Isolation, Identification and Screening of Cellulolytic Fungi from Soil in Maharashtra Nature Park.

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INTRODUCTION:

Fungi are eukaryotic heterotrophic organisms diversely present in various habitats. The majority of the fungi are filamentous, while some of the lower fungi are unicellular. As they lack chlorophyll, they depend on an external source for food. Fungi secrete digestive enzymes on the surface where they are growing. These digestive enzymes convert organic molecules externally into simpler forms, which are absorbed by the fungal hyphae for their growth and reproduction. These extracellular enzymes are essential in the environment for recycling natural waste and also play significant roles in industries (Kwon et al., 2007).

Enzymes are biological catalysts that play crucial roles in biological reactions and facilitate commercial processes. These enzymes are widely present in all the other forms of living organisms. But enzymes obtained from fungal sources are cost-effective, efficient, faster, scalable, and more stable than those from other sources. Filamentous fungi are especially considered as an interesting source of enzymes as they have the potency for high production of enzymes, and the enzymes produced are easily purified and separated (El-Gendi *et al*., 2021).

Fungi are known to produce a wide range of extracellular enzymes. In particular, hydrolase enzymes like cellulases, amylases, lipases, and proteases are the most widely studied group of enzymes as they have several applications in industrial sectors.; thus, a continuous effort to improve the production efficiency of enzymes with cost effectiveness is very important (El-Gendi *et al*., 2021).

Filamentous fungi extensively produce cellulase enzymes that catalyze the hydrolysis of cellulosic biomass (Sharada R. *et al*., 2013). Cellulase is a complex of three different enzymes: endocellulase, exocellulase, and β-glucosidase. Cellulase, with all its three components, converts cellulose to monosaccharide glucose. Most of the cellulolytic fungi found are *Trichoderma, Humicola, Aspergillus, Penicillium, Myceliophthora,*etc. (Vaishnav *et al*., 2018). Cellulases are widely used fungal enzymes for their significant applications in textile processing, detergent improvement, animal feed, biofuels, baking, beverages, pulp and paper processing, cosmetics, leather, and pharmaceuticals. Thus, considering the extensive applications of cellulase in industries, this research aims to screen cellulase-producing fungi from soil and determine their enzyme activity.

MATERIALS AND METHODS:

1. Collection of soil samples:

The soil samples were collected from Maharashtra Nature Park (MNP). The ground surface was dug 8 cm deep to collect the soil samples and emptied into a sterilised polyethylene bag. The sample bags were labelled appropriately and brought to the lab for further work.

2. Sterilization :

The laminar air flow was completely wiped and sterilised with 70% alcohol before and after use to avoid contamination. All the glassware and media were autoclaved before use.

3. Preparation and pouring of media:

The culture media used for the isolation of fungi was Potato dextrose agar and for the screening of cellulolytic fungi was Czapek's Dox Agar with Cellulose (Bagool, 1982), Czapek's Dox Agar with Filter paper strips (Subba Rao, 1977), and Reese liquid medium (Mandels and Weber, 1969) were used.

3. Serial dilution (Waksman, 1927):

The method followed was the Soil Plate Dilution Method (Waksman,1927) with sterilized 0.85% saline solution.

4. Spread plate method:

100 µl of selected dilution factor (10^{-6}) was pipetted out in Petri plates containing PDA and spread evenly with the help of a spreader. The petri plates were incubated at room temperature for 7 days. The fungus was subcultured and maintained in Petri plates containing PDA for further experiments.

5. Identification of fungus:

The fungal species was identified by morphology and microscopy characters. For microscopic examinations, the mycelium of fungus was stained with lactophenol blue and observed under a light microscope at 10x and 40x magnification. The fungi were identified with the help of relevant literature and experts.

