purification of phytase enzyme from Saccharomyces cerevisiae, study of their kinetic properties and estimation of their molecular weights

ميس قاسم محمد، احمد حامد مهدي

Mais Qasem Mohammed, Ahmed Hamed Mahde Shugran

Department of Chemistry, College of Education for pure. sciences/Tikrit university, Tikrit, Iraq.

mais.moh@ntu.edu.iq

Abstract

The current study aimed to extract and purify the phytase enzyme from dried Saccharomyces cerevisiae yeast (of Iranian origin), determine its specific activity during the various purification stages, and study the optimum conditions for enzyme activity in terms of pH and temperature. The aim was to evaluate its efficiency and potential use in industrial or food applications. A single sample of dried Saccharomyces cerevisiae yeast of Iranian origin was obtained from the local market in Tikrit during September ٢٠٢٣ and used to extract and purify the phytase enzyme and study its specific activity and protein concentration. The purification process was carried out through several sequential stages, including preparation of the crude extract, precipitation with ammonium sulfate, ion exchange chromatography, and gel filtration chromatography. The results showed that the total activity of the crude extract was ٧٤,٩١٩ enzyme units (IU) and the specific activity was ٥,٢٣ IU/mg. After precipitation with ammonium sulfate, the total activity increased to ١٩٥,٣ IU and the specific activity to ٦,٠٣ units/mg, with a ١,١٥-fold increase in enzyme purity. Following ion-exchange purification, the specific activity increased to ١٤,٨ units/mg, a ٢,٨٣-fold purification ratio and a yield of ٢٠٥,٦٪. Finally, the gel filtration stage significantly increased the enzyme purity, with the specific activity reaching ٦٦,٨ units/mg, a ١٢,٧٥-fold purification ratio compared to the crude extract, with a total yield of ٢٣١,٨٪. The study showed that the purified phytase enzyme had its highest activity at pH ٧,٥, while the optimum temperature for its activity was ٣٦°C, where enzyme activity gradually increased with increasing temperature up to this

point and then began to decline. Conclusion: The study results demonstrate that Saccharomyces cerevisiae (Iranian type) is an effective source for phytase production, as it can be efficiently purified through successive stages to achieve high specific activity and a clear purity index. The purified enzyme also showed the best activity at nearly neutral acidity (pH ٧,٥) and an optimum temperature (٣٦°C), indicating its suitability for industrial or food applications operating under these conditions.

Keywords: *Saccharomyces cerevisiae*, phytase, purification, kinetic properties

المستخلص:

هدفت الدراسة الحالية الى استخلاص وتنقية إنزيم الفايتاز من خميرة *Saccharomyces cerevisiae* المجففة (من منشأ إيراني)، وتحديد النشاط النوعي له خلال مراحل التنقية المختلفة، إضافة إلى دراسة الظروف المثلى لفعالية الإنزيم من حيث درجة الحموضة (pH) ودرجة الحرارة، وذلك بهدف تقييم كفاءته وإمكانية استخدامه في التطبيقات الصناعية أو الغذائية. تم الحصول على عينة واحدة من خميرة *Saccharomyces cerevisiae* المجففة من السوق المحلي في تكريت خلال شهر أيلول ٢٠٢٣، وهي من منشأ إيراني، واستخدمت لغرض استخلاص وتنقية إنزيم الفايتاز ودراسة نشاطه النوعي وتركيز البروتين. تمت عملية التنقية من خلال عدة مراحل متتابعة شملت: تحضير المستخلص الخام، الترسيب بكبريتات الأمونيوم، الكروماتوغرافيا بالتبادل الأيوني، وكروماتوغرافيا الترشيح الهلامي. أظهرت النتائج أن النشاط الكلي للمستخلص الخام بلغ ٧٤,٩١٩ وحدة إنزيمية (IU) والنشاط النوعي ٥,٢٣ وحدة/ملغم. بعد الترسيب بكبريتات الأمونيوم ارتفع النشاط الكلي إلى ١٩٥,٣ IU والنشاط النوعي إلى ٦,٠٣ وحدة/ملغم، مع زيادة في نقاوة الإنزيم بمقدار ١,١٥ مرة. أما بعد التنقية بالتبادل الأيوني فقد ارتفع النشاط النوعي إلى ١٤,٨ وحدة/ملغم بنسبة تنقية ٢,٨٣ مرة، وبمردود ٢٠٥,٦٪. وأخيرا، أدت مرحلة الترشيح الهلامي إلى زيادة كبيرة في نقاوة الإنزيم حيث بلغ النشاط النوعي ٦٦,٨ وحدة/ملغم، بمعدل تنقية ١٢,٧٥ مرة مقارنة بالمستخلص الخام، وبمردود كلي بلغ ٢٣١,٨.% . أظهرت الدراسة أن إنزيم الفايتاز المنقى يمتلك أعلى نشاط عند درجة pH تساوي ٧,٥، بينما كانت درجة الحرارة المثلى لنشاطه ٣٦°C، حيث يزداد النشاط الإنزيمي تدريجيا مع ارتفاع درجة الحرارة حتى هذه النقطة ثم يبدأ بالانخفاض بعدها . الاستنتاج: تظهر نتائج الدراسة أن خميرة *Saccharomyces cerevisiae* (النوع الإيراني) تعد مصدرا فعالا لإنتاج إنزيم الفايتاز، إذ أمكن تنقيته بكفاءة عالية من خلال المراحل المتتابعة حتى الوصول إلى نشاط نوعي مرتفع ودليل نقاوة واضح. كما تبين أن الإنزيم المنقى يتميز بأفضل نشاط عند درجة حموضة متعادلة تقريبا (pH ٧,٥) ودرجة حرارة مثلى (٣٦°C)، مما يشير إلى ملاءمته للتطبيقات الصناعية أو الغذائية التي تعمل ضمن هذه الظروف.

