 5ml of sample obtained from I.E.C. was poured gently on the surface of the gel, then it was washed by Ammonium carbonate mono hydrate buffer pH 9.0 with flow rate of 20 ml/hour (5ml for each fraction). Optical density (at 280nm), and enzyme activity (U/ml) were determined in each fraction. Fractions represents phytase activity were pooled and kept at 4 C for further studies.

Characterization of Purified Phytase

Determination the Molecular Weight

The molecular weight of the purified phytase was determined by Sodium dodosyl sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE) which was carried out according to the method of [15].

Effect of Cations

A. One ml of purified phytase was mixed with 1ml of metal ion

solution including $FeSo_4$, ZnCl, KI, $CuSo_4$, and $MgSo_4$, and incubated at 30°C for 30min.

B. Reaction solution was added to the mixture and incubated at 30°Cfor 30min then, the enzyme activity was measured .

Optimal Temperature for Phytase Activity

Optimal temperature for maximal phytase activity was determined by incubating the enzyme at various temperatures in the range of 20 to 50 ºC with increments of 5 under assay conditions already mentioned, except temperature. Enzyme activity was calculated as the above describtion.

Optimal pH for Phytase Activity

Optimum pH for maximal phytase activity was determined by conducting enzyme assay at various levels of pH in the range of 4-8 with increments of 1 . phytase activity was calculated. Citrate buffer (pH 3-6), Phosphate buffer (pH 7), Tris buffer (pH 8-9), Carbonate bi carbonate buffer (pH 10).

Enzyme Stability at Different Temperatures

Thermal stability of phytase was determined by incubating the enzyme at various temperatures ranging from 20-80ºC with increments of 10, and the enzyme assay was conducted after 1h. Phytase activity of the other sample kept at 4ºC and was taken as control. Residual activity of enzyme was calculated.

Stability of Phytase at Various pH

Stability of the phytase over a range of pH was determined by measuring the residual activity after incubating the enzyme in different buffer systems of pH 4-8 with increments of 1 for1h.**Residual activity**

Residual activity is the percent enzyme activity of the sample with respect to enzyme activity of control sample.

Residual Activity (%) = Activity of sample (U/ml) / Activity of control (U/ml)x100

Results and Discussion

Phytase extraction and purification

Phytase extraction

Phytase was extracted from the homogenizing of fruiting bodies of *P. ostreatus*11 L which cultivated in wheat straw medium and supplemented by 3% of wheat bran with fruiting at 17°C at mature fruit bodies stage using ammonium mono hydrogen carbonate buffer pH6 for extract phytase enzyme which was intracellular enzyme, and regarded as a crud enzyme, according to these conditions. Results in table (1) showed that phytase activity was 0.88 unit/ml with specific activity 0.38 u/mg protein. Extraction of the phytase enzyme from solid state media by suitable buffer as many studies [16,17, 18].

Purification

Phytase produced from an edible mushroom *P.ostreatus*11L was purified in three steps, in precipitation with ammonium Sulfate (saturation ratio70%), the specific activity of phytase increased from 0.38u/mg protein in crude extract to 0.77 u/mg protein with purification of 2.03 fold and the enzyme yield was 92.05% table (1). Precipitation of the proteins usually carried out in the early stages of the purification of the enzyme and that the disposal of a large proportion of the water and get the degree of purity and often used for this purpose salts such as ammonium sulfate because of the good solubility in water where sedimentation salts occurs as a result of the equation protein charged with salt, leading to reduced solubility protein and deposition and this

