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The Enzyme Activity of Dual-Domain β-Propeller Alkaline Phytase as a Potential Factor in Improving Soil Phosphorus Fertility and *Triticum aestivum* Growth

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Abstract: The widespread use of non-renewable phosphate fertilizers in agriculture poses a significant pollution threat to soil, necessitating the exploration of sustainable alternatives for phosphate fertility. Releasing phytate phosphorus through microbial phytases presents an eco-friendly solution for sustainable phosphate fertility in agriculture. This study directly inoculated dual-domain β-propeller alkaline phytase (phyHT) derived from *Bacillus* sp. HJB17 into the soil. The study analyzed the impact of inoculated phyHT on the physicochemical properties of the soil, assessed the variations in enzyme activity of phyHT within the soil, and examined the effects of the treated soil on wheat growth. Additionally, the study explored the enhancement of the available phosphorus in the soil through the inoculation of phyHT in both crop residues and organic fertilizer. PhyHT exhibited the highest catalytic activity at 37 °C and pH 8.0. After soil adsorption, phyHT maintained stable enzymatic activity. PhyHT markedly boosted the available phosphorus in the soil while reducing the soil phytate content by about 20%, increasing the phosphorus levels and enhancing soil fertility. PhyHT effectively degraded phytates in an organic fertilizer and crop residues, increasing the available phosphorus. PhyHT supplementation enhanced growth, biomass, and phosphorus content in both the shoot and root weights of *Triticum aestivum*. This study establishes phyHT as a viable and eco-friendly method to enhance phosphorus fertility in soil. The direct application of microbial phytases can serve as a sustainable source of phosphate fertility in soil.

Keywords: microbial enzymes (phyHT); phosphorus fertility; phytase; soil fertility; sustainable agriculture

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1. Introduction

Phosphorus (P) is a fundamental plant element, and its deficiency significantly limits plant production. Soil phosphorus deficiency limits agricultural production, and the main cause of phosphorus deficiency is a deficiency in effective phosphorus in the soil. Meanwhile, applying high-phosphorus fertilizers causes phosphorus accumulation, leading to serious pollution problems [1]. In soil, the cycling and utilization of P in organic phosphorus is more relevant than the mere addition of inorganic phosphorus [2]. Organic phosphorus accounts for 40–95% of the total P, of which, phytate is the major constituent, and it accounts for 50–80% of the organic phosphorus in soil [3]. Sources of phytate in soil include synthesis in plants, animal waste residues, transformation from inorganic phosphorus (Pi) in fertilizer, sewage sludge, and hay grass. As a predominant phosphorus type, phytate represents a potential source for plant nutrition [4]. The hydrolysis of phytate by phytases, leading to the production of lower inositol phosphate derivatives and inorganic phosphate, is a prerequisite for making phytate accessible to plants [5]. The application of phytases offers a means to address phosphorus source shortages, reduce environmental pollution, and promote agricultural development [6].

While phytases play a vital role in the soil phosphorus cycle due to their abundance [7], their effectiveness can be compromised by various factors in the soil environment, including

soil proteinases, microbial-mediated degradation, sorption on soil particles, and substrate availability [8]. Soil adsorption of phytase introduces two contrasting effects on enzyme activity. Initially, phytase adsorption onto soil particles may reduce the enzyme–substrate affinity, decreasing its activity. However, it is noteworthy that adsorption may also be crucial for the enzyme's prolonged stability in soil [9]. The impact of bio-decomposition on phytase activity can be confirmed through autoclaving [10]. This study showed that sterilization did not impair phytase activity, indicating a potential temporal stability and resistance to microbial degradation for phyHT. Phytase activity is also influenced by substrate availability [11]. The low availability of phytate in soils hinders its hydrolysis by phytases [12]. Increasing the solubility of phytate is essential to improve the availability of phytases for the sustainable use of phosphorus in soils [13]. Furthermore, inorganic P supplementation can be substituted with phytases to enhance the available P content in soil [14]. Notably, phytases demonstrate superior efficacy in promoting plant growth than directly enhancing the available phosphorus in the soil, offering a practical and effective approach for optimal plant development [15].

The β-propeller phytase (BPP) from *Bacillus* sp. is a microbial phytase and is regarded as the most diverse phytase [16]. Alkaline phytases, with an optimal pH range of 6.0 to 8.0, exhibit properties such as strict phytate specificity, resistance to proteolysis, Ca^{2+}-dependent catalytic efficiency, and high thermostability [17]. In a study conducted by Lu [18], the phytase derived from *Bacillus* sp. HJB17, designated as phyHT, was categorized within the alkaline phytase group. This enzyme demonstrated distinctive properties that are commonly observed in Beta-Propeller Phytases (BPP).

The traditional focus in phytase research has been on soil microbes secreting phytases, involving tasks such as screening microbes for high phytase secretion. However, this study took a different approach by bypassing such screening steps. It explored the feasibility of directly applying phytases to soil to enhance the available phosphorus content. This novel method aims to improve soil fertility by circumventing the conventional microbial screening processes (Figure 1).

