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Harnessing the Phytase Production Potential of Soil-Borne Fungi from Wastewater Irrigated Fields Based on Eco-Cultural Optimization under Shake Flask Method

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Abstract: Indigenous fungi present in agricultural soils could have synchronized their inherent potentials to the local climatic conditions. Therefore, the fungi resident in the untreated wastewater irrigated agricultural field might develop their potential for producing various enzymes to handle the induced full organic load from domestic wastewater and toxic chemicals from the textile industry. Around 53 various fungal isolates were grown and separated from the soil samples from these sites through soil dilution, soil-culture plate, and soil-culture plate methods. All the purified fungi were subjected to a phosphatase production test, and only 13 fungal strains were selected as phosphatase producers. Among them, only five fungi identified as *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Penicillium purourogenum,* and *Mucor rouxii* based on morphological similarities, showing higher phosphate solubilizing indices, were utilized for eco-cultural fine-tuning to harness their full production potential under shake flask (SF) method. Among various media, orchestral tuning, 200 µM sodium phytate as substrate with 1.5 mL of inoculum size of the fungi, pH 7, temperature 30 °C, glucose, and ammonium nitrate as carbon and nitrogen additive with seven days of incubation were found to be the most appropriate cultural conditions to harness the phytase production potential of the selected fungi. *Aspergillus niger* and *Aspergillus flavus* showed initial phytase activity (5.2 Units/mL, 4.8 Units/mL) and phytase specific activity (2.85, 2.65 Units/mL per mg protein) during screening to be enhanced up to 17 ± 0.033 (Units/mL), 16 ± 0.033 (Units/mL) and (13 ± 0.012), 10 ± 0.066 (Units/mL per mg protein), respectively, with the above-mentioned conditions. The phytase enzyme produced from these fungi were found to be almost stable for a wide range of pH (4–8); temperature (20–60 °C); insensitive to Ca^{2+} and Mg^{2+} ions, and EDTA, Ni^{2+}, and Ba^{2+} inhibitors but highly sensitive to Mn^{2+}, Cu^{2+}, and Zn^{2+} ions, and Co^{2+}, Cr^{3+}, Al^{3+}, Fe^{2+} and Ag^{1+} inhibitors. It was suggested that both phytase-producing strains of *A. niger* and *A. flavus* or their crude phytase enzymes might be good candidates for application in soils to release phosphates from phytate and a possible valuable substitute of phosphate fertilizers.

Keywords: fungal phytase; eco-cultural optimization; soil born fungi; enzyme stability analysis; *Aspergillus niger*; *Aspergillus flavus*

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Citation: Sadaf, N.; Haider, M.Z.; Iqbal, N.; Abualreesh, M.H.; Alatawi, A. Harnessing the Phytase Production Potential of Soil-Borne Fungi from Wastewater Irrigated Fields Based on Eco-Cultural Optimization under Shake Flask Method. *Agriculture* **2022**, *12*, 103. https://doi.org/10.3390/agriculture12010103

Academic Editor: Ofir Degani

Received: 5 October 2021
Accepted: 8 January 2022
Published: 12 January 2022

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1. Introduction

Phytases are an enzyme that hydrolyze phytic acid into two sub group myo-inositol, Hexakisphosphate, or phytate (salt form). It is a type of phosphatases that hydrolyze the phosphomonoester bonds present in phytates. Phytate is a primary storage form of phosphorus in plant tissues, such as cereal grains, pollen, and oilseed; these are sources

of phosphorus and are known as macro elements for both plants and animals [1–3]. Phytic acid/phytate is the predominant/primary form of phosphorus (60–90%) present in plants [4] and is not available to plants for direct utilization. Therefore, phosphate supplementation is required for optimum growth of plants in the form of fertilizers. Furthermore, it is also considered as an antinutrient responsible for phosphorus pollution present in animal manure [5,6]. In addition, phytate is found in different forms in soils, such as adsorbed to clays, insoluble salts of iron, and aluminum precipitation in acidic soils, or in alkaline soils as insoluble calcium salts [7]. Moreover, phytate, as a strong chelating agent, filches out essential metal ions responsible for soil fertility and optimum plant growth [8]. Therefore, plants, along with monogastric animals, could not utilize phytate–phosphorus, thus enhancing the utilization of phytase as a supplement would be useful to tap this important phosphorus source [9].

While phosphatase from various sources in soil is responsible for liberating P in rhizospheric areas of soil, phytases are required to hydrolyze phytic acid in the soil and are widely produced in nature by many fungi, yeast, plants, animals, and bacteria. However, among these microorganisms, phytase enzymes were reported in *Aspergillus* species, especially in *A. niger* and *A. ficuum* [1,2,10]. Interestingly, the first fungal phytase was identified in 1964 from *A. niger*, and was marketed in 1991. Similarly, many fungi, including *A. niger*, *A. ficuum* [11], *A. oryzae* [12], *A. fumigatus* [13], *A. niger* van *teighem* [14], *Rhizopus oligosporus* [15], *R. oryzae*, *Neurospora sitophila* [12] and *Penicillium purpurogenum* [16] were reported as phytase enzyme producers.

Different cultural conditions increase or decrease phytase production from different fungal strains [11,17,18]. These conditions include pH, temperature, substrate, substrate concentration, carbon sources, and nitrogen sources [18–20]. Fungal strains during enzyme assay could face many problems, such as insufficient oxygen, mass transfer, low and high broth nutrients level, and clumpy growth, which can affect phytase production [21]. In the literature, phytase produced from the fungi are showing a broad spectrum of substrate specificity, pH range between 1.3 to 8.0 and temperature ranges from 37 °C to 67 °C [17].

With the utilization of fungal phytase, improvement in the soil fertility, increase in a plant's nutritional quality, and agricultural productivity could be achieved by the reduction in the use of chemical fertilizers, hence, could be economically beneficial. Fungal phytases are a cheap method to tap the unused phytate, a phosphorus reservoir, in agricultural soils. The available form of the phosphorous present in the soil is always limited, and could cause potential future crisis in agriculture [22]. Experiments suggested that one gram of inorganic phosphate supplements in soil could be replaced with approximately 500–1000 U of phytase, reducing 30–50% total phosphorus excretion at the end [23,24].

The irrigation of fields with untreated wastewater, fully loaded with organic matter and textile industrial waste, might be very competitive among the indigenous fungal strains and force them to equip themselves with various kinds of primary and secondary metabolites, and a wide array of enzyme production over the years. The study aimed to screen the suitable fungal candidates with inherent potential of phytase enzyme production from untreated wastewater (mixture of urban and textile industry effluents) irrigated (twice in a crop season from more than 10 years) soils. After the isolation of fungi, purified cultures were maintained and utilized for the optimum phytase production by fine-tuning of eco-cultural conditions and with simple media additives. The fine-tuned orchestral nodes of fungal culture medium were utilized for optimum phytase enzyme production from A. niger and A. flavus fungal strains. These fungal strains' crude phytase enzyme products were further subjected to enzyme stability analysis.