الكلمات المفتاحية: خميرة *Saccharomyces cerevisiae* ، فيتاز، تنقية، خصائص حركية

Introduction

Phytases are hydrolytic enzymes (phosphatases) that are part of the histidine acid phosphatase subfamily. They facilitate the hydrolysis of phytate phosphomonoester linkages (salts of myo-inositol hexakisphosphate) or myo-inositol ١, ٢, ٣, ٤, ٥, ٦-hexakis dihydrogen phosphate (phytic acid), yielding

derivatives such as tetra, tri, di, and inositol monophosphate, in addition to inorganic phosphate (Pi) [١,٢].

Phytase producing microorganisms include filamentous fungi of the genus Aspergillus. In various studies, these fungi were found to produce the most active extracellular enzyme with the most suitable characteristics of both pH and temperature stability. Hence, microorganisms of the genus Aspergillus, are the most used in the industrial production of this enzyme [٣,٤]. Solid-state fermentation is a process commonly applied for the production of extracellular enzymes [٥].

As a result of the favorable characteristics of phytase, as well as its practical application as an additive in the diets of non-ruminants, this enzyme has taken a position of great interest in biotechnological applications to reduce the phytate content of fodder and commercial foods [٦]. Phytases used as additive must be effective in releasing phosphate from phytic acid and must demonstrate their effectiveness at the digestive tract level, as well as withstand the conditions of pH and temperature [٧,٨].

Materials and methods

A single sample of S. cerevisia dried yeast, which was taken from the local market in Tikrit in September ٢٠٢٣. It is an Iranian variety, with one replicate to test the specific activity and protein concentration during the extraction and precipitation phase. Local yeast is prepared using the common traditional methods, using only flour and water without any additives. A certain amount of flour, ٢٥ grams, is placed in a glass bottle with a tight lid. The same amount of distilled water (٢٥ ml) is added to the flour and mixed well. The container is covered, leaving a small opening for air to enter for respiration. The mixture is left for ٢٤ hours at room temperature (٢٥°C). On the second day, ٢٥ grams of flour and water are added, mixed, and left under the same conditions. On the fifth day, after all the additions are complete, the container is tightly closed and placed in the refrigerator at ٤°C for later use.

Phytase Enzyme Extraction and Production

Weigh ١٠٠ g of prepared baker's yeast and mix it with ٥٠ ml of organic toluene solvent. Place the mixture in a water bath at ٤٥°C for one hour. Leave the mixture at room temperature at ٢٥°C for ٢-٣ hours, then add ١٠٠ ml of cold distilled water. Place the mixture in a glass separating funnel and shake well for half an hour. Then, incubate at ٤°C for ١٨ hours. Filter the mixture, collect the separated aqueous phase, and centrifuge it in a refrigerated centrifuge at ١٠,٠٠٠ rpm at ٤°C for ٢٠ minutes. The filtrate was taken and the precipitate discarded. Phytase activity and protein concentration were measured.

Measuring the activity of the phytase enzyme from baker's yeast

٠,٢ml of the crude extract prepared was added to ٠,٣ ml of the basic solution and ٠,٥ ml of the phytase solution. Phytase activity was evaluated as described by [٩].

Estimation of Total protein

The method of [١٠] was followed in estimating the amount of protein in the enzyme solution extracted from baker's yeast S. cerevisiae, as well as in all stages of extraction, purification and characterization. This method depends on the reaction of the basic copper solution (basic copper sulfate) present in the contents of the reagent solution with the nitrogen atoms present in the peptide bonds of the protein to produce a complex with a purple color.

Phytase Enzyme Purification

Ammonium Sulfate Precipitation:

Ammonium sulfate ($SO_4^{٢}$)($NH_4^{٤}$) was used in the crude enzyme extract at saturation levels ranging from ٦٠٪ to ٧٠٪. These levels were tested individually to achieve the best saturation levels. These weights were added gradually to the extract in an ice bath under continuous stirring using a magnetic stirrer for an hour. The components were separated using a refrigerated centrifuge at ١٠,٠٠٠ rpm at ٤°C for ٢٠ minutes. This process was repeated by taking the filtrate and adding ammonium sulfate to reach ٧٠٪ saturation under the same conditions as before. The filtrate was discarded, and the precipitate was dissolved in ١٥ ml of

٠,١ M basic buffer at pH ٧. Absorbance readings were taken at ٢٨٦ nm to measure enzyme activity [١١].