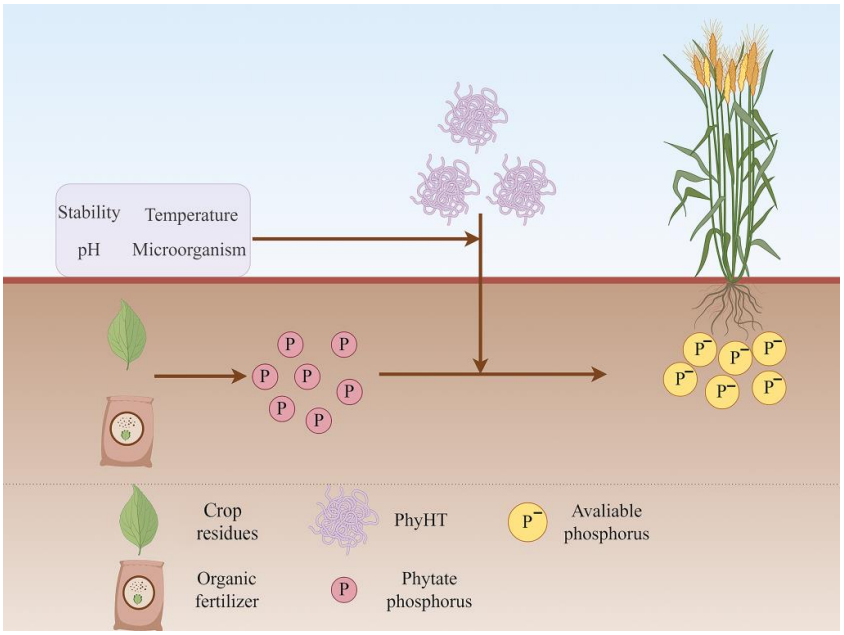

Figure 1. Direct application of microbial phytases can serve as a sustainable source of phosphate fertility in soil.

This paper reports the expression, purification, and characterization of phyHT from *Bacillus* sp. HJB17, highlighting the stability and resistance of phyHT when inoculated into the soil. Furthermore, the impact of phyHT on soil properties, the content of available P

in the soil, and plant growth were investigated. This study aimed to evaluate the effect of phytases on the degradation of phytate P in soil. A hypothesis was formulated and we verified that the exogenous addition of dual-domain β-propeller alkaline phytase could significantly degrade phytate in soil, increase the available phosphorus content in soil, and promote plant growth. This study establishes a theoretical basis for applying microbial phytases to soil.

2. Materials and Methods

2.1. PhyHT Construction, Expression, and Purification

The dual-domain β-propeller phytase (phyHT) gene (HM003049.1) used in this study was from the strain *Bacillus* sp. HJB17. The recombinant vector pET-28b(+)-phyHT was produced as described by Lu [19] and was transformed into *E. coli* BL21 (DE3) competent cells. Positive transformants were grown in 5 mL of LB medium containing 50 g L^{-1} kanamycin overnight at 37 °C. Ten-milliliter aliquots of the overnight culture were subcultured into 1 L of fresh LB medium containing kanamycin (50 g L^{-1}). Protein expression was induced by the addition of 0.2 mmol L^{-1} isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 4–6 h at 37 °C when the A 600 reached 0.6–0.8. The cell pellet was resuspended and lysed by sonication on ice for 10 min with phenylmethylsulfonyl fluoride (PMSF) added to a final concentration of 1 mmol L^{-1}. The inclusion body was refolded using a dialysis method shown in the patents of Gao [20]. The clear supernatant of the protein solution was purified using Ni^{2+}-chelating chromatography on a His-Trap HP column (GE Healthcare, Chicago, IL, USA) and Size-Exclusion Chromatography (SEC) on a Superdex G200 column (GE Healthcare, USA). All the purification procedures described above were conducted at 4 °C. Protein purification and identity were assessed by 15% SDS–PAGE. The protein concentration was analyzed by a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, WA, USA) and the sample was directly used to measure phytase activity.

PhyHT was eluted as a symmetrical peak from a Superdex G200 SEC column, and the target peak was observed at 12.73 mL (Figure 2b) after the Ni^{2+}-chelating chromatography and size-exclusion chromatography (SEC) steps. SDS-PAGE was used to detect the obtained protein; its molecular weight was 69.3 kDa, as shown in Figure 2a. The activity of phyHT was 62.11 U mg^{-1} under optimal conditions.

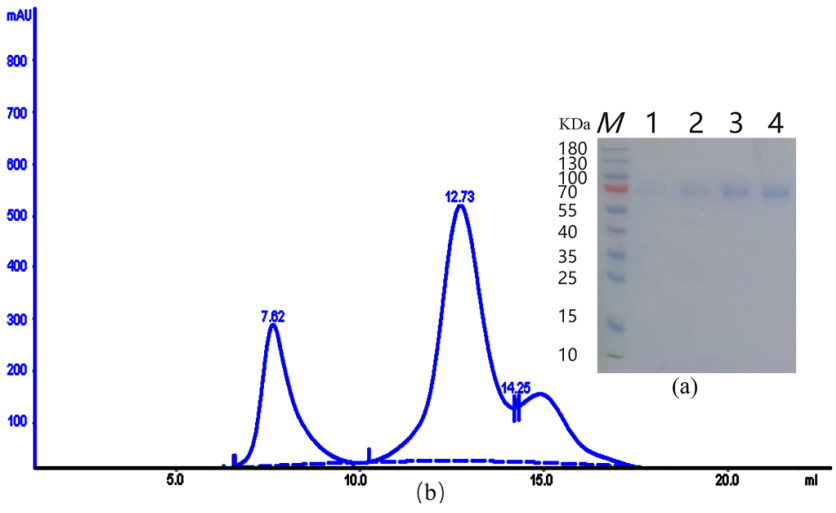

Figure 2. Purification of phyHT on gel filtration column and SDS polyacrylamide gradient gel analysis: (**a**) 12% SDS–PAGE gel stained with Coomassie Brilliant Blue. Lane M, protein marker; lanes 1–4, phyHT, which correspond to the peak on the gel filtration profile (Figure 2b). (**b**) Purification profile of phyHT, which eluted as a symmetrical peak from the SEC Superdex G200 column (blue peak). The vertical coordinate is the absorbency value (mAU), and the horizontal coordinate is the volume of the solution (mL).

