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BIODIVERSITY OF XYLANASE PRODUCING FUNGI PRESENT IN THE LEAF LITTER SOIL OF MUNNAR HILLS, KERALA

T. Shankar[1*], B. Harinathan[2], S. Palpperumal[2], S. Sankaralingam[2], B. Jamunadevi[3]

1. PG and Research Department of Microbiology, Vivekanandha College of Arts & Sciences for Women, Elayampalayam, Periyar University, Tamil Nadu.
2. PG and Research Department of Botany, Saraswathi Narayanan College, Madurai Kamaraj University, Madurai, Tamil Nadu.
3. PG and Research Department of Microbiology, Ayya Nadar Janaki Ammal College, Madurai Kamaraj University, Madurai, Tamil Nadu.

ABSTRACT: Enzymes play crucial roles in producing the valuable commodities in our day to day routine life and also producing fuel for automobiles. Enzymes are also important in reducing both energy consumption and combating environmental pollution. Enzymes are a type of protein in all biological entities and perform as biological catalysts. Several fungi and bacteria are capable of producing multiple groups of enzymes. The increasing consciousness among the people to use chemical free food has made the production of these enzymes very valuable as such enzymes in turn can be used in the processing of various food substances. Xylan is the most widely used as a valuable substrate for enzyme production next to cellulose so this investigation has got a prime focus on xylanase enzyme. Leaf litter soil samples were collected from ten different sites of Munnar hill, Kerala. The soil samples were serially diluted and the potential fungal strains were isolated from the soil. These fungal strains were designated as SJ1 to SJ12. Among the twelve strains xylanase producing fungi were confirmed by Congo red method. The potential xylanase producing fungal strain was conformed as *Fusarium* sp. by morphology on Sabouraud's Dextrose agar plate and Lacto Phenol Cotton Blue staining. The *Fusarium* sp. was sequenced by 18S rDNA and the fungal gene sequence was submitted in the NCBI with accession number KX092008 and confirmed as *Fusarium sporotrichoides*.

KEYWORDS: Xylanase, Leaf litter, 18S rDNA sequencing, *Fusarium sporotrichoides*

***Corresponding Author: Dr. T. Shankar** Ph.D.
PG and Research Department of Microbiology, Vivekanandha College of Arts & Sciences for Women, Elayampalayam, Periyar University, Tamil Nadu.
Email Address: drmicroshankar@gmail.com

1. INTRODUCTION

Enzymes are the capable of bio catalysis for a wide variety of chemical reactions. Although enzymes are produced from animal and plant sources, the world market for enzymes remains in excess of $4500 million in 1985 [15]. Enzyme production is a growing field of biotechnology and annual world sale are close to a billion dollars [9] and it is expected to double by 2005 [25]. Microorganisms represent the most common candidates as sources of new enzymes because of their broad biochemical diversity, feasibility of mass culture and alleviation of genetic manipulation. They are the preferred sources of these enzymes in fermentation bioprocesses because of their fast growth rate and also that they can be genetically engineered to generate new enzyme with desirable abilities [17]. Enzymes are the catalytic cornerstones of metabolism and as such are the focus of worldwide intense research not only in the biological community, but also with the process designers, chemical engineers and researchers working in other scientific fields [4]. Xylan is one of the key elements of hemicelluloses found in plant cell wall is the second most abundant polysaccharide next to cellulose [1, 28]. The term hemicelluloses refer to plant cell wall polysaccharides that occur in close association with cellulose and glucans. In fact, the plant cell wall is a composite material in which cellulose, xylan and lignin are closely linked [3]. Usually, hydrolytic enzymes like cellulases and xylanases are produced by fungi for hydrolysis of complex substrates such as lignocelluloses) as carbon source for their growth. Fungi are highly diverse in nature; they have been recognized as a target for screening to find out the appropriate source of enzymes with constructive and novel characteristics [2]. *Trichoderma* spp. and *Aspergillus* spp. have most widely been used for production of these enzymes [12]. Cellulose degrading fungi produce a battery of cellulases which act synergistically to degrade cellulose [10]. Cellulases and hemicellulases are the focus of several investigations for their use in the bioconversion of agricultural wastes and to replace pumice in the manufacture of stone washed denim in the textile industry [7].

2. MATERIALS AND METHODS

1.1. Collection of sample

In the present study, the litter soil samples were collected from the area of Munnar Hill. Munnar is located in India at the longitude of 77.07 and latitude of 10.09 and it is situated 105 km (618.37 meter) from Sivakasi. The town is about 4500 feet above the sea level.

1.2. Sample collection

Soil samples were collected from ten different locations in leaf litter degrading soil environment of Munnar Hills (Kerala). Using a sterilized spatula, 250 g of soil sample was collected from each location into 300 g capacity plastic container which was previously washed and rinsed with 70% alcohol. The samples from the ten different sites were conveyed to the Microbiology Laboratory within a day, where they were analyzed (Fig. 1a, 1b).

