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Screening of plant growth promoting attributes and extracelluar hydrolytic enzyme production by soil dwelling fungi

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Abstract

Plant growth promoting (PGP) activities are symbiotic relationship of plant and microorganisms where microbes produce bioactive molecules which help in plant growth, and in return plant gives nutrition and habitat. In the present study, 25 fungal cultures were isolated from agricultural soil. The screening for PGP and extracellular enzyme production revealed two fungal cultures MA2 and TV1 as promising candidates. The fungal isolates were identified by morphological and cultural characteristics. The molecular level identification showed the isolate MA2 as *Aspergillus ochraceus* and TV1 as *Trichoderma yunnanens.* The *in vitro* screening of PGP activity by both the fungal isolates demonstrated positive results for the production of siderophore, ammonia, indole acetic acid (IAA) and gibberellic acid. The isolates were found to be incapable of phosphate solubilization. The extracellular enzyme production by fungal isolates was carried out on solid media incorporated with specific substrates. The isolates displayed amylase, lipase, protease, cellulase and chitinase production capacity.Therefore, the fungal isolates *Aspergillus ochraceus* and *Trichoderma yunnanens* can be explored as potential candidates for the preparation of plant growth promoting and enzyme formulations for agriculture and industrial application.

Key words: Screening, plant growth promotion, *Aspergillus ochraceus*, *Trichoderma yunnanens*, extracelluar enzymes

INTRODUCTION:

The microorganisms are considered as an integral part of agriculture and industry as the preparation of several economic products is possible due to diverse metabolic activities of the microorganisms. Fungi represent an important soil dwelling microorganisms owing to their dynamic growth requirements and excellent capacity to adopt with adverse or unfavorable conditions.They produce an array of extracellular enzymes which mediate breakdown and decomposition of complex molecules into simpler one, and thereby enhancing soil quality and fertility (Zifcakova *et al*., 2016). Based on their roles, soil fungi can be classified as biocontrol agents, ecosystem regulators, and decomposing and transforming agents (Frac *et al*., 2018). The interaction of fungi with plants can be beneficial (mycorrhizal symbiosis) or harmful (fungal diseases). For nutrient acquisition, both symbiotic and most pathogenic fungi penetrate their host plants without breaking the cellular plasma membrane (Chanclud and Morel, 2016).

Successful plant growth is the result of a favorable combination of several abiotic factors and microbial interactions. Rhizosphere, the region around the plant root, is comparatively rich in nutrients; around 40% of plant photosynthesis products are released from the roots and accumulate in this region (Bais *et al*., 2006). The rhizosphere is the site where a large number of diverse groups of microorganisms show intense metabolic activity. Moreover, it provides an ideal habitat for the microbial populations exhibiting positive, neutral or detrimental effects on plant growth. Plant growth promoting fungi (PGPF) are a heterogenous group of nonpathogenic fungi (true fungi and oomycetes) that are associated with plant roots and provoke enhancement in plant growth or health (Bent, 2006). Mostly, the PGPF includes nonsymbiotic

and saprotrophic free living fungi present in rhizosphere soil or on the plant root surface. Plant growth promoting and biocontrol activities of several members of genera *Aspergillus*, *Fusarium*, *Penicillium*, *Phoma* and *Trichoderma* are frequently reported (Brazhnikova et al., 2021). Arbuscular mycorrhizal fungi (AMF) belonging to *Glomeromycotina* phylum have been considered as one of the most important components of the soil ecosystem, as they possess several beneficial characteristics for plant growth in abiotic and biotic stress conditions (Wagg et al., 2019; Yu et al., 2022). *Aspergillus* species among the rhizosphere organisms produce a variety of secondary metabolites which promote plant growth and protect against several soil borne plant pathogens (Akhtar and Siddiqui 2006). *Aspergillus fumigates* (Pradhan and Sukla, 2006) and *Aspergillus* sp. (Rashid *et al.*, 2004) are known for their capability to solubilize insoluble phosphate and make it available to plants. Another candidate is the *Trichoderma* spp., which are free-living fungus commonly found in soil, rhizosphere and foliar environments. This fungus has an excellent plant growth promoting attributes as well as induction of systemic resistance in plants (Yedidia et al. 1999). Different species of *Trichoderma* are widely utilized as biocontrol agents in agriculture. Apart from that, fungi are also known for the production of certain compounds having similarity with plant hormones, such as auxins, gibberellic acids, cytokinins, jasmonic acid, ethylene, salicylic acid and abscisic acid. The role of these hormones in overall plant growth and development as well as in triggering important plant signaling events during biotic and abiotic stresses is well documented (Chanclud and Morel, 2016). The extracellular enzymes produced by fungi have an important role in nutrient acquisition by the plants and defense against plant pathogens.

