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Cellulase Activity of Soil Fungi (*Aspergillus, Fusarium, Penicillum, Trichoderma*) Isolated from Rhizosphere Region of Iron Ore Mine Overburden Soil

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Abstract—*A study was carried out to determine cellulase activity of 92 fungi was isolated from different age of iron ore mine overburden soil which contains a very low level of organic matter. These fungi belong to 27 different genera. Among 54 fungal isolates were identified as cellulase producer. Among them four genera, namely, Aspergillus, Fusarium, Penicillum and Trichoderma with 26 fungal isolate were dominant cellulase producers while rest of the fungi were only able to grow in basal salt media but not shown enzymatic activity. Among them Trichoderma spp. showed the highest cellulase activity. Therefore, Trichoderma spp. may be applied in mine overburden soil along with amendment of organic matter to improve its fertility.*

Keyword*: rhizosphere microorganism, cellulase activity, Iron ore, mining, soil fertility*

1. INTRODUCTION

Fungi are an important component of the microbiota typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions (Ainsworth and Bisby, 1995). The role of fungi in the soil is an extremely complex one and is fundamental to the soil ecosystem. They perform ecological services that strongly impact the quality of human life and have enormous potential for providing economic benefits (Hawksworth and Rossman, 1997; Hawksworth, 2001). These fungi play an important role in the decomposition of organic matter and maintenance of soil nutrients. Soil enzymes are important for life processes of microorganism and stabilization of soil structure (Dick *et al*., 1994). Microbial biomass and soil enzyme activity can potentially provide an integrated biological assessment of soil quality (Dick *et al*., 1997). The relationship between soil organic matter, microbial biomass and microbial activity has been proposed as indicators of soil maturity (Anderson and Domsch, 1989). After mining several change occurred in physical, chemical and microbiological properties of soil. Physico-chemical properties of mine spoil overburden was analysis and recorded that organic carbon, N, P, K content was lower as compare to natural soil (Unpublished data). Due to these reason plant was not able to grow and establish.

Soil texture was improved by adding chemical fertilizer, But it's create environment pollution. In present investigation fungi was isolated from rhizosphere region of mine overburden soil after that test out enzymatic activity of these fungi. Screened fungi were able to secrete cellulase enzyme which degrade the dead plants materials, root, rhizome and help in stimulating the soil fertility.

2. MATERIALS AND METHODS

2.1 Study Site

Dalli-Rajhara is located on a hill range bounded by 20° 33' 0" and 20° 34' 30" N latitude and 81° 1' 0" and 81° 4' 30" E longitude under Balod (District) in Chhattisgarh. The climate of Balod is tropical and the whole year is divisible in to three well marked seasons viz., summer (March to June), monsoon (July to October) and winter (November to February). Temperature rises up to even 47ºC in the month of May/June and comes down to 6.5ºC in December/January. The mean annual rainfall is 1400 mm, 75-80% of which occurs during the monsoon period.

2.2 Soil vegetation

The vegetation of natural forest is dominated by teak (*Tectona grandis* Linn. F.) and other tree constituents of vegetation is consisting of *Acacia nilotica* (Linnaeus) Delile., *Aegle marmelos* (L.), *Acacia catechu* Roxb., *Albizia procera* (Roxb.) Benth., *Bauhinia retusa* L., *Boswellia serreta* Triana and Planch., *Butea monosperma* (Witt.) Maheswari, *Dalbergia paniculata* Roxb., *Diospyros melanoxylon* Roxb., *Dendrocalamus strictus* (Roxb.), *Haldina cordifolia* (Roxb.), *Lagerstroemia parviflora* (Linnaeus), *Pongamia pinnata* (L.)

Pierre., *Shorea robusta* Roth, *Terminalia tomentosa* Roxb (ex DC) Wight & Arnbelonging, and *Ziziphus mauritiana* Lamk. (Banerjee *et al*., 1997).