called Salting out [19]. Saturation ratios with ammonium sulfate was different according to enzyme source and the nature of its composition. extracting crude phytase of *Aspergillus niviues* is precipitated with ammonium sulfate(60%) [20], while its precipitated from Oakbug Milkcap (*Lactarius Quietus*) with ammonium sulfate (70%) [21]. The ammonium sulphate was used in enzyme precipitation because it is highly soluble and cheap compared with the others salts, unaffected in pH and enzyme stability. The concentration by ammonium sulfate depending on equilibrate charges found in protein surface and disrupt of the water layer surrounding it leads to precipitate it [18]. In second step, ion exchange chromatography using DEAE-cellulose, fig.(1) showed that there are 4 protein peaks resulted from this step, one peak of phytase resulted within second protein peak, phytase activity was appear in fractions between 40-45 (maximum at 42). In ion exchange chromatography step, specific activity was 3.6 u/mg protein with purification of 9.47 fold with a yield of 71. 82%.Ion exchange chromatography method by using the risen Cellulose A50 DEAE Di Ethyl Amino Ethyl cellulose as this matrix used for purification because it has high capacity for bio separation, easy to prepare, multiple use, in addition to simplicity to separate different biomolecules [22]. Ion exchange chromatography was the one of many methods which used for fractionation and purification enzymes depending on the principle of difference in charge of fractions where was obtain a one fraction [22, 23]. Ion exchange was used successfully in phytase purification from mushrooms like *V. volvacea*, [24], *F. velotipes* [14], *Lintinus edodes* [12].

Table (1) Purification steps of phytase from *P. ostreatus* L 11 fruitbodies

Step	Volume (ml)	Activity (Uint/ml)	Protein conc. (mg/ml)	Specific activity (U/mg protein)	Total activity (unit)	Purification (Fold)	Yield %
Crude enzyme	10	0.88	20.33	0.38	8.8	1	100
Ammonium sulfate (70%)	10	0.81	1.05	0.77	8.1	2.03	92.05
ion exchange (DEAE-cellulose)	8	0.79	0.22	3.6	6.32	9.47	71. 82
Gel filtration (Sephadex G75)	5	0.75	0.1	7.5	3.75	19.74	42.61

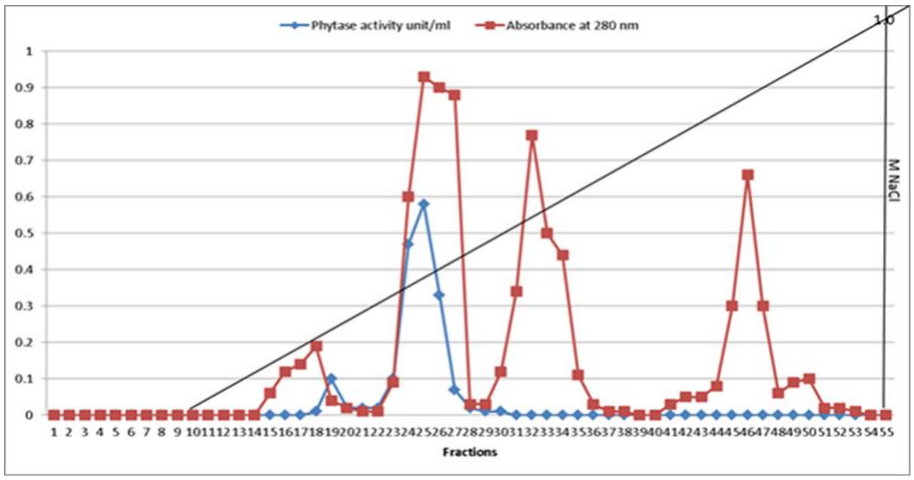
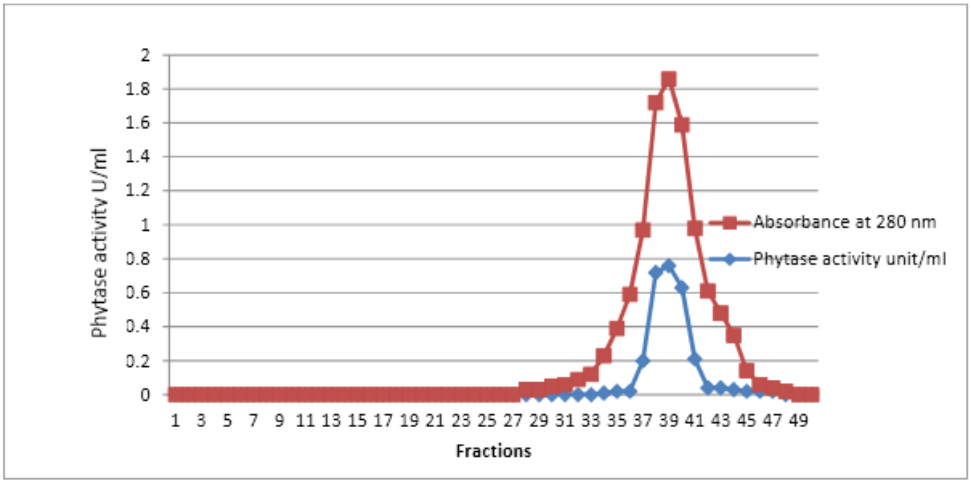


