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# Screening of Wild Soil Fungi for Production of Extracellular Enzymes, Organic Acids, Phytohormones and their Role in Soil Fertility

***Sindhushri Chauhan and Chethan J. Dandin****
*Department of Biotechnology and Microbiology,*
*Karnatak University, Dharwad (Karnataka), India.*

*(Corresponding author: Chethan J. Dandin*)*
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**ABSTRACT: Fungi are essential eukaryotic microorganisms that contribute to decomposition, organic matter recycling, nutrient cycling, and ecosystem balance. They adapt to various stress, environmental niches, survive pH and temperature ranges, and in consortia play crucial roles in biological cycles like mineral and water absorption, chemical transition, stomatal activity, and substance biosynthesis. Fungi with their own unique physiology, help plants cope with adaptive challenges like drought, salinity, humidity, etc. Implantation of fungi as a soil quality booster is essential, as they produce extracellular enzymes essential for nutrient cycling in natural and disturbed ecosystems. The study highlights the role of plant growth promoting wild fungi can impart in enhancing the soil fertility by producing various extracellular enzymes, phytohormones, and organic acids, which supports plant growth and enhance resistance to various abiotic stresses. And their variable, adoptive behaviour in natural, wild environment.**

**Keywords:** Soil fertility, phytohormones, extracellular enzymes, organic acids, PGPF.

## INTRODUCTION

'Soil fertility' is defined as the ability of soil to provide the conditions required for plant growth where the physical, chemical, and biological processes act together to provide nutrients, water, aeration, and stability to the plant, as well as freedom from any substances that may inhibit growth (Stockdale *et al.*, 2002). Soil fungi play a crucial role in maintaining soil fertility and promoting plant growth. Fungi with the armoury of their unique physiology and metabolites are capable of adopting and producing many important molecules necessary for supportive enhancement of bio-productivity. They produce organic acids, which aid in the solubilization of phosphorus, iron, calcium, and other minerals other, and also secrete enzymes that break down organic matter and recycle nutrients for bioavailability. Typically, fungi grow on solid substrates, breaking down the substrate and taking use of the resources around them with the help of extracellular enzymes and physical force. Fungal cells possess an advanced secretome that enables them to get nutrients, even from highly polymerized and sometimes extremely hydrophobic substances like lignin or cellulose, an extremely challenging task for other microorganisms (Richards & Talbot 2013; Hiscox *et al.*, 2018). Filamentous fungi naturally absorb nutrients from their surroundings, which can be dead plant and animal debris or living organisms, depending on their lifestyle. They play a crucial role in breaking down complex materials releasing nutrients back into the environment. High-through put techniques /Next-generation sequencing (NGS), helps to investigate the molecular mechanisms underlying the heterogeneity within fungal populations, which could provide insights into how fungi adapt to changing environments and nutrient availability. The majority of fungal species exist as mycelial thallus, which are cylindrical syncitiums with infinite apical growth that are covered in chitinous cell walls and frequently divided into compartments by perforated septa (Richards *et al.*, 2017). They rely on external organic carbon sources because they are heterotrophic, but many of them are capable of absorbing inorganic nitrogen and other nutrients for, thereby they convert them for various growth and metabolic functions. They are able to produce all the various amino acids required using inorganic nitrogen sources. Primary and secondary metabolism are the typical divisions of fungal metabolism, and mushrooms have the ability to biosynthesize a wide range of chemicals with industrial uses, the majority of which are released into the environment by the mycelium. Many of these substances are used by organisms in nature to fight off other organisms or recover nutrients, such as secondary metabolites and antibiotics. In addition to being used for nutrient recovery, substances like organic acids and enzymes can compete with other organisms for nutrients. Fungal enzymes have garnered significant attention due to their remarkable production potency, ease of purification, and efficient catalytic activity, particularly among filamentous fungi. These attributes make fungal enzymes highly desirable for various industrial applications. Notably, their robustness and stability against harsh conditions further enhance their utility/applications across diverse sectors.

