

Bioprospecting for Superior Biomass Hydrolysing Fungi from Diverse Habitats

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Abstract

Bioprospecting activities tap vast sources of microbes from diverse and extreme habitats. Microbial hydrolases are important in the development of sustainable biomass energy or biofuels. To achieve full potential of this resource, there is a need to discover novel, unexploited microbes and their enzymes. So, a bioprospecting survey was conducted to identify fungal sources of biomass hydrolysing enzymes from different environments. Out of the total isolates, 39 were found to exhibit cellulolytic, hemicellulolytic and ligninolytic activities. Qualitative enzyme assays were performed using specific soluble substrates. Submerged fermentation of paddy straw/carrot grass (*Parthenium hysterophorus*) was then done to quantify the lignocellulolytic potential of all the isolates. The isolate CM20, identified to be *Aspergillus terreus*, was found to be a superior producer of cellulases and xylanases, while the isolate LG7, identified as *Myrothecium roridum*, was found to produce ligninolytic enzymes in high amounts. The study, therefore, resulted in the identification of cellulolytic and ligninolytic fungi which could be used in biorefineries for the development of greener energy.

Keywords: Biomass; Bioprospecting; Cellulase; Submerged fermentation; Paddy straw; *Parthenium*

Introduction

The acceptance of biomass energy as a promising alternative energy source has led to an exhaustive search for new and superior microorganisms and hydrolytic enzymes involved in biomass deconstruction. Biomass contains about 40–50% cellulose, 25–35% hemicellulose and 15–20% lignin, along with lesser amounts of minerals, oils, soluble sugars, and other components [1]. Biomass, as a source of renewable energy, can be converted to methanol, ethanol or may be mixed partially with diesel [2].

Lignocellulosic biomass, as substrate for bioethanol, needs to be broken down into sugars which are then fermented to ethanol. The most efficient method of biomass hydrolysis is through enzymatic saccharification, accomplished by a concerted action of several enzymes including cellulases, hemicellulases and other accessory enzymes. The most prominent of these are the cellulases, comprising of several different enzymatic classes. Primarily, cellulases are classified into three main groups: the exoglucanases, endoglucanases (cleaving β -1,4 glycosidic bonds from chain ends and internally within chains, respectively) and β -glucosidases (cleave the final β -1,4 linkage of cellobiose or small polysaccharides) [3]. Hydrolysis of hemicelluloses involves enzymes like glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, and endohemicellulases. These include endo-1,4- β -xylanase, β -xylosidase, β -mannanase, β -mannosidase, α -glucuronidase, α -L-arabino furanosidase, acetyl xylan esterase and other enzymes [4].

Cellulose and hemicellulose, being polysaccharides, can be hydrolysed into sugars. Lignin is not a polysaccharide and is not fermented to produce liquid biofuels, but instead can be recovered and used as a fuel for heat and electricity at an ethanol production facility [5]. Three kinds of extracellular phenol oxidases, namely, lignin

peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac), are responsible for initiating the depolymerization of lignin, the expression pattern of which depends on the organisms [6]. Hydrolytic enzymes produced by filamentous fungi, yeasts and bacteria are implicated in biomass degradation and hence, these organisms establish a key link in the carbon cycle [7]. Although several microorganisms have been found to produce a variety of hydrolytic enzymes for the degradation of lignocellulosic biomass, the yields and levels of individual enzyme components are not often satisfactory for commercial biomass saccharification [8].

Bioprospecting to uncover potential microorganisms as sources of superior key enzymes is an important strategy to improve the natural diversity of enzymes and to tailor the enzyme mixtures of the future [4]. The systematic search for new natural resources, known as bioprospecting, helps in tapping the innate power of native organisms in biomass degradation and hence, biodiversity assumes importance as the reserves of future [9]. The present study investigates the lignocellulolytic potential of isolates of filamentous fungi obtained from diverse habitats, with the overall aim of identifying efficient hydrolytic systems with high catalytic efficiency on insoluble substrates, a characteristic better suited for use in biorefineries.

