Tianjin Daxue Xuebao (Ziran Kexue yu Gongcheng Jishu Ban)/
Journal of Tianjin University Science and Technology
ISSN (Online):0493-2137
E-Publication: Online Open Access
Vol: 56 Issue: 11:2023
DOI: 10.5281/zenodo.10152981

EXPLORING PHOSPHATE-SOLUBILIZING *ASPERGILLUS SP*: ISOLATION AND OPTIMIZATION OF EXTRACELLULAR ACID PHOSPHATASE ACTIVITY

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Abstract

Background: The global phosphorus crisis is an evolving concern as the world's phosphorus sources are running out and demand for agricultural production is rising. Phosphorus shortages undermine food security and demand long-term management practices to assure phosphorus availability. **Methods:** *Aspergillus* strains were first screened on PVK media to get phosphorus solubilizing species and then the best strain was used to produce acid phosphatases. Finally, the effect of various assay conditions was analyzed to characterize extracellular acid phosphatases. **Results:** Out of four isolated strains, only *A. niger, A. oryzae,* and *A. flavus* were able to solubilize phosphate on PVK, of which *A. niger* stood out with a maximum solubility index of 2.38. The optimal pH and temperature for enzyme activity were recorded as 4.5 and 30°C. Furthermore, the substrate concentration of 100 mM with a 40-minute incubation period was found as a standard condition to achieve maximum specific activity i.e. 12 U/mg and a 17% higher free phosphorus level. After optimization, a 43.8% increment in the activity of extracellular acid phosphatases was observed. **Conclusion:** The study concludes that *A. niger* was the prominent performer, with improved phosphate solubilization and enzyme activity, as well as higher phosphorus release, demonstrating its potential for enhancing phosphorus availability not only in agricultural and environmental fields but in setups with lower phosphate availability too.

Keywords: Bio-fertilizer, Enzyme production, Fungal phosphatases, Phosphorus solubility, Phosphorus.

INTRODUCTION

Subsequent to nitrogen, phosphorus (P) is one of the most important limiting macronutrients validating ideal plant production and quality. It is the core of some major molecular components including proteins, nucleic acids, coenzymes, etc., as well as carries out various significant biological functions, be it photosynthesis, respiration, reproduction, storage, or energy transfer [1]. Bioavailable P can be found in the soil body in both organic (20-1000 mg P kg^{-1} in very productive soils,) and inorganic (0.01-1 mg L^{-1}) forms, of which, about 80% of the total soil P loading is made up of organic P, while only 20% comes from inorganic sources [2,3]. Despite being present in soils, plants still have to strive for phosphorus because the main form of it that plants assimilate is very limited in most soil and needs to be converted into an accessible form. So, in order to meet cellular P requirements, not only plants have developed an intricate network of genes and regulatory systems but soil microorganisms also use a variety of techniques

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to get rid of the inorganic P (Pi) deficiency in rhizospheric regions. Such a strategy involves the production and release of acid phosphatases [4].

Acid phosphatases (EC 3.1.3.2) enzymes are a class of extracellular phosphatases that catalyze the hydrolysis of phosphate monoesters under acidic conditions to mobilize Pi from organic sources and enhance soluble phosphate availability in P-depleted soil [5]. However, the expression of the acid phosphatases is governed by the concentration of phosphate via the regulatory mechanism of repression and de-repression [6]. The enzymes from a perspective of structural diversity, accept a wide variety of substrates in vitro, ranging from tiny chemical molecules to phosphoproteins [7]. Based on the presence or absence of a binuclear metal center, the acid phosphatase family typically splits into two primary categories and can be obtained from plants, fungi, animals, and bacteria. The first class of enzymes, known as metallo-hydrolases, has a distinctive purple color as a result of charge transfer from tyrosine residue to Fe(III), awarding them the common name purple acid phosphatases (PAP). The next is tartrate-resistant acid phosphatases (TRAP) which can be distinguished from the previous group by their insensitivity to tartrate inhibition [8,9].