2. Materials and Methods

2.1. Sample Collection and Fungal Identification

For the collection of soil samples, the untreated wastewater (mixture of urban and textile industry effluents) irrigated (twice in a crop season from more than 10 years) crop fields from the vicinity (fields near Chakira) of Faisalabad, Pakistan was visited. These

samples were obtained vertically from 10-15 cm depths by removing the topsoil to access the rhizospheric zone. Samples were kept at 4 ºC until use. The soil dilution method, described by [25] was used for the preparation of soil microbial suspension. Dilutions (10^{-3} to 10^{-5}) from soil microbial suspension were utilized for the isolation of fungi. Autoclaved culture media (Potato Dextrose Agar, PDA) supplemented with 1% streptomycin was added to sterile Petri dishes and made triplicate for each dilution. The inoculated Petri dishes were incubated at 35 °C and were observed every day for 3 days. The growing fungal colonies were counted and individual fungal colonies were purified with successive sub-culturing on PDA plates. Pure fungal cultures were maintained on PDA and preserved on PDA-slants for further utilization [26,27] For identification of purified fungi, slides were prepared from the small amount of freshly growing fungi in a drop of lecto-phenol cotton blue stain. Fungal morphological features were observed under calibrated Labomed microscope (LX-400) coupled with the camera (iVU-1500). Identification up to species level was carried out using standard fungal monographs by consulting [28,29], index of fungal fungorum (CABI), and other web resources.

Different media compositions were used to screen the phosphate solubilization efficiency of fungi like Pikovskaya's medium (PVK) broth [30] supplemented with tricalcium phosphate and the National Botanical Research Institute's phosphate growth medium [31].

Phosphate solubilizing efficiency (S.E) for each fungus was calculated according to the following formula [32,33].

$$\%\ solubilizing\ efficiency = \frac{Diameter\ of\ clear\ zone\ around\ colony}{Diameter\ of\ colony} \times 100$$

2.2. Phytase Enzyme Assay

For measuring the phytase enzymes, the following method was used while considering the known concentration of KH_2PO_4 as standard. Briefly, 0.5 mL of sodium phytate (0.007 M), 0.1 mL of $MgSO_4$ (0.05 M), 0.1 mL of sodium acetate buffer (0.2 M), and enzyme solution (0.1 mL) was added to the above mixture and the mixture was incubated at 50 °C for 30 min. 1 mL of 10% tricarboxylic acid (TCA) and 2.0 mL of distilled water were mixed well with above the mixture following the addition of 5.0 mL of Taussky–Schoor reagent and reading the absorbance at 660 nm by using a spectrophotometer. One unit of phytase activity was defined as "the amount of enzyme that liberates one µmol of inorganic phosphate per mL under assay conditions". The phytase-specific activity was also calculated by dividing phytase activity with the concentration of protein in that specific assay reaction. The protein estimation was performed with Bradford reagent according to Bradford protein assay [34].

2.3. Eco-Cultural Conditions for Optimization of Phytase Production

2.3.1. Substrates and Their Concentrations Optimization

Different substrates, including Glucose-1-6-phosphate, phenyl phosphate, sodium phytate, glucose-1-phosphate, naphthyl phosphatases were used with different concentrations for optimization of phytase production from the selected fungal strains. Moreover, the effects of various carbon (lactose, glucose, galactose, maltose, fructose, sucrose, starch, and xylose) and nitrogen (casein, yeast extract, sodium nitrate, urea, ammonium nitrate, ammonium sulfate, and sodium nitrate) media additive were also monitored during the study. These parameters were optimized separately and successively in triplicates [35,36].

2.3.2. Culture Media PH, Temperature, and Inoculum Size Optimization

Different inoculum sizes (0.5–4 mL), a range of pH (2–9), and temperature (30–60 °C) were used to optimize the phytase production from the selected fungal strains. Each of these parameters was optimized separately and successively in triplicates.

2.4. Phytase Enzyme Stability Analysis

After optimization of eco-cultural conditions, the selected fungi were cultured, and the stability of their products (crude phytase) was estimated at various temperatures (10–90 °C) and pH (2–9). Moreover, the effects of various metals ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}) and enzyme inhibitors (EDTA, Ni^{2+}, Cr^{3+}, Ba^{2+}, Ag^{1+}, Al^{3+}, Co^{2+}, and Fe^{2+}) on the functionality of crude phytase enzyme was also analyzed by their addition into assay conditions. The phytase-specific activity was also calculated by dividing phytase activity with the concentration of protein in that specific assay reaction.

2.5. Phytase Test in Soil

The crude phytase enzymes from *Aspergillus niger* and *Aspergillus flavus* were added in 10 g of autoclaved garden soil in Petri dishes and incubated under room condition in the dark in a triplicate manner. The autoclaved soil without any addition served as a control. After incubation of 7 days, 2 g of soil was removed from these Petri dishes and used for measuring the phosphate contents through spectrophotometric method [37]. Standard curve was prepared with known concentrations of KH_2PO_4.

2.6. Statistical Analysis

The basic statistical analyses were carried in Microsoft Excel 2016 software. Data collected were evaluated using a two-way analysis of variance (ANOVA). Fisher's least significant difference (LSD) post hoc (Student-Newman–Keuls) test (at 0.05 significance level) was used to verify differences among means, with the values mentioned on each figure.

3. Results and Discussion

From various soil samples, 53 fungal isolates were grown on the PDA culture media and, in the beginning, simply counted with the difference in their colony color and its apparent texture through dilution plate method and direct plate method. All these isolates were successively sub-cultured repeatedly to obtain their pure cultures. All purified fungal isolates were plated on the Pikovskaya agar medium (PKV) supplemented with 0.5% tri-calcium phosphate (TCP) to estimate their phosphate solubilizing potential. Among them, only 13 fungal isolates showed the formation of a clear zone around their colonies representing the solubilizing of the insoluble tri-calcium phosphate present in the culture media. These fungi were identified based on the colony color, texture, and their microscopic morphological similarities (Table S1) with the standard fungal monographs and named accordingly. The phosphate solubilizing indices (Si) indicated that only five fungal isolates naming *Aspergillus niger* (5.3), *Aspergillus flavus* (4.3), *Aspergillus fumigatus* (4.7), *Penicillium purourogenum* (4.5) and *Mucor rouxii* (4.3) had shown the significantly higher solubilizing index for TCP (Figure 1a). These selected fungi were monitored for the days (3–11 days) required for maximum phytase specific activity and phytase enzyme production (Figure 1b,c). The same letter on bars were representing the non-significant difference at 0.05 significance level among the means of the treatment in each group of bars (Figure 1). The days (time) significantly ($p < 0.001$) affected the production of phytase enzyme in all the studied fungi (Table 1). Almost all the fungal strains showed maximum phytase activity and phytase-specific activity at day 7, which afterward decreased under standard conditions. This decrease might be due to denaturation of enzyme protein or/and reduction in the nutrient level of medium or/and fungal mycelium autolysis or/and catabolic repression [38].