Materials and Methods

Isolation of fungi

Filamentous fungi were isolated from diverse habitats in India. These included the soils of Andaman and Nicobar coastal region, the wetlands of Bhitarkanika, Odisha, the thermal springs of Bakreshwar, West Bengal, the acidic soils of Kerala, degraded plant residues from Delhi ridge, an extension of Aravali hills, New Delhi, and compost pits in the farms of Indian Agricultural Research Institute, New Delhi, India. Isolation of cellulolytic fungi was carried out on Reese's mineral medium [10] with acid swollen cellulose (1%) or CMC (1%) as sole

carbon source (Table 1). Ligninolytic fungi were isolated on basal medium supplemented with alkali lignin (0.1% w/v), defined by Janshekar et al. [11]. The fungal isolates were maintained on Potato Dextrose Agar slants, stored at 4°C and periodically sub-cultured.

SI No.	Isolation medium	Isolate	Habitat
Cellulolytic fungi			
1	Reese's mineral medium with 1% CMC	CM1	Compost pits in the farms of IARI, New Delhi
2		CM2	Wetlands of Bhitarkanika, Odisha
3		CM3	Wetlands of Bhitarkanika, Odisha
4		CM4	Compost pits in the farms of IARI, New Delhi
5		CM5	Compost pits in the farms of IARI, New Delhi
6		CM6	Compost pits in the farms of IARI, New Delhi
7		CM7	Wetlands of Bhitarkanika, Odisha
8		CM8	Compost enriched with 1% cellulose
9		CM11	Compost pits in the farms of IARI, New Delhi
10		CM12	Jatropha compost enriched with 1% cellulose
11		CM13	Compost enriched with 1% cellulose
12		CM14	Vermicompost enriched with 1% cellulose
13		CM15	Vermicompost enriched with 1% cellulose
14		CM21	Compost enriched with 1% cellulose
15		CM22	Compost pits in the farms of IARI, New Delhi
16		CM23	Acidic soil, Kerala
17		CM45	Wetlands of Bhitarkanika
18		CM46	Wetlands of Bhitarkanika
19	Reese's mineral medium with 1% swollen cellulose	CM10	Compost pits in the farms of IARI, New Delhi
20		CM16	Compost pits in the farms of IARI, New Delhi
21		CM20	Soils of Andaman and Nicobar coastal region
22		CM37	Compost pits in the farms of IARI, New Delhi
23		CM40	Compost pits in the farms of IARI, New Delhi
24		CM43	Bakreshwar thermal spring
25		CM44	Bakreshwar thermal spring
Ligninolytic fungi			

26	Basal medium supplemented with alkali lignin (0.1% w/v)	LG1	Degraded plant residues from Delhi ridge
27		LG2	Degraded plant residues from Delhi ridge
28		LG3	Degraded plant residues from Delhi ridge
29		LG4	Degraded plant residues from Delhi ridge
30		LG5	Degraded plant residues from Delhi ridge
31		LG6	Degraded plant residues from Delhi ridge
32		LG7	Degraded plant residues from Delhi ridge
33		LG8	Degraded plant residues from Delhi ridge
34		LG9	Degraded plant residues from Delhi ridge
35		LG10	Degraded plant residues from Delhi ridge
36		LG11	Degraded plant residues from Delhi ridge
37		LG12	Degraded plant residues from Delhi ridge
38		LG13	Degraded plant residues from Delhi ridge
39		LG14	Degraded plant residues from Delhi ridge

Table 1: Isolates of cellulolytic and ligninolytic filamentous fungi obtained from different habitats.

Qualitative assays of lignocellulolytic activities

All the isolates were screened for their cellulolytic, hemicellulolytic and ligninolytic abilities on the basis of qualitative tests.

Cellulolytic and hemicellulolytic abilities

The cellulolytic ability of the cultures was assessed by their ability to produce zones of hydrolysis on CMC (carboxy methyl cellulose) agar (Congo-red test) [12]. After five days of incubation, the plates were flooded with congo-red solution (1 mg/ml in distilled water). The dye was drained off after 15 minutes and the plates were washed thrice with 1M sodium chloride solution. The organisms producing CMCase (endoglucanase) showed yellow zones around growth. The isolates were checked for their xylanolytic activity by their growth on Reese's mineral medium containing xylan (0.1% w/v) as sole carbon source [13].

