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Phytase Producing Fungi and Their Efficiency in Hydrolyzing Phytin-P Compounds

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Abstract: Five most efficient phytase producing fungi belonging to genus *Aspergillus, Emericella, Gliocladium, Penicillium* and *Trichoderma* were isolated from arid and semi-arid soils and tested for their efficiency in hydrolyzing phytin-P compounds. *Penicillium purpurogenum* accumulated more biomass closely followed by *Trichoderma harzianum* during the growth period. A strong negative correlation ($r = -0.91$, $n = 20$, $P < 0.01$) was observed between development of fungal mat and pH of the media. The extracellular phytase released by the organisms was 12.7 times more than their intracellular phytase. The extracellular phytase activity was more in *Emericella rugulosa*, whereas intracellular phytase activity was more in *Trichoderma harzianum*. *Emericella rugulosa* was found to be the most efficient in hydrolyzing phytin-P (98.82 $\mu g\ g^{-1}$). The results indicated that *Emericella rugulosa* can be used as a biofertilizer under arid and semi-arid environments for native P mobilization.

Key words: Fungi, efficiency, phytase, phytin-P, hydrolysis.

Phosphorus is one of the most essential macronutrients required for the growth and development of plants, but in most agricultural soils its content is about 0.05%, of which only 0.1% is available to plants. Deficiency of soil P is one of the most important nutrient restricting plant growth. Therefore, large quantity of soluble forms of P fertilizers is applied to achieve maximum plant productivity. However, the applied soluble forms of P fertilizers are easily precipitated into insoluble forms and are not efficiently taken up by the plants, which lead to an excess application of P fertilizers to cropland (Omar, 1998). In general, 20-80% of the total P in all agricultural soils is present in organic form, which includes a range of inositol phosphate esters, phospholipids, nucleic acids, phosphate linked to sugars and derivatives of phosphoric acid. More than 50% of the organic P in soil is present in phytin form, which could be hydrolyzed by phytase. Plants can use phosphorus from organic sources generally only after hydrolysis of the C-O-P ester bond by phosphatases or phytase and the release of plant available P ($H_2PO_4^-$, $HPO_4^=$) as inorganic phosphate. Several types of phosphatases, such as phytases, are able to increase the rate of the hydrolysis of organic P. Phosphatases in the rhizosphere may arise from plant roots and soil microorganisms (Tarafdar *et al.*, 2001). Soil microorganisms that solubilize mineral P can significantly affect phosphorus cycling in both natural and agricultural ecosystems. Microbial acid phosphatase was found to be more efficient in hydrolysis of organic P compounds than plant sources (Tarafdar *et al.*, 2001). However, the potential role of phytases in increasing the availability of P from phytate in soils remains to be established. During an examination of the breakdown of organophosphorus compounds it was found necessary to determine the potential phytase activity for a large number of soil microbes.

The aim of this study was to examine the efficiency of phytase produced by different organisms to hydrolyze phytin-P compounds so that the amount of P mobilized by different fungi could be quantified and the most efficient fungus could be used as inoculum to exploit native soil organic phosphorus for plant growth as more than 70% of total organic P under arid and semi-arid soils is present as phytin.

Materials and Methods

Fungi were isolated from twenty-seven diverse types of Indian soils from arid and semi-arid areas (Table 1), using a dilution plate technique on Martin's Rose Bengal agar containing streptomycin sulphate (Allen, 1959). Twenty phytase-producing fungi were isolated, purified from the single spore in slants, identified by Agharkar Research Institute, Pune, India. The pure cultures were maintained on potato dextrose agar (PDA) medium. Based on their intra- and extra-cellular phytase activity