Purification by membrane screening (dialysis)

The enzyme produced from the ammonium sulfate precipitation step was dialysis. The enzyme was placed in a dialysis bag, which allows the passage of substances less than ١٢٠ kDa. The bag was placed in a container containing basic buffer solution for ٢٤ hours, which separated the saline solution from the enzyme, leaving the enzyme inside the bag. The solution was concentrated using sucrose. Enzyme activity, specificity, and protein concentration in the sample were determined. The sample was stored at -٢٠°C until further purification steps were performed[١٢]

Ion-exchang chromatography

This purification was done in this way based on what was mentioned by [١١].

Sephadex G-٢٠٠

Sephadex G-٢٠٠ column was prepared and filled according to the manufacturer's instructions (Pharmacia Fine chemicals). ١٠ g of Sephadex powder was suspended in ٢٠٠ ml of distilled water to remove preservatives. The column was then heated in a water bath for ٥ hours at ٩٠°C, then left to cool and washed twice with ٠,٢٥ M tetraacetic acid-base buffer solution, pH ٧,٢. Degassing was performed using a laboratory vacuum pump. The gel was filled into a glass column with dimensions of (١,٥ × ٨٠) cm and the material was left to settle. The column was then calibrated using tetraacetic acid-base buffer solution at a flow rate of ٣٠ ml/h and ٥ ml per part [٩].

Kinetic study

Determining the pH

To determine the optimum pH for enzyme stability, buffer solutions with a concentration of ٠,١ M and varying pHs ranging from ٢ to ١٢ were prepared according to paragraph (٣-٥-٢). Fifty microliters of purified enzyme were added

to the buffer solutions with varying pHs. The solutions were incubated in an incubator at ٣٧°C for ٣٠ minutes. The enzyme activity was then measured spectrophotometrically. The enzyme activity was calculated, and the relationship between the optimum pH for enzyme stability and the percentage of residual activity was determined by plotting the **curve [١٢]**.

Determining the optimum temperature for enzyme activity:

To determine the optimum temperature for enzyme activity, ٥٠ microliters of purified enzyme were added to test tubes containing ٠,١ M basic buffer solution at pH ٧. The tubes were incubated for ١٠ minutes at temperatures of ٥, ٢٠, ٣٠, ٤٠, ٤٠, and ٦٠°C [٩].

Result

The purification process of phytase enzyme from Saccharomyces cerevisiae was carried out through several sequential steps, including crude extract preparation, ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration chromatography. The results of the purification steps are summarized in Table (١). The crude extract showed a total activity of ٧٤,٩١٩ IU and a specific activity of ٥,٢٣ U/mg. After ammonium sulfate precipitation, the total activity reach ١٩٥,٣ IU, while the specific activity increased to ٦,٠٣ U/mg. The yield at this stage was ١,١٥-fold increase in enzyme purity. Subsequent purification by ion-exchange chromatography resulted in a marked increase in specific activity to ١٤,٨U/mg, corresponding to a ٢,٨٣-fold purification, with recovery yield of ٢٠٥,٦. The final purification step, gel filtration chromatography, further enhanced the enzyme purity, yielding a specific activity of ٦٦,٨ U/mg and a ١٢,٧٥-fold purification compared to the crude extract. However, the overall recovery at this stage was ٢٣١,٨% as shown in table (١) and Figure(١).

Table (١) Purification of phytase enzyme from Saccharomyces cerevisiae

Purification Steps	Total Volume mL	Activity IU/mL	Total Activity	Protein g/mL	Total Protein	Specific Activity U/mg	Recovery Yielld %	Fold of Purification Total Protein
Crude	١٣	٥,٧٦٣٠٠	٧٤,٩١٩	١,١٠	١٤,٣	٥,٢٣٩٠٩١	١٠٠,٠٠	١
Precepitation	١٨	١٠,٨٥٠٠٠	١٩٥,٣٠٠	١,٨٠	٣٢,٤	٦,٠٢٧٧٧٨	١٨٨,٢٧	١,١٥
Ione-Exchange	٥	١١,٨٥٠٠٠	٥٩,٢٥٠	٠,٨٠٠٠٠	٤	١٤,٨١٢٥٠	٢٠٥,٦٢	٢,٨٣
Gel Filtration	٥	١٣,٣٦٠٠٠٠	٦٦,٨٠٠	٠,٢٠٠٠٠	١	٦٦,٨٠٠٠	٢٣١,٨٢	١٢,٧٥