Present study deals with the isolation and identification of soil dwelling fungi. The *in vitro* screening of plant growth promoting attributes as well as extracellular enzymes production by two fungal isolates *Aspergillus ochraceus* and *Trichoderma yunnanens* is included in this study.

2. Material and Methods:

2.1 Collection of soil sample and isolation of fungal cultures

The soil samples were collected from agricultural farms (21˚59'11.9"N 70˚46'32.7"E) located in Gondal city, District Rajkot, Gujarat. All the soil samples were collected in sterile plastic bags and transferred to the laboratory for further processing. The soil samples were serially diluted with sterile distilled water and plated on potato dextrose agar (PDA) plates with the help of sterile glass spreader. The plates were incubated at 28± 2° C for 4-5 days. The growth of fungi was observed in terms of characteristic colony morphology. The pure cultures of fungi were transferred to PDA slants and stored at 4° C.

2.2 Identification of fungal isolates

The fungal isolates were identified by colony morphology, texture, color and reverse pigmentation. The microscopic analysis was done for the identification of mycelia type and spore arrangements. The molecular level identification was performed by 18S rRNA ITS gene sequencing from GeneXplore Diagnostics & Research Center Pvt. Ltd., Ahmedabad, Gujrat.

2.3 Screening of plant growth promoting attributes of fungal isolates

2.3.1 Phosphate solubilization

Phosphate solubilization capacity of fungal isolates were screened on the Pikovskaya's medium containing (g/l): glucose, 10; yeast extract, 0.5; $Ca_3(PO4)$, 5; $(NH_4)_2SO_4$, 0.5; KCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; $MnSO_4 \cdot H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; agar-agar, 15; pH, 7.0±0.2. The isolates were spot inoculated on the medium and incubate at 28±2 °C for seven days. Clear zone around fungal growth indicated phosphate solubilization.

2.3.2 Siderophore production

Blue agar CAS (chrome azurol S) assay was carried out to test siderophore production as described by Schwyne and Neilands (1987). The basic principle of this test is that when a strong ligand L (siderophore) is added to a colored dye-Fe^{+3} complex, the color changes due to free dye release as the ligand- Fe^{+3} complex is formed. The fungal isolates were spot inoculated on a PDA plate containing CAS and $FeCl_3$. The plates were incubated at 28±2°C for seven days. Orange halo around fungal growth indicated siderophore production.

2.3.3 Ammonia production:

The fungal isolates were inoculated into the test tubes containing 2% peptone water and incubated at 28± 2°C for 3 days. After incubation, 0.5 ml Nessler's reagent was added into each tube. Development of brown to yellow color indicated positive test for ammonia production.

2.3.4 Indole acetic acid (IAA) production:

The potato dextrose broth supplemented with L-Tryptophan (1 µg/ml) was inoculated with fungal isolates and incubated at 28± 2°C for three days. After incubation, the tubes were centrifuged at 10,000 rpm for 10 min. The clear supernatant (1 ml) was mixed with 2 ml of Salkowski's reagent and incubated for 15 min at room temperature. Development of pink color indicated IAA production by the isolates.

2.3.5 Gibberellic acid production:

The screening of gibberellic acid production was carried out using peptone-glucose Czepak's medium. The fungal isolates were inoculated into the medium and incubated at 28±2°C for seven days. The culture broth was centrifuged at 10,000 rpm for 10 min and clear supernatant was separated. The pH of the supernatant was adjusted to 2.5 using 1 M HCl and the extraction of gibberellic acid was done by adding equal volume of ethyl acetate to the culture supernatant. After 1 h, the aqueous phase was discarded and an ethyl acetate layer was collected and evaporated at 65°C in a rotary evaporator. The residues were dissolved in a small volume of acetone and spotted on the TLC plate. The mobile phase used was isopropanol-ammonia-water (10:1:1). The plates were sprayed with developing reagent containing 50 mg $FeCl_3$ and 3% H_2SO_4 in methanol, and incubated at 80°C for 10 min. The TLC plates were observed under UV light. Greenish fluorescence indicated the presence of gibberellic acid.

