

## Research Article



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# Partial purification, characterization and wheat bran degradation studies of a new phytase from the *Bacillus megaterium* EBD 9-1 strain

## *Bacillus megaterium* EBD 9-1 suşundan yeni bir fitazın kısmen saflaştırılması, karakterizasyonu ve buğday kepeğini parçalama çalışmaları

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### Abstract

**Objective:** The present study was designed to report the bacterial identification and characterization of a new phytase enzyme from a *Bacillus* sp. strain isolated from soil.

**Methods:** *Bacillus* sp. strain was identified based on 16S rRNA analysis. The phytase was partially purified through ammonium sulfate precipitation and Sephadex G100 gel filtration steps, and characterized for its activity and stability.

**Results:** The new isolate EBD 9-1 showed 100% sequence identity with *Bacillus megaterium*. The partially purified enzyme had the maximum activity at pH 7.0 and 60°C. The activity of the enzyme was stimulated in the presence of Ca<sup>2+</sup>, V<sub>max</sub> and K<sub>m</sub> for enzyme were found to be 333 U/mL and 2 mM, respectively. The estimated molecular weight of enzyme was 45 kDa. The storage stability of phytase was 93% of the initial activity after 6 months at 4°C and –20°C. This study represents the partial purification,

characterization and wheat bran degradation studies for *B. megaterium* phytase.

**Conclusion:** Consequently, due to the characteristics such as significant stability at higher temperatures, alkaline pH and storage of the novel phytase enzyme produced by *B. megaterium* EBD 9-1, the enzyme may be suitable for supplementing animal feeds to improve the availability of phosphorus from phytates.

**Keywords:** *Bacillus megaterium*; Phytase; Partial purification; Characterization; Animal feeds.

### Özet

**Amaç:** Bu çalışma, topraktan izole edilmiş bir *Bacillus* sp. suşundan yeni fitaz enziminin karakterizasyonunu ve bakteriyel adlandırmayı rapor etmek için tasarlanmıştır.

**Metod:** *Bacillus* sp. suşu 16S rRNA analizinin ile tanımlanmıştır. Fitaz bir dizi basamak ile saflaştırılmış, aktivite ve stabilite için karakterize edilmiştir.

**Bulgular:** Yeni izolat EBD 9-1 *Bacillus megaterium* ile % 100 sekans benzerliği göstermiştir. Kısmen saflaştırılmış enzim, pH 7.0 ve 60°C maksimum aktiviteye sahipti. Enzim aktivitesi Ca<sup>2+</sup> varlığında uyarılmıştır. Enzim için V<sub>max</sub> ve K<sub>m</sub> sırasıyla, 333 U/mL ve 2 mM olarak bulunmuştur. Kısmen saflaştırılmış enzimin tahmini molekül ağırlığı 45 kDa'dır. Fitaz depolama stabilitesi 4°C'de ve –20°C'de altı aydan sonra başlangıç aktivitesinin % 93'dir. Bu çalışma *B. megaterium* fitaz için kısmen saflaştırma, karakterizasyonu ve buğday kepeğini parçalanmasını göstermektedir.

**Sonuç:** Sonuç olarak, *Bacillus megaterium* EBD 9-1 tarafından üretilen yeni bir fitaz enziminin daha yüksek

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sıcaklıklarda, alkalın pH'da stabil olması ve depolama özellikleri nedeniyle, enzim fitatlardan fosforun kullanılabilirliğini artırmak için hayvan yemlerine ilave için uygun olabilir.

**Anahtar Kelimeler:** *Bacillus megaterium*; Fitaz; Kısmen saflaştırma; Karakterizasyon; Hayvan yemi.

## Introduction

The main constituents of feedstuff for poultry are derived from plants (wheat, corn, or rye). Up to 80% of the phosphorus in the grain is bound to phytic acid [1, 2]. Phytate (Phytic acid) is known as a food inhibitor that chelates micronutrients and prevents them from being bioavailable for monogastric animals, including humans, because they lack the enzyme phytase in their digestive tract. Several methods have been developed to reduce the phytic acid content in food and improve the nutritional value of cereal that becomes poor due to this antinutrient [3]. Despite improved phosphorus uptake by animals, the quantity of excreted phosphorus increases environmental pollution, especially eutrophia of rivers and lakes in countries with high animal populations. Recent phytase research has been driven by the urgent need to improve the utilization of phytate-phosphorus in diets for simple-stomached animals to reduce their phosphorus excretion to the environment. Phytases (E.C.3.1.3.8. inositol hexaphosphate phosphohydrolase) have been one of the focal enzymes for nutrition, environmental protection, and human health during the past two decades. These enzymes sequentially cleave orthophosphate groups from the inositol core of phytate [4].

