Original Research Article

Studies on Cellulolytic Fungal Flora from Forests of Maharashtra

ABSTRACT

The present investigation was conducted to isolate effective cellulolytic fungal flora from soil samples collected from various forests in Maharashtra (India). The study aimed to determine hydrolytic zones and relative enzyme activity for selecting most efficient fungal isolates. Further, the isolates were identified using morphological identification technique.

Altogether 137 fungal isolates were obtained in the present study, collected from forests of Lonavala, Chandrapur, Mahabaleshwar, Gadchiroli and Bhandardara. The screening of these fungal isolates for cellulase production revealed that 41 isolates had varying degrees of cellulolytic activity. However, isolate C77 produced the largest clearance zone (4.8 cm) on carboxymethyl cellulose (CMC) medium. The relative enzyme activity, expressed as the cellulolytic index, ranged from 1.1 to 3.5 among the 41 fungal isolates. Notably, C65 displayed the highest cellulolytic index, indicating superior enzyme activity, while other isolates like G5X, B24 and M145 also exhibited significant cellulolytic activity. From the present investigation it is concluded that *Aspergillus terreusThom*, *Trichoderma* spp*. Aff. T. harzianum Rifai*, *Aspergillus fumigatus*, and *Aspergillus* spp*. Aff. A. flavus Link* isolated from the forests of Chandrapur, Gadchiroli, Bhandardara and Mahabaleshwar, respectively, were the most efficient cellulose degrading fungi.

Keywords: [Cellulolytic fungi, Hydrolytic zones, forests, Relative enzyme activity, Trichoderma, Aspergillus]

1. INTRODUCTION

In a world where the demand for food is rapidly increasing, agricultural production has surged, resulting in a significant accumulation of leftover materials [1]. In India, farming activities contribute to the production of approximately 500 million tons of crop residues annually [2], referred to as agricultural stubbles, rich in cellulose, hemicelluloses, and lignin.

Unfortunately, improper disposal practices, notably the burning of residues, have become prevalent, causing alarming levels of air pollution in northern regions, particularly impacting areas like Delhi [3]. This practice not only harms human health, leading to symptoms such as heart disease and respiratory issues,but also adversely affects soil quality.

The burning of agricultural residues raises environmental concerns, leading to a decline in soil organic matter, disruption of nutrient cycles, and harm to beneficial soil organisms. Despite the known detrimental effects, a significant number of farmers continue this practice, resulting in elevated soil temperatures and the loss of valuable moisture [4].

Chemically, continuous burning disrupts nutrient cycles within the soil, causing a loss of organic carbon, increased soil pH, and reduced nutrient levels. This decline in organic matter is particularly harmful to soil fertility indicators such as cation exchange capacity [5].

Biologically, while burning crop residues may offer a temporary boost in nutrient availability, it comes at a cost. The heat generated during the burning process adversely affects crucial soil organisms, creating a double-edged sword scenario where some problems are eliminated, but essential bacterial and fungal populations necessary for sustaining fertile soil are wiped out [6].

Recognizing the urgency of the situation, there is a critical need for proper residue disposal methods and increased awareness among farmers. Contemporary technologies are emerging as effective means to utilize agricultural waste, offering avenues for residual conversion into revenue-generating resources. These opportunities not only address environmental concerns but also provide farmers with the potential for additional income by selling previously discarded waste [7].

The concept of a circular economy has gained traction, focusing on nutrient recycling through composting or converting residues into organic fertilizers. This sustainable approach not only closes the nutrient loop but also reduces the environmental impact of traditional farming practices [8].

Central to these processes is the breakdown of cellulose, the most prevalent carbohydrate in plant cell walls. Despite its abundance, cellulose is notably resistant to degradation due to its complex structure. Enzyme-driven reactions, specifically cellulases, play a pivotal role in converting intricate cellulose compounds into simple sugars, such as glucose [9].

Microorganisms, particularly fungi like *Trichoderma* and *Aspergillus*, are crucial contributors to the intricate process of cellulose degradation. These microorganisms act as nature's recyclers in natural environments, breaking down cellulose-rich plant material, releasing vital nutrients back into the soil, and nourishing the growth of fresh plants [10].

