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Isolation and Qualitative Selection of Fungi for Production of Lignocellulolytic Enzymes

Ajit Kaur[1]* and Urmila Gupta Phutela[2]

[1]Department of Microbiology, College of Basic Sciences and Humanities, Punjab Agricultural University, Ludhiana-141004, Punjab, India
[2]School of Energy Studies for Agriculture, College of Agricultural Engineering and Technology, Punjab Agricultural University, Ludhiana-141004, Punjab, India
**Corresponding author*

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A B S T R A C T

Microorganisms especially Fungi are well known for their capability to excrete industrially important enzymes into the environment. In this purposed study, thirty four mesophilic as well as thermophilic fungal species were isolated from different sources like, decaying wood, decaying kinnow fruit, compost and soil. The isolated species were then purified and qualitatively selected for their cellulolytic, lignolytic and hemicellulose degrading potential using agar plate assay method. Carboxy Methyl Cellulose was used as substrate for cellulase activity, xylan was used as a substrate for xylanase activity; Remazol brilliant blue (RBB) dye and guaiacol were used as substrate to evaluate lignolytic activity in the isolated fungi. A total of 15 fungal isolates and the standard cultures namely *Phanerochaete chrysosporium* MTCC 787, *Trichoderma reesei MTCC 164, T. harzianum* MTCC 792, *Coriolus versicolor* MTCC 138, *Pleurotus ostreatus* MTCC 142, *Aspergillus oryzae* NCIM 1212, *Penicillium roquefortii* NCIM 712, *Thermoascus aurantiacus* MTCC 375, *Humicola fuscoatra* MTCC 1409 showed redness zone on guaiacol. Two mesophilic isolates namely A4 and A5; two thermophilic isolates A25 and A31 are the potential cellulose, hemicelluloses and lignin degraders which can further be used for enhancing biogas production. Among standard cultures *P. chrysosporium* and *T. aurantiacus* produced remarkable amounts of lignocellulolytic enzymes.

Introduction

Lignocellulose describes the three major constituents of biomass, namely cellulose, hemicelluloses and lignin. Cellulose is the major chemical component of the fiber wall of plant, which is a homopolysaccharide composed entirely of D-glucose linked to each other by β-1,4-glycosidic bonds by degree of polymerization ranging from 1,000 in bleached kraft pulps to 10,000 in native wood. Cellulose is degraded by the enzymes which belong predominantly to hydrolases, cleaving the glycosidic bonds (Schmidt, 2006). Hemicelluloses are complex heterogeneous polysaccharides made up of different monomeric residues,

such as D-xylose, D-glucose, D-arabinose, D-mannose and D-glucuronic acid. They are classified according to the monomeric sugar in the backbone of the polymer, e.g. mannan (β-1,4-linked mannose) or xylan (β-1,4-linked xylose) hemicelluloses. The third main constituent is lignin, which is a complex macromolecule formed by the dehydrogenative polymerization of three phenyl propane units namely p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol joined through ether bonds (Hofrichter, 2002).