In contrast, it has also been reported that lower inositol phosphate derivatives can have health benefits in the protection against colon cancer [5], arteriosclerosis, neural tissue, and coronary heart disease [6] Technical improvements by adding phytases during food processing have been reported for bread making, the production of plant protein isolates, corn wet milling and the fractionation of cereal bran [5]. These enzymes have been isolated from fungi, yeast, bacteria and protozoa. Bacterial phytases and phytase-producing bacteria, as well as their potential biotechnological applications. Particularly, *Bacillus* sp., *E. coli*, *Pseudomonas* sp., and *Citrobacter* sp., are the best alternative bacteria to produce the enzyme. *Bacillus* phytases have been studied extensively because of the immense potential of these enzymes with unique characteristics, feasibility of mass market production and applicability in animal feed [7].

The first step of microbial enzyme production is the selection of appropriate microorganisms that have desirable enzyme activity. At the same time, enzyme production is related to the culture medium and fermentation conditions. The level of enzyme activity produced by an organism from the natural environment is frequently low and needs to be raised for industrial production [8].

In our previous study, we screened phytase-producing *Bacillus* species in soil samples, and have isolated a good producer. The effects of some physical and nutritional parameters were studied for the optimum production of phytase enzyme [9]. In the present study, we report the partial purification, characterization of phytase enzyme produced from a new isolate *Bacillus megaterium* EBD 9-1 strain, and its ability to degrade wheat bran.

## Materials and methods

### Materials

In this study, *Bacillus* sp. EBD 9-1, which was isolated in our previous study, was used [9]. For the bacterial identification and phylogenetic analysis, genomic DNA was extracted from strain EBD 9-1 using the Qiagen Blood & Tissue kit (Qbiogene, Montreal, PQ, Canada) according to the manufacturer's protocol. The 16S rRNA gene amplification was performed using the universal primers. 27F : 5'AGAGTTTGATCCTGGCTCAG-3' and 1492R : 5' ACG-GCTACCTTGTTACGACTT-3'. The amplified sequences were determined by an automated ABI 3100 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). The resulting sequences were compared with the GenBank database (NCBI) using BLAST [10]. The 16S rRNA gene sequence of strain EBD 9-1 was aligned with those of *Bacillus* species by using the CLUSTAL W program [11]. By using MEGA 6.0 software [12] the phylogenetic analysis was done, and the tree obtained by means of neighbor-joining method was analyzed for related organisms [13].

### Enzyme production and partial purification

The bacterial strain was cultured in the optimized medium [9]. The growth medium used for phytase production was composed of 5 g/L lactose, 8 g/L meat extract, 1 g/L CaCl<sub>2</sub>·2H<sub>2</sub>O, and 5 g/L Na-phytate, and the sample was rotated at 150 rpm for 48 h at 37°C, pH 7.5. After the removal of the cells by centrifugation, the enzyme in the culture supernatant was partially purified using a series

of steps. The supernatant was brought to 70% saturation with ammonium sulfate. The precipitate was collected (12,300 × g, 30 min at +4°C), dissolved in 20 mM Tris-HCl buffer (pH 7.0) containing 1 mM CaCl<sub>2</sub>, dialyzed overnight in the same buffer. The dialyzed enzyme was applied to Sephadex G-100 column (1.5 × 20) pre-equilibrated with the same buffer. The flow rate was 30 mL/h, and was collected into fraction tubes (5 mL/tubes). Protein content and phytase activity were determined. Active fractions were collected, and concentrated using ultrafiltration through a Centriprep-10 concentrator (Amicon).