Fig.(1) Ion exchange chromatography for purification of phytase produced from *P. ostreatus*(L11)

In the last step it was with gel filtration chromatography using Sephadex G75 , fig.(1) showed there is only one protein peak resulted from this step, and one peak of phytase resulted within this peak , phytase activity was appear in fractions between 22-28(maximum at 25). Gel filtration chromatography step gave the highest specific activity reached 7.5 U/mg with the purification folds arrived to 19.74 and enzyme yield was 42.61% Table (1).

Furthermore, gel filtration is the simplest and mildest of all the chromatography techniques and separates molecules on the basis of differences in molecular size (Eli and Co, 2001). Gel filtration chromatography was used in phytase purification from different mushrooms such as [12] In the last step of purification phytse from Shiitake Mushroom *Lentinus edodes* used gel filtration chromatography by Superdex 75 which given highest specific activity reached 3.11 unit/mg, thepurificationfolds arrived to 31.6 and enzyme Yield was 27.1mg. [14] also used some chromatographic procedures for purification of the phytase from winter mushroom, *Flammulina velutipes*, CM-cellulose, Q-Sepharose, affinity chromatography on Affi-gel blue gel and fast protein liquid chromatography-gel filtration on Superdex 75.



Fig(2) Gel filtration chromatography for purification of phytase produced from *P. ostreatus*(11L)

Purity of phytase

Fig. (3-B) showed there is only one band appeared in SDS-PAGE technique after last step of phytase purification compared to 6 bands appeared from crude enzyme (fig. 3-A) Purified enzyme shows that only one protein band indicating a high purity of phytase enzyme. the position of phytase band was near to the fifth protein bands in crude extract, that means there is a correspondence between them. The appearance of one band of purified phytase using SDS-PAGA purification Technique with the disappearance of the rest bands (another proteins) that appeared in the crude extract line indicates enzyme purity and success the purification process.

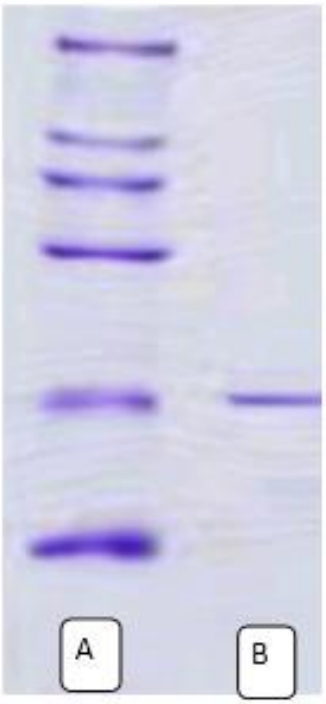


Fig. (3). Purity monitoring of the phytase by SDS-PAGE: A- Crude extract; B – purified phytase

Characterization of purified enzyme

Phytase molecular weight

Fig. (4) showed the relationship between Log Mw of 4 standard proteins and the R_f by SDS-PAGE for determination of purified phytase molecular weights, the purified phytase behaved as a monomeric protein with a molecular mass of about 25.12 kDa. The principle of electrophoresis by SDS-PAGE depends on the molecular weight and enzyme charge since the enzyme band was separated individually. Phytase differs in molecular weight according to its source, molecular weights of phytase produced from filamentous fungi ranged (49-85) kDa [25, 26, 27]. whereas in some mushrooms molecular weights of phytase ranged (14-45) kDa [12, 24, 21]. In the present study, Molecular weight of purified phytase from *P. ostreatus*11L was differed compared with other phytases that led to be regarded it as unique enzyme has molecular weight 25.12 kDa.