Fig.1a. Map shows the sampling site - (Munnar Hill, Kerala)

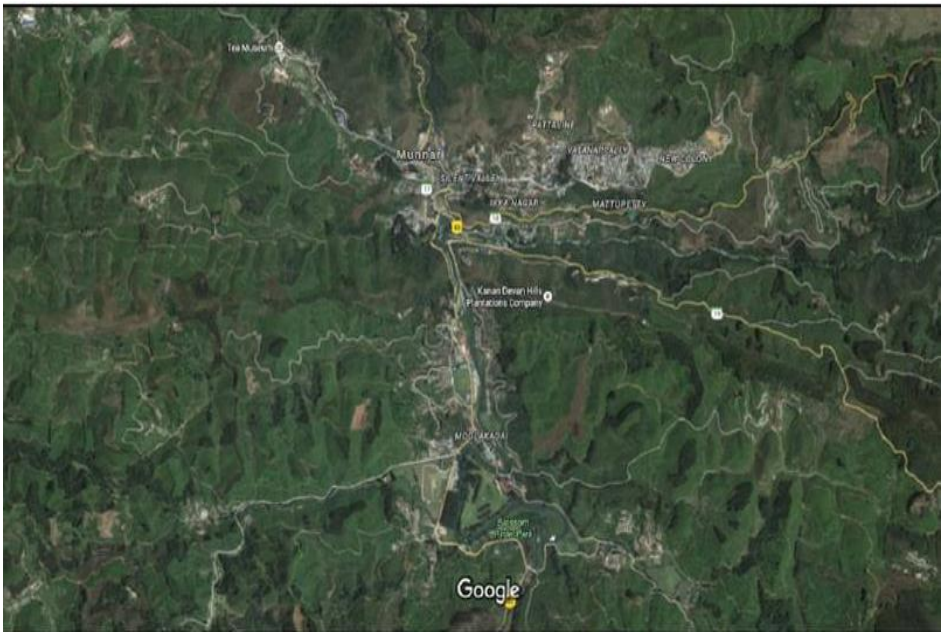

Fig.1b. Map shows sampling site from A to J

1.3. Sterilization

Glassware's and culture media were sterilized by autoclave in 15 lbs pressure at 121ºC for 15 minutes. Pipettes were sterilized at 160ºC for 2 hours in hot air oven. Inoculation works were carried out under aseptic conditions in laminar air flow chamber and the plates were incubated at different temperature depending upon the microorganisms.

1.4. Isolation and identification of fungal isolates

Serial dilutions of soil samples from each site were prepared and inoculated in duplicates into Sabouraud's Dextrose Agar (SDA) plates by spread plate methods. The plates were incubated at 30^0C for 3 days and observations were made daily to determine the presence of filamentous fungi. Pure cultures of isolates were obtained by repeated sub-culture on SDA [13]. The fungal isolates were identified based on the basis of their cultural and morphological characteristics. The cultural characteristics were determined by their appearance on culture plates while the morphological features were determined microscopically. The isolates were identified with reference to the work of [6].

1.5. Determination of percentage frequency of occurrence of isolates

The percentage frequency of occurrence for each species of fungus was determined by the method of using the formula: A/B X 100; where, A=Number of plates in which species appear and B=Total number of plates incubated for each site [20].

1.6. Xylanase production medium

Strains were screened for xylanase production in liquid culture which contained Oat spelt xylan-0.5g; peptone- 0.5g; yeast extract-0.5g; K_2HPO_4-0.1g; $MgSO_4.7H_2O$ - 0.02 g and Agar-2 g in Distilled water 100ml [5].

1.7. Selection of strain for xylanase production

The fungal isolates SJ1 to SJ12 (12 fungal isolates) strains were inoculated in production broth and incubated at 30°C for 3 days and then analyzed for the xylanase production.

1.8. Isolation and identification of fungal strains

Serial dilutions were performed by using the collected leaf litter soil sample to isolate the fungi. The isolated colonies were examined by lactophenol cotton blue technique and identified according to their morphological characteristics [11].

1.9. Identification of fungi by 18S rDNA sequencing analysis DNA Extraction from fungi

DNA was extracted from the sample as described by [19]. In this method, 0.5 ng – 3.0 ng of microbial mat material in 0.5 ng increments were mechanically lysed using a mini bead beater (Bio 101, Thermo Savant, Holbrook, NY) at speed 6.5 for 2-45 second cycles and then centrifuged for 3 minutes at 13,200 rpm. After centrifugation, 490 µl of 100% isopropanol was added to the supernatant and samples were centrifuged in a cold room for an additional 10 minutes. The samples were allowed to precipitate overnight at 4°C and were centrifuged at maximum rpm for 1 hour, after

which the supernatant was discarded and the samples were vacuum dried for 30 minutes at 45°C. Samples were then stored at -20°C until re-suspended for PCR amplification. DNA extraction was done using the following kit; Mo Bio soil DNA extraction kit, (Mo Bio Laboratories, Inc. Carlsbad, CA).