Among the sources of these enzymes microbial sources are preferred industrially because of the short generation times of the microbes and so large volumes of enzymes can be obtained within a short time. Microorganisms such as bacteria and fungi are capable of degrading these complex polymers. Among these microorganisms, filamentous fungi appear to be the most efficient degraders (Jørgensen, 2003, Hakala *et al.*,2006; Jiménez-Tobon *et al.*, 2003; Kamitsuji *et al.*, 2004; Lankinen *et al.*, 2005; Nuske *et al.*, 2002; Palma *et al.*, 2000; Petruccioli *et al.*,2009; Steffen *et al.*,2002; Susla *et al.*,2008; Silva *et al.*,2008; Sklenar *et al.*,2010; Singh *et al.*,2011; Taboada-Puig *et al.*,2011; Wang *et al.*,2008). These fungi include species from the ascomycetes (e.g. *T. reesei*), basidiomycetes including white-rot fungi (e.g. *P. chrysosporium*), brown-rot fungi (e.g. *Fomitopsis palustris*) and finally a few anaerobic species (e.g. *Orpinomyces* sp.) (Ljungdahl, 2008; Yoon *et al.*, 2007; Dashtban *et al.*, 2009). Lignocellulose degradation by these fungi is performed by complex mixtures of cellulases (Dashtban *et al.*,2009; Weng *et al.*, 2008) hemicellulases (Yoon *et al.*,2007) and ligninases (Weng *et al.*, 2008; Sanchez, 2009) reflecting the complexity of the materials. Nagendran *et al.*, (2009) cited that, a multitude of enzymatic activities are required for the conversion of lignocellulosic biomass into useful (fermentable) products. Known essential activities to degrade crystalline cellulose include cellobiohydrolase (CBH), endo-β1,4-glucanase (EG), and β-glucosidase (BG). A larger suite of enzymes is necessary to depolymerize hemicelluloses, including endo-β1,4-xylanase (EX), β-xylosidase (BX), α-arabinosidase, α-glucuronidase, and esterase. The ability to produce specific enzymes for degradation of different carbon and nitrogen sources are due to the diverse habitat where they are found (Prathumpai, 2003). Over the last few years, scientific investigations on such industrial enzyme production from microorganisms have intensified, mainly because of the world wide interest in exploiting renewable resources of biomass as a source of chemicals and liquid fuels. There has been a growing interest in studying the lignin-modifying enzymes of a wider array of white rot fungi for use in various biotechnological applications.

For the year 2010, demands for thermostable, strong, as well as highly specific industrial enzymes in the global market were valued at $3.6 billion; it was estimated that the value will grow at a compounded annual growth rate (CAGR) of 9.1% and reach $6 billion by 2016 (Dewan, 2012). Around 75% of these enzymes of the global enzyme market are lignocellulases. But, one of the bottlenecks of this strategy is the high preparation cost of pure commercial enzymes, and relatively low conversion efficiency, which make it less practicable for a vast industrial scale (Kang *et al.*, 2004). For this, many innovative efforts like genetic modification, microbiological selection, enzymes immobilization and protein engineering have been made to innovate an alternative tuning tool to enhance the features of the enzymes and

their production process (Barbosa *et al.*, 2013). For this, the recombinant production and regulation of lignolytic enzymes has also been intensively studied in filamentous fungi (Conesa *et al.*,2000; Irie *et al.*,2001; Li *et al.*,2001), some yeasts (Jiang *et al.*,2008a), some novel strains of bacteria (Whitwam & Tien ,1996; Bharagava *et al.*,2009; Mishra & Thakur 2010; Yadav *et al.*,2011) and alson in some insect (Johnson *et al.*,1992; Pease *et al.*,1991) hosts with successful production but modest yields of active enzyme. Filamentous fungi secrete these enzymes into the medium and have higher activities in contrast to yeasts and bacteria (Krisana *et al.,* 2005). Therefore, the present work reports the isolation and primary screening of lignocelluloses degrading fungi.

Materials and Methods

Isolation and Purification of Lignocellulolytic Fungi

Different fungal cultures were isolated from different sources like decaying wood, decaying kinnow fruit, soil, compost, digested slurry and plant debris. One gram of sample was vortexed with 99 ml of sterilized distilled water to make uniform suspension. Heavy particles were allowed to settle and clear supernatant was used for serial dilution. One ml of serially diluted sample was pour plated on potato dextrose agar medium (PDA), each containing chloramphenicol (50 mg/l) and incubated at 30±2°C for mesophilic and 45±2°C for thermophilic cultures. Further, the isolated colonies were sub cultured again on fresh agar plates to purify the cultures.

