Asian Journal of Agriculture and Biology
https://doi.org/10.35495/ajab.2026.050

Original Article

Acid phosphatase from *Trichoderma asperellum* and its potential role in phosphorus mobilization for sustainable soil fertility

Zair Shakirov[1], Iskandar Yakubov[2], Khursheda Khamidova[1], Saidakhon Zakiryaeva[1], Nodira Azimova[1], Sardarkhodja Kurganov[3], Cheng Gao[4], Husniddin Karimov[1]*

[1]Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan, Tashkent, Uzbekistan
[2]National University of Uzbekistan, Tashkent, Uzbekistan
[3]Republican Scientific Specialized Center of Allergology, Tashkent, Uzbekistan
[4]State Key Laboratory of Microbial Diversity and Innovative Utilization, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China

**Corresponding author's email:* husniddin263@gmail.com
Received: 25 January 2026 / Revised: 04 May 2026 / Accepted: 20 May 2026 / Published Online: 12 June 2026

Abstract

Extracellular acid phosphatase plays a key role in the mobilization of organic phosphorus in soil ecosystems. In this study, acid phosphatase produced by *Trichoderma asperellum* Uz-A4 was isolated, purified and biochemically characterized. Maximum enzyme activity (1534 μM min^{-1} mg^{-1}) was observed on the sixth day of cultivation in Czapek broth. Zymogram analysis revealed two extracellular acid phosphatase isoforms with molecular masses of approximately 175 and 115 kDa. The dominant isoform (ACP1) was purified by ammonium sulfate precipitation followed by DEAE–TSK ion-exchange and Phenyl–Sepharose hydrophobic interaction chromatography. SDS–PAGE analysis showed that ACP1 is a homodimer composed of two identical subunits of 85 kDa. The enzyme exhibited optimal activity at pH 5.0 and 50 °C and retained high activity after incubation at 60 °C for 1 h, indicating pronounced thermostability. Zn^{2+} and Mn^{2+} ions significantly inhibited enzyme activity, whereas Ca^{2+}, Co^{2+}, Mg^{2+}, EDTA and reducing agents had no substantial effect. The biochemical properties of ACP1 highlight the potential of *T. asperellum* Uz-A4 to contribute to organic phosphorus mineralization and to improve phosphorus availability and soil fertility under sustainable agricultural systems.

Keywords: Acid phosphatase, Phosphate-solubilizing microorganisms, Phosphorus mobilization, Soil fertility, *Trichoderma* spp.

How to cite this article:

Shakirov Z, Yakubov I, Khamidova K, Zakiryaeva S, Azimova N, Kurganov S, Gao C and Karimov H. Acid phosphatase from *Trichoderma asperellum* and its potential role in phosphorus mobilization for sustainable soil fertility. Asian J. Agric. Biol. 2026: e2026050. DOI: https://doi.org/10.35495/ajab.2026.050

Introduction

Phosphorus (P) is the second most important macronutrient after nitrogen for plant growth and development. It is a key structural component of nucleic acids, phospholipids and energy-transfer molecules, playing a crucial role in the regulation of metabolic, physiological and biochemical processes that determine plant productivity and yield (Mishra et al., 2024). Despite generally sufficient total phosphorus levels in most soils, only a small fraction is readily available for plant uptake, as phosphorus is predominantly present in insoluble inorganic minerals or bound within organic compounds (Khan et al., 2023).

Moreover, a large proportion of applied chemical phosphorus fertilizers are rapidly immobilized in soil through fixation processes, forming poorly soluble complexes that are inaccessible to plants. Under these conditions, the availability of phosphorus in the rhizosphere largely depends on biological and chemical transformations mediated by soil microorganisms and root-associated processes (Mwende, 2019; Pang et al., 2024). These transformations are particularly important in saline and degraded soils, where nutrient availability is further limited and sustainable soil fertility management is required.

Phosphate-mobilizing microorganisms play a central role in the transformation of insoluble phosphorus into plant-available forms. Members of the genera *Azotobacter*, *Burkholderia*, *Rahnella*, *Pseudomonas*, *Bacillus*, *Rhizobium*, *Enterobacter* and *Pantoea*, as well as filamentous fungi such as *Trichoderma* and *Aspergillus*, are known to solubilize inorganic phosphorus through the production of organic acids and other metabolites (Karimov et al., 2024; Landa-Acuña et al., 2023; Shakirov et al., 2022; Wang et al., 2023; Bononi et al., 2020). In addition to acidification mechanisms, many of these microorganisms contribute to phosphorus cycling by secreting extracellular phosphatases that hydrolyze organic phosphorus compounds in soil.

Acid phosphatases (EC 3.1.3.2) are phosphomonoesterase enzymes that catalyze the hydrolysis of phosphomonoester bonds, releasing inorganic phosphate (PO_4^{3-}) and free hydroxyl groups from organic substrates. These enzymes are widely distributed in plants, animals and microorganisms, although their activity and biochemical properties vary considerably among organisms and environmental conditions. The production of extracellular acid phosphatases by soil microorganisms represents an important biological pathway for the mineralization of organic phosphorus and serves as an additional phosphorus source for plant nutrition (Pratibha et al., 2021; Tian et al., 2021; Wang et al., 2023; Rejsek et al., 2012).

Among phosphate-mobilizing fungi, species of the genus *Trichoderma* are of particular interest due to their ecological adaptability, high enzymatic activity and widespread use in agriculture as biofertilizers and biocontrol agents (Odoh et al., 2020; Jawad et al., 2023). However, detailed information on the physicochemical properties and functional characteristics of extracellular acid phosphatases produced by *T. asperellum* Uz-A4 remains limited.

The present study aimed to isolate, purify and characterize extracellular acid phosphatase isoforms produced by *T. asperellum* Uz-A4, with a particular focus on their biochemical properties and potential role in phosphorus mobilization processes relevant to sustainable soil fertility management.

Material and Methods

Cultivation of *T. asperellum* Uz-A4

To select the optimal nutrient medium for the cultivation of *T. asperellum* Uz-A4 (Shavkiev et al., 2022), several commonly used liquid (broth) media were tested, including Mandels medium (Turaeva et al., 2021), Czapek broth, Sabouraud broth, and potato dextrose broth (PDB). In Mandels and Czapek broth media, wheat bran was used as an alternative carbon source instead of conventional sugars (Azimova et al., 2020).

The fungus was cultivated in 500-ml Erlenmeyer flasks containing 200 ml of liquid medium. Incubation was carried out on a rotary shaker at 150 rpm at a temperature of 24–28 °C for 6 days.

Determination of acid phosphatase activity

T. asperellum Uz-A4 was cultivated on Mandels, Czapek, Sabouraud and PDB media at 28 °C for 6 days. After cultivation, the culture was filtered through filter paper to remove fungal biomass. The resulting culture filtrates were used as crude enzyme extracts for the determination of acid phosphatase activity.

The reaction mixture for the acid phosphatase assay consisted of 2.0 ml of sodium acetate buffer (100 mM, pH 5.5), 30 µl of p-nitrophenyl phosphate (65 mM) as

https://doi.org/10.35495/ajab.2026.050

the substrate, and 0.1 ml of $MgCl_2$ solution (10 mM). The enzymatic reaction was initiated by adding 0.1–0.4 ml of enzyme extract to the reaction mixture. The mixture was incubated at 42 °C for 15–30 min.

The reaction was terminated by the addition of 0.6 ml of 1 M NaOH, and the amount of p-nitrophenol released was measured spectrophotometrically at 405 nm. A reaction mixture without the enzyme extract served as a control. Acid phosphatase activity was calculated using a standard calibration curve of p-nitrophenol.

