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Isolation and evaluation of cellulolytic potency of fungal isolates recovered from undisturbed forest localities

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Abstract
The study focused on the evaluation of cellulolytic fungi isolated from forest litter and soil samples for use in the biodegradation of waste products into valuable biomass. Out of 59 fungal isolates recovered from 24 samples by using dilution and pour plate technique, 5 selected isolates *viz*., Aspergillus niger, Aspergillus flavus, Trichoderma viride, Fusarium oxysporum and Penicillium sp. had highest cellulolytic index were selected and were identified by observing cultural and morphological characteristics along with microscopic examination. The qualitative plate assay technique was employed by using PDA medium in the investigation. The zone of clearance produced by cellulolytic fungi was recorded at 7 days after inoculation in each isolate by staining with 1% Congo red solution. The diameter of clear zone on fungal plates, gave an approximate indication of cellulose activities. In all, 45 fungal isolates showed clearing zones of varying diameter around the colonies. The zone of clearance ranged from 0.2 mm to 20.03 mm. However, among all the fungal isolates, maximum zone of clearance was detected in *A.* Niger (20.03 mm) followed by *A.* favas (19.33 mm), T. viride (14.67 mm), Penicillium sp. (10.33 mm) and F. oxysporum (10 mm) in comparison with other fungal isolates. The highest cellulolytic index to the tune of 0.564 was detected in Aspergillus Niger. Second position was occupied by the Aspergillus flavus (0.476) followed by Penicillium sp. (0.428), T. viride (0.395) and F. oxysporum (0.331) as compared to other fungal isolates. This study showed that the fungi isolates have appreciable cellulose degradation property.

Keywords: Cellulolytic index, zone of clearance, T. viride, screening

Introduction
Sahyadri Mountain of India is coroneted with dense canopy of evergreen forest. These hills are important forest resource of Western India. The Mahabaleshwar, one of the hill stations located in the Sahyadri mountain range in Satara district of Maharashtra at 17^0 56' N and 73^0 40' E. Total area of the forest is 137.15 sq.km possess lateritic soil and undulating topography with steep escarpments. Considerable amount of precipitation received in this locality which is mostly confined to the month of June to September. The natural vegetation belongs to the Western sub-tropical broad leaved hill forest type with forest ranges in height from 5 m to 20 m. The major tree species of the forest are Jambul (Syzygium cumini), Parjamb (Olea dioica), Kavla (*Symplocos beddomei*), Hirda (*Terminalia chebula*), Anjan (*Memecylon umbellatum*), Pisa (*Actinodaphne angustifolia*), Gela (*Xeromphis spinosa*), Tambat (*Flacourtia ramontchi*), Bhoma (*Glochidion hohenackeri*). In the herbaceous stratum mostly Karvi (*Carvia callosa*) and Waiti (*Thelapaepale ixiocephala*); climber species *viz*., Ambulki (*Elaegnus conferata*), Chimat (*Scutia myrtina*) and Kamgoni (*Celastrus paniculata*) are dominant. The large volume of cellulosic waste in the form of leaves, fruits, dried branches etc. is generated consistently in the forest. Many species of micro flora *viz*., fungi, bacteria, actinomycetes, algae and protozoa etc. are present in the soil. Among those, fungi are known to play a major role in decomposition and humus formation in the soil. Fungi colonize the lignocellulose matrix by decomposing it in litter, which is harder for other microorganisms (Swift *et al*., 1979; Kjoller and Struwe, 1982 and Cooke and Rayner, 1984) [26, 26, 17, 6, 6], Cellulose is the most common and abundantly found structural component of plant cell wall. Cellulase and other hydrolytic enzymes involve in cellulose degradation in to smaller sugar components like glucose. These enzymes mainly secreted by many soil dwelling fungi and bacteria (Onsori *et at*., 2004) [21], However, fungi are more effecient in degrading cellulosic material, since it can grow either on the surface or penetrate into the cellulosic material (Boe *et al*., 2004) [2], It also proved that application of cellulolytic fungi improve the decomposting potentials of cellulose waste where

the C:N ratio was not optimal. In addition, fungal inoculation Improves water holding capacity. Various Biological studies have been, carried out to identify the major microbial agents responsible for biodegradation. Now a days, environmental policies and regulation progress support to the development of biodegradation process for conversion of organic waste into valuable resource by potential microbes. Thus, the present study was carried out as a first step to find more efficient cellulose producing strain capable of degrading.