Phosphate-solubilizing microorganisms (PSMs) are a diverse group of soil and rhizospheric microorganisms, capable of efficiently releasing P from the soil by solubilizing and mineralizing it, and are extensively harbored as bio-fertilizers to boost plant development and the production of several significant crops [10]. Furthermore, previous studies have exposed that the prolonged use of P fertilizers results in eutrophication, soil acidification, and water contamination [11]. Therefore, one of the main objectives of agricultural and forestry development is to increase the bioavailability of soil-insoluble phosphate for plants. Due to their incentives, such as environmental safety, low cost, and outstanding efficacy, PSMs have drawn interest [12].

To date, most illustrative species of *Aspergillus* have been extensively published as phosphate solubilizers [13, 14, 15]. Generally, species of the genus *Aspergillus* solubilizes P by making various organic acids like succinic, glycolic, gluconic, and oxalic acids [16,17]. Therefore, the administration of P Solubilizing fungi into soil had been stated to enhance the quantity of accessible P in the soils by activating fixed P that causes better plant growth [18]. Multiple investigations have already revealed *A. niger's* ability to enhance P uptake. For example, Tian and colleagues recently discovered that administering *Aspergillus niger* fermentation broth can ameliorate low P stress-induced oxidative damage, reduce oxidative stress, and improve the resilience to low P stress in maize seedlings. Furthermore, it indicates *A. niger's* ability to boost development and opens up new study avenues on beneficial plant-fungal interactions [19]. The objective of the research was to isolate and subsequently screen *Aspergillus* sp. from rhizospheric soil for the synthesis and characterization of acid-phosphatases. Moreover, the potential of *Aspergillus* APase to solubilize phosphorous was explored.

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METHODS

All the chemicals (Martin's agar, α-naphthyl acid phosphate, bovine serum albumin, ammonium sulfate, etc.) used during the investigation were obtained from E-Merck and Sigma Aldrich. However, rhizospheric soil samples were collected from different agricultural sites of Lahore, Pakistan. The study was conducted at the IIB labs of GC University Lahore, Pakistan.

Isolation of Phosphofungi isolates

Different *Aspergillus* sp. were isolated from the soil samples using the serial dilution method and maintained on PDA slants followed by their incubation for almost 3–4 days at 30±2°C and then screened for phosphate-solubilizing isolates [20].

Identification of isolated *Aspergillus sp.*

Four of the isolates that showed higher growth were preserved on slants at 4°C and further characterized through microscopic as well as macroscopic examinations. Slide culture is a traditional technique for identifying filamentous fungi based on microscopic features. The macroscopic properties of the isolated fungi, including color, appearance, and colony diameter (following their growth on PDA plates) were also used to identify the isolates [21]. Moreover, for microscopic examination, a glass slide was put into a petri dish that already had filter paper inside of it, and two applicator sticks were placed underneath the slide. Before it solidifies, spread fungal culture spores on a drop of molten sterile PDA (pH 5.6) that was placed on a glass slide. Once the agar spot on the glass plate has solidified, cover it with a cover slip. For 3 to 7 days, incubate it at 30 °C. After the incubation period, slides were examined under a light microscope, and the findings were contrasted with the typical structure of *Aspergillus sp.* as described by Robert and Ellen [22].

Preliminary screening of tri-calcium phosphate solubilizing isolates of *Aspergillus*

To screen phosphate-solubilizing *Aspergillus* isolates, chosen isolates were initially examined on Pikovskaya (PVK) agar medium for their capacity to solubilize tri-calcium phosphate **(**TCP) as insoluble inorganic phosphate sources. The following were included in one liter (1 L) of PVK agar (g/L): 0.5 g of $(NH4)_2SO_4$, 0.1 g of $MgSO_4$_$7H_2O$, 0.02 g of NaCl, 0.02 g of KCl, 0.003 g of $FeSO_4.7H_2O$, 0.003 g of $MnSO_4.H_2O$, and 5 g of $Ca_3 (PO_4)_2$, 10 g of glucose, 0.5 g of yeast extract, 15 g of agar, and 1000 mL of distilled water [23]. The autoclaved PVK agar was added to the sterile Petri plates. With the use of a sterile Eppendorf tube, fungus mycelium plugs of each isolate were cut from the borders of each colony that was actively growing throughout the seven-day PDA at 28°C cultivation. After that, fungus mycelium plugs were put on Petri plates with PVK agar supplemented with 0.5% TCP for 7 days at 28°C. Whereas, plugs of controls were made of sterile PDA.