## Enzyme and protein assays

Phytase activity was determined according to the method described by Choi et al. [14]. 0.1 mL of enzyme solution with 0.9 mL of 2 mM sodium phytate in 0.1 M Tris-HCl buffer (pH 7.0) was carried out at 37°C for 10 min and then the reaction was stopped by adding 0.75 mL of 5% trichloroacetic acid. The liberated phosphate was measured at 700 nm after adding 1.5 mL of color reagent, which is prepared freshly before using by mixing four volumes of 2.5% ammonium molybdate solution in 5.5% sulfuric acid and one volume of 2.5% ferrous sulfate solution. One unit of phytase activity was defined as to liberate 1 μmol of phosphate per minute under the assay condition. A standard curve (using KH<sub>2</sub>PO<sub>4</sub>) was made by treating standard phosphate solutions without added phytase under the same conditions.

The protein concentration was measured by a method described previously [15] with bovine serum albumin (BSA) as the standard. The absorbance at 546 nm was monitored.

## Enzyme characterization

The effects of pH, temperature and the presence of various metal ions on the phytase activity were determined. The enzyme samples were incubated for 30 min between 35 and 80°C (pH 7.0). For thermal stability, the enzyme was kept at 60°C for various time intervals (30–70 min) at pH 7.0, and the residual activities were determined. The optimum pH for the enzyme was determined using the following buffers: 0.1 M glycine-HCl (pH 2.0 and 3.0), 0.1 M sodium acetate (pH 4.0–6.0), 0.1 M Tris-HCl (pH 7.0 and 8.0), and 0.1 M glycine-NaOH (pH 9.0 and 10.0) at 37°C. The pH stability of the enzyme was determined by incubation at various time intervals in the same buffers (pH 6.0–8.0) at 37°C.

The enzyme samples were incubated with some cations and potential inhibitors at the concentrations of 1 and 5 mM. The relative activities were expressed as percentages of the activity of the untreated control taken as 100%.

The kinetic constants ( $K_m$  and  $V_{max}$ ) were calculated from the Lineweaver-Burk plot by incubating the enzyme with various concentrations of the substrate. Phytase activity was estimated over a range of substrate concentrations (0.28–2.85 mM).

For the determination of the molecular weight and zymogram analysis, electrophoresis was carried out in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) according to the Laemmli method [16]. Zymogram analysis of the partially purified enzyme was demonstrated on polyacrylamide gels. After electrophoresis, the gel was washed twice with 0.5% (v/v) Triton X-100 for 15 min to remove SDS. The band corresponding to phytase activity was visualized using substrate overlay gels containing phytic acid and then was stained.

To show wheat bran degradation by the enzyme, wheat bran was washed 5 times with Milli-Q water followed by 0.1 M Tris-HCl buffer (pH 7.0). The wheat bran was desiccated, and 5% wheat bran was treated with 500 μL of enzyme solution. The enzyme substrate mixture was incubated for 1, 3, 12, and 24 h and then was centrifuged. The wheat bran was washed twice with pure ethanol and t-butyl alcohol. After lyophilization, scanning electron microscopy (SEM) was performed using a Carl Zeiss AG-EVO 40 SEM instrument, and the images were taken accordingly.

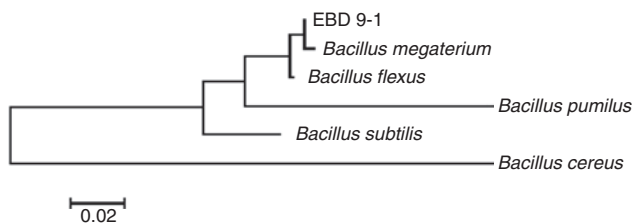
To evaluate the storage stability of the crude phytase, it was stored at room temperature, 4°C, and –20°C for 6 months, and the residual activity was determined every 15 days. The residual activity (%) was calculated according to the initial activity (100%). All of the assays were carried out in triplicate.

## Results

A phylogenetic tree based on the 16S rRNA gene sequences from the *Bacillus* strains showed that the new isolate EBD 9-1 shared 100% sequence identity with *Bacillus megaterium* (Figure 1). Thus, it was named *Bacillus megaterium* EBD 9-1.