2.2. Analysis of Phytase Properties

The phytase activity was determined by measuring the amount of phosphate released from phytate using a modified ferrous sulfate molybdenum blue method [21]. A 100 µL volume of the protein solution was measured by adding phytate into the substrate solution at a ratio of 1:4 and incubated for 15 min at 37 °C. Approximately 500 µL of 10% TCA was added to stop the reaction. Phosphate in the supernatant solution was measured by adding 500 µL of ammonium molybdate–ferrous sulfate buffer (50 mM Tris-HCl, 5.5% sulfuric acid, 1.5% ammonium molybdate, 2.7% ferrous sulfate). After centrifugation at 4 °C and 13,000× *g* for 5 min using a Benchtop High-Speed Refrigerated Centrifuge 3K30 (Sigma, Darmstadt, Germany), the absorbance of the supernatant was measured at 700 nm. One unit of phytase activity was defined as 1 µmol inorganic P in 1 min under certain conditions. Blanks were generated by adding a stop solution into the protein solution before adding the substrate.

The enzyme activity–temperature profile was obtained by performing phytase activity assays at temperatures ranging from 10 to 65 °C. The available pH range of phytase was studied at the optimum temperature by measuring the residual phytase activity after 30 min of incubation in a range of pH levels. The thermal stability of the phytase was evaluated by measuring the residual phytase activity after 5 h of incubation at different temperatures (20–60 °C) at the optimum pH. Each treatment and incubation period were prepared in triplicate.

Evaluation of enzymatic properties of purified phyHT showed that the optimum temperature of phyHT was 37 °C (Figure 3a), and it has strong thermal stability (Figure 3c). The enzymatic activity varied as a function of pH. The highest activity was recorded at pH 8.0 (Figure 3b). PhyHT was stable at neutral and alkaline pHs (Figure 3d) and showed high activity in a relatively broad pH range between 6.0 and 12.0.

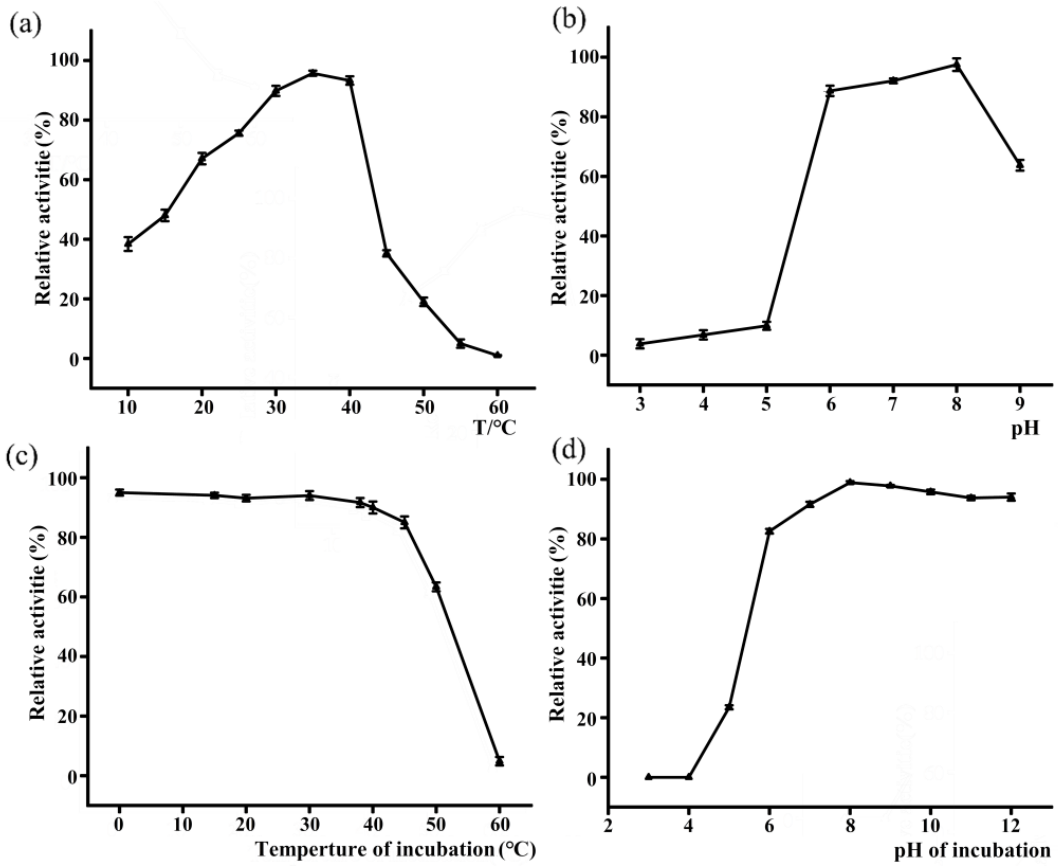

Figure 3. Analysis of enzymatic properties of phyHT: (**a**) the optimal temperature of phyHT; (**b**) the optimal pH of phyHT; (**c**) the temperature stability of phyHT; (**d**) the pH stability of phyHT.

2.3. Soil Samples and Their Chemical Properties, Organic Fertilizer Composition, and Crop Residue Samples

Soil samples were collected following the guidelines specified by George [9]. The soil samples were obtained from the mixed forest of *Pinus tabuliformis* and *Juniperus chinensis* at Beijing Forestry University, Haidian District, Beijing (40°0′5″ N, 116°20′28″ E, at 50 m altitude), at a depth of 0–20 cm. It was air-dried, sorted from coarse organic residues, ground until it passed through a 2 mm sieve, and stored at room temperature. Detailed characterization of the soil was performed. The soil type was fluvo-aquic soil, and the soil exhibited the following physical and chemical properties: pH of 8.52; organic matter content of 38.78 g kg^{-1}; 0.26 ms cm^{-1} exchangeable cations; and the available N, P, and K levels were 79.91, 17.98, and 122.46 mg kg^{-1}, respectively.

The crop residues at the four distinct growth stages of *Sorghum bicolor* were sampled. Appropriate amounts of the *Sorghum bicolor* samples were stored at −80 °C, and a grinder was used to thoroughly break them up.

Beijing Steiner Biodynamic Agriculture Co., Beijing, China, provided the organic fertilizer. The total organic matter content was 54.63%, the total nitrogen (N) content was 0.23%, the total P content was 3.08%, and the total potassium (K) content was 6.43%. It was de-hybridized, autoclaved, ground until it passed through a 2 mm sieve, and stored at room temperature.