Material and Method

Collection of Soil Sample

Sampling of soil was done from forest of Mahabaleshwar in Satara district of Maharashtra state. Twenty four soil samples were collected from different sample points from the undisturbed localities employing soil auger, hand trowel and polythene bags. The soil was dug out using augers up to 20 cm depth and was immediately scooped into sterile polythene bag using the hand trowel. The sample was collected from 2 spots in each site and then were mixed together in order to obtain representative sample. Field moist samples were composited, 2 mm sieved and then were stored at -10 ^{0}C until isolation of fungi.

Isolation of Fungi

Serial dilution and plating technique (Warcup, 1950) [30], method was used for isolation of cellulolytic fungi from the soil samples. Ten gram composite sample was suspended in 90 ml of sterilized water blanks. Serial dilutions were made from 10^{-1} to 10^{-5}. After solidification of the plates poured with culture medium, the plate were kept at 28 + 2 °C in BOD incubator for 4 to7 days. Single isolated colonies of microorganisms were picked up, numbered and maintained on the respective PDA slant were maintained at 4 ^{0}C for further studies.

Screening of Fungal Isolates *in vitro*

The fungal isolates were screened by using qualitative plate assay for their ability to produce cellulases complex by using method given by Teather and Wood (1982) [27, 27], Formation of clear zones around the fungal colonies on Czapek- Dox agar medium containing carboxy methyl cellulose (CMC) used as base for determination of cellulolytic activity of the fungal isolates. The detailed procedure is as below.

1. The sterilized Czapek's mineral salt agar medium (autoclaving at 121 ^{0}C and 15 lbs pressure) was poured into petri plates and allowed to solidify.
2. Cavities of 5 mm size were made in solidified medium and inoculated with 1 ml of spore suspension was prepared by suspending 7 days old fungal growth in 5 ml distilled water in test tubes.
3. The inoculated plates were incubated at room temperature (28 + 2 °C) for three days to allow fungal growth. Then again incubated for 18 hr at 50 ^{0}C which is the optimum temperature for cellulose activity.
4. At the end of incubation, the medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min to visualize the hydrolysis zone.
5. The Congo red solution was then poured off, and the plates were further flooded with 1N NaCl solution for 15 min.
6. The ratio of the clear zone diameter to colony diameter was measured in order to select the highest cellulase activity producer. The largest ratio was assumed to express the highest activity.

Identification of Fungi

Identification of efficient cellulolytic fungi was made with the help of standard mycological books and manuals by studying colony characters, morphology of reproductive structures, conidia, spore arrangement etc. on PDA. (Gilman, 1957; Booth, 1971; Subramanian, 1971 and Aneja, 2003) [11, 3, 24, 1],

Results and Discussion

Collection of Soil Sample

The soil samples rich in decomposing cellulosic material have been collected in this investigation as the habitat which were rich in cellulosic substrate were the best sources for isolation of cellulolytic microorganism (Haung and Monk, 2004) [13, 13], This type of study had been undertaken by Geethadevi, *et al*., (1978) [10], Sudha, *et al.*, (2018) [25], and many other past workers by collecting soil, litter or compost samples

Table 1: Collection of soil samples from undisturbed localities of the forest

Sample No.	Latitude	Longitude	No. of isolates
1	17^0 56' 15"N	17^0 40' 40"E	2
2	17^0 55' 23"N	17^0 40' 03"E	2
3	17^0 55' 57"N	17^0 41' 10"E	1
4	17^0 56' 05"N	17^0 40' 50"E	3
5	17^0 57' 27"N	17^0 40' 07"E	4
6	17^0 57' 30"N	17^0 42' 10"E	1
7	17^0 56' 06"N	17^0 41' 14"E	3
8	17^0 56' 18"N	17^0 41' 33"E	1
9	17^0 56' 05"N	17^0 40' 50"E	2
10	17^0 55' 42"N	17^0 40' 00"E	2
11	17^0 55' 29"N	17^0 39' 36"E	3
12	17^0 55' 47"N	17^0 43' 36"E	4
13	17^0 56' 17"N	17^0 40' 39"E	3
14	17^0 56' 35"N	17^0 41' 20"E	1
15	17^0 55' 34"N	17^0 41' 11"E	3
16	17^0 56' 10"N	17^0 34' 39"E	4
17	17^0 55' 25"N	17^0 39' 30"E	3
18	17^0 57' 50"N	17^0 37' 40"E	2
19	17^0 57' 42"N	17^0 37' 11"E	4
20	17^0 55' 10"N	17^0 34' 42"E	1
21	17^0 56' 50"N	17^0 34' 12"E	4
22	17^0 57' 05"N	17^0 34' 00"E	2
23	17^0 57' 00"N	17^0 40' 18"E	3
24	17^0 57' 40"N	17^0 40' 15"E	1
Total			59

