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Research Article

Potential Fungal Phytase Producers from Rhizosphere Soils

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ABSTRACT

The objective of this study is to isolate, screen phytate-degrading fungi and to select potential organism that could secrete extracellular phytase which convert insoluble form of phosphorous(phytic acid) to an accessible form. About sixty three different fungal strains were isolated from different sites of rhizosphere soil. The isolate S2 was selected from positive strains, and checked for the quantitative production of phytaseenzyme both in static and submerged fermentation conditions. S2 was found to produce highest halo zone with phytase activity of 4.6U/ml in submerged state and 3.1U/ml in static state of fermentation at 4th day of incubation. The total biomass (1350mg/100ml) and total protein content (1280µg/ml) was also high in submerged state. The isolate was further characterized and identified as *Aspergillusniger*.

Keywords:*Rhizosphere soil,Phytic acid,Phytase, submerged state,Aspergillusniger.*

[1] INTRODUCTION

The animal feed industry is an extremely important part of the world's agro industrial activities. Of the various additives used by the feed industry, enzymes are a relatively new development but one which is anticipated to grow rapidly. Enzyme like phytase, mainly improves the availability of the organic phosphorus (phytic acid or phytate) found in cereals and vegetable proteins.Phytic acid is the principal storage form of phosphorus and inositol. It represents ~60-90% of the total phosphorus content in cereals, legumes, and oilseeds [13]. In spite of being a rich source of phosphorus, the bound phosphorus in phytate is poorly utilized by monogastric animals such as pigs, poultry and fish because these animals have very low levels of phytate-degrading enzymes *i.e*. phytase (myo-inositol hexakisphosphatephosphohydrolase) in their digestive tracts which requires addition to the forage of sources with $Ca_3(PO_4)_2$. Besides this phytate is also considered as anti-nutritional compound because it forms complexes with several divalent cations of major nutritional significance, such as Ca^{2+}, Mg^{2+},Zn^{2+}, Cu^{2+}, Fe^{2+} and Mn^{2+} and with proteins under both acidic and alkaline pH conditions which affect its structure, resulting in decrease in the enzymatic activity, protein solubility and proteolytic digestibility [11].

The undigested phytate excreted by the animals is degraded by microorganisms in the soil and the released phosphorus at high concentrations gets into the rivers where it causes eutrophication. The importance of phytic acid as a source of phosphorus, its ability to cause undesirable ecological effects and antinutritive properties has stimulated research into ways for its dephosphorylation. Phytases are enzymes that catalyze the breakdown of phytate into inorganic phosphorus and *myo*-inositol phosphate derivatives. Hence from last few decades, phytases have attracted substantial interest of scientists and entrepreneurs in the areas of nutrition, environmental protection and biotechnology.

[II] MATERIALS AND METHODS

2.1. Collection of soil samples

Soil samples were collected in sterile polythene bags from different agricultural fields of Chittoor district, Andhra pradesh. Samples were stored in sterile polythene bags at 4^0C in a refrigerator for further use.

2.2. Isolation

About one-gram of soil sample was suspended in 10 ml of 0.9% saline solution and agitated (200rpm) at30^{o}C on incubator shaker for 10min. The soil suspension was then diluted up to 10^{-5} dilutions. 1ml of each dilution was poured on Potato Dextrose Agar (PDA) plates and incubated at 30^0C for 2-7days. After sufficient growth on plates, the fungal colonies that were grown were maintained on PDA slants and maintained at 4^0 C by sub culturing every four weeks.

2.3. Screening of phytase producing fungi:

The qualitative screening was carried out for extracellular phytase production on phytase screening medium (PSM) containing 1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.5% agar, 0.01% NaCl, 0.01% $CaCl_2.2H_2O$, 0.001% $FeSO_4.7H_20$, 0.001% $MnSO_4.H_20$, pH 6.5 with 0.5% sodium phytate[4]. Medium was sterilized by autoclaving (15 psi, 121 °C, 20 min), with the exception of sodium phytate, which was sterilized by membrane filtration (Millipore, 0.45 µm) and added aseptically to cooled autoclaved media. Spore inoculum was prepared from freshly raised 7 days old PDA slant culture. To fully sporulated agar slope culture, 10 ml of sterile distilled water with 0.1% Tween-80 was added. The spores were then scrapped using an inoculation needle and shaken gently to break the clumps of conidia. Plates were inoculated with $2x10^6$ /ml inoculum and kept for incubation at 30 °C for 5 days. After incubation, zone of clearing around the fungal growth on PSM agar plates were observed. The samples which showed clear zone were considered as positive samples. The zone forming fungi were selected for quantitative screening.

