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CELLULOLYTIC POTENTIAL OF FUNGAL ISOLATES FROM DEGRADING LITTER OF ORCHHA WILDLIFE SANCTUARY, M.P., INDIA

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ABSTRACT

Cellulases are a family of hydrolytic enzymes that may breakdown the organic polymer, cellulose to smaller constituents like glucose subunits. Cellulases have a wide range of applications in pulp, paper, textile, laundry, food and animal feed industries. The current study was executed with an objective to screen out cellulolytic ability of fungi isolated from degrading litter samples collected from Orchha wildlife sanctuary, M.P. Firstly, isolation of fungi has been carried out on Potato dextrose agar media then after, cellulolytic activities of isolated fungi were screened out on Czapek's Dox agar medium having 1% carboxymethyl cellulose as substrate. Fungal colonies were stained with 0.1% congo red dye, then observation for the appearance of clear zone was done. Thirty two fungal species were used for the present study of which twenty seven were recorded as positive for cellulolytic activity. The maximum activity was achieved by *Rhizopus stolonifer, Penicillium decumbens, Penicillium chrysogenum* with cellulolytic index 1.66, 1.6 and 1.44 respectively.

Keywords: Cellulases, Litter, Fungi, Potato dextrose agar

INTRODUCTION

Enzymes are biocatalyst which enhances rate of metabolic reactions including fermentations. Enzyme cellulase is one of the most widely used enzyme for working with cellulosic materials. Cellulose is a linear polysaccharide of glucose residues with 1, 4-glycosidic connections that is the most abundant form of biomass on this planet **[1]**. Lignocellulosic material consists of cellulose (40-50%), hemicelluloses (25-30%) and lignin (15-20%) as their main constituents **[2]**. In nature, polysaccharide degrading enzymes such as cellulases, hemicellulases and ligninases are pervasively found in various microbial systems including bacteria, fungi, actinomycetes, algae, plants etc. Among them fungi is the best source to produce the cellulases extracellularly for biological degradation of cellulose **[3]**. Microorganisms that recycle cellulose, a lot of carbohydrate produced by plants through photosynthesis, serve a vital function in the biosphere. Cellulases are enzymes that operate together in the three categories – Endoglucanases that hydrolyzes β-1, 4-glycosidic bonds in a random fashion over a cellulose chain. As a result there is a rapid change in chain length and a slow increase in reducing end groups; exoglucanases that help in the release of cellobiose from the cellulose polymer and β-glucosidases which take part in the transformation of cellobiose to glucose **[4, 5]**. Microorganisms and their respective enzymes can transform raw lignocellulosic waste materials into value added products. Nature (forest litter soil) is said to be the richest and most diverse hotspot for a wide range of microorganisms that produce an endless supply of enzymes useful in various industrial operations. Researchers have strong interest in cellulases because of their applications in various industries like for biopolishing of cotton and other cellulosic fabrics, starch processing, brewing and malting, extraction of fruits and vegetables juices and also used as feed additives **[6, 7]**. The most striving of these is the conversion of lignocelluloses to alternative energy sources like ethanol, butanol, methanol etc. Forest litter and agro-industrial waste have a considerable quantity of unused cellulose that causes environmental problems. By using hydrolyzing enzymes such as cellulases, sugars, biofuels, animal feed and human nutrition are now being produced. Considering the importance and applications of cellulases, the purpose of the investigation was to isolate and identify fungi from degrading litter that could hydrolyze the cellulose.

MATERIALS AND METHODS

Sample collection

The samples of degraded litter were collected from Orchha Wildlife Sanctuary (25° 21' 6.91" N and 78° 38' 25.19" E), Madhya Pradesh. In order to isolate fungi, the samples were brought to the laboratory in plastic bags.