The historical use of fungi in traditional practices, such as brewing and baking, provides a solid foundation for their contemporary applications. This long-standing history underscores the safety and reliability of fungal-

based preparations, instilling confidence in their utilization in modern contexts (Kango *et al.,* 2019). Enzymes are essential in various fields, including industrial processes, healthcare, and environmental remediation. Fungal enzymes, primarily from genera *Aspergillus, Trichoderma, Rhizopus*, and *Penicillium,* dominate the global enzyme market, accounting for over 50% of total production. These fungi have robust enzymatic machinery and adaptability to large-scale cultivation processes, making them a valuable solution for sustainable practices and resource conservation (Kumla *et al.,* 2020).

In essence, the widespread adoption of fungal enzymes underscores their pivotal role in industrial biotechnology and underscores their potential to drive innovation and address evolving societal needs across diverse sectors. A variety of fungal enzymes that are secreted extracellularly and work in concert to break down different insoluble plant and insect polymeric substrates into soluble sugar nutrients facilitate the adaptability of these organisms. Fungi have been utilized in food and beverage production since ancient times, with their use in alcoholic beverages and bread it increasing rapidly over the past 50 years. Fungi are morphologically complex organisms, differing in structure at different times in their life cycle, differing in form between surface and submerged growth, differing also with the nature of the growth medium and physical environment. Many genes and physiological mechanisms are involved in the process of morphogenesis. In submerged culture, a large number of factors contribute to the development of any particular morphological form. Factors affecting morphology include the type and concentration of carbon substrate, levels of nitrogen and phosphate, trace minerals, dissolved oxygen and carbon dioxide, pH and temperature. Physical factors affecting morphology include fermenter geometry, agitation systems, rheology and the culture modes, whether batch, fed-batch or continuous. In many cases, particular morphological forms achieve maximum performance. It is a very difficult task to deduce unequivocal general relationships between process variables, product formation and fungal morphology since too many parameters influence these interrelationships and the role of many of them is still not fully understood.

## MATERIALS AND METHODS

*A. Sample collection*

In order to assess and contrast the fertility status of soil, samples were taken from two geographically distinct ecosystems. The first is located in the Uttara Kannada district and is composed primarily of highly fertile forest regions with undisturbed soil ecosystems. The other region is located in the Chitradurga district and is known as Bayaluseme (in Kannada), a disturbed region that is not used for agriculture due to a lack of fertility (Fig. 1).