Fungi, being primary cellulase-producing microorganisms, offer potential solutions for agricultural applications. Studies on cellulolytic enzyme production through biowaste degradation processes, involving a diverse range of microorganisms, hold promise for sustainable waste management and enzyme production [11].

Recognizing the importance of cellulolytic fungi in recycling plant nutrients, investigations into the "Studies on cellulolytic fungal flora from forests of Maharashtra" aim to isolate efficient cellulose-degrading fungi from forest soils. Obtaining such efficient strains can revolutionize the management of agricultural waste on farms, aiding in the quick production of compost and potentially generating income from waste.

In conclusion, urgent action is imperative to address the pressing issue of agricultural residue management. By embracing contemporary technologies, recognizing the value of agricultural waste, and incorporating sustainable practices, we can contribute to environmental conservation and economic development, viewing agricultural residues not as byproducts but as potential sources of innovation.

2. MATERIAL AND METHODS

2.1 Collection of Soil Sample

Forty soil samples were collected from various undisturbed locations in selected forests (Table 1) using soil augers, hand trowels, and polythene bags. The soil was excavated up to 20 cm depth and immediately placed in sterile polythene bags with hand trowels. Two spots per site were sampled, and the collected samples were mixed for a representative composite. Field moist samples were then brought to the laboratory, stored at -10°C, and awaited cellulolytic fungi isolation.

Table 1 Location of soil samples collected

Sr. No.	Soil sample code	Location	Coordinates
1.	L1	Lonavala	18.696991, 73.387006
2.	L2	Lonavala	18.688033, 73.386270
3.	L3	Lonavala	18.653908, 73.389111
4.	L4	Lonavala	18.644679, 73.381298
5.	L5	Lonavala	18.680440, 73.388299
6.	L6	Lonavala	18.694085, 73.386302
7.	L7	Lonavala	18.622470, 73.353609
8.	L8	Lonavala	18.712314, 73.388969
9.	C1	Chandrapur	20.487215, 79.930562
10.	C2	Chandrapur	20.492949, 79.903210
11.	C3	Chandrapur	20.487152, 79.880816
12.	C4	Chandrapur	20.538557, 79.924056
13.	C5	Chandrapur	20.563338, 79.874953
14.	C6	Chandrapur	20.454882, 79.879757
15.	C7	Chandrapur	20.479591, 79.855062
16.	C8	Chandrapur	20.462700, 79.822257
17.	C9	Chandrapur	20.450499, 79.919669
18.	C10	Chandrapur	20.481233, 79.935069
19.	G1	Gadchiroli	20.507077, 79.994653
20.	G2	Gadchiroli	20.438342, 80.072833

21.	G3	Gadchiroli	20.432663, 80.083224
22.	G4	Gadchiroli	20.452831, 80.059721
23.	G5	Gadchiroli	20.437067, 80.035229
24.	G6	Gadchiroli	20.446108, 80.054278
25.	G7	Gadchiroli	20.455728, 79.995769
26.	G8	Gadchiroli	20.564981, 79.972390
27.	G9	Gadchiroli	20.536488, 79.978699
28.	G10	Gadchiroli	20.477515, 79.954825
29.	M1	Mahabaleshwar	17.935438, 73.687512
30.	M2	Mahabaleshwar	17.935188, 73.687916
31.	M3	Mahabaleshwar	17.940980, 73.674627
32.	M4	Mahabaleshwar	17.940230, 73.675962
33.	M5	Mahabaleshwar	17.938844, 73.675477
34.	M8	Mahabaleshwar	17.938767, 73.676185
35.	M10	Mahabaleshwar	17.918402, 73.667069
36.	M11	Mahabaleshwar	17.919361, 73.667558
37.	M14	Mahabaleshwar	17.917111, 73.666680
38.	M16	Mahabaleshwar	17.916935, 73.665223
39.	B1	Bhandardara	19.533864, 73.839297
40.	B2	Bhandardara	19.553413, 73.627504