Zymogram analysis of acidic phosphatase isoforms

Acidic phosphatase isoforms produced by *T. asperellum* Uz-A4 were analyzed using the zymogram method following polyacrylamide gel electrophoresis. After electrophoresis, gels were incubated in a staining solution containing 30 mg naphthol AS-MX phosphate as the substrate, 50 ml of incubation buffer, 0.25 ml of 0.1 M $MgCl_2$, 0.25 ml of $MnCl_2$, 5 ml of 20% NaCl solution and 30 mg of Fast Blue salt. Enzyme activity bands were visualized as colored zones after incubation at room temperature until clear bands appeared (Gadallah et al., 2017).

Purification of acid phosphatase from *T. asperellum* Uz-A4

Acid phosphatase from *T. asperellum* Uz-A4 was purified using a combination of ammonium sulfate precipitation, ion-exchange chromatography and hydrophobic interaction chromatography. In the first step, solid ammonium sulfate was gradually added to the cell-free culture supernatant to reach 80% saturation and completely dissolved. The mixture was incubated at 4 °C for 18 h and then centrifuged at 12,000 rpm for 15 min. The resulting precipitate was dissolved in 15 ml of distilled water and dialyzed against 50 mM Tris–HCl buffer (pH 7.0).

The concentrated enzyme preparation was applied to a DEAE–TSK ion-exchange chromatography column (30 × 2.5 cm) previously equilibrated with 50 mM Tris–HCl buffer (pH 7.0). Bound proteins were eluted using a linear NaCl gradient ranging from 0 to 0.6 M. Fractions exhibiting high acid phosphatase activity were pooled and dialyzed against 20 mM Tris–HCl buffer (pH 7.0) for 24 h. This fraction was subsequently used for further purification by hydrophobic interaction chromatography.

Hydrophobic chromatography was performed on a Phenyl–Sepharose column (15 × 1.5 cm) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.0) containing 2 M ammonium sulfate. Solid ammonium sulfate was added to the pooled DEAE–TSK fraction to achieve a final concentration of 2 M, and the sample was loaded onto the column. The column was initially washed with 2 M ammonium sulfate solution, followed by elution of bound proteins using a decreasing ammonium sulfate gradient from 1 M to 0 M. Fractions retaining acid phosphatase activity were pooled and dialyzed against Tris–HCl buffer for subsequent analyses.

Determination of the molecular mass of acid phosphatase

The molecular mass of the purified acid phosphatase was estimated by SDS–PAGE. Electrophoretic analysis was performed using a 12% polyacrylamide gel under denaturing conditions. SDS–PAGE was used to assess both the purity and the molecular mass of the purified enzyme by comparison with standard protein molecular weight markers.

Physicochemical properties of acid phosphatase

Acid phosphatase activity was determined in 0.1 M sodium acetate buffer within a pH range of 3.0–7.0. The optimum temperature for enzyme activity was evaluated over a temperature range of 30–80 °C using 0.1 M sodium acetate buffer (pH 4.5). Substrate dependence of the enzyme was assessed using *p*-nitrophenyl phosphate as the substrate. To evaluate the time dependence of enzyme activity, the reaction was monitored for up to 70 min under standard assay conditions.

Thermostability of the acid phosphatase was determined by incubating the purified enzyme in 0.1 M sodium acetate buffer (pH 4.5) at temperatures ranging from 50 to 70 °C for 10 min, followed by immediate cooling on ice. Residual enzyme activity was subsequently measured under standard assay conditions.

Effect of metal ions and inhibitors on enzyme activity

The effect of metal ions and inhibitors on acid phosphatase activity was investigated by preincubating the purified enzyme with selected reagents at final concentrations of 0.1, 1.0 or 5 mM in

0.1 M sodium acetate buffer (pH 4.5) for 30 min. Enzymatic reactions were initiated by the addition of the substrate (*p*-nitrophenyl phosphate), and residual activity was determined as described above.

Statistical analysis

All experiments were performed in triplicate, and results are presented as mean ± standard deviation (SD). Differences among treatments were evaluated using one-way ANOVA, with significance set at $p < 0.05$. All statistical analyses were performed using Microsoft Excel (Microsoft Corporation, USA).

Results and Discussion

Growth and biomass production of *T. asperellum* Uz-A4 in different culture media

The growth and biomass production of *T. asperellum* Uz-A4 were evaluated in Mandels, Czapek, Sabouraud and PDB liquid media. The fungus was cultivated at pH 5.0 and 28 °C on a rotary shaker at 150 rpm for 6 days.

Following inoculation into the different culture media, active fungal growth was observed from the third day of cultivation. At this stage, dry biomass production ranged from 83 to 101 µg mL^{-1}, depending on the medium used (Table 1).

Table-1. Dry biomass production of *T. asperellum* Uz-A4 during cultivation (µg mL^{-1}).

Culturemedium	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Mandels	0	11±0,8	91±0,6	243±0,3	307±0,6	509±0,9
Czapek	0	9±0,9	92±0,6	231±0,9	295±0,9	456±0,9
Sabouraud	0	6±0,6	83±0,3	196±0,9	253±0,3	321±0,9
PDB	0	8±0,6	101±0,8	201±0,6	257±0,6	338±0,6

Note: Values represent mean ± SD (n = 3). Differences among treatments were evaluated using one-way ANOVA ($p < 0.05$).

No significant differences in biomass accumulation were detected among the tested media during the early growth phase. After 6 days of cultivation, dry biomass values increased to 321–509 µg mL^{-1} across all media. By the sixth day of cultivation, clear differences in biomass production became evident. The highest biomass yield was recorded in Mandels medium (509 µg mL^{-1}), followed by Czapek broth (456 µg mL^{-1}). In contrast, lower biomass production was observed in Sabouraud broth (321 µg mL^{-1}) and PDB (338 µg mL^{-1}) media. These results indicate that the composition of the culture medium significantly influences the growth performance of *T. asperellum* Uz-A4 during prolonged cultivation.

Acid phosphatase activity of *T. asperellum* Uz-A4 in different culture media

The activity of acid phosphatase secreted into the culture filtrates of *T. asperellum* Uz-A4 varied depending on the nutrient medium used for cultivation. Although the strain produced comparable amounts of biomass in the different media, pronounced differences were observed in acid phosphatase activity levels in the corresponding culture filtrates.

The highest acid phosphatase activity (1534 µM min^{-1} mg^{-1}) was detected during the growth of *T. asperellum* Uz-A4 in Czapek broth, where enzyme activity reached its maximum on the sixth day of cultivation (Figure 1). In contrast, lower levels of enzyme activity were observed in Mandels, Sabouraud and PDB media throughout the cultivation period.

Based on these results, the culture filtrate obtained from *T. asperellum* Uz-A4 grown on Czapek broth was selected as the most suitable source of extracellular acid phosphatase for subsequent purification and characterization studies.

https://doi.org/10.35495/ajab.2026.050

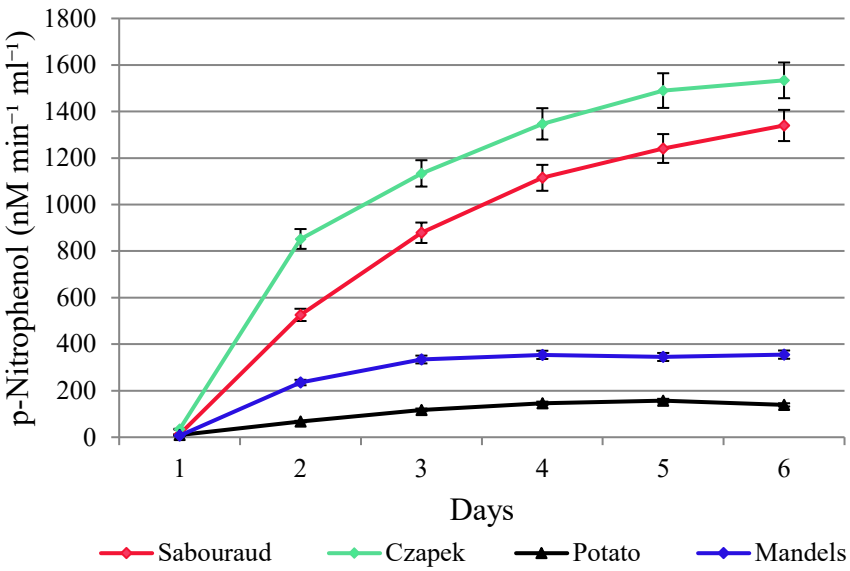

Figure-1. Acid phosphatase activity of *T. asperellum* Uz-A4 during cultivation in Mandels, Czapek, Sabouraud, and PDB media.