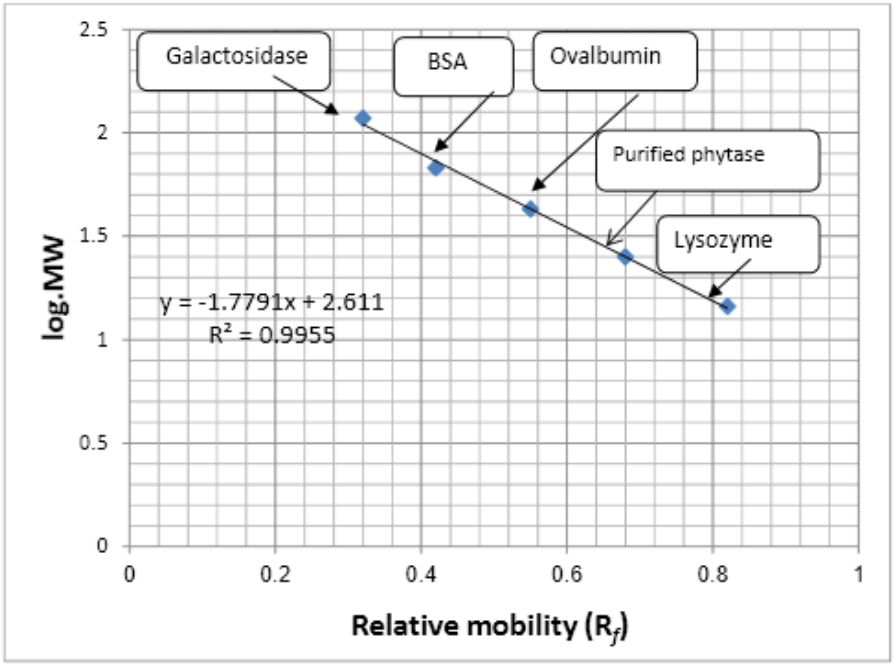


Fig. (4) Standard curve of relationship between log. MW and R_f of the standard proteins using SDS-PAGE technique

Effect of reaction time on phytase enzyme activity and stability

Results in fig (5) showed the effect of reaction time in phytas activity, the enzyme activity increased with the increase of reaction time until the period reached to 20-30 min in which, phytase activity was stable at 0.91u/ml.

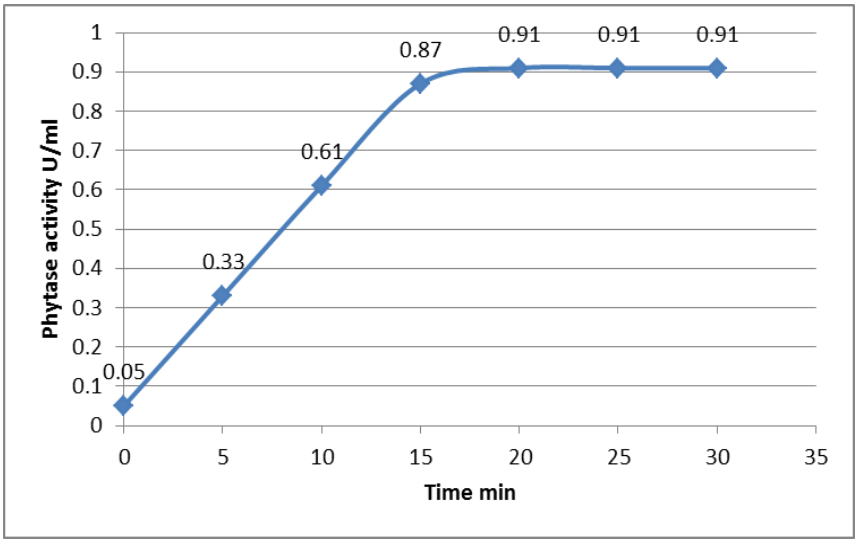


Fig. (5) Effect of reaction time on phytase enzyme activity

[28]. reported that time-scale is an important factor in determining the enzyme activity, and it was preferred to use methods with short time incubation to determine the enzyme activity. In addition, the short time of phytase activity plays an important role in an industrial applications.