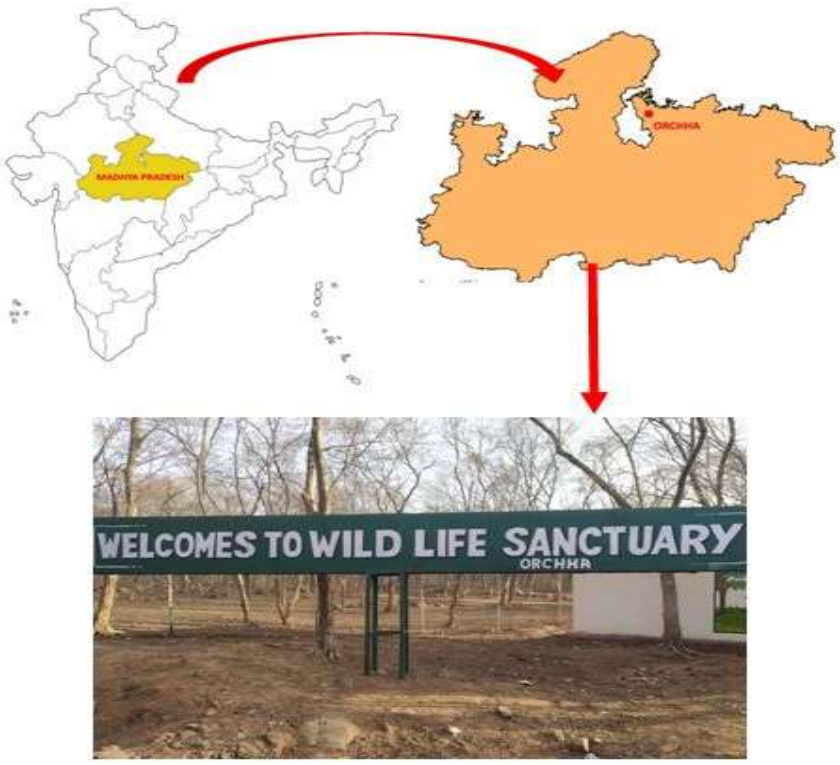


Figure 1: Location of Orchha Wildlife Sanctuary, M.P., India

Isolation and Identification of fungi

Litter suspension was prepared by suspending 1.0 g of sample in 9.0 ml sterile distilled water to make up volume of 10.0 ml and its dilutions were made up to 10^{-4} **[8]**. Each dilution was streaked over agar plates having Potato Dextrose Agar (PDA) medium contained (g/L): Potato – 200.0, dextrose – 20.0, agar – 15.0, pH - 5.6. Inoculated petriplates were incubated at 30°C for fungal growth. Fungal cultures were purified by repeated subcultures. The pure cultures were maintained at 4°C as PDA slants for further work.

Fungal isolates were identified on the basis of morphological as well as microscopic characteristics. For morphological study such as growth pattern, texture, color and appearance of colonies were considered. For microscopic identification, mycelium with its

fruit body was placed on glass slide with addition of a drop of mounting fluid i.e. lactophenol cotton blue and covered with a clean cover slip. Then, the slide was observed under microscopic with X40 objective lens.

Screening cellulolytic activity of fungi

To screen out cellulose degrading capability of fungal isolates, well diffusion method was used. Cellulolytic activity of isolated fungi was examined on Czapek-Dox Agar (CzA) medium contained (g/L): Sucrose – 30; $NaNO_3$ - 2.0; K_2HPO_4 – 1.0; $MgSO_4$ – 0.05; KCl – 0.5; $FeSO_4$ – 0.01, Agar – 20.0; Carboxymethyl cellulose – 1%; pH – 5.0 **[9]**. After autoclaving at 121°C and 15 lbs/in^2 pressure, the medium was transferred into sterile petriplates and allowed to solidify. Cavities with 6 mm diameter were prepared after solidification with the help of sterile cork borer. Fungal suspension of 0.1 ml was poured into cavities and these inoculated petriplates were incubated at 30° for fungal growth. Once mycelial growth appears, petriplates were again incubated at 50°C for 18 hours for screening of cellulase activity. After incubation, agar plates were treated with 10 ml of 1% congo red solution and allowed to stand for 15 min at room temperature. After 15 min, congo red solution was discarded. 1M NaOH solution was added to the plates for counterstaining **[10]**. Clear zone appeared around growing fungal colonies indicating that the fungus is capable of producing cellulase enzyme. The measurement of clear zone around each fungal colony was made and cellulolytic activity index was determined by using following expression **[11, 12]**.

$$\textbf{Cellulolytic Activity Index} = \frac{\textbf{Diameter of clear zone}}{\textbf{Diameter of colony}}$$

Results presented in the table no. 1 are the mean of three replicates. For each fungal isolate, the average enzymatic was calculated together with standard deviation (SD).