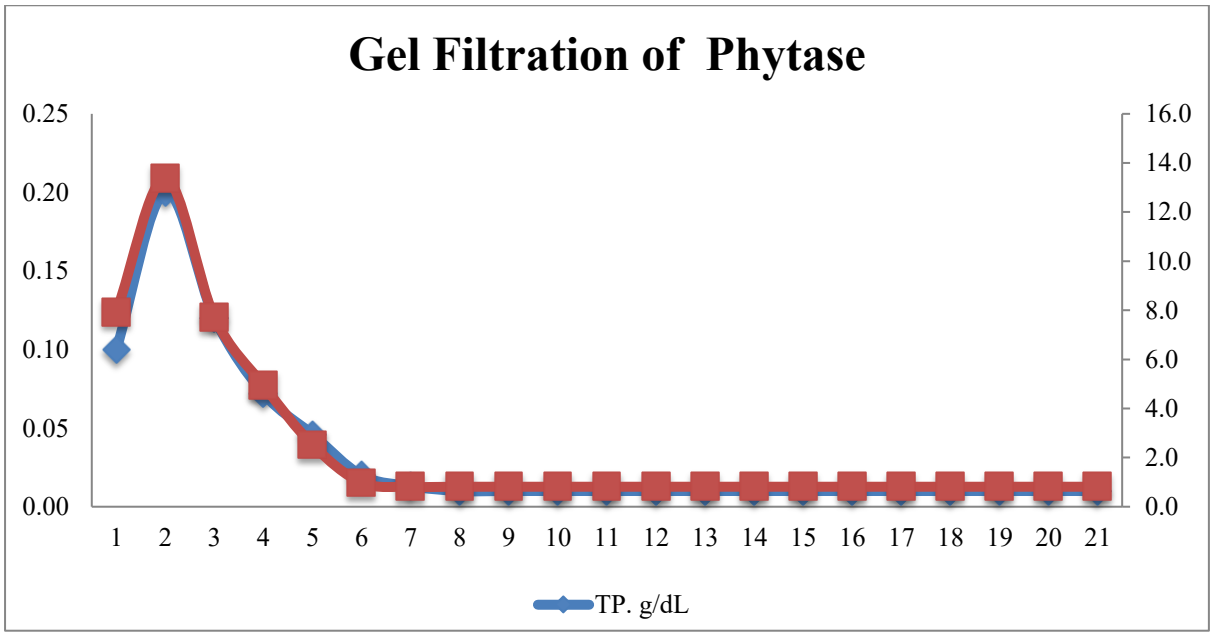

Figure (٢) Gel filtration chromatography of phytase using Sephadex G-١٠٠.

The effect of the substrate concentration on the reaction rate of the partially purified phytase enzyme from Saccharomyces cerevisiae was studied. The optimal substrate concentration is used when estimating the enzyme activity in order to obtain the maximum speed. Therefore, different concentrations were used as shown in Figure (١).

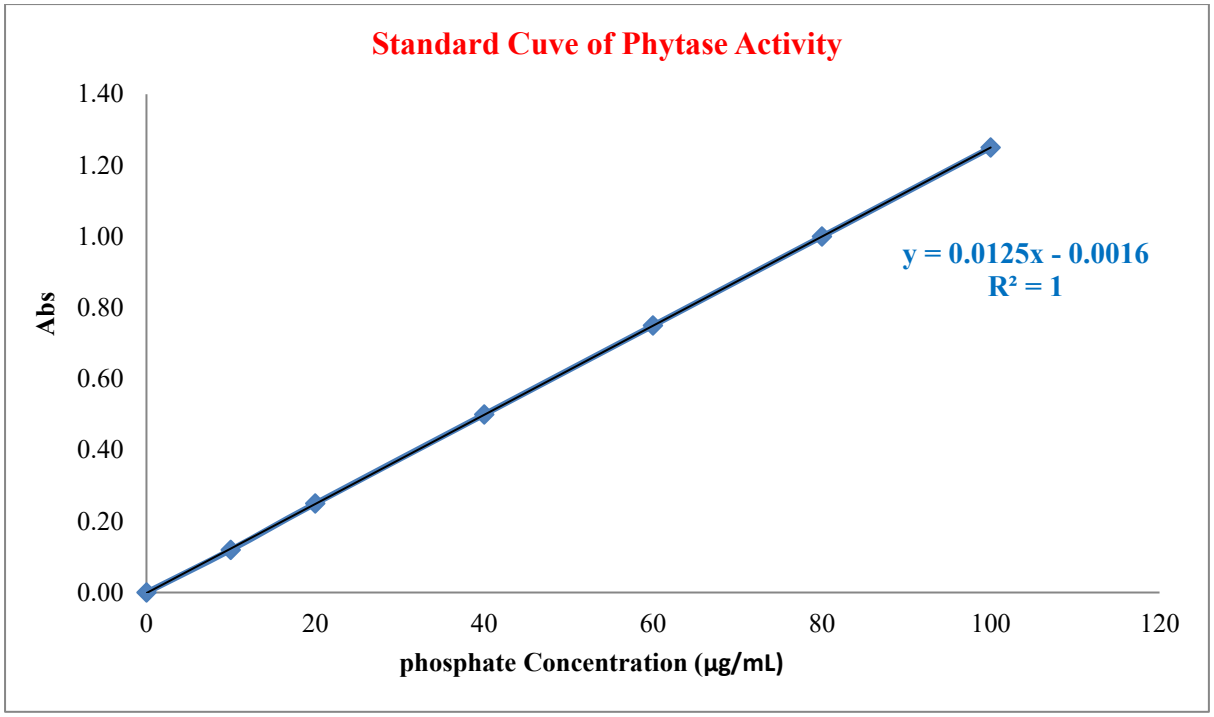

Figure (١) shows the Michaelis-Menten diagram showing the effect of substrate concentration on phytase enzyme activity.