T. asperellum Uz-A4 strain, the culture fluid of the culture grown for 6 days on Czapek broth served as an acid phosphatase enzyme extract for further studies. Values represent mean ± SD (n = 3). Differences among treatments were evaluated using one-way ANOVA at $p < 0.05$.

Zymogram analysis of acid phosphatase isoforms in the culture filtrate of *T. asperellum* Uz-A4

Zymogram analysis of acid phosphatase activity in the culture filtrate of *T. asperellum* Uz-A4 was performed using native polyacrylamide gel electrophoresis (PAGE). Enzyme extracts exhibiting high phosphatase activity were subjected to electrophoresis on an 8% polyacrylamide gel. Following separation, enzymatic activity was visualized by incubating the gel at 45 °C for 30 min in a reaction mixture containing a specific substrate for acid phosphatase. As a result, two distinct activity bands were detected on the gel, indicating the presence of multiple extracellular acid phosphatase isoforms (Figure 2).

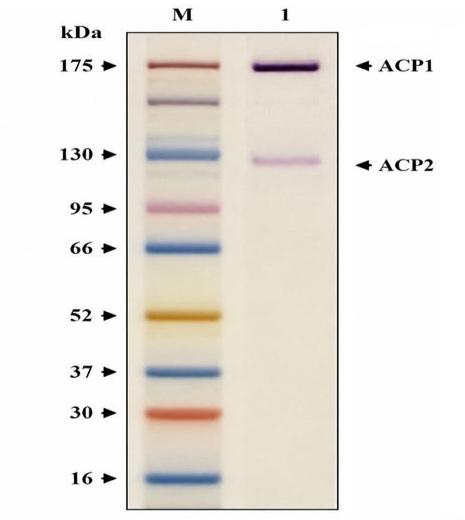

Figure-2. Zymogram analysis of extracellular acid phosphatase isoforms produced by *T. asperellum* Uz-A4.

https://doi.org/10.35495/ajab.2026.050

Zymogram analysis revealed that *T. asperellum* Uz-A4 produces at least two native acid phosphatase isoforms with markedly different molecular masses. The estimated molecular mass of the first isoform (ACP1) was approximately 175 kDa, whereas the second isoform (ACP2) had a molecular mass of about 115 kDa. The occurrence of multiple acid phosphatase isoforms has also been reported in other filamentous fungi.

Purification of acid phosphatase from *T. asperellum* Uz-A4

Previous studies have reported the purification of acid phosphatase enzymes from different *Trichoderma* species (Leitao et al., 2010). In the present study, extracellular acid phosphatase from *T. asperellum* Uz-A4 was purified using a multistep procedure involving ammonium sulfate precipitation, ion-exchange chromatography and hydrophobic interaction chromatography.

In the first purification step, proteins from the fungal culture filtrate were precipitated with solid ammonium sulfate at 80% saturation. This step resulted in an 8.48-fold increase in specific activity, with 57% recovery of the total acid phosphatase activity (Table 2). After precipitation, the total protein content was 4.36 mg, and the specific activity of acid phosphatase reached 3.05 μM min^{-1} mg^{-1}.

Table-2. Purification summary of extracellular acid phosphatase from *T. asperellum* Uz-A4.

Purificationstep	Total protein (mg)	Total activity (U)	Specificactivity (U mg^{-1})	Purificationfold	Yield (%)
Cell-free supernatant[a]	64,5	23,22	0,36	1	100
Ammonium sulfate precipitation[b]	4,36	13,30	3,05	8,48	57
DEAE–TSK anion-exchange chromatography[c]	0,27	7,54	27,51	76,43	32,5
Phenyl–Sepharose chromatography[d]	0,09	4,16	45,98	127,72	17,9

[a]Cell-free culture supernatant
[b] Protein fraction precipitated with 80% ammonium sulfate
[c] DEAE–TSK anion-exchange chromatography
[d] Hydrophobic interaction chromatography on Phenyl–Sepharose

Following dialysis, the ammonium sulfate–precipitated proteins were subjected to high-performance anion-exchange chromatography on a DEAE–TSK column. Acid phosphatase activity was eluted at a NaCl concentration of approximately 0.25 M (Figure 3). Enzymatic activity was detected in two fractions (HP1 and HP2); however, the fraction containing the dominant acid phosphatase isoform (ACP1) was selected for further purification. The elution profile suggests that ACP1 possesses a moderate content of negatively charged amino acid residues. After this step, a 76.43-fold purification was achieved, with 32.5% recovery of the total enzyme activity (Table 2).

https://doi.org/10.35495/ajab.2026.050

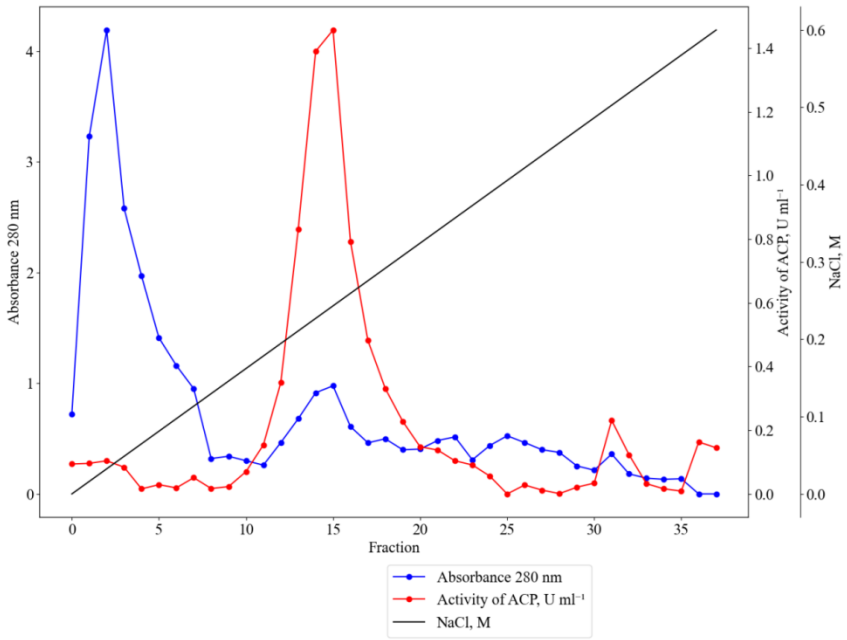

Figure-3. Ion-exchange chromatography of extracellular acid phosphatase from *T. asperellum* Uz-A4 on a DEAE-TSK column. The concentrated enzyme preparation was applied to a DEAE–TSK ion-exchange chromatography column (30 × 2.5 cm) previously equilibrated with 50 mM Tris–HCl buffer (pH 7.0). Bound proteins were eluted using a linear NaCl gradient ranging from 0 to 0.6 M. Fractions exhibiting high acid phosphatase activity were pooled and dialyzed against 20 mM Tris–HCl buffer (pH 7.0) for 24 h.