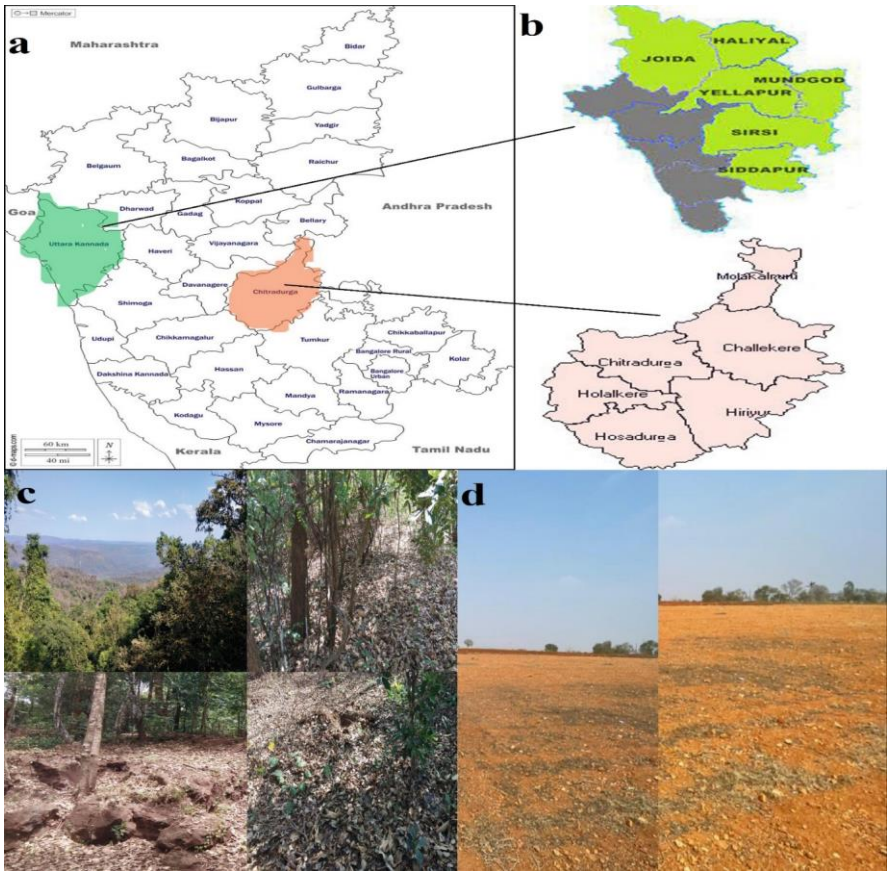



**Fig. 1.** Map depicting (a and b), the two locations Challakere Tq. of Chitradurga Dist. and Joida Tq. of Uttara Kannada Dist (c and d) of Karnataka state from where the comparative soil samplings were done for the present study (Chauhan *et al.,* 2023).

*B. Isolation of fungi*

Soil fungi were isolated using sterile media like PDA, CZA, YPDA, MEA, RA and SDA (Table 1). The technique is a variation on the Warcup soil plate technique, which involves spreading soil in water suspension on agar surfaces and incubating for up to 7 days. Daily observation, dilutions, and re-culturing were performed to obtain pure cultures. The morphological and microscopically identified fungal colonies were then used to determine their taxonomic identity.

**Table 1: Different media used for isolation of soil fungi.**

| Sr. No. | Growth media | Composition (g/L) of Distilled water. |
|---|---|---|
| 1. | SDA | Dextrose 40, peptone 10, agar 15, pH 5.6± 0.2. |
| 2. | PDA | Potato extract 4, Dextrose 20, agar 15, pH 5.6± 0.2. |
| 3. | CZA | Sucrose 30, $NaNo_3$ 2.0, $K_2$ $HPO_4$ 1.0, Magnesium sulphate 0.5, KCl 0.5, Ferrous sulphate 0.01, agar 20, pH 7.3± 0.2. |
| 4. | MEA | Malt extract 30, Mycological peptone 5, agar 15, pH 5.4± 0.2 |
| 5. | YPDA | Yeast extract 10, peptone 20, dextrose 20, agar 15, pH 7.3± 0.2. |
| 6. | RA | $KN0_3$ - l, $KH_2P0_4$ - 50, $MgS0_4$, $7H_2O$ -0.25, FeCH - 0.002, Sucrose- 3.00, Agar- 2.00, pH 7.3± 0.2. |
| 7. | YDA | Yeast extract- 7.50, Dextrose- 20.00, Agar- 15.00, pH 7.3± 0.2. |

*C. Microscopic identification of isolates*

The fungal mycelia were meticulously examined under a compound microscope, employing various magnifications (10X, 40X and 100X), following staining with lacto-phenol cotton blue and dispersion onto microscopic slides. Through this method, the colonies were scrutinized, and their characteristics were discerned to the genus level by visually assessing their morphology and structures under bright field microscopy. Identification of the isolated fungal colonies up to genus level was accomplished through visual inspection of morphology and structures using bright field microscopy (Aneja, 2006) and partially identified using reference key manual (Watanabe, 2018).

*D. Screening of fungal isolates for different physiological functions*

**(i) Screening for Amylase activity.** PDA medium was amended with 2% soluble starch, final pH was adjusted to 5.0 and incubated at room temperature, after incubation the plate was flooded with iodine solution (2-3 drops of 10% iodine solution). A clear zone around the colony indicated amylase activity other way showing opaque blue zone, indicating no amylase activity.

**(ii) Screening for protease activity.** PDA medium was amended with skimmed milk (2%), final pH was adjusted to 5.0 and incubated at room temperature, after incubation the plates were examined for the formation of the clear zone by flooding them with a solution of 10% trichloroacetic acid (TCA) or 10% tannic acid. Observed for the presence of a clear zone around the fungal colony indicated lysis of protein there by indicating protease activity other way showing white opaque zone, indicating no protease activity.