The wheat (*Triticum aestivum* L.) grains used in the experiments were obtained from the local market, and the variety is ND399.

2.4. Factors Affecting Phytase Activity of phyHT in Soil

In accordance with the procedures described by George [9], a 0.5 g soil sample was weighed into a 15 mL screw cap polyethylene tube containing 5 mL of a phyHT solution (0–5 U g^{-1} of soil). The suspension was shaken on a flatbed shaker (200 oscillations min^{-1}). Phytase activity in the soil suspension and solution fractions was measured after different incubation periods.

The effects of time and the presence of microorganisms on the phytase activity were examined at 1, 3, 5, 12, and 24 h after phytase (5 U g^{-1} of soil) inoculation into the sterile soil and non-sterile soil suspensions. The temperature stability assessment of phyHT–soil complexes involved testing at various temperatures (20, 25, 30, 35, 40, and 45 °C) in a 20 mM Tris-HCl solution at pH 8.0. The phytase activities in soil suspension and solution fractions were subsequently measured following 24 h of agitation on a flatbed shaker (SKY-2012C, Shanghai Sukun Industrial Co., Ltd., Shanghai, China) at a speed of 200 revolutions per minute. The pH dependence of the phyHT–soil complex activity was measured in solutions and suspensions at a pH of 5, 6, 7, 8, and 9 after incubation for 24 h.

Total enzyme activity (phytase activities in soil suspension) was measured in a 100 µL sub-sample. The soil and solution were separated by centrifugation at 13,000× *g* for 15 min to measure phytase activity in the solution (supernatant) phase. Each treatment and incubation period were prepared in triplicate.

2.5. Effect of phyHT on Available P Content in Soil

A 0.5 g soil sample was weighed into a 100 mL conical flask with 50 mL of a solution of phyHT (0–100 U) to study the effect of phyHT on the available P content in the soil. The solution underwent incubation in a shaking incubator (SPH-211B, Shanghai Sukun Industrial Co., Ltd., Shanghai, China) under constant conditions (37 °C) for 12 h. Subsequently, the solution was filtered through phosphorus-free quantitative filter paper (9 cm, Henan Ruinong, Zhengzhou, China), employing a filtration method to separate the supernatant. The isolated supernatant was subsequently utilized for available phosphorus analysis using the modified ferrous sulfate molybdenum blue method [21].

2.6. Effect of phyHT on Soil Physical and Chemical Properties

The efficacy of the phytase as a soil amendment was studied. A 50 g soil sample was thoroughly mixed with phyHT (250 U), along with a control process without phyHT. The pots were incubated in a greenhouse at normal temperature (~20 °C) and 60% of field water holding capacity for 7 days, after which it was collected, dried, and used for further physical and chemical analyses.

The pH values were measured using the potentiometric method. Electrical conductivity (EC) was determined using the conductometric method. Soil organic matter content (OM) was assessed by employing the potassium dichromate oxidation spectrophotometric method. The soil available N, available P, and available K were determined by the alkaline hydrolysis diffusion method, molybdenum antimony colorimetry, and the ammonium acetate extraction–flame photometry method, respectively. The calcium carbonate ($CaCO_3$) content was assessed using the gasometric method. Phytate content was assessed using the ferric chloride colorimetric method. The selection of the above methods was based on a study by Li [22].

2.7. Effect of phyHT on the Available P Content in the Samples of Crop Residues and Organic Fertilizer

The impact of phyHT on the available phosphorus content in the crop residue and organic fertilizer samples was assessed using the modified ammonium molybdate method for determining the release of phosphate from phytate salts [21]. Samples (0.5 g) of four *Sorghum bicolor* plants were used as samples of crop residues and were weighed into 15 mL screw cap polyethylene tubes containing 5 mL of a solution of phyHT (0–5 U g^{-1} of soil). They were labeled as H-T, H-O, UH-T, and UH-O, respectively, and each sample was set up with a control group. The control and experimental groups were placed in a flatbed shaker (SKY-2012C, Shanghai Sukun Industrial Co., Ltd., Shanghai, China) at 200 rpm for 5 h. After the reaction, the samples were filtered through phosphorus-free quantitative filter paper (9 cm, Henan Ruinong, Zhengzhou, China), and the effective phosphorus content in the filtrate pipe was determined using the method described in Section 2.2 of the manuscript.

A 0.5 g sample of the organic fertilizer was weighed into a 15 mL screw cap polyethylene tube containing 5 mL of solution of phyHT (0–5 U g^{-1} of soil). A control group without the addition of protein was also set up. In the subsequent experimental steps, the control samples were treated the same way as the plant samples.

2.8. Effect of phyHT on Wheat Growth

Wheat (*Triticum aestivum*) seeds of the ND399 variety were surface sterilized with 1% sodium hypochlorite for 30 s, followed by three rinses with distilled water. Soil (500 g) was distributed equally in a 12 × 10 nutrient tray, and wheat seeds were sown at a depth of 2 cm. Fifty seeds of wheat were sown in each pot in three replicates. The pots were supplemented with A (phytate 6.6 mg), B1 (phyHT 250 U), B2 (phytate 6.6 mg with phyHT 250 U), C1 (organic fertilizer 10.0 g), C2 (organic fertilizer 10.0 g with phyHT 250 U), or D (P-KH_2PO_4, 5.98 mg). Seedlings treated with water only were used as a negative control (CK). After watering, pots were kept in a growth chamber. The growth conditions were as follows: 25/21 °C day/night temperatures, 60% relative humidity, and a 16 h photoperiod. The nutrient tray maintained a data water holding capacity of 80% during the experiment. Seedlings were watered periodically and monitored for plant height and growth. After 20 days of growth, the treated seedlings were harvested. Their root and shoot lengths and root and shoot dry weights were measured. The P contents in roots and shoots were measured separately. The soil in the nutrient tray was collected and air-dried, and subsequently used for available P and total P analyses.