Optimal temperature for phytase activity

The results recorded in Fig. (6) indicated that the phytase was active over a broad range of incubation temperature (20 - 50ºC) , maximal activity was 0.83 unit/ml when phytase incubated at 30ºC followed by 0.81 and 0.78 unit/ml when phytase incubated at 35 and 40ºC respectively, (without significant differences between these values).It was also noted that the phytase was active even at 50ºC resulting in 0.48 unit/ml.

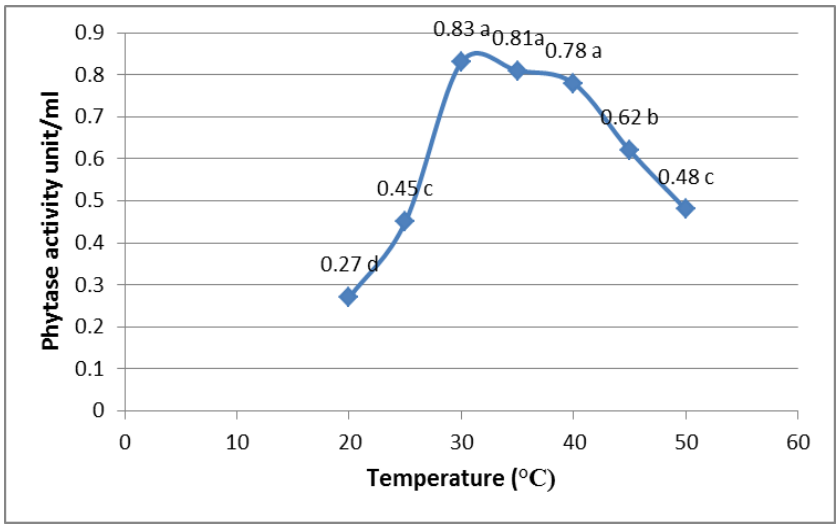


Fig. (6) Effect of temperature on phytase activity

Enzymes have an optimum degree of the temperature that is equal, more or less than the temperature that includes them. The velocity of enzyme interaction with temperature increases when it reaches the optimum degree of interaction. Then being declined gradually. This can be attributed to the process of reducing or destructing the enzyme molecule that this decline in the degree of interaction leads to the reduction or loss of the enzyme activity. This decrease can be construed (explained) by the influence of high temperatures on the state of ionization of the groups found on the enzyme surface and its substrate. Due to the fact that the enzyme are complex protein molecules whosecatalyst activity is affected by regular tertiary structure. High temperature changes the enzyme structure which causes the loss of its activity[29].

Thermostability of phytase

From the results presented in Fig.(7) for the temperature stability studies conducted with phytase it is inferred that the enzyme has thermal stability over a broad range of temperatures (20-80ºC) for one h since more than 50 % of the relative enzyme activity was retained after incubation. The never less even at 70ºC 22.2% of activity was retained after 1 h. full phytase activity was recorded at 20 and 30°C, according to these results, it may be suggested that the phytase purified from *p.ostreatus* is thermostable up to 60ºC.