**(iii) Screening for cellulase activity.** PDA medium was supplemented with (CMC) final pH was adjusted to 5.0 and incubated at room temperature (~32°C to 35 °C), at room temperature. The presence of a clear zone around the fungal colony indicates the cellulase activity, degrading the CM cellulose, other way showing opaque white zone, indicating no cellulase activity.

**(iv) Screening for urease activity.** To prepare the urea base, dissolve the ingredients (Peptone 1 g, Dextrose 1 g, Sodium chloride 5 g, Potassium phosphate, monobasic 2 g, Phenol red 0.012 g) in 100 ml of distilled water and filter sterilized (Urea 20 g, using 0.45-mm pore size, as urea denatures in to ammonia and isocyanic acid on heating above 1600°C). Suspend the agar in 900 ml of distilled water, boil to dissolve completely, and autoclave at 121°C and 15 psi for 15 minutes. Cool the agar to 50 to 55°C. Aseptically add 100 ml of filter-sterilized urea base to the cooled agar solution and mix thoroughly. Prepared media will have a yellow-orange colour. Once prepared, do not reheat the medium as the urea will decompose. Change in the colour of the medium from pink to yellow indicates urease activity, other way showing pink zone, indicating no urease activity.

**(v) Screening for organic acid production.** The production of organic acids and enzymes by fungi is influenced by various factors such as pH, temperature, nutrient availability, and the type of carbon source. Fungi produce higher levels of organic acids and enzymes under acidic conditions, with a pH range of 4.5 to 5.5 being optimal for organic acid and enzyme production. Temperature also plays a crucial role in organic acid and enzyme production by fungi, with higher temperatures resulting in increased production**.** Isolated cultures were subjected for screening of organic acid production. A loopful of fungal spores were inoculated on the Petri plates containing PDA supplemented with bromophenol blue as an indicator and incubated for the formation of yellow zone around the mycelial growth. Yellow zone is the resultant of pH change due to the presence of different organic acids produced by the isolates.

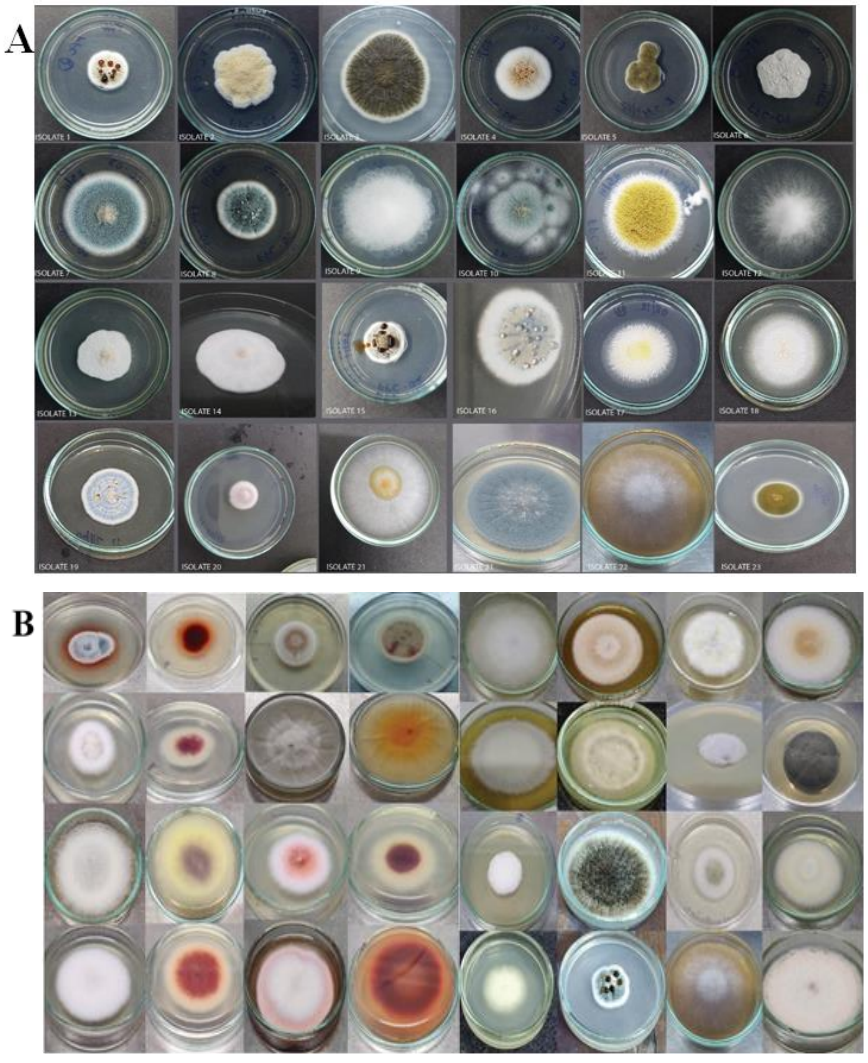
**(vi) Indole acetic acid (IAA) production.** To determine the production of indole-3-acetic acid (IAA), the fungal strains were cultured in Czepak Dox (CD) broth supplemented with 0.1% (1000 µg/ml) L-tryptophan. Following incubation, the fungal broth was filtered and centrifuged at 10,000 rpm for 10 min. One millilitre of the filtrate was mixed with 2 mL of Salkowski's reagent and incubated in the dark for 30 min. A pink coloration indicated the presence of IAA