2.9. Data and Statistical Analyses

The presented data represent the mean of three replicates, with error bars depicting one standard error on either side of the mean. Analysis of variance (ANOVA) was employed to

assess significant differences, and pairwise comparisons were conducted using Duncan's multiple range test with a significance level set at $p < 0.05$. Line graphs were constructed utilizing Origin software for precise visualization.

3. Results and Discussion

3.1. The Implications for Temporal Stability and Resistance to Microbial Degradation of phyHT in Soil

The microorganisms in the soil were inactivated after autoclaving, thereby allowing us to compare sterilized and non-sterilized soils to distinguish between abiotic and biotic effects. The activity of phyHT in the soil suspension and solution is presented in Figure 4. No significant difference was observed in the sterilized and non-sterilized soil at any time. The stability of phyHT in the soil, as indicated by the unaffected enzyme activity in sterilized and non-sterilized soils, aligns with the findings of Kedi [10]. The stable adsorption of phytases into the soil provides the possibility of long-term enzyme activity in the soil. Adsorption may help protect the enzymes from degradation by various microorganisms in the soil, leaving the added phytase unaffected or slightly affected by biological effects [9], which is consistent with the conclusions obtained in this study. However, it is important to note that a single sterilization treatment cannot eliminate the effects of microorganisms. Sterilization can only kill microorganisms in the soil, but it cannot eliminate the effect of microbial products, such as some proteases and organic acids, which may impact phyHT efficacy. In conclusion, while the microbial effect on phyHT activity is limited, the influence of microbial products on enzyme efficacy cannot be disregarded.

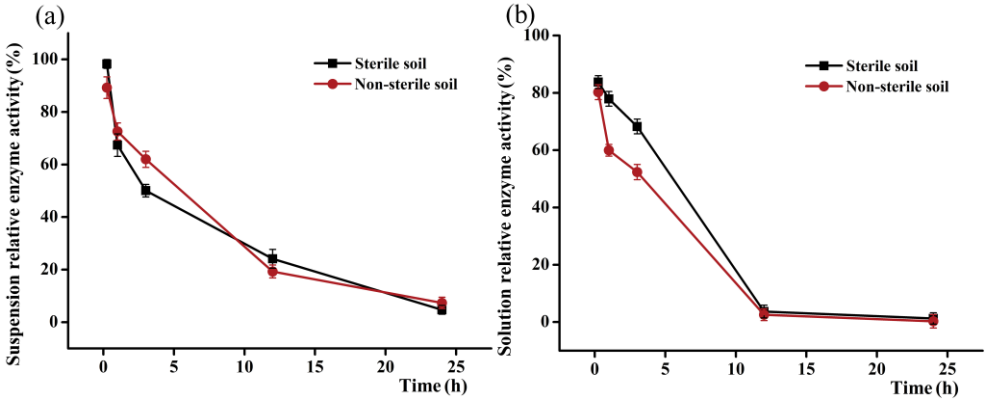

Figure 4. Effect of sterilization on phyHT activity in soil (the amount of phyHT was 0.4 U g^{-1}): (**a**) relative retention rate of phyHT activity in soil suspension; (**b**) relative retention rate of phyHT activity in soil solution.

The relative enzyme activity of phyHT in the soil solution showed a significant decline over time; the tendency was fast over the first 12 h and then it slowed. When inoculated into soil, phyHT was quickly degraded, and its relative enzyme activity was reduced. When phyHT is in contact with the soil, the soil may contain components, such as some proteases, organic acids, and heavy metals, which reduce its enzyme activity. After a 12 h incubation in the soil, the relative enzyme activity of phyHT in the suspension and the supernatant decreased to less than 10% when the amount of phyHT was 0.4 U g^{-1}. At this concentration, the activity of phyHT can be maintained for at least 12 h. By increasing the amount of phyHT inoculated, it was found that activity was maintained, which increased after inoculating phyHT into the soil. When the phytase concentration was increased to 1 U g^{-1}, the relative enzyme activity of phyHT was about 34% after 12 h, which shows that the amount of phytase added affects the maintenance of phyHT activity in the soil.

3.2. Effect of Temperature and pH on Inoculated phyHT Activity in Soil

The enzyme activity of phyHT in the soil suspension and solution was investigated under different temperature conditions (20 to 45 °C). For both the soil solution and soil

suspension at pH 8.0, the temperature-dependent activity curves are presented in Figure 5a. Notably, the enzyme's adsorption to soil exhibited the optimal effectiveness at 35 °C, suggesting an optimal balance between adsorption and activity.

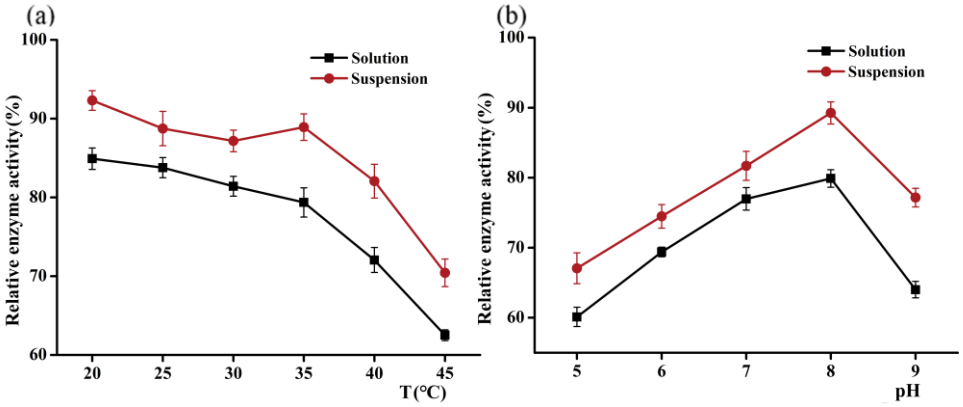

Figure 5. Effect of different temperatures and pHs on phyHT activity in soil: (**a**) effect of different temperatures on phyHT activity in soil; (**b**) effect of different pHs on phyHT activity in soil.

