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Isolation of Potential Cellulolytic Fungal Isolate from Soil Samples

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Abstract – The current ethanol production processes using crops such as sugar cane and corn are well established; however, utilization of a cheaper substrate such as lignocellulosic biomass (LB) could make bioethanol (= ethanol derived from biomass) more competitive as well as without the ethical concerns associated with the use of potential food resources. Hence, the lignocellulosic biomasses are considered a future alternative for the agricultural products that are currently used as a feedstock for ethanol production. Furthermore, the use of LB is more attractive not only in terms of energy balance and emission of greenhouse gases but also because of its degradation by many naturally occurring cellulolytic fungi which can be potentially screened for bioethanol production from the LB.

In purview of the above, various mesophilic fungi were isolated from different soil samples collected from different regions of Rohtak in Haryana (INDIA). A total of 20 soil samples were collected and processed for isolating the different cellulolytic fungi. Out of 20 soil samples, 82 fungal isolates were isolated by serial dilution (10^{-3} - 10^{-7}) method and their pure cultures were maintained on potato dextrose agar (PDA) media. Then, all fungal isolates were screened primarily for cellulase production by plate assay method. Out of 82 fungal isolates, only 8 having maximum zone of hydrolysis were selected for carboxymethyl cellulase (CMCase) activity under submerged cultivation by making use of 1% carboxymethyl cellulose (CMC) acting as a carbon source. The CMCase activity was found to be maximum in *Trichoderma* sp. R-4 and *Aspergillus* sp. R-30 among all isolates of the genera *Trichoderma* and *Aspergillus* respectively.

Keywords: Isolation, Screening, Fungi, Lignocellulosic Biomass, Carboxymethylcellulase (Cmcase) Activity, Submerged Fermentation, *Trichoderma*, *Aspergillus*

INTRODUCTION

It has been estimated that lignocellulose accounts for about 50% of the biomass in the world (Claasen et al., 1999) but its fermentation is proving to be immensely challenging owing its complex physico-chemical structure which needs to be degraded into reducing sugars before fermentation. Hence, three major steps involved are: pretreatment process to release cellulose, hemicellulose and lignin from lignocellulose matrix, hydrolysis to produce reducing sugars and fermentation to convert sugar mixtures to ethanol. One of these three steps is accomplished by many naturally occurring bacterial and fungal microorganisms that can saccharify/hydrolyse the major components of lignocellulose, cellulose, a homopolymer of glucose units linked by β -(1,4)-glucosidic bonds (Gielkens et

al., 1999; Han et al., 1995) and hemicellulose with the help of extracellular hydrolytic enzymes, viz., cellulase and hemicellulase respectively.

Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds (Chellapandi and Himansu, 2008). The cellulase system in fungi is considered to comprise three hydrolytic enzymes: endo-(1,4)- β -D glucanase [endoglucanase, endocellulase, CMCase (EC 3.2.1.4)] which cleaves β - linkage at random, exo-(1,4)- β -D glucanase [cellobiohydrolase, exocellulase (EC 3.2.1.91)] which releases cellobiose from non-reducing or reducing end, generally from the crystalline parts of cellulose and β -glucosidase [cellobiase (EC 3.2.1.21)] which releases glucose from cellobiose and short chain

cello-oligosaccharides (Bhat and Bhat, 1997). The purpose of this study was to isolate and screen the potential fungi with better cellulases production so that the wasteful lignocellulosic biomass (LB) could be converted into useful products.

MATERIALS AND METHODS

Isolation of Fungal Strains

Cellulases producing fungal strains were isolated from 20 soil samples collected from various sites of district Rohtak (30°1' N and 75°17' E) in Haryana (INDIA). One gram of soil from each sample was suspended in 100mL normal saline solution in a 250mL flask and incubated for 30 minutes at 180rpm. The serially diluted samples (10^{-3} - 10^{-7}) were spread on the surface of potato-dextrose agar (PDA) media (prepared by adding potato peels 200g, dextrose 20g, agar agar 20g and streptomycin sulphate 70µg/mL to inhibit bacterial growth) and incubated for 3 days at 30°C. The colonies were picked up and subcultured to obtain pure cultures. Stock cultures were maintained on PDA media at 4°C.

