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ISOLATION OF ASPERGILLUS STRAINS FROM WASTEWATER-IMPACTED SOILS AND OPTIMIZATION OF ACID PHOSPHATASE PRODUCTION

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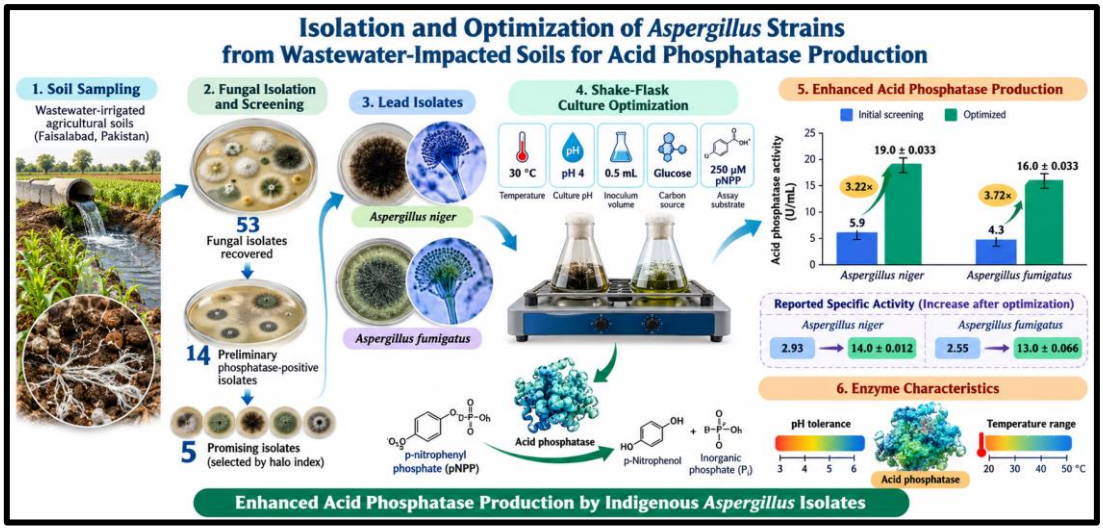
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Abstract

Wastewater-impacted agricultural soils may harbor fungi capable of producing extracellular phosphatases that participate in organic phosphorus turnover. This study screened indigenous fungal isolates recovered from agricultural soils irrigated with untreated wastewater in the Faisalabad region of Pakistan and evaluated shake-flask culture variables affecting acid phosphatase activity. Of 53 recovered isolates, 14 were described as preliminary phosphatase-positive and five candidates were prioritized by a plate-based halo index. The leading isolates were morphologically assigned to Aspergillus niger and Aspergillus fumigatus. Assays using p-nitrophenyl phosphate indicated initial acid phosphatase activities of 5.9 and 4.3 U/mL, respectively. Following the reported optimization procedures, these values increased to 19.0 ± 0.033 and 16.0 ± 0.033 U/mL, equivalent to approximately 3.22- and 3.72-fold increases. The experiments identified 250 µM substrate, an inoculum volume of 0.5 mL, pH 4, and 30 °C as favorable settings, with glucose supporting activity; however, the source contains conflicting descriptions of the optimal incubation period and nitrogen supplement. Crude preparations retained measurable activity across an acidic pH interval and a broad temperature range, with altered activity in the presence of several metal ions and inhibitors. These results support further evaluation of indigenous Aspergillus isolates as acid phosphatase sources, while molecular identification, assay normalization, reproducibility, and application-level validation remain necessary.

Graphical Abstract



INTRODUCTION

Phosphorus is essential for plant growth, yet much of the phosphorus in agricultural soils exists in forms that are not immediately accessible to roots. Soil microorganisms influence phosphorus cycling through organic-acid production, mineral dissolution, and extracellular enzymatic hydrolysis. Acid phosphatases (EC 3.1.3.2) release inorganic phosphate from suitable phosphomonoester substrates under acidic reaction conditions; they should be distinguished from phytases, which specifically act on phytate and require a phytate-based assay for direct activity claims (Tarafdar & Chhonkar, 1979; Kushwaha & Kumar, 2022).