## Partial purification of phytase enzyme

The phytase from the *B. megaterium* EBD 9-1 strain was partially purified 9.9 fold with a 21.2% yield. The results of the



**Figure 1:** Phylogenetic relationship of the 16S rRNA sequences of *Bacillus* sp. EBD 9-1 with other type strains of *Bacillus*. Bar, 0.02 substitutions per nucleotide position.

partial purification steps are summarized in Table 1. The chromatogram of gel filtration on Sephadex G-100 showed one protein peak (Figure 2).

### Effects of temperature and pH on phytase activity

The activity of the partially purified phytase was optimal at 60°C. The phytase was stable at temperatures between 40°C and 60°C (Figure 3). Thermostability studies have shown that the enzyme activity was retained at 69% for

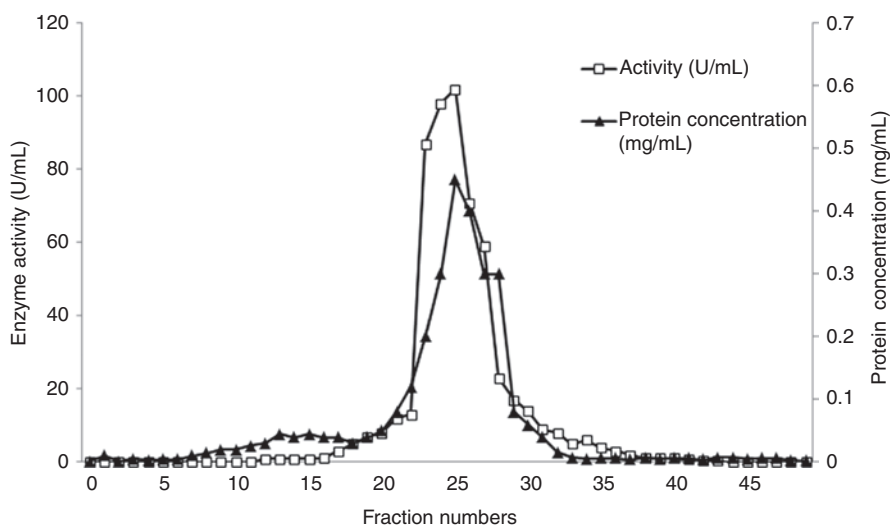
50 min at 60°C, therefore, this might be a thermostable enzyme (Figure 4). The enzyme was the most active in the pH range of 5.0–8.0 (Figure 5) with maximum activity at pH 7.0. The enzyme was active under alkaline conditions, retaining 81% of its activity at pH 8.0. However, the enzyme remained at 50% residual activity during 40 min at pH 8.0 (Figure 6). Following incubation for 60 min at pH 7.0 and 8.0, the residual enzyme activities were 76% and 20%, respectively. Optimal pH of most of the phytases were reported to be at alkaline regions.

### Effect of metal ions and chemical reagents

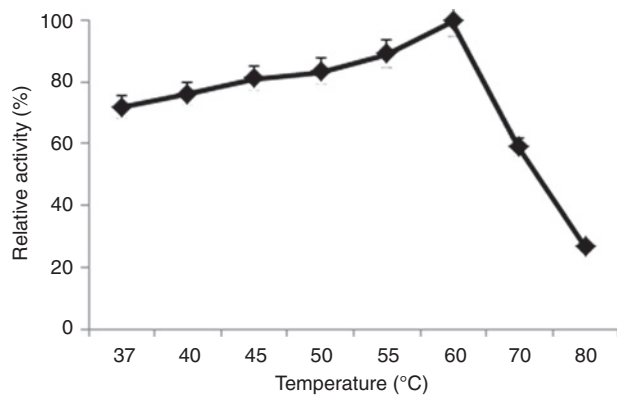
In this study, 1-mM concentration of metal ions was more effective than 5 mM. The effects of various metal ions and chemical reagents were examined in order to find which are stimulators and which are inhibitors of the catalytic process. Among metal ions,  $\text{Ca}^{2+}$  stimulated phytase activity up to 14% at 1 mM concentration. Most of the metal ions and chemical reagents were shown to reduce phytase activity compared with the control (Table 2). The phytase activity was strongly inhibited by SDS, which may have affected the enzyme's active site.