Primary Screening for Cellulase Production

Each fungal isolate was spot inoculated on solidified Mandels and Reese (1954) media plates containing 1% carboxymethyl cellulose (CMC) and incubated for three days at 30°C. After 3 days, the plates were flooded with 0.1% Congo red solution for 15 minutes and then destained with 1M sodium chloride (NaCl) solution for 15 minutes. The diameter of zone of decolorization around each colony was measured.

Secondary Screening for Cellulase Production under Submerged Cultivation (SmC)

The fungal isolates with larger zone of decolorization were selected for cellulase production in submerged cultivation. In the test tube, the Mandels and Reese medium (pH 5.0) supplemented with 1% CMC was used for enzyme production. A fungal disc of 4.0mm diameter was inoculated in 250mL Erlenmeyer flask containing 50mL Mandels and Reese medium with 1% CMC as carbon source. The flasks were incubated at 30°C, 180rpm for 3 days. After 3 days, the culture was centrifuged at 10,000rpm for 10 minutes at 4°C and supernatant was used as crude enzyme.

Carboxymethyl Cellulase (CMCase) Assay

The endoglucanase/carboxymethyl cellulase (CMCase) activity was measured according to IUPAC (Ghose, 1987). In the test tube, 0.5mL carboxymethyl cellulose (1%, 4.8pH, and 0.05M citrate buffer) was added with 0.5mL appropriately diluted enzyme and incubated at 50°C for 30 minutes. The reducing sugar concentration was estimated by DNSA method (Miller, 1959). At the end of incubation period, the reaction was stopped by adding 3.0mL DNSA reagent. The tubes were incubated for 5 minutes in boiling water

bath for colour development and the optical densities (OD) were taken at 540nm. The CMCase activity were calculated following the concept that 0.185 units of enzymes will liberate 0.5mg of glucose under the assay conditions and was expressed as U/mL.

Identification of Fungal Isolates

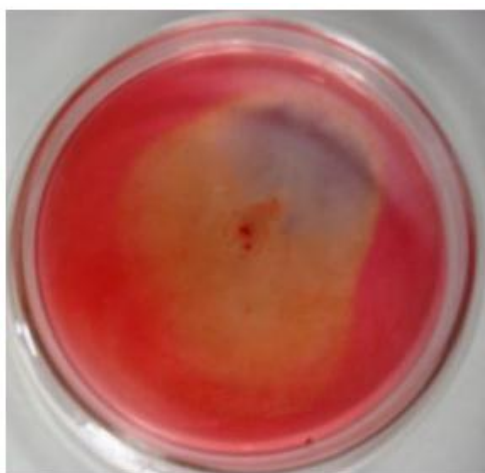
The fungal isolates with higher zone of decolorization were identified at genera level by staining the mycelium, spores and conidia with lactophenol cotton blue, observing under the microscope and by colony morphology characteristics.

RESULTS AND DISCUSSION

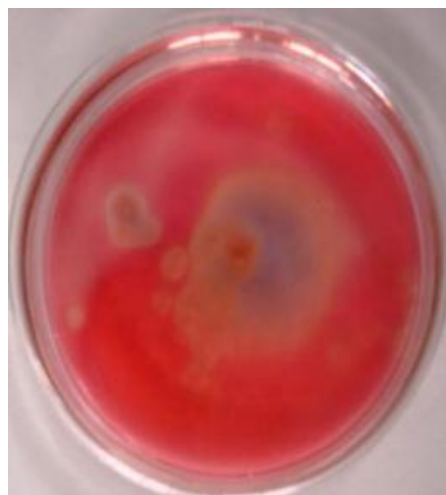
In the present study, a total of 82 fungal isolates were isolated from 20 soil samples collected from various regions of Rohtak from the sites rich in decomposing organic matter. The fungal isolates with larger zones of decolorization (Figure 1) belonged to genera *Trichoderma* and *Aspergillus* which were identified by microscopic examination of conidia, spore, mycelium structure and colony morphology characteristics. On the basis of zone of decolorisation (Table 1), the fungal isolates 2 (8.3cm), 4 (9.0cm), 12 (8.5cm) and 15 (8.6cm) belonging to genera *Trichoderma* and 30 (5.9cm), 38 (5.7cm), 47 (5.7cm) and 80 (5.6cm) belonging to genera *Aspergillus* were selected for CMCase production in submerged cultivation. The CMCase activities of all the selected fungal isolates were analysed on third day. Among all the selected *Trichoderma* and *Aspergillus* isolates, the CMCase activities of isolates 4 and 30 named *Trichoderma* sp. R-4 (0.193IU/mL) and *Aspergillus* sp. R-30 (0.0971IU/mL) respectively were found to be maximum (Figure 2).