Fungal communities, including members of Aspergillus and Penicillium, are known contributors to extracellular phosphatase production. Local isolation is attractive because strains recovered from cultivated or wastewater-influenced environments may be suited to the nutrient profiles and physicochemical conditions encountered there. However, environmental provenance alone does not establish pollutant tolerance, remediation capacity, or application safety. Such functions must be demonstrated by independent tests (Sadaf et al., 2022; Zafar et al., 2026).

Untreated and reclaimed wastewater can modify soil nutrient dynamics and microbial communities. Its mixed contaminants also create a need for cautious interpretation of any organisms isolated from these settings. Recent work on Aspergillus-associated dye degradation emphasizes the broader biotechnological potential of filamentous fungi, whereas reviews of microbial biodegradation and water reuse highlight the need to separate laboratory enzyme-production findings from direct environmental performance claims (Ghafoor et al., 2024; Sial et al., 2025; Zafar et al., 2026). Sustainable biotechnological uses of environmental microorganisms have also been considered in the wider context of resource recovery and environmental health (Javed et al., 2024; Shafiq et al., 2025).

The present work aimed to recover indigenous fungi from wastewater-impacted agricultural soils, rank isolates through preliminary plate screening, examine acid phosphatase activity in crude fungal preparations, and identify culture variables associated with improved enzyme output. The study specifically describes two high-performing morphotypes as A. niger and A. fumigatus. The results are presented as an exploratory isolation and optimization study rather than evidence of field-scale phosphorus mobilization or wastewater treatment.

2. Materials and Methods

2.1. Sampling sites and soil collection

Soil samples were obtained from agricultural land influenced by wastewater irrigation in the Chakara/Chakra area near Faisalabad, Punjab, Pakistan. The original records describe collections during the October–December periods of 2017–2019. Samples were taken at approximately 20 cm depth, with three samples reported per sampling location, and transferred in sterile containers for processing at the

Plant–Microbe Interactions Laboratory, Department of Botany, Government College University Faisalabad. The exact number of sites, the sampling coordinates, the number of distinct collection events, and whether the three samples represented biological replicates were not specified in the supplied manuscript.

2.2. Isolation, purification, and presumptive identification

Fungi were recovered using soil-dilution and soil-culture plate procedures. Growth and purification were conducted on potato dextrose agar (PDA); the source describes incubation of morphology plates for approximately seven days at 37 °C. Repeated subculturing was used to obtain visually pure colonies. Colony traits (pigmentation, growth form, and surface characteristics) and microscopic features (hyphae, septation, conidiophores, and conidial morphology) were examined on stained mounts under light microscopy. Taxonomic names were assigned from morphological keys and comparative descriptions. In the absence of an ITS, calmodulin, or β-tubulin sequence report, species assignments should be regarded as provisional morphological identifications, particularly within species-complex genera such as Aspergillus.

2.3. Preliminary phosphatase screening

Purified isolates were assessed in a plate-based screen reported to employ Pikovskaya (PVK) agar amended with 0.5% p-nitrophenyl phosphate (pNPP). Plates were incubated at approximately 32 °C for seven days. The ratio of the halo-related diameter to colony diameter was described as a solubilization index (SI). Because pNPP hydrolysis and dissolution of insoluble mineral phosphate are distinct processes, the SI values are retained here as screening indices rather than direct proof of mineral-phosphate solubilization. Five isolates displayed relatively high indices and were advanced for culture optimization.

2.4. Shake-flask cultures and optimization design

Selected fungal isolates were cultured in shake flasks while varying incubation duration, test substrate, substrate concentration, inoculum volume, culture temperature, initial pH, and carbon and nitrogen supplements. The incubation-time series was described as 3, 5, 7, 9, and 11 days. Concentrations of pNPP investigated were approximately 50–400 μM; environmental or culture temperature assessments were described over approximately 10–50 °C, and the production-pH screen focused on an acidic range. Carbon supplements included glucose, maltose, xylose, galactose, fructose, sucrose, starch, and lactose. The nitrogen-source screen included ammonium nitrate, yeast extract, casein, sodium nitrate, ammonium sulfate, urea, and ammonium chloride. A complete fixed medium recipe was not included, flask working volume, agitation speed, inoculum spore concentration, or whether factor effects were assessed one-at-a-time or in a controlled factorial arrangement; these values must be recovered from laboratory records before independent reproduction.

