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Assessment of the potential of promising fungal isolates for Lignocellulosic biomass utilization under controlled conditions

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Abstract
An experiment was conducted to evaluate the Lignocellulolytic potential of four fungi isolated from soil samples collected from different ecological niches on malt extract agar medium. Out of four fungi isolated, two isolates showed significant cellulolytic activity with I_{CMC} varying from 0.745 to 0.805 and all the four isolates showed lignolytic activity with a zone of clearance varying from 0.99 to 1.20. Fungal isolates produced a maximum amount of Lignocellulolytic enzymes at 30 ℃ with HST16 showing maximum CM Case, laccase and lignin peroxidase activity, HST14 showing maximum F Pase activity and HST11 showing maximum manganese peroxidase activity. Lignocellulolytic enzyme production was found more when fungal isolates were grown in medium broth with pH 6 and at stationary condition. Maximum enzyme production occurred on 7th day of incubation. These results suggested that the environmental conditions could be optimized for growth of Lignocellulolytic fungi and these fungal isolates could be exploited for compost preparation, in paper industry, textile industry and for improvement of soil texture and fertility.

Keywords: Lignocellulolytic fungi, F Pase, CM Case, laccase, lignin peroxidase, manganese peroxidase

1. Introduction
Land plants direct most photosynthetic ally fixed carbon into lignocellulose, a cluster of the polymers cellulose, hemicellulose, pectin and lignin. During the life of the plant, this complex matrix provides structural integrity and resistance to herbivores and pathogens. The Lignocellulosic biomass can be used as a feedstock for biofuel generation, paper and pulp industry, textile industry, compost and many more. But it is recalcitrant to enzymatic dispensation due to presence of barriers to enzyme access that are because of the paracrystallinity of cellulose, the intricacy of the hemicellulose coating and the interpenetration and encapsulation of cellulose and hemicelluloses by lignin. In industrial processes, this recalcitrance is overcome by severe chemical and physical pre-treatments that require high temperature and pressure as well as highly acidic or alkali treatment. But, it was observed that under physiologically tolerable conditions, microorganisms may also assist lignocellulose deconstruction (Cragg *et al*., 2015) [5].
Some members of all classes of microorganisms, *viz*., fungi, actinomycetes and bacteria are known to produce ligninase and cellulase isoforms (Kubicek, 2008) [9] but, filamentous fungi have proved to be the most persuasive degraders of Lignocellulosic biomass as they produce a high number and a broad range of enzymes having different and complementary catalytic activities (Lundell *et al*., 2010) [10]. Among wood-decaying basidiomycetes, a wide range of enzymes such as lignin peroxidases and glycosyl hydrolases is secreted by the white-rot fungi *Phanerochaete chrysosporium* (GHs) (Martinez *et al*., 2004, Ravalason *et al*., 2008) [11, 19]. Many ascomycetes species have been identified as good candidates for the release of monosaccharide such as *Trichoderma reesei*, which is extensively used in industry due to its ability to secrete high level of cellulases. In addition to cellulose and lignin, white-rot fungi are also able to degrade a variety of persistent environmental pollutants such as chlorinated aromatic compounds, heterocyclic aromatic hydrocarbons, various dyes and synthetic high polymers (Bennett *et al*., 2002) [2].
Different enzymes involved in cellulose degradation are collectively known as cellulases. Cellulases hydrolyze the β-1, 4-glycosidic linkages of cellulose. Endoglucanases break internal bonds in cellulose chains; preferably in cellulose amorphous regions releasing new terminal ends whereas exoglucanases break cellulose bonds at the ends of cellulose resulting in