Table 1: Plate Assay Screening of Fungal Isolates

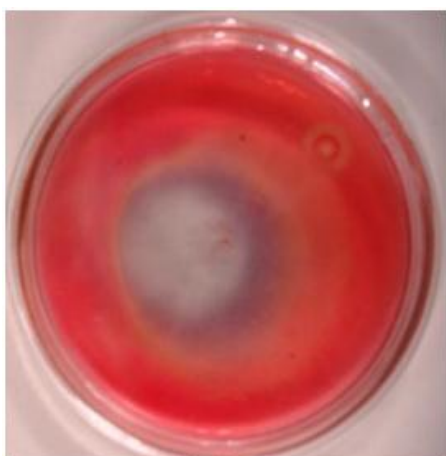
Fungal Isolate	Zone of Decolourization (cm)	Fungal Isolate	Zone of Decolourization (cm)	Fungal Isolate	Zone of Decolourization (cm)
1	6.5	28	3.3	55	4.1
2	8.3	29	3.5	56	3.9
3	4	30	5.9	57	4.8
4	9	31	3.6	58	5
5	3	32	3.3	59	3.3
6	3.8	33	3.6	60	4
7	6.1	34	3.5	61	3.1
8	5.5	35	3	62	4
9	8	36	3.4	63	3.7
10	6.2	37	3.2	64	4.5
11	3.2	38	5.7	65	6.6
12	8.5	39	3	66	4.4
13	3.4	40	3.2	67	3.4
14	5.3	41	3.1	68	5.7
15	8.6	42	4.2	69	4.6
16	3.5	43	3.4	70	4.2
17	3	44	7.2	71	6.1
18	3.2	45	3.1	72	4.6
19	7.9	46	4	73	3
20	3.8	47	5.7	74	4.3
21	6.4	48	3.3	75	4.6
22	7	49	4.7	76	5.1
23	3.6	50	8	77	4.1
24	3.3	51	6.3	78	3.8
25	3.4	52	4.6	79	5.4
26	3.8	53	3	80	5.6
27	4	54	7	81	7
				82	4.8