1.10. PCR amplification of DNA

The 18S rRNA gene sequences were PCR amplified using the universal eukaryotic primers CDMF (5‟-GTCAGAGGTGAAATTCTTGGATTTA-3‟) and CDMR (5‟-AAGGGCAGGGACGTAATC AACG-3‟). This primer set was used in the first PCR-based phylogenetic analysis of 18S rRNA gene sequences in SJ1 fungal isolate. This primer set has been used in more recent analysis of 18S rRNA genes of SJ1 that were PCR amplified directly from samples. These primers were originally designed to amplify the 18S rRNA gene from "all" eukaryotic organisms. The primer set amplifies a region of the 18S rRNA gene from nucleotides of SJ1. PCR amplifications were performed using *Taq*DNA polymerase and reagents according to the manufacturer's instruction (Promega, Madison, WI). We used 10-100 ng of template DNA per reaction. PCR was performed in an iCycler (Bio-Rad, Hercules, CA) using the following settings; initial 94ºC for 5 minutes, followed by 30 cycles of 94ºC for 30 seconds, 60ºC for 1 minute and 74°C 1 minute, then a final extension step at 74ºC for 10 minutes.

1.11. Sequence analysis

Sequences were aligned and edited using Sequencher (Gene Codes Corporation, Ann Arbor, MN). Identification of sequences determined using the BLAST algorithm at the NCBI web site (National Center for Biotechnology Information, www4.ncbi.nlm.nih.gov). Phylotypes for this study were defined as 18S rRNA gene sequences that shared ≥ 97% sequence homology. Dendrograms were used to depict 18S rRNA sequence similarity between fungi sequences detected in our survey and those of related organisms. Best nucleotide substitution models were selected using the Model test version 3.06 [16]. Dendrograms were constructed using the neighbor joining algorithm in software.

1.12. Primary screening

The fungal isolates were screened for their abilities to produce extracellular xylanase during their growth on xylan agar medium containing xylan as the sole carbon source. The inoculated plates were incubated for 7 days at 30ºC. Then the plates were flooded with 0.1% (w/v) Congo red. After 30 min of incubation, plates were washed with 1M NaCl. The fungal isolates, which produced distinct clearing zones around their colonies, were selected. Isolated fungi were identified using standard reference manuals by wet mount preparation. *Fusarium sporotrichoides* was preserved on PDA slants for further studies. The isolates were cultured on standard substrate like birch wood xylan. Protein content of the culture supernatant was assayed by Lowry's method using Bovine serum albumin as standard [27].

3. RESULTS AND DISCUSSION

Identification of xylanase producing fungi conformed by Congo red method

The clear zone produced by *Fusarium sporotrichoides* confirmed the xylanase production. The zone produced by *Fusarium sporotrichoides* was 16 mm after 72 hours (Fig.2 - 14).