Ligninolytic ability

The preferential degradation of lignin by the fungi was tested by the assays of lignin peroxidase and laccase. The isolates were grown on 2% malt extract agar plates for one week at 30°C. Wells of 6-8 mm diameter were cut in the plates with the help of sterile cork borer and the bottoms of wells were sealed with molten agar. For the detection of laccase, one ml of freshly prepared solution of guaiacol (2-methoxyphenol; 1% v/v, prepared by mixing 1 ml of guaiacol in 95% ethanol) was added in the wells and the plates kept for 10 hours in the dark [14]. The appearance of red to purple colour indicated the presence of laccase. For the detection of lignin peroxidase, aqueous solution (0.5 ml) of pyrogallol (1.0% w/v, prepared by dissolving 1 g of Pyrogallol in 100 ml of distilled water) and 0.5 ml of freshly prepared hydrogen peroxide (0.4% v/v, prepared by mixing 0.4 ml of hydrogen peroxide in 100 ml of distilled water) were added to the wells [15]. The plates were kept in the dark at room temperature for 10 hours. The

development of a golden yellow to brown colour indicated the presence of lignin peroxidase activity.

Quantitative assays of lignocellulolytic activities

Extracellular levels of lignocellulolytic enzymes produced by the fungal isolates under submerged fermentation with paddy straw/carrot grass (*Parthenium hysterophorus*) as carbon source (1% w/v) were estimated. Paddy straw of the aromatic rice variety Pusa Sugandh 5, cultivated in the farms of Indian Agricultural Research Institute (IARI), New Delhi and carrot grass collected from the premises of IARI were used as substrates for fermentation. One gram dried and chopped substrate suspended in 100 ml Reese's mineral medium (RMM) was inoculated with mycelial discs (6.0 mm) and incubated at 30°C.

Cellulase and hemicellulase activities

The activity of cellulases (FPase, CMCase and β -glucosidase) was estimated in the culture filtrates after seven days of submerged fermentation by the methods described by Ghose [16] and Wood and Bhat [17]. One unit of filter paper activity or CMCase corresponded to 1 μ M of glucose formed per minute during hydrolysis. β -glucosidase assay was performed using p-nitrophenyl- β -D-glucopyranoside as substrate and the activity was calculated in terms of μ M of p-nitrophenol produced per ml of culture filtrate per minute. Xylanase was estimated by the method of Ghose and Bisaria [18] and one enzyme unit was expressed as 1 μ M of glucose formed per minute during hydrolysis.

Ligninase activity

The H₂O₂ dependent oxidation of Azure-B was used for determination of lignin peroxidase (LiP) activity as described by Kirk et al. [19]. Absorbance was recorded at 651 nm for 180 seconds at 30 seconds interval. Laccase activity was determined by measuring the change in absorbance at 436 nm with 5 mM ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) as the substrate [20]. One enzyme unit was defined as 0.01 unit change in absorbance per minute per ml of the culture filtrate.

All experiments were carried out in triplicates. Statistical analyses, such as least square difference and mean standard error were performed using SPSS 16.0 software package.

Results

Qualitative lignocellulolytic enzyme assays

The isolates of filamentous fungi were screened for production of cellulose, hemicellulose and lignin degrading enzymes qualitatively, on agar media containing specific substrates. Table 2 presents the cellulolytic/ligninolytic capacity of the isolates as observed from the zones produced on selective agar media. The isolates were adjudged for their cellulolytic abilities based on the zone of hydrolysis on CMC agar, which is indicative of endoglucanase (CMCase) activity. Those producing CMCase showed yellow zones around growth (Figure 1a). The isolates, CM20 and LG4 produced zones of greater diameter, followed by the isolates CM45 and CM46 (Table 2).

Xylan is the major constituent of the hemicellulose present in plant biomass and hence utilization of xylan by an organism is indicative of its hemicellulolytic capability. Several isolates, including CM20, LG9,

LG10, CM2, CM1 and CM12, were found to be highly xylanolytic based on the zone of clearance on xylan containing medium (Table 2). Lignin degrading capability of the isolates, especially LG7, LG9, CM3, LG12, LG6 etc. were evident from the production of red coloured zone on guaiacol containing malt extract agar and brown coloured zone on agar with pyrogallol (Figure 1c and 1d).