Isolation of Fungi

By using dilution and pour plate technique various fungal species were isolated from the collected soil samples (Table 1). These fungal isolates were maintained in pure form and further subjected under screening for cellulose production assay. Out of 59 fungal isolates, *A. Niger*, *A. favas*, *T. viride*, *F. oxysporum* and *Penicillium* sp. are well known potent fungi to degrade cellulosic crop residues. The material containing cellulosic matter in high proportion can be decomposed by combination of physical, chemical and biological processes. Fungi are known to be the main cellulose producing microbes. Some bacteria and actionmycetes have also been reported to yield cellulose activity. The decomposition of organic matter by the fungi particularly cellulosic material has been reported by Lynd, *et al*., (2002) [19], Especially *Aspergillus* and

Trichoderma as efficient cellulose yielding genera. (Peij, *et al*., 1998) [22], On the basis of earlier report, it can be narrated that fungi can utilize wide range of cellulosic material. Therefore, efforts for isolation of cellulase yielding efficient fungal species have been undertaken. These results of present study are in the agreement of earlier workers who tried to isolate efficient cellulose yielding microbe's *viz*., More, *et al*., (1980) [20], Limtong, *et al*., (1990) [18], Gawade (2001) [9], Gautam, *et al*., (2010) [8], Khokhar, *et al*., (2012) [16], and had isolated the similar fungal species from crop residues, compost or solid waste material.

Screening of Fungal Isolates *in vitro*

In the present study, 59 fungal isolates were screened *in vitro* to assess their ability to degrade cellulose with the help of qualitative plate assay (Table 2). Synthesis of extracellular cellulose in different proportion was found in 45 fungal isolates which was evident from formation of clearing zones of varying diameter around the colonies. The zone of clearance ranged from 0.2 mm to 20.03 mm. However, among all the fungal isolates, maximum zone of clearance was detected in isolate 3 (20.03 mm) followed by isolate 31 (19.33 mm), isolate 41 (14.67 mm), isolate 52 (10.33 mm), and isolate 17 (10 mm) as it indicate maximum efficiency of fungal isolates for hydrolyzing cellulose (Table 2).Colony diameter was ranged from 1 mm to 50.4 mm in various fungal isolates. However, maximum colony diameter was found in isolate 5 (50.4 mm) followed by isolate 4 (45.3 mm), isolate 31 (40.59 mm), isolate 41 (37.08 mm), and isolate 3 (35.46 mm). The relative enzyme activity of all the fungal isolates was computed individually by using the formula given by Jamroo, *et al*., 2015 [15], which was expressed as cellulolytic index (Table 2).

$$\text{Cellulolytic index} = \frac{\text{Diameter of clearing (mm)}}{\text{Diameter of colony growth (mm)}}$$

The cellulolytic index of all the 59 fungal isolates ranged between 0 and 0.564 (Table 2). However, highest cellulolytic index to the tune of 0.564 was obtained in the isolate 3. Fungal isolate 31 was stood second, which displayed a cellulolytic index of the value of 0.476. Isolate 52, 41, and 17 were the next best set of isolates, which had cellulolytic index of 0.428, 0.395 and 0.331, respectively. Investigation to find out relative capacity to generate enzyme cellulose has been undertaken by past workers. The fungus *Aspergillus* sp. able to carry out biodegradation of cellulose by producing significant amount of cell free cellulose capable of hydrolyzing cellulose into fermentable soluble sugars especially glucose (Wainwright, 2010) [29], In all 45 fungal isolates could synthesize extra cellular cellulose in different proportion which was evident from formation of clearing zones around the colonies. *A. Niger* was found the most efficient fungus in hydrolyzing cellulose. The other fungal isolates *A. favas* and *T. viride* were also found to be effective in degrading cellulose. The results of this evaluation are in conformity with past reports of Hao, *et al*., (2005) [12], who reported the *Trichoderma* sp. was efficient decomposer of cellulose while *Aspergillus fumigates* was efficient decomposer of lignocellulose. Devi and Kumar (2012) [7, 7], and Chandel, *et al*., (2013) [5], isolated different fungal strains and found *Aspergillus* and *Penicillium* had