RESULTS AND DISCUSSION

Saprophytic fungi are key decomposers in ecosystems because they break down complex organic material into basic inorganic forms. They are a key component of soil microbial ecosystems, which decompose organic substrates in the litter to provide energy and nutrients **[13]**. In this study, a total of 33 fungi were isolated from serially diluted degrading litter samples collected from different areas of Orchha Wildlife Sanctuary, M.P. Fungal isolates were identified on the basis of colony morphology i.e. by observing their culture, growth pattern and microscopic examination. Microscopic study includes hyphae structure, conidia, conidiophores and spore structure

etc. Identification of fungal isolates were confirmed according to consult keys given in standard books of mycology **[14, 15]**. The fungal isolates were identified as *Acremonium implicatum, Alternaria alternata, Aschochyta, Aspergillus flavipes, Aspergillus flavus, Aspergillus fischeri, Aspergillus fumigatus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus niger, Aspergillus terreus, Aspergillus tamarii, Beltraniella humicola, Ceratocystis paradoxa, Chaetomium salami, Cladosporium sphaereospermum, Colletotrichum capsici, Cunninghamella blakesleeana, Curvularia lunata, Emericella nidulans, Fusarium oxysporum, Gibberella zeae, Humicola fuscoatra, Mucor hiemalis, Paecilomyces lilacinus, Paecilomyces variotii, Pestalotiopsis mangiferae, Penicillium chrysogenum, Penicillium decumbens, Rhizopus stolonifer, Rhodotorula glutinis, Scopulariopsis brumptii and Trichoderma viride.* Our results are consistent with previous reports that found five genera *Aspergillus, Fusarium, Penicillium, Stachybotrys* and *Trichoderma* which are commonly present on soil samples **[16]**.

Cellulose degradation in soil is generally carried out by cellulolytic microbes like bacteria and fungi. Fungi play a critical role in the degradation of lignocellulose polymer by secreting necessary enzymes involved in lignocellulose depolymerization **[17]**. The results of this study revealed that all cellulolytic-active fungal isolates were screened out. For screening of fungal isolates for the cellulose degradation was performed on Czapek-Dox Agar (CzA) medium supplemented with 1% Carboxymethyl cellulose (CMC) as a sole carbon source. CMC is a pure cellulose substrate that is applied as indicator for cellulase enzyme production. CMC is a cellulose derivative that can be dissolved in water at any temperature. Fungal isolates producing cellulase enzyme was marked by the presence of clear zone formation around the fungal growth around the wells. Carboxymethyl cellulose containing agar plates for the screening of cellulase producing fungal isolates through formation of zone of substrate hydrolysis have been reported by several workers **[18-21]**. To make clear zone more visible, plates were flooded with 1% congo red solution. The clear zone of fungal isolates is proportional to their cellulolytic activity. Significant isolates were defined as those with a clearing zone of greater than 1.0. It was presented in the table no. 1 that out of 33 fungal isolates, 30 isolates were capable of producing

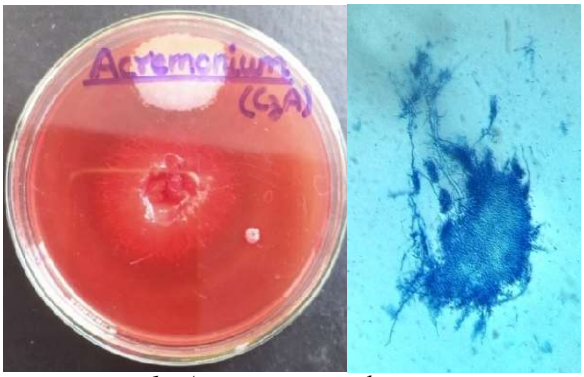
cellulase enzyme with cellulolytic index more than 1.0. They are - *Acremonium implicatum, Alternaria alternata*, *Aschochyta, Aspergillus flavipes, Aspergillus flavus, Aspergillus fischeri, Aspergillus fumigatus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus niger, Aspergillus terreus, Aspergillus tamarii, Beltraniella humicola, Cladosporium sphaereospermum, Colletotrichum capsici, Curvularia lunata, Fusarium oxysporum, Humicola fuscoatra, Paecilomyces lilacinus, Paecilomyces variotii, Pestalotiopsis mangiferae, Penicillium chrysogenum, Penicillium decumbens*, *Scopulariopsis brumptii and Trichoderma viride* belongs to class deuteromycetes*, Ceratocystis paradoxa, Chaetomium salami, Gibberella zeae* belongs to class ascomycetes, *Mucor hiemalis* and *Rhizopus stolonifer* belongs to class zygomycetes while 2 another isolates i.e. *Chaetomium salami, Emericella nidulans* blelongs to class acomycetes and *Rhodotorula glutinis* belongs to basidiomycetes showed cellulolytic index equal or less than 1, considered as negative for cellulose production. **[22]** isolated microorganisms from lake area containing water hyacinth for enzymatic hydrolysis of cellulose. Eight positively fungal isolates *Basidiobolus sp., Aspergilllus sp., Anthroderma sp., Penicillium sp., Geotrichum sp., Arthrographis sp., Alternaria sp.* and *Fusarium sp.* were found to have cellulolytic activity.

