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Isolation, screening and identification of enzyme producing fungi isolated from degraded wood, fruits and animal dung

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Keywords

Cellulase, xylanase, screening, *Emericella nidulans*, qualitative, quantitative.

Abstract

Present study is centered towards finding of efficient fungi for cellulase and xylanase activity. For this, isolation was performed using degraded wood and fruits, animal dung cake. A total of 40 fungal isolates which were qualitatively and quantitatively screened out. All fungal isolates showed cellulolytic activity in qualitative screening but bigger clearance zone were found in 14M, 15M and 26M fungal isolates with diameter of 9.05±0.04 cm, 8.23±0.02cm and 6.89±0.04 cm for cellulase and 7.45±0.01 cm, 7.20±0.01 cm and 5.78±0.04 cm for xylanase respectively. After quantitative screening, it was found that among the three fungal isolates, fungal isolates 14 having maximum cellulase and xylanase activity 4.78±0.2 U/ml and 28 ±0.1 U/ml respectively on the 3rd day. Thus fungal isolates 14 was identified by the process of DNA sequencing and phylogenetic analysis of similar species. The screened fungal isolate 14 was closely related to *Emericella nidulans* with 99% similarity. Based on this similarity the isolated new fungal strain was identified as *Emericella nidulans.*

Introduction

Lignocellulosic biomass containing cellulose, hemicellulose and lignin present in abundant amount in nature (Kumar et al., 2016). For lignocellulosic biomass degradation, a group of hydrolytic enzymes (cellulase and xylanase) has capability to hydrolyse cellulose and hemicellulose into smaller hexose and pentose components (Álvarez et al., 2016). Cellulase and xylanase enzymes plays a significant role in natural biodegradation processes of cellulosic materials produced by fungi, bacteria, actinomycetes and protozoa (Lynd et al., 2002, Peciulyte, 2007). But, fungi are well known microorganism for decomposition of organic matter (Lynd et al., 2002). Filamentous fungi are industrially important producers of enzyme due to extracellular release of xylanases, higher yields compared to yeast and bacteria, and production of several auxiliary enzymes that are necessary fordisbranching of the substituted xylans (Haltrich et al., 1996). Cellulases have enormous potential in industries and are used in food, beverages, textile, laundry, paper and pulp industries etc (Jahangeer et al. 2005, Cavaco-Paulo and Gübitz2003). Celllulase and xylanase producing fungi was area of interest because these enzymes hydrolyse the biomass into sugars which can be fermented to produce ethanol. Thus demand for more thermo stable, highly active and specific cellulases and xylanase producing fungi is on the increase, therefore, local fungi should be investigated for enzyme production. This investigation deals with the isolation, screening and identification of enzyme producing fungi.

Materials and Methods

Collection of sample : Sample of degrading wood were collected from G.J.U.S. & T., Hisar, H.A.U., Hisar, Campus, distillery spent wash (DSW) and decaying fruits. Samples of domestic and wild animal dung manure soils (cow, black deer, and camel) from Dear Park in Hisar.

Isolation of fungi colonies using petriplate method: The degraded wood and dung samples were washed using sterilized deionized water for removing dust and contaminants. One gram of the sample was taken in a conical flask with 100 ml sterilized deionized water and shake for an hour on rotary shaker. After sedimentation, the supernatant was then serially diluted as 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} into separate test tube (Brown and Young, 1947, Thomas et al., 2015). Fungal colonies were supported by Rose Bengal agar medium (Martin, 1950). All other essential requirements i.e. micro tips, petriplates along with media were sterilized in autoclave for 15 min 121°C and 15 psi pressure. Pour the media into petriplates in laminar flow and let the media solidify in petriplate. Inoculums (100 µl of each) from $10^{-1,}$ 10^{-2}, 10^{-3}, 10^{-4} dilution were spread with sterile glass rods over the solidify media agar plates. Incubate the petriplates in incubator set at temperature of 30°C for 48 hours. Different fungal colonies were grown on medium and further isolation was done by pure culturing streak plate method as shown in fig. 1.