Screening of Lignocellulolytic Fungi

The agar plate assay method (Okino *et al.*, 2000) also recommended by International Union of Pure and Applied Chemistry (IUPAC) was used in the investigation for the qualitative selection of the isolated and purified cultures for their lignocellulose degradation potential. Fungal species were grouped as high and low celluloytic isolates on the basis of cellulase activity using Index of Relative Enzyme Activity (ICMC). Cellulolytic fungi grown on paddy straw agar (PSA) medium supplemented with Carboxy methyl cellulose were evaluated after 7 days for the production of cellulolytic enzymes by flooding with 0.15% I_2 solution. The diameter of clear zone around the fungal colony, gave an approximate indication of cellulase activities. Hemicellulose degrading fungi were anylzed by supplementing the paddy straw agar (PSA) medium with xylan and flooding the colonies with 1% congo red. The clearance zone around the fungal colony indicates xylanase activities. Remazol brilliant blue (RBB) and guaiacol were used as indicator dyes for lignin degradation and potency index was calculated by the following formula:

Potency index = size of clearance zone (cm^2)/size of colony (cm^2)

The concentration of RBB used was 0.05% and that of guaiacol was 0.075ml/l. The clearance zone on RBB plates indicates the presence of lignin degrading enzymes which may be lignin peroxidase (LiP), manganese peroxidase (MnP) or laccase. The presence of red zone on guaiacol containing medium indicates the presence of lignin peroxidase and presence of clearance zone indicates either manganese peroxidase or laccase.

Growth Profile of Lignocellulolytic Isolates

Growth profiles of lignocellulolytic isolates were studied by measuring the colony size

(cm^2) on potato dextrose agar medium up to 5 days of incubation period.

Results and Discussion

Results from Table 1, 2 and 3 showed potency index of isolated cultures (A1 to A34) and standard cultures i.e. Cellulase activity, RBB activity, Guaiacol activity and xylanase activity. The potency index for cellulase activity ranges from 1.05 to 4.71, forming three different groups i.e. high, moderate and low cellulase producing cultures. The potency index for high cellulase producers ranges from 3.01 to 4.71 which includes isolates no. A4, A5, A7, A9 and A10 among the mesophilic isolates; A21, A25 and A31 among the thermophilc isolates; and *Phanaerochaete chrysosporium* and *Thermoascus aurantiacus* from the standard cultures. *Phanaerochaete chrysosporium* showed maximum cellulase activity with potency index of 4.7. The potency index for moderate cellulase producers ranges from 2.01 to 3.00 including mesophilc isolate A2,A3 and A8; thermophilc isolates A16, A17 and A18; standard cultures namely *Pleurotus ostratus, Trichoderma reesei, Trichoderma harzianum, Humicola fuscoatra and Penicillium roquefortii*. The potency index for low cellulase producers ranges from 1.05 to 2.0. Mesophilic isolates A1, A6, A11, A12, A13, A14; Thermophilic isolates A15, A19, A20, A22, A23, A24, A26, A27, A28, A29, A30, A32, A33, A34; Standard cultures namely *Coriolus versicolor* and *Aspergillus oryzae* are included in this category. The potency index for RBB ranges from 0.26 to 2.89. *Phanaerochaete chrysosporium* showed maximum RBB activity having potency index 2.89 followed by thermophilic isolate A25 (PI = 2.71). For guaiacol activity, many isolates gave negative results. Mesophilc isolate number A10 gave good result. Other isolates which gave positive result includes: A1, A4, A5, A7, A8, A9, A12, A13, A14 (mesophilic); and A20, A21, A25, A31, A34 (thermophilc). Isolate number A2, A3, A6, A11, A15, A16, A17, A18, A19, A22, A23, A24, A26, A27, A28, A29, A30, A32, A33 gave negative result. Among the standard cultures, all standards except *Penicillium roquefortii* gave positive results on guaiacol containing media i.e. presence of redness zone on guaiacol plates, thus indicating presence of lignin peroxidase.

Results from Table 4, 5 and 6 showed growth profile of all the 14 mesophilc, 20 thermophilc isolates and standard cultures. Maximum colony size of 31.1 cm^2 was observed by thermophilc isolate A31 and A28 followed by thermophilc isolate A25 having colony size of 30.6 cm^2 while mesophilic isolate A7 showed minimum colony size of 4.1 cm^2 on 5^{th} day of incubation.