2.2 Isolation of Fungi

The pour plate technique was used for the isolation of cellulolytic fungi from the forest soil samples. One gram of soil from each sample was suspended separately in 9 mL of distilled water in test tube. Further, the dilutions were made up to 10^{-6} and 1 mL of soil suspension from each sample was poured separately in to Petri plates. The sterilized Czapek-Dox agar mediumhaving a temperature of 42-45^{0}C was poured to Petri plates [12]. After solidification of the medium, the inoculated Petri plates were kept in an incubator at 28^{o}C for 4 days. The discrete and typical fungal colonies growing on the medium were transferred onto PDA slants and were maintained at 4^{o}C for further studies.

2.3Screening of Fungal Strains for Cellulase Using Qualitative Plate Assay

Cellulase activity of the fungal isolates was determined by using 1% CMC (Carboxymethyl cellulose) agar medium. Agar block (6 mm in diameter) from each one-week-old fungal colony grown on plates was cut and inoculated separately in centre of the plates containing 1% CMC agar. Three replicates were maintained for each isolate. Further, the plates were incubated at 30^{o}C for 72 hours. After growth of the fungal isolates, the plates were flooded with Gram's Iodine (0.133g KI and 0.067g Iodine dissolved in 20 mL distilled water) for 3 to 5 min [13]. The fungal isolates that were able to decompose CMC were indicated by the formation of a clear zone around the colony after being tested by Gram's Iodine. The strains that showed a clearing zone around the colony were maintained as potential cellulase producing fungi. The diameter of zone of hydrolysis was measured for each isolate.The fungal isolates were selected on the basis of diameter of the cultivated colonies on the basic medium with carboxymethyl cellulose as a carbon source in the Petri dishes and Index of Relative Enzyme Activity (ICMC) [14], which is the ratio between the diameter of the hydrolysis zone and the diameter of the colony. The strains with indices near or greater than 1 were selected for further studies.

3. RESULTS AND DISCUSSION

3.1 Screening of Fungal Isolates for Cellulase Production Using Qualitative Plate Assay

Results of the present investigation pertaining to screening of the fungal isolates for cellulase production ability, estimated qualitatively using the plate assay method, are presented in Table 1 and 2,Fig 1 and 2, Plate 1.
In the present study, 137 fungal isolates obtained from samples of forest soils, collected from Lonavala, Chandrapur, Mahabaleshwar, Gadchiroli and Bhandardara forests located in Maharashtra, India, were appraised for their ability to produce cellulase. Through *in vitro* screening, using a qualitative plate assay, we evaluated their capacity to degrade

cellulose. The cellulolytic activity of these fungal strains was gauged by the formation of clear zones (hydrolysis zone) around their colonies in carboxymethyl cellulose (CMC) medium and by computing relative enzyme activity.
Out of 137 fungal isolates obtained in the study, 41 isolates could degrade cellulose which was evident from the formation of hydrolysis zone around their colonies. The rest of the 96 isolates were found to be inefficient in degrading cellulose as they did not show a hydrolysis zone around them.