2.4. Phytase enzyme production and assay:

Isolates that produced clear zones on screening medium were tested for phytase production both in static and submerged fermentation conditions in PSM broth with 0.5% sodium phytate as substrate. For submerged fermentation, aliquots of 100 ml PSM broth medium were taken in 500ml Erlenmeyer flask and autoclaved for 20 minutes at 121^0C and 15 lbs pressure. The media were cooled to room temperature and inoculated with a spore suspension of 2×10^6spores/mL. The flasks were incubatedat 30 °C on an orbital shaker at 200 rpm shaking for 7 days. The same conditions were maintained for static fermentation except shaking.
The crude enzyme extracted from both static state and submerged fermentation media was quantitatively assayed for phytase enzyme as described byEngelen[2]. Standard curve was prepared using potassium dihydrogen phosphate (KH_2PO_4) in the range 0–1000 µmol. One unit of phytase activity was defined as 1M mol of phosphate produced per mL of culture filtrate under the assay condition (pH 5.5, temperature 37^0C and substrate concentration, sodium phytateat 0.0051 mol/L).

2.5. Estimation of Protein:

Protein concentration was determined by the method of Lowry *et al.*,[6]with Bovine serum albumin (BSA) as the standard.

2.6. Biomass estimation:

To determine the fungal biomass, the culture was filtered through Whatman No.1 filter paper. The filtered mycelium was dried at 600c for 24 hrs in a pre- weighed dry petridish to constant weight and expressed as mgdryweight/100ml.

2.7. Molecular Characterization

Identification of fungi using 18S rRNA gene analysis

Fungal mycelium or spores were cultured on potato dextrose agar medium. The plates were incubated at 30 °C for 2 to 3 days. The fungal mycelium was used for DNA isolation. Total genomic DNA from the fungi was isolated by N-Cetyl- N, N, N-trimethyl- ammonium bromide (CTAB) method. The quantity of the isolated DNA was checked in UV-VIS spectrophotomer (VivaspecBiophotometer, Germany). From the stock 1µl DNA was mixed with 49-µl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

PCR amplification of 18S rRNA gene

PCR amplification of fungal small-subunit rDNA (18S *rRNA* gene) was carried out using the primer set ITS4 and ITS6[8]. The ITS4 and ITS6 primers amplified a 500-600bp section of the 18S *rRNA* gene. Primer sequences were as follows: ITS4 (5′-TCCTCCGCTTATTGATATG -3′) and ITS6 (5′-GACACTCAAACAGGTGTACC -3′). PCR amplification was performed in a 20µL reaction containing 5 U of *Taq* DNA polymerase (Sigma), a 10 X dilution of the manufacturer's buffer (Sigma), 2.5mM concentrations of each deoxynucleoside triphosphate (dNTPs), and 10 pM of primers ITS4 and ITS6 and 50 ng of genomic DNA. The reaction conditions were as follows: initial denaturation at 94 °C for 2 min, 30 amplification cycles of denaturation at 94 °C for 50 sec, annealing at 52 °C for 30 sec and primer extension at 72 °C for 1 min 30 sec; followed by a final extension at 72 °C for 6 min. PCR amplifications were carried out using a Thermo-Hybaid PCR thermal cycler (Thermo Fisher Scientific USA). Aliquots of the PCR products (5 µL) were analyzed in 1% (w/v) agarose gels (Sigma, USA) by horizontal gel electrophoresis. DNAwas visualized by UV excitation after staining with ethidium bromide (0.5 mg/L). The PCR product was purified by PCR purification kit (Bangalore Genie) following the manufacturer's instruction. The 18S *rRNA* nucleotide sequence was determined by PCR-direct sequencing done by Credora Life sciences Pvt. Ltd., Bangalore, India.

[III] RESULTS AND DISCUSSION

Twenty five soil samples were collected from rhizosphere soils of different agricultural fields. A total of 63 isolates were isolated from collected samples and were named as S1—S64. All of these isolates were screened for phytase production on PSM agar medium. Out of 63 isolates, 45 isolates of *Aspergillus*spexhibited extracellular phytase activity(Table1). Among all the positive isolates, 7 isolates were found to be potential phytase producers basing on the clear zone formed around the colonies. The isolate S2 was selected to test phytase production in liquid medium as it has highest zone on plates (Fig.1). All these findings indicate that *Aspergillus*species are widely distributed in soil and have great potential of enzyme production [5,10] .