**Table 1:** Summary of the partial purification steps of phytase produced by *B. megaterium* EBD 9-1.

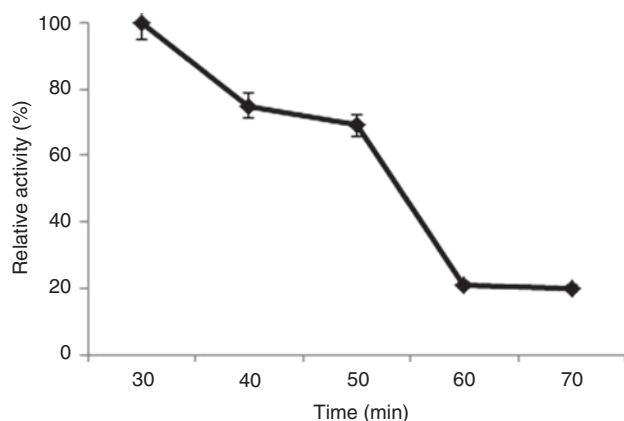
Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	233	3396	14.5	100	1
( $\text{NH}_4$ ) <sub>2</sub> SO <sub>4</sub> precipitation (70%)	130	1578	12.1	46.4	0.83
Dialysis	57	1348	23.6	39.6	1.62
Ultrafiltration after SephadexG-100 gel filtration	5	720	144	21.2	9.9



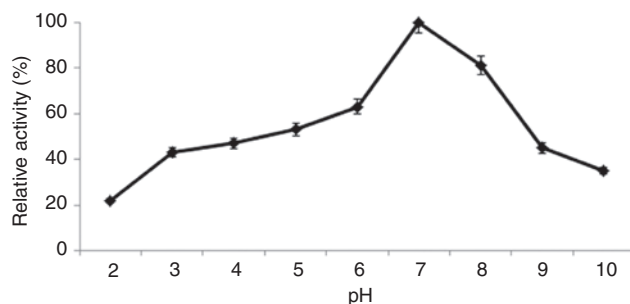
**Figure 2:** Elution profile of phytase on Sephadex G-100.



**Figure 3:** Effect of temperature on enzyme activity. Maximum enzyme activity (100%) was 209 IU/mL.



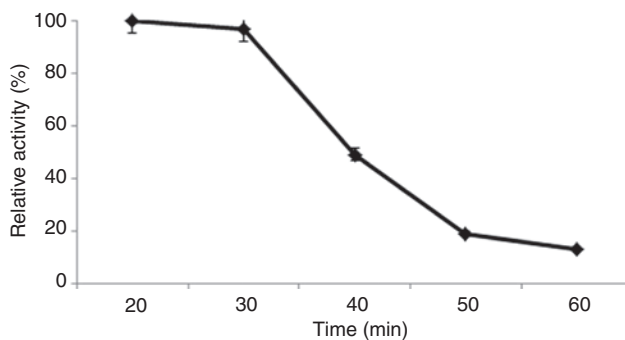
**Figure 4:** Thermostability profile of partially purified phytase. Maximum enzyme activity (100%) was 209 IU/mL at 60°C for 30 min.



**Figure 5:** Effect of pH on the activity of the enzyme. Maximum enzyme activity (100%) was 150 IU/mL.

## Enzyme kinetics

Substrate concentration from 0.28–2.85 mM was taken and enzyme activity at each substrate concentration was checked to study kinetics (Figure 7). The  $K_m$  value of phytase from *B. megaterium* EBD 9-1 was 2 mM, and the maximum reaction rate ( $V_{max}$ ) was calculated as 333 U/mL.



**Figure 6:** Stability for the partially purified phytase enzyme at pH 8.0. Maximum enzyme activity (100%) was 125 IU/mL at 37°C for 20 min.

**Table 2:** Effect of metal ions and chemical reagents on relative activity (%) of the partially purified phytase.

	Relative activity (%)	
	1 mM (%)	5 mM (%)
None(Control)	100	100
MnSO <sub>4</sub>	89	81
MgSO <sub>4</sub>	101	86
FeSO <sub>4</sub>	62	54
ZnSO <sub>4</sub>	55	45
CuSO <sub>4</sub>	52	32
CaCl <sub>2</sub>	114	101
LiSO <sub>4</sub>	80	66
BaCl <sub>2</sub>	50	27
NaCl	80	79
KCl	62	53
SDS	37	25
EDTA	51	48
2-Mercaptoethanol	52	39

Enzyme activity of control was 150 IU/mL.