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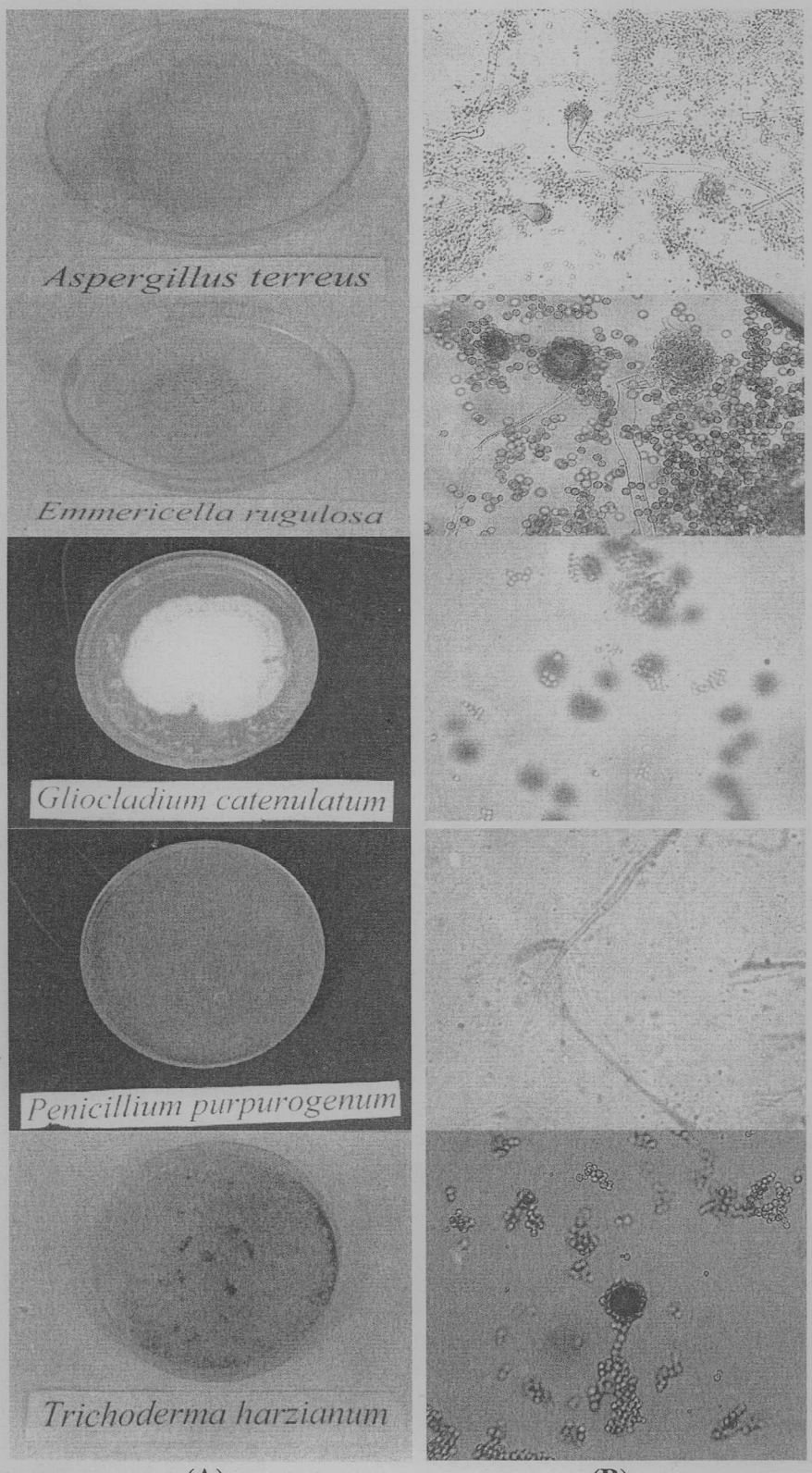

Fig. 1. Different identified phytase releasing fungi along with spores (A) growth in Petri plate (B) spore (x 312.5).

Table 1. Physico-chemical characteristics of soils used

Place	No. of samples	Soil Characteristics			Vegetation
		pH	EC	OC (g kg^{-1})	
Arid region					
Jodhpur	2	8.7±0.20	0.23±0.05	0.32±0.03	Pearl millet, clusterbean
Bilara	2	9.1±0.15	0.85±0.09	0.34±0.03	Pearl millet, mung bean
Ossian	2	8.6±0.24	0.15±0.02	0.36±0.04	Mung bean, moth bean
Tiwari	2	8.5±0.22	0.16±0.02	0.36±0.04	Mung bean, vegetables
Semi-arid region					
Udaipur	2	7.9±0.24	0.48±0.03	6.9±0.12	Maize, sorghum
Chittorgarh	2	7.8±0.22	0.46±0.04	5.8±0.09	Maize, sugarcane
Banswara	2	7.6±0.23	0.45±0.03	6.2±0.13	Maize, rice
Nagpur	5	8.7±0.85	0.19±0.08	4.5±0.54	Fruit orchard, sorghum, cotton
Dharwad	4	8.9±0.19	0.18±0.03	3.2±0.85	Rice, sugarcane, cotton
Banglore	4	8.4±0.45	0.76±0.08	3.6±0.29	Rice, cotton, sugarcane

(data not shown) the best five fungi were selected for further study.

