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Screening of Protease Producing Fungi for Microbial Digestion of Seed Proteins and Synthesis of Amino Acids-metalnutrient Chelates

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Abstract: The problem of metalnutrient deficiency is becoming more serious with the introduction of modern agricultural practices. As a result, metalnutrient deficiency is recognized as one of the critical yield limiting factors. Metalnutrients are generally offered in their sulphate or oxide forms. However, it is reported that organically bound minerals generally have a higher bioavailability than inorganic minerals. Chelation makes otherwise unavailable metalnutrients plant available. Amino acids are well known among various chelating agents. In present investigation the fungus *Paecilomyces variotii* PR-4 was isolated from soil and was used for production of protease and determination of its activity. Proteins from germinating seeds of chick pea, mung bean, soybean and cowpea were hydrolyzed for the production of amino acids. Amino acids were recovered, estimated and utilized for chelation of metalnutrients viz., Zn, Cu, Fe, Mn, Mg, B and Mo. The resultant chelates were employed to detect with Fourier Transform Infra-Red Spectrophotometer (FTIR) analysis. The peaks of most intensive bands in the IR spectra of ligands recorded were present in the intervals of the wave numbers 3500-3300 and 1720-1700 cm^{-1}. Chelation of metalnutrients led to the broadening of peak and changes of the peak position of hydroxyl groups, which indicated the binding of the carboxylic groups and primary amine groups of amino acids to the metalnutrients. The resultant amino acids-metalnutrient chelates can be utilized as organic fertilizer.

Key words: Chelate, FTIR, metalnutrients, *Paecilomyces variotii* PR-4, seed amino acids, seed proteins

INTRODUCTION

It is well known that plants require 17 essential elements for healthy growth. Nutrients are grouped into two main categories: macronutrients and micronutrients. These nutrients must be available at the proper time of the crop cycle relative to growth, bloom, fruit setting and fruit maturation. Micronutrients are usually required in minute quantities, nevertheless are vital to the growth of plant (Benepal, 1967). They improve general condition of plants and are known to act as catalysts in promoting organic reactions taking place in plant (Patil *et al*., 2008). Metalnutrients are generally offered to the plants by adding to soil or as foliar spray. Still it is realized that productivity of crop is being adversely affected in different areas due to deficiencies of metalnutrients (Bose and Tripathi, 1996) recently, which has been increased markedly due to intensive cropping, loss of top soil by erosion, loss of metalnutrients by leaching, inorganic nature of metalnutrients, liming of soil and decreased availability (Fageria *et al*., 2002).

The term chelate was first coined by Morgan and Drew (1920), they define it as, "A chemical compound in the form of a heterocyclic ring, containing a metal ion attached by coordinate bonds to at least two nonmetal ions" and the phenomenon is known as 'Chelation'. Chelation makes otherwise unavailable metalnutrients plant available (AAPFCO, 1998). Chelated nutrients are more plant available than complexed nutrients. If a metal cation is joined with an organic compound at two or more exchange sites to form a ring structure, then that structure is considered a metal chelate (Meister, 1999). There are some synthetic chelating agents that have the ability of making strong chelate being used widely. EDTA and EDDHA are well known as synthetic chelating agents. Natural chelators as normal molecular weight compounds e.g., humic and fulvic acid, amino acids, polyflavanoids that have long organic chains and low molecular weight compounds e.g., citric acid, ascorbic acid, tartaric acid that have short organic chains can diffuse easily to cell cytoplasm according to their chemical structure. These natural chelators are not phytotoxic to plants.

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Two or three amino acids can bind to a metal to form a chelate (Ashmead, 1986). The amino acids used to complex or chelate cation metalnutrients include glycine, lysine, glutamic acid (Miller, 1998), cysteine and histidine (Baker and Ammerman, 1995). The amino acids can be produced from natural sources like proteinaceous seeds, egg albumin, milk, meat etc.; using enzymatic hydrolysis. Leguminous seeds are considered as the most important source of dietary proteins. Normally, leguminous seeds have large protein contents, ranging from 20% to as much as 40% of their dry matter, according to species, genotypes within species and environments (Norton *et al*., 1985). Storage proteins in leguminous seeds are mainly located in the cotyledonary tissues. Also embryonic axis and testas contribute little to the total seed protein content, mainly because those components represent small proportions of the seed mass. Globulins are the major storage proteins in legume seeds which usually account for about 70% of the total protein after that remaining are glutelins (10-20%) and albumins (10-20%). The principal storage globulins in most legumes are legumin and vicilin, the latter predominating in common bean (Jansman, 1996).

