Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(8):581-586

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Isolation and screening of xylanolytic fungi from natural habitat in Khandesh region of Maharashtra, India

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ABSTRACT

Xylanases are important class of hydrolytic enzyme involved in degradation of xylan- a back bone of hemicellulose. It has wide applications in biotechnological processes such as food, feed, baking, pulp and paper, bio fuel, fruit waste treatment. In the present investigation xylanase producing fungi were screened from the samples collected from various natural habitats such as forest, farm soil and compost pile. Plate assay technique with basal media containing 0.5 % [w/v] beech wood xylan was used for the primary screening. Thirty one fungal strains were obtained though this screening which has xylanase production ability. The fungal isolate MR 57 has showed highest potential in xylanase production with 4745.45 U/ml after 6th day of incubation using submerged fermentation. The isolate was further identified as Aspergillus heteromorphus using morphological as well as molecular approach and sequence was submitted to gene bank.

Keywords: Xylanase, *Aspergillus heteromorphous*

INTRODUCTION

Microbial enzymes are widely used for hydrolysis of complex substrates and are preferred to others since they are specific biocatalysts which can operate under much milder reaction conditions and do not produce undesirable products and hence eco friendly [1]. Xylanase [EC 3.2.1.8] are a group of enzymes that catalyzes hydrolysis of 1, 4-β-D-xylosidic linkages in xylan, a constituent of hemicelluloses [2-4]. It comprises Xylanase [endo-1, 4-β-xylanase] and β- xylosidase [β- D-xyloside xylohydrolase] as a main constituent of enzyme which convert xylan into a more readily fermentable pentose sugars [5-6]. In recent years, xylanases have been extensively studied with respect to production of hemicellulosic hydrolysate for sustainable production of clean and efficient bio-fuels [7]. It has been reported for bioconversion of the agro-industrial wastes, in nutritional improvements of lignocellulosic feeds which increases animal feed digestibility, biobleaching of paper pulp [1; 8], processing of food like clarification of fruit juices and wine [9], the extraction of plant oil, coffee and starch [10-11], textile industry for biopolishing of fabrics in household laundry detergents for improving fabric softness and brightness [7]. Xylanases have also been applied for the production of xylooligosaccahrides, which are used as moisturizing agents for food, sweeteners, and specific health food [12-13]. Xylanolyitc enzymes have also opened new possibilities for the bioconversion of agricultural wastes in to easy fermentable sugars [14-15]. Xylanase are produced by diverse genera and species of bacteria, actinomycetes and fungi Viz. *Trichoderma* spp., *Cryptococcus* spp., *Aspergillus* spp., *Penicillium* spp., *Aureobasidium* spp., *Fusarium* spp., [16] *Haetomium* spp., *Phanerochaete* spp., *Rhizomucor* spp., *Humicola* spp., *Talaromyces* spp., *Bacillus* spp, *Thermomonospora* spp., *Streptomyces* spp. [17-19]. The fungi are preferred source of cellulases and hemicellulases since they secrete these enzymes extracellularly with higher enzyme activities in contrast to yeasts and bacteria [1]. In view of the abundance fungal diversity there is incredible scope for screening and isolation of newer potential microbes with diverse property hence it is a continues process [20]. To expand the applications of xylanase, cost of enzyme is one of the main factors which determine the economics of the process.

Reduction in the cost for enzyme production by optimizing the fermentation medium and isolation of potential strains from diverse habitat is a basic strategy for industrial application [2]. The present study was an attempt to screen potent xylanase producing fungi from natural habitat.

EXPERIMENTAL SECTION

Materials

Chemicals used in the study are of reagent grade prepared in double distilled water stored at room temperature. All media and chemicals were used in the present work Himedia unless otherwise mentioned specifically.

Collection and Enrichment of soil samples

Samples were collected from different natural habitats like agriculture soil, forest soil, agri waste material from nearby location around the Jalgaon, Khandesh region, Maharashtra, India. During the sampling, upper 1 inch soil was removed with the help of sanitized spatula and samples were collected from 10 to 15 cm depth. The collected samples were processed in the laboratory within 24 hrs. Initially, one gram of soil from each location was enriched with media containing 0.5% beach wood xylan [Sigma] [w/v] and incubated at 30^0 C for 7 days. The enriched soil samples were used for the isolation and screening of the xylanase producing fungi [21].