3.2 Hydrolysis Zone Diameter

Data pertaining to the diameter of hydrolysis zone around the fungal colonies, growing on 1% CMC agar medium, indicated that the hydrolysis zone diameter with different fungal isolates varied between 0.77 cm and 4.8 cm (Table 3) Notably, amongst the 41 fungal isolates showing varying degrees of cellulose degradation capability, isolate C77 exhibited the highest zone of clearance, measuring 4.8 cm on carboxymethyl cellulose (CMC) medium. Fungal isolate C64 and C75 were the next best isolates in cellulose hydrolysis, generating a substantial clearance zone of 4.1 cm and 4.0 cm, respectively. In the order of effectiveness, fungal isolate M34 was the next best cellulose hydrolyser, which recorded a clearance zone measuring 3.7 cm. Other isolates, including M23 (3.65 cm), C65 (3.6 cm), G310 (3.33 cm), C66 (3.26 cm), C68 (3 cm), M110 (2.83 cm), M811 (2.73 cm), C73 (2.77 cm), G44 (2.76 cm), M27 (2.7 cm) and so forth, demonstrated varying degrees of clearance zones in CMC medium. Subsequently, three fungal isolates *viz.,* L12 (0.77 cm), B16 (0.97 cm), and B21 (1.17 cm) were found to be the least effective in cellulose hydrolysis in comparison with the other isolates in the study.
The results from the study, specifically regarding the clearance zones produced by different isolates in CMC medium, unequivocally indicated that among all the isolates examined, C77 was the most efficient fungus in cellulose degradation. Conversely, isolates L12, B16 and B21 demonstrated the least efficacy in comparison to the rest of fungal isolates. Many of the earlier scientists have recorded the zone of hydrolysis of varying diameters. Kumar *et al.* (2013) [15] recorded a zone of clearance of 0.42 cm. Moreover, Gomashe*et al.* (2013)[16], Ahmed *et al.* (2018)[12], Nhan *et al.* (2021) [17] and Das *et al.* (2023) [18]obtained various values of hydrolytic zones. Results obtained in the present study were in agreement with the findings of these researchers.

Table 2 Hydrolysis zone diameter (cm)

Sr. No.	Culture code	Diameter of hydrolyzed zone (cm)	Sr. No.	Culture code	Diameter of hydrolyzed zone (cm)
1.	G34	2.06	22.	M145	2.30
2.	G310	3.33	23.	M110	2.83
3.	G44	2.76	24.	M815	1.77
4.	G5X	2.40	25.	M23	3.65
5.	G66	2.00	26.	M811	2.73
6.	L13	2.63	27.	L12	0.77
7.	L84	2.30	28.	L55	2.30
8.	L51	1.43	29.	L44	1.80
9.	LXX	2.13	30.	M152	1.70
10.	B14	2.10	31.	M31	1.70
11.	B24	1.40	32.	M112	2.20
12.	B16	0.97	33.	M27	2.70
13.	B17	2.30	34.	M815	2.60
14.	B22	1.64	35.	C77	4.80
15.	B21	1.17	36.	C66	3.26
16.	B23	2.17	37.	C73	2.77
17.	M165	2.23	38.	C65	3.60
18.	M106	2.00	39.	C75	4.00
19.	M111	1.97	40.	C68	3.00
20.	M34	3.70	41.	C64	4.10
21.	M19	1.57			