The active ACP1 fraction obtained after DEAE–TSK chromatography was further purified by hydrophobic interaction chromatography on a Phenyl–Sepharose column (Figure 4). The enzyme was eluted at an ammonium sulfate concentration of approximately 0.12 M. This final purification step resulted in a 127.72-fold increase in specific activity, with a recovery of 17.9% of the total activity. At this stage, the total protein content was reduced to 0.09 mg, while the specific activity of acid phosphatase increased to 45.98 µM min^{-1} mg^{-1}.

https://doi.org/10.35495/ajab.2026.050

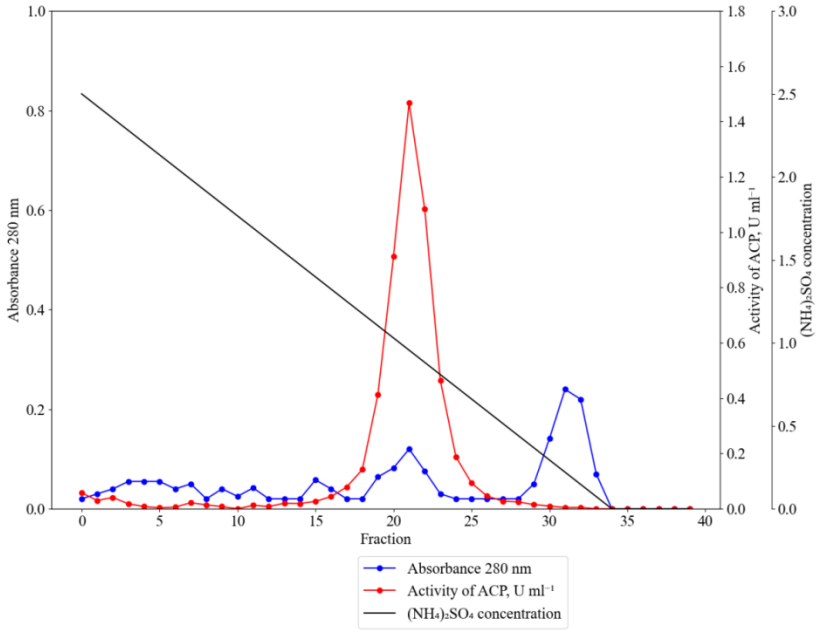

Figure-4. Hydrophobic interaction chromatography of ACP1 from *T. asperellum* Uz-A4 on a Phenyl–Sepharose column equilibrated with 50 mM Tris–HCl buffer (pH 7.0). Proteins were eluted using a decreasing ammonium sulfate gradient from 2.5 to 0.0 M.

Purity of the enzyme preparation was confirmed by SDS–PAGE analysis using a 12% polyacrylamide gel. The purified acid phosphatase migrated as a single protein band when 10 µg of protein was loaded, indicating homogeneity of the enzyme preparation (Figure 5).

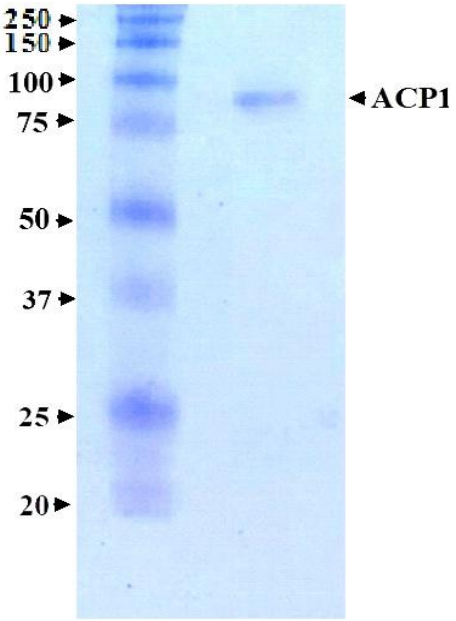

Figure-5. SDS–PAGE analysis of the purified acid phosphatase (ACP1) from *T. asperellum* Uz-A4. From left Lane 1, molecular weight markers; Lane 2, purified ACP1. Protein bands were stained with Coomassie Brillant blue R-250 solution.

https://doi.org/10.35495/ajab.2026.050

Based on comparison with molecular weight standards, the apparent molecular mass of the acid phosphatase monomer from *T. asperellum* Uz-A4 was estimated to be approximately 85 kDa. This value differs from a previously reported molecular mass of 57.8 kDafor acid phosphatase isolated from *T. harzianum* (Souza et al., 2016), suggesting possible strain-specific or isoform-related differences.The molecular mass value of *T. asperellum* Uz-A4 (85 kDa) is close to that of ACPase II (90 kDa) from the fungus *T. harzianum*.

Physicochemical properties of acid phosphatase (ACP1) from *T. asperellum* Uz-A4

The time dependence of ACP1 activity was examined using *p*-nitrophenyl phosphate as the substrate. As shown in Figure 6, the amount of *p*-nitrophenol released increased progressively with incubation time over the entire reaction period. Enzyme activity increased almost linearly during the initial 60 min of incubation, indicating that the reaction rate remained constant under the applied assay conditions and that substrate depletion did not occur during this time interval.

The effect of pH on ACP1 activity is presented in Figure 7. The enzyme exhibited maximal activity within a relatively narrow pH range of 5.0–5.5. At pH values below 4.5 and above 6.0, enzyme activity declined markedly, indicating strong pH dependence of catalytic performance.

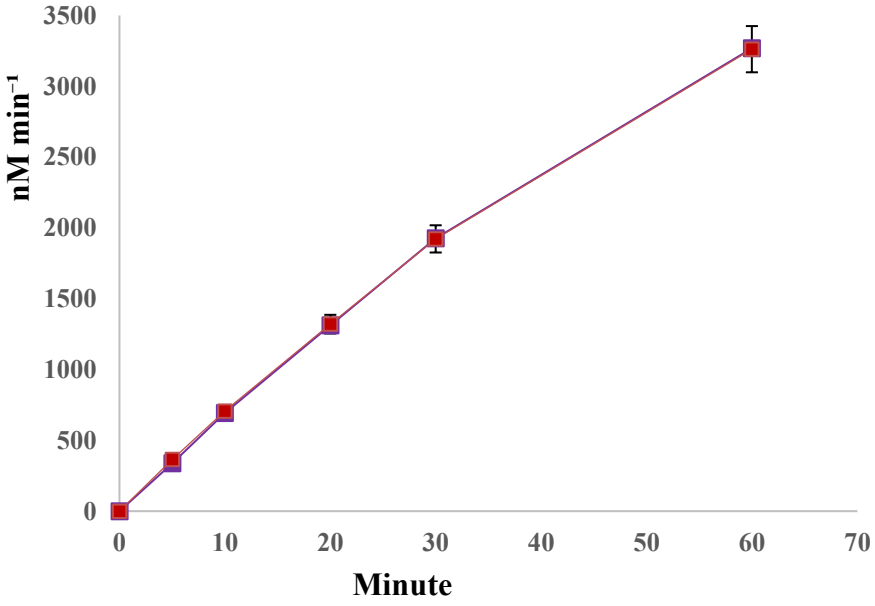

Figure-6. Time-dependent activity of acid phosphatase (ACP1) from *T. asperellum* Uz-A4 using p-nitrophenyl phosphate as substrate. Values represent mean ± SD (n = 3).

https://doi.org/10.35495/ajab.2026.050

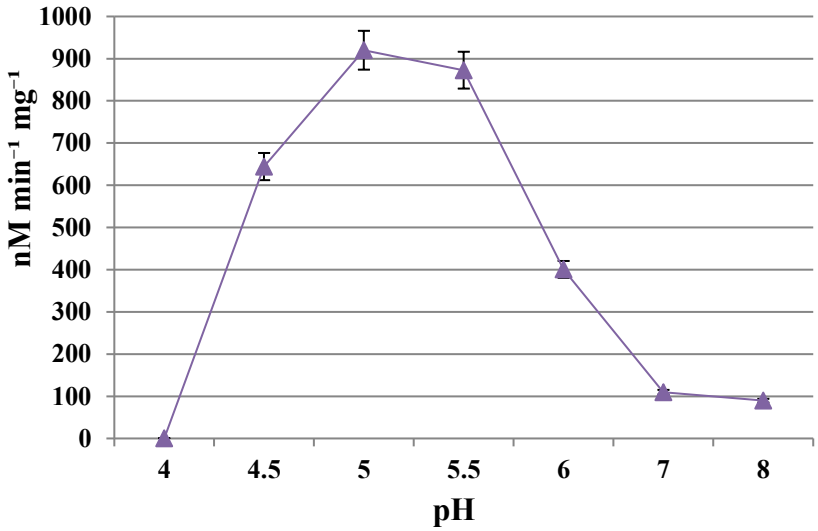

Figure-7. Effect of pH on acid phosphatase (ACP1) activity from *T. asperellum* Uz-A4. Optimum pH was estimated using 100 mM acetate buffer over a pH range of 4.0-8.0. Values represent mean ± SD (n = 3).