2.4 Screening of extracellular hydrolytic enzymes production

2.4.1 Amylase production:

The production of amylase by the fungal isolates was evaluated by streaking the isolates on nutrient agar plates containing 2% starch. All the plates were incubated at 28± 2°C for five days. After the incubation, the plates were flooded with gram's iodine solution. The formation of clear zones around fungal growth indicated amylase production.

2.4.2 Cellulase production:

The fungal isolates were streaked on a minimal agar medium containing 5% carboxymethyl cellulose (CMC). The plates were incubated at 28± 2°C for five days. Growth of the isolates indicated hydrolysis of CMC by the production of cellulase.

2.4.3 Chitinase production:

The screening of fungal isolates for chitinase production was performed on the solid medium containing 2% chitin and 0.5% peptone. The fungal isolates were streaked on the medium and incubated at 28± 2°C for five days. After incubation, the formation of clear zones around fungal growth indicated the production of chitinase.

2.4.4 Lipase production:

The lipase production capacity of the fungal isolates was checked on tributyrin agar plate. The fungal isolates were streaked on the plates and incubated at 28± 2°C for five days. After incubation, the formation of clear zones around fungal growth indicated the production of lipase by the isolates.

2.4.5 Protease production:

The production of protease by the fungal isolates was screened on solid medium containing 3% skimmed milk powder and 1% glucose. The isolates were inoculated on the plates and incubated at 28± 2°C for five days. The formation of a clear zone around fungal growth was indicated as a positive result for protease production.

3. Results and Discussion:

3.1 Isolation and identification of fungal isolates:

The serial dilution of soil samples and standard plate method resulted in isolation of 25 fungal cultures. The growth of the isolates on PDA indicated formation of white mycelium during the initial phase. The colored spores developed in later stages of fungal growth. The growth of the isolate MA2 was granular mycelial mat with brown colored pigmentation (Fig. 1A). The microscopic observation of the isolate MA2 revealed septate mycelia containing fungal

nuclei. The fruiting body contained a large spherical vesicle. The phialides were arranged on the vesicle in the circular pattern. The conidiospores were produced from the phialides in the form of long chains (Fig. 1B). The growth of the isolates TV1 was in the form of white mycelial mat on the PDA plate. The formation of conidiospores was evident in the form of loose and fluffy greenish colored growth with distinct concentric rings (Fig. 1A). Under microscope, the septate mycelium with lateral branches was observed. These branches were further divided at an angle of 90° to form secondary branches. These branches contained clusters of phialides. The phialides were enlarged in the middle and the tip containing conidiospores (Fig. 1B). The 18S rRNA sequence was used to carry out BLAST with the database of NCBI GenBank. Based on the maximum identity score, first fifteen sequences were selected and aligned using multiple sequence alignment software programs. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.19439419 (MA2) and 1.08478630 (TV1) is shown (Next to the branches). The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 428 (MA2) and 485 (TV1) positions in the final dataset. Evolutionary analysis was conducted in MEGA7. Based on molecular data the isolates MA2 and TV1 were identified as *Aspergillus ochraceus* and *Trichoderma yunnanens* respectively (Fig. 2).

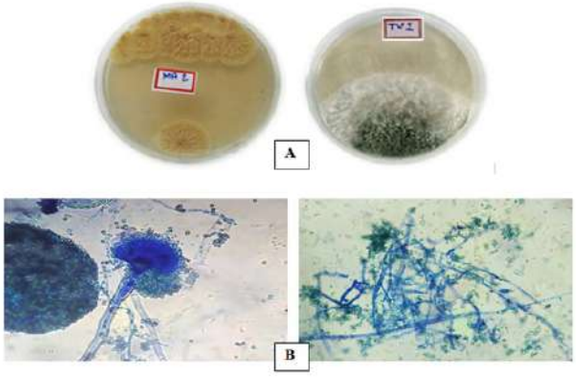


Figure 1: Growth and morphological characteristics of fungal isolates MA2 and TV1 on PDA medium (A), and typical characteristics of fungal isolates MA2 and TV1 under 40X objective of microscope (B)