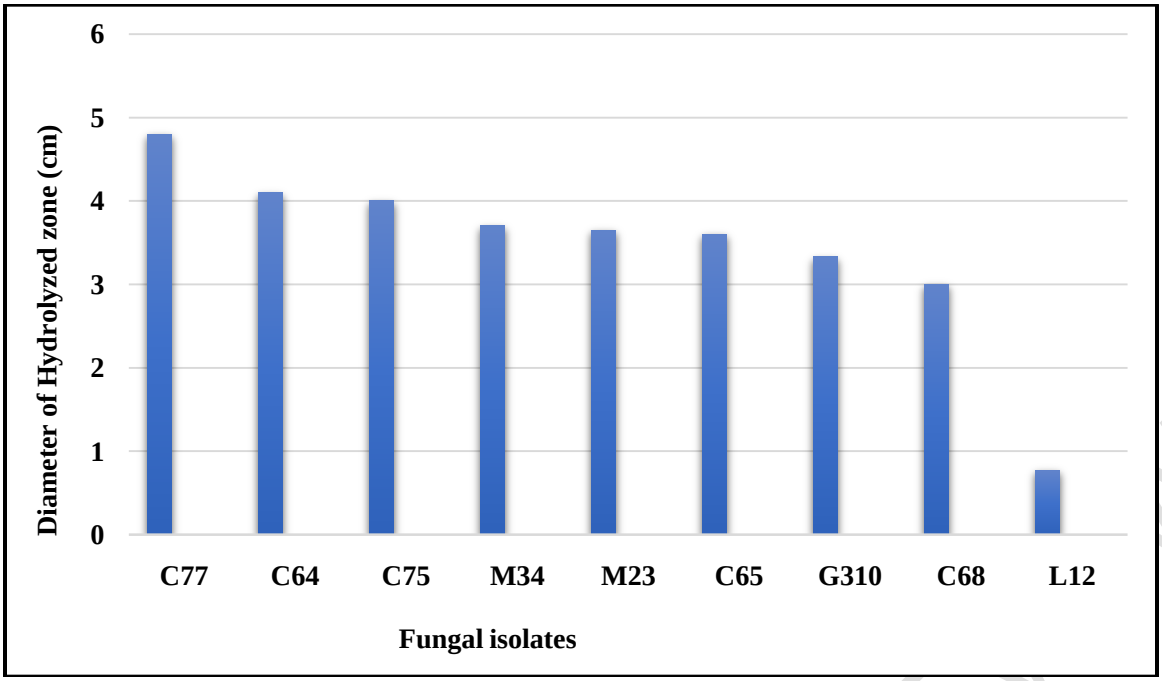

Fig 1 Zone of hydrolysis by different fungal isolates.

3.3 Relative Enzyme Activity

The results depicted in Table 3, about the relative enzyme activity index (ICMC) of all the fungal isolates showing hydrolysis zone, revealed a range in cellulolytic index across all 41 fungal isolates, spanning from 1.1 to 3.5.
A perusal of the data explicitly unveiled that, the most distinguishable cellulolytic index, to the tune of 3.5, was obtained in the fungal isolate C65, establishing this particular fungal isolate as distinctly superior in displaying the highest enzyme activity compared to its counterparts. Following closely in the rankings, fungal isolate G5X secured the second position with a cellulolytic index of 3.16. Subsequent isolates, namely B24, M145, C73, and C68 formed the next tier of top performers, each displaying a cellulolytic index of 2.5, 2.2, 2.2, and 2.07, respectively. Rivetingly, these four fungal isolates displayed almost identical relative enzyme activity, with minute differences in cellulolytic indices. Conversely, the remaining isolates, including C77 (2.00), G66 (2.00), B16 (1.94), M27 (1.93), M112 (1.83), L55 (1.8), B17 (1.8), L12 (1.79), LXX (1.75), B14 (1.7) and others, exhibited varying degrees of cellulolytic index. The lowest recorded cellulolytic index, of 0.59, was with B21. Additionally, the cellulolytic index values for B23 (0.74), G44 (1.1), L51 (1.19) and M811 (1.2) indicated lower ICMC activity. These isolates were found to have the least relative enzyme activity compared to the other isolates.
The findings unequivocally suggested that C65 stood out the best with the highest relative enzyme activity among all the isolates under study. On the contrary, B21, B23, G44, L51, and M811 exhibited the least ICMC activity in comparison to the rest of the fungal isolates. Hitherto, many researchers have conducted identical studies including Khokhar *et al.* (2012) [14]. Sun *et al.* (2020) [19] found that the relative enzyme activity index ranged from 1.89 to 3.19. Wisdawati*et al.* (2021) [20] reported ICMCindex ranging from 0 to 1.66. Similarly, Gat *et al.* (2022) [21] obtained ICMC values ranging from 0 to 0.56. The results of the present investigation are in line with the results of these scientists.

Table 3Relative enzyme activity index (ICMC) of cellulose-degrading fungal isolates isolated from forest soils of Maharashtra

Sr. No.	Strain Code	ICMC	Sr. No.	Strain Code	ICMC
1.	G34	1.34	22.	M145	2.20
2.	G310	1.39	23.	M110	1.30
3.	G44	1.10	24.	M815	1.30
4.	G5X	3.16	25.	M23	1.23
5.	G66	2.00	26.	M811	1.20
6.	L13	1.30	27.	L12	1.79
7.	L84	1.50	28.	L55	1.80
8.	L51	1.19	29.	L44	1.59
9.	LXX	1.75	30.	M152	1.45