6. Qualitative screening of Cellulase (Wang et al., 2003):

Extracellular enzymes were measured by the agar-plate based method. The fungus were subcultured on the Czapek Agar media with cellulose [3g $NaNO_3$, 1g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, 0.01g $FeSO_4$, 1% Cellulose, 15g Agar in 1000 ml distilled water pH 5.5 (Bagool, 1982)]. After 7 days of incubation, the plates were flooded with 0.1% Congo red solution and allowed the plates to stand for 30 min at room temperature and for 20 min with 1M sodium chloride solution. The formation of clear zones around the colony indicated the presence of cellulase activity (Mohammed *et al.,* 2016). The enzymatic index (EI) was calculated for each enzyme to evaluate its activity. The formula for **EI = R/r;** where, R is the diameter of the hydrolysis zone (cm) and r is the diameter of the colony (cm).

7. Quantitative screening of Cellulase:

The fungal isolates that showed positive cellulase activity were inoculated in the sugar test tubes containing Czapek Agar media with Filter paper strip (2*10 cm) (Subba Rao, 1977) for further quantitative estimation of cellulase. After 8 days, in each test tube 2-3 ml of sterile distilled water were added. Then 1 ml of water with culture was transferred into 500 ml Erlenmeyer flask with 250 ml Reese liquid media with Whattman filter paper no. 1 filter paper strips (5 gm) as substrate (Mandels and Weber, 1969). The flasks were incubated at room temperature on a rotary shaker at 180 rpm for 8 days. After 8 days the broth was filtered using glass wool. The supernatant was used further for enzyme assay.

7. Assay of Cellulase:

Carboxymethyl assay (CMCase) and Filter paper assay (FPA) was carried out according to Mandels et al. (1976). Test tubes containing Reaction mixture (0.5 ml CMC solution +0.5 ml of culture supernatant), Enzyme blank (0.5 ml CMC solution +0.5 ml distilled water) and Substrate blank (0.5 ml supernatant+0.5 ml distilled) was used for both the assay.
For CMCase, 1% CMC dissolved in 0.05 M citrate buffer was used as substrate. For FPA Whattman filter paper no. 1 (1x6 cm) strip was used as a substrate. All the test tubes were incubated at 50 ℃ for 1 hour. After incubation, the reaction was terminated by adding 3 ml Dinitrosalicylic acid (DNSA) where colour of reaction was developed and the reducing sugar was measured according to Miller (1959) at 540 nm against the blank. One unit of enzyme is defined as the amount of enzyme that releases 1 micromole of reducing sugars, expressed as glucose, per minute under given conditions.

RESULT AND DISCUSSIONS:

1. Isolation and Identification of soil fungi:

Soil is an oligotrophic medium for fungal growth (Raja M. *et al.,* 2017). Fungi are an important component of the soil microbiota as they secrete digestive enzymes that convert complex molecules into simpler forms making it available for higher plants as well as to support their

own growth (Kaiding, R. K. P *et.al*., 2015). These extracellular enzymes are essential in the environment for recycling natural waste and also plays significant roles in industries (Kwon *et al*., 2007). Thus, soil is an excellent source to screen various enzymes producing fungi. Hence, in this study the fungi were isolated from the soil. The isolated fungi from the soil were *Aspergillus sp.*, *Penicillium sp.*, *Fusarium sp.*, and *Rhizopus sp.*(Fig 1). Among all the isolate *Aspergillus* and *Penicillium* was dominant in the soil.

2. Qualitative screening of Cellulase:

The agar plate-based method was done to screen cellulase-producing fungi for all four isolates among which only two isolates showed positive result by the formation of clear zones around the colony when Congo red dye indicator was used. Based on the colony diameter and hydrolysis zone diameter the enzymatic index was calculated. *Penicillium sp.* showed an enzymatic index of 1.57 cm higher than the *Aspergillus flavus* which showed an enzymatic index of 1.33 cm.[Table-1] The halo zone formation indicates the positive result for the cellulolytic activity, in contrast the Congo red dye that remains in the plate reveals the presence of non-hydrolysed β-1,4-D-glucosidic bonds (Lamb *et al*., 2005). *Aspergillus flavus* and *Penicillium sp.* showed good growth on Filter paper strips in Czapek Agar media(Fig 3).