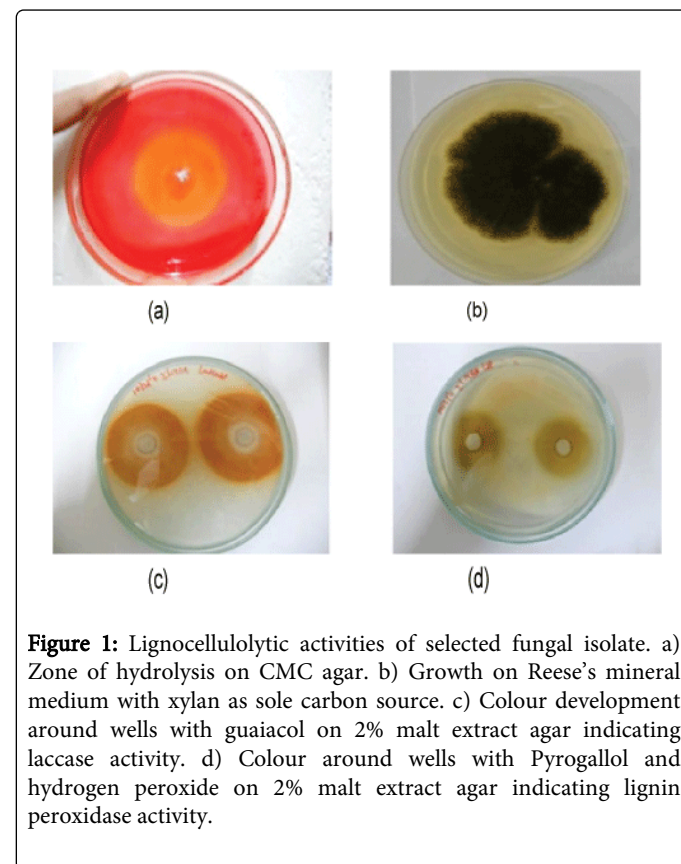


Figure 1: Lignocellulolytic activities of selected fungal isolate. a) Zone of hydrolysis on CMC agar. b) Growth on Reese's mineral medium with xylan as sole carbon source. c) Colour development around wells with guaiacol on 2% malt extract agar indicating laccase activity. d) Colour around wells with Pyrogallol and hydrogen peroxide on 2% malt extract agar indicating lignin peroxidase activity.

Isolates	Zone of hydrolysis on CMC agar	Growth on Reese's medium with xylan	Zone around wells with guaiacol on 2% malt extract agar (laccase activity)	Zone around wells with Pyrogallol and H ₂ O ₂ on 2% malt extract agar (lignin peroxidase activity)
CM1	-	++++	+	+
CM2	++	++++	-	+
CM3	++	+	++	+++
CM4	++	++	-	++
CM5	+	++	-	+
CM6	++	+	-	++
CM7	-	-	-	++
CM8	++	++	-	+
CM10	-	+++	-	++
CM11	+	++	-	+

CM12	-	++++	-	+
CM13	+	+	-	+
CM14	+	-	-	++
CM15	+	+++	-	+
CM16	-	-	-	-
CM20	++++	++++	-	
CM21	++	++	-	+++
CM22	++	+	-	-
CM23	+	+	-	+
CM37	++	++	-	+
CM40	+	+	-	+++
CM43	-	-	-	++
CM44	-	-	-	+
CM45	+++	+	-	++
CM46	+++	+	-	+
LG1	++	+	++	+++
LG2	+	+	+	+
LG3	++	+	-	+
LG4	++++	+	-	+
LG5	+	-	-	++
LG6	-	++	+	++
LG7	+	-	++++	++++
LG8	+	-	+	++
LG9	++	++++	++	+++
LG10	+	++++	++	+
LG11	++	+	+++	+
LG12	+	-	+++	++
LG13	-	-	+	++
LG14	+	+	-	+

* + denotes a zone of >1 mm diameter, ++ denotes a zone of 2 mm diameter, +++ denotes a zone of 3 mm diameter, ++++ denotes a zone of >3 mm diameter, - denotes absence of a zone

Table 2: Qualitative assays of lignocellulolytic abilities of the fungal isolates*.