According to Machado *et al.,* (2005), the potency index of a culture cannot be correlated with the amount of lignocellulose degrading enzymes present. But the intensity of clearance zone may be correlated with the activity of the enzymes. From the literature, many reports can be cited, where potency index is used as criteria for qualitative selection of lignocelluloses degrading cultures (Mtui and Masalu, 2008; Okino *et al.,* 2000). Thirty ligno-cellulolytic fungi from various sources like soil, compost, plant straw and decaying plant twigs were isolated by Barrasa *et al.,* (2009). They identified the isolated fungal cultures on the basis of their morphological characteristics.

Table.1 Potency index of isolated Mesophilic cultures

Isolate No.	Cultural characteristics	Potency index			
		Cellulase activity	RBB activity	Guaiacol activity	Xylanase activity
A1	Black biomass, spores, fast growing, no pigmentation	1.65	1.44	+ve	1.60
A2	Greenish black hyphal biomass, fluffy cottony appearance	2.77	0.87	-ve	1.98
A3	Initially white, then dark greenish hyphal biomass, fast growing	2.14	0.26	-ve	1.75
A4	Light greenish hyphal biomass, no pigmentation, fast growing	3.51	2.01	+ve	2.16
A5	Dark green beautiful colony, spore forming, fast growing, no pigmentation	3.05	1.67	+ve	2.32
A6	Light green colored growth, powdery, small spores	1.51	1.56	-ve	1.42
A7	Black biomass, soft, fast growing, no pigmentation	3.06	1.36	+ve	1.11
A8	Brown spores, fast growing, wrinkled colonies	2.78	1.25	+ve	1.44
A9	Black spores in centre, whitish ends, axial look from lower surface	3.02	1.56	+ve	1.33
A10	White, cottony growth, pale coloured from lower surface	3.24	1.02	+ve (Good)	3.07
A11	Dark green, small in size, fast growing, no pigmentation	1.14	1.31	-ve	1.44
A12	Exact white, pulled up colonies, soft look, no pigmentation	1.69	1.07	+ve	1.14
A13	Brown spores, fast growing, no pigmentation,	1.72	1.96	+ve	1.8
A14	Black coloured spores, small in size, soft mat/grassy look	1.89	2.04	+ve	2.24

Media used: MEA + Paddy straw (1%), incubated at 30±2^{o}C for 5-7 days, RBB: Remazol Brilliant Blue (0.05%), +ve values of RBB plates indicates lignin degradation, -ve value of RBB plates indicates no lignin degradation; Guaiacol i.e. O- methoxy phenol (0.075ml/l), +ve/-ve values shows presence/absence of redness zone which indicates the presence of lignin peroxidise

Table.2 Potency index of isolated Thermophilic cultures

Isolate No.	Cultural characterisitics	Potency index			
		Cellulase activity	RBB activity	Guaiacol activity	Xylanase activity
A15	Green colonies, soft matty/grassy look, no pigmentation	1.12	1.56	-ve	1.51
A16	White hyphal biomass, fluffy, no pigmentation	2.94	0.92	-ve	1.84
A17	Emarald green, cottony biomass, filled the plate on 4th day	2.56	0.69	-ve	1.69
A18	Black hyphal biomass, small spores, later on fluffy biomass	2.25	0.68	-ve	1.14
A19	Green colony, soft matty look, no pigmentation	1.12	1.03	-ve	1.52
A20	Light green colony, powdery, fast growing	1.56	+	+ve	1.38
A21	Black spores, appears as dots of black colour, fast growing	3.3	+	+ve	1.23
A22	Concentric rings of light and dark green colour, slow growing,	1.92	0.73	-ve	1.19
A23	Green with maroonish, powdery, no pigmentation	1.39	0.45	-ve	1.09
A24	Light greenish, clumped colonies,fast growing	1.2	0.68	-ve	1.11
A25	Yellowish green, thick mass, no pigmentation	3.17	2.74	+ve	2.25
A26	Light green, wrinkled, no pigmentation	1.19	0.88	-ve	1.21
A27	Dark green, initially whitish, flat layer,	1.05	0.47	-ve	1.02
A28	Whitish green, fast growing, no pigmentation	1.12	1.04	-ve	1.28
A29	Creamish white colony, centre of colonies pulled up/pointed, no pigmentation	1.31	2.35	-ve	1.32
A30	Creamish white colony, wrinkled and clumped, no pigmentation	1.62	2.0	-ve	1.11
A31	Black spores with white ends, soft growth, axial pattern on lower surface	3.36	2.23	+ve	2.06
A32	Green, soapy soft powdery, no pigmentation	1.05	1.96	-ve	1.87
A33	Green small spores, powdery, fast growing	1.11	0.34	-ve	1.45
A34	Yellowish mat, soft hyphal biomass, no pigmentation	1.32	0.21	+ve	1.16