3. Quantitative screening of Cellulase:

The isolates that showed positive results were further subjected for the quantitative estimation of cellulase. After the culture of isolates in the Reese liquid media for 8 days, the filtrate was collected and was used to study the CMCase and FPA activities. *Penicillium sp.* showed higher CMCase (13.24) and FPA (19.68) activities than *Aspergillus flavus* [Table-2]. Deschamps *et al*., (1985) noticed that *Trichodermna harzianum* produced maximum cellulolytic activity after 3 days of incubation by using filter paper as substrate. De Sousa Santos *et al*., (2021) studied that *Penicillium sp.* FSDE15 is a good cellulase producer. In the study by Singhal, A *et al*., (2022) and Sajith, S *et al*., (2013) *Aspergillus flavus* showed high cellulase activity when lignocellulosic waste was used as substrate.

Fungal enzymes have been used from ancient times till the present day for brewing, baking, cheese making, leather manufacturing, and many other purposes. (El-Gendi *et al*., 2021)
These enzymes are better than chemical processes as they are specific, stable, require less energy and increase product efficiency (El-Gendi *et al*., 2021). Thus demand for enzymes is increasing day by day. The marketing of fungal enzymes has pushed continuously to research novel enzyme producers with all the industrial characteristics and also cost-effective techniques for the production and purification of enzymes.

Thus, in this study, we conclude that *Penicillium sp.* and *Aspergillus flavus* isolated from the soil have the potential to produce cellulase enzymes when Whatman filter paper no. 1 filter paper strips were used as substrate. These fungal isolates isolated from the environment can

play an important role in industries as efficient cellulase producers. The production of cellulase can be increased further by optimizing the media or the incubation period.

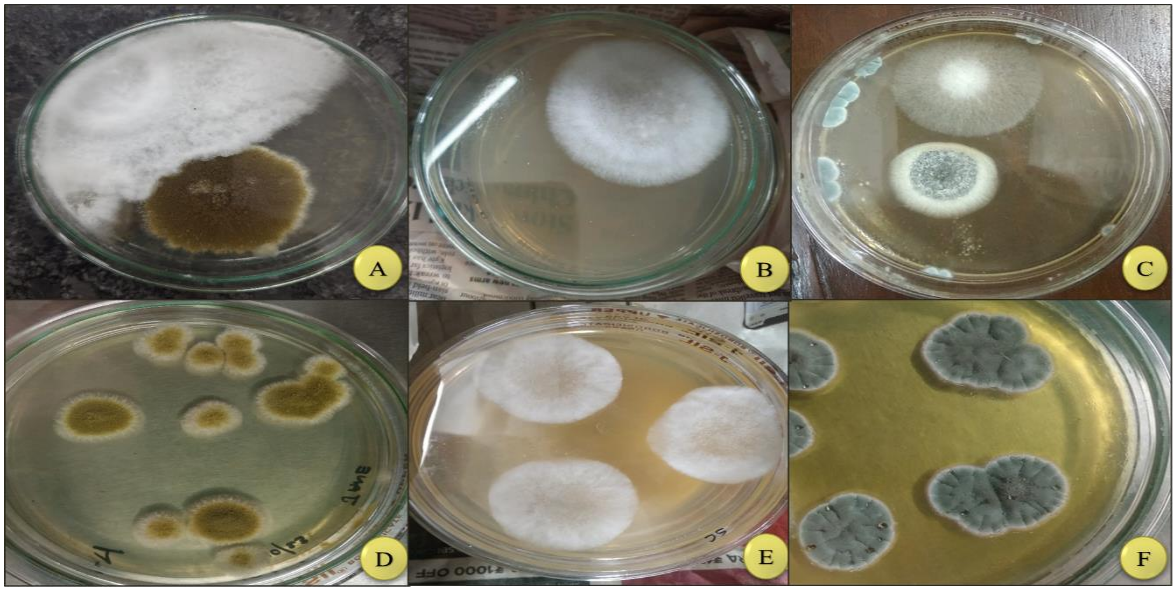


Fig 1: (A-C) Soil fungi isolated from serial dilution agar plating method [10-6 Dilution] and (D-F) Pure cultures of isolated fungi.