production, and the absorbance was measured at 530 nm using a UV spectrophotometer (Ahmad *et al.,* 2005; Bose *et al.,* 2013). The concentration of IAA was calculated by comparing it to the standard calibration curve of IAA (Fig. 4). The high-IAA-producing strains were selected for further study in defining their role in soil fertility.

## RESULTS AND DISCUSSION

A total of 359 fungal colonies were cultivated across diverse media types, as detailed in table 1. Following sub-culturing, 98 isolates were procured and meticulously identified up to the genus level, employing a fungal key manual (Fig. 2). Notably, the fertile region of Uttara Kannada yielded the highest number of isolates, to talling 79 while the barren region of Chitradurga produced 27 isolate. Among the cultured fungal colonies, *Penicillium, Aspergillus*, *Trichoderma, Rhizopus, Mucor, Candida, Alternaria* spp. were prominent, followed by *Absida*, *Fusarium*, *Mortierella, Cladosporium,* etc. The study's findings underscore a notable discrepancy in fungal diversity between *Rhodotorula, Gliocephalotrichum* forested and barren soil regions. Forest zones exhibited a richer fungal diversity compared to barren soils, suggesting a correlation with variations in abiotic factors. This observation aligns with previous research endeavors aimed at elucidating the influence of abiotic and biotic factors on soil microorganism diversity. After the isolation of fungi, they were subjected to screening for various extracellular enzyme's activity (amylase, protease, cellulase, and urease). The isolates showing the positive response to the above assays were chosen for co-culture tests and pot experimental studies.

Isolated fungi from soil samples was inoculated on PDA medium supplemented with different substrate to screen the various extracellular enzymes. Zone developed around the colonies is indication of the fungal isolate's ability of producing that particular metabolities and those were taken for the enzyme production in broth culture. The screening results indicating the color formation in the medium for the respective substrate confirm that isolated fungus is capable of producing different extracellular enzyme (Fig. 3). Plants face abiotic and biotic stress, affecting their physiology and disrupting homeostasis. As population growth and climate change increase, developing plant varieties resistant to these stresses is crucial to ensure crop quality and productivity (Yaman and Prasann Kumar 2021).