2.3 Soil sampling

Soil sample were collected from planted trees in iron ore mined overburden dumps. Sample uses for fungal quantification were taken from rhizosphere zone by removing one cm soil from surface. A soil auger used which as washed thoroughly before starting of sampling procedure. Sampling done in 10-20 cm depth in soil horizon and carefully collected in polyethylene bags and their mouth were tied with rubber bands. In lab sample were homogenized and spread on paper to remove plant material, they are air dried, sifted with 2mm mesh sieve and stored at 4ºC used for experiment (Parkinson, 1979).

2.4 Preparation of Serial dilution

1g of each sample was grinded in pestle-mortar these samples were dissolved in 9 ml of sterile distilled water and thoroughly shake with vortex shaker to obtain 10^{-1} dilution. 1 ml of this solution is transferred to a second tube containing a 9ml of sterile water resulting in a 10^{-2} dilution of the spore mass in the original material. The process was repeated to yield dilution of 10^{-3}, 10^{-4} and 10^{-5} up to 10^{-9} from each dilution was prepared (Agrawal and Hasija, 1986).

Warcups soil plate method: 1 ml of the sample was placed in a sterile Petri-dish and 10 ml of sterile cooled (40°C) PDA was added. The contents were thoroughly mixed and the plates were incubated (Warcup, 1950). Incubate the plate at 27ºC for 3 to 7 days in BOD incubator.

2.5 Identification of Fungi

After incubation distinct colonies were identified. The cultures were identified on the basis of macroscopic (colonial morphology, color, texture, shape, diameter and appearance of colony) and microscopic characteristics (spore bearing fruiting body, spore size, growth rate hyphae, septation in mycelium, presence of specific reproductive structures, shape and structure of conidia and presence of sterile mycelium). Pure cultures of fungi isolates were identified with the help of literature (Raper and Thom (1949); Gilman, (1957); Barnett (1962); Booth (1971); Ellis (1971); Barnett and Hunter, (1972); Booth, (1977); Alexopolous *et al*., (1996); Nagmani *et al*., (2006); Verma *et al*., (2008) and Expert in Forest Pathology Division, T.F.R.I., Jabalpur were referred for identification of fungi. After the identification pure culture was stored in refrigerator for further use and preservation.

2.6 Screening by enzymatic assay of isolated soil fungi

Prepared the Basal salt medium (for cellulase activity), pour into the sterile Petri dishes. Allow it to solidify. Use sterile loop; make a single streak inoculation of each organism into the center of its appropriately labeled plate (Hankin and Anagnostakis, 1977) then incubated plate for 72 – 96 hours at 25ºC in an inverted position. After incubation flood the surface of basal salt medium with 1% Congo red dye (30 min), followed by de-staining with 1 M NaCl solution for 20 min. clear zones could be observed only around colonies of the active fungal strains.

2.6.1 Index of Relative enzyme activity

The enzymatic activities were estimated according to the method reported by Hankin and Anagnostakis (1975) who proposed an Index of Relative Enzyme Activity Index (REA) (Goldbeck *et al*., 2012; Choudhary and Jain, 2012; Bradner *et al*., 1999; Rajamani, and Hilda, 1987).

$$\text{Clear zone ratios} = \frac{\text{Clear zone diameter}}{\text{Colony diameter}}$$

2.6.2 Growth simulation/inhibition index

Different isolates will be cultured on growth media (Potato Dextrose Agar) and enzymatic activity test media and observed growth simulation/inhibition index (Teather and Wood 1982; Bradner *et al*., 1999).

$$\text{Growth simulation/ inhibition index} = \frac{\text{Colony diameter on basal salt media}}{\text{Colony diameter on potato dextrose agar}}$$

3. RESULTS AND DISCUSSION

138 soil samples were collected from different plantation and natural soil in three replications. A total of 92 fungal forms were obtained from the samples. Cellulase activity of test fungi was determined using carboxymethylcellulose as cellulose substrate on solid media.