Media used: MEA + Paddy straw (1%), incubated at 45±2^{o}C for 5-7 days, RBB: Remazol Brilliant Blue (0.05%), +ve values of RBB plates indicates lignin degradation, -ve value of RBB plates indicates no lignin degradation; Guaiacol i.e. O- methoxy phenol (0.075ml/l), +ve/-ve values shows presence/absence of redness zone which indicates the presence of lignin peroxidise

Table.3 Potency index of standard cultures

Std. Culture	Cultural characterisitics	Potency index			
		Cellulase activity	RBB activity	Guaiacol activity	Xylanase activity
Phanaerochaete chrysosporium MTCC 787	Whitish, creamy, powdered, no pigmentation, fast growth	4.71	2.89	+ve (Very Good)	2.44
Pleurotus ostreatus MTCC 142	Whitish, cottony growth, no pigmentation	2.83	1.07	+ve	1.21
Coriolus versicolor MTCC 138	Creamy whitish, thick mass, no pigmentation	1.89	0.98	+ve (very good)	2.8
Trichoderma reesei MTCC 164	Emerald green coloured spores, fast growing, light yellowish green in beginning	2.71	0.67	+ve	1.61
Trichoderma harzianum MTCC 792	Dark green coloured sporous growth, radial pattern beneath	2.78	1.07	+ve	2.25
Penicillium roquefortii NCIM 712	Green coloured, small spores,	2.31	2.06	-ve	1.44
Aspergillus oryzae NCIM 1212	Green colour, powdery growth,	1.23	0.59	+ve	1.69
Humicola fuscoatra MTCC 1409	Light yellowish green, matty grassy look,	2.12	1.1	+ve	1.09
Thermoascus aurantiacus MTCC 375	Soft white, thick cottony biomass, no pigmentation	3.07	2.84	+ve	3.51

Media used: MEA + Paddy straw (1%), incubated at 30±2^{o}C (mesophilic) and 45±2^{o}C (thermophilic) for 5-7 days, RBB: Remazol Brilliant Blue (0.05%), +ve values of RBB plates indicates lignin degradation, -ve value of RBB plates indicates no lignin degradation; Guaiacol i.e. O- methoxy phenol (0.075ml/l), +ve/-ve values shows presence/absence of redness zone which indicates the presence of lignin peroxidise

Table.4 Growth profile of mesophilic isolates

Sr. No.	Isolate No.	Colony size (cm^2)				
		Day 1	Day 2	Day3	Day4	Day 5
1.	A1	1.4	2.8	4.3	5.6	6.9
2.	A2	3.4	6.6	9.4	13.2	17.7
3.	A3	3.6	5.2	9.7	16.9	20.1
4.	A4	4.1	8.7	13.4	17.3	21.0
5.	A5	3.5	6.1	9.8	14.3	18.9
6.	A6	0.7	3.2	6.4	9.9	12.5
7.	A7	0.5	1.2	2.0	2.8	4.1
8.	A8	0.3	3.4	8.7	12.8	15.7
9.	A9	1.5	3.2	4.3	6.2	8.1
10.	A10	0.8	3.4	7.7	10.1	14.1
11.	A11	2.4	3.8	4.9	6.8	9.6
12.	A12	0.5	2.7	6.9	11.8	13.1
13.	A13	1.4	3.9	7.1	10.2	13.5
14.	A14	Negligible	1.6	4.5	8.2	13.4