2.5. Acid phosphatase activity and specific activity

The enzyme assay was described using pNPP as a chromogenic phosphomonoester substrate, with absorbance measured at 420 nm. Hydrolysis of pNPP yields p-nitrophenol, enabling spectrophotometric activity estimation when a suitable product calibration curve and reaction conditions are specified (Kushwaha & Kumar, 2022). The assay report contains a calibration plot (R^2 = 0.9745) but describes pNPP concentrations as standards even though the plot is labeled for p-nitrophenol (pNP). This discrepancy prevents independent confirmation of molar product formation. Accordingly, the activity values are preserved exactly as reported (U/mL), without recalculation. The original notation for specific activity is also ambiguous (U/mL per mg protein), and no independently verifiable assay-volume, reaction-time, or protein-normalization workflow was provided.

2.6. pH, thermal, metal-ion, and inhibitor responses

Crude enzyme preparations were examined for activity under different pH and temperature conditions and in the presence of selected added salts and chemical inhibitors. The experiment recorded measurable activity at 20–50 °C and pH 3–6, as well as responses to Mg^{2+}, Ca^{2+}, Cu^{2+}, EDTA, Ba^{2+}, Ni^{2+}, and Fe^{2+}.

Because the source does not distinguish clearly between pre-incubation stability and direct activity under assay conditions, these experiments are described as pH/temperature responses rather than definitive thermostability or storage-stability determinations. Metal and inhibitor concentrations and appropriate no-additive controls must be confirmed.

2.7. Data reporting and statistical considerations

Values with reported uncertainty are transcribed from the submitted manuscript. The type of error term, sample size per comparison, and definition of an independent biological replicate were not consistently specified. Multiple supplied ANOVA tables contain discrepancies among degrees of freedom, sums of squares, mean squares, and F statistics; therefore, no unverified p values or inferential significance claims are reproduced. A reanalysis of the original replicate-level observations is needed for journal-ready inferential statistics.

3. Results

3.1. Recovery, Morphological Characterization, and Screening of Fungal Isolates

A total of 53 indigenous fungal isolates were recovered from wastewater-irrigated agricultural soils in the Faisalabad region of Pakistan. Preliminary screening identified 14 isolates exhibiting phosphatase-associated activity, while 13 fungal morphotypes were reported to have undergone morphological characterization. Morphological identification was performed based on colony appearance, pigmentation, hyphal structure, conidiophores, and conidial characteristics observed through microscopic examination. The recovered fungi were provisionally assigned to several genera, including *Aspergillus*, *Rhizopus*, *Mucor*, *Fusarium*, and *Penicillium*. Representative microscopic structures, hyphal characteristics, and colony morphologies are presented in Figure 1 (panels A–K). Among these, *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus fumigatus* exhibited distinct morphological characteristics, as illustrated in Figure 1C–E. These observations supported preliminary morphological identification of the recovered fungi, although molecular confirmation remains necessary for definitive species-level assignment.

Based on the preliminary plate-screening results, five fungal isolates were prioritized for further evaluation of acid phosphatase production. Among these, *A. niger* exhibited the highest reported solubilization index (SI = 6.00 ± 0.05), followed by *A. fumigatus* (5.00 ± 0.87), *Rhizopus oligosporus* (4.00 ± 0.94), *A. flavus* (4.00 ± 0.67), and *Mucor rouxii* (4.00 ± 0.62). The comparatively higher indices of *A. niger* and *A. fumigatus* indicated stronger plate-based screening responses, supporting their selection as the leading candidates for subsequent shake-flask optimization and quantitative acid phosphatase assays. Overall, these findings demonstrate variation in preliminary phosphatase-associated responses among indigenous soil fungi and identify two promising *Aspergillus* isolates for further enzyme-production studies.