High temperatures accelerated the degradation of phyHT, leading to a reduction in its activity. In a soil environment, the stability of phytases is related to their enzymatic properties on the one hand and the adsorption into the soil on the other hand. Adsorption allows the enzyme to be rapidly immobilized upon addition to the soil. The studies on enzyme stability, particularly the work conducted by Kedi [10], have suggested that the loss of enzyme activity in the adsorbed state was attributed to degradation and conformational changes. However, it was noted that these factors would only inactivate a small fraction of the enzyme. Changes in conformation may cause some degree of decrease. In addition, when the temperature is higher, the adsorption characteristics of phyHT to soil may be enhanced, which inhibits the catalytic activity of phyHT.

Moreover, the study conducted by Menezes-Blackburn showed that elevated temperatures enhance the adsorption of phytases into the soil, consequently influencing its stability [23]. This observation aligns with the findings of the present study. At higher temperatures, the adsorption characteristics of phyHT into the soil may intensify, potentially hindering the catalytic activity of phyHT.

The enzyme activity of phyHT was assessed in the soil suspension and solution at various pH levels (pH 5 to 9). The experimental data are shown in Figure 5b. Both in the soil solution and filtered supernatant, the enzyme activity increased from pH 5 to 8 and sharply decreased at pH 9. A comparative analysis revealed that at pH 9, the disparity between enzyme activity in the soil suspension and filtered supernatant was more pronounced, indicating enhanced enzyme adsorption into soil at this pH. Consequently, this study posit that the optimal adsorption efficacy occurred at pH 9, while enzyme activity peaked at pH 8. This observation aligns with the consistent patterns in the activity vs. pH curves, where phyHT exhibited relatively high enzyme activity in the soil solution and the highest activity in solution at pH 8.0, close to the natural pH of the soil, as depicted in Figure 5b and supported by the enzyme properties observed in Figure 3b. These results collectively suggest that phyHT can maintain a relatively stable enzyme activity in the soil, with the retention rate of the activity being mainly related to the enzymatic properties of phyHT. The temperature- and pH-dependent behaviors highlight the interplay between enzyme activity, adsorption, and environmental conditions. These findings provide crucial insights into the adsorption and activity dynamics of phyHT in soil, supporting the hypothesis that phyHT can be effectively adsorbed into soil and retain enzymatic activity.

3.3. Efficacy of phyHT in Hydrolysis of Phytate in Soil

The effect of phyHT on the available P content in soil is shown in Figure 6. Compared with the control group without phyHT, the available P content in the soil increased gradually as the amount of phyHT increased. When the amount of phyHT inoculated reached 1.6 U g^{-1}, the available P showed a significant difference compared with the soil without phyHT ($p < 0.05$). When the amount of phyHT inoculated reached 5 U g^{-1}, the available P in soil was the highest, an increase of 43.09% compared with the treatment without phyHT. After that, with increasing addition of phyHT, the available P content no longer increased. The amount of phyHT can effectively increase the available P content in soil. The efficiency of increasing the effective P was related to the amount of phyHT inoculated. When the amount of phyHT reached 5 U g^{-1}, the available P content in the soil reached a maximum, and after which, it was no longer affected by the amount of phyHT inoculated.

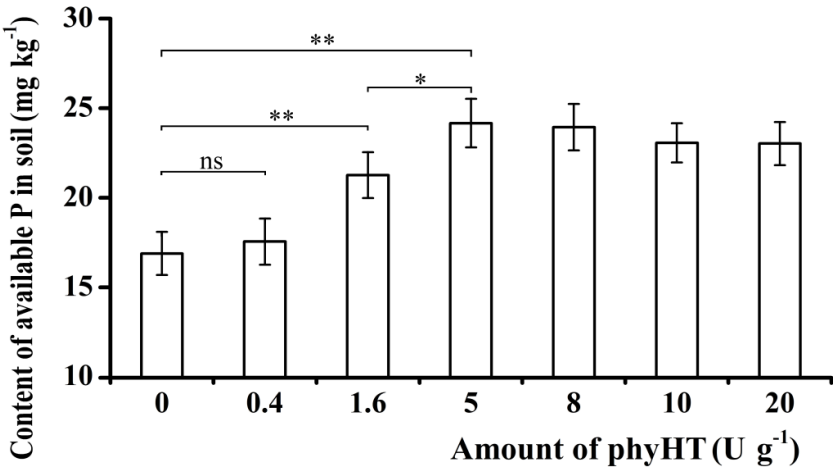

Figure 6. Effect of phyHT on available P content in soil. **, *: significant differences at the 0.01 and 0.05 levels, respectively; ns: not significant.

3.4. Effects of phyHT on Soil Physical and Chemical Properties

In this study, following the addition of phyHT to the soil, the physical and chemical properties of both the treated and untreated groups were assessed, as presented in Table 1. Introducing phyHT into the soil resulted in a noticeable decrease in pH and a significant increase in electrical conductivity. These changes were conducive to the hydrolysis of certain mineral elements. Notably, N availability, potassium availability, and phosphorus content in the soil significantly increased, which can foster plant growth. PhyHT reduced the soil phytate content by approximately 20.48% while concurrently elevating the phytate phosphate availability by 1.22-fold. This highlights that incorporating phyHT can enhance the utilization of phytate in the soil, thereby improving phosphorus fertility.

Table 1. Physical and chemical analyses of soil.

Parameter	Control	phyHT Treatment
pH (1:2.5)	8.78	8.16 **
EC [(a)] (dS m^{-1})	0.29	0.68 **
OM [(b)] (g kg^{-1})	38.78	42.87 *
Available N (mg kg^{-1})	78.59	93.04 **
Available P (mg kg^{-1})	17.98	22.03 **
Available K (mg kg^{-1})	122.49	156.17 *
$CaCO_3$ (%)	13.67	10.12 *
Phytate (mg kg^{-1})	597.56	495.98 **
phytase activity (mg P_2O_5 $(g \times d)^{-1}$)	0.25	0.61 **

[(a)] EC: exchangeable cations. [(b)] OM: organic matter content. **, *: represent significant differences at the 0.01 and 0.05 levels, respectively.