The results in our study showed that by plotting the reciprocal of the initial velocity versus the reciprocal of the substrate concentration, a linear relationship was obtained with the values of Km and Vmax for the enzyme, which were ١٠٢,١٤٥ IU/L (- ٠,٠٠٠٢٠٤) Mm for the phytase enzyme. As shown in Figure(٢).

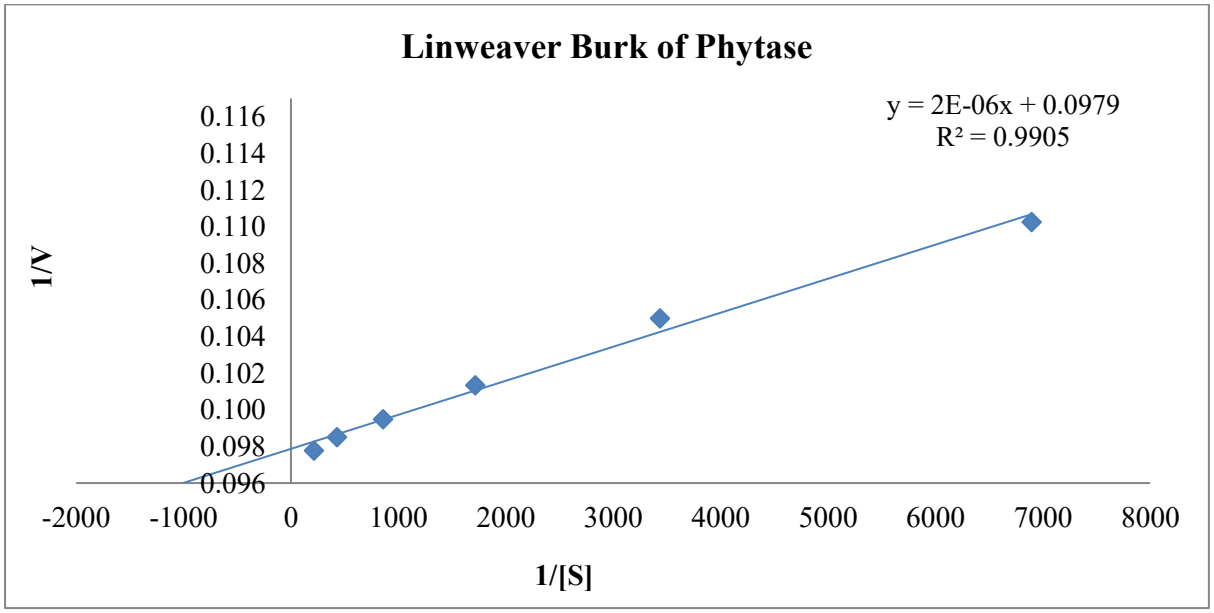

Figure (٢) Lineweaver–Burk diagram of partially purified phytase enzyme

Different temperatures were tested to determine the optimum temperature for phytase activity (٢٠-٣٠-٤٠-٥٠-٦٠°C). The optimum temperature was ٣٦°C. The results showed that increasing the temperature led to an increase in enzyme activity, followed by a decrease in their activity, as shown in the Figure.(٣)