For determination of extracellular phytase activity, fungi were grown in 100 mL Czapek-Dox broth in 250 mL Erlenmeyer flasks. The medium was inoculated with 8 mm discs of 4-day-old fungal growth (on PDA medium) and the 12 flasks of each culture were incubated at 30±1°C. At the end of 7, 14, 21 and 28 days after incubation, three flasks of each fungal culture were harvested. The flasks were chilled in ice and the contents were filtered through Whatman No. 1 filter paper into another flask kept in ice. The final volume of each filtrate was made upto 50 mL using sterilized cold distilled water and the filtrate was used for assaying the extracellular phytase activity.

For determination of intra-cellular phytase activity, fungal mats at each sampling were washed at least 10 times with ice-cold distilled water (10 mL each time) to remove traces of extra-cellular enzymes. Washed fungal mats were weighed after lightly pressing them between sheets of filter paper. A small part of the mat was dried to express the results on dry weight basis. The remaining part was ground with acid-washed quartz sand in a mortar. Ice-cold sterilized distilled water was added to obtain a fine suspension of fungal mat. The extract obtained was centrifuged at 12,000 rpm for 20 minutes to settle the fungal debris. A clear supernatant containing the intra-cellular enzymes was obtained and made up to a known volume.

Phytase activity was assayed by measuring inorganic phosphate (Pi) hydrolyzed from sodium phytate in acetate buffer (pH 4.5) incubating at 37°C for 1h (Ames, 1966). The activity was expressed in terms of enzyme unit (EU). One unit of phytase activity was defined as the amount of enzyme, which liberated 1M Pi per second.

Phytase enzymes generated from selected fungi were tested towards hydrolysis of phytate (phytin-P) compounds. Exactly 1 mL of 300 µg mL^{-1} (50 g mL^{-1} in case of soil solution) solutions of phytin-P, were added into 150 mL of Erlenmeyer flasks containing 50 mL of phytase of known concentration originated from fungal sources. The flasks were incubated at 30±1°C for 24 h and the release of inorganic P was determined at 2, 4, 6 and 24 h by standard method (Jackson, 1967).

Results and Discussion

The five most efficient phytase producing fungi isolated belonged to the genera: *Aspergillus, Emericella, Gliocladium, Penicillium* and *Trichoderma* (Fig. 1). The pH of the medium declined progressively with time after inoculation of five phytase producing fungi. The more proton release (pH changes from 7.3 to 2.26) was observed with *Penicillium purpurogenum* followed by *Trichoderma harzianum* (7.3 to 5.72) and less proton release was observed with *Emericella rugulosa* (7.3 to 6.20) closely followed by *Gliocladium catenulatum* (7.3 to 6.16) after 4 weeks of growth (Table 1). In general, the proton release in the medium followed the order: *Penicillium purpurogenum* > *Trichoderma harzianum* > *Gliocladium catenulatum* > *Emericella rugulosa*. The change in pH is an important criterion as it regulates the P release. The reduction of culture pH with time is due to the release of

Table 2. pH reduction, biomass accumulation and phytin-P hydrolysis by efficient isolated phytase producing fungi

Fungal species	pH* (after 4 weeks)	Biomass (g) (after 4 weeks)	Phytin-P hydrolysis ($\mu g\ g^{-1}$)
Aspergillus terreus	5.80	4.25	45.26
Emericella rugulosa	6.20	2.16	98.82
Gliocladium catenulatum	6.16	2.26	89.18
Penicillium purpurogenum	2.96	4.50	46.10
Trichoderma harzianum	5.72	4.30	46.14
LSD (P=0.05)	0.33	0.57	6.43

* Original media pH was 7.3.

different organic acids by different fungi, as production of organic acid such as malate, citrate and oxalate by microorganisms is well known (Cunningham and Kuiack, 1992; Illmer and Schinner, 1995; Jones, 1998; Reyes *et al.*, 1999).