10.	B14	1.70	31.	M31	1.28
11.	B24	2.50	32.	M112	1.83
12.	B16	1.94	33.	M27	1.93
13.	B17	1.80	34.	M815	1.59
14.	B22	1.59	35.	C77	2.00
15.	B21	0.59	36.	C66	1.63
16.	B23	0.74	37.	C73	1.54
17.	M165	1.30	38.	C65	3.50
18.	M106	1.60	39.	C75	2.07
19.	M111	1.50	40.	C68	2.20
20.	M34	1.54	41.	C64	1.50
21.	M19	1.68			

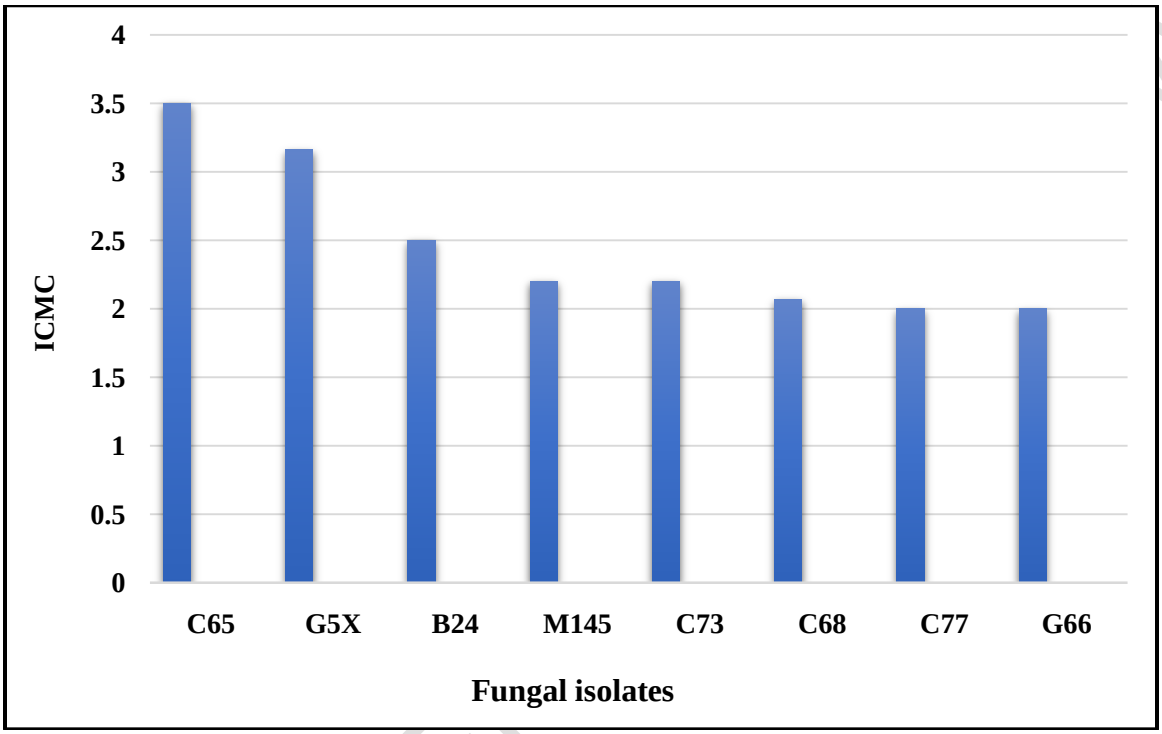

Fig 2 Relative enzyme activity index (ICMC) of cellulose degrading fungal isolates isolated from forest soils of Maharashtra, India.