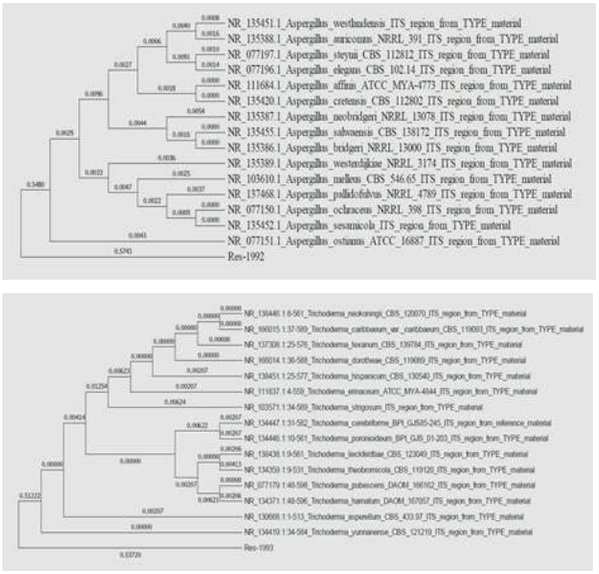


Figure 2: Phylogenetic analysis of ITS gene sequence displaying the place of fungal isolates MA2 (A) and TV1 (B) and its relationship among other species of related genera

3.2 Evaluation of plant growth promoting activities of fungal isolates

The isolates *Aspergillus ochraceus* and *Trichoderma yunnanens* exhibited orange halo around their growth indicating siderophore production. However, the isolate *Aspergillus ochraceus* showed an excellent siderophore production capacity on CAS agar plate in terms of larger size halo (Fig. 3A). Siderophores are low molecular weight compounds produced from microorganisms and some plants. They offer an excellent iron chelating capacity; their production is the part of strategy to obtain iron from the environment containing low amounts of available iron (Ferreira *et al.*, 2019). The biosurfactant producing fungus *Xylaria regalis* was able to produce siderophores which belonged to hydroxamate-type (Adnan *et al.,* 2018). In another study plant growth promoting endophytic fungus *Asprgillus fumigatus* TS1 exhibited siderophore production on CAS agar plate (Bilal *et al*., 2018).

The screening of IAA production was carried out by mixing culture supernatents with Salkowski's reagent. Positive result was indicated by the formation of pink. Both the fungal

isolates *Aspergillus ochraceus* and *Trichoderma yunnanens* exhibited IAA production capacity (Fig. 3B). It is well known that IAA regulates several physiological processes in the plant such as cell growth, tissue differentiation and tactic response. Therefore, IAA production by our fungal isolates adds an advantage of promoting plant growth. The IAA production capacity was found in *Xylaria regalis* (Adnan *et al*., 2018), *Aspergillus flavus* CSH1 (Asaf *et al*., 2018) and *Asprgillus fumigatus* TS1and *Fusarium proliferatum* BRL1 (Bilal *et al*., 2018).

The TLC plates observed under UV light exhibited greenish fluorescence in both the fungal isolates indicating gibberellic acid production capacity (Fig. 3C). Similar to IAA, gibberellic acid also has several developmental and regulatory roles in the plant such as germination of seed and development of seedling, growth of stem and leaf, floral initiation, and flower and fruit growth (Davies 2010; Pharis and King 1985). Moreover, it also helps in the plant root growth and root hair development, inhibition of floral bud differentiation, vegetative and reproductive bud dormancy as well as delay of senescence (Bottini and Luna 1993; Fulchieri *et al*. 1993; Reinoso *et al*. 2002; Tanimoto 1987). Two endophytic fungi, *Asprgillus fumigatus* TS1 and *Fusarium proliferatum* BRL1 isolated from the roots of *Oxalis corniculata* exhibited gibberellic acid production capacity (Bilal *et al.*, 2018). The fungal culture *Aspergillus niger* CSR3 isolated from *Cannabis* sativa exhibited multiple PGP traits like production of siderophores, indole acetic acid (IAA), gibberellins, and phosphate solubilization (Lubna *et al*., 2018).

The culture supernatents of both fungal isolates *Aspergillus ochraceus* and *Trichoderma yunnanens* reacts with Nessler's reagent and produced brownish yellow color indicating ammonia production. The production of ammonia is one of the important features of PGPR microorganisms for enhanced plant growth. In general, ammonia produced by such organisms serve as nitrogen source, which has enhancing effect on plant root and shoot elongation, and biomass production (Marquese *et al*., 2010).

The ability of fungal isolates to solubilize phosphate was evaluated on Pikovskaya's agar medium. In our study, none of the two fungal isolates showed phosphate solubilization as there was no evidence of a clear zone around their growth. The organic acids produced by fungi are responsible for the solubilization of calcium phosphate and liberation of phosphate, which is further utilized for the fungal growth. Solubilization and mineralization are main microbial mechanisms of releasing phosphate from both organic and inorganic phosphate pools in the soil (Bhattacharya and Jha, 2012). The fungi belonging to genera *Aspergillus* and *Penicillium* are principal microorganisms to carry out phosphate solubilization (Nelofer *et al*., 2016). Pradhan and Sukla (2006) reported that *Aspergillus fumigatus* and *Penicillium* sp. isolated from the rice field soil had good phosphate solubilization potential.