Since amino acids are building blocks of proteins, it is possible to hydrolyze proteins with protease enzymes to their constituent amino acids. Earlier to 1950, most of amino acids were produced by the denaturing and hydrolysis of various protein sources (Araki and Ozeki, 1991).

A variety of microorganisms such as bacteria, fungi, yeast and actinomycetes are known to produce protease enzymes (Madan *et al*., 2002). Potential use of fungal proteases is being increasingly realized (Joo *et al*., 2001); they offer a distinct advantage over bacterial enzymes in terms of downstream processing. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products (Kalisz, 1988). Fungi of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases (Sandhya *et al*., 2005). Hajji *et al*. (2008) have been recently identified *Aspergillus clavatus* ES1 as an extracellular bleaching stable alkaline protease producer.

The aim of present investigation was to screen protease producing fungus, hydrolysis of seed proteins and synthesis of amino acids - metalnutrient chelates.

MATERIALS AND METHODS

All chemicals required were procured from Qualigens Fine Chemicals, Thermo Electron LLS India Pvt. Ltd., Navi Mumbai (India). Protein rich leguminous seeds required for production of amino acids viz. chickpea, mung bean, soybean and cowpea were procured from Sadguru Seeds Pvt. Ltd., Market Yard, Pune (India).

Isolation of *Paecilomyces variotii* PR-4: Protease producer, *Paecilomyces variotii* PR-4 was isolated from soil samples collected near Government Dairy Scheme, Pune. Soil dilution (10^{-4}) was inoculated on potato dextrose agar (PDA) medium by spread plate technique and inoculated at 37°C for 5 days. After 5 days the fungal colonies were inoculated on modified nutrient agar containing 0.4% gelatin.

After incubation of 48 h plates were flooded with 1% tannic acid (Naidu and Devi, 2005). Colonies showed clear halo zone were isolated, purified and identified on the basis of morphological characters and authenticated from Agharkar Research Institute (ARI), Pune.

Estimation of protease activity (EC 3.4.21.14): The protease activity was assayed by casein digestion method (Manachini *et al*., 1988) at 37°C, pH 7.5 in 50 mM phosphate buffer. Dialyzed sample (1 mL) was added to 5.0 mL of casein (0.65%) solution and incubated at 37°C for 10 min. After incubation the reaction was arrested by adding 5.0 mL of Trichloro Acetic acid (110 mM). The residual protein precipitated after 30 min of incubation was pelleted by centrifugation at 1000 xg for 5 min. The supernatant (2 mL) was utilized for color reaction using Na_2CO_3 (5 mL) and Folin-Ciocalteau reagent (1 mL). The blue color developed was read at 660 nm on UV-visible spectrophotometer (Shimadzu-1700). One unit of protease activity is defined as the amount of which liberates 1 μg of tyrosine min^{-1} under experimental conditions.

Production of free amino acids: Spore suspension of *Paecilomyces variotii* PR-4 (approx. 1.1×10^6 spores mL^{-1}) was inoculated in modified production media (pH-7.5) suggested by Srinubabu *et al*. (2007) which contained; malt extract (1 gL^{-1}), glucose (6 gL^{-1}), yeast extract (1 gL^{-1}), peptone (2 gL^{-1}), K_2HPO_4 (0.5 gL^{-1}), $MgSO_4.7H_2O$ (0.5 gL^{-1}), $FeSO_4.7H_2O$ (0.01 gL^{-1}) and incubated on rotary shaker (120 xg) 37°C for 9 days. The broth was filtered through Whatman no. 1 filter paper; ammonium sulphate (70.7 g) was added to 100 mL of filtrate for protein precipitation (100% saturation). The precipitated proteins were recovered by centrifugation in at 10,000 xg, 4°C for 20 min. The protein pellet was suspended in 2 mL of 0.15 M NaCl (Amersham, 1998). The protein suspension was transferred to dialysis membrane bags and dialyzed against 200 mL physiological saline solution with two changes. The protein solution was kept overnight. The solution was centrifuged at 5,000 xg

for 10 min. It was further tested for its protease activity (Manachini *et al*., 1988) and utilized for digestion of seed proteins.