disaccharides called cellobiose; act on the existing or endoglucanases generated chain ends, preferably in cellulose crystalline regions. Fungi degrade lignin by secreting enzymes collectively termed as "ligninases". Ligninases can be classified as heme peroxidases or phenol oxidases (Martinez *et al*., 2005) [12]. If lignolytic enzyme activity would be supplemented to a Lignocellulosic substrate undergoing cellulolytic hydrolysis, then the added enzyme activity might boost the conversion of the cellulose (Sitarz *et al*., 2013) [23].
The appropriate physicochemical conditions play an important role in the enzyme production by microorganisms. Media composition and fungal growth conditions strongly affect the production of ligninolytic and cellulolytic enzymes and the extent of lignocellulose degradation (Tanvi *et al*., 2018) [24]. Therefore, there are immense possibilities for further increasing the production of lignocellulolytic enzyme by fungi under highly optimized conditions. In this study, lignocellulolytic fungi were isolated and their cultural conditions were optimized for enhanced production of lignocellulolytic enzymes.

2. Materials and Methods

2.1 Isolation of fungal cultures from soil samples

Soil samples were collected from different sites like mushroom cultivation area, compost, vermicompost and garbage dumping sites of CCS Haryana Agricultural University campus, Hisar and nearby areas. From each location, samples were collected from six different sites. Four samples were collected from each site and pooled together to make the composite sample. Fungal isolates were obtained from these soil samples by serial dilution plate method using Potato Dextrose Agar (PDA) media (300 ml potato extract, 20.0g dextrose and 20.0g agar) in 1L of deionised water. The serial dilutions of the soil samples were made up to 10^{-5} and 0.1 ml of diluted soil suspension was spreaded on PDA medium plates. The plates were incubated at 28±2 °C in biological oxygen demand (BOD) incubator for 7 days. Morphologically different fungi were selected. The fungal isolates were maintained by periodic transfer on PDA slants. These isolates were stored at 4 °C for further use.

2.2 Screening of fungal isolates for cellulolytic and ligninolytic activity.

Cellulolytic activity of fungal isolates was studied on Carboxy Methyl Cellulose (CMC) agar plates by the I_{CMC}, Eq. (1) method. Plates of CMC agar medium (5.0g carboxymethyl cellulose, 5.0g yeast extract, 20.0g glucose and 20.0g agar) in 1L deionised water were prepared. Hyphae of 72 hour old isolate were spotted on above prepared plates. Plates were incubated at 28±2 °C for 7 days. Degradation of cellulose was visualized as a clear zone of hydrolysis around the fungal colony when after incubation the plates were flooded with 0.1% aqueous solution of congo red for 15-20 minutes followed by destaining with 1 M NaCl for 15-20 minutes. Diameter of the clear zone and colony were measured. Isolates were selected on the basis of the Index of Relative Enzyme Activity (I_{CMC}). I_{CMC} was calculated by the method of Bradner *et al.* (1999) [3] and Peciulyte (2007) [16].

$$I_{CMC} = \frac{\text{clear zone diameters surrounding the colony}}{\text{colony diameter}} \quad (1)$$

Ligninolytic activity of fungal isolates was also studied on malt extract agar medium (30.0g malt extract, 5.0g mycological peptone and 20.0g agar) (Pinasthika *et al*., 2018) supplemented with 0.04% aniline blue dye. Fungal hyphae (72 hour old) were spotted on the media plates and the decolorization zone, Eq. (2) was calculated by taking the ratio of total diameter (zone + colony) to colony diameter.

$$\text{Zone of clearance} = \frac{\text{total diameter (zone + colony)}}{\text{colony diameter}} \quad (2)$$

2.3 Quantitative estimation of lignocellulolytic activity

Fungal isolates were inoculated into 25 ml malt extract broth in 50 ml capacity flask. All the inoculated flasks were incubated at 28±2 °C for 7 days. The growth suspension was centrifuged at 4,000 g for 10 minutes in the REMI Research Centrifuge to separate the supernatant from the hyphal growth and other debris. Five enzyme activities namely, laccase (lac), lignin peroxidase (Li P), manganese peroxidase (Mn P), exoglucanases (filter paper degrading activity) and endoglucanases (carboxymethyl cellulase activity) were measured. Laccase (Shandilya and Munjal, 1983) [22], lignin peroxidase (Tien and Kirk, 1988) and manganese peroxidase (Paszczynski *et. al.* 1988) production were measured with alkali lignin as substrate. CM Case and F Pase production were measured by the method described by Ghose (1987) [6] with CMC and Whatman filter paper no. 1 respectively as substrate. Reducing sugars produced due to enzymatic actions were determined by 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) [13]. One International Unit (IU) of enzyme was defined as the amount of enzyme required to produce 1 µmol of reducing sugars per minute under the assay conditions.