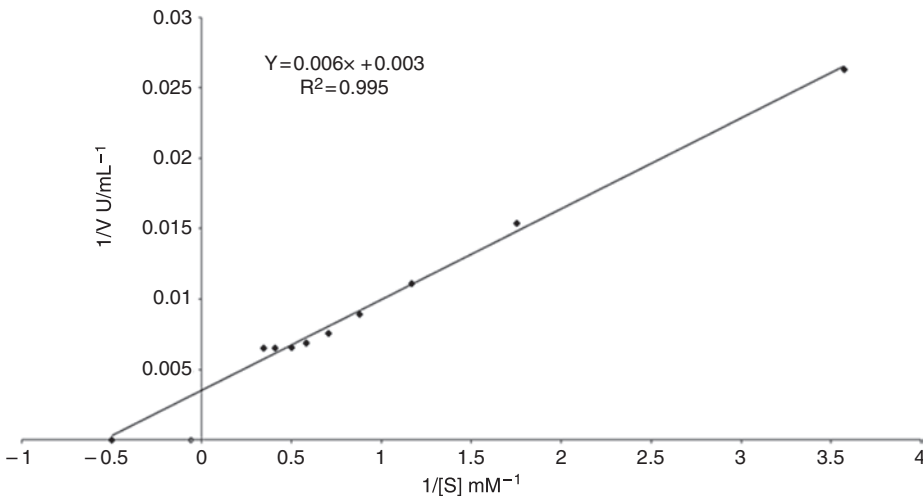
## Molecular weight determination and zymogram analysis

The partially purified phytase was analyzed by SDS-PAGE and showed a single band. Consequently, this enzyme is a monomeric protein. The molecular weight of the enzyme was estimated at 45 kDa (Figure 8). Zymogram analysis of the enzyme was also performed, and its homogeneity was confirmed.

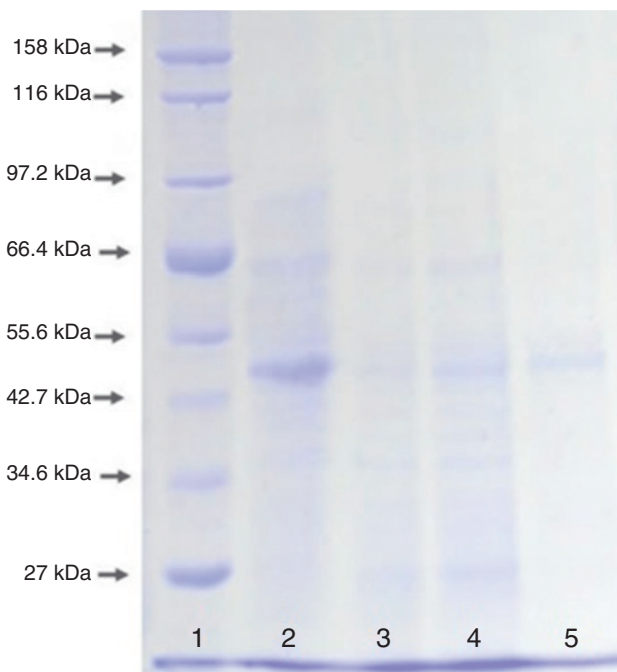
## Wheat bran degradation by the enzyme

The degradation patterns of the enzyme on wheat bran were observed by SEM (Figure 9). The surface structure of the wheat straws before the enzymatic treatment was





**Figure 7:** Lineweaver-Burk plot used to estimate kinetic constants of partially purified phytase.



**Figure 8:** SDS-polyacrylamide gel electrophoresis of phytase. 1. Protein markers, 2. Crude sample, 3. Ammonium sulfate precipitate, 4. Dialysis sample, 5. Ultrafiltration after SephadexG-100 gel filtration.