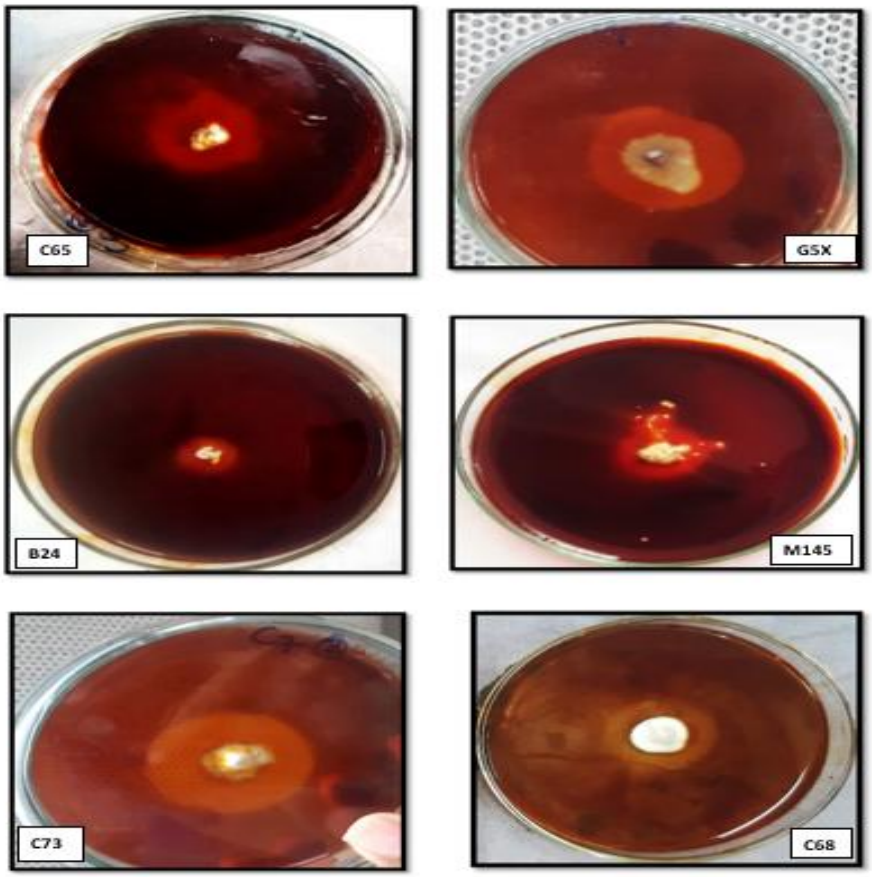

Plate 1.Screening of fungal isolates for cellulose degradation (Hydrolysis zone)

3.4 Identification of Efficient Fungal Isolates

Four fungal isolates showing the highest relative enzyme activity (ICMC) of 3.5, 3.16, 2.5 and 2.2 *viz.*, C65, G5X, B24 and M145 respectively, were selected further for identification. The fungal isolates were identified by studying spores and mycelia and this data was used for identification as described in Domsch*et al.* (1980)[22]. The cellulolytic fungal isolates C65, G5X, B24 and M145, having highest relative enzyme activity, were identified as *Aspergillus terreus*Thom*, Trichoderma sp.* aff. *T. harzianum*Rifai*, Aspergillus fumigatus* and *Aspergillus sp.* aff. *A. flavus* Link*,* respectively.

4. CONCLUSION

In this study, 137 fungal isolates from forest soil samples across different locations were qualitatively assessed for cellulase production through a plate assay method. Out of these isolates, 41 demonstrated cellulose degradation ability based on the formation of hydrolysis zones in carboxymethyl cellulose (CMC) medium. The diameter of hydrolysis zones varied between 0.77 cm and 4.8 cm, with isolate C77 exhibiting the highest clearance zone. Subsequent analysis of relative enzyme activity (ICMC) across these isolates revealed a range from 1.1 to 3.5, with isolate C65 standing out as the most efficient cellulolytic fungus. Further identification of efficient isolates—C65, G5X, B24, and M145—revealed them as *Aspergillus terreusThom*, *Trichoderma* sp*. aff. T. harzianum Rifai*, *Aspergillus fumigatus*, and *Aspergillus* sp*. aff. A. flavus Link*, respectively. These findings align with previous studies and highlight the cellulolytic potential of these fungal isolates in cellulose degradation.

REFERENCES

1. Ritchie H, Rosado P, Roser M. Agricultural Production. Our world in data. 2003

2. Meena HN, Singh SK, Meena MS, Narayan R, Bheem Sen.Crop residue: waste or wealth? Technical Bulletin 2022; 1-30.

3.ChananaI, Sharma A, Kumar P, Kumar L, Kulshrestha S,Kumar S, et al. Combustion and stubble burning: a major concern for the environment and human health. Fire. 2023; 6(2):79.