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Estimation of Solubilizing Capacity

Subsequently, the phosphorus solubilizing strains were identified based on halo zones on media plates post-incubation. For each fungal isolate, three replicates were tested. After the third, fifth, and seventh days of incubation, the diameter of the clear zones surrounding each isolate's colony was measured. Finally, the solubilization index was calculated by the method of Premono [24] using the below formula.

$$Solubilization\ Index = \frac{Colony\ diameter\ +\ Clearing\ zone\ diameter}{Colony\ diameter}$$

Culture medium for the production of extracellular acid phosphatases from selected strains of *Aspergillus*

The Shake flask fermentation method was used to produce APase in a culture medium containing (g/L): corn starch (50), glucose (50), KCl (0.5), $NaNO_3$ (8.6), MgSO4.7H_2O (0.5), $FeSO_4.7H_2O$ (0.1), K_2HPO_4 0.1 g P; pH 5. Erlenmeyer flasks (300 mL) containing 30 mL media were inoculated with 50-d conidia (final concentration 20-30/nL, i.e. 2-3 107/mL) and shaken for 7 days at 30°C, 222 rpm [25].

Optimizing culture conditions

The Shake Flask cultures were performed using the protocol of Trinci, 1972 [26]. Continuous flow cultures were achieved with the help of the methodology opted by Wiebe and Trinci [27]. The foam was regulated by the addition of a 4% PEG (Polypropylene Glycol Suspension) [28]. However, the pH was constantly optimized by adding two chemicals, 1.0 M NaOH or 0.5M H_2SO_4 as per requirement.

Enumerating fungal biomass and growth

After culturing, the samples were reserved for biomass evaluation. Biomass dry weight was primarily confirmed by filtering 10 mL on filter paper and pre-washing with 30mL fresh deionized water followed by drying at 75℃ until a constant weight of fungal mass was achieved from batch cultures. The complete dry weight measurements were executed in triplicates. The Turbidity of Culture was also evaluated using an EEL Colorimeter equipped with a green filter (540–560 nm).

Assessment of enzymatic activity

According to the method outlined by Ullah and Cummins [29], APase activity was measured. An aliquot (0.2 ml) of the enzyme solution was incubated at 40°C for 20 min with 100 mM p-nitrophenyl phosphate (PNPP) in 40 mM acetate buffer (pH 4.0). By adding 1 M NaOH, the process was stopped. The absorbance at 430 nm was used to calculate the amount of emitted p-nitrophenol (PNP), a yellow color complex with the help of a standard curve. The amount of enzyme releasing 1 pmol of PNP per minute under the assay conditions used was considered to be one unit of APase activity.

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Partial characterization of Acid Phosphatases

Produced Acid phosphatases were characterized by analyzing the factors that affect their activity. Therefore, the effect of the temperature (20, 30, 40 50, 60^{0}C), pH (3, 3.5, 4, 4.5, 5, and 5.5), substrate (50, 75, 100, 125, 150 mM), and incubation period (10-50 min), were investigated to find the best suitable assay conditions to achieve the maximum enzymatic activity.

Protein assay

Protein concentrations were determined by the Lowry [30] modification of the phenol method, taking bovine serum albumin (BSA) as standard.

Preparation of standard curve of BSA

Initially, BSA stock solution of concentration 1mg/ml was prepared. Ten dilutions were made from the stock solution, starting at 0.1 mg/ml, while changing distilled water's volume. Each dilution had an addition of 5 ml of Bradford reagent before being incubated at room temperature for 5 minutes. Last but not least, their optical density at 595nm was calculated in comparison to a blank that contained 1ml of distilled water. The standard curve method using the equation of a straight line, y=mx+c, was used to estimate the total protein produced (Fig.1).