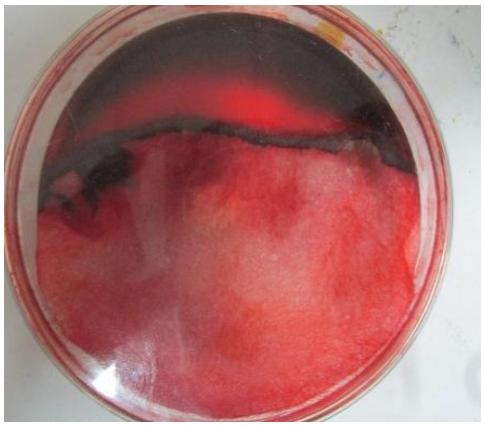

Fig.2.Clear zone produced by *Fusarium sporotrichoides* on Xylan agar

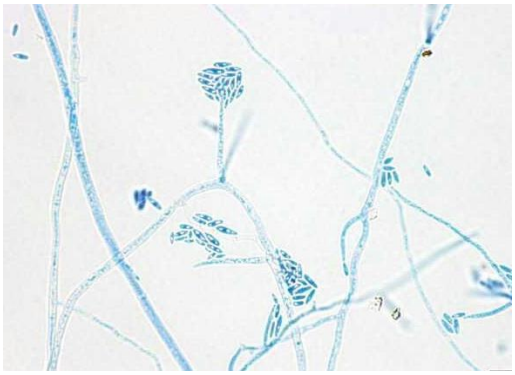

Fig.3a.SJ1-Lactophenol cotton blue mount of *Fusarium sporotrichoides*

Fig3b. SJ1 = *Fusarium sporotrichoides* on Sabouraud's Dextrose Agar (SDA) plate

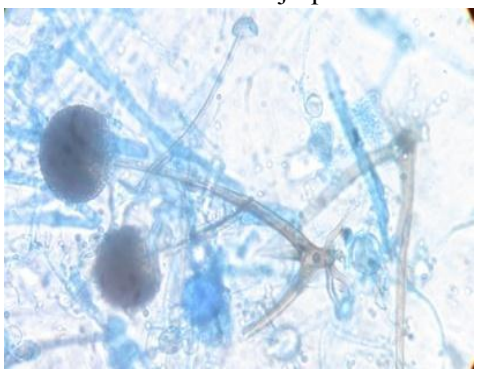

Fig.4a. SJ2 = Lactophenol cotton blue mount of *Rhizopus microspores*

Fig.4b. SJ2= *Rhizopus microsporus* on Sabouraud's Dextrose Agar (SDA) plat

Fig.5a. SJ3 = Lactophenol cotton blue mount of *Aspergillus tamari*

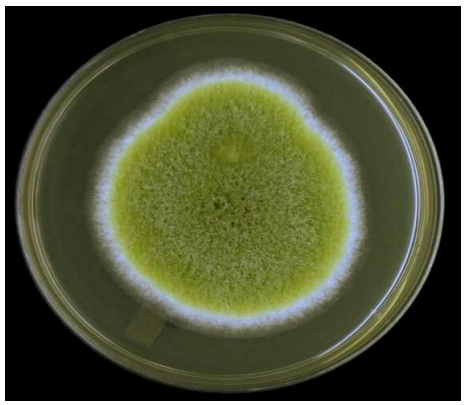

Fig.5b. SJ3 = *Aspergillus tamari* on Sabouraud's Dextrose Agar (SDA) plate

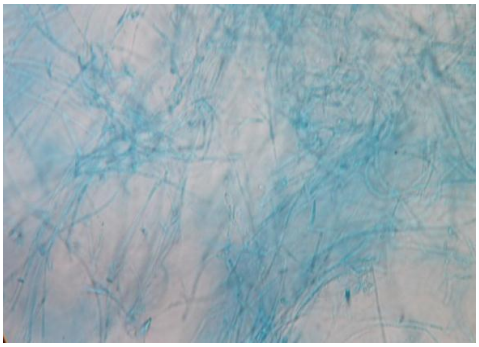

Fig.6a. SJ4 = Lactophenol cotton blue mount of *Fusarium oxysporum*

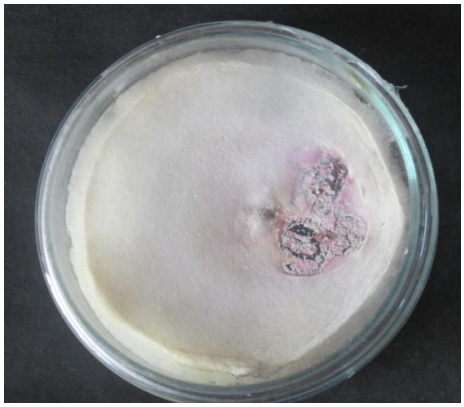

Fig.6b. SJ4 = *Fusarium oxysporum* on Sabouraud's Dextrose Agar (SDA) plate

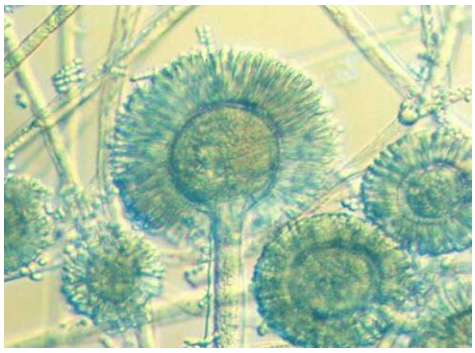

Fig.7a. SJ5 = Lactophenol cotton blue mount of *Syncephalastrum racemosum*

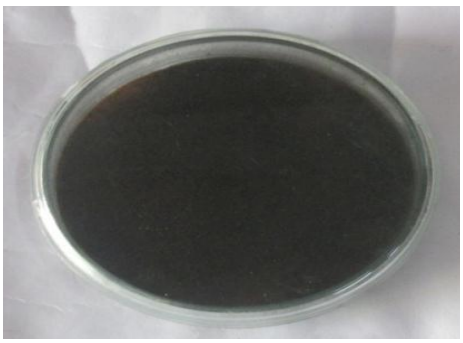

Fig.7b.SJ5 = *Syncephalastrum racemosum* on Sabouraud's Dextrose Agar (SDA)