The rhizospheric fungi are least explored for their PGP potential as compared to rhizobacteria. Moreover, these fungi have no stringent host specificity, and therefore it provides an added advantage of selection and preparation of fungal inoculants for diverse crops. Therefore, our study was focused on isolation of free-living rhizospheric fungi and *in vitro* screening for their PGP potential. The role of soil fungi is not limited to PGP activity; they play vital roles in nutrient mineralization, biogeochemical cycling of the elements, decomposition of a vast variety of complex organic materials into humus, and thereby improvement of soil fertility and productivity. Organic acids produced by a number of fungi are responsible for mineral mobilization, and thus accelerates mineral uptake process by plant roots (Imran *et al*., 2021).

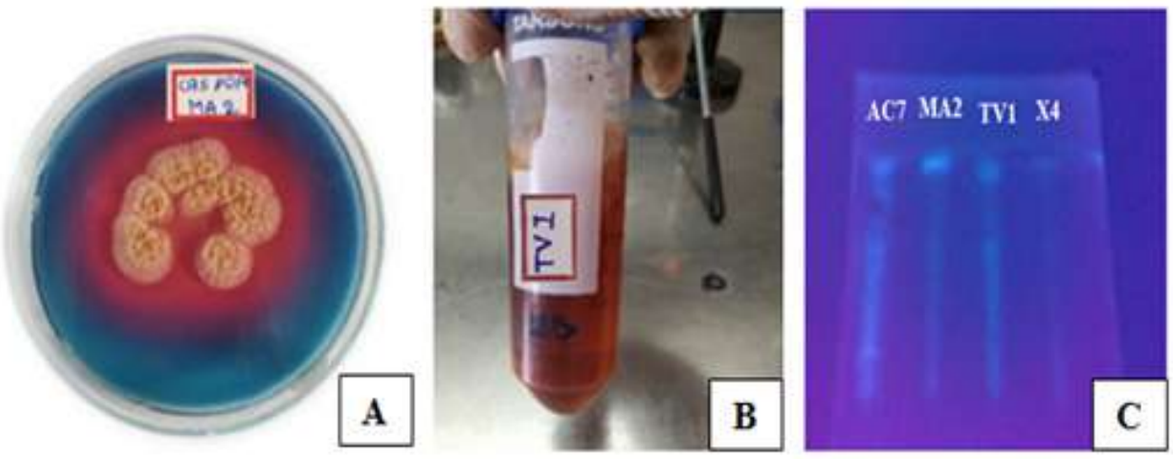


Figure 3: Plant growth promoting activities by fungal isolates: Orange halo around the growth of MA2 (*Aspergillus ochraceus)* indicating siderophore production (A), isolate TV1 (*Trichoderma yunnanens*) showing positive result for indole acetic acid (IAA) production (B) greenish fluorescence under UV light showing positive result for gibberellic acid production by both the fungal isolates (C)

3.3 Screening of extracellular hydrolytic enzymes production by fungal isolates

The production of amylase enzymes was indicated by formation of clear zones around fungal growth as a result of hydrolysis of starch. The amylase enzyme production capacity was observed with both the fungal isolates *Aspergillus ochraceus* and *Trichoderma yunnanens* (Fig. 4A). Fungal amylase is responsible for hydrolysis of starch into sugars, which can be taken up by fungi for their growth. In accordance with our findings, *Aspergillus fumigates* isolated from soil in Eastern Nigeria showed amylase production on solid media (Nwagu and Okolo 2011).

The growth and formation of clear halo on tributyrin agar plate was the indication of lipase enzyme production. In our study, the fungal isolates *Aspergillus ochraceus* and *Trichoderma yunnanens* produced lipase enzymes on solid media (Fig. 4B and 4C). Many researchers have documented lipase production capacity of fungal isolates. For instance, Takó et al., (2012) screened 35 fungal isolates for lipase production, and all the *fungal isolates* were found positive. Similarly, a strain of *Trichoderma harzianum* IDM14D isolated from soil in Turkey was found positive for extracellular lipase production (Ülker et al. 2011).