Isolation and screening of Xylanase producing fungi

Fungi were isolated from enriched samples, using a streak plating technique on potato dextrose agar [PDA] plates. The plates were incubated at 30 ± 2^0C. The cultures were observed daily and fungal growth was sub cultured onto fresh plates of PDA until pure isolates were obtained. The pure cultures were transferred to PDA slants and maintained at 4^0C with sub culturing after every four weeks [22]. Isolates were screened for xylanase production ability using minimal basal agar [gm/ 100 ml: KH_2PO_4- 0.6, K_2HPO_4-0.2, $MgSO_4.7H_2O$- 0.05, $[NH_4]SO_4$- 0.1, Yeast extract-0.1] containing 0.5% beach wood xylan as a selective carbon source. After incubation at 30^0C for 5 days, xylanolytic isolates were selected based on the observation of clear zones of hydrolysis around the colony after addition of 0.1% aqueous Congo red followed by repeated washing with 1 M sodium chloride. The zone of xylan degradation around the colonies appeared as an opaque area against reddish colour for undergirded xylan [7, 23]. In secondary screening, Xylanase production ability was studied using medium and condition describe above with application of iodine reagent to visualized clear zones of xylan hydrolysis against adjacent reddish brown colour [10].

Quantitative screening of Xylanase

The plate assay techniques discussed above are qualitative approach hence the isolate were further screened using quantitative techniques as suggested by [24]. The isolates were grown in submerged fermentation and evaluated for the ability of production of xylanase enzymes. After incubation, broth was centrifuged at 10,000 rpm at 4^0C for 10 min [Remi CPR-30 plus model] and supernatant was used as crude enzyme to determine xylanase activity [10].

Enzyme Assay

Xylanase activity was assayed by measuring the amount of reducing sugar liberated from beech wood xylan as a substrate using 3, 5-dinitrosalicylic acid method [25]. The reaction mixture contains 1.0 ml of 0.01 % beech wood xylan [prepared in 100mM sodium phosphate buffer having pH 6.7] and 1.0 ml supernatant. It was incubated at 50^0C for 10 min and the reaction was terminated with addition of 1.0 ml of 3, 5- dinitrosalicylic acid reagent. The reaction mixture containing tubes were placed in boiling water bath for 10 min. After cooling to room temperature, the absorbance of the resulting red colour was measured against the control at 540 nm using a double beam spectrophotometer [Shimadzu UV-1800]. The amount of reducing sugar liberated was quantified using xylose as standard. One unit of xylanase activity was defined as the amount of enzyme that liberates 1 μmole of xylose equivalents per minute per ml under the assay conditions [10, 26].

Measurement of soluble protein

The soluble protein released was measured using bovine serum albumin as standard by using Lowery method [27; 10].

Morphological identification

The potential fungal isolate was identified on the basis of microscopic observations of mycelium microstructure characteristics. Smears of the isolated fungi were prepared in Lactophenol stain and examined with the 40X objectives of a compound binocular microscope [Lawrence & Mayo model: NLCD-307B]. The observations were compared with the standard atlas of fungi for identification [28, 1].

Molecular identification

DNA extraction

Fungal genomic DNA was extracted from pure cultures grown in Petri plates using QIAamp DNA Mini Kit (Qiagen, Inc., Valencia CA) following manufacturer's instructions and quantified by NanoDrop spectrophotometer (NDe1000, Thermo scientific, USA) [29].

PCR amplification

Successfully amplified PCR products [30] were purified using the polyethylene glycol (PEG)–NaCl method [31-32] and were sequenced bi-directionally on an ABI 3730 xl DNA analyser using the Big Dye terminator kit (Applied Biosystems, Inc., Foster City, CA).Raw sequences were manually edited and assembled using Chromas Pro v 1.34 (Technelysium Pty Ltd, Tewantin QLD, Australia). Sequence homology was checked using BLASTn search tool [33-34].

Phylogenetic analysis

Related sequences were aligned, trimmed and phylogenetic tree of ITS (data not shown) and D1/D2 LSU region with bootstrap value more than 50% was constructed using MEGA 5.0 [34] using Neighbour-Joining method. Phylogenetic analysis was also conducted using Bayesian inference of phylogeny by using the program MrBayes 3.1.2 as describe by Salunke *et al.,* [35]. The selected model of nucleotide substitution was 'GTR+I+G'. Three independent runs were performed. In phylogenetic trees, levels of confidence for each node are shown in the form of Bayesian posterior probabilities (BPP). BPP below 0.50 are not shown. GenBank accession numbers are shown after each species name in parenthesis. Scale bar represents substitutions per site.