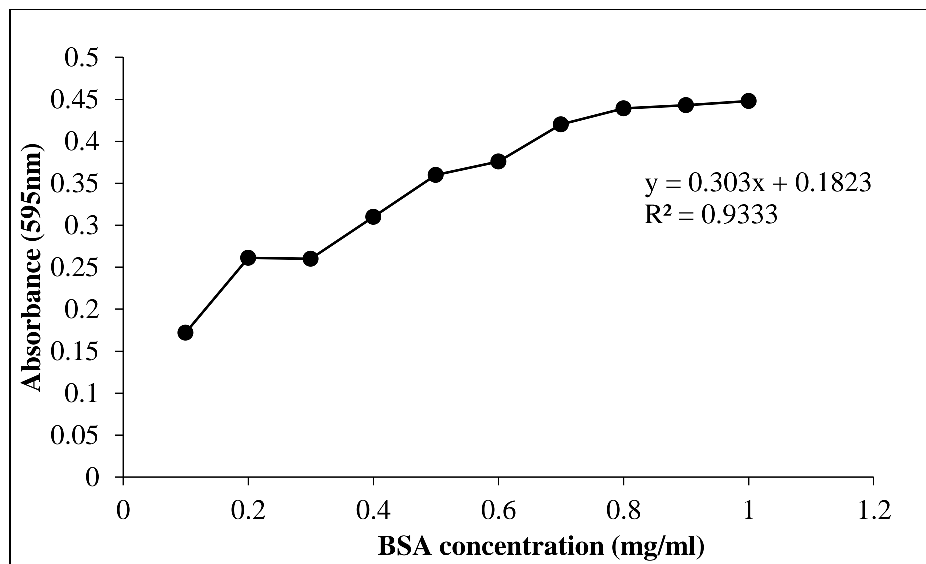

Fig-1: Standard curve of BSA

Statistical Analysis

Collected data was evaluated statistically by one-way ANOVA, and paired t-test using IBM SPSS 20 [31]. Furthermore, the significant difference between replicates was given as Duncan's multiple ranges through probability values (<p>).

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RESULTS

Isolation of *Aspergillus sp.*

Using serial dilutions of rhizospheric soil samples, multiple *Aspergillus* species were isolated on PDA medium. Four days of incubation resulted in the growth medium containing colonies of almost seven *Aspergillus* species. From these obtained species, pure cultures were prepared by re-culturing technique. Only four cultures that have shown good fungal growth after re-culturing were further studied for characterization.

Morphological identification of isolated *Aspergillus sp.*

All *Aspergillus* species share certain characteristic microscopic structures that allow for identification at the genus level based on their morphology e.g. vesicles, hyphae, conidia, and conidiophores. The slide culture technique was utilized to classify the microscopic traits of isolated *Aspergillus sp*. *Aspergillus niger* features colorless, smooth conidiophores and spores. However, a glubose vesicle with a slightly rough-thin walled structure, and a colorless, coarsely walled conidiophore were the two main structural components of *Aspergillus flavus*. *Aspergillus oryzae* has septate mycelium, spherical or ellipsoidal conidia, and unbranched or sparsely branching conidiophores, frequently in green or yellow. Moreover, *Aspergillus fumigatus* presents features like a glubose vesicle, spherical conidia, and a conidiophore with a smooth, greyish surface which is also known as stipe. It does not have metulae, instead, phialides are directly attached to vesicles. Moreover, on the basis of the morphology of the colony of each isolate, the identification was verified.

Selection of *Aspergillus* strains having phosphate solubilizing capacity

Results of the primary screening revealed that only three of the isolates had solubilized tri-calcium phosphate **(**TCP) on selective media, Pikovskaya (PVK), and formed positive halo zones. Whereas *Aspergillus fumigatus* could not solubilize TCP. The zones of solubilization appeared by phosphate solubilizing *Aspergillus* species after 60 h of incubation and up to the seventh day. There was constant observation of the halo zone in increasing order. The diameter of the clear zones surrounding each isolate's colony was measured using the Premono et al. (1996) formula. Zone diameters displayed by *A. niger, A. oryzae, A. flavus,* were 8.2, 5.1, and 6.3 mm, respectively (Table 1). The isolate with the highest Solubility index as shown in Fig. 2 was chosen for the production of acid phosphatases.