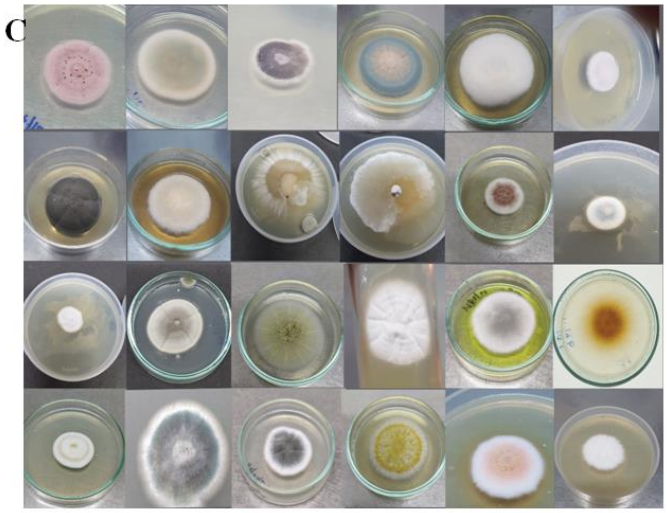



**Fig. 2.** Diversity of soil fungi. A and B – Pure fungal cultures isolated from soil samples from Uttara Kannada (D) of Joida (T) forest region. C –Fungal isolates from Chitradurga (D) of Challakere (T) barren region (Chauhan *et al.*, 2023).

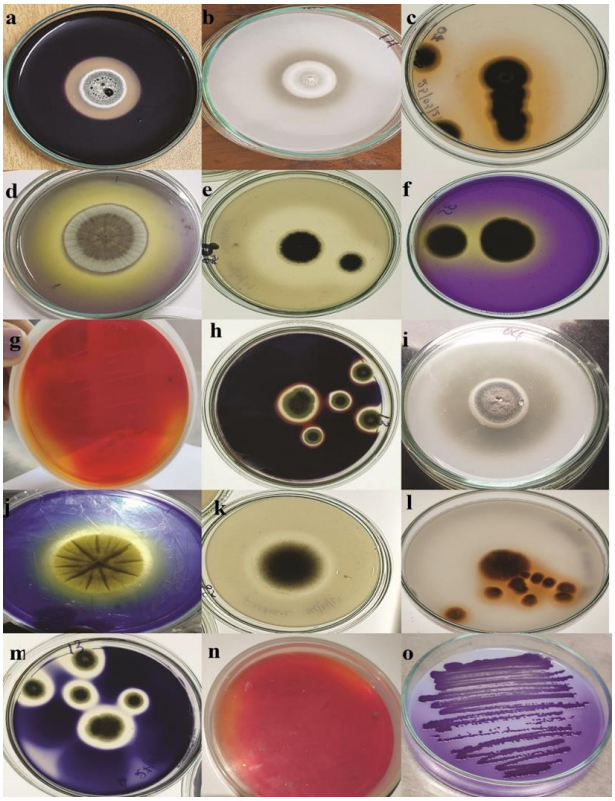



**Fig. 3.** Illustrates the process wherein each distinct colony, characterized by varying shapes and colours upon initial incubation, underwent sub-culturing and maintenance on fresh agar plates. Initially, identification relied on morphological and microscopic characteristics, as outlined in the fungal key manual (a, m and h- amylase activity, e and k-protease, b and i-cellulase, g and n urease, c and i- pvk).