Gradual increase in fungal biomass was observed with time (28 days) with all the phytase producing fungi tested. *Penicillium purpurogenum* accumulated maximum biomass (4.50 g), and was closely followed by *Trichoderma harzianum* (4.30 g) and *Aspergillus terreus* (4.25 g). The rate of increase in accumulation of fungal biomass declined with time. The less biomass (2.16 g) was accumulated by *Emericella rugulosa* among the phytase releasing fungi isolated (Table 2). A strong negative correlation ($r = -0.91$, $n = 20$, $P < 0.01$) was observed between development of fungal mat and pH of the media which may be due to the affinity of the fungi towards acidity. Similar relationship was also observed with pH verses phytase activities in the soil ($r = -0.68$, $n = 20$, $P < 0.01$). However, there was marginal correlation with the biomass accumulation and phytase activity ($r = 0.42$, $n = 20$) indicating isolated organisms are not equally efficient in releasing phytase.

The selected fungi released more phytase (both intra- and extra-cellular) at 21 days of growth (Fig. 2). The extra-cellular phytase released was 12.7 times higher than their intra-cellular counterpart. *Emericella rugulosa* released more extracellular phytase followed by *Trichoderma harzianum*, whereas *Trichoderma harzianum* and *Emericella rugulosa* released more intra-cellular phytase. Phytase released (both intra- and extra-cellular) was less noticed by *Gliocladium catenulatum* among all the five best phytase releasing fungi isolated. Higher extra-cellular than intra- cellular phytase activity may suggest a higher membrane

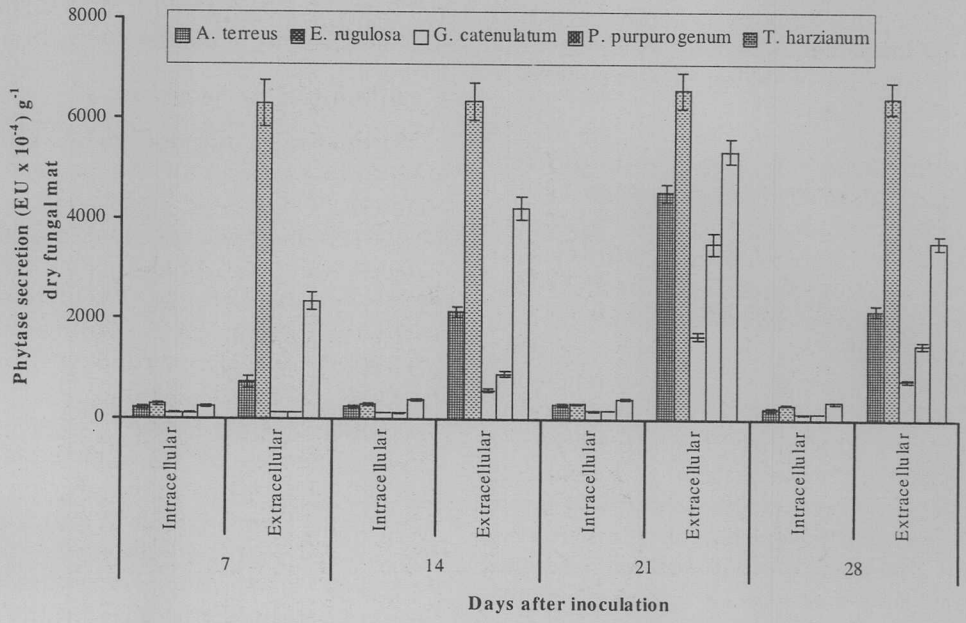

Fig. 2. Secretion of phytase by different efficient fungi at different time intervals. Vertical bars are LSD (P=0.05).