Each seed samples viz. chick pea, mung bean, soybean and cowpea (10 g each) were germinated with sterile distilled water for 48 h in incubator at constant temperature (37°C) and humidity (80%) and extracted in distilled water. The seeds were crushed together using blender and crushed material was adjusted to pH-7.5 with 0.1 N NaOH and subjected to *Paecilomyces variotii* PR-4 protease of for enzymatic digestion (Vanlada *et al*., 2006) at 37°C temperature for 168 h to recover maximum amount of amino acids. The digested material were treated with 95% ethanol for 30 min and centrifuged at 10000 xg for 5 min and extracted with distilled water (1 L). Seeds crush without digestion were used as control. Total amino acids (Sadasivam and Manickam, 2003) were estimated from the filtrate and utilized for synthesis of metalnutrient chelate.

Estimation of amino acids: Total free amino acid content was estimated according to the Ninhydrin method given by Sadasivam and Manickam (2003). Undigested seed crush and seeds crush digested with fungal protease were treated with 95% ethanol and extracted in distilled water. It was centrifuged for 10,000 xg for 10 min and supernatant was saved. The extraction was repeated with the residue and both the supernatants were pooled. The volume was reduced to approximately 5.0 mL by evaporating the supernatant on boiling water bath. Final volume of extract was adjusted to 10 mL with distilled water. The reaction was arranged in test tubes, where 0.1 mL of extract was diluted to 1.0 mL with distilled water and 1.0 mL of Ninhydrin reagent was added. The purple colour developed in the reaction mixture after 20 min of incubation in a boiling water bath was read at 570 nm on UV-visible spectrophotometer (Shimadzu-1700). L-lysine was used as standard amino acid.

Production and analysis of amino acid- metalnutrient chelates: The amino acids (chelating solution) recovered from enzymatic (*Paecilomyces variotii* PR-4) digestion were utilized for production of chelate. One gram each of metalnutrients viz., zinc sulphate, copper sulphate, ferrous sulphate, manganese sulphate, magnesium sulphate, boric acid and ammonium molybdate was added separately to 100 mL of chelating solution and incubated for 2 h on rotary shaker at 37°C temperature and 120 xg (Jeppsen, 1991). After 2 h of incubation it was employed to detect chelate in accordance to Nakamoto (1986) using FTIR spectrophotometer (Thermo Nicolet-4200).

Statistical analysis: Data were subjected to single way Analysis of Variance (ANOVA) and critical differences were calculated at p = 0.05 level.

RESULTS

Screening of *Paecilomyces variotii* PR-4: Fungal isolate *Paecilomyces variotii* PR-4 was isolated from soils near Government Dairy Scheme, Pune. Eleven fungal isolates were screened for protease production and three of them were selected for further procedure (viz., PR-4, PR-5 and PR-10). After 48 h of incubation highest values for total diameter and colony diameter were recorded by isolate PR-4 (84.2 and 50.4 mm) followed by isolate PR-10 (61.3 and 46.7 mm) and isolate PR-5 (42.3 and 21.3 mm), respectively. While, highest value for zone of clearance was recorded by isolate PR-4 (33.8 mm) followed by isolate PR-5 (21.0 mm) and PR-10 (14.6 mm), respectively (Table 1). On the basis of morphological characters, the isolate PR-4 was identified as *Paecilomyces variotii* PR-4 and authenticated from Agharkar Research Institute, Pune.

Protease production: *Paecilomyces variotii* PR-4 showed significant drop in pH during incubation, however the protease activity was found to be promoted with incubation. Gradual drop was recorded for pH with incubation period; however, hasty drop was recorded after 72 h of incubation over again followed by regular drop. Protease activity was increased progressively with increase in incubation time. Maximum protease activity was recorded after 96 and 120 h (53 U mL^{-1}) of incubation, then after slight drop was recorded. Whereas, sudden increase in protease activity was recorded after 48 up to 72 h (i.e., 19 to 42 U mL^{-1}) of incubation (Table 2).