In the present study, it was observed that from 30 positive isolates for cellulase activity, only three fungi named *Rhizopus stolonifer, Penicillium decumbens, Penicillium chrysogenum* have exhibited considerable activity to degrade the cellulose. Highest zone of clearance has been recorded for *Rhizopus stolonifer* (1.66), *Penicillium decumbens* (1.6), and *Penicillium chrysogenum* (1.44). Previously it has been observed that out of 115 fungal isolates only 78 were cellulolytic acivity and those fungal isolates belonged to genera *Aspergillus, Trichoderma, Fusarium, Alternaria* and *Penillium, Cladosporium* and *Rhizopus* **[1]**.

Table 1: Showing Enzymatic index of fungal isolates

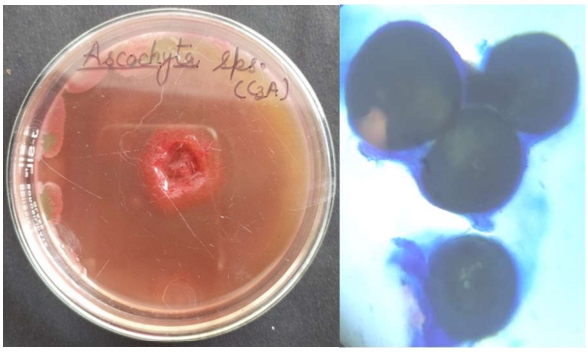
FUNGAL ISOLATES	COLONY DIAMETER (Cm)	HALO AREA DIAMTER (Cm)	ENZYMATIC INDEX ± SD
Acremonium implicatum	2.9	3.4	1.17 ± 0.036056
Alternaria alternata	3.2	3.6	1.12 ± 0.020817
Aschochyta graminicola	2.4	2.6	1.04 ± 0.040415
Aspergillus flavipes	3.3	3.7	1.12 ± 0.01
Aspergillus flavus	3.9	4.3	1.10 ± 0.011547
Aspergillus fischeri	3.4	3.6	1.05 ± 0.015275
Aspergillus fumigatus	3.5	3.9	1.11 ± 0.01
Aspergillus japonicus	2.3	2.8	1.22 ± 0.01
Aspergillus nidulans	3.4	3.7	1.08 ± 0.01
Aspergillus niger	3.1	4.0	1.27 ± 0.015275
Aspergilus terreus	3.7	4.4	1.18 ± 0.02
Aspergillus tamarii	3.1	3.7	1.19 ± 0.020817
Beltraniella humicola	2.3	2.5	1.05 ± 0.03
Ceratocystis paradoxa	2.3	2.6	1.1 ± 0.025166
Chaetomium salami	0	0	0
Cladosporium sphaerospermum	2.9	3.4	1.17 ± 0.02
Colletotrichum capsici	2.4	2.9	1.20 ± 0.015275
Cunninghamella blakesleeana	3.0	3.2	1.02 ± 0.017638
Curvularia lunata	2.2	2.7	1.22 ±0.015275
Emericella nidulans	2.6	2.6	1.0 ± 0.0
Fusarium oxysporum	3.0	3.3	1.1 ± 0.01
Gibberella zeae	3.6	4.4	1.22 ± 0.026458
Humicola fuscoatra	3.3	4.1	1.24 ±0.035119
Mucor hiemalis	3.5	4.6	1.22 ± 0.020817
Paecilomyces lilacinus	2.3	2.6	1.13 ± 0.13
Paecilomyces variotii	3.6	4.0	1.11 ± 0.015275
Pestalotiopsis mangiferae	3.6	4.1	1.13 ± 0.025166
Penicillium chrysogenum	2.7	3.9	1.44 ± 0.030551
Penicillium decumbens	2.5	4.0	1.6 ± 0.02
Rhizopus stolonifer	2.4	4.0	1.66 ± 0.036056
Rhodotorula glutinis	0	0	0
Scopulariopsis brumptii	3.2	3.6	1.12 ± 0.02
Trichoderma viride	4.0	5.1	1.27 ± 0.02