The soil sample contained considerable population of the cellulase producing fungi. The Fungi grown on the selective media supported the growth of the fungi. 92 fungal species belonging to 27 genera i.e. *Absidia, Acremonium, Alternaria, Aspergillus, Biopolarus, Botryotr ichum, Cephalosporium, Cladosporium, Clamydomyces, Curularia, Emericella, Eupenicillum, Fusarium, Gliocladium, Memmoniella, Mucor, Nigrospora,* Non sporolating hypomyctes, *Oidiodendron, Paecilomyces, Penicillum, Periconia, Phoma, Scytalidium,* Sterile fungi, *Trichoderma* and *Tritriachium* were isolated from different age overburden. To check enzymatic activity four species which was highly dominated in iron ore mine was selected. Screening of fungal isolates was performed by plate method. Among 54 fungal isolates, 26 fungal isolates were identified as cellulose producer. Most of the cellulase producers belonged to *Aspergillus* (11), *Fusarium* (8) and *Penicillum* (6) followed by *Trichoderma* (1). However, rest of the isolates: *Aspergillus* (11), *Penicillum* (10) and

Trichoderma (6) and *Fusarium* (1) did not show any cellulolytic activity, but able to grown in basal salt medium (Table 1).

Cellulase activity on CMC agar was recorded as the Index of Relative Enzyme Activity (REA) was recorded as clear zone ratios. Maximum ratio was observed in *Penicillum commune* (1.375), *Penicillum* sp. 1 (1.356), *Fusarium solani* (1.259), *Fusarium chlamydosporum* (1.229), *Aspergillus flavus var. oryzae* (1.224) and *Aspergillus* sp. 3 (1.209) followed by *Penicillum adametzi* (1.198). Minimum was recorded in *Trichoderma* sp. (0.76) followed by *Fusarium poae* (0.935).

Growth simulation/inhibition index was computed as the colony diameter on carboxymethyl agar/colony diameter on control agar ratio. The index value <1, represented substrate inhibited fungal growth, while the index value >1, exhibited substrate rendered growth stimulation. The hydrolysis zone diameter compair with the colony diameter on carboxymethyl agar medium. However, hydrolysis zone diameters were not greater than colony diameter in case some isolates. Therefore, hydrolysis activity indices were found to be >1 in case of *Trichoderma viride* (1.789), *Penicillum aurantiogriseum* (1.742), *Aspergillus flavus* (1.646), *Penicillum* sp. 3 (1.613), *Penicillum roseopurpureum* (1.588), *Aspergillus flavus var. columnaris* (1.492), *Penicillum adametzi* (1.372), *Penicillum oxalicum* (1.351), *Penicillum* sp. 2 (1.21), *Aspergillus* sp. 1 (1.182), *Fusarium udum* (1.177), *Aspergillus niger* (1.172), *Penicillum asperum* (1.14), *Fusarium oxysporum* (1.129), *Aspergillus clavatus* (1.097), *Aspergillus* sp. 4 (1.094), *Trichoderma strctipilis* (1.079), *Aspergillus restrictus* (1.043), *Fusarium solani* (1.028), *Trichoderma pseudokoningii* (1.016). While, some colony was show < 1 *Aspergillus flavus var. oryzae* (0.997), *Penicillum novae* (0.996), *Aspergillus terreus* and *Aspergillus parasiticus* (0.992), *Aspergillus candidus* (0.932), *Penicillum nigricans* (0.924), *Fusarium roseum* (0.903), *Penicillum citreonigrum* and *Trichoderma* sp. (0.89), *Penicillum funiculosum* (0.799), *Trichoderma reesi* (0.797), *Trichoderma polysporum* (0.79), *Aspergillus janus* (0.781), *Fusarium javanicum* (0.778), *Aspergillus nidulars var. echinulatus* (0.749), *Penicillum restrictum* (0.744), *Penicillum citrinum* (0.731), *Aspergillus versicolor* (0.728), *Aspergillus* sp. 2 (0.727), *Fusarium chlamydosporum* (0.716), Asp*ergillus awamori* (0.686), *Aspergillus* sp. 3 (0.682), *Aspergillus unguis* and *Fusarium poae* (0.679), *Aspergillus repens* (0.672), *Fusarium oxysporeum* (0.629), *Aspergillus humicola* (0.616), *Trichoderma aureoviride* (0.612), *Aspergillus sydowii* (0.609), *Fusarium moniliforme* (0.545), *Aspergillus fumigates* (0.481), *Penicillum commune* (0.414) and *Penicillum rugulosum* (0.351) followed by *Penicillum* sp. 1 (0.005).