Figure 1: Morphological Diversity of Indigenous Fungal Isolates Recovered from Wastewater-Irrigated Agricultural Soils. Representative microscopic, hyphal, and colony characteristics of morphologically identified fungal isolates: (A) Mucor rouxii; (B) Rhizopus stolonifer; (C) Aspergillus niger; (D) Aspergillus flavus; (E) Aspergillus fumigatus; (F) Aspergillus ficuum; (G) Aspergillus oryzae; (H) Aspergillus nodulus; (I) Fusarium oxysporum; (J) Fusarium solani; and (K) Penicillium citrium. Each group presents representative microscopic, hyphal, and colony features. Species assignments are based on morphological characterization.

3.2. Selection of assay substrate and substrate concentration

Several phosphorylated substrates were compared and reports the highest apparent phosphatase activity using p-nitrophenyl phosphate. At the reported substrate optimum of 250 µM, nominal activities among the five candidates were approximately 4–5 U/mL in the sequential screen, with A. niger and A. fumigatus among the highest performers. The original plots also depict a decline above 250 µM. The comparison supports selection of pNPP as the analytical substrate in this screening workflow, but it does not establish selective phytase activity because sodium phytate was not used to define the final enzyme-production endpoint.

3.3. Optimization of Culture Conditions for Acid Phosphatase Production

The effects of physicochemical and nutritional parameters on extracellular acid phosphatase production were investigated under shake-flask cultivation conditions. Enzyme activity varied with inoculum volume, culture pH, incubation temperature, substrate concentration, carbon and nitrogen sources, and cultivation duration. An inoculum volume of 0.5 mL was associated with favorable enzyme production, while pH 4 and 30 °C were identified as the most suitable culture conditions among those evaluated. Substrate screening revealed that *p*-nitrophenyl phosphate (pNPP) supported the highest measured acid phosphatase activity, with an optimal assay concentration of 250 µM. Among the tested carbon sources, glucose produced comparatively higher enzyme activity. Nitrogen supplementation also influenced enzyme production, although the reported findings differed between casein and ammonium nitrate, preventing definitive identification of the most effective nitrogen source. Incubation duration further

affected enzyme activity, with the detailed time-course results showing a progressive increase up to day 7, followed by a decline on days 9–11. However, the initial experimental summary identified day 5 as the optimum incubation period. Overall, the findings indicate that acidic pH, moderate cultivation temperature, appropriate substrate concentration, and nutrient supplementation favor extracellular acid phosphatase production by the selected fungal isolates, particularly *Aspergillus niger* and *Aspergillus fumigatus*. The optimal nitrogen source and incubation period require confirmation from the original experimental records.

3.4. Enhancement of Acid Phosphatase Activity and Specific Activity Following Culture Optimization

The influence of culture optimization on extracellular acid phosphatase production was assessed in two selected indigenous fungal isolates, *Aspergillus niger* and *Aspergillus fumigatus*, by comparing their initial screening activities with the values obtained following optimization (Figure 2A–B). Both isolates exhibited considerable improvements in enzyme activity, although differences in their initial production capacities and relative responses were observed. During preliminary screening, *A. niger* demonstrated higher extracellular acid phosphatase activity (5.9 U/mL) than *A. fumigatus* (4.3 U/mL), indicating variation in their baseline enzyme-producing capacities. Following culture optimization, acid phosphatase activity increased to 19.0 ± 0.033 U/mL in *A. niger* and 16.0 ± 0.033 U/mL in *A. fumigatus* (Figure 2A). These values represent absolute increases of 13.1 and 11.7 U/mL, corresponding to approximately 3.22-fold and 3.72-fold increases over the respective initial activities. Although *A. niger* maintained the highest overall extracellular enzyme activity, *A. fumigatus* exhibited a comparatively greater proportional improvement, indicating differences in the responses of the two isolates to the evaluated cultivation conditions.