2.4 Optimization of growth conditions for efficient lignocellulolytic enzyme production

Lignocellulolytic enzyme production was determined in malt extract agar medium at neutral pH and 28±2 °C temperature. The variation in temperature, pH and incubation time as well as aeration condition were made to find out optimum conditions for efficient enzyme production. To determine the effect of incubation temperature, malt extract medium broths were inoculated with the fungal isolates. Cultures were incubated at different temperatures i.e., 25 ℃ and 35 ℃ along with 30℃ for 7 days. To study the effect of pH on lignocellulolytic enzyme production, the malt extract medium broths were prepared in different pH range i.e., 4, 5, 6 and 7 using N/10 HCl or N/10 Na OH. After inoculation of bacterial strains, broth were incubated at 28±2 ℃ for 7 days. To check the relationship between enzyme activity and incubation period, malt extract medium broth were inoculated with fungal isolates and incubated at 28±2 ℃. Samples from the inoculated broth were withdrawn daily for 10 days and analyzed for enzyme production using standard protocols. To understand the effect of aeration condition on enzyme production, malt extract medium broth inoculated with fungal isolates was incubated at 28±2 ℃ under shaking as well as stationary condition.

3. Results and Discussion

In this study, lignocellulolytic fungi were isolated from different soil samples collected from various ecological niches of CCS Haryana Agricultural University campus, Hisar and nearby areas. Fungal isolates were examined for their ability to produce lignocellulolytic enzymes. The efficient lignocellulolytic fungi were further subjected to varying

incubation conditions to understand the release of lignocellulolytic enzymes from them.

3.1 Isolation of lignocellulolytic fungi

Four fungal isolates were obtained from samples collected from 8 cm deep layer of soil or compost from CCS Haryana Agricultural University campus, Hisar (Table 1). Serial dilutions of soil samples were made up to10^{-5} and dilutions were spreaded on malt extract agar medium and carboxymethyl cellulose medium.

Table 1: Fungal isolates with their respective isolation site

Fungal Isolates	Ecological Niche
HST11	Mushroom waste
HST14	Vermicompost
HST15	Waste from dumping site
HST16	Compost

3.2 Screening of fungi for production of lignocellulolytic enzymes

Primary screening of 4 fungal isolates was done on malt extract agar medium containing aniline blue dye (0.04%) and carboxy methyl cellulose agar plates. It was found that out of these 4 fungal isolates, only 2 isolates were cellulolytic whereas all the isolates conferred lignolytic activity by producing zone of clearance (Table 2). In a similar study, Kale and Zanwar in 2016 [7] studied four fungal species belonging to two genera i.e. *Trichoderma* and *Aspergillus* which were isolated from different sources, screened and compared their ability to degrade cellulose. They also used the plate screening assay recommended by International Union of Pure and Applied Chemistry (IUPAC) for cellulolytic fungi. Cellulolytic fungi were screened after 7 days for the production of cellulolytic enzymes by staining with 1% congo red and after that washing with 1M NaCl. The diameter of clear zone on fungal plates was measured as it gave an approximate indication of cellulose. However, to identify white rot fungi with high potential in biological pretreatment of Lignocellulosic biomass, Xu *et al.* in 2015 also screened the fungal isolates on plates by testing different strains for their ability to oxidize guaiacol and decolorize the dyes azure B and Poly R-478. Out of the 86 strains screened by them, 16 were selected for secondary screening for their ligninolytic ability.