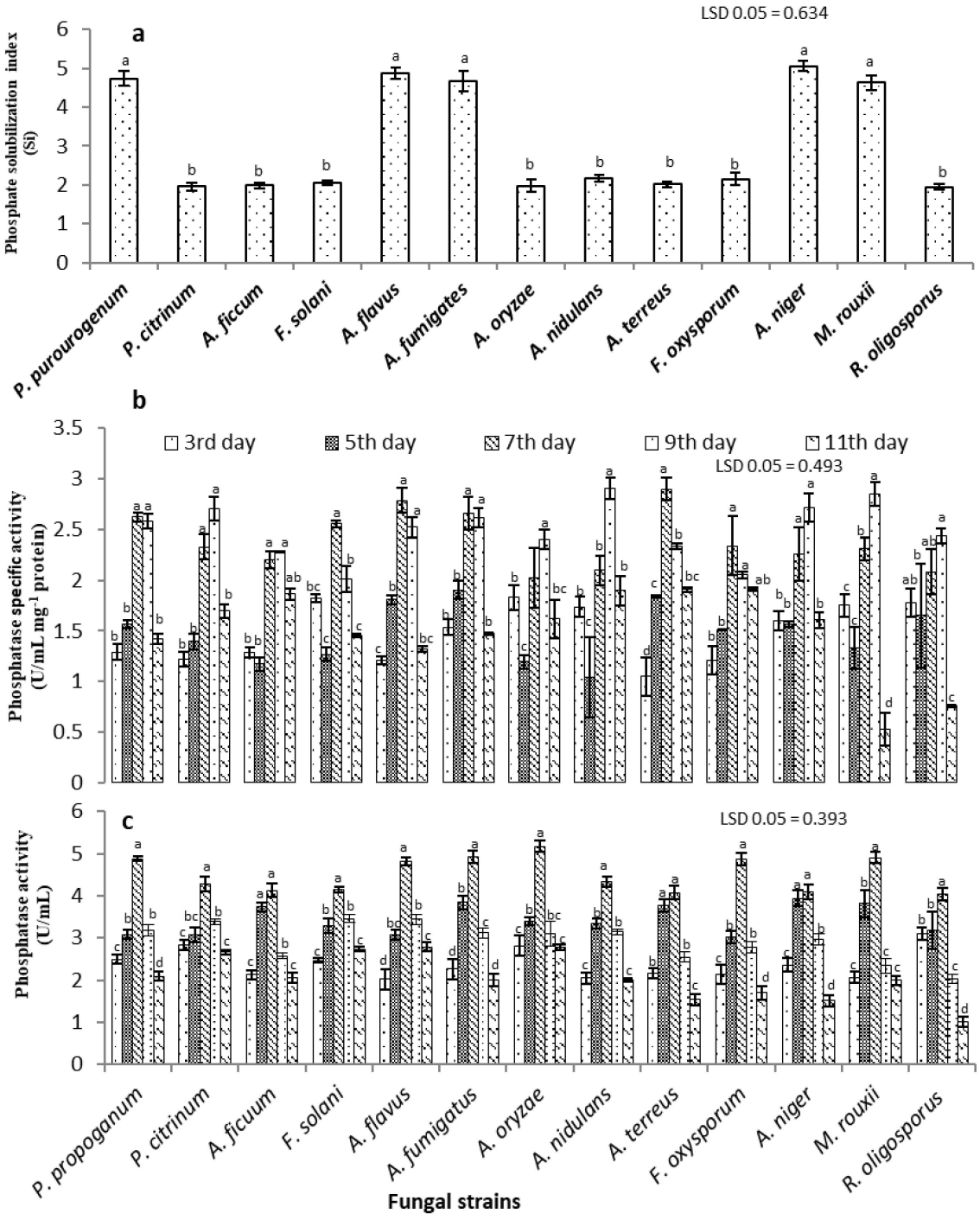

Figure 1. Screening of the 13 selected fungal strains for phosphate solubilizing index (**a**) calculated based on halo zone formation and comparison of phosphatase specific activities (**b**), phosphatase activities (**c**) at various time intervals (3–11 days). *P. = Penicillium; A. = Aspergillus; F. = Fusarium, M. = Mucor; R. = Rhizopus.* The given LSD value at 0.05 level refers to the significant differences among mean values of each treatment.

Table 1. Mean squares from analysis of variance (ANOVA) of data for phytase activity and phytase specific activity of the selected fungi during the independent experiments for each treatment. df = degree of freedom; x = interaction between the studied parameters and the selected fungi; MS = mean squares.

Nature of Study	Main Effects	Df	Phytase Activity (MS)	Phytase Specific Activity (MS)	Nature of Study	Main Effects	Df	Phytase Activity (MS)	Phytase Specific Activity (MS)
Optimization of days	Fungi	12	15.591 ***	3.795 ***	**Optimization of carbon sources**	Fungi	4	9.781 **	2.171 ns
	Days	4	31.144 ***	30.90 ***		Carbon sources	7	66.94 ***	2.171 ns
	Fungi × Days	48	5.014 ***	3.088 ***		Fungi × Carbon source	28	6.465 ***	2.171 ns
Optimization of substrates	Fungi	4	1.302 ***	0.609 ***	**Optimization of nitrogen sources**	Fungi	4	1.336 ns	1.280 ns
	Substrate	7	10.485 ***	2.327 ***		Nitrogen sources	7	14.52 ***	8.357 ***
	Fungi × Substrate	28	0.698 ***	0.361 ***		Fungi × Nitrogen sources	28	3.784 ***	1.574 ***
Optimization of substrates concentrations	Fungi	4	2.833 ***	0.038 ***	**Stability analysis with inhibitors**	Fungi	1	0.886 ns	52.290 ***
	Substrate concentrations	7	2.913 ***	0.020 ***		Inhibitor	7	2.873 **	1.540 ns
	Fungi × Substrate concentrations	28	3.206 ***	0.003 ***		Fungi × Inhibitor	7	2.344 **	4.413 ***
Optimization of inoculum size	Fungi	4	2.382 ***	0.060 **	**Stability analysis with metal ions**	Fungi	1	7.535 ***	15.978 ***
	Inoculum size	7	14.142 ***	16.65 ***		Metal ions	4	1.517 *	4.241 ***
	Fungi × Inoculum size	28	29.458 ***	0.209 ***		Fungi × Metal ions	4	0.925 ns	5.408 ***
Optimization of Temperatures	Fungi	4	2.886 **	0.562 ***	**Stability analysis at various temperatures**	Fungi	1	0.890 ns	0.071 ns
	Temperature	3	229.76 ***	1.055 ***		Temperature	8	4.941 ***	2.458 ***
	Fungi × Temperature	12	1.040 *	0.215 ***		Fungi × Temperature	8	0.197 ns	0.526 ns
Optimization of pH	Fungi	4	1.105 *	6.761 ***	**Stability analysis at various pH**	Fungi	1	2.163 **	12.682 ***
	pH	7	39.502 ***	29.30 ***		pH	7	16.83 ***	0.982 ***
	Fungi × pH	28	1.240 ***	5.127 ***		Fungi × pH	7	1.094 **	1.247 ***