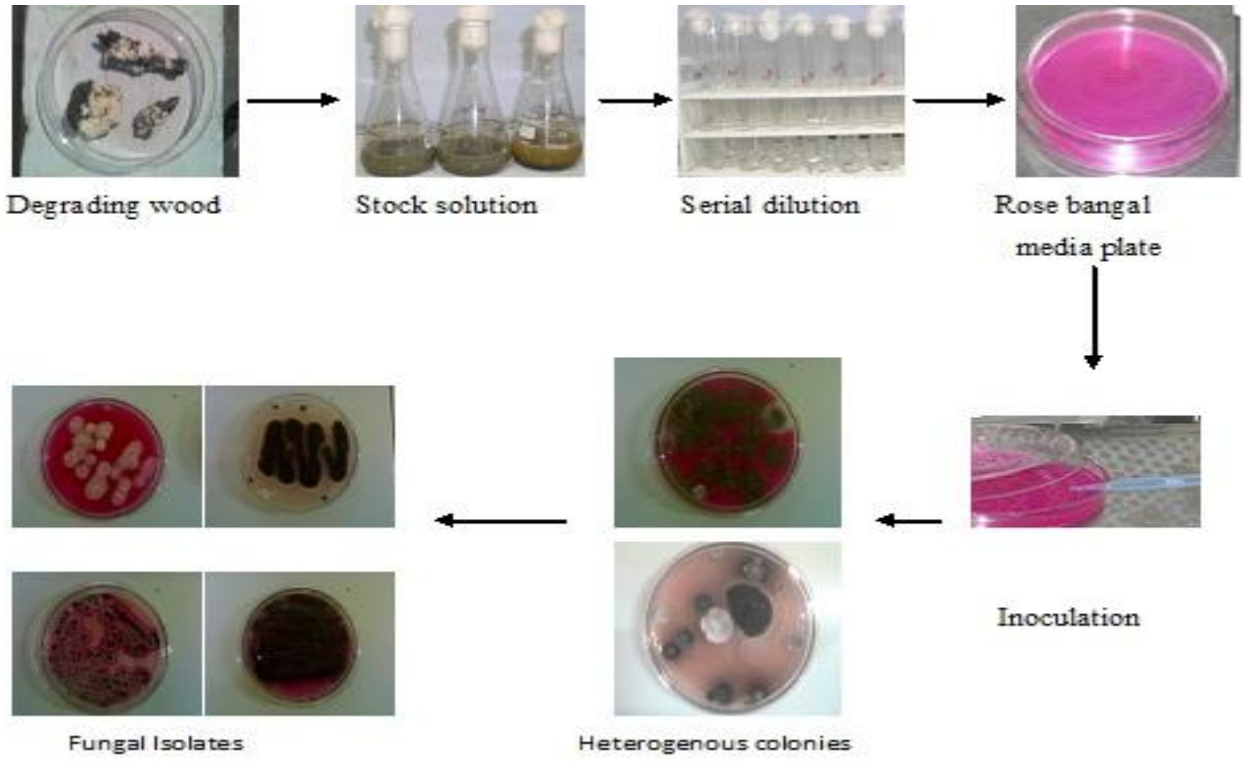

Fig. 1 Isolation and purification of fungal isolates

Purification of fungi

For purification of fungi, one particular colony was picked and streak out on the top of petriplate containing Rose Bengal medium, incubate at 30°C till growth appeared. Pure colonies were obtained by repeating the above process.

Screening of enzyme producing fungi

Totally 40 fungal species were isolated from collected samples (degrading wood, distillery spent wash, animal dung and decaying fruits). These isolated species were qualitatively and quantitatively screened for enzyme producing fungi.