The growth of both the fungal isolates was evident on the CMC agar plate. The cellulase enzyme produced by the fungal isolates hydrolyzes CMC, and such hydrolyzed products can be further utilized for fungal growth. The fungal cellulase has an important role in nature as well as in the industry, as it degrades complex insoluble cellulose into simple sugars. For instance, in the process of bioconversion of lignocellulose into simple sugars, *Trichoderma reesei* finds its potential owing to production and secretion of large quantities of cellulase-rich secretome (Berrin *et al*., 2014). Reddy *et al*., (2014) screened 23 fungal isolates for cellulase production; nine fungal isolates displayed positive results for cellulase production.

The screening of extracellular chitinase production showed positive results with both the fungal isolates *Aspergillus ochraceus* and *Trichoderma yunnanens*. The chitinase production was indicated by the formation of clear zones around fungal growth due to hydrolysis of chitin. The production of chitinase has a protective role, as it acts and hydrolyses the cell wall of plant pathogenic fungi (Castillo et al. 2016, Suman *et al*. 2016). Our results are in accordance with previous study, wherein seven fungal isolates isolated from Egyptian soil were positive for chitinase production (Sherief *et al.* 1991).

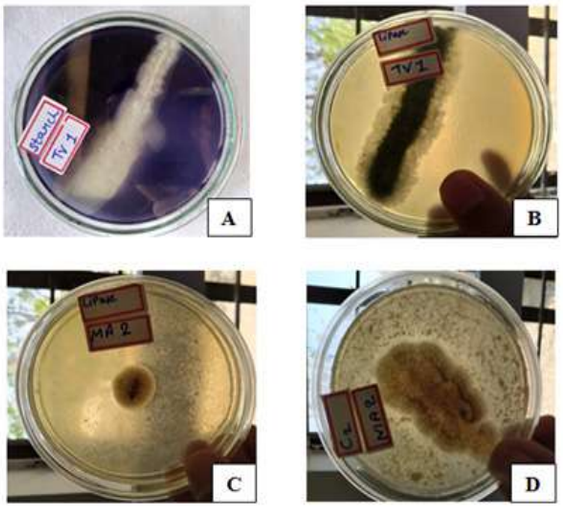


Figure 4: Screening of fungal isolates for extracellular enzyme production: Positive results of amylase (A) and lipase (B) production by TV1 (*Trichoderma yunnanens)*; lipase (C) and cellulase (D) production by MA2 (*Aspergillus ochraceus)*

The extracellular production of protease was indicated by the formation of a clear zone on skimmed milk agar plate. In our study, both the fungal isolates were found to be positive for extracellular protease production. Protease enzyme mediates proteolysis, the breakdown of larger proteins into smaller polypeptides and single amino acid residues. Such amino acids are further utilized for the synthesis of new protein products. Similar to our findings, Sharma et al., (2015) carried out screening of five fungal isolates for protease production on gelatin agar and skimmed milk agar; three isolates among five showed positive results for protease production. In another study, *Panicillium bilaiae* Pb14, *Panicillium bilaiae* C11, *Talaromyces pinophilus* T14, *Aspergillus* sp. D1 and *Beauveria bassiana* T7 displayed production of different extracellular enzymes like amylase, protease and lipase (Brazhnikova et al., 2021).

Numerous industrial as well as biotechnological applications, directly or indirectly are linked with fungal extracellular enzymes. The extracellular enzymes of fungal origin have a potential role in the decomposition and degradation of numerous organic residues in the soil and formation of humus. The degraded metabolites released in the soil serve as source of nutrients for other organisms as well as for plants (Frąc *et al*. 2018; Wösten 2019). Therefore, fungal isolates having capacity to produce large arrays of extracellular enzymes are quite promising candidates for enhancing soil fertility and thereby agricultural crop yield (Imran *et al*., 2021). In our study, soil dwelling fungal isolates *Aspergillus ochraceus* and *Trichoderma yunnanens* possess both PGP as well as extracellular enzyme production capacity, which ultimately help in the improvement of soil fertility and productivity.

4. Conclusion

In the present study, two fungal strains were isolated from agricultural soil and tested for plant growth promoting activities and extracellular hydrolytic enzymes production capacity. The isolates demonstrated positive results for the production of siderophore, ammonia, indole acetic acid (IAA) and gibberellic acid. The fungal isolates also produced extracellular hydrolytic enzymes, mainly amylase, lipase, protease, chitinase and cellulase. Therefore, the fungal isolates *Aspergillus ochraceus* and *Trichoderma yunnanens* hold potential as plant growth promoting fungi as well as source of extracellular enzymes, and can be explored for fungal bioinoculant formation.

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