ns = non-significant; *, **, *** significant at 0.05, 0.01, and 0.001 level, respectively.

For the eco-cultural optimization, various substrates Ca phytate, glucose-1-6-phosphatase, sodium phytate, glucose-1-phosphatate., AMP ADP, and ATP were used to enhance the phytase production in selected fungi (Figure 2a,b). Among all of these substrates, sodium phytate showed optimum phytase production. Following the selection of suitable substrate i.e., sodium phytate, its various concentration (50 µM to 200 µM) were also screened for maximum phytase production. Almost all the fungi were shown optimum phytase activity with sodium phytate as a substrate with 200 µM concentrations (Figure 2c,d). Increasing the sodium phytate concentration from 200 µM showed the decrease in phytase production among all the five selected fungi. The sequence of maximum phytase activity and phytase-specific activity could be represented as Na-phytate > ADP > glucose-1-phosphate > glucose-1-phosphate > AMP > glucose-1-6-phosphate > ATP > Ca-phytate. The different letter on bars were representing the significant difference at 0.05 significance level among the means of the treatment in each group of bars (Figure 2). The different substrates and concentrations of the selected substrates were significantly ($p < 0.001$) affecting the production of phytase enzyme in all the studied fungi (Table 1). The suitability of sodium phytate was also reported by [39]. Furthermore, the sequence of maximum phytase activity and phytase specific activity in relation to sodium phytate concentration was found as 50 µM $\leq$ 100 µM, $\leq$150 µM $\leq$ 200 µM $\geq$ 250 µM $\geq$ 300 µM.

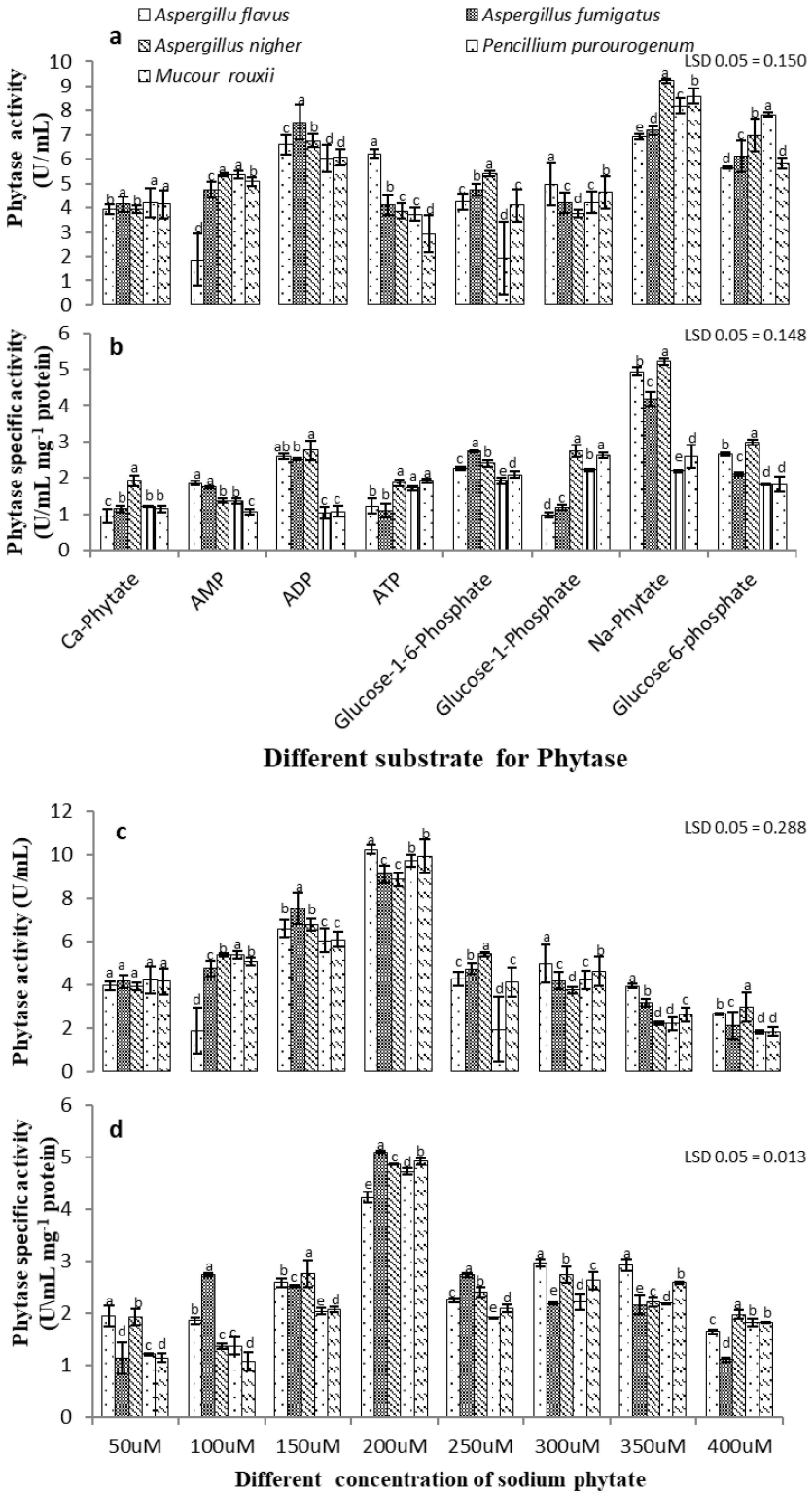

Figure 2. Different substrates for phytase and different concentrations of sodium phytate: modulation with substrates in (**a**) phytase activity and (**b**) phytase specific activity; and with different concentration of the best substrate in phytase activity (**c**) and phytase specific activity (**d**) of the selected fungi (*Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Penicillium purourogenum* and *Mucor rouxii*). The given LSD value at 0.05 level refers to the significant differences among mean values of each treatment.