Quantitative lignocellulolytic enzyme assays

Submerged fermentation of paddy straw/carrot grass by the 39 fungal isolates was carried out for quantitative estimation of their hydrolytic potential. When grown on Reese's mineral medium with 1% paddy straw as sole carbon source, all the fungal isolates showed higher CMCase and xylanase activities while no clear trend was seen for FPase activity (Figure 2a 2b and 2d). However, β -glucosidase

activity of most isolates was lower than when 1% carrot grass was used as the substrate (Figure 2c). Results showed the inducible nature of cellulolytic and hemicellulolytic activities in presence of complex lignocellulosic substrates. Among all the 39 isolates, CM20 exhibited the highest values of CMCase (1.0 IU/ml), FPase (0.2 IU/ml) and xylanase (3.4 IU/ml), when paddy straw was used as the substrate for fermentation. Similar results were obtained with carrot grass as substrate. The β -glucosidase activity of CM20 (1.2 IU/ml) was comparable with the other isolates when carrot grass was used as the substrate. The isolate LG4 showed CMCase activity at par with CM20, with paddy straw as substrate, but the yields of other enzymes were lower. High xylanase activity was also reported by CM2, CM12 and LG9, but with lesser activities of cellulolytic enzymes.

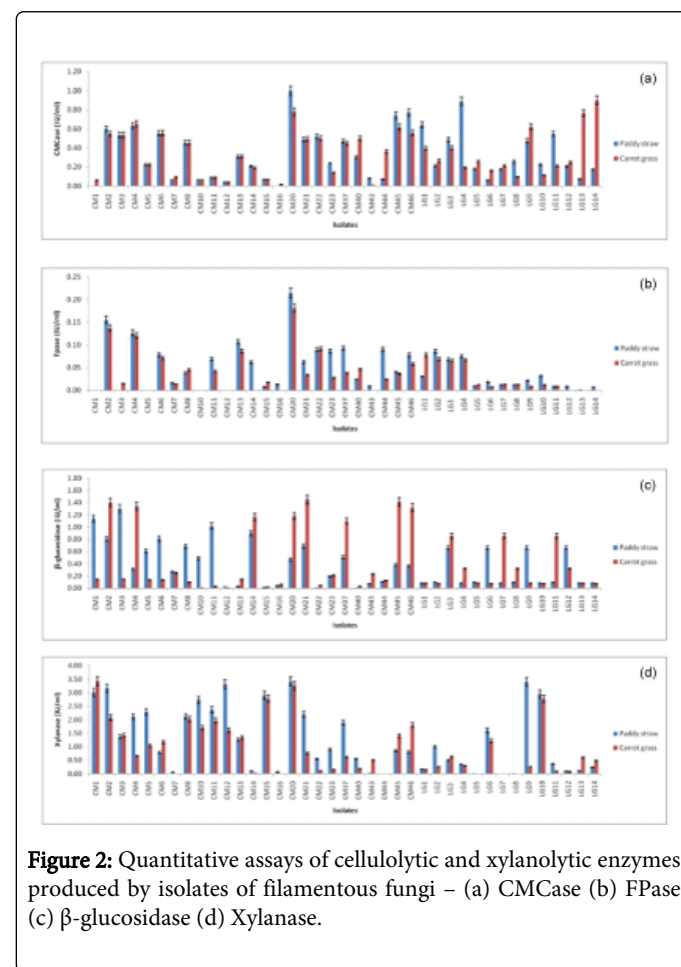


Figure 2: Quantitative assays of cellulolytic and xylanolytic enzymes produced by isolates of filamentous fungi – (a) CMCase (b) FPase (c) β -glucosidase (d) Xylanase.

Action of lignin degrading enzymes such as lignin peroxidases and laccases play an important role in bringing about degradation of lignin present in biomass. Lignin degrading ability of the isolates was evident from the high activities of lignin peroxidase. The activities ranged from 2.4–21.0 IU/ml in various isolates (Figure 3a). The isolate LG7 showed the highest activity (21.0 IU/ml), followed by LG1 (12.4 IU/ml), when paddy straw was the substrate. However, when carrot grass was used, the isolate, LG6 showed a high activity of 11.0 IU/ml. Laccase activity was detected, qualitatively, in only 12 isolates, CM1, CM3, LG1, LG2, LG6, LG7, LG8, LG9, LG10, LG11, LG12 and LG13. Quantification of laccase activities of these isolates on paddy straw showed that LG7, LG11 and LG12 were high producers, with activities of 32.2 IU/ml, 17.7 IU/ml and 14.2 IU/ml, respectively (Figure 3b).