* Media used: Malt Extract Agar with 1% paddy straw, incubation temperature: 30±2^oC

Table.5 Growth profile of Thermophilic isolates

Sr. No.	Isolate No.	Colony size (cm^2)				
		Day 1	Day 2	Day3	Day4	Day 5
1.	A15	0.7	5.8	9.1	13.4	16.2
2.	A16	5.2	6.5	9.2	12.4	15.8
3.	A17	5.8	7.1	10.7	14.1	17.7
4.	A18	5.6	8.3	11.9	15.2	19.3
5.	A19	1.1	5.4	9.8	13.5	17.1
6.	A20	0.8	4.1	8.6	14.2	19.1
7.	A21	1.7	4.2	7.9	12.6	16.7
8.	A22	0.8	6.4	11.7	21.7	24.7
9.	A23	0.5	1.4	3.4	8.1	11.2
10.	A24	1.0	3.5	10.7	19.9	22.1
11.	A25	0.8	3.5	11.1	19.8	30.6
12.	A26	0.4	3.4	12.7	25.8	33.7
13.	A27	0.4	8.1	15.2	23.1	30.3
14.	A28	0.3	3.2	11.3	26.2	31.1
15.	A29	0.7	4.6	8.1	12.5	16.3
16.	A30	1.4	4.2	6.5	8.1	10.2
17.	A31	1.2	3.2	11.3	26.2	31.1
18.	A32	0.2	2.4	4.7	7.1	9.3
19.	A33	0.4	3.3	6.9	10.4	14.1
20.	A34	1.2	3.6	7.2	10.2	12.4

* Media used: Malt Extract Agar with 1% paddy straw, incubation temperature: 45±2^oC

Table.6 Growth profile of Standard Cultures

Sr. No.	Std. Cultures	Colony size (cm^2)				
Mesophilic Cultures:		Day 1	Day 2	Day3	Day4	Day 5
1.	*P. chrysosporium* MTCC 787	3.2	6.4	10.1	13.7	19.4
2.	*P. ostreatus* MTCC 142	0.5	2.5	4.1	11.1	13.2
3.	*C. versicolor* MTCC 138	0.3	2.2	3.5	6.2	7.1
4.	*T. reesei* MTCC 164	2.0	2.8	3.9	4.8	5.5
5.	*T. harzianum* MTCC 792	1.0	2.1	3.4	4.7	5.9
6.	*P. roquefortii* NCIM 712	0.4	2.9	4.6	7.3	10.1
7.	*A. oryzae* NCIM 1212	1.2	2.5	3.8	5.1	7.2
Thermophilic Cultures:						
8.	*H. fuscoatra* MTCC 1409	0.8	2.5	5.3	7.6	10.5
9.	*T. aurantiacus* MTCC 375	0.4	2.3	3.9	5.8	9.1

* Media used: Malt Extract Agar with 1% paddy straw, incubation temperature: 30±2°C (mesophilic) and 45±2°C (thermophilic).

The isolated cultures were compared for their ability to decolorize reactive black 5 and reactive blue 38 at concentration of 75 and 150 mg/L respectively to evaluate lignolytic activity. *Aspergillus sp, Paecilomyces sp* and *Sporotrichum sp* were identified as most efficient lignocellulose degraders by Mandhulika *et al.*, (1993). Remazol Brilliant Blue R (RBBR) dye was used as a substrate to evaluate lignolytic activity in 125 basidiomycetes fungi isolated from tropical ecosystem and higher level of peroxidase and laccase could not be related to high RBBR decolorization, though intensity of decolorization was in correlation to enzyme production (Machado *et al.*, 2005). Sadaf *et al.*, (2005) reported that majority of *Aspergillus* and *Penicillium* sps. were found to posses cellulolytic activity.

From the above studies it is concluded that the mesophilic isolate numbers A4 and A5; thermophilc isolates A25 and A31; and standard cultures namely *Phanaerochaete chrysosporium* and *Thermoascus aurantiacus* are the potential lignocellulolytic cultures.

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