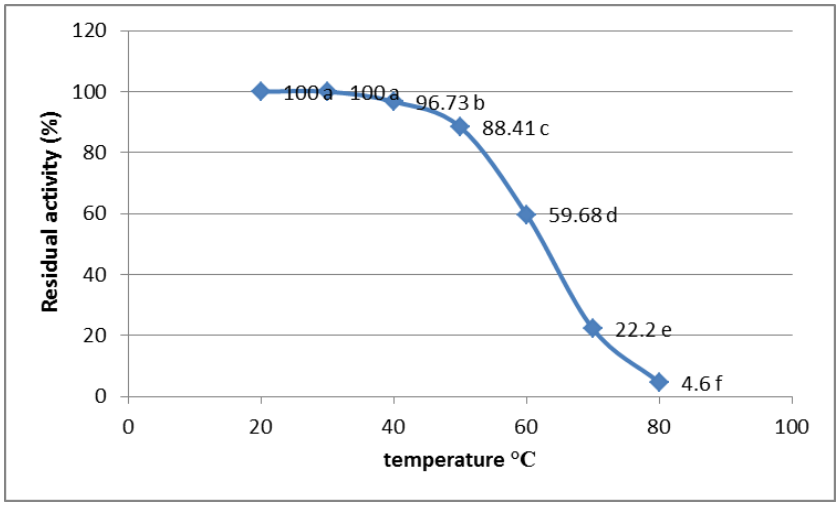


Fig. (7) Stability of phytaseat different temperature

The decrease in the enzyme activity with the increase in temperature may lead to the denaturation of enzyme by destructing the three dimensional structure of protein that causes a change in the active site which leads to inactivation of the enzyme at high temperatures [30]. Temperature may effect on the protein structure by breaking the bonds that stabilizes secondary and tertiary structure of protein which results in denaturation [31].

Optimal pH for phyase activity

The results recorded in Fig.(8) indicated that the phytase was active over a broad range of pH (4-8) , maximal activity was 0.81unit/ml when phytase incubated at pH6 followed by 0.79 unit/ml when phytase incubated at pH5 without significant differences between them.

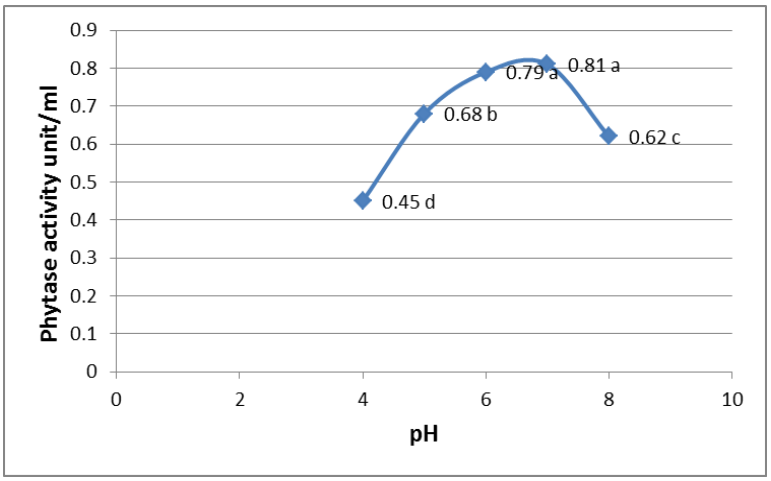


Fig. (8) Effect of pH on phytase activity

The pH of a solution can have several effects on the structure and activity of enzymes, pH can have an effect on the state of ionization of acidic or basic amino acids. Acidic amino acids have carboxyl functional groups in their side chains. Basic amino acids have amine functional groups in their side chains. If the state of ionization of amino acids in a protein is altered then the ionic bonds that help to determine the 3-D shape of the protein can be altered. This can lead to altered protein recognition or an enzyme might become inactive. Changes in pH may not only affect the shape of an enzyme but it may also change the shape or the properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis [31].

Stability of phytase at different pH

The data presented in Fig.(9) showed that the enzyme was full stable at pH 5 and 6 for 1 h of incubation at 37^{o}C.phytase activity do not reached under 70% at the other pH values, hence it is inferred that the phytase was active over a broad range of pH 4-8.