Table 2: Phosphate solubilization index of PVK-positive *Aspergillus* isolates

Isolate	Diameter of zones (mm)	Solubility index
Aspergillus oryzae	5.1	2.20
Aspergillus niger	8.2	2.38
Aspergillus flavus	6.3	2.13

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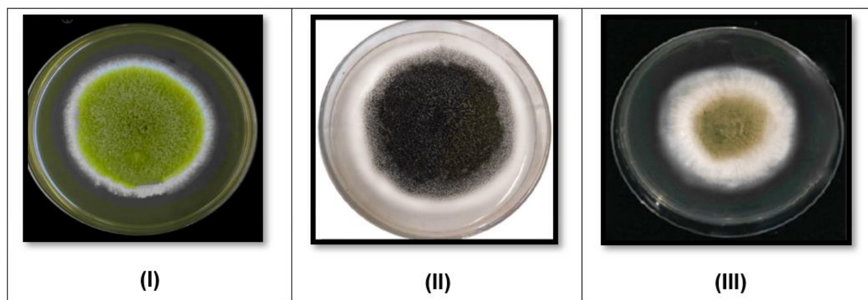

Fig 2: Pictorial view of solubilization zones of different *Aspergillus sp.* on PVK medium. (I) *Aspergillus flavus* (II) *Aspergillus niger* (III) *Aspergillus oryzae*

Growth and biomass production

Results have shown that when *Aspergillus niger* was grown in shake flask culture with potassium phosphate as a phosphorous source, an average of 2.5±0.17 g/L of dry fungal biomass was gained under-regulated shake flask culture conditions; pH 5 and temperature 30°C**,** exhibiting an enzymatic activity of 11.5±0.55 U/ml. To further optimize the activity of acid phosphatases, the effect of the incubation period, temperature, pH, and substrate were varied, and ideal assay conditions were investigated to achieve the maximum enzymatic activity.

Temperature affecting APase activity

The effect of different assay temperatures (20, 30, 40 50, 60 ^{0}C) was determined on APase activity. As shown in Fig. 3, the maximum enzyme activity of 12.1±0.64 U/ml was obtained at 30°C. At this ideal temperature, the enzyme hydrolyzed maximum PNPP and liberated free phosphorus (393 µg/ml) while exhibiting a specific activity of 67±1.4 U/mg. However, once the temperature was raised above 30°C, the enzyme activity gradually began to decline. Finally, the enzyme activity decreased by 52%, drastically at the temperature of 60°C, showing that APase was losing its structural integrity and catalytic function at increasing temperatures over the studied range.

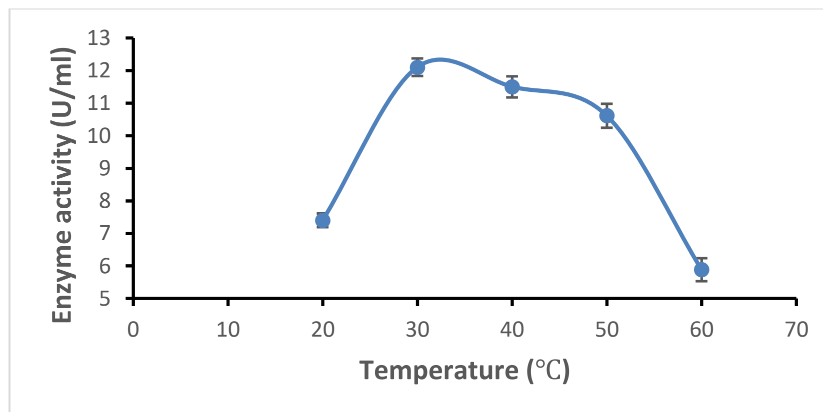

Fig 3: Influence of different temperatures on the activity of acid phosphatases obtained from *A. niger*

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pH affecting APase activity

The pH of the reaction mixture was changed ranging from 3, 3.5, 4, 4.5, 5 to 5.5 while maintaining a constant temperature of 30°C in order to maximize the APase activity. As depicted in Fig. 4, The best pH for the reaction was shown to be 4.5, which had the greatest enzyme activity of 13.2±0.57 U/ml and a specific activity recorded as 73±1.2 U/mg, illustrating that extremely acidic conditions slow down catalysis. Moreover, a decrease in the enzyme's activity occurred when the pH elevated beyond 4.5, and it lost its capacity to tolerate acid in more alkaline conditions outside of its pH stability range.