After selection of suitable substrate with its appropriate concentration, optimization of fungal inoculum size (0.5 mL, 1 mL, 1.5 mL, 2 mL, 2.5 mL, 3 mL, 3.5 mL, and 4 mL) was carried out. The different levels of inoculum significantly ($p < 0.001$) affected the production of phytase enzyme in all the studied fungi (Table 1). Out of all studied fungal inoculum sizes, 1.5 mL showed appropriate starting material for phytate production (enzyme activity and specific activity) in all the selected fungi (Figure 3a,b). The observed sequential effects on phytase activity and phytase specific activity were 0.5 mL $\leq$ 1 mL $\leq$ 1.5 mL $\leq$ 2 mL $\geq$ 3 mL $\geq$ 3.5 mL $\geq$ 4 mL. *A. niger* (9 U/mL) showed the highest phytase activity while *M. rouxii* showed the lowest activity (7 U/mL) with 200 µM of sodium phytate with 1.5 mL of inoculum size. Similarly, the specific activity of *A. niger* (7 Units/mL per mg protein) and lowest *M. rouxii* (5 Units/mL per mg protein) with 200 µM of sodium phytate with 1.5 mL of fungal inoculum size. The same letters on bars represented the non-significant difference at 0.05 significance level among the means of the treatment in each group of bars (Figure 3). Similar results were also reported by [40] with the fungus *A. niger*.

Following the selection, 200 µM of sodium phytate with 1.5 mL of fungal inoculum size, different temperatures (30–60 °C), and pH (2–9) were also optimized, one after the other. The different temperatures and pH levels were significantly ($p < 0.001$) affecting the production of phytase enzyme in all the studied fungi (Table 1). Among the various temperatures, the maximum phytase activity was found in fungi *A. flavus* (10 ± 0.621 Units/mL), *A. fumigatus* (10 ± 0.691 Units/mL), *A. niger* (13 ± 0.8622 Units/mL), *P. purourogenum* (10 ± 0.421 Units/mL), and *M. rouxii* (9 ± 0.321 Units/mL) at 30 °C (Figure 3c; Table 1). Similarly, the specific activities were recorded from the fungi: *A. flavus* (7 ± 0.339 Units/mLper mg protein), *A. fumigatus* (6 ± 0.664 Units/mL per mg protein), *A. niger* (8 ± 0.871 Units/mL per mg protein), *P. purourogenum* (5 ± 0.471 Units/mL per mg protein), and *M. rouxii* (4 ± 0.321 Units/mL per mg protein) at 30 °C temperature with the above optimized conditions (Figure 3d). The *A. niger* showed significantly higher phytase activity and phytase specific activity at 50 °C while *A. flavus* showed significantly higher phytase activity and phytase specific activity at 30 °C. Thus, from these two contrasting temperatures, the lowest was selected for further optimization.

The effect of pH changes (2–9) was also studied in five selected fungal stains (*A. flavus, A. niger, A. fumigates, P. purourogenum*, and *M. rouxii*). Phytase enzyme production was found to be a maximum at pH 7 in all the studied fungi. *A. niger* showed the highest phytase activity (14 ± 0.17 Units/mL) and phytase specific activity (9 ± 0.992 Units/mL per mg protein) at pH 7 with the above optimized eco-cultural parameters. Moreover, the phytase activity was also recorded in *A. flavus* (12 ± 0.056 Units/mL), *A. fumigatus* (10 ± 0.849 Units/mL), *P. purourogenum* (10 ± 0.027 Units/mL), and *M. rouxii* (10 ± 0.801 Units/mL), which also represented enhanced production of the enzymes. While the phytase specific activities of *A. flavus* (5 ± 0.059 Units/mL per mg protein), *A. fumigatus* (6 ± 0.039 Units/mL per mg protein), *A. niger* (6 ± 0.790 Units/mL per mg protein), *P. purourogenum* (6 ± 0.027 Units/mL per mg protein), and *Mucor rouxii* (6 ± 0.080 Units/mL per mg protein) were also found to be representing the order of phytase production as follows: *A. niger* > *A. flavus* > *A. fumigatus* > *P. purourogenum* > *M. rouxii* (Figure 3e,f) at pH 7. The different letters on bars were representing the significant difference at 0.05 significance level among the means of the treatment in each group of bars (Figure 3).