RESULTS AND DISCUSSION

Sample collection and Isolation

In the present work 23 samples were collected from 3 different natural habitats viz. compost pile, forest and field soils. The detail of sample collection and isolates is given in table 1. Sixteen isolates from four different compost piles, 25 from forest soil and 20 from 2 different banana field soils were obtained.

Table 1: Profile of sample collection and isolates

Sr. no	Type of sample	Location	No. of samples	No. of isolates
1	Compost pile 1	Sirsoli	03	06
	Compost pile 2	Domgaon	02	04
	Compost pile 3	Mehrun	01	04
	Compost pile 4	Eklagna	01	02
2	Forest soil	Manu Devi	08	25
3	Banana field soil	Adavad	04	09
	Banana field soil	Vidgaon	04	11
	Total		**23**	**61**

Figure 1: Screening of xylanase producer a] Congo red test b] Iodine test

Primary and secondary screening of Xylanase producing fungi

All the isolates were subjected for primary as well as secondary screening procedures. Amongst 61, thirty one isolates are found to be xylanase positive using both Congo red and Iodine indicator reagent techniques [36]. The zone of clearance due to the hydrolytic action of xylanases is shown in Figure 1. Similar attempts were made by various researchers to isolates and screen xylolytic fungi from forest soil of Eastern Ghats, India. Ramanjaneyulu *et al*., have isolated 450 fungal culture form forest soil and report maximum xylanase activity 4560 U/ml after optimization for *A. Niger* [7]. The another researcher Parihar and Rai, has obtained 50 fungal isolate from soil out of which 17 isolates were positive for xylanase activity [37].

Quantitative screening for xylanase

The Xylanase production from isolates MR 50 and MR 55 was comparatively higher with enzyme activity around 3500 U/ml, where as isolate MR 57 have produce xylanase 4745.45 U/ml with specific activity of 23.074 mg/ml of protein. The detail of xylanase produced by different fungal isolates is summarized in the table 2. Amongst the different samples collected soil from Banana field has given a highest number of xylanase producing isolates. In the literature reported xylanase activity for fungal and bacterial cultures is within a range of 182 to 3060 U/ml [38-40].

Table 2: Quantification of xylanase activity of screened isolates

Code	Enzyme activity (U/ml)	Specific activity (mg/ml)
MR01	840.00	5.217
MR02	341.82	3.007
MR02 [A]	1054.54	7.550
MR02 [B]	1154.54	9.784
MR03	392.73	0.881
MR04	1156.36	9.129
MR05	603.64	1.865
MR06	1605.45	11.149
MR12	1014.54	9.423
MR12 [A]	850.91	8.315
MR12 [B]	869.091	9.447
MR17	1367.27	7.624
MR19	1274.54	7.439
MR21	1130.91	8.881
MR37	687.27	3.890
MR38	116.36	0.639
MR41	3721.82	17.501
MR42	3289.09	19.894
MR43	3425.45	17.687
MR44	2429.09	5.649
MR45	2545.45	12.856
MR46	3283.64	16.149
MR47	338.182	1.208
MR48	3361.82	15.759
MR49	3434.54	13.665
MR50	4416.36	21.972
MR52	974.54	4.533
MR54	376.36	1.153
MR55	3800.00	16.309
MR56	172.73	1.134
MR57	**4745.45**	**23.074**

Similar approach for quantitative screening of xylanase producing fungal strains from soil samples was used by Sathiyavathi and Parvatham, for *Trichoderma* spp. [41].

Morphological and Molecular Identification of fungi

The colony of fungal isolate MR 57 was circular, black, cottony mycelium with white periphery. The reverse morphology was circular, sulcate, cream colour and the size of colony was 6.0 cm in diameter after 5^{th} day of incubation. The microscopic observations [figure 2] show globose conidia with vesicle, primary and secondary sterigmate and spores. The observations were comparable with the colony characteristics of *Aspergillus* spp. [28; 42]. Further molecular and Phylogenetic identification has supported the morphological observations and helped in species identification.