Gujar has provided insights into the soil-improving potential of phytases [24]. The specific hydrolysis action of phytases is efficient in breaking down phytate and releasing effective phosphorus. The application of phytases has been shown to enhance soil fertility and conditions by increasing the effective phosphorus content, aligning with the findings of the current study. These collective results suggest that the exogenous addition of phyHT protein as a soil amendment exhibits stability in soil, effectively decomposes phytate, and significantly boosts the effective phosphorus content.

3.5. Effectiveness of phyHT for Hydrolysis of Phytate in Crop Residues and Organic Fertilizer

The accumulation of phosphorus in *Sorghum bicolor* differs among different growth stages. The longer the growth time, the more phosphorus will be accumulated. The study demonstrated the effective hydrolysis of phytate in *Sorghum bicolor* by phyHT, leading to an increase in the available phosphorus content across all growth states (Figure 7a). The most substantial increase, at 7.88%, was observed for the UH-O state.

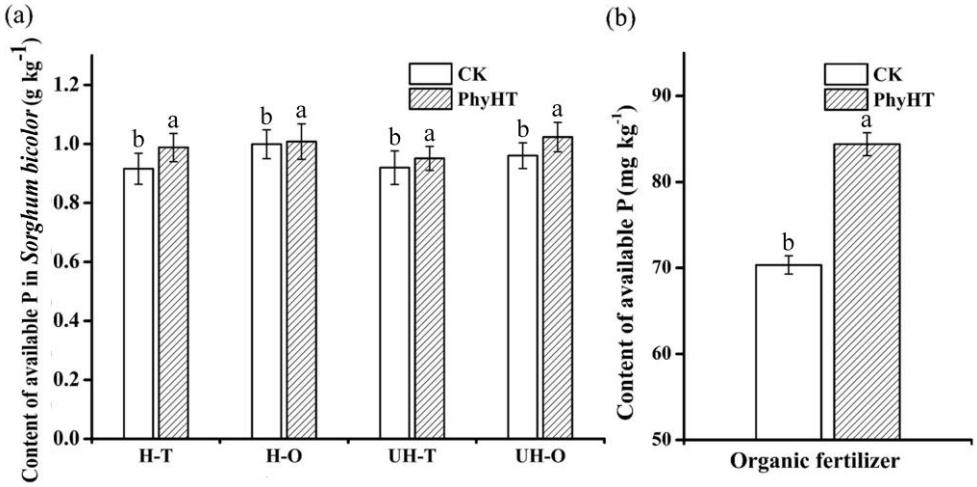

Figure 7. Effect of phyHT on available P content. Different letters indicate significant differences ($p < 0.05$) among treatments applied to the same sample. (**a**) Available P Content in *Sorghum bicolor*; H-T, H-O, UH-T, and UH-O represent the four different growth states of *Sorghum bicolor*. "H" means heading, when *Sorghum bicolor* has already emerged; "UH" means that the *Sorghum bicolor* has not produced any ears; "T" means tender; and "O" means old. (**b**) Available P content in organic fertilizer.

Crop residues primarily consists of the post-harvest remnants of agriculture, including stems and stalks, leaves, and seed pods. These contain phosphorus within the range of 0.45% to 2% [25]. The treatment of phytate accumulation in *Sorghum bicolor* plant tissues by a phytase can play a significant role. The direct application of phyHT in the soil may be beneficial for the degradation of crop residues, particularly in the breakdown of phytate salts. In this process, it can contribute to an increase in the soil's available phosphorus content.

In animal feed production, there is a need to improve the utilization efficiency of phytate [4]. The application of phyHT shows promise in enhancing the available phosphorus content in plant samples. This finding suggests potential applications in the processing and production of animal feed. Using phyHT can elevate the available phosphorus content in animal feed, facilitating better absorption of phosphorus nutrients by animals. This, in turn, can reduce the environmental impact associated with high phosphorus levels in animal manure.

The phyHT effectively increased the content of free phosphorus in the organic fertilizer, effectively improving its phosphorus fertility. After phyHT treatment, the effective phosphorus content of the organic fertilizer increased by 19.97% (Figure 7b). When the organic fertilizer is applied to soil supplemented with phyHT, the availability of phosphorus increased. Given the worldwide scarcity of phosphate rock, which are the raw materials

for the production of P fertilizers, it is necessary to utilize phytate in animal manures [4]. Organic fertilizer, as an environmentally friendly fertilizer, can promote plant growth if the addition of phyHT can increase its effective phosphorus content.

3.6. Effect of phyHT on Plant Growth

As shown in Figure 8, supplementing phyHT and organic fertilizer can improve wheat seedlings' growth (shoot and root lengths) and dry weight. The addition of inorganic P fertilizer significantly ($p < 0.05$) increased plant growth, indicating that P is a major limiting factor for plant growth (Figure 8a, combination D). The lengths of the shoots and roots of the wheat seedlings were longer when supplemented with phyHT or phytate alone compared to that of control (Figure 8a combination A and B1). The supplementation with the combination of phyHT and phytate was the best for plant growth among all the combinations studied (Figure 8a combination B2). Organic fertilizer as an all-purpose fertilizer presented significantly better results than the control group (Figure 8a, combination C1). Compared with C2 group (Figure 8a), the length of the seedlings was more greater when given both phyHT and the organic fertilizer (Figure 8a, combination C2). The soil treatment with the inorganic P fertilizer did promote a significant increase in root dry weight (Figure 8b, combination D). This indicates that inorganic P does not promote the growth of wheat roots. The phyHT and phytate treatments significantly increased the dry weight compared to the control group (Figure 8b). Moreover, as shown in Figure 8a, the combination of phyHT and the organic fertilizer had a significantly superior effect compared to all the other groups.