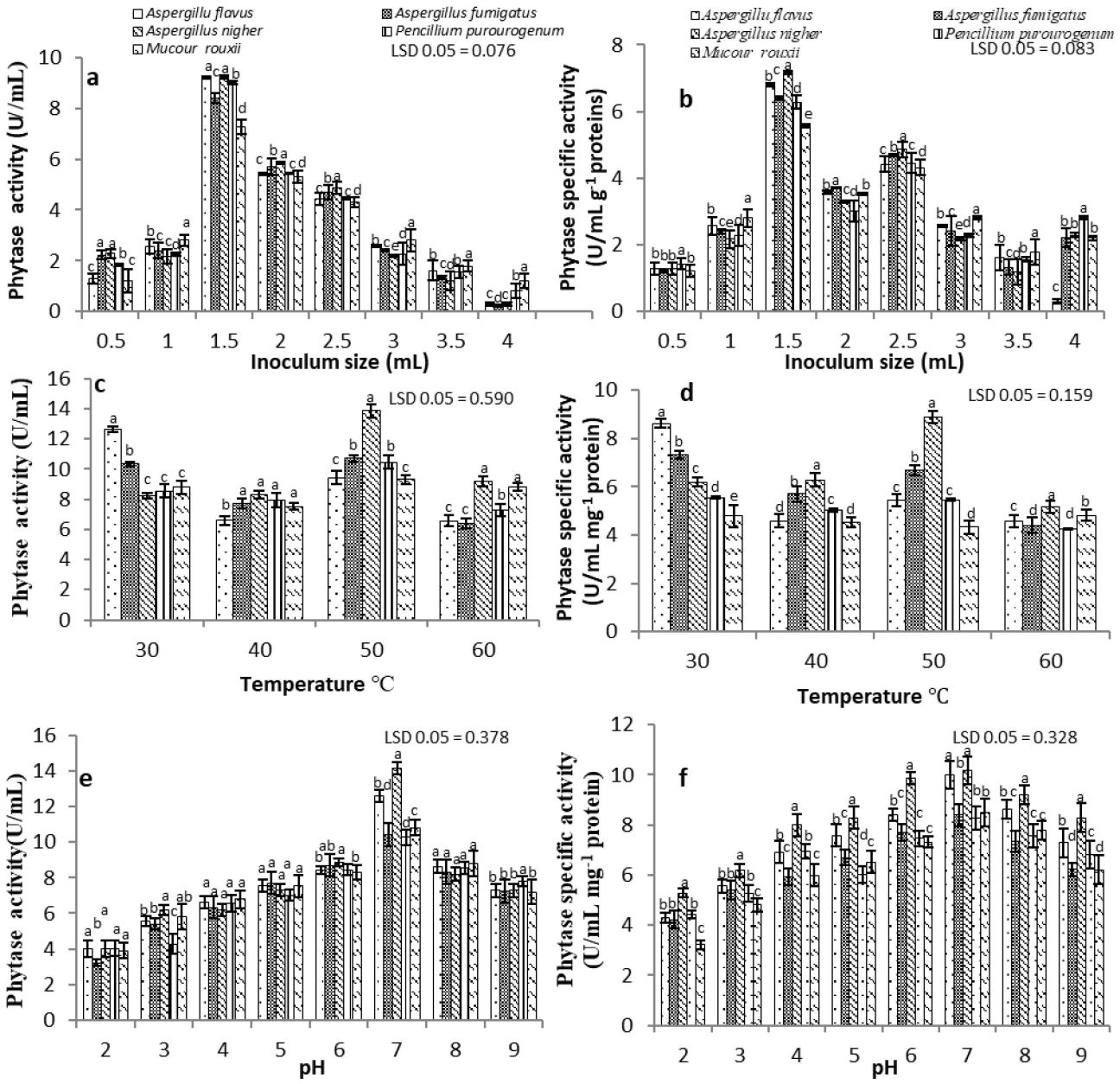

Figure 3. Effect of various eco-cultural conditions (inoculum size, temperature, and pH) on phytase activity (**a**,**c**,**e**) and phytase specific activity (**b**,**d**,**f**) from selected fungi (*Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Penicillium purourogenum*, and *Mucor rouxii*) having higher inherent phytase production potential. The given LSD value at 0.05 level refers to the significant differences among mean values of each treatment.

Furthermore, in addition to the above optimized conditions, the effect of various carbon (lactose, glucose, galactose, maltose, fructose, sucrose, starch and xylose) and nitrogen (casein, yeast extract, sodium nitrate, urea, ammonium nitrate, ammonium sulfate, and sodium nitrate) as media additive on phytase production was also studied (Figure 4). The addition of different carbon sources significantly ($p < 0.001$) affected the production of phytase enzyme while showing non-significant effects of phytase specific activity in all the studied fungi (Table 1). The obtained results indicated that among carbon sources, addition of glucose showed optimum phytase activity with *A. niger* (16 ± 0.33 Units/mL), *A. flavus* (14 ± 0.33 Units/mL), *A. fumigatus* (13 ± 0.33 Units/mL), *P. purourgenum* (12 ± 0.33 Units/mL), and *M. rouxii* (10 ± 012 Units/mL) while the lowest

come with the addition of xylose in the media (Figure 4a). A similar trend was also found in the case of phytase-specific activity (Figure 4b). The same letters on the bars represented the non-significant difference at 0.05 significance level among the means of the treatment in each group of bars (Figure 4). Glucose is the simplest carbon source, and was reported by many authors to yield higher fungal biomass corresponding to higher phytase production with *A. niger* [18,41,42].

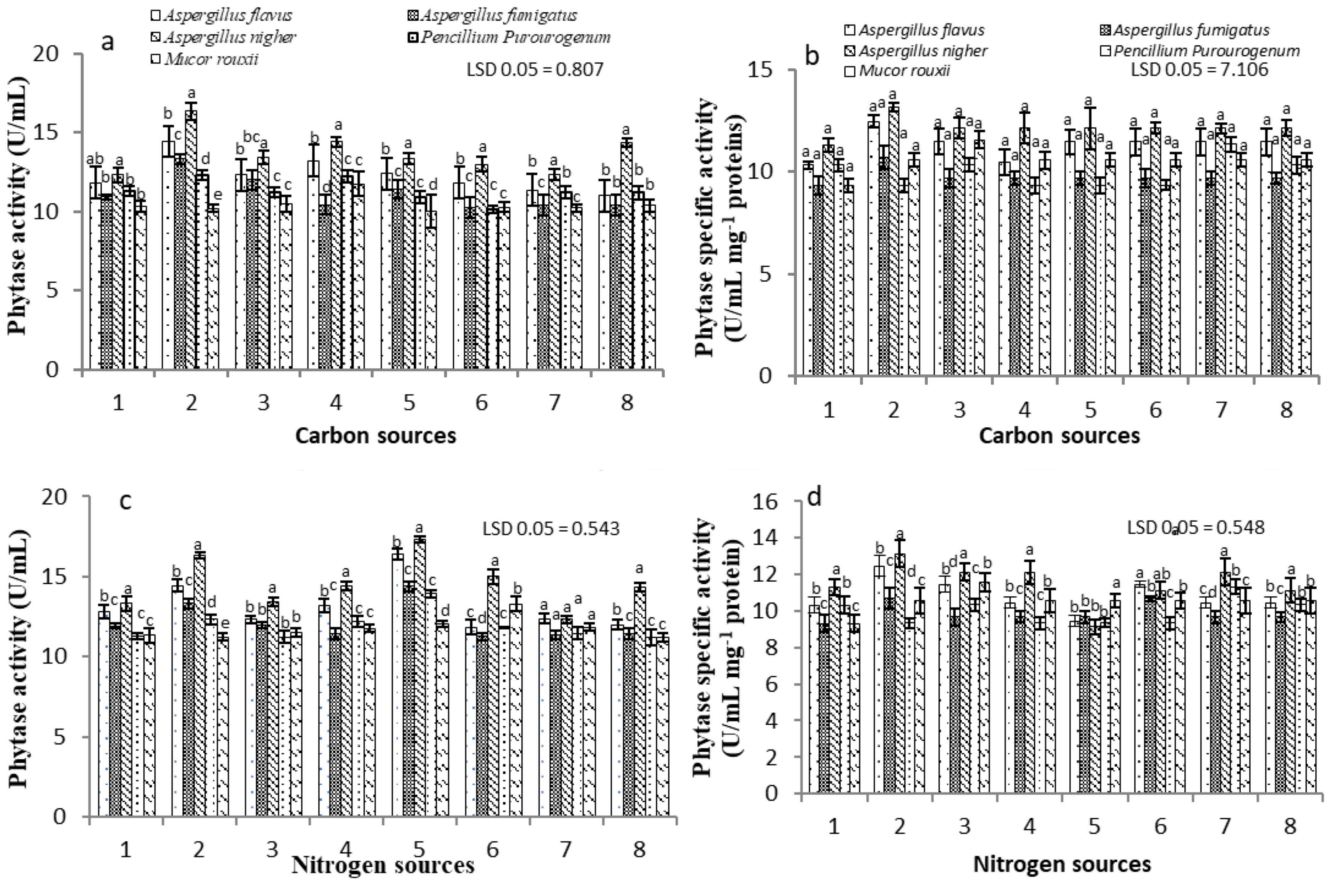

Figure 4. Effect of various culture medium additives, such as carbon (1–8), lactose, glucose, galactose, maltose, fructose, sucrose, xylose, and starch; and nitrogen sources: (1–8) casein, yeast extract, sodium nitrate, urea, ammonium nitrate, ammonium sulfate, and sodium nitrate, respectively, on phytase activity (**a**,**c**) and phytase specific activity (**b**,**d**) of selected fungi (*Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Penicillium purourogenum,* and *Mucor rouxii*) having inherent phytase production potential. The given LSD value at 0.05 level refers to the significant differences among mean values of each treatment.