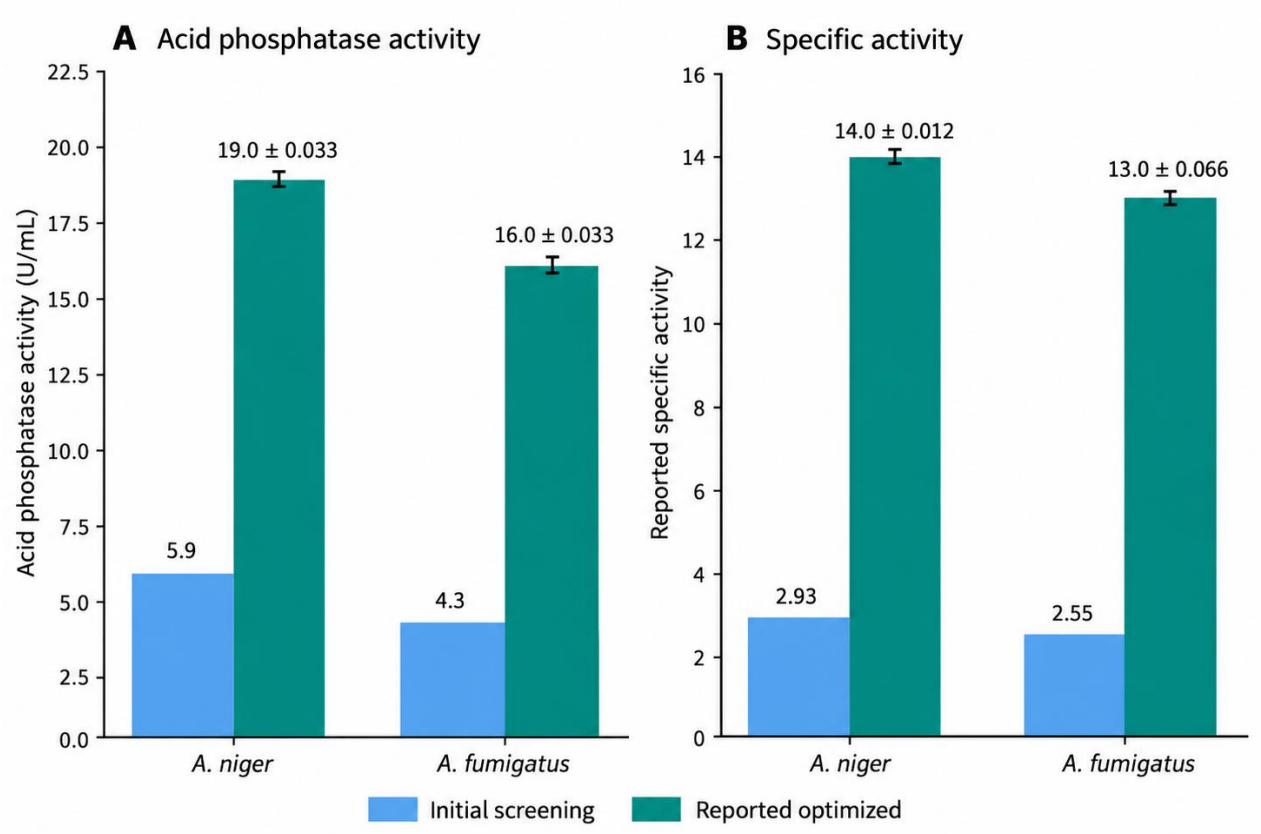


Figure 2. Enhanced Acid Phosphatase Activity and Specific Activity in Indigenous Aspergillus Isolates Following Culture Optimization. Figure 2A: Enhancement of Extracellular Acid Phosphatase Activity. Figure 2B: Improvement in Acid Phosphatase Specific Activity

A similar improvement was observed in the reported protein-normalized acid phosphatase activity of both fungal isolates (Figure 2B). Initially, *A. niger* exhibited a specific activity of 2.93, whereas *A. fumigatus* showed a comparatively lower value of 2.55. Following optimization, the corresponding specific activities