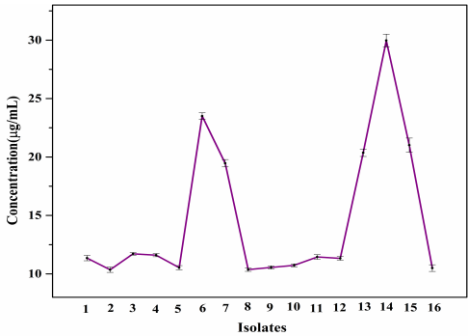



**Fig. 4.** Concentration of IAA produced by 16 isolates collected across two locations.

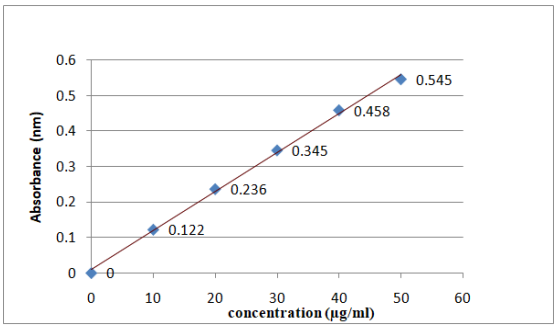



**Fig. 5.** IAA standard curve.

Amylase is an enzyme that hydrolyzes polysaccharides in soil, breaking them down into glucose or oligosaccharides. It is typically extracellular and inducible, depending on the substrate type (Alexander, 1978). Soil amylase is crucial for the conversion of starch into glucose or oligosaccharides, providing nutrients for microbes and plant's growth (Singaram and Kamalakumari 2000). The decomposition of organic matter by plants, animals, and microorganisms is crucial for nutrient availability. This process is facilitated by extracellular enzymes, including protease, which hydrolyzes the protein part of organically bound nitrogen into single amino acids. Proteolysis is a key component of the nitrogen cycle and a limiting factor in soil nitrogen mineralization (Buckley *et al.,* 2019). Cellulases are crucial for soil fertility by accelerating the breakdown of plant residues, resulting in improved soil fertility. They degrade cellulose to glucose, promoting plant growth performance, seed germination, and protective effects (Han and He 2010). Urease, can enhance soil fertility by converting urea into ammonium, which plants can use as a nitrogen source. Its activity is influenced by soil chemical properties like pH and nutrient availability (Hasan, 2000). PGPF stimulate enzymes, such as inorganic acids, organic acids, phosphotases, and phytase, for complex organic phosphorous solubilization and mineralization into various small molecules (Malgioglio *et al.*, 2022).

**Detection and quantification of IAA:** Among 16 isolates, only 5 showed the highest IAA concentration. The highest IAA was detected by isolate number 14 (30 µg/ml), followed by 23, 19.5, 20, and isolate 20.94 µg/ml, respectively (Fig. 4). The mineralization of nutrients in the soil mediated by PGPF carriy out a special function in plant growth improvement. It reduces the breakdown of the substrate into a soluble molecule that can be assimilated by plants. Phosphorus (P) is an important macroelement that promotes plant growth. Fungus, *Ceriporia lacerata*was reported by Sui *et al*. (2022) to solubilize the phosphates by the activity of organic acid and phytase. In addition to this, *Aspergillus awamori* and *Penicillium digitatum* were also reported to solubilize phosphates.One key area of focus will be elucidating the biochemical properties of the identified fungal species. This entails exploring their enzymatic capabilities, metabolic pathways, and interactions with surrounding soil components. By investigating these biochemical attributes, we can gain insights into the mechanisms through which fungi contribute to nutrient cycling and organic matter decomposition in the soil. The screening process revealed several fungal and yeast strains with distinct capabilities such as *Rhodotorula*, *Gliocephalotrichum*. Some strains exhibited a high enzymatic activity, indicating their potential to decompose organic matter and release essential nutrients. Others showed significant organic acid production, suggesting their role in mineral withering and nutrient solubilization. Phytohormones were detected in certain cultures, indicating their potential for plant growth promotion. Additionally, some strains displayed efficient phosphate solubilization, enhancing the availability of phosphorus to plants.

## CONCLUSIONS

Soil fungi contribute to improved soil fertility by producing organic acids, extracellular enzymes, and phytohormones. Understanding these processes can inform sustainable agricultural practices aimed at optimizing soil health and plant performance. Understanding the physiological roles of fungal species in soil colonization is of paramount need. This involves examining their growth patterns, reproductive strategies, and responses to environmental cues. By deciphering the physiological characteristics of these fungi, we can elucidate their adaptive strategies for thriving in diverse soil conditions and niches. Ultimately, the extended outcomes of this research will provide detailed insights into the prevalence and ecological significance of widely adopted fungal species in soil ecosystems of different kinds (fertile and barren). By elucidating their roles in bioconversion, nutrient bioavailability, and recycling processes, we can discern their impact on soil fertility dynamics. This knowledge will not only enhance our understanding of soil microbial ecology but also inform sustainable soil management practices aimed at optimizing agricultural productivity and ecosystem's health.

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**Conflict of Interest.** None.

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