Fig. 1 showed that *Aspergillus* was present in higher number and more numbers of species were able to produced enzyme, but hydrolysis zone was the minimum. Similar result was observed by *Fusarium,* but its hydrolysis zone was highest as compare to *Aspergillus.* In case of *Penicillum* number of species was reduced but zone size was increased. *Trichoderma* spp. were present in lesser number (6) but their cellulase hydrolysis capacity were maximum.

In the present study, fungal isolation was done from iron ore mine soil. All fungi were able to grow in the medium with the sole carbon source – cellulose. Among 54 fungal isolates only 26 fungal isolates were identified as cellulase producer (Fig. 2). Most of the cellulase producers identified belonged to genera *Aspergillus, Fusarium, Penicillum* and *Trichoderma.* On the contrary Khokhar *et al*., (2012) observed maximum in *Trichoderma* and *Penicillium* followed by *Aspergillus* which have minimum number of species present in soil. They also reported that some isolates of *Aspergillus* and *Penicillium* did not show any cellulolytic activity. The most common and most effective cellulase producers are *Trichoderma ressei, T. koningii, Fusarium* sp., *Aspergillus* and *Penicillium* sp. (Yalpani 1987). Gautam *et al*., (2010) isolated fungi from municipal solid waste and observed cellulase activity of *Aspergillus fumigatus* and *Trichoderna* sp.1 were found relatively towards the higher side and *A. niger, A. flavus, A. nidulans, Alernaria* sp., *Penicillium* sp. moderate range while *Fusarium* sp., *Humicola* sp. and *Torula* sp. showed low cellulase activity. Fungi were also isolated from coal-mine spoil soil and their cellulolytic activities were reported (Stewart and Walah, 1972). At Homestake gold mine, Lead in South Dakota, USA soil bacteria belonging to the genera *Brevibacillus, Paenibacillus, Bacillus* and *Geobacillus* were isolated and their cellulase-degrading capacity were reported (Rastogi *et al*., 2009). Ten fungi *Aspergillus niger, A. flavus, A. glaucus, A. ustus, Aspergillus* sp., *Mucor* sp., *Alternaria alternata, Sarcinella* sp., *Cladosporium* sp. and Unidentified sp. (1). were isolated from municipal solid waste dumping region of Jabalpur city. Out of these 10 species 6 showed secretion of high amount of enzyme such as *A. flavus, Aspergillus sp., A. glaucus, A. ustus, Mucor sp.,* Unidentified sp. (1) (Verma *et al*., 2015).

4. CONCLUSION

Cellulase production by filamentous fungi differs amongst fungi isolated from mine overburden soil. *Trichoderma* spp. showed the highest cellulase activity, which may be applied in mine overburden soil along with amendment of organic matter to improve fertility of mine spoil soil.

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Table 1: Screening of isolated soil fungi on the basis of cellulase enzymatic assay (relative enzymatic activity index)

S. No	Fungal isolates	HZ (cm)	CD (cm)	REA Index	CDP (cm)	GS/ I I
1	*Aspergillus awamori*	0	5.67	0	8.27	0.686
2	*Aspergillus candidus*	4.93	4.43	1.113	4.75	0.932
3	*Aspergillus clavatus*	0	4.77	0	4.35	1.097
4	*Aspergillus flavus*	6.06	5.53	1.096	3.36	1.646
5	*Aspergillus flavus var. columnaris*	0	6.46	0	4.33	1.492
6	*Aspergillus flavus var. oryzae*	3.83	3.13	1.224	3.14	0.997