On the other hand, the effects of different nitrogen sources as cultural media additives (casein, yeast extract, sodium nitrate, urea, ammonium nitrate, ammonium sulfate and sodium nitrate) showed that the highest phytase activity (17 ± 0.033 Units/mL) and phytase specific activity (13 ± 0.012 Units/mL per mg protein) were obtained with the addition of ammonium nitrate by *A. niger* followed by *A. flavus* (16 ± 0.033 Units/mL) with phytase specific activity (10 ± 0.066 Units/mL per mg protein), *A. fumigatus* (14 ± 0.033 Units/mL) with phytase specific activity (10 ± 0.056 Units/mL per mg protein), *Mucor rouxii* (13 ± 0.031 Units/mL) with phytase specific activity (10 ± 0.56 Units/mL per mg protein), and *P. purourogenum* (12 ± 0.012 Units/mL) with its phytase specific activity (10 ± 0.031 Units/mL per mg protein). The different nitrogen additives significantly ($p < 0.001$) affected the production of phytase enzyme while showing non-significant effects within the studied fungi (Table 1). The different letters on the bars represented the significant difference at 0.05 significance level among the means of the treatment in each group of bars (Figure 4). The utiliza-

tion of ammonium nitrate as the nitrogen source in culture medium for maximum phytase production was also reported by experiments with *A. niger* and *Rhizopus oligosporus* [40,43] but with solid-state fermentation (SSF). In conclusion, the *A. niger* and *A. flavus* were selected on the basis of their optimum enzyme production under optimized eco-cultural conditions (i.e., 200 µM sodium phytate with 1.5 mL of fungal inoculum, pH 7, temperature 30 °C, glucose, and ammonium nitrate as carbon and nitrogen media additives) for the crude phytase stability analysis.

Various parameters responsible for proper enzyme functionality were studied, including pH (2–9), temperature (10–90 °C), the addition of metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+}), and enzyme inhibitors (EDTA, Ni^{2+}, Cr^{3+}, Ba^{2+}, Ag^{1+}, Al^{3+}, Co^{2+} and Fe^{2+}) during the enzymatic reaction of unpurified/crude phytase enzyme. The different letters on the bars represented the significant difference at 0.05 significance level among the means of the treatment in each group of bars (Figure 5). The results showed that phytase activity and specific phytase activity by *A. niger* and *A. flavus* were highest at pH 7, representing its highest stability (Figure 5a,b). The changes in pH significantly ($p < 0.001$) affected the production of phytase enzyme and its specific activity while also showing significant ($p < 0.01$) effects within studied fungi (Table 1).

The stable temperature for phytase reaction was found to be 50 °C for *A. niger* phytase and 30 °C for phytase produced by *A. flavus* indicated by their phytase activity and specific activity (Figure 5c,d). The stability trend of phytase enzymatic reaction (phytase activity and phytase specific activity) of *A. niger* was found to be 10 °C ≤ 20 °C ≤ 30 °C ≤ 40 °C ≤ 50 °C ≥ 60° C ≥ 70 °C ≥ 80 °C ≥ 90 °C while for the product of *A. flavus*, it was found to be 10 °C ≤ 20 °C ≤ 30 °C ≥ 40 °C ≥ 50 °C ≥ 60 °C ≥ 70 °C ≥ 80 °C ≥ 90 °C. The temperature variations significantly ($p < 0.001$) affected the production of phytase enzyme while showing non-significant effects within the studied fungi (Table 1).

Enzymatic reactions are e susceptible to various metabolic ions. To check the stability of crude phytase enzymes from both of the sources, different metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}) were studied under assay conditions. Results showed that phytase activity and its specific activity was higher with the addition of Ca^{2+}, Mg^{2+}, and Mn^{2+} as compared with Cu^{2+} and Zn^{2+} (Figure 5e,f). The metallic ion addition to the enzymatic reaction significantly ($p < 0.05$) affected the production of phytase enzyme while showing highly significant ($p < 0.001$) effects on phytase specific activity within the studied fungi (Table 1). The order of the effects with the addition of metals ions (lowest to highest) was Ca^{2+} > Mg^{2+} > Mn^{2+} > Cu^{2+} > Zn^{2+}. These metal ions were also reported as playing a significantly important role in fungal growth and development [44,45].

The effects of various enzymes inhibitors were also observed: Ba^{2+}, EDTA (ethylenediaminetetraacetic acid), and Ni^{2+} were mild inhibitors, whereas Fe^{2+}, Al^{+3}, and Ba^{2+} were found to be moderate inhibitors, while Cr^{3+} and Co^{2+} strongly inhibited the phytate activity and phytate specific activity of the enzyme produced by *A. niger* and *A. flavus* (Figure 5g,h). The maximum and minimum phytase activity for *A. niger* was found as 15 ± 0.66 and 5 ± 0.078 (Units/mL), and similarly for *A. flavus* as 14 ± 0.090 and 5 ± 0.096 (Units/mL), by the addition of EDTA and Co^{2+} in the enzymatic reaction. The order (lowest to highest) of the inhibition of phytase activity was observed as EDTA > Ba^{2+} > Ni^{2+} > Fe^{2+} > Al^{3+} > Ag^{1+} > Cr^{3+} > Co^{2+} as well as in case of phytase specific activity of these two selected fungi with the addition of inhibitors. The inhibitors' addition to the enzymatic reaction significantly ($p < 0.01$) affected the production of phytase enzyme, while showing non-significant effects on phytase specific activity as well as on the studied fungi (Table 1). The inhibitory effects of metal ions on phytase enzyme activity were also reported by [46]. These inhibitory effects of metallic ions on phytase activity and its specific activity might be due to their function as strong chelation with phytate by blocking the phytate functional groups, resulting in strongly decreased phytase activity [46]. The addition of crude phytase enzymes of *A. niger* and *A. flavus* in sterilized soil significantly increased the phosphate concentrations as compared with control soils after incubation for 7 and 40 days (Figure S1). The involve-

ment of microbial phytases in phytate solubilization in soils was also reported [47,48] and essentially requires further exploration for sustainable agricultural production.