Temperature dependence analysis demonstrated that ACP1 activity increased with rising temperature up to an optimum range of 45–50 °C (Figure 8). Beyond this range, enzyme activity decreased rapidly, and only residual activity was detected at 60 °C. At 70 °C, ACP1 activity was almost completely lost.

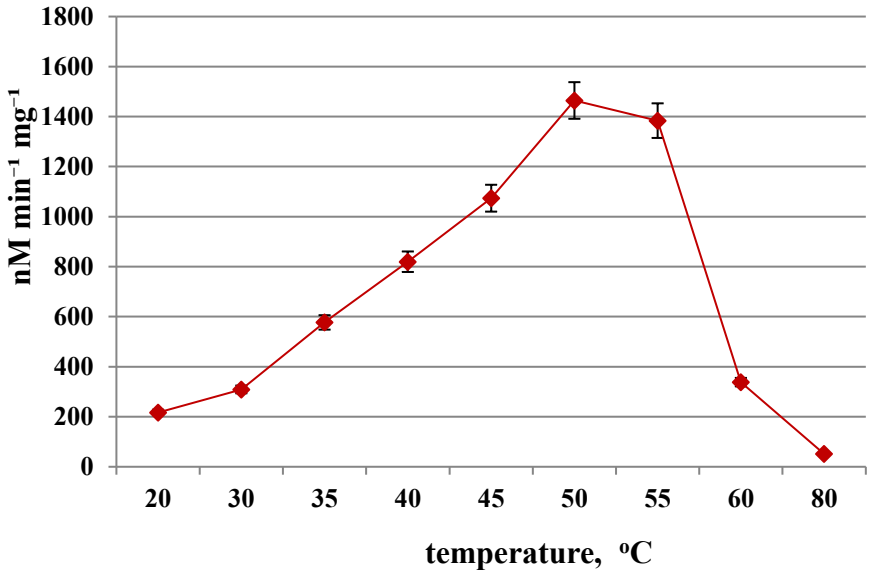

Figure-8. Effect of temperature on acid phosphatase (ACP1) activity from *T. asperellum* Uz-A4. Optimal temperature of 50°C was estimated from 20 to 80°C under standard assay conditions and 100 mM sodium acetate buffer pH 5.5 was used.Values represent mean ± SD (n = 3).

Thermostability of ACP1 was evaluated by monitoring residual activity after incubation at different temperatures for various time intervals (Figure 9). The enzyme retained 96–100% of its initial activity after incubation at 50 and 60 °C for 60 min. In contrast, incubation at 70 °C for 30 min resulted in a reduction of residual activity to approximately 42%. Further increases in temperature led to a pronounced loss of activity, with only 14% and 2% of the initial activity remaining after incubation at 75 and 80 °C, respectively.

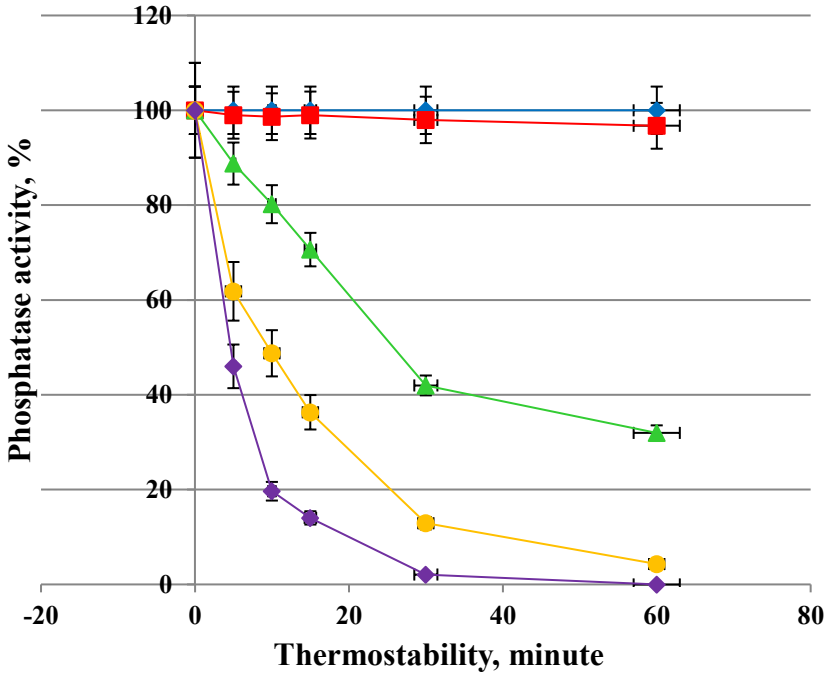

Figure-9. Thermostability of acid phosphatase (ACP1) from *T. asperellum* Uz-A4 at different temperatures over time. The enzyme was pre-incubated at 50, 60,70 and 80°C in 100 mM sodium acetate buffer (pH 3.8) for 10 min, and residual activity was measured at the indicated time points. Values represent mean ± SD (n = 3).

Substrate concentration dependence of ACP1 activity is shown in Figure 10. Enzyme activity increased with increasing *p*-nitrophenyl phosphate concentration and approached saturation at higher substrate concentrations, indicating Michaelis–Menten-type kinetics.

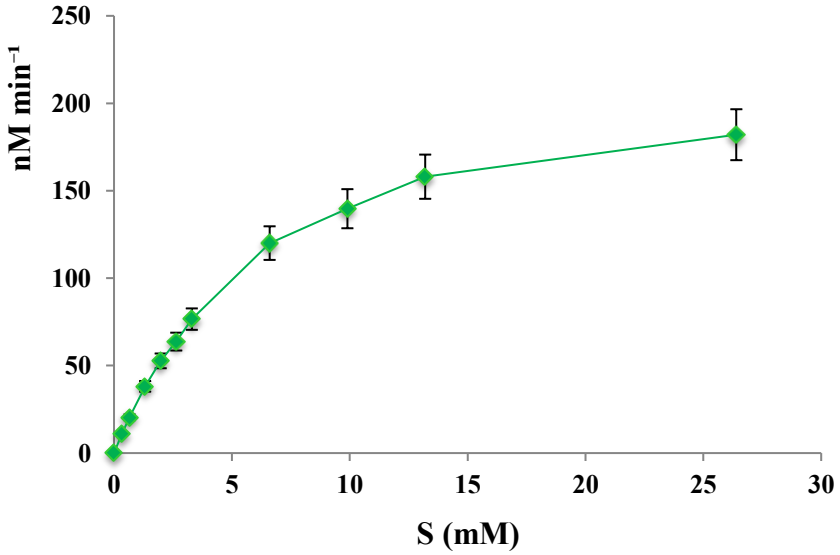

Figure-10. Dependence of acid phosphatase (ACP1) activity from *T. asperellum* Uz-A4 on ***p***-nitrophenyl phosphate concentration, presented in Michaelis–Menten coordinates. Values represent mean ± SD (n = 3).

https://doi.org/10.35495/ajab.2026.050

Lineweaver–Burk transformation of the kinetic data allowed estimation of the kinetic parameters. The Michaelis constant (K_m) was determined to be 33 nM, while the maximum reaction velocity (V_{max}) reached 218 nM/min.These results were similar to the respective $K_m$and V_{max} values 165 nM and 237 nM min-1 for acid phosphatase from *Trichoderma Harzianum* (Leitao et al., 2010) andsimilar to $K_m$results for ACPase II from *T.harzianum*(Souza et al., 2016). Chinese researchers purified acid phosphatase from *Trichoderma asperellum* Q1, but did not study the enzymatic properties of the enzyme (Lei et al., 2017). The effects of various metal ions and inhibitors on ACP1 activity are summarized in Figure 11.