7	*Aspergillus fumigatus*	0	2.23	0	4.63	0.481
8	*Aspergillus humicola*	0	4.46	0	7.24	0.616
9	*Aspergillus janus*	0	3.86	0	4.94	0.781
10	*Aspergillus nidulars var. echinulatus*	3.86	3.56	1.084	4.75	0.749
11	*Aspergillus niger*	6.15	5.23	1.176	4.46	1.172
12	*Aspergillus parasiticus*	0	2.33	0	2.35	0.992
13	*Aspergillus repens*	4.56	3.85	1.184	5.73	0.672
14	*Aspergillus restrictus*	5.47	5.06	1.081	4.85	1.043
15	*Aspergillus* sp. 1	0	4.53	0	3.83	1.182
16	*Aspergillus* sp. 2	0	3.39	0	4.66	0.727
17	*Aspergillus* sp. 3	3.53	2.92	1.209	4.28	0.682
18	*Aspergillus* sp. 4	6.23	5.73	1.087	5.24	1.094
19	*Aspergillus sydowii*	0	3.13	0	5.14	0.609
20	*Aspergillus terreus*	4.73	4.23	1.118	4.26	0.992
21	*Aspergillus unguis*	0	3.36	0	4.95	0.679
22	*Aspergillus versicolor*	4.53	4.13	1.097	5.67	0.728
23	*Fusarium oxysporum*	6.33	6.03	1.049	5.34	1.129
24	*Fusarium chlamydosporum*	6.71	5.46	1.229	7.63	0.716
25	*Fusarium javanicum*	4.13	3.83	1.078	4.92	0.778
26	*Fusarium moniliforme*	3.66	3.13	1.169	5.74	0.545
27	*Fusarium oxysporeum*	3.43	3.03	1.132	4.81	0.629
28	*Fusarium poae*	2.29	2.45	0.935	3.61	0.679
29	*Fusarium roseum*	0	5.76	0	6.38	0.903
30	*Fusarium solani*	5.01	3.98	1.259	3.87	1.028
31	*Fusarium udum*	5.66	5.46	1.037	4.64	1.177
32	*Penicillum adametzi*	7.82	6.53	1.198	4.76	1.372
33	*Penicillum asperum*	0	5.84	0	5.12	1.14
34	*Penicillum aurantiogriseum*	0	4.46	0	2.56	1.742
35	*Penicillum citreonigrum*	3.93	3.35	1.173	3.76	0.89
36	*Penicillum citrinum*	0	1.98	0	2.71	0.731
37	*Penicillum commune*	2.93	2.13	1.375	5.14	0.414
38	*Penicillum funiculosum*	3.87	3.45	1.121	4.32	0.799
39	*Penicillum nigricans*	0	3.65	0	3.95	0.924
40	*Penicillum novae*	0	4.93	0	4.95	0.996
41	*Penicillum oxalicum*	0	4.23	0	3.13	1.351
42	*Penicillum restrictum*	0	2.87	0	3.86	0.744
43	*Penicillum roseopurpureum*	7.36	6.73	1.094	4.24	1.588
44	*Penicillum rugulosum*	0	1.85	0	5.27	0.351
45	*Penicillum* sp. 1	3.58	2.64	1.356	5.25	0.005
46	*Penicillum* sp. 2	0	6.83	0	5.64	1.21
47	*Penicillum* sp. 3	0	7.76	0	4.81	1.613
48	*Trichoderma aureoviride*	0	3.27	0	5.34	0.612
49	*Trichoderma polysporum*	0	3.36	0	4.25	0.79
50	*Trichoderma pseudokoningii*	0	6.96	0	6.85	1.016
51	*Trichoderma reesi*	0	4.35	0	5.46	0.797
52	*Trichoderma* sp.	3.47	4.56	0.76	5.12	0.89
53	*Trichoderma strctipilis*	0	6.61	0	6.13	1.079
54	*Trichoderma viride*	0	7.53	0	4.21	1.789