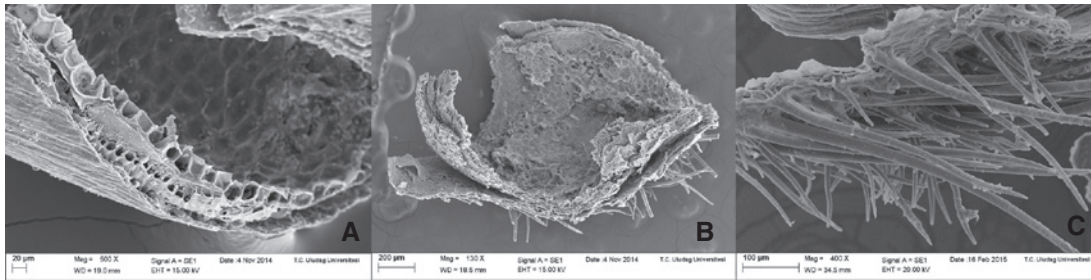
tight, and the texture was relatively hard. Compared with the sample that was not subjected to enzymatic hydrolysis, the size and number of the crimps on the surface of the treated wheat straw was increased during the time period of enzymatic treatment from 12 h to 24 h (Figure 9). After enzymatic hydrolysis for 12 h, the crimps were more slight and smaller than the enzymatic treatment for 24 h.

## Storage stability of the crude enzyme

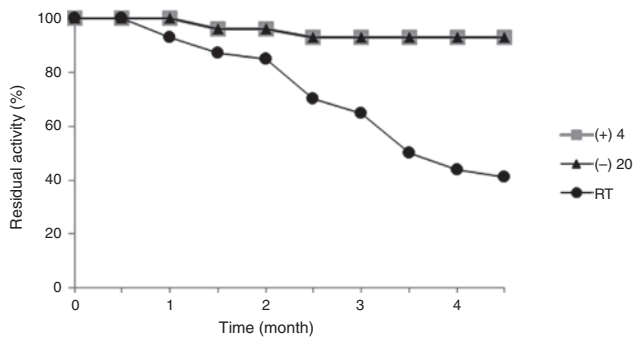
There are several phytases available in the market; however, few scientific studies have been carried out to examine the influence of the manner and storage period on enzyme activity. This information is very important for broiler producers because the phytase needs to be stored, as well as the other ingredients of the feed [17]. In this study, the phytase demonstrated high stability at +4°C and -20°C. After 6 months, the enzyme retained 93% of its initial activity. In contrast, at room temperature, the enzyme showed 65% residual activity after storage for 3 months (Figure 10). Therefore, phytase storage will be important for the application of the enzyme.

## Discussion

The phytase-producing *Bacillus* strain EBD 9-1 was identified phylogenetically as *B. megaterium* owing to its 100% similarity in the 16S rRNA gene sequence. The phytase from the *B. megaterium* EBD 9-1 strain was partially purified 9.9 fold with a 21.2% yield. The partially purified enzyme exhibited maximum activity at pH 7.0 and 60°C. The enzyme retained 69% of its initial activity after a 50 min incubation at 60°C. The enzyme activity was fairly stable from pH 5.0 to 8.0. The optimum temperature and pH of phytase for most microorganisms according to other researchers have been reported to be in the range of 50°C–77°C and 2.0–7.5, respectively [18–21]. In studies using *Bacillus* strains, the optimum temperature and pH for phytase activity have been observed to be 60°C and pH 6.5 for *Bacillus subtilis* (natto) N-77 [22], 55°C and



**Figure 9:** Scanning electron microscopy (SEM) of wheat bran which was treated with phytase enzyme. A: control sample without enzyme treatment, B: after treatment for 12 h, C: after treatment for 24 h.



**Figure 10:** Storage stability of phytase solution. Initial enzyme activity (100%) was 150 IU/mL for all conditions.

pH 7.0 for *Bacillus subtilis* VTT E-68013 [23]. Incubation of the phytase enzyme at a temperature of 70°C for 10, 20, 30, 40 and 50 min resulted in inhibition of its activity by 34%, 27%, 24%, 20% and 2%, respectively [24]. The phytases obtained from different microorganisms had higher thermal stability. The phytase from *Aspergillus* sp. L117 showed totally stable activity after 20 min of exposure between 30°C and 90°C, and even at 100°C [25]. The enzyme from *Aspergillus niger* 11T53A9 lost no activity in 10 min at temperatures up to 65°C [18]. The phytase enzyme from *E. coli* did not lose any activity after heating for 1 h at 62°C, and 27% residual activity was reported after 10 min at 85°C [26]. Most of the corresponding microbial enzymes were rather stable even at pH values above 8.0 and below 3.0 [27].