Table 1: Total diameter, colony diameter and zone of clearance (mm) of fungal isolates incubated on modified Gelatin agar medium at 37°C for 48 h

	Fungal Isolates		
Parameters	PR-4	PR-5	PR-10
Total diameter (mm)	84.2±0.6	42.3±0.9	61.3±0.7
Colony diameter (mm)	50.4±0.9	21.3±0.7	46.7±0.5
Zone of clearance (mm)	33.8±1.2	21.0±0.2	14.6±0.3

Table 2: Effect of incubation on pH and protease production by *Paecilomyces variotii* PR-4

Incubation time (h)	pH	Protease activity (U mL^{-1})
24	6.50	11.00
48	6.20	19.00
72	4.90	42.00
96	4.60	53.00
120	4.50	53.00
144	4.10	52.00
168	3.80	51.00
SEM±	0.18	1.07
CD at 0.05	0.29	1.21

SEM: Standard error of mean, CD: Critical difference

Table 3: Effect of incubation on production of amino acids in undigested seeds crush and seeds crush digested with protease of *P. variotii* PR-4

Incubation time (h)	Undigested seeds crush Amino acids (mg mL^{-1})	Seeds crush digested with protease of *P. variotii* PR-4 Amino acids (mg mL^{-1})
0	1.11	1.29
24	1.29	4.98
48	1.36	5.77
72	1.43	5.80
96	1.49	5.82
120	1.56	5.85
144	1.61	5.84
168	1.79	5.84
SEM±	0.21	0.10
CD at 0.05	0.41	0.11

SEM: Standard error of mean, CD: Critical difference

Production of amino acids: Results pertaining to amount of amino acids released from undigested seeds crush and seeds crush digested with protease of *Paecilomyces variotii* PR-4 registered gradual increase in amino acid with increase in incubation period.

Amino acids released from untreated seeds crush was recorded at regular interval and observed that the amount of amino acids released was significant over zero hours but comparatively less than enzyme digest. On the other hand data recorded in seeds crush digested with protease of *P. variotii* PR-4 showed sudden increase after 24 h of incubation. In case of untreated seed crush maximum release of amino acids 1.79 mg mL^{-1} was recorded at 168 h of incubation and in seeds crush digested with protease of *P. variotii* PR-4 maximum production i.e., 5.85 mg mL^{-1} of amino acids was recorded at 120 h of incubation (Table 3).

Organic chleates: The metalnutrients like Cu^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}, Mg^{2+}, B^{3+} and Mo^{2+} chelated with organic chelating agents (amino acids) were vary in colors. In present investigation, detection of metalnutrient chelate has been confined to the higher frequency region in which internal vibrations of the ligands (chelating agents) were observed. The IR spectra of ligands before and after addition of individual metalnutrients show that a new complexes or chelates were formed after 2 h of incubation (Fig. 1 and 2). The peaks of most intensive bands in the IR spectra of ligands recorded were present in the intervals of the wave numbers 3500-3300 and 1720-1700 cm^{-1}. Chelation of metalnutrients led to the broadening of peak and changes of the peak position of hydroxyl groups, which indicated the binding of the carboxylic groups and primary amine groups of amino acids to the metalnutrients.

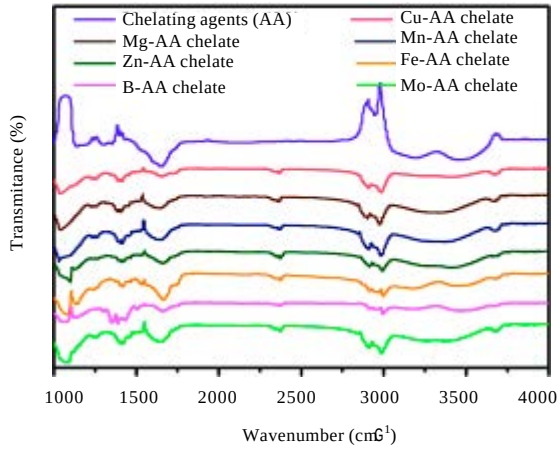

Fig. 1: Comparative FTIR spectra of chelating agents (seed amino acids) and micronutrient-seed amino acid chelate