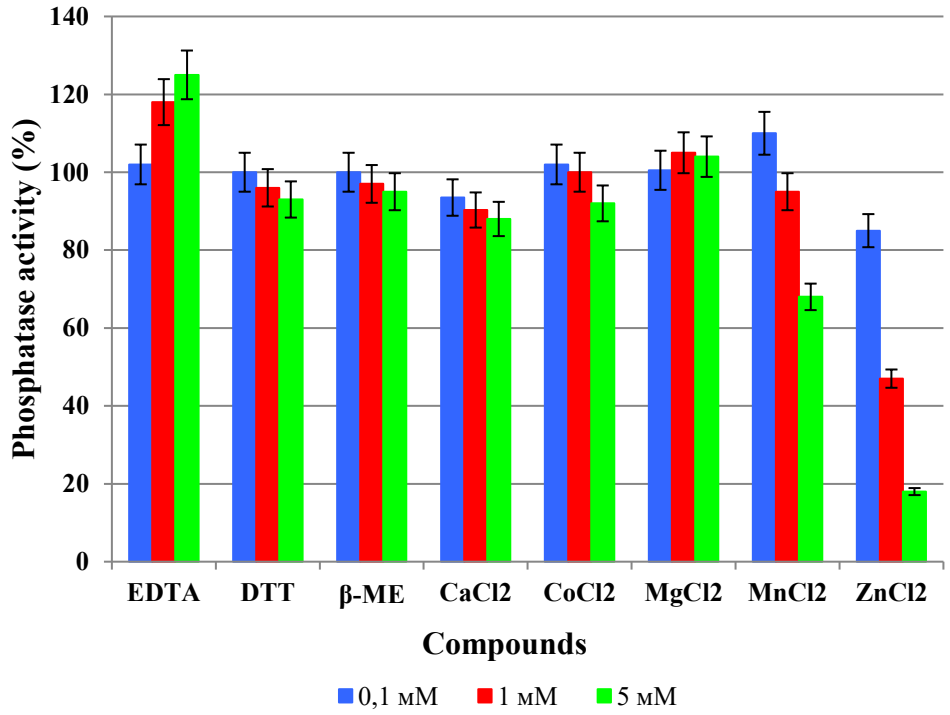

Figure-11. Effect of various metal ions and inhibitors on acid phosphatase (ACP1) activity from *T. asperellum* Uz-A4.

At a concentration of 0.1 mM, most tested compounds had no pronounced effect on enzyme activity. Increasing the concentration to 1.0 and 5.0 mM resulted in differential effects. Mn^{2+} and Zn^{2+} caused a strong inhibition of ACP1 activity, whereas Ca^{2+}, Co^{2+} and Mg^{2+} showed only minor effects. Reducing agents (DTT and β-mercaptoethanol) and EDTA did not substantially affect enzyme activity across the tested concentration range.

Effect of inhibitors, reducing agents and metal ions on acid phosphatase (ACP1) activity

The effects of various metal ions, reducing agents and inhibitors on the activity of acid phosphatase (ACP1) from *T. asperellum* Uz-A4 are shown in Figure 12. Enzyme activity was examined in the presence of Zn^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+} and Mg^{2+} ions at concentrations of 0.1, 1.0 and 5.0 mM.

Zinc ions caused a pronounced concentration-dependent inhibition of ACP1 activity. At the highest tested concentration (5 mM), Zn^{2+} markedly reduced enzyme activity. Manganese ions also inhibited ACP1 activity, resulting in a 32% decrease at a concentration of 5 mM.

In contrast, Ca^{2+}, Co^{2+} and Mg^{2+} ions had little effect on ACP1 activity. In some cases, a slight increase in enzymatic activity (up to approximately 10%) was observed in the presence of these ions. Reducing agents, including dithiothreitol (DTT) and β-mercaptoethanol, did not significantly affect enzyme activity across the tested concentration range.

The metal chelator ethylenediaminetetraacetic acid (EDTA) at concentrations of 1.0 and 5.0 mM caused a

slight increase in ACP1 activity, indicating that divalent metal ions are not strictly required for catalytic function under the assay conditions.

Discussion

The present study demonstrates that *T. asperellum* Uz-A4 is capable of producing a thermostable extracellular acid phosphatase with distinct physicochemical properties, highlighting its potential role in phosphorus mobilization processes relevant to sustainable soil fertility management.

The role of microbial phosphatases in phosphorus mobilization is well documented, particularly in soil systems where organic phosphorus requires enzymatic mineralization (García-Berumen et al., 2025). In this context, the activity observed for *T. asperellum* Uz-A4 is consistent with previously reported functions of phosphate-mobilizing microorganisms.

Although biomass production of *T. asperellum* Uz-A4 did not differ markedly among the tested culture media during the early stages of growth, pronounced differences were observed in acid phosphatase activity in the corresponding culture filtrates. This observation indicates that extracellular enzyme production is not directly correlated with fungal biomass accumulation but is strongly influenced by the composition of the nutrient medium. In particular, Czapek broth promoted the highest acid phosphatase activity, suggesting that defined mineral media may favor the induction of phosphatase synthesis over complex organic substrates. Similar patterns have been reported for other phosphate-mobilizing fungi, where enzyme secretion is regulated primarily by nutrient availability rather than growth intensity (Midgley and Phillips, 2019; Zhang et al., 2023; Brazhnikova et al., 2022).

Zymogram analysis revealed the presence of two extracellular acid phosphatase isoforms with molecular masses of approximately 175 and 115 kDa. The occurrence of multiple phosphatase isoforms has been widely documented in filamentous fungi and higher plants and is commonly associated with functional adaptation to varying environmental conditions, including changes in pH and substrate availability (Della Mónica et al., 2018). For example, *Aspergillus caespitosus* was shown to produce acid phosphatases with molecular masses of 186 and 190 kDa (Guimara˜es et al., 2004), while *Serendipita indica* and *Metarhizium anisopliae* were reported to possess two extracellular acid phosphatase isoforms (Kushwaha and Kumar, 2022; Li et al., 2007). In higher plants, the number of extracellular acid phosphatase isoforms may be even greater, with up to five or six isoforms described in different species (Malboobi et al., 2022).

Purification of the dominant isoform (ACP1) resulted in a 127.7-fold increase in specific activity, confirming the effectiveness of the applied multistep purification strategy. SDS–PAGE analysis showed that ACP1 consists of identical subunits with an apparent molecular mass of 85 kDa. This value differs from previously reported molecular masses of acid phosphatases isolated from other *Trichoderma* strains, which may reflect strain-specific structural differences or the presence of distinct isoenzymatic forms, a characteristic feature of fungal phosphatases (Leitao et al., 2010).

The optimal pH and temperature observed for ACP1 are within the range reported for fungal phosphatases, which are typically active under moderately acidic conditions (Poussereau et al., 2001).