increased to 14.0 ± 0.012 and 13.0 ± 0.066, respectively. These changes represent approximately 4.78-fold and 5.10-fold increases in specific activity for *A. niger* and *A. fumigatus*, respectively. Although *A. niger* retained the highest final specific activity, *A. fumigatus* demonstrated a slightly greater relative improvement. The parallel increases in extracellular enzyme activity and reported specific activity suggest that the optimized cultivation conditions were associated with improved acid phosphatase production relative to the measured protein content of the crude enzyme preparations. Nevertheless, differences in protein concentration, fungal biomass, or enzyme secretion could also contribute to the observed responses and cannot be distinguished from the available measurements. Overall, the optimization results demonstrated consistent increases in both extracellular acid phosphatase activity and reported specific activity in the two selected *Aspergillus* isolates (Figure 2A–B). The highest final enzyme activity and specific activity were recorded for *A. niger*, whereas *A. fumigatus* exhibited the greater proportional improvements in both parameters. These findings identify *A. niger* as the stronger absolute producer under the reported optimized conditions, while highlighting the substantial improvement achieved by *A. fumigatus*. The observed enhancements support the potential of these indigenous fungal isolates as candidates for further acid phosphatase production studies, including enzyme purification, biochemical characterization, and evaluation of production reproducibility.

3.5. pH, temperature, and chemical-response profiles

The crude enzyme assays were reported to exhibit measurable acid phosphatase activity across pH 3–6 and temperatures of approximately 20–50 °C, with strong performance near pH 4 and 30 °C. The manuscript also documents changes in apparent activity with added ions, including Mg^{2+}, Cu^{2+}, and Ca^{2+}, and under treatments with EDTA and several transition-metal or divalent-metal salts. However, the metal-ion and inhibitor section contains inconsistent rankings, valence notation, and incompatible activity units. Quantitative percent inhibition, activation, and resistance cannot be determined reliably without a common control reference and concentration-specific raw measurements. Taken together, the screening indicates potentially useful acidic-enzyme production under moderate cultivation conditions, but stability, mechanism of metal sensitivity, enzyme purity, and industrial robustness remain open questions. In particular, these observations do not establish that the whole organisms can treat textile wastewater, remove dissolved inorganic phosphorus, or increase crop yield.

4. Discussion

The emphasis on native fungi from wastewater-irrigated fields is biologically reasonable because contaminated or nutrient-enriched soils can host diverse decomposer communities. Screening these isolates for extracellular phosphorus-hydrolyzing activity provides a practical route to identify laboratory candidates. The identification of A. niger among the stronger producers accords with earlier investigations showing pronounced phosphatase production by fungi from Aspergillus and related genera (Tarafdar & Chhonkar, 1979). More recent methodological work has reinforced the utility of pNPP-based assays for quantifying fungal acid phosphatase, while also emphasizing the differences between extracellular, intracellular, and isoform-specific activity (Kushwaha & Kumar, 2022). It is important to separate acid phosphatase from phytase. A published investigation using wastewater-irrigated soil fungi focused on phytate-degrading enzymes and reported growth-condition optimization in selected Aspergillus species (Sadaf et al., 2022). The present manuscript, in contrast, uses a pNPP-oriented activity endpoint and therefore should not borrow phytase terminology or infer phytate mineralization from the reported final activities. Direct sodium-phytate hydrolysis measurements would be needed to establish any additional phytase function.

A modestly acidic medium is compatible with expression and assay of many fungal acid phosphatases, while a temperature near 30 °C provides a reasonable growth setting for mesophilic Aspergillus cultures. The available data suggest that activity varied with inoculum quantity, supplied carbon and nitrogen, and cultivation time. Such changes could reflect enzyme expression, biomass accumulation, secretory capacity, culture pH drift, or more than one of these factors simultaneously. As the original record lacks biomass-normalized secretion rates and a fully documented experimental design, causation should not be attributed to a particular regulatory mechanism. The apparent threefold or greater increase in the lead

isolates is the clearest quantitative outcome in the submitted summary. Nevertheless, the direct interpretation of optimized activity depends on whether the initial and final measurements used equal assay volumes, substrate concentrations, culture ages, and calibration conditions. Without these details, the fold increases are informative descriptions of the reported values rather than independently validated process gains.