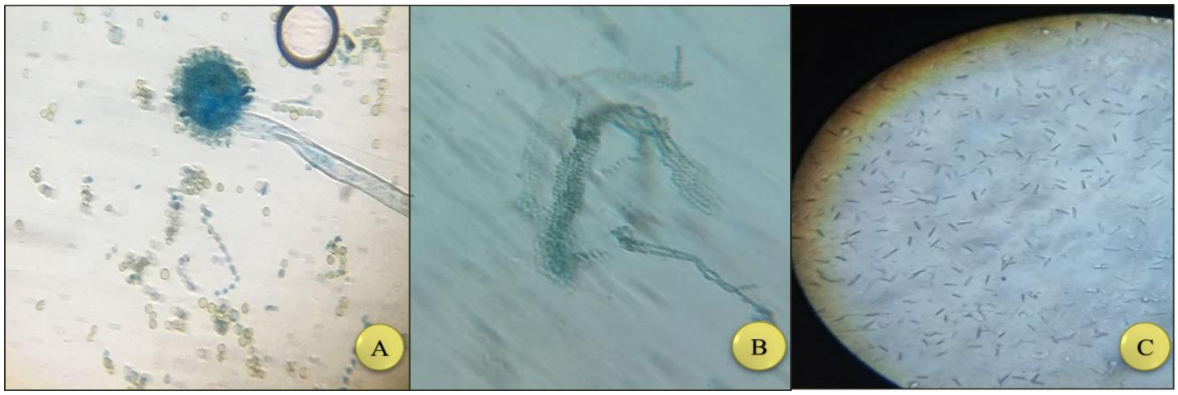
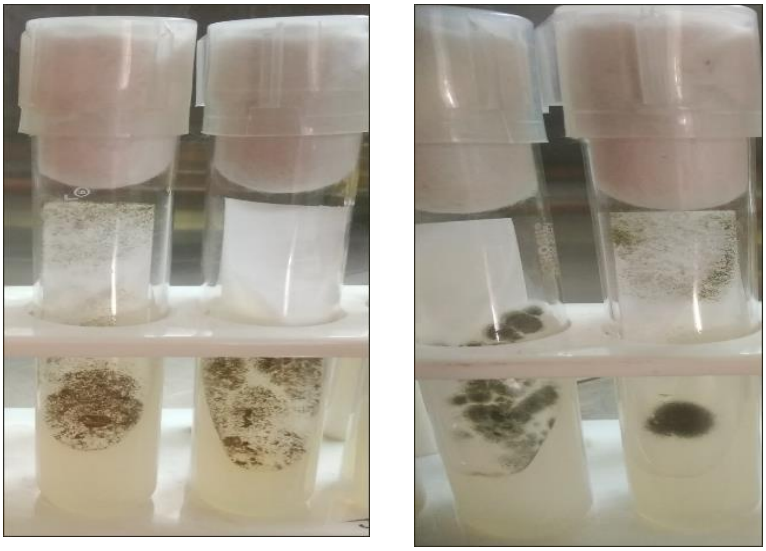


Fig 2 : A- *Aspergillus flavus*, B- *Penicillium sp.*, and C- *Fusarium sp.*



A **B**

Fig 3: A- *Aspergillus flavus and B-Penicillium sp.* growing on filter paper

Isolates	*Aspergillus flavus*	*Penicillium sp*
Colony diameter	1.5	1.4
Zone diameter	2.0	2.2
Enzymatic index	1.33	1.57

Table 1 – Enzymatic indices of fungal isolates.

Fig 5: CMCase and FPA activity of *Aspergillus flavus* and *Penicillium sp.*

Fungal culture	FPA (mg/ml)	CMCase (mg/ml)
Aspergillus flavus	18.84	12.32
Penicillium sp.	19.68	13.24

Table 2 – Quantitative estimation of cellulase by DNSA method(mg/ml)

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