These cultures were further screened for their ability to produce five different enzymes namely laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP), exoglucanase (filter paper degrading activity) and endoglucanases (carboxymethyl cellulase activity). Table 3 shows that isolate HST11 and HST15 were not at all producing cellulase enzymes however; HST14 and HST16 were showing significantly different cellulolytic activities. Further, maximum laccase (6 IU/ml) activity was shown by HST15 and HST16, maximum LiP activity (28.00 IU/ml) by HST16 and manganese peroxidase activity (3.48 IU/ml) by HST 11 (Table 3). Similarly, Xu *et al*., 2015 selected 16 fungal strains out of 86 strains for secondary screening. They found that upon fermentation no LiP activity was detected in any of the strains. MnP activity was detected in 12 strains of the 16 strains tested; however, the activities were very low. For Laccase, five of the sixteen strains were able to produce detectable extracellular laccase.

Table 2: Formation of degradation zone by different fungal isolates

Fungal Isolate	Ligninolytic Activity	Zone of clearance	Cellulolytic Activity	I_{CMC}
HST11	++	1.10	-	0
HST14	++	0.99	+	0.805
HST15	++	1.08	-	0
HST16	++	1.20	+	0.745

Table 3: Different enzyme activities of selected fungal isolates

Fungal isolate	Cellulolytic activities (IU/ml)		Ligninolytic activities (IU/ml)		
	FP ase	CM Case	Lac	LiP	Mn P
HST11	-	-	2.00	10.00	3.48
HST14	0.012	0.007	1.75	5.00	0.75
HST15	-	-	6.00	21.00	1.60
HST16	0.010	0.010	6.00	28.00	1.50

3.3 Optimization of conditions for efficient lignocellulolytic enzyme production

These fungal strains were further tested for optimization of conditions for efficient lignocellulolytic enzyme production under varying conditions of temperature, pH, incubation period and aeration. At 30 ℃, the fungal isolate HST14 showed maximum FPase activity of 0.012 IU/ml and HST16 showed maximum CM Case activity of 0.010 IU/ml. Maximum laccase activity (6.00 IU/ml) was shown by HST15 and HST16, manganese peroxidase activity (3.48 IU/ml) was observed for HST11 whereas, HST16 showed maximum lignin peroxidase activity of 28.00 IU/ml (Table 4). It was found that lignocellulolytic enzyme activities decreased after increasing or decreasing the incubation temperature from 30 ℃. Kaur and Phutela (2017) [8], optimized cultural conditions for production of lignocellulolytic enzymes using *Phanerochaete chrysosporium* MTCC 787. They studied the effect of different incubation temperature (25, 30 and 35 °C) on Lignocellulosic bioconversion and found that at 30 ℃, maximum enzyme production was obtained. The maximum activities of endoglucanase (0.997U/ml), exoglucanase (0.785U/ml), β-glucosidase (4.321U/ml); xylanase (128.12U/ml), mannanase (72.5U/ml), lac case (6.67U/ml), lignin peroxidase (32.5U/ml) and manganese peroxidase (167.5 U/ml) were recorded at 30 °C.

The pH of the substrate plays a critical role in production of lignocellulolytic enzymes by affecting either the microbial growth or denaturating the enzymes (Ramesh and Lonsane, 1991) [18]. Most of the fungi prefer acidic pH for their growth. Therefore to determine the pH on lignocellulolytic enzyme production, selected fungal isolates were grown under different pH conditions. It was found that enzyme production was maximum when fungal strains were grown in a medium with pH 6.0 (Table 5). Enzyme activities increased with

increase in pH from 4 to 6; however, there were only slight differences in enzyme activities at pH 6 and 7. Maximum FPase (0.014 IU/ml) and CM Case (0.015 IU/ml) activities were shown by the isolate HST16. Maximum Lac activity (4.00 IU/ml) and LiP activity (26.00 IU/ml) was shown by HST16 and Mn P activity (1.64 IU/ml) were observed for HST11. Chmelova *et al*., 2011 grew *C. subvermispora* at different initial pH values of cultivation medium (2.0; 3.0; 5.0; 6.0 and 7.0) and found that the maximum growth of *C. subvermispora* was obtained when the initial pH was adjusted to 5.0.