Qualitative screening of enzyme production fungi

Cellulase and Xylanase producing fungi: Cellulase activity was tested using low viscosity CMC 0.5% carboxymethylcellulose (CMC) in mendal and reese agar media, which is a substrate for its activity. Particular isolated fungi were inoculated as dot in the center of petriplates which consisted of carboxymethyl cellulose medium. Put in incubator at 30°C without light. After 2- 5 days, when culture colony was grow up to diameter of 30 mm. Plates were stained by flooding with 2 % (w/v) congo red solution and run off after 15 min by washing with distilled water softly. Destain the plate by flooding with 1 M NaCI for 15 min. CMC degradation is visible as a yellow coloured opaque area against red coloured CMC. Xylan was used as a substrate in mandel and reese media (table 1) for xylanase activity.

Table 1 Composition of Mandel and Reese agar media for cellulase and xylanase production

Ingredients	Amount (g/L)
Peptone	1.0
KH_2PO_4	2.0
Urea	0.3
$(NH_4)_2SO_4$	1.4
$CaCl_2$	0.3
$MgSO_4.7H_2O$	0.3
$FeSO_4$	0.005
$MnSO_4$	0.0016
ZnCl	0.0017
CMC	5.0 (for cellulase)
Xylan	5.0 (for xylanase)
pH	5.3
Agar	20

Quantitative screening of enzyme production fungi

For this, 5g cellulosic biomass of water hyacinth with 100 ml of Czapeck- Dox inorganic medium (CMS) at pH 5.0 were sterilized in 250 ml conical flask by autoclaving at 121°C temperature for 15 min. After this, sterilized flasks were inoculated with 1 ml of 10^7 spores/ml suspension of test fungi. The inoculated flasks materials were mixed thoroughly by tapping and incubated under controlled temperature (30°C) with shaking in incubator shaker (Remi) at 120 rpm for 7 days. After regular interval of time, solids were separated out using double muslin cloth for filtration and then centrifugation (7200*x*g; 15 min) at 4°C. The supernatants achieved were crude enzymes which are further used in analysis. The experiments were run in three replicates and mean values were presented.

Spore suspension

Screened isolates were cultivated on PDA (potato dextrose agar) at 28°C for 7 days. Spores were dislodged from petriplate within the peptone water solution containing tween 80 and were counted using haematocytometer. This suspension was used where required and stored at 4°C.

Enzyme Assay

Cellulase and xylanase activity was determined using CMC and xylan (Ghosh 1987, Bailey et al. 1992). A 0.5 ml enzyme dilution was added to 2 ml substrate solution i.e. CMC solution (2.0 %; pH 5.0; 50 mM citrate buffer) for cellulase and xylan solution (1.0 %; pH 5.0; 50 mM citrate buffer) for xylanase, incubate at 50 °C. After 30 min, 3 ml 3, 5-dinitrosalicylic acid reagent was added to stop the reaction, and the amount of reducing sugar released in the reaction was estimated by using the dinitrosalicylic acid reagent method (Miller 1959). One unit (1 IU) of enzyme activity is defined as the amount of enzyme required to release 1 µmol glucose/xylose per minute under the assay conditions.

Characterization and Identification of screened fungi

The most powerful tool to identify the unknown microorganism is to sequence the gene (DNA) coding for 18S rRNA. The gene coding for the 18S rRNA was amplified using the PCR and the amplified product was subjected to sequencing and the sequence obtained was compared with the sequence obtained from the Nucleotide Database of NCBI.

Results and Discussion

Screening of fungal colonies

Screening of different isolated fungal colonies was performed in order to find out maximum enzyme producing fungi which can be used as appropriate starting material. Screening was performed in two ways and results were presented both qualitatively and quantitatively.

Qualitative screening of enzyme producing fungi

Qualitatively results were analyzed by measuring the diameter of hydrolysis zone. The cellulase and xylanase producing colonies were recognized by observing colourless zone in the region of colonies against the red background.