Metallic cofactors are important in the enzymatic reaction, because their presence or absence regulate enzyme activity. The activity of phytase was accelerated by some metal ions and chemical reagents. In this study, the activity of the enzyme was stimulated in the presence of  $\text{Ca}^{+2}$  and was strongly inhibited by SDS. The effect of metal ions on phytase activity has been demonstrated in many studies [28]. Similar to our findings, the enzymatic activity of the phytate-degrading enzymes of *B. subtilis* [22, 23] *B. amyloliquefaciens* [29] and *Bacillus*

*licheniformis* PFBL-03 [30] were reported to be  $\text{Mg}^{+2}$  or  $\text{Ca}^{+2}$  dependent. Most of the phytate-degrading enzymes characterized thus far are greatly inhibited by EDTA,  $\text{Zn}^{+2}$ ,  $\text{Cd}^{+2}$ ,  $\text{Ba}^{+2}$ ,  $\text{Cu}^{+2}$ ,  $\text{Fe}^{+2}$ ,  $\text{Al}^{+3}$  and SDS [23]. Metal ions have been shown to modulate phytate-degrading activity, but it is difficult to determine whether the inhibitory effect of various metal ions is the result of binding to the enzyme or formation of poorly soluble metal ion-phytate complexes [27].

The rate of activation and inactivation of enzymes and their functional efficiency together constitutes the kinetic data of an enzyme. The determination of  $K_m$  and  $V_{max}$  values of enzyme system is necessary to determine the kinetic potentiality of enzymes and their specificity. It is important to choose an enzyme which has fastest reaction rate, efficiency and stability for industrial scale production. Hence, enzyme kinetics play an important role in enzymology. In the present study, the kinetic values of  $V_{max}$  and  $K_m$  for the partially purified enzyme were 333 U/mL and 2 mM, respectively. The maximum hydrolysis rate ( $V_{max}$ ) and apparent Michaelis-Menten constant ( $K_m$ ) were 1074 IU/mL and 606  $\mu\text{M}$ , respectively [21]. The estimated molecular weight of the partially purified enzyme was 45 kDa. Thus far, most of the characterized phytate-degrading enzymes behave like monomeric proteins with molecular masses between 40 kDa and 70 kDa [27]. The molecular masses of the partially purified phytase enzymes were estimated to be approximately 45 kDa [31], 60 kDa [32]. The degradation patterns of the enzyme on wheat bran were observed by SEM (Figure 9). We obtained distinct fibril structures and crimps even with a short period of an enzymatic treatment of 12 h (B in Figure 9). In many studies, several pretreatment methods, as well as alkali [33], hydrothermal [34] and acidic [35] treatments, were applied before the enzymatic treatment of wheat bran for various purposes such as bioethanol production, animal feeding, dietary products [36] and applications to prepare the wheat bran for enzymatic hydrolysis. In our study, without any

pretreatment method, the enzymatic treatment of wheat bran was successfully fulfilled.

The storage is very important for broilers producers because the phytase needs to be stored, as well as the other ingredients of the feed. It is necessary to determine the maximum storage period. In this study, the storage stability of phytase was 93% of the initial activity after 6 months at 4°C and –20°C but was 65% after 3 months at room temperature. This result was quite good in comparison with that of other researchers. The storage stability of phytase from *Aspergillus* sp. L117 was preserved at over 96% of its initial activity for 60 days at 4, –20, and –70°C and retained even 70% of the initial activity at room temperature [25]. It has been reported that the phytases of *A. oryzae* and *A. niger* can be stored in the pure forms for up to 53 and 135 days at room temperature, respectively, to ensure 80% of the initial activity [17].

Consequently, due to the characteristics of the novel phytase enzyme produced by *B. megaterium* EBD 9-1, such as its significant stability at higher temperatures and alkaline pH and also storage, the enzyme may be suitable for supplementing animal feeds to improve the availability of phosphorus from phytates. On the other hand, an increase in enzyme levels is often achieved using recombinant organisms. Therefore, Cloning studies of *B. megaterium* EBD 9-1 phytase is in progress.

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**Conflict of interest statement:** The authors declare that there is no conflicts of interest.

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