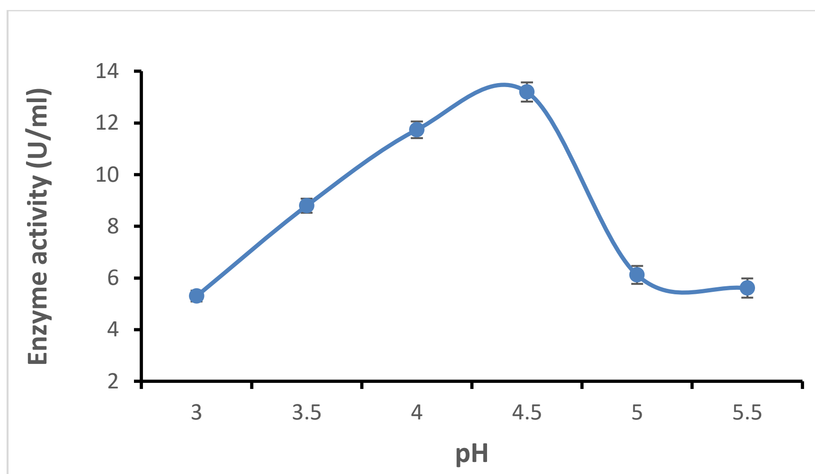

Fig 4: Influence of different pH values on the activity of acid phosphatases obtained from *A. niger*

Substrate concentration affecting APase activity

By adjusting the p-nitrophenyl phosphate substrate concentration utilized in the experiment, which ranged from 50 to 150 mM, the APase activity was maximized. When 100 mM of the substrate was present in the reaction mixture, the maximum enzyme activity of 15.8±0.61 U/ml was seen at this optimal concentration (Fig. 5). However, lower enzyme activity was seen for substrate concentrations both lower and higher than the optimal concentration, indicating that the substrate concentration tested was the one that best supported the enzyme's catalytic capabilities and kinetics. The specific activity calculated at the optimal substrate concentration (100 mM p-nitrophenyl phosphate) was 87.7±2.4 U/mg.

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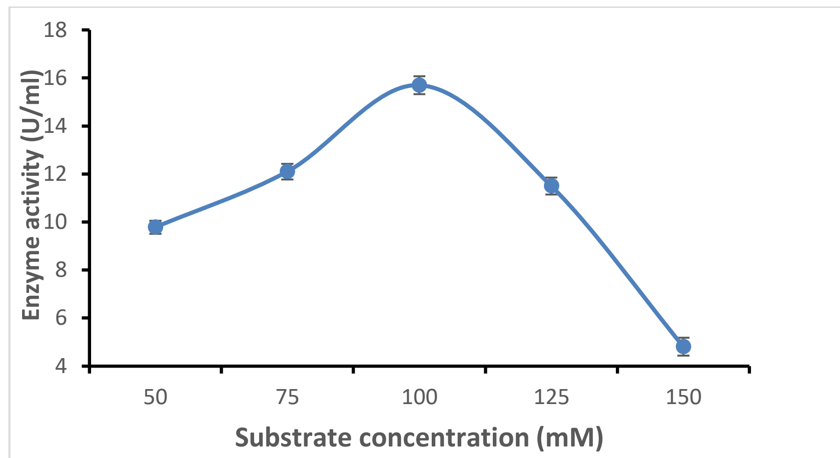

Fig 5: Influence of different concentrations of substrate on the activity of acid phosphatases obtained from *A. niger*

Incubation period affecting APase activity

Enzyme activity was performed by incubating the enzyme with p-nitrophenyl phosphate at varying time periods (10,20,30,40, and 50 min) as shown in Fig. 6, in order to identify the ideal time needed for the acid phosphatase-catalyzed reaction. After 40 minutes of incubation, the enzyme activity as well as specific activity reached its peak and was measured to be 17.4±1.15 U/ml and 97.8±3.1 U/mg, respectively. At this stage, the amount of released P was 462 µg/ml. Any further advancement in time had a negative impact on activity.