± SD from Mean value



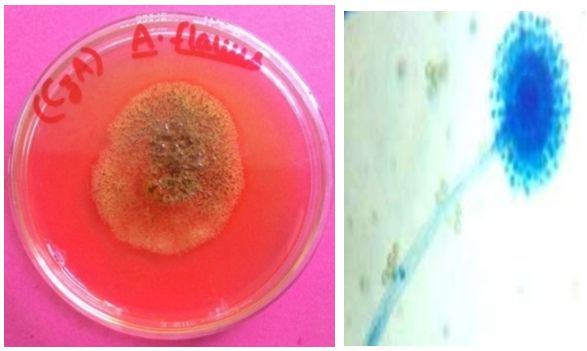
1. Acremonium implicatum

2. Alternaria alternata

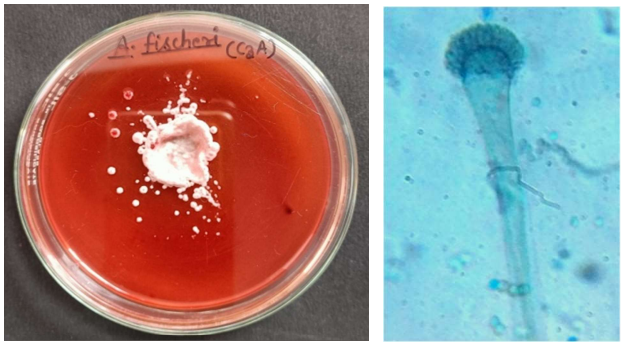


3. Aschochyta graminicola

4. Aspergillus flavipes



5. Aspergillus flavus

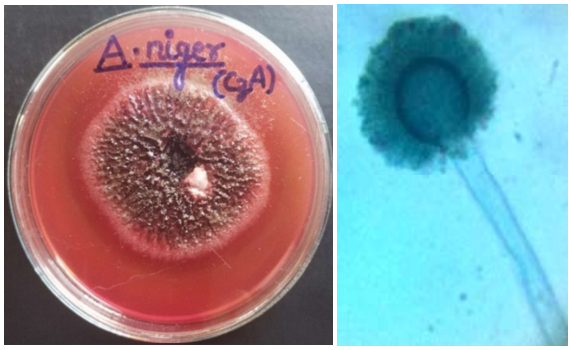


6. Aspergillus fischeri

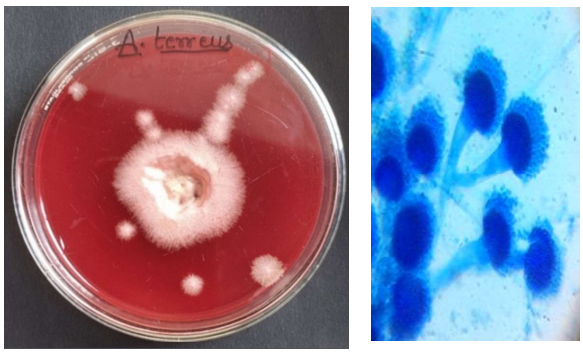
7. Aspergillus fumigatus

8. Aspergillus japonicus

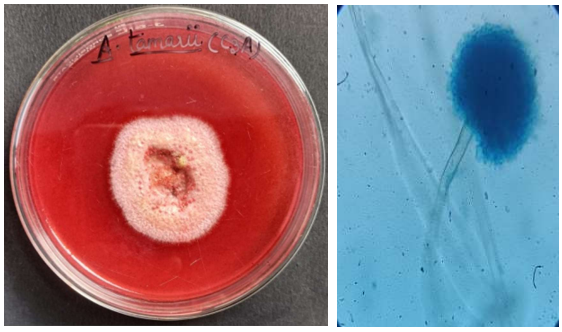
9. *Aspergillus nidulans*



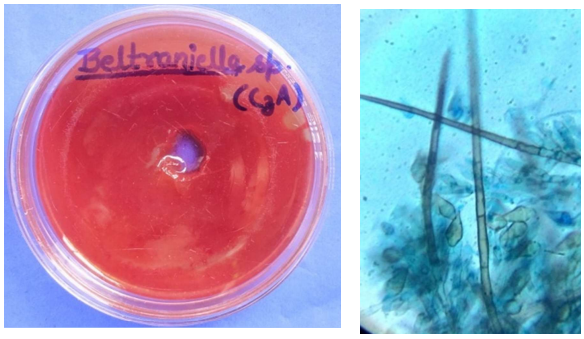
10. *Aspergillus niger*



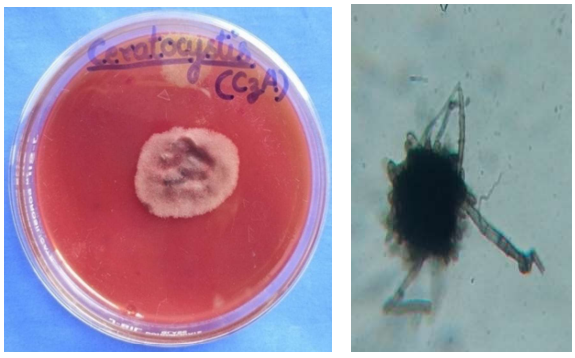
11. *Aspergillus terreus*



12. *Aspergillus tamarii*

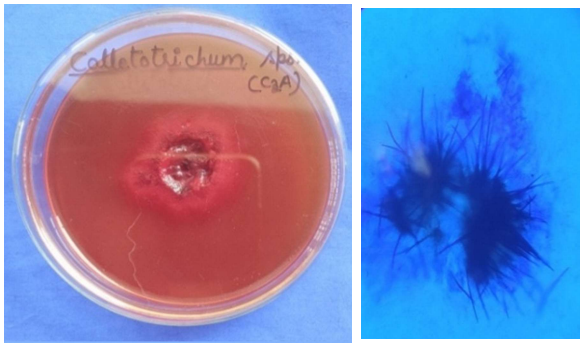


13. *Beltraniella*