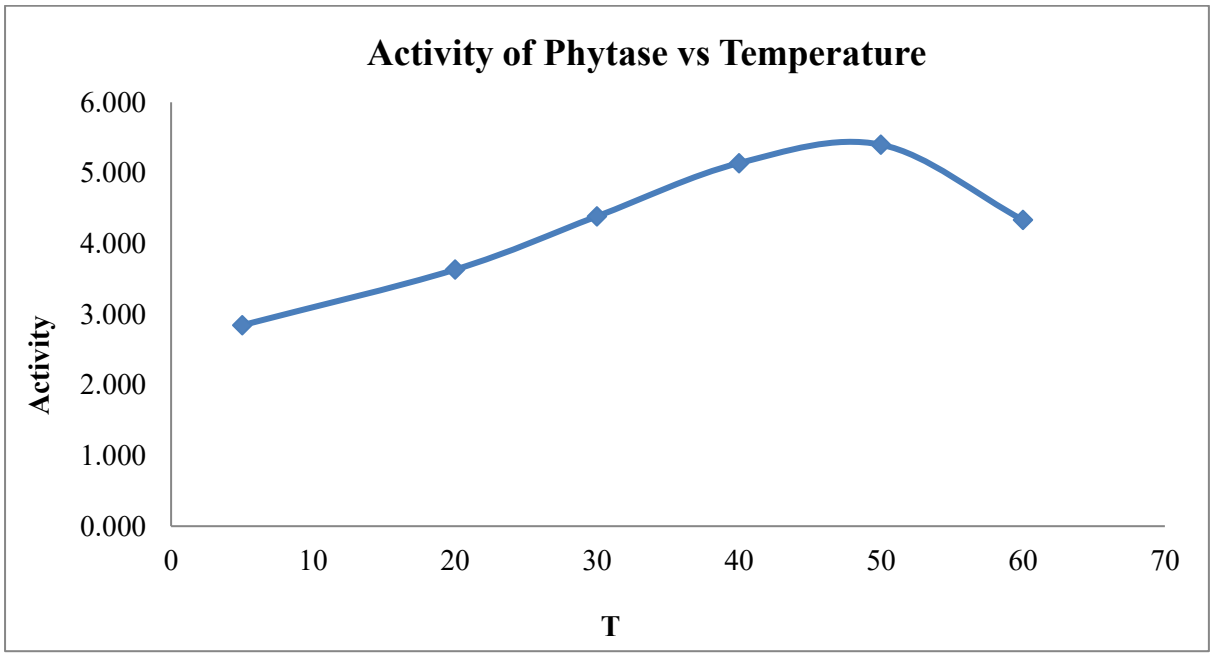

Figure (٣) Effect of temperature on phytase enzyme activity

The different effects of pH on phytase activity were studied as shown in Figure (٤). For the pure enzyme, it was found that the highest enzyme activity was achieved at pH.(٧،٥)

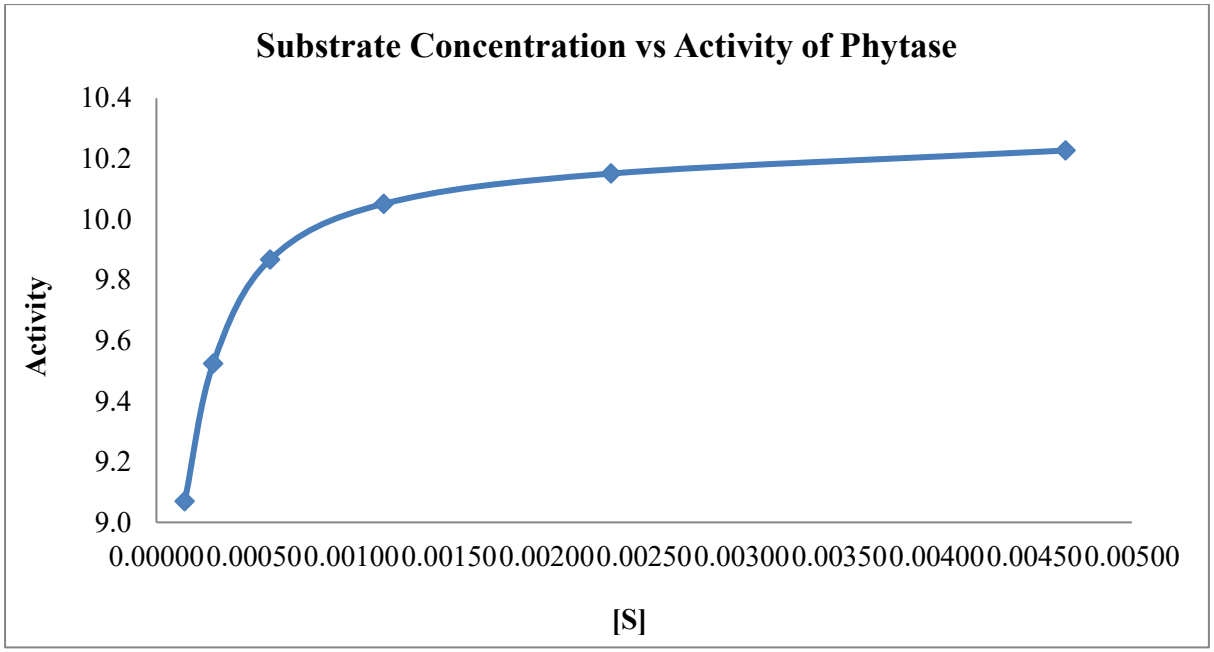

Figure (٤) Effect of pH on phytase activity

Discussion

The present study successfully demonstrated the extraction and purification of the phytase enzyme from Saccharomyces cerevisiae (Iranian origin), revealing progressive increases in enzyme purity and specific activity across successive purification steps. The crude extract initially exhibited a specific activity of ٥,٢٣ IU/mg, which increased to ٦,٠٣ IU/mg after ammonium sulfate precipitation, indicating partial removal of non-protein impurities. This initial purification step is consistent with previous findings that ammonium sulfate precipitation effectively concentrates phytase by selective protein precipitation, enhancing enzyme recovery and stability [١٣].