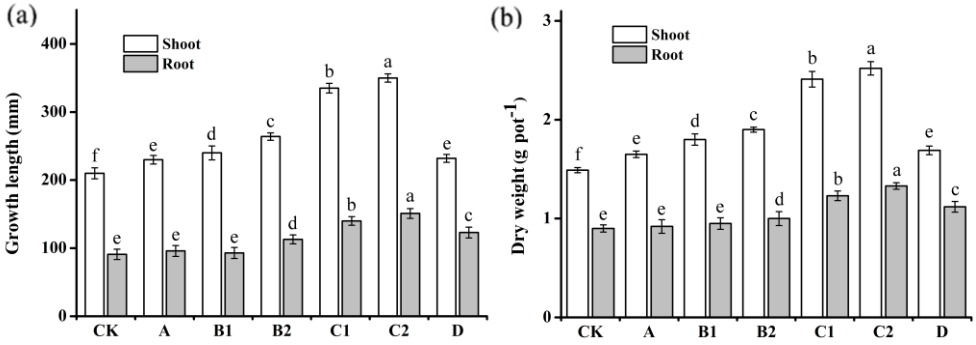

Figure 8. Effect of phyHT on length (**a**) and dry weight (**b**) of shoots and roots. A (phytate 6.6 mg), B1 (phyHT 250 U), B2 (phytate 6.6 mg with phyHT 250 U), C1 (organic fertilizer 10 g), C2 (organic fertilizer 10 g with phyHT 250 U), D (KH_2PO_4, 5.98 mg). Different letters indicate significant differences ($p < 0.05$) among treatments applied to the same sample.

The tissue P concentration was only significantly higher in the phyHT and phytate treatment combination than in the control (Figure 9 A and B1), indicating that adding phytate and phyHT can increase the absorption of P by wheat. When phyHT was added to organic fertilizer, the phosphorus concentration in plant tissues was significantly higher than in the group only given organic fertilizer (Figure 9, combinations C1 and C2).

Adding phytate, phyHT, and organic fertilizer increased the absorption of P by wheat (Figure 9). The tissue P concentration was significantly higher in the phyHT and organic fertilizer treatment combination than in the control (Figure 9, combination C2). These results suggest that the phyHT maintained its activity and progressively hydrolyzed organic P from the soil and organic fertilizer during cultivation. The increased inorganic soil P concentration from the phyHT treatment increased plant P nutrition.

Low P stress is one of the major limiting factors for crop yield. Extracellular phytases can improve the P nutrition of plants when presented in the rhizosphere [26–29]. The enzyme activity of plant phytases showed great differences depending on their source, with the enzyme activity of the phytase from rye seeds being much higher than that of phytases for wheat, corn germ, and sorghum seeds [30]. Although plant-derived phytases have a wide range of sources, their overall content is not high, and it is not easy to separate and

purify them from plants. Their enzyme activity stability is insufficient, and these enzymes are easily affected by processing, storage, and other processes [31]. Adding phytases to the soil may be a more effective P biofertilizer to increase agricultural productivity.

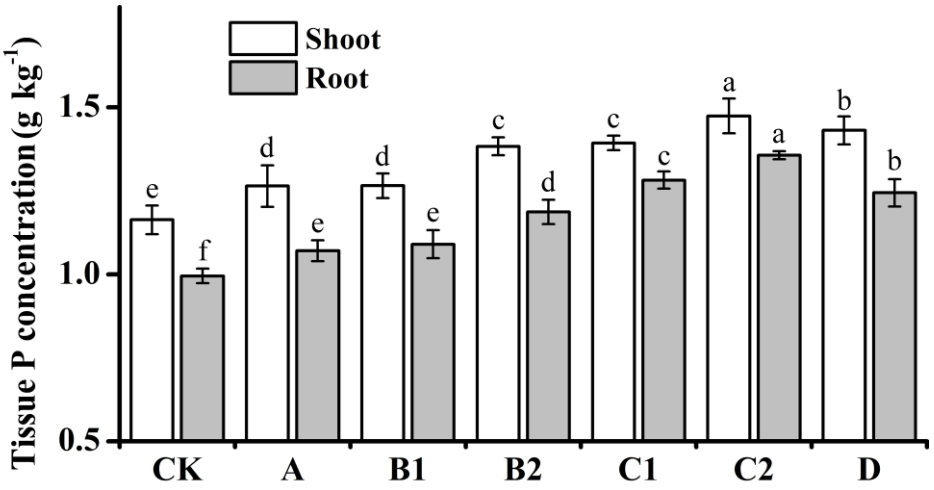

Figure 9. Effect of phyHT on P concentration in shoot and root tissues of wheat. Different letters indicate significant differences ($p < 0.05$) among treatments applied to the same sample. A (phytate 6.6 mg), B1 (phyHT 250 U), B2 (phytate 6.6 mg with phyHT 250 U), C1 (organic fertilizer 10 g), C2 (organic fertilizer 10 g with phyHT 250 U), and D (KH_2PO_4, 5.98 mg).

4. Conclusions

PhyHT exhibited high catalytic efficiency, strong thermal stability, and resistance to pH changes. The exogenous addition of dual-domain β-propeller alkaline phytase, phyHT, significantly degraded the phytate in the soil, increased the content of available phosphorus in the soil, and promoted plant growth. This study contributes to the theoretical foundation for the application of microbial phytases to soils. The results highlight the stability and functionality of phyHT in soil, providing insights into its potential applications in diverse environmental conditions and contributes to the theoretical foundation for the direct application of microbial phytases to soils.

The application of phyHT in soil showed promising results in terms of increasing the available phosphorus content, enhancing soil fertility, and promoting plant growth. The study contributes to our understanding of microbial phytases and their behavior in soil environments and provides practical implications for sustainable agricultural practices.

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