Table 2: Screening of fungal isolates *in vitro*

Isolate No.	Zone of clearance (mm)	Colony diameter (mm)	Cellulolytic index	Isolate No.	Zone of clearance (mm)	Colony diameter (mm)	Cellulolytic index
1	5.02	40	0.125	31	19.33	40.59	0.476
2	1	5	0.2	32	1.07	3.25	0.329
3	20.03	35.46	0.564	33	1.02	7	0.145
4	5.1	45.3	0.112	34	0	3.25	0
5	3.5	50.4	0.069	35	0	3.1	0
6	1.03	6	0.171	36	1.05	30	0.035
7	0	2.2	0	37	3.06	5.1	0.6
8	1.03	3.25	0.316	38	1.05	3.3	0.318
9	7.04	32.2	0.218	39	1	3.25	0.307
10	4.01	25.3	0.158	40	1.16	4.2	0.276
11	1.01	4.25	0.257	41	14.67	37.08	0.395
12	1.05	6.1	0.172	42	1.06	3.4	0.311
13	1.18	4	0.295	43	4.2	50	0.084
14	1.1	4.35	0.252	44	0	1.5	0
15	8.1	35.4	0.228	45	2.2	7.2	0.305
16	1.08	3.5	0.308	46	1	2.5	0.04
17	10	30.2	0.331	47	0	3.4	0
18	3.08	9.4	0.327	48	0	2.2	0
19	1.11	3.5	0.317	49	1.09	4.5	0.242
20	1.04	4.45	0.233	50	0	0.5	0
21	0	1	0	51	1	4.5	0.222
22	1.02	3.1	0.329	52	10.33	24.1	0.428
23	0	2	0	53	0	2.1	0
24	0.9	4.1	0.219	54	1.08	3.5	0.308
25	1.08	5.1	0.211	55	0	3.2	0
26	0	21	0	56	0.2	1.3	0.153
27	0	11	0	57	1.8	20	0.09
28	0	11.1	0	58	1.7	5.4	0.314
29	1	22.3	0.044	59	2.1	20.25	0.103
30	2.25	20.4	0.11				

Significantly more cellulolytic activity over other fungal strains. Similar results presented by Jahangeer, *et al*., (2005) [14], and Toyama (2001) [28], who identified the fungal isolates *viz*. *A. Niger*, *A. favas* and *Trichoderma* sp. and were able to yield highest cellulase in their study. In the research of Reanprayoon and Pathomsiriwong (2012) [23, 23], fungal strains isolated from agricultural soil had high efficiency for biodegradation of cellulosic material especially *Trichoderma* sp. and *Aspergillus* sp. *F. oxysporum* was also one of the fungal species in the present investigation which indicated better cellulolytic activity. It is in the conformity of the results put forth by Bowen and Harper (1988) [4, 4], who reported *Fusarium* sp. was one of the most frequently isolated cellulose decomposer from decomposing wheat straw

Morphological Identification of Fungi

Best performing five fungal isolates were selected from 59 fungal isolates for further study. These isolates were identified on the basis of its morphological characteristics (Gilman, 1957) [11], and found the most efficient fungal isolates as indicated in Table 3

Table 3: The most efficient fungal isolates on the basis of cellulolytic index.

Isolate No.	Identified fungal sp.	Cellulolytic index
3	A. niger	0.564
31	A. flavus	0.476
52	Penicillium sp.	0.428
41	T. viride	0.395
17	F. oxysporum	0.331

Conclusion

In the present study 24 soil samples collected from undisturbed localities of dense forest from which 59 fungal isolates were recovered, out of which 5 isolates had shown maximum zone of clearance on Czapek - box agar medium amended with Carboxyl methyl cellulose were *A. Niger* (20.03 mm) followed by *A. favas* (19.33 mm) and *T. viride* (14.67 mm). Maximum colony diameter was found in isolate 5 (50.4 mm) followed by isolate 4 (45.3 mm). The highest cellulolytic index to the tune of 0.564 was found in the *A. Niger* followed by *A. favas*, as an indication of maximum cellulose enzyme activity.

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