4. Turmel SL, Speratti A, Baudron F,Verhulst N,Govaerts B.Crop residue management and soil health: A systems analysis. Agric. Syst. 2015;134(C): 6-16.

5. Butterly CR,Kaudal B, Baldock JA,Tang C.Contribution of soluble and insoluble fractions of agricultural residues to short-term pH changes. Euro. J. Soil Sci. 2011;62(5):718-727.

6. Thakur JK, Prajapati S, Mandal A, Manna MC, Somasundaram J.Crop residue burning: consequences on soil microbes. Harit Dhara. 2019;2(2): 8-11.

7. BentsenNS, Felby C, andThorsen BJ. Agricultural residue production and potentials for energy and materials services. Prog. Energy Combust. Sci. 2014;40: 59-73.

8. WaqasM, Hashim S,Humphries UW, Ahmad S, Noor R,Shoaib M, et al. Composting Processes for Agricultural Waste Management: A Comprehensive Review. Processes. 2023;11:731.

9. Wood TM, Garcia-Campayo V. Enzymology of cellulose degradation. Biodegradation. 1990;1:147-161.

10. McDonaldJE, Rooks DJ, McCarthy AJ.Methods for the isolation of cellulose-degrading microorganisms. Meth. Enzymol. 2012;510: 349-374.

11. Mandels M, and Reese ET. Fungal cellulase and microbial decomposition of cellulosic fibres. Dev. Ind. Microbiol. 1985;5: 5-20.

12. Ahmed MM, El-Zayat SA, El-Syed MA. Cellulolytic activity of cellulose-decomposing fungi isolated from Aswan hot desert soil, Egypt. J. Biol. Sci. 2018;1(2): 35-48.

13. GohelHR, Contractor CN,Ghosh SK, Braganza VJ. A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates. Int.J.Curr.Microbiol.App.Sci. 2014;3(5): 261-266.

14. KhokharI, Haider MS, Mushtaq S, Mukhtar I. Isolation and screening of highly cellulolytic filamentous fungi. Scholarly J. Agric. Sci. 2012;2(7): 126-129.

15.KumarU, Tapwal A, Kalkal P, Varghese S, Chandra S. Isolation and screening of cellulase producing fungi from forest waste. IJPBA. 2013;5(1): 56-59.

16. GomasheAV, Gulhane PA, Bezalwar PM. Isolation and screening of cellulose degrading microbes from nagpur region soil. Int. J. of Life Sciences. 2013;1(4): 291-293.

17. NhanNT, Anh DLH, Tri NM. Isolation of cellulolytic fungi and their application for production of organic fertilizer from water hyacinth (*Eichhornia crassipes*). Biotech Studies. 2021;30(1): 16-21.

18. DasS, Kumari BDR, Kumar TS, Harinikumar KM,Banu AS, Sellstedt A.Collection, Isolation and Characterization of Rot Fungus from isolated from Pushpagiri and Brahmagiri Hills, Western Ghats of Karnataka. Acta Biomed (Internet). 2023;94(1):e2023110.

19.Sun, S, Weng Y,Di X,Liu Z, Yang G.Screening of Cellulose-degrading fungi in forest litter and fungal effects on litter decomposition. BioResources. 2020;15(12): 2937-2946.

20. WisdawatiE, Kuswinanti T, RosmanaA, Nasruddin A. Screening and identification of cellulolytic fungi at rhizosphere of safira taro plant. IOP Conf. Ser. Earth Environ. Sci. 2021;807(2):022041.

21. Gat SD, Mahajan SB, Shinde TB and Deshmukh DP. Isolation and evaluation of cellulolytic potency of fungal isolates recovered from undisturbed forest localities. Pharma innov. 2022;11(3): 112-116.

22. DomschK, GamswH, Aderson TH. Compendium of soil fungi, vol.1. Academic press, London. 1980.