Species	No of cultures tested	Phytase activity
*Aspergillus*sp	45	++++
*Penicillum*sp	2	++
*Trichoderma*sp	5	+
*Rhizopus*sp	7	-
*Mucor*sp	4	-

Table: 1 Screening of microbial cultures for phytase production

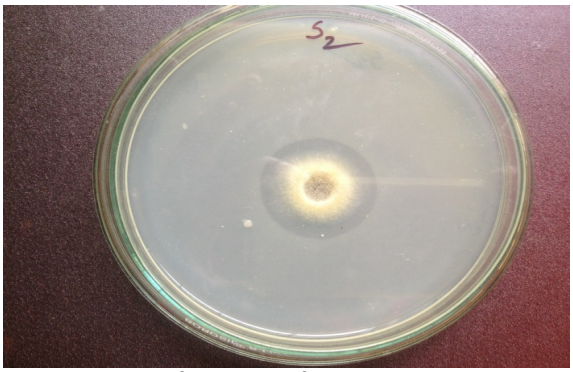

Fig 1: Halo zone formation of S2 isolate

The production of phytase by *Aspergillus*sp under submerged and static conditions at different days of incubation were studied in the present study and the results were depicted in the Table. 2& 3. The highest activity of phytase was more at 4th day of fermentation with 4.5U/ml in submerged state and with 3.1U/ml in static condition (Table2). There is decrease in phytase activity as the days of incubation increased. The total biomass and secretion of extracellular protein during the fermentation time was also recorded. There is an increase in biomass content while, the protein secretion was high at 4th day of fermentation later it was declined (Table2&3).It was interesting to note that the cell growth entered in stationery phase after 96 hrs of cultivation. It was found that enzyme production takes place during the period of logarithmic phase. There was no much variation in fungal biomass after 5th day of incubation. The reason may be cell lysis due to exhaustion of nutrients[8,10].

S.N	Incubation time (Hrs)	Phytase activity (U/ml)	Protein content (µg/ml)	Biomass (mg/100ml)
1	24	0.7	265	680
2	48	2.3	650	994
3	72	2.6	937	1192
4	96	3.1	1100	1200
5	120	1.4	965	1254
6	144	1.1	904	1260
7	168	0.6	889	1272

Table 2: Estimation of phytase activity, protein content and biomass in static condition

S. N	Incubation time (Hrs)	Phytase activity (U/ml)	Protein content (µg/ml)	Biomass (mg/100ml)
1	24	1.0	385	760
2	48	2.4	700	980
3	72	2.8	926	1250
4	96	4.5	1280	1298
5	120	1.9	1035	1350
6	144	1.3	979	1359
7	168	0.9	905	1364

Table 3: Estimation of phytase activity, protein content and biomass in submerged condition

The isolate S2 was further subjected to characterization and was identified as *Aspergillusniger*. Several workers have also reported characterization of fungi based on 18S rRNA gene sequence analysis [9,10]. On the basis of 18S rRNA gene similarity isolate S2 showed 99% sequence homology with *Aspergillusniger*[14]. *A. niger* is well known for its phytase activity. Phytase activity from *A.niger* has been extensively studied and reported by several workers[5, 12, 3, 9].

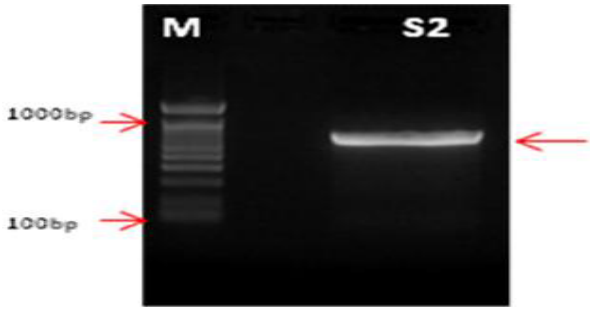

Fig: 2 PCR amplification of 18SrRNA gene

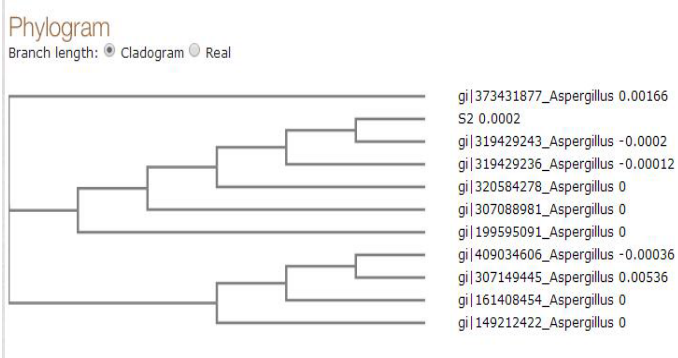

Fig: 3 Phylogenetic tree showing the relationships of the isolates to closely related fungi

[IV] CONCLUSION

The results indicated that *Aspergillusniger* is a potent producer of phytase under submerged fermentation and has higher activity in comparison with fungi cited in literature. Further work on optimization and characterization of the enzyme is in progress.

[V]ACKNOWLEDGEMENTS

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