REA= Relative enzyme activity index (values more than 0 showed positive cellulase enzymatic activity); HZ=Hydrolysis zone; CD=Colony Diameter; CDP= Colony diameter on potato dextrose agar; GS/ I I= Growth simulation/inhibition index

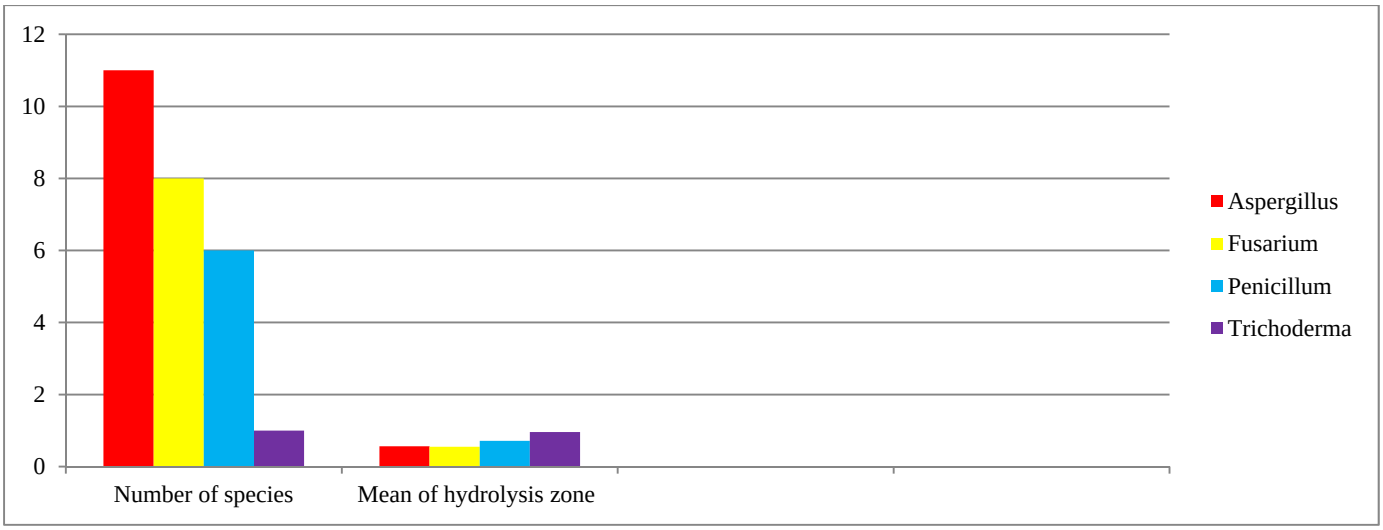

Fig. 1: Number of fungal species and mean of hydrolysis zone of fungi showing positive enzymatic activity

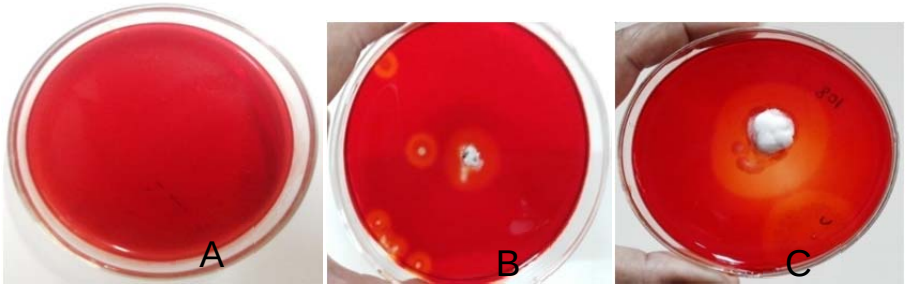

Fig. 2: Cellulase enzymatic activity: (A) Control plate (B) and (C) Zone of hydrolysis activity