Following ion exchange chromatography, a notable rise in specific activity to ١٤,٨ IU/mg was observed, corresponding to a ٢,٨٣-fold purification increase. This step likely removed additional negatively charged contaminants, as phytase typically binds to anion-exchange matrices under low-salt conditions due to its acidic nature [١٤]. Similar trends were reported by [١٥], who found that DEAE-cellulose ion exchange significantly increased the specific activity of yeast phytase. The elevated yield (٢٠٥,٦٪) observed after this step may result from the

removal of inhibitors or enzyme activation during partial purification, a phenomenon also reported by [١٦].

The final purification stage—gel filtration chromatography—achieved the highest enzyme purity, with a specific activity of ٦٦,٨ IU/mg and a ١٢,٧٥-fold purification relative to the crude extract. Gel filtration separates proteins based on molecular weight, thus effectively eliminating low-molecular-weight contaminants and aggregates [١٧]. Similar purification folds were obtained in previous studies purifying S. cerevisiae and Aspergillus niger phytases [١٨], confirming the reliability of this multi-step approach.

Regarding enzyme characterization, the purified phytase exhibited maximum activity at pH ٧,٥ and ٣٦°C, aligning with the near-neutral pH range optimal for yeast phytases. This finding corresponds with earlier reports showing that S. cerevisiae phytase is active under slightly neutral to mildly acidic conditions (pH ٦,٥–٧,٥), which is favorable for food and feed processing environments [١٩]. The optimal temperature of ٣٦°C also matches the mesophilic nature of S. cerevisiae, suggesting the enzyme's stability under physiological and moderate industrial temperatures [٢٠]. Beyond this temperature, the observed decline in enzyme activity may be attributed to conformational denaturation, as phytase's tertiary structure is sensitive to heat [١٦].

Phytase activity is highly sensitive to both pH and temperature, as both factors affect the enzyme's spatial structure and reaction rate. Studies indicate that most fungal phytases, particularly those extracted from Aspergillus niger, operate at their maximum efficiency in relatively acidic environments, with optimal activity recorded at pHs between ٤,٥ and ٥,٥ [١٩, ٢١]. This property makes them suitable for use in the acidic environment of the human and animal stomachs, which explains their widespread adoption in the poultry and pig feed industries to improve phosphorus release from phytate.

Bacterial phytases, on the other hand, have demonstrated broader pH optimum ranges, with good activity observed in neutral and even slightly alkaline media

(pH ٦,٠–٧,٥) [١١]. This broad pH optimum range makes them an important choice for industrial applications requiring stability under diverse processing conditions, such as food production or the bioremediation of agricultural waste. Regarding temperature, most studies agree that the optimal activity of most fungal phytases is found at temperatures between ٤٥ and ٦٠°C [٢٢]. However, the stability of activity at high temperatures varies depending on the source. Bacterial phytases, particularly those from the genus Bacillus, have been shown to retain a significant portion of their activity at ٧٠–٨٠°C, making them suitable for heat-processing feeds [٢٣]. In contrast, plant phytases experience a marked decline in activity at temperatures above ٥٠°C due to thermal decomposition and loss of the enzyme's tertiary structure [٢٤].

Conclusion

The results confirm that Saccharomyces cerevisiae is an efficient and eco-friendly source of phytase. The enzyme’s biochemical properties—neutral pH and moderate temperature optima, make it highly suitable for use in food processing and feed applications to enhance mineral bioavailability and reduce anti-nutritional phytate content.

References

١. **BILGIÇLI, Nermin; ELGÜN, Adem; TÜRKER, Selman. Effects of various phytase sources on phytic acid content, mineral extractability and protein digestibility of tarhana.** ***Food chemistry*****, ٢٠٠٦, ٩٨,٢: ٣٢٩-٣٣٧.**

٢. **ALBARRACÍN, Micaela; GONZÁLEZ, Rolando J.; DRAGO, Silvina R. Effect of soaking process on nutrient bio-accessibility and phytic acid content of brown rice cultivar.** ***LWT-food Science and Technology*****, ٢٠١٣, ٥٣,١: ٧٦-٨٠.**

٣. **HAEFNER, Stefan, et al. Biotechnological production and applications of phytases.** ***Applied microbiology and biotechnology*****, ٢٠٠٥, ٦٨,٥: ٥٨٨-٥٩٧.**

٤. **BHAVSAR, K.; KUMAR, V. Ravi; KHIRE, J. M. Downstream processing of extracellular phytase from Aspergillus niger: Chromatography process vs. aqueous two phase extraction for its simultaneous partitioning and purification.** ***Process Biochemistry*****, ٢٠١٢, ٤٧,٧: ١٠٦٦-١٠٧٢.**

٥. **GAIND, Sunita; SINGH, Surender. Production, purification and characterization of neutral phytase from thermotolerant Aspergillus flavus ITCC ٦٧٢٠.** ***International Biodeterioration & Biodegradation*****, ٢٠١٥, ٩٩: ١٥-٢٢.**