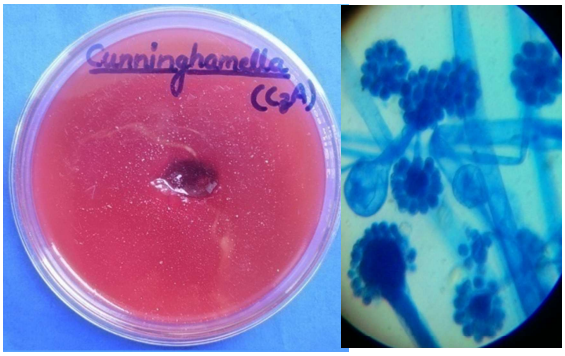


14. *Ceratocystis paradoxa*

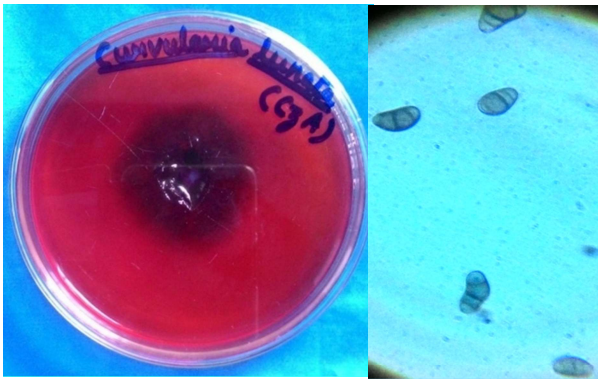
15. *Cladosporium sphaerospermum*



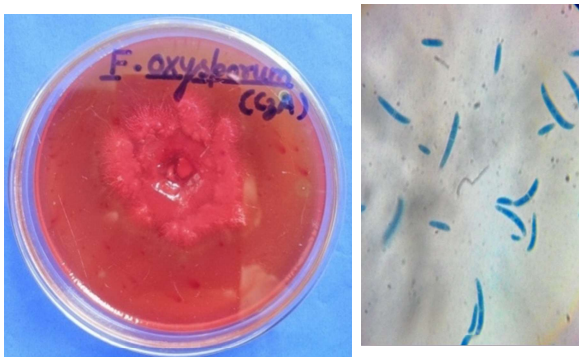
16. *Colletotrichum capsici*



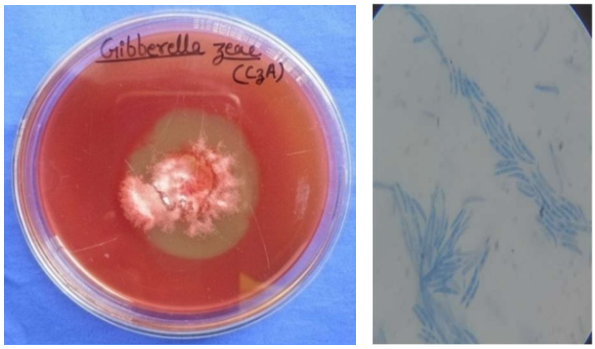
17. *Cunninghamella blakeesleeana*



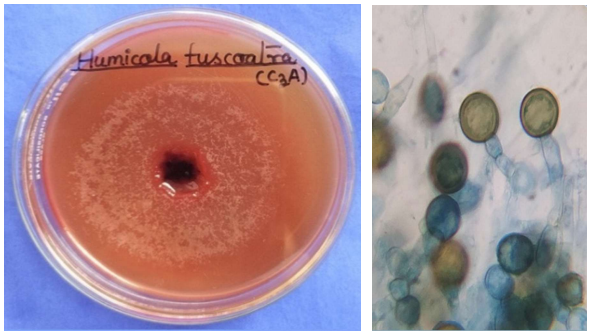
18. *Curvularia lunata*



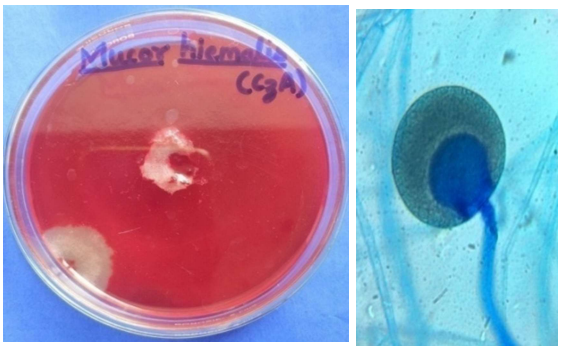
19. *Fusarium oxysporum*



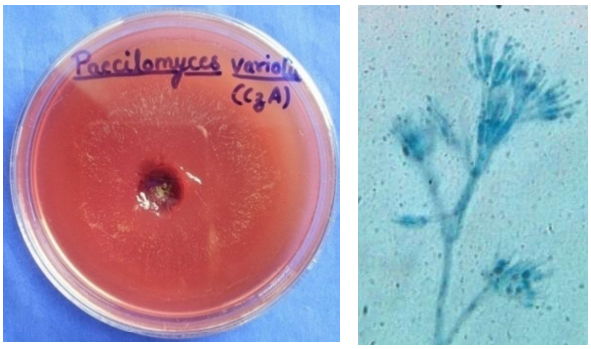
20. *Gibbrella zeae*



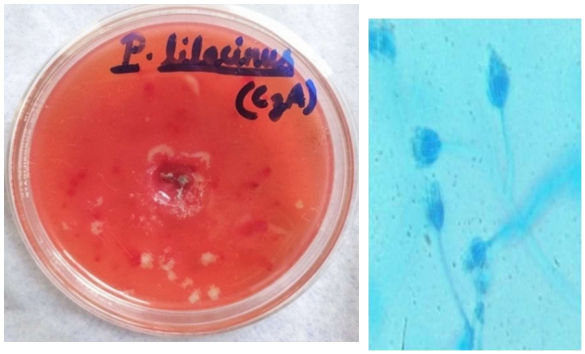
21. Humicola fuscoatra



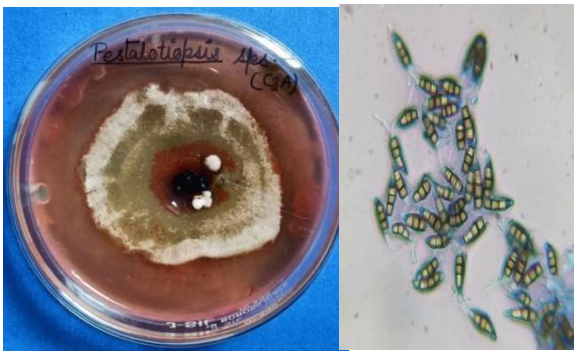
22. Mucor hiemalis



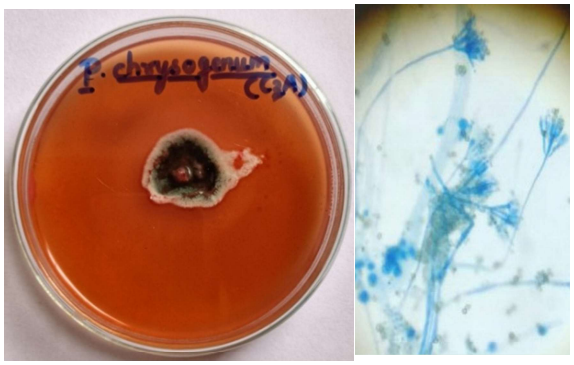
23. Paecilomyces lilacinus