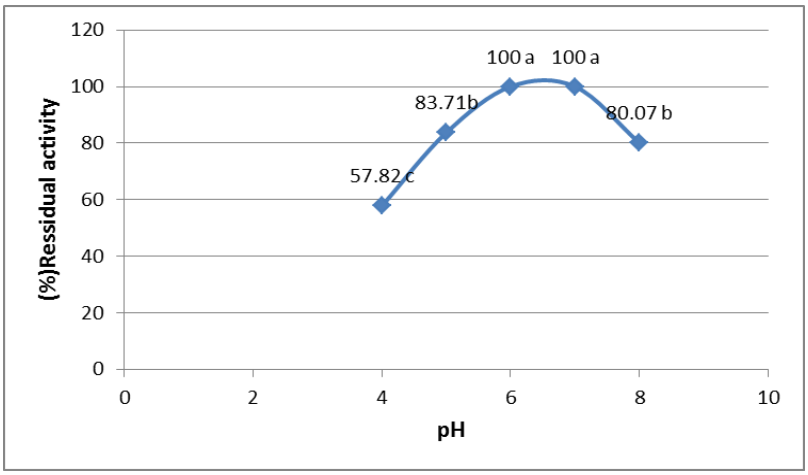


Fig (9) Stability of phytase at different pH

[32] found that most enzymes undergo irreversible denaturation in strong acidic or alkaline conditions. Enzymes like other proteins, are stable over only a limited range of pH. Outside this range, changes in the charges on ionisable residues result in change in secondary and tertiary structure of the ionic state of active site of the enzyme and substrate [35].The pH of the buffer of an enzyme may affect the activity of the enzyme in several ways. First each enzyme has its pH optimum, at which the enzyme is most active, but the enzyme is stable within certain limits on each side of the optimum. Secondly, the pH of the enzyme may influence its stability, and at extremes of acidity or alkalinity the enzyme may be denatured. Thirdly, the pH of the reaction mixture may cause dissociation of the substrate and so by its action on the substrate influence the character of the pH activity curve and the pH optimum. These factors have been discussed by [36].

modifications of the tertiary structure of the protein and eventually cause denaturation[33,34]. The lowering in enzymatic activity in extreme acidic and extreme basic conditions may be due to

Effect of cations on phytase activity

The effect of some metal ions on the phytse activity was recorded in fig(10). Only MgSO4 has a syregenic effect with phytase activity in which activity

increased as residual activity was 117.36% , while CuSO4 and ZnCl were the most inhibitory agents for *P.ostreatus* phytase, in which the remaining activities were 7.21 and 11.75%, respectively, it is inferred from these results that the phytase needs Mg ion or other ions not tested in the present study as cofactor for full activity. Some minerals cause decreasing or complete inhibition of enzyme because it may be preventing the suitable substrate connected with phytase. From these results, it can be concludedthat metal ions vary in their effect on phytase depending on the kind of ion, and this may give some knowledge about the activation or inhibition of *P. ostreatus* phytase by monovalent and divalent cations. This step agrees with[21] found that Hg^{2+} and Mg^{2+} ions increased the activity of immobilized phytase, Fe^{2+}, Cu^{2+} and Zn^{2+}aproximately had not affected it. Because of this feature of enzyme, immobilized phytase could be found to be highly suitable for industrial application. Metal ions have been shown to modulate phytase activity. However, it is difficult to determine whether the inhibitory effect of various metals is due to direct binding to the enzyme, or whether the metal ions form poorly soluble complexes with phytic acid and therefore decrease the active substrate concentration.