To study the effect of incubation period on enzyme production, the enzyme activities of selected isolates were measured daily for 10 days. No enzyme activity was observed on 1st day however, the enzyme activities increased with incubation period upto 7 days reaching a maximum value of 0.014 IU/ml FP ase and 0.010 IU/ml CM Case for HST16 and became almost constant afterwards. Similar trend was observed for lac case and lignin peroxidase activities with maximum values of 6.50 IU/ml and 27.68 IU/ml respectively by HST16 and manganese peroxidase activity (3.64 IU/ml) by HST11on 7th day of incubation. Therefore, all the enzyme activities were measured on 7th day of incubation (Table 6). Usually, the optimum temperatures and incubation time for lignocellulolytic enzymes production vary with the use of different strains. Sharma *et al*., 2016 observed that the maximum enzyme production was mainly recorded in the mid values of incubation time with decreased trend after prolonged incubation time of 90 h. This type of behavior is mainly attributed to the inefficient transport across membranes and denaturation of enzyme activity at longer incubation times.

Fungal isolates were grown at stationary as well as shaking conditions. It was found that the cellulolytic and ligninolytic activities of selected fungal isolates under stationary conditions were better as compared to their activities in shaking conditions. The isolate HST14 showed maximum FP ase activity (0.012 IU/ml) as well as CM Case (0.011 IU/ml) activity. HST16 gave maximum Lac (6.00 IU/ml) and LiP activity (28.10 IU/ml). on the other hand, HST11showed maximum Mn P activity (3.48 IU/ml) (Table7). Some of the researchers have reported that shake conditions are effective in the production of more enzymes. On the contrary, few reports showed better enzyme production under static culture conditions (Roushdy & Abdel 2011; Balaji *et al*. 2012; Muthukumaran *et al*. 2014) [20, 1, 14].

Table 4: Cellulolytic and ligninolytic activities of selected fungal isolates at different temperatures

Fungal isolate	Cellulolytic activities (IU/ml)		Ligninolytic activities (IU/ml)		
	FP ase	CM Case	Lac	LiP	Mn P
25℃					
HST11	-	-	1.75	7.30	3.11
HST14	0.010	0.005	1.30	3.84	0.60
HST15	-	-	4.91	16.49	1.05
HST16	0.006	0.004	2.50	23.50	1.00
30℃					
HST11	-	-	2.00	10.00	3.48
HST14	0.012	0.007	1.75	5.00	0.75
HST15	-	-	6.00	21.00	1.60
HST16	0.010	0.010	6.00	28.00	1.50
35℃					
HST11	-	-	1.75	9.10	2.70
HST14	0.010	0.005	0.60	4.75	0.70
HST15	-	-	5.43	20.00	1.00
HST16	0.008	0.007	3.00	20.00	1.00

Table 5: Cellulolytic and ligninolytic activities of selected fungal isolates at different pH

Fungal isolate	Cellulolytic activities (IU/ml)		Ligninolytic activities (IU/ml)		
	FP ase	CMC ase	Lac	LiP	Mn P
pH 4					
HST11	-	-	0.90	0.50	0.44
HST14	0.005	0.005	0.45	0.00	0.10
HST15	-	-	1.39	1.65	0.08
HST16	0.004	0.003	1.00	1.00	0.50
pH 5					
HST11	-	-	0.98	0.50	0.47
HST14	0.011	0.010	0.54	0.65	0.29
HST15	-	-	1.30	1.98	0.17
HST16	0.009	0.010	3.00	1.500	0.500
pH 6					
HST11	-	-	1.50	9.00	1.64
HST14	0.012	0.010	0.96	4.15	0.75
HST15	-	-	1.85	21.40	0.30
HST16	0.014	0.015	4.00	26.00	0.50
pH 7					
HST11	-	-	1.21	8.00	1.43
HST14	0.010	0.003	0.63	4.00	0.64
HST15	-	-	1.05	20.98	0.23
HST16	0.007	0.004	3.28	22.50	0.50