The results of the clearing zones of hydrolysis for cellulase and xylanase enzymes in different fungal isolates are shown in table 2. From the table it is very clearly revealed that fungal isolates 14 M, 15 M and 26 M were possessing bigger clearing zone for cellulase and xylanase as compared to all other isolates and screening plates are shown in fig. 2 and 3. In the study of Sukumaran et al., 2009, CMC was used as a substrate in mandel and reese media (table 1) for endoglucanase activity. Shahriarinour et al., (2011) also found out CMC as a positive carbon source for cellulolytic fungi screening. This is a well established procedure; results are clearly visible which makes interpretation easy. After fungal culture growth over CMC, plate was stained using congo red dye in order to differentiate between whole and degraded CMC.

Table 2 Plate assay screening of fungal isolates for cellulase and xylanase enzyme

Serial no.	Fungal isolates	Zone of hydrolysis (cm)		Serial no.	Fungal isolates	Zone of hydrolysis (cm)	
		Cellulase	Xylanase			Cellulase	Xylanase
1	1M	4.57±0.02	4.28±0.02	21	21M	4.29±0.01	4.12±0.03
2	2M	3.28±0.03	3.21±0.03	22	22M	4.24±0.04	3.43±0.01
3	3M	2.57±0.03	2.15±0.02	23	23M	2.50±0.02	2.23±0.02
4	4M	3.67±0.04	3.57±0.04	24	24M	3.05±0.03	3.11±0.02
5	5M	1.96±0.02	1.52±0.01	25	25M	3.94±0.03	3.22±0.02
6	6M	4.78±0.01	4.19±0.03	26	26M	**6.89±0.04**	**5.78±0.04**
7	7M	4.56±0.04	3.01±0.03	27	27M	2.61±0.02	2.10±0.03
8	8M	3.78±0.02	2.34±0.04	28	28M	2.13±0.01	1.56±0.02
9	9M	3.45±0.04	2.57±0.01	29	29M	3.76±0.02	3.23±0.01
10	10M	4.38±0.03	3.23±0.02	30	30M	3.28±0.02	4.32±0.01
11	11M	4.35±0.02	4.98±0.01	31	31M	2.71±0.04	2.14±0.02
12	12M	2.94±0.01	1.67±0.03	32	32M	4.62±0.02	4.25±0.01
13	13M	2.67±0.01	2.20±0.04	33	33M	2.73±0.03	2.40±0.03
14	14M	**9.05±0.04**	**7.45±0.01**	34	34M	2.58±0.01	2.14±0.04
15	15M	**8.23±0.02**	**7.20±0.01**	35	35M	3.02±0.01	2.67±0.01
16	16M	4.78±0.02	4.13±0.02	36	36M	1.90±0.02	2.18±0.01
17	17M	2.57±0.03	2.11±0.04	37	37M	1.56±0.04	1.11±0.02
18	18M	3.20±0.02	3.45±0.03	38	38M	4.71±0.04	4.28±0.01
19	19M	3.40±0.02	4.37±0.02	39	39M	4.82±0.02	4.12±0.03
20	20M	1.37±0.04	1.05±0.01	40	40M	3.79±0.03	3.28±0.04

Earlier Voget et al., 2006 had demonstrated the carboxymethyl cellulase activity with 0.2% Congo red. According to Kasana et al., (2008) gram iodine plate assay was the most widely used method for the screening of cellulase activities. Bhalerao et al. (1990) also observed the xylanase positive colonies by using 0.1% congo red, followed by washing with 1M NaCl.