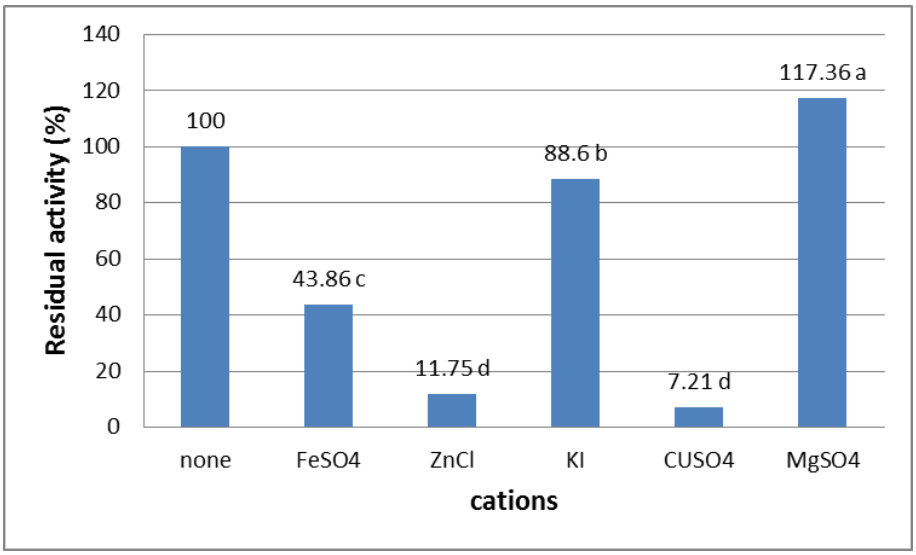


Fig. (10) Effect of cations on phytase residual activity

Many cations such as Mg^{+2} increase the activity of some enzymes due to their role in protection of enzyme structure and hence creating a suitable reaction state. Reducing of enzyme activity by Cu^{+2}, may indicate that groups are involved in the catalytic sites or that these groups are essential for maintenance of the three-dimensional structure of the active protein is called competitive inhibitor [37].

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تنقية وتوصيف الفايتيز من الاجسام الثمرية للفطر *Pleorotus ostreatus* والمنمى تحت ظروف تخمر الاوساط الصلبة

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الملخص

اجريت تنقية الفايتيز من الفطر *P.ostreatus* (11L) بثلاث خطوات شملت الترسيب بملح كبريتات الامونيوم (نسبة الاشباع 70%) وفيها ارتفعت الفعالية النوعية من 0.38 وحدة/ملغم بروتين في المستخلص الخام الى 0.77 وحدة/ ملغم بروتين بعدد مرات التنقية 2.03 مرة وبحصيلة انزيمية 92.05% ، وعند خطوة كروماتوغرافيا التبادل الايوني باستخدام DEAE-cellulose بلغت الفعالية النوعية 3.6 وحدة/ملغم بروتين بعدد مرات التنقية 9.47 مرة وبحصيلة انزيمية 71.82% وفي اخر خطوة باستخدام كروماتوغرافيا الترشيح الهلامي باستخدام الهلام Sephadex G 75 وصلت اعلى فعالية نوعية عند 7.5 وحدة/ ملغم بوتين بعدد مرات التنقية 19.74 مرة وبحصيلة انزيمية 42.61 %, اظهرت نتائج الترحيل الكهربائي باستخدام هلام الاكريلامايد المتعدد وجود حزمة بروتينية واحدة دليل على نقاوة الانزيم، بلغ الوزن الجزيئي للفايتيز المنقى 25.12 كيلودالتون، ابدى الفايتيز المنقى فعالية بمدى حراري 20–50م وبلغت اعلى فعالية انزيمية 0.83 وحدة/مل عند درجة حرارة 30م , بينما كان الانزيم ثابت عند مدى 20–60م لمدة ساعة واحدة اذ لم تنخفض الفعالية عن 50% ، كما كان الفايتيز المنقى فعالا عند مدى 4–8 من الرقم الهيدروجيني واعلى فعالية بلغت 0.81 وحدة/مل عند الرقم الهيدروجيني 6 تليها 0.79 وحدة/مل عند التحضين بالرقم الهيدروجيني5 مع عدم وجود فرق معنوي بينهما كما وكان الانزيم ثابت تماما عند قيم 5 و6 من الرقم الهيدروجيني عند التحضين مدة ساعة واحدة بدرجة حرارة 37م ولم تنخفض الفعالية عند اقل من 70% عند قيم الرقم الهيدروجيني الاخرى وعليه فيعد ثابت عندى المدى 4–8. بين مختلف المواد المعدنية سجلت كبريتات المغنيسيوم فقط تاثير تازري في زيادة فعالية الانزيم اذ بلغت الفعالية المتبقية 117.36% بينما ادت كل من كبريتات النحاس وكلوريد الخارصين الى اعلى تثبيط في فعالية الانزيم.

الكلمات مفتاحية: تنقية الفايتيز, الفطر *P. ostreatus* فايتيز داخل خلوي, توصيف الفايتيز