Physicochemical characterization demonstrated that ACP1 exhibited optimal enzymatic activity at pH 5.0–5.5 and at temperatures of 45–50 °C. These conditions are compatible with many soil environments, particularly within the rhizosphere, where microbial activity and organic acid production often lead to localized pH reductions. Comparable pH optima have been reported for acid phosphatases from *Penicillium chrysogenum*, *Metarhizium anisopliae* and *Aspergillus niger*, whereas enzymes from *Aspergillus ficuum* and *Aspergillus fumigatus* display optimal activity under more acidic conditions (Vera-Morales et al., 2023; Wyss et al., 1998). Similarly, optimal temperatures reported for acid phosphatases from *Mucor hiemalis* and *Aspergillus niger* are higher than those observed in the present study, while enzymes from *Metarhizium anisopliae* and *Aspergillus caespitosus* exhibit even greater thermal optima (Gebremariam et al., 2022; Gargova et al., 2006).

Species of the genus *Trichoderma* are known to contribute to phosphorus availability through enzymatic activity and organic acid production (Liu et al., 2020), which supports the observed phosphatase activity in the present study.

A notable feature of ACP1 is its high thermostability. The enzyme retained nearly full activity after prolonged incubation at 50–60 °C and maintained measurable activity even at higher temperatures. Such thermostability may provide an ecological advantage by enabling sustained phosphorus mineralization under fluctuating environmental conditions and during

prolonged cultivation periods, as observed in the present study.

Kinetic analysis revealed relatively high K_m and high V_{max} values for ACP1 when *p*-nitrophenyl phosphate was used as the substrate, indicating moderate substrate affinity and high catalytic efficiency. These kinetic properties are characteristic of enzymes involved in the hydrolysis of organic phosphorus compounds and support the functional role of ACP1 in efficient phosphorus release from organic sources (Zaman et al., 2023).

Although no soil-based or plant growth experiments were conducted in this study, previous research has demonstrated that microbial phosphatases contribute to phosphorus mobilization in the rhizosphere (Senkovs et al., 2021).

These results are consistent with previous reports indicating thatmicrobial extracellular enzymes play a key role in nutrient cycling and soil fertility (Luo et al., 2017).

Taken together, these results suggest that efficient extracellular secretion, favorable kinetic parameters and pronounced thermostability suggests that the acid phosphatase produced by *T. asperellum* Uz-A4 plays an important role in phosphorus cycling. From an agronomic perspective, these characteristics support the potential application of this strain, or its enzyme, in the development of biofertilizers aimed at improving phosphorus availability and restoring fertility in degraded or nutrient-limited soils.

Conclusions

In the present study, Czapek broth was identified as the most suitable for the production and purification of extracellular acid phosphatase from *T. asperellum* Uz-A4. Zymogram analysis of the culture filtrate revealed the presence of two native acid phosphatase isoforms with molecular masses of approximately 175 and 115 kDa. The dominant isoform (ACP1), purified to homogeneity by ion-exchange and hydrophobic chromatography, appeared as a single protein band with an apparent molecular mass of 85 kDa on 12% SDS–PAGE, indicating that the native enzyme consists of two identical subunits.

The purified ACP1 exhibited optimal catalytic activity at pH 5.0 and 50 °C and demonstrated high thermostability, retaining full activity after incubation at 60 °C for 1 h. Enzyme activity was strongly inhibited by Zn^{2+} and Mn^{2+} ions, whereas Ca^{2+}, Co^{2+} and Mg^{2+}ions, as well as reducing agents (DTT and β-mercaptoethanol) and EDTA, had no significant effect on catalytic performance.

The physicochemical properties of ACP1 suggest that this enzyme is well adapted to soil and rhizosphere conditions, where moderately acidic pH and fluctuating temperatures prevail. The ability of *T. asperellum* Uz-A4 to produce a stable extracellular acid phosphatase capable of hydrolyzing organic phosphorus compounds highlights its potential role in phosphorus mobilization. From an agronomic perspective, the application of this strain may have potential to contribute to improving phosphorus availability in soils, enhancing plant growth and productivity, and supporting environmentally sustainable agricultural practices.

Acknowledgements

This work was funded and carried out at the Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan, under the main scientific project.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: This work was funded and carried out at the Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan, under the main scientific project.

Contribution of Authors

Shakirov Z: Conceived and designed the study, supervised the experimental work, and critically revised the manuscript.

Karimov H: Performed the enzyme purification experiments and biochemical analyses.

Yakubov I: Contributed to data analysis and interpretation of results.

Khamidova K & Zakiryaeva S: Carried out cultivation experiments and enzyme activity assays.

Azimova N: Assisted in statistical analysis and figure preparation.

Kurganov S & Gao C: Contributed to manuscript editing and literature review.

All authors read and approved the final version of the manuscript.

References

Azimova NS, Khamidova KM, Turaeva BI, Karimov HK and Shakirov ZS, 2020. Properties of the celllase and xylanase enzyme complexes of *Trichoderma harzianum* UzCF-28. *EurAsian Journal of Biosciences, 2020, Vol 14, Issue 2, p5803*

Bononi L, ChiaramonteJB, Pansa CC, Moitinho MA and Melo IS, 2020. Phosphorus-solubilizing *Trichoderma* spp. from Amazon soils improve soybean plant growth. Scientific Reports 10:2858https://doi.org/10.1038/s41598-020-59793-8

Brazhnikova YV, Shaposhnikov AI, Sazanova AL, Belimov AA, Mukasheva TD and Ignatova LV, 2022. Phosphate mobilization by culturable fungi and their capacity to increase soil p availability and promote barley growth. *Current microbiology*, *79*(8), 240.https://doi.org/10.1007/s00284-022-02926-1

Della Mónica IF, Godoy MS, Godeas AM and Scervino JM, 2018. Fungal extracellular phosphatases: their role in P cycling under different pH and P sources availability. Journal of Applied Microbiology, *124*(1), 155-165.

Gadallah AA, El-Ghawet HA and Sabry DA, 2017. Deca Durabolin (*Nandrolone Decanoate*) impair structure and function of liver, prostate and pituitary of Male Wistar Rats. J Pharma Reports 2: 137.

García-Berumen JA, de la Torre JAF, de Los Santos-Villalobos S, Espinoza-Canales A, Echavarría-Cháirez FG and Gutiérrez-Bañuelos H, 2025. Phosphorus dynamics and sustainable agriculture: The role of microbial solubilization and innovations in nutrient management. Current Research in Microbial Sciences, 8, 100326.

Gargova S, Sariyska M, Angelov A and Stoilova I, 2006. Aspergillus niger pH 2.1 optimum acid phosphatase with high affinity for phytate. Folia Microbiol. (Praha). 51(6):541-555. doi: 10.1007/BF02931618.

Gebremariam A, Chekol Y and Assefa F, 2022. Extracellular enzyme activity of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* and their pathogenicity potential as a bio-control agent against whitefly pests, Bemisiatabaci and Trialeurodesvaporariorum (Hemiptera: Aleyrodidae). *BMC research notes*, *15*(1), 117.

Guimara˜es LHS, Terenzi HF, Jorge JA, Leone JA and Polizeli MDTM, 2004. Characterization and properties of acid phosphatases with phytase activity produced by *Aspergillus caespitosus*. Biotechnol Appl Biochem, 40:201–207.

Jawad M, Jabeen A, Gurmani AR and Rafique M, 2023. Phosphate-mobilizing Mycorrhizal-based Bio-fertilizers. In *Biofertilizers for Sustainable Soil Management* (pp. 89-106). CRC Press.

Karimov H, Azimova N, Khaytbayeva N, Kobilov F, Khamidova K, Shukurov O and Razzokov J, 2024. Isolation of antagonistic bacterial strains against fungi. Regulatory Mechanisms in Biosystems, 15(4), 782-788.