(a) **(b)** **(c)**

Fig 2(a-c) Cellulase screening plates

(a) **(b)** **(c)**

Fig. 3 (a-c) Xylanase screening plates

Quantitative screening for enzyme production

The fungal colonies creating significant zone in plate assay method were tested for quantitatively or secondary screening for enzyme production in flask. Only three 14M, 15M and 26M isolates were able to synthesize high cellulolytic activity and xylanolytic activity in plate assay method. Therefore, to screen out potential enzyme producing fungi, submerged fermentation with these three fungal isolates was carried out to measure their enzyme activities. All the three fungi were tested for extracellular cellulase and xylanase activity on Czapeck- Dox inorganic medium (CMS) containing water hyacinth as sole carbon source and incubated at 30°C under shaking conditions at 120 rpm. Samples were taken after a regular interval of time for enzyme assay. Bharat, (2012) also used CMS media for xylanase production under submerged condition from *Aspergillus flavus*.

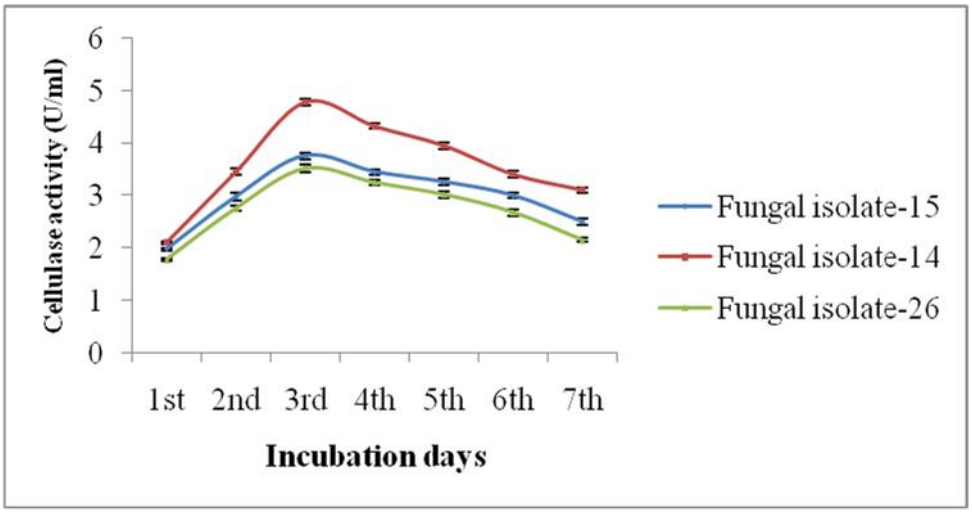

Fig. 4 Cellulase production in SmF by different fungal strains under unoptimized conditions

It was found that almost all the fungal isolates showed increase in cellulase and xylanase production with the increasing time period up to 3rd day; thereafter slight decrease in cellulase and xylanase producing ability was observed (Fig. 4-5). Maximum cellulase and xylanase activity was observed in case fungal isolate number 14, 4.78±0.2 U/ml and 28 ±0.1 U/ml respectively as shown in figs. 4 and 5. There was a decrease in enzyme activity of these three fungal isolates with further increase in incubation period. The impact of time of fermentation on the cellulase and xylanase activities revealed that at the start of trials the organism was in acclimatizing stage, so could not produce sufficient activities of the enzyme.