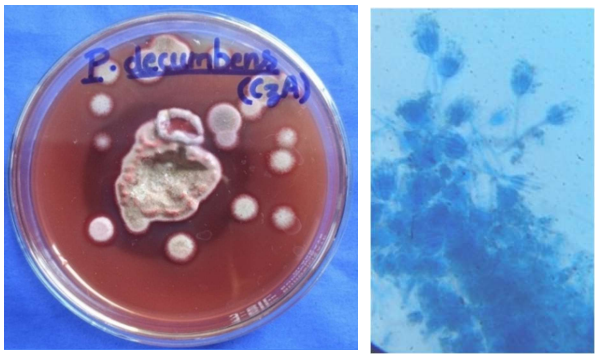
24. Paecilomyces variotii



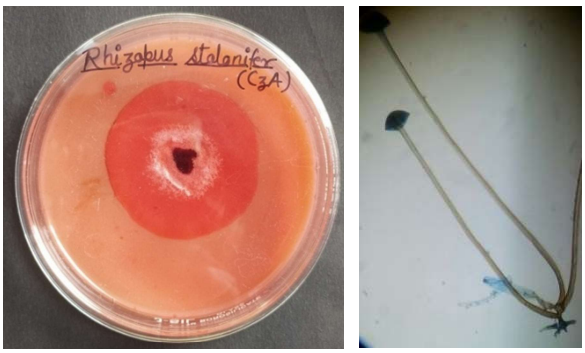
25. Pestalotiopsis mangiferae



26. Penicillium chrysogenum

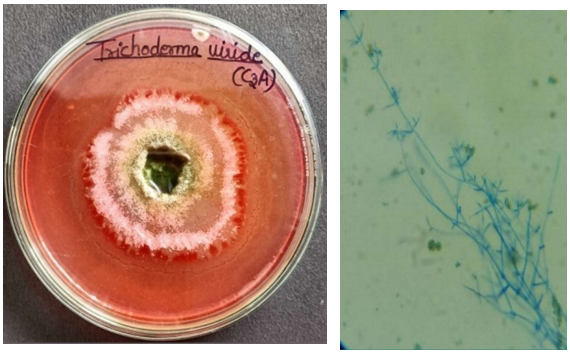


27. Penicillium decumbens



28. Rhizopus stolonifer

29. Scopulariopsis brumptii



30. Trichoderma viride

Figure 2: Identified Fungal colonies showing their cellulolytic activities on media containing CMC

CONCLUSION

Recent study aims to isolate and identify important fungi, capable of producing enzymes with unique features and the potential for future industrial uses. The present study was undertaken to isolate fungi from degrading litter. In this work it was found that 33 fungi which were isolated from Orchha Wildlife Sanctuary. Out of which 30 fungal isolates were found positive for cellulolytic activity that were categorized based on their capacity to produce cellulase enzyme and formed clear zone around their growth. Among all these, 3 isolates *Rhizopus stolonifer, Penicillium decumbens* and *Penicillium chrysogenum* were selected with maximum cellulase activity. This was determined as their ratio of colony diameter and clear zone diameter was higher than others. These three fungal isolates may be

further studied to develop processes for producing cellulases on cheaper substrates that is demanded by various industries.

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