Table 6: Cellulolytic and ligninolytic activities of selected fungal isolates at different days of incubation

Fungal isolate	Cellulolytic activities (IU/ml)		Ligninolytic activities (IU/ml)		
	FP ase	CMC ase	Lac	LiP	Mn P
Day 2					
HST11	-	-	0.00	0	0.50
HST14	0	0	0.55	2.00	0.00
HST15	-	-	0.96	4.00	0.00
HST16	0	0.001	0	4.00	0.00
Day 3					
HST11	-	-	0.99	5.00	0.50
HST14	0.001	0.000	0.58	2.50	0.00
HST15	-	-	1.44	5.30	0.05
HST16	0	0.001	0.50	4.50	0.10
Day 4					
HST11	-	-	1.09	6.70	1.00
HST14	0.003	0.001	0.60	3.00	0.50
HST15	-	-	1.54	6.00	0.10
HST16	0.001	0.004	0.50	15.50	1.00
Day 5					
HST11	-	-	1.20	7.50	1.00
HST14	0.007	0.002	0.63	3.88	0.50
HST15	-	-	1.69	6.50	0.19
HST16	0.001	0.004	1.00	16.00	1.00
Day 6					
HST11	-	-	1.50	9.00	1.43
HST14	0.010	0.003	0.96	4.15	0.64
HST15	-	-	1.80	7.40	0.23
HST16	0.007	0.009	4.50	27.00	1.50
Day 7					
HST11	-	-	1.21	8.00	3.64
HST14	0.013	0.013	0.94	4.00	0.75
HST15	-	-	1.85	7.98	0.30
HST16	0.014	0.010	6.50	27.68	1.50
Day 8					
HST11	-	-	1.19	7.50	1.00
HST14	0.013	0.013	0.86	3.96	0.50
HST15	-	-	1.83	7.50	0.21
HST16	0.010	0.008	6.00	5.90	1.00
Day 9					
HST11	-	-	1.10	7.00	1.00
HST14	0.013	0.012	0.82	3.90	0.50
HST15	-	-	1.79	7.43	0.20
HST16	0.010	0.008	5.64	5.30	1.00
Day 10					
HST11	-	-	1.06	6.98	0.50
HST14	0.011	0.012	0.83	3.00	0.50
HST15	-	-	1.72	5.22	0.20
HST16	0.010	0.007	5.12	4.50	1.00

Table 7: Cellulolytic and ligninolytic activities of selected fungal isolates under different aeration conditions

Fungal isolate	Cellulolytic activities (IU/ml)		Ligninolytic activities (IU/ml)		
	FP ase	CMC ase	Lac	LiP	Mn P
Shaking conditions					
HST11	-	-	1.00	6.00	1.69
HST14	0.007	0.003	0.25	1.75	0.50
HST15	-	-	2.50	1.00	0.89
HST16	0.002	0.002	3.00	23.00	1.00
Stationary conditions					
HST11	-	-	2.00	10.00	3.48
HST14	0.012	0.011	1.75	5.00	0.75
HST15	-	-	5.60	21.00	1.60
HST16	0.010	0.010	6.00	28.10	1.50

4. Conclusions

The Lignocellulosic plant biomass can be utilized for many different applications in various industries like in paper and pulp industry, textile industry, biofuel generation, compost preparation and many more. All these applications require digestion of Lignocellulosic biomass to simple monomer units but the main hurdle in this process is the complex structure of lignocelluloses which could be overcome by severe chemical

and physical pretreatment. With time these pretreatments have proven to be harsh on environment as they require high temperature and pressure as well as use of very strong acids and alkalis. As a substitution of these treatments, biodegradation of Lignocellulosic material has emerged as a boon for the industries as well as environment. In this study, four lignocellulose degrading fungi were isolated from soil samples. They were further examined for optimization of conditions and it was found that maximum lignocelluloses degradation occurred at 30 ℃, pH 6, on 7th day of incubation and at stationary condition. These results suggested that efficient lignocelluloses degrading strains could be further exploited for their use under field conditions.

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