With carrot grass, isolates LG7 and CM3 produced 18.0 IU/ml and 12.0 IU/ml of laccase, respectively. The selective lignin degrading ability of the isolates (LG series) is evident from the results.

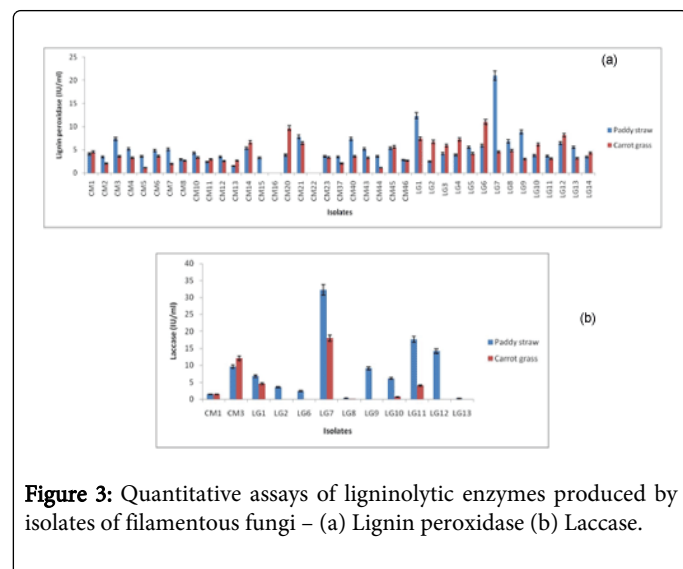


Figure 3: Quantitative assays of ligninolytic enzymes produced by isolates of filamentous fungi – (a) Lignin peroxidase (b) Laccase.

Morphological and molecular characterization

The most promising fungal isolates were identified by Indian Type Culture Collection (ITCC), New Delhi on the basis of their morphological characteristics. The isolate CM20 which was able to grow on cellulose as sole carbon source produced white mycelium on potato dextrose agar with numerous brownish spores. The isolate was identified as *Aspergillus terreus* (NCBI GenBank accession number KM401596) through sequencing of the ITS region followed by BLAST search. Similarly, based on the morphology and sequencing of ITS region, the isolate LG7 was identified as *Myrothecium roridum*. The partial sequence was submitted to NCBI GenBank with the Bankit ID 1547819.

Hence, our study identified fungal strains, which are suitable for biotechnological applications, especially in bioconversion of less effectively decomposable waste products such as agro-residues.

Discussion

Biomass energy, in the form of biofuels, offers a carbon neutral alternative to the depleting energy sources. They contribute to economic development and to the environment through climate change mitigation. The exploitation of biomass as an energy resource enables the conservation of non-renewable sources of energy and more importantly, the preservation of biodiversity [21]. Bioconversion of lignocellulosic biomass using cellulases and other accessory hydrolytic enzymes is the most important step towards realization of high sugar yields in the bioethanol industry.

Many microorganisms in nature are capable of producing biomass hydrolyzing enzymes. However, the complex nature of biomass and the enzyme regulations lead to variations in type of secreted enzymes and enzyme titers. Researchers are now focusing on developing new enzyme sources that offer more desirable features for use in the biofuel and bioproduct industries, including higher specific activities with more balanced synergism, better thermal stability, better resistance to environmental inhibitors and improved combination of various

enzymes [4,22]. Enzyme and microbe combinations vary in different biomass-degrading ecosystems depending on the initial biomass source and environmental factors [22]. Natural degradation of lignocellulosic biomass involving arrays of enzymes have been demonstrated by many bacterial and fungal plant pathogens, microorganisms from termite gut, leaf litter, forest floor etc. [23]. The search for potential sources of hydrolytic enzymes is, therefore, key to the biomass to bioenergy process.