Khan A, Siddique AB, Shabala S, Zhou M and Zhao C, 2023. Phosphorus Plays Key Roles in Regulating Plants' Physiological Responses to Abiotic Stresses. *Plants,12*(15), 2861; https://doi.org/10.3390/plants12152861

Kushwaha AS and Kumar M, 2022. An effective in-gel assay protocol for the assessment of acidphosphatase (ACPase) isoform expression in the fungus *Serendipita indica.* Biotech 12:40. https://doi.org/10.1007/s13205-021-03103-0

Landa-Acuña D, Toro M,Santos-Mendoza R and Zúñiga-Dávila D, 2023. Role of *Rahnella aquatilis* AZO16M2 in Phosphate Solubilization and Ex Vitro Acclimatization of Musa acuminata var. Valery. Microorganisms 11, 1596. https://doi.org/10.3390/microorganisms11061596

Lei Z, Qun L, Ya-qing Z, Qing-yu C and Yuan-cun L, 2017. Effect of acidphosphatase produced by *Trichoderma asperellum* on growth of *Arabidopsis* under salt stress. Journal of Integrative Agriculture 16(6): 1341–1346

Leitao VO, Lima RCM, Vainstein MH and Ulhoa CJ, 2010. Purification and characterization of an acid phosphatasefrom*Trichodermaharzianum.* BiotechnolLett 32:1083–1088.

Li Z, Wang Z, Peng G, Yin Y, Zhao H, Cao Y and Xia Y, 2007. Regulation of extracellular acid phosphatase biosynthesis by culture

conditions in entomopathogenic fungus *Metarhizium anisopliae* strain CQMa102. Annals of Microbiology 2007. 57 (4) 565-570.

Liu Q, Meng X, Li T, Raza W, Liu D and Shen Q, 2020. The growth promotion of peppers (Capsicum annuum L.) by *Trichoderma guizhouense* NJAU4742-based biological organic fertilizer: Possible role of increasing nutrient availabilities. Microorganisms, 8(9), 1296.

Luo L, Meng H and Gu JD, 2017. Microbial extracellular enzymes in biogeochemical cycling of ecosystems. Journal of Environmental Management, 197, 539-549.

Malboobi MA, Sabet MS, Zamani K, Lohrasebi T, Fathi Z and Zamani J, 2022. Acid Phosphatases Roles in Plant Performance. In *Agricultural Biocatalysis* (pp. 117-157). Jenny Stanford Publishing.

Midgley MG and Phillips RP, 2019. Spatio-temporal heterogeneity in extracellular enzyme activities tracks variation in saprotrophic fungal biomass in a temperate hardwood forest. *Soil Biology and Biochemistry*, *138*, 107600.

Mishra S, Levengood H, Fan J and Zhang C, 2024. Plants under stress: exploring physiological and molecular responses to nitrogen and phosphorus deficiency. Plants, 13(22), 3144.

Mwende ME, 2019. Understanding soil phosphorus. International Journal of Plant and Soil Science 31(2), 1-18. DOI:10.9734/IJPSS/2019/v31i230208

Odoh CK, Eze CN, Obi CJ, Anyah F, Egbe K, Unah UV and Adobu US, 2020. Fungal biofertilizers for sustainable agricultural productivity. In *Agriculturally Important Fungi for Sustainable Agriculture: Volume 1: Perspective for Diversity and Crop Productivity* (pp. 199-225). Cham: Springer International Publishing.

Pang F, Li Q, Solanki MK, Wang Z, Xing YX and Dong DF, 2024. Soil phosphorus transformation and plant uptake driven by phosphate-solubilizing microorganisms. Frontiers in Microbiology, 15, 1383813.

Poussereau N, Creton S, Billon-Grand G, Rascle C and Fevre M, 2001. Regulation of acp1, encoding a non-aspartyl acid protease expressed during pathogenesis of Sclerotinia sclerotiorum. Microbiology, 147(3), 717-726.

Pratibha R, Sudeshna D, Deepti S and Shankhdhar SC, 2021. Phosphate-solubilizing microorganisms: mechanism and their role in phosphate solubilization and uptake. *Journal of Soil Science and Plant Nutrition* 21.1 49-68.

Rejsek K, Vranova V, Pavelka M and Formanek P, 2012. Acid phosphomonoesterase (EC 3.1. 3.2) location in soil. *Journal of Plant Nutrition and Soil Science*, *175*(2), 196-211.

Senkovs M, Nikolajeva V, Makarenkova G and Petrina Z, 2021. Influence of *Trichoderma asperellum* and *Bacillus subtilis* as biocontrol and plant growth promoting agents on soil microbiota. Annals of microbiology, 71(1), 34.

Shakirov ZS, Mamanazarova KS, Yakubov IT, Zakiryaeva SI and Khamidova KM, 2022. Nitrogen-fixing, phosphate-potassium-mobilizing ability of *Rahnella* bacteria isolated from wheat roots. Regulatory Mechanisms in Biosystems, 13(4), 379-384.

Shavkiev J, Karimov HK, Turaeva BI, Azimova NS, Nazirbekov NK and Khamidova KM, 2022. Some volatile metabolites produced by the antifungal-*Trichoderma asperellum* UZ-A4 micromycete. (2022): International Journal of Phytopathology, 2022, Vol. 11, No. 3, 239-251 ref.

Souza AA, Leitão VO, Ramada MH, Mehdad A, Georg RDC, Ulhoa CJ and Freitas SMD, 2016. *Trichoderma harzianum* produces a new thermally stable acid phosphatase, with potential for biotechnological application. PLoS One, 11(3), e0150455.

Tian J, Ge F, Zhang D, Deng S and Liu X, 2021. Roles of phosphate solubilizing microorganisms from managing soil phosphorus deficiency to mediating biogeochemical P cycle. Biology, 10(2), 158.

Turaeva BI, Soliev AB, Karimov HK, Azimova NS, Kutlieva GJ, Khamidova KM and Zuxritdinova NY, 2021. Disease causing phytopathogenic micromycetes in citrus in Uzbekistan. Pakistan Journal of Phytopathology, 2021, Vol 33, Issue 2, p383

Vera-Morales M, López Medina SE, Naranjo-Morán J, Quevedo A, Ratti MF, 2023. Nematophagous fungi: A review of their phosphorus solubilization potential. Microorganisms, *11*(1), 137.

Wang S, Walker R, Schicklberger M, Nico PS, Fox PM, Karaoz U, Chakraborty R, Wang Y, Luo D, Xiong Z, Wang Z and Gao M, 2023. Changes in rhizosphere phosphorus fractions and phosphate-mineralizing microbial populations in acid soil as influenced by organic acid exudation. Soil and Tillage Research, *225*, 105543.

Wyss M, Pasamontes L, Rémy R, Kohler J, Kusznir E, Gadient M and van Loon AP, 1998. Comparison of the thermostability properties of three acid phosphatases from molds: Aspergillus fumigatus phytase, A. niger phytase, and A. niger pH 2.5 acid phosphatase. Applied and environmental microbiology, 64(11), 4446-4451.

Zaman U, Khan SU, Hendi AA, Ur Rehman K, Badshah S, Refat MS and Wahab A, 2023. Kinetic and thermodynamic studies of novel acid phosphatase isolated and purified from Carthamus oxyacantha seedlings. International journal of biological macromolecules, 224, 20-31.

Zhang J, Zhao Y, He H, Wang H and Wu F, 2023. Extracellular Enzyme Activity and Nutrient Characteristics of Pinus massoniana Lamb. Families with Different Growth Levels: Insights into the Ectomycorrhizal Fungal Community and Rhizosphere Soil. Forests, 14(7), 1447.

https://doi.org/10.35495/ajab.2026.050