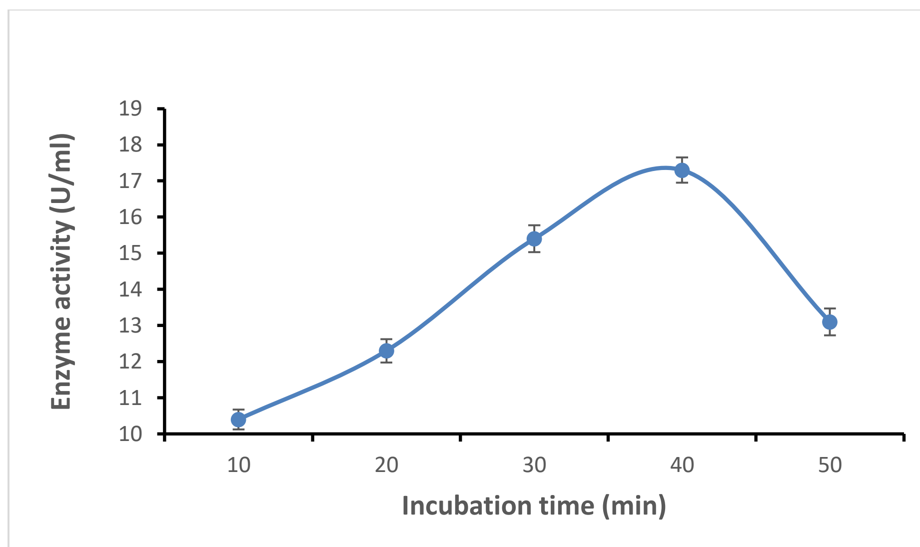

Fig 6: Influence of different incubation periods on the activity of acid phosphatases obtained from *A. niger*

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In a nutshell, optimization experiments outlined that the ideal setting for achieving peak enzymatic turnover (17.4 U/ml) was 30°C, pH 4.5, 100 mM substrate concentration, and an incubation time of 40 minutes.

DISCUSSION

Phosphorus plays an important part in many plant activities, but its scarcity limits agricultural production. Chemical phosphate fertilizers only showcase up to 25% efficacy, given their soil-associated problems such as insoluble complexes formation. Moreover, rock reserves of phosphate are near to deplete. So, scrutinizing phosphorus using biological machinery is a need of the hour due to rising concerns about the phosphorus crisis [32]. As per estimates, approximately 70–90% of phosphorus fertilizers given to soils are fixed by cations and transformed into inorganic P [33]. Contrarily, phosphate-solubilizing microorganisms provide a long-term solution since they are sustainable, cost-effective, and improve nutrient accessibility. Inoculating plants with potent phosphatases has been proven to increase plant yield and phosphorus uptake. As a result, contributes to plant growth by assisting with nitrogen fixation, phytohormone production, and vitamin availability. Besides their agricultural benefits, APases serve plenty of roles in a variety of domains. They aid in medical diagnostics for the detection of prostate cancer and bone problems, industrial operations such as detergent and food production, and environmental monitoring for the assessment of water quality and pollution [34]. This study focused on the screening of the best phosphate-solubilizing species of *Aspergillus* from the soil samples of Lahore agricultural area to produce acid phosphatase. Results of the study have outlined *A. niger* as the most efficient strain with a TCP-solubilization zone of 2.38 mm. These results are in agreement with the findings of LA [35], who also determined that on PVK, *A. niger* was the most efficient phosphate solubilizer of all 12 isolated fungi, with a solubilization index (PSI) of 2.4. In contrast, no solubility of *A. fumigatus* on PVK was observed in our case. Our study was also in line with the study of Yasser and his fellows, who described that *Aspergillus niger*, *Penicillium variable* and *Trichoderma harzianum* 301 are the dominant Phosphate-solubilizing genera present in the soil of the Beni-Suef, Egypt. The study further disclosed that *A. niger* had the highest solubility potential i.e. 1.67 followed by *P. variable* and *T. harzianum* on Pikovskaya's medium [36]. In the same vein, two *A. niger* strains displayed effective tricalcium phosphate (TCP) solubilization in Svalbard Arctic soils. In seven days, strain-1 released 285 g/ml of inorganic P and strain-2 released 262 g/ml of 0.5% TCP, indicating their potential as effective biofertilizers [37]. Isolation of phosphorus hydrolyzing *Aspergillus* has been reported in numerous studies [38,39].