We isolated several filamentous fungi from various diverse habitats in India and screened them to determine their ability to degrade the polysaccharides and lignin present in biomass. Commercially, emphasis is laid on the use of enzymes developed from fungi because of their ability to produce copious amounts of hydrolases which are secreted to the medium for easy extraction and purification [4]. Initial qualitative assays demonstrated high cellulolytic, xylanolytic and ligninolytic activities of the fungi. Lignocellulose, being insoluble, qualitative screening for the assay of lignocellulolytic activities of microorganisms is widely done using soluble substrates [24,25]. However, in order to fit the biorefinery applications, higher activity on insoluble substrates with high hydrolysis rate is a prerequisite [4]. The methodologies of agar plate screening and fermentative production of cellulase have been demonstrated to be in good agreement and positively correlated [26].

Hence, submerged fermentative production of hydrolytic enzymes in medium composed of agricultural waste products (paddy straw and the weed, carrot grass (*Parthenium hysterophorus*) was carried out using the 39 fungal isolates. The enzyme activities were quantified after 7 days of incubation. When paddy straw was used as the substrate, the isolate CM20 (*Aspergillus terreus*) was found to be a prolific producer of cellulase and xylanase. *Aspergillus terreus* has been used for cellulolytic enzyme production by various research groups [27,28]. Mishra et al. [29] attempted lignocellulolytic enzyme production from submerged fermentation of paddy straw using various microorganisms. In their study, *Cellulomonas uda* NCIM 2353 was found to produce the highest CMCase activity (0.38 IU/ml), while *Cellulomonas cellulans* MTCC23 demonstrated higher FPase (0.72 IU/ml) and β -glucosidase (1.05 IU/ml) activities. Our fungal isolates reported higher activities on paddy straw. Comprising of cellulose, hemicellulose, and lignin in approximate concentrations of 41.4%, 20.4% and 12.1% respectively on weight basis, paddy straw is one of the most abundant lignocellulosic crop residues in India [30]. For complete hydrolysis of insoluble cellulosic substrates, synergistic action of the various components of the enzyme complex is required [31]. When carrot grass was used, the extracellular levels of enzymes were lower. Chemical composition of *Parthenium* biomass has been reported as around 13–17% lignin, 21% hemicelluloses, and 28% cellulose [32]. The lower contents of polysaccharides may have had a less effect on the cellulolytic enzymes, which are mostly inducible.

The isolate LG7 (*Myrothecium roridum*) produced higher levels of lignin peroxidase and laccase. The capability of the plant pathogenic fungus, *Myrothecium* sp. to produce extracellular enzymes in submerged culture and solid-state culture has been reported [33]. In biorefineries high lignolytic and low cellulolytic organisms are important for delignification [34]. Lignin degrading enzymes find wide applications industrially, including delignification of pulp, textile dye decolorization, effluent detoxification etc. [35].

Results of qualitative and quantitative assays show the potential of native fungal isolates for targeted deconstruction of biomass, the most abundant and renewable biopolymer on earth. Biomass bioconversion

depends on the enzymatic hydrolysis of biomass carbohydrates. Saccharification of polysaccharides from paddy straw and other lignocellulose complex to fermentable sugars requires enzyme cocktails with synergistically acting components. Moreover, the use of microorganisms for selective degradation/removal of lignin and hemicellulose hold immense importance in biomass pretreatment, to make biomass digestible by enzymes or acid without losing too much of structural sugars. Hence, to achieve full potential of biorefineries, there is an increasing need to discover novel and unexploited microbes and their enzyme arsenals.

Conclusions

Biomass-based technology has a significant impact in driving sustainable bioproducts production and to meet the energy needs of the future. The innate potential of microorganisms in the natural degradation of lignocellulosic biomass is utilized for the development of more potent enzyme preparations economically. In our study, filamentous fungi were isolated from diverse environments and were screened for their biomass hydrolysing abilities. Thirty nine isolates were found to be lignocellulolytic, both qualitatively and quantitatively. We were able to isolate two promising fungal strains, *Aspergillus terreus* CM20, a potent cellulase and hemicellulase producer and *Myrothecium roridum* LG7, a superior ligninolytic strain, for utilization in biorefinery applications. Through optimization and detailed insights into the biomass bioconversion process, an economical and environment friendly conversion system utilising these organisms can be developed. Moreover, the unique set of hydrolytic enzymes they possess may also complement commercial cellulases for faster and more complete saccharification of biomass polysaccharides. The identification of efficient hydrolytic enzyme systems is, therefore, of utmost importance and bioprospecting continues to identify opportunities to unwrap novel sources for biotechnological applications.

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