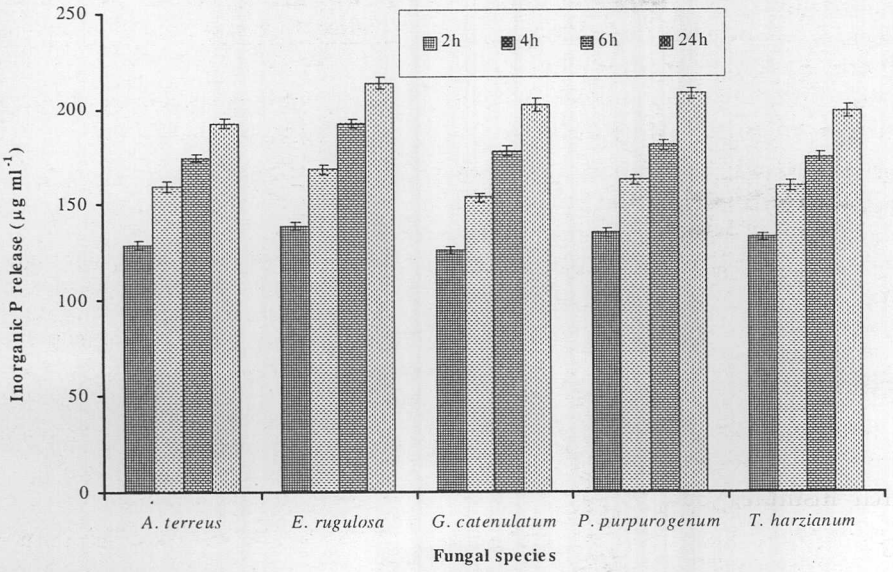

Fig. 3. Release of inorganic P with time from phytin-P by the action of phytase secreted by different fungi. Vertical bars are LSD (P=0.05).

permeability for phytase. The other reason would be that part of the phytases may be mainly located at/near the surface, which resulted in their higher release as extra-cellular enzyme. The decline in the activity of the enzymes after 21 days might be due to the on set of stationary phase in fungal culture. The results obtained in the present study support the hypothesis proposed by Yadav and Tarafdar (2003).

The efficiency of fungal phytase to hydrolyze phytin-P compounds increases with time up to 24 h of incubation (Fig. 3). *Emericella rugulosa* was the most and *Aspergillus terreus* the least efficient in hydrolyzing phytin-P (Table 2). After 24 h of incubation *Emericella rugulosa* hydrolyzed maximum (67%) of the available phytin-P present in soil solution followed by *Penicillium purpurogenum* (65%) and *Gliocladium catennulatum* (63%) (Fig. 4). There was significant increase in release of phytin-P with increase in incubation time in all the fungi tested till 24 h. The different fungi had differences in capable of hydrolyzing

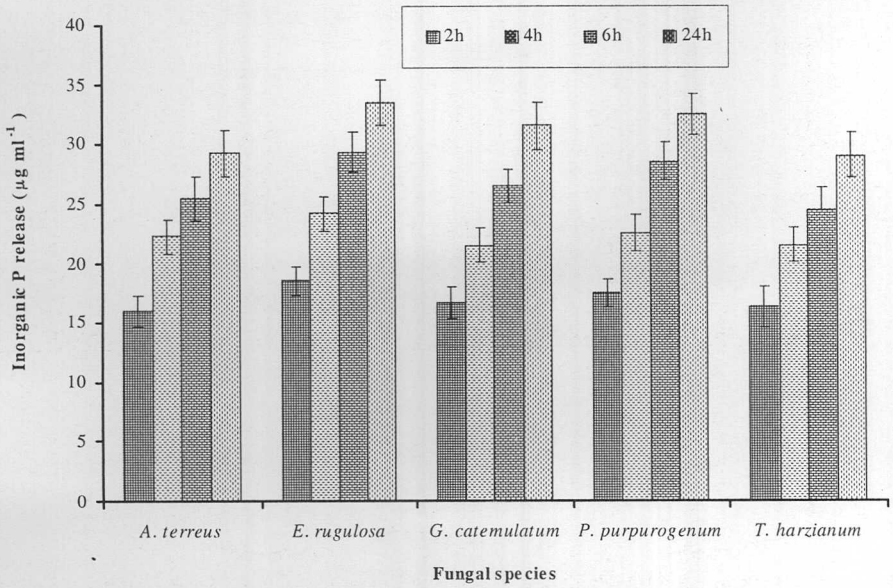

Fig. 4. Release of inorganic P with time from phytin-P present in soil solution by phytase secreted by different fungi. Vertical bars are LSD (P=0.05).

phytin-P with the application of same amount of phytase (Figs. 3, 4). Besides the cleavage of C-O-P ester bond by fungal phytase the fungi may also produce different organic acids, which may possibly help in greater release of Pi.

The present results clearly demonstrated that *Emericella rugulosa* hydrolyses 98.82 μg g^{-1} phytin-P and may be used as biological phosphorus fertilizer under arid and semi-arid areas where more than 70% organic P present is in phytin form.

Acknowledgements

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