Industrial and environmental use of extracellular phosphatases may be explored through substrate conversion, immobilization, and evaluation under complex matrices. Fungal biomass has also been investigated for phosphorus recovery from dairy manure wastewater, although that application differs from the acid phosphatase production endpoint examined here (He et al., 2019). Related fungal studies have demonstrated that Aspergillus species possess additional extracellular enzyme activities relevant to dye transformation, although dye-degrading laccases and phosphate-hydrolyzing acid phosphatases catalyze different chemical reactions (Ghafoor et al., 2024). Broader biotechnological research highlights opportunities for microbial and enzyme systems, alongside requirements for containment, consistency, and environmental risk assessment (Javed et al., 2024; Sial et al., 2025). Because A. fumigatus is an opportunistic human pathogen and species-level identity was based on morphology alone, use of live fungal biomass in an open agricultural or food-related system should not be recommended from these data. A more defensible next step would be characterization of purified or cell-free enzyme preparations and safety assessment of the producing organism. Environmental health and wastewater reuse also demand attention to downstream exposure, antimicrobial resistance, and multiple contaminant classes, issues emphasized in recent wastewater and One Health literature (Shafiq et al., 2025; Zafar et al., 2026).

4.4. Study Limitations

Despite demonstrating enhanced acid phosphatase production by indigenous fungal isolates, the present study has several limitations. Fungal identification was primarily based on morphological characteristics without molecular confirmation, potentially limiting the accuracy of species-level classification. The optimization experiments were conducted under laboratory-scale shake-flask conditions, restricting direct extrapolation to large-scale enzyme production. Inconsistencies in the reported optimal incubation period and nitrogen source, together with incomplete replicate-level data, limit the interpretation of experimental reproducibility and statistical significance. Furthermore, enzyme purification, detailed kinetic characterization, and quantitative assessments of long-term stability were not performed. The absence of direct phosphorus-mobilization and wastewater-treatment experiments also limits conclusions regarding practical environmental applications.

4.5. Future Directions

Future investigations should focus on molecular identification of the selected fungal isolates using internal transcribed spacer (ITS) sequencing and complementary molecular markers to establish reliable taxonomic identities. Advanced statistical optimization approaches, including response surface methodology (RSM), could be employed to refine cultivation parameters and improve enzyme productivity. Further studies should investigate enzyme purification, kinetic properties, substrate specificity, thermal stability, pH tolerance, and the effects of metal ions and inhibitors to establish biochemical performance. Scale-up experiments using controlled bioreactor systems, enzyme immobilization, and repeated-batch production could help evaluate industrial feasibility. Additionally, the phosphate-releasing potential of the purified enzymes should be assessed under environmentally relevant conditions, particularly in phosphorus-deficient agricultural soils and wastewater matrices. Comprehensive biosafety assessments, especially for *Aspergillus fumigatus*, will be essential before considering agricultural or environmental applications.

5. Conclusion

The present study demonstrated the potential of indigenous fungal isolates recovered from wastewater-irrigated agricultural soils as promising sources of extracellular acid phosphatase. Among 53 recovered fungal isolates, five were prioritized based on their preliminary phosphatase-associated screening responses, with *Aspergillus niger* and *Aspergillus fumigatus* emerging as the leading enzyme-producing

candidates. Culture optimization substantially enhanced acid phosphatase activity from 5.9 to 19.0 ± 0.033 U/mL in *A. niger* and from 4.3 to 16.0 ± 0.033 U/mL in *A. fumigatus*, representing approximately 3.22-fold and 3.72-fold improvements, respectively. Both isolates also exhibited considerable increases in reported specific activity, highlighting their responsiveness to modified cultivation conditions. Favorable enzyme-production conditions included pH 4, a cultivation temperature of 30 °C, a 0.5 mL inoculum volume, and glucose supplementation, while the highest reported assay activity was observed at a *p*-nitrophenyl phosphate concentration of 250 µM. The crude enzyme preparations also exhibited activity across acidic pH conditions and a relatively broad temperature range, indicating potentially useful biochemical characteristics. Collectively, these findings highlight the value of exploring indigenous soil fungi for acid phosphatase production and provide a foundation for further enzyme-focused biotechnological investigations. However, molecular identification, confirmation of optimal culture conditions, comprehensive biochemical characterization, and biosafety evaluation remain essential before considering industrial or environmental applications.

Declarations

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Ethics approval: Not indicated as applicable for soil sampling; confirm institutional and site permissions where relevant.
Data availability: Raw replicate-level enzyme-activity and assay records should be deposited or made available in accordance with the target journal's policy; current availability was not specified.
Conflicts of interest: To be declared by the authors.

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