٦. **OMOGBENIGUN, F. O.; NYACHOTI, C. M.; SLOMINSKI, B. A. The effect of supplementing microbial phytase and organic acids to a corn-soybean based diet fed to early-weaned pigs.** ***Journal of Animal Science*****, ٢٠٠٣, ٨١,٧: ١٨٠٦-١٨١٣.**

٧. **JONDREVILLE, Catherine, et al. Effects of additional zinc and phytase on zinc availability in piglets and chicks fed diets containing different amounts of phytates.** ***Livestock Science*****, ٢٠٠٧, ١٠٩,١-٣: ٦٠-٦٢.**

٨. **MONTEIRO, Paulo S., et al. Isolation of a thermostable acid phytase from Aspergillus niger UFV-١ with strong proteolysis resistance.** ***Brazilian Journal of Microbiology*****, ٢٠١٥, ٤٦: ٢٥١-٢٦٠.**

٩. **Pandey, A., Szakacs, G., Soccol, C. R., Rodriguez-Leon, J. A., & Soccol, V. T. (٢٠٠١). Production, purification and properties of microbial phytases.** ***Bioresource technology*****, *77*(٣), ٢٠٣-٢١٤.**

١٠. **CANAN, Cristiane, et al. Studies on the extraction and purification of phytic acid from rice bran.** ***Journal of food composition and analysis*****, ٢٠١١, ٢٤,٧: ١٠٥٧-١٠٦٣.**

١١. **Dokuzparmak, E., et al. (٢٠١٧). Purification and biochemical characterization of thermostable phytase from Aspergillus niger.** ***Biocatalysis and Agricultural Biotechnology*****, ١٢, ٢٤٧–٢٥٣. https://doi.org/١٠,١٠١٦/j.bcab.٢٠١٧,١٠,٠١٠**

١٢. Yao, M. Z., et al. (٢٠١٢). Biochemical characterization of a phytase from Bacillus subtilis with low Km value. *Journal of Agricultural and Food Chemistry*, ٦٠(٤٦), ١١٦٠٠–١١٦٠٧. https://doi.org/١٠,١٠٢١/jf٣٠٣٩٧٨u

١٣. Bhavsar, K., Shah, P., & Soni, S.K. (٢٠١٨). Production, purification, and characterization of a novel thermostable phytase from Aspergillus niger NCIM ٥٦٣. Biocatalysis and Agricultural Biotechnology, ١٣, ١٧٦–١٨٢.

١٤. Lei, X.G., Weaver, J.D., Mullaney, E., Ullah, A.H., & Azain, M.J. (٢٠١٣). Phytase, a new life for an "old" enzyme. Annual Review of Animal Biosciences, ١, ٢٨٣–٣٠٩.

١٥. Vohra, A. & Satyanarayana, T. (٢٠٠٣). Phytases: microbial sources, production, purification, and potential biotechnological applications. Critical Reviews in Biotechnology, ٢٣(١), ٢٩–٦٠.

١٦. Rani, K. & Ghosh, S. (٢٠١١). Purification and characterization of phytase from Pichia anomala. Journal of Agricultural and Food Chemistry, ٥٩(١٥), ٨٢٣٣–٨٢٣٩.

١٧. Selle, P.H. & Ravindran, V. (٢٠٠٨). Phytate-degrading enzymes in pig nutrition. Livestock Science, ١١٣(٢-٣), ٩٩–١٢٢.

١٨. Shivange, A.V., Serwe, A., Dennig, A., & Schwaneberg, U. (٢٠١٢). Phytases in biotechnology: recent trends and applications. Applied Microbiology and Biotechnology, ٩٥(٢), ٤٧١–٤٨٠.

١٩. Greiner, R. & Konietzny, U. (٢٠١١). Phytase: biochemistry, enzymology and characteristics relevant to animal feed use. Biotechnology Advances, ٢٩(٦), ٦٨٢–٦٨٩.

٢٠. Wyss, M., Brugger, R., Kronenberger, A., Rémy, R., Fimbel, R., Oesterhelt, G., Lehmann, M., & van Loon, A.P.G.M. (١٩٩٩). Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): catalytic properties. Applied and Environmental Microbiology, ٦٥(٢), ٣٦٧–٣٧٣.

٢١. Singh B, et al. Properties and applications of phytases: A review. Crit Rev Biotechnol. ٢٠١٦;٣٦(٤):٩٧٣–٩٨٧.

٢٢. Yao MZ, et al. Biochemical characterization of a phytase from *Bacillus subtilis* with low Km value. J Agric Food Chem. ٢٠١٢;٦٠(٤٦):١١٦٠٠–١١٦٠٧.

٢٣. Chauhan RS, et al. Thermostable phytases: Characteristics and applications in animal feed. Enzyme Microb Technol. ٢٠١٨;١١٦:٤٤–٥٤.

٢٤. Brinch-Pedersen H, et al. Engineering crop plants: getting a handle on phosphate. Trends Plant Sci. ٢٠٠٢;٧(٣):١١٨–١٢٥.