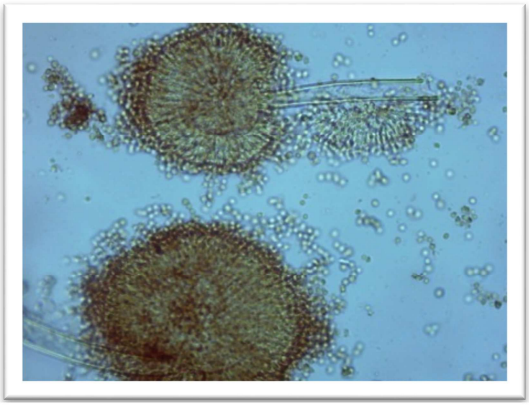

Figure 2 Microscopic observation of MR 57 using Lactophenol stain

Molecular identification of MR 57

BLST result of ITS sequence confirmed the affiliation of strain MR57 to genus *Aspergillus*. Hence for further species level discrimination phylogenetic analysis was conducted using D1/D2 LSU rDNA region (Figure4). The tree topologies obtained using Bayesian inference method clearly suggested the strong clustering of strain MR57 with *Aspergillus heteromorphus* (KM434330 and JN048503) supported by a strong statistical support (BPP 0.80). Hence, this analysis confirms that the strain MR57 from this study is *Aspergillus heteromorphus.*Gene sequence of the strain MR 57submitted to GeneBank with accession number *Apergillus* sp. KF201645.

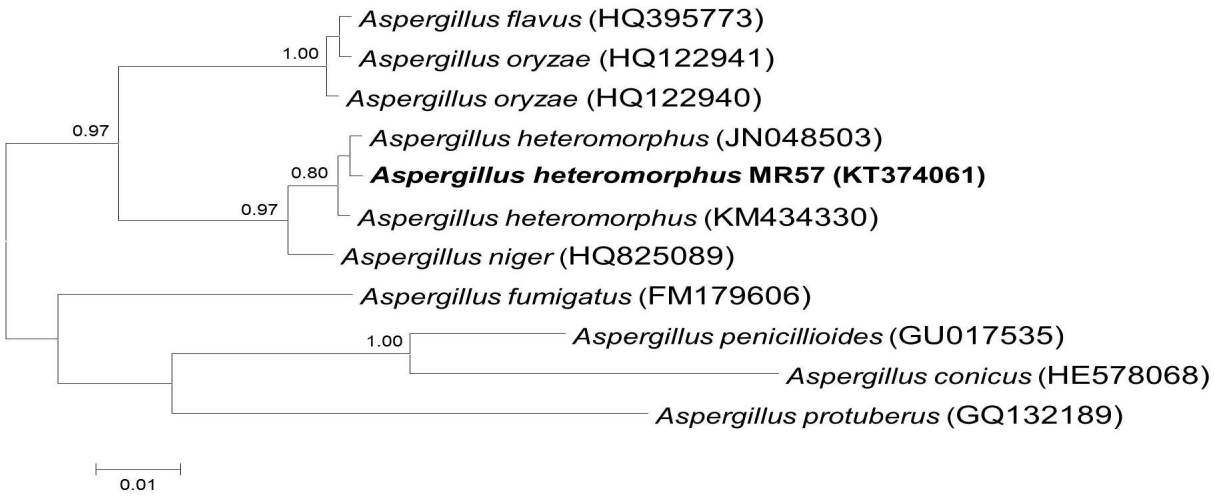

Figure 3 Phylogenetic analysis of isolate MR 57

CONCLUSION

In conclusion, this study reports screening and isolation of potential Xylanolytic fungal strain MR57 from banana field soil sample belonging to Jalgaon, Khandesh region, Maharashtra, India. On the basis of morphological and molecular identification it was identified as *Aspergillus heteromorphus*. It gave maximum Xylanase activity of 4745.45 U/ml after 6th day of incubation which is comparatively higher. Even though species *Aspergillus* has been reported earlier for xylanase, the *Aspergillus heteromorphous* is never explore for xylanase production and was only restricted for cellulase production on this background the current Xylanaoytic isolate can be further explored for agricultural waste management, enzymatic hydrolysis for bio ethanol production, fruit juice processing and pharmaceutical applications.

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