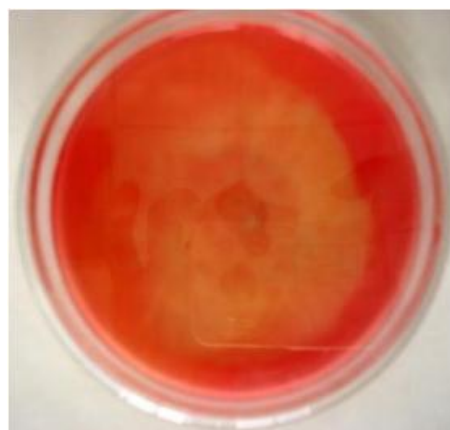
Aspergillus sp. R- 30



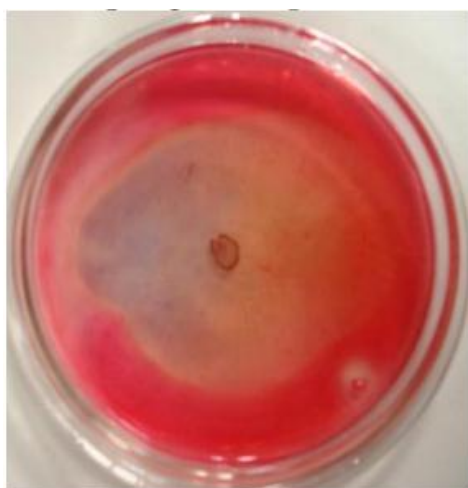
Aspergillus sp. R- 80



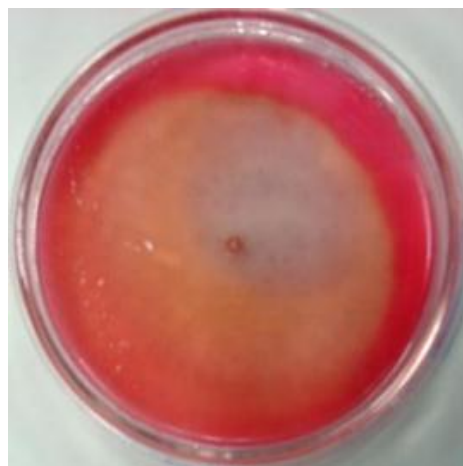
Aspergillus sp. R- 38



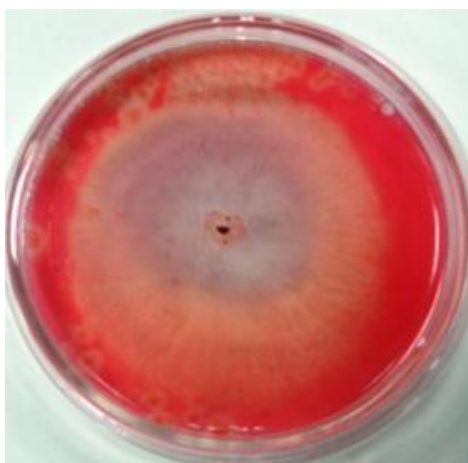
Trichoderma sp. R- 2



Aspergillus sp. R- 47



Trichoderma sp. R- 4



Trichoderma sp. R- 12



Trichoderma sp. R- 15

Figure 1: Zone of decolorisation of different fungal isolates of *Aspergillus* and *Trichoderma*

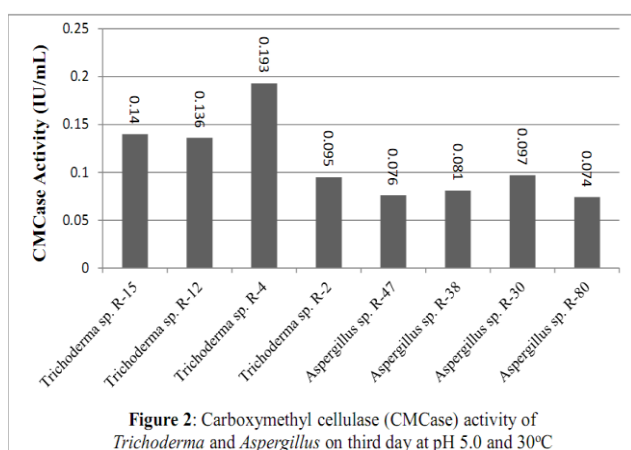


Figure 2: Carboxymethyl cellulase (CMCase) activity of *Trichoderma* and *Aspergillus* on third day at pH 5.0 and 30°C

The results obtained during this study indicated that cellulase activity of tested *Trichoderma* sp. R-4 and *Aspergillus* sp. R-30 were found relatively higher which was found to be commensurate with the results of Upedgraff (2004), Klueczek-Turpeinen *et al.* (2005) and Makut and Godiya (2010). This investigation may also lead to the development of strains of soil fungi that would be used locally for the biodegradation of

cellulose materials. So these organisms are recommended as source of cellulases which may not only be harnessed for industrial production of the enzyme but also for the management of solid wastes containing cellulose. The cited results may also be conducive to the industries that use cellulases, *viz.*, textile, detergents, laundry, pulp, paper, solid waste management and biofuel industry. However, further studies are required to be carried out to determine quantitatively the optimum catalytic activity of the cellulases produced by each of the cellulases producing fungal isolates so that the tested highly efficient fungal strains could be used optimally in a H effective manner for the protection of environment through solid waste management by the environmental agencies as the solid waste is mostly in the form of cellulose, the world's most common organic substance (Ruttloff, 1987), which can be decomposed easily by the investigated potential cellulolytic fungal strains, *i.e.*, *Trichoderma* sp. R-4 and *Aspergillus* sp. R-30.

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