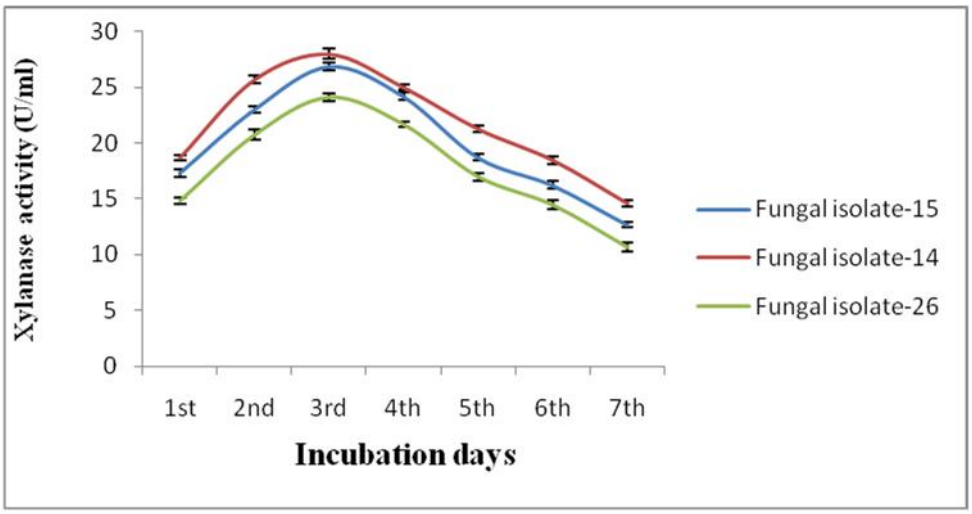

Fig. 5 Xylanase production in SmF by different fungal strains under unoptimized conditions

At 72 hrs the culture was most vigorous and secreted maximum enzymes activities, while after 72 hrs, the cellulase and xylanase activities decreased due to depletion of the nutrients from the culture medium. The decrease in the productivities with prolonged incubation period might also be due to the release of proteases in the growth medium or due to the release of secondary metabolites during the stationary phase that might have inactivated the enzymes.

Identification of new fungal strain

From the primary and secondary screening it was found that fungal isolates 14 were shown maximum cellulase and xylanase activity. So, DNA extraction, amplification using PCR and sequencing was performed. Sequence of screened fungal isolate 14 (5' to 3' sequence of leading strand) were given below:

CCCTCCCATTGCGCATATCGAGTTGTTGCAGTTAAAGCTCGTAGTTGAACCTTGGGTCTGGCTGGCCGGT
CCGCCTCACCGCGAGTACTGGTCCGGCTGGACCTTTCCTTCTGGGGAATCTCATGGCCTTCACTGGCTGT
GGGTGGAACCAGGACTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATACATTA
GCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATA
GGGATAGTCGGGGGCGTCAGTATTCAGCTGTCAGAGGTGAAATTCTTGGATTTGCTGAAGACTAACTAC
TGCGAAAGCATTCGCCAAGGATGTTTTCATTAATCAGGGAACGAAAGTTAGGGGATCGAAGACGATCA
GATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGGCGGTGTTTCTATGATGACCCGC
TCGGCACCTTACGAGAAATCAAAGTTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAA
GAAATTGACGGAAGGGCACCACAAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTC
ACCAGGTCCAGACAAAATAAGGATTGACAGATTGAGAGCTCTTTCTTGATCTTTTGGATGGTGGTGCAT
GGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGCTTAATTGCGATAACGAACGAGACCTCGGCCCTTAAAT
AGCCCGGTCCGCATCTGCGGGCCGCTGGCTTCTTAGGGGGACTATCGGCTCAAGCCGATGAAGTGCGCG
GCAATAACAGCGAGGGCCCCTCCGCCCCGGCAGGA

Phylogenetic analysis

Phylogenetic tree was constructed from neighbor joining program, using bootstrap consensus test with 500 in MEGA 6 and the branch lengths are in the same as those of the evolutionary distances used to infer the phylogenetic tree. The screened fungal isolate 14 was closely related to *Emericella nidulans* with 99% similarity. Based on this similarity the isolated new fungal strain was identified as *Emericella nidulans*. Fig. 6 showed the phylogenetic tree derived from the 18srRNA.

Fig. 6 Phylogenetic tree of screened fungal isolate 14, showing relationship with other worldwide known species

Conclusion

The present study was conducted in an attempt to find out efficient fungal species for enzymes production. From the study it was found that among all fungal isolates, fungal isolates 14 having maximum cellulase and xylanase activity 4.78±0.2 U/ml and 28 ±0.1 U/ml respectively on the 3rd day. The screened fungal isolate 14 was closely related to *Emericella nidulans* with 99% similarity. Based on this similarity the isolated new fungal strain was identified as *Emericella nidulans* which have significant capability to produce enzymes.

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