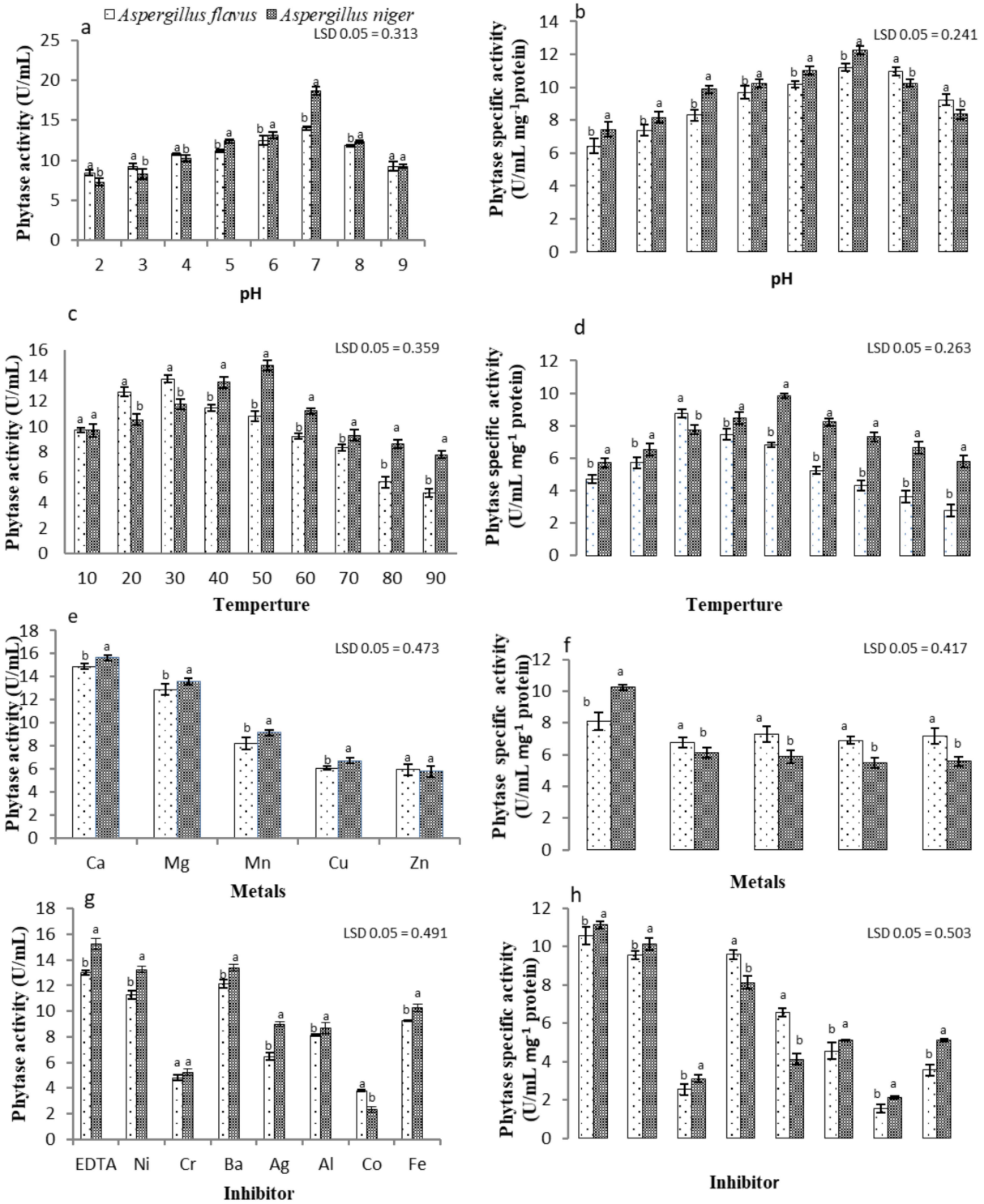

Figure 5. Stability analysis of crude phytase enzyme from two selected hyper producing fungal strains of *Aspergillus flavus* and *Aspergillus niger* under optimized eco-cultural conditions. The effects of different pH (**a**,**b**), temperature (**c**,**d**), addition of metal ions (**e**,**f**), and inhibitors (**g**,**h**) on phytase activity and phytase specific activity under standard assayed conditions. The given LSD value at 0.05 level refers to the significant differences among mean values of each treatment.

4. Conclusions

In conclusion, results have shown that *A. niger* and *A. flavus* were selected based on their optimum enzyme production under optimized eco-cultural conditions. The optimized cultural conditions were as follows: 200 µM sodium phytate with 1.5 mL of fungal inoculum, pH 7, temperature 30 °C, glucose, and ammonium nitrate as carbon and nitrogen media additives for the crude phytase stability analysis. Moreover, the results of enzyme stability analysis suggested that the phytase enzyme obtained from these fungi were found to be almost stable for a wide range of conditions, including pH (4–8), temperature (20–60 °C), insensitive to Ca^{2+} and Mg^{2+} ions, and EDTA, Ni^{2+} and Ba^{2+} inhibitors, but highly sensitive to Mn^{2+}, Cu^{2+}, and Zn^{2+} ions, and Co^{2+}, Cr^{3+}, Al^{3+}, Fe^{2+} and Ag^{1+} inhibitors. This enzyme stability suggests them to be good candidates for their application in the complex continuum of soil to degrade phytate, an untapped phosphorus source, and to liberate phosphate, which could be used by the plants. Hence, this could be a good substitution for artificial fertilizers, which are considered as a source of environmental pollution and responsible for the loss of natural soil fertility.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12010103/s1, Figure S1: Effects of crude phytase enzymes of *Aspergillus niger* and *Aspergillus flavus* on phosphate mineralization in sterilized garden soil under room conditions, Table S1: Characteristic morphological features of fungal strains based on colony appearance and microscopic structures.

Author Contributions: Conceptualization, N.S.; data curation, N.S., A.A. and N.I.; formal analysis, M.Z.H. and N.I.; funding acquisition, M.Z.H., M.H.A. and A.A.; investigation, M.Z.H. and A.A.; methodology, N.S. and N.I.; project administration, M.H.A.; resources, M.Z.H.; writing—original draft, N.I., M.H.A. and A.A.; Writing—review and editing, N.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was partially funded by National Research program for Universities (NRPU) of HEC, Pakistan, grant number 10309/Punjab/NRPU/R&D/HEC/2017.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The dataset generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: The reported study is a part of the doctoral thesis of Naila Sadaf, Reg. No. 2011-GCUF-05137 from the Department of Botany, Government College University, Faisalabad. The authors would like to express their deepest gratitude to the University of Tabuk for providing technical support for this study.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

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