Maximal APase activity was observed at 30°C. The declining trend of enzyme activity at further increase in the incubation temperature suggests that the enzyme might be destabilized at high temperatures. These results corroborated with the study of Bhattacharya and his teammates [40] as their work showed that maximum P hydrolysis (468 µg/ml) by APase was recorded when the temperature was kept at 30°C. Likewise, [35] also prospected 30°C as the optimal temperature to achieve the highest phosphate

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solubilization rate (58.53 g/ml) using APase isolated from *A. niger* MPF-8. Further outcomes of our findings to optimize pH have defined that at pH 4.5, enzyme activity was improved and gained the maximum level of free phosphorus in the assay medium and decreased at slightly acidic pH. Perhaps, due to the change in the pH, amino acids of the active site deteriorated which in turn affected the activity of the enzyme [41]. A parallel investigation has indicated that 4.5 is the ideal pH exhibited by extracellular acid phosphatases [42]. However, the results are not aligned with the study of Hidayat, 2006 [6] who At PH 6.5, attained the greatest activity of *A.niger* acid phosphatase. Correspondingly, Chen et al., (2019) investigated the hydrolysis of 2-Phospho-L-ascorbic acid (AAP) and concluded that pH 4.5 was the best pH to yield higher titers of ascorbic acid and orthophosphate ion [43]. Further optimizations revealed that where increasing the time of incubation supported the hydrolysis at the same time the boosted concentration of substrate beyond 100 mM led to a decrease in the activity of the enzyme. According to available data, the combined effects of end-product inhibition by released phosphate ions, substrate inhibition involving non-productive binding that changes the enzyme structure, and saturation of the catalytic reaction kinetics maximum rate cause the APase activity to decline at higher substrate concentrations [44]. The abovementioned results help us to infer that acid phosphatases work better in lower phosphate soils and P-depleted environments.

Conclusion: Phosphorus is the second most essential nutrient for plants. This study aimed to isolate the best phosphate-hydrolyzing species of *Aspergillus* to produce potent acid phosphatases followed by their characterization. To achieve the purpose, three of the seven isolated *Aspergillus* strains; *A. niger, A. oryzae* and *A. flavus,* were identified to be promising phosphorus solubilizers on PVK media, with *A. niger* having the highest solubility index of 2.38. Subsequently, this strain (*A. niger*) was employed to produce the extracellular acid phosphatase (APases) enzyme under shake flask method. The characterization of APases involved optimizing various assay conditions, revealing that the enzyme exhibited optimal activity at 30°C, pH 4.5, with a 100 mM substrate concentration and a 40-minute incubation time, resulting in the highest enzyme activity and specific activity values of 17.4±1.15 U/ml and 97.8±3.1 U/mg, respectively. The study's findings revealed that under optimized settings, 17% more phosphorus was released than under unoptimized conditions. In addition, the enzyme's activity was also increased by 43.8%. These findings imply that *A. niger* and its extracellular acid phosphatase enzyme have the potential to be reliable tools for boosting phosphorus availability in agricultural, environmental, and other applications, with implications for improved nutrient uptake and sustainable agriculture practices.

Conflict of interest

The author declares that there is no conflict of interest.

Funding sources

This study is not part of any sponsored research project. No other financial support was provided from the university or any other source.

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E-Publication: Online Open Access
Vol: 56 Issue: 11:2023
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