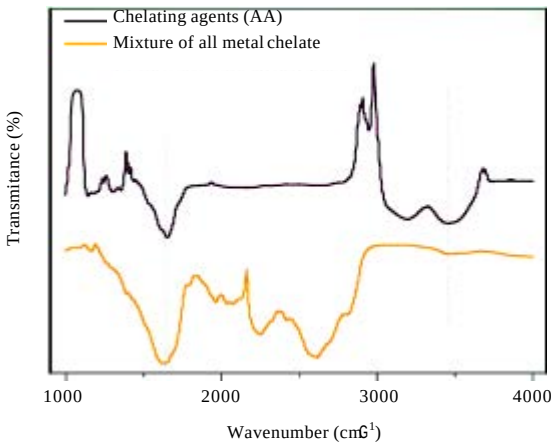

Fig. 2: FTIR spectra of chelating agents (seed amino acids) and mixture of all micronutrient-seed amino acid chelate

DISCUSSION

In present study 1% tannic acid was used to determine the protease producing capability of the organisms because, tannic acid is a relatively non-toxic substance that forms an insoluble complex with proteins. Tannic acid has been basically used to detect proteins in polyacrylamide gels (Aoki *et al*., 1979) and in tissue samples after counter-staining with an iron compound (Foster, 1934). It has also been used to complex proteins in solid media to assess protease that remain functional in the presence of tannin (Osawa and Walsh, 1993). In

present study clear zone around the colony considered as indicative of the conversion of proteins to amino acids.

It is reported that protease production by microbial strains depends on the extra-cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production (Ellaiah *et al*., 2002). In present investigation, the isolate *P. variotii* PR-4 was grown in modified production media of slightly basic pH suggested by Srinubabu *et al.* (2007). The enhancement in protease production was recorded up to 120 h of incubation there after insignificant drop was recorded. Decrease in enzyme activity with increasing incubation period clearly suggesting the enzyme's role as a primary metabolite, being produced in the log phase of the growth of the fungus for utilization of nutrients present in the solid substrate (Sumantha *et al*., 2006). The subsequent decrease in the enzyme units could probably be due to inactivation of the enzyme by other constituent proteases (Paranthaman *et al*., 2009).

In present investigation the legume seeds were germinated overnight because it has been previously reported that protein and thiamin (Sattar *et al*., 1989), mineral bioavailability (Ghanem and Hussein, 1999) and protein and starch digestibility (Preet and Punia, 2000) increased, whereas phytic acid (Kataria *et al*., 1989; Sattar *et al*., 1989) and tannin (Ayet *et al*., 1997) decreased during germination of legumes. Protein and thiamin contents after germination increased which could be due to biosynthesis during germination (Vanderstoep, 1981; Sattar *et al*., 1989). These partially digested proteins were further treated with protease enzyme of *P. variotii* PR-4 for maximum yield of amino acids.

On comparison of IR spectrum of the parent ligands with that of its each metalnutrient chelates certain characteristics differences were revealed. One of the significant differences to be expected between the IR spectrum of the parent ligands and its metalnutrient chelates is the presence of more broadened bands in the region of 3500-3200 cm^{-1} for the metalnutrient chelates as the oxygen of the O-H group and hydrogen from N-H group of the ligands forms a coordination bond with the metal ions and (Kemp, 1998). Another evident difference is that the stretching of bands at 1600 cm^{-1} due to the COO^{-} anion coordination bond with metal cations in the IR spectrum of the each metalnutrient chelates (Nakamoto, 1986).

CONCLUSION

In present investigation, we conclude that *P. variotti* PR-4 can be used as potential protease producer and the amino acids procured from leguminous seeds can chelate almost all metalnutrients. This amino acids-metalnutrient chelates can be utilized as liquid organic fertilizer which can be useful to overcome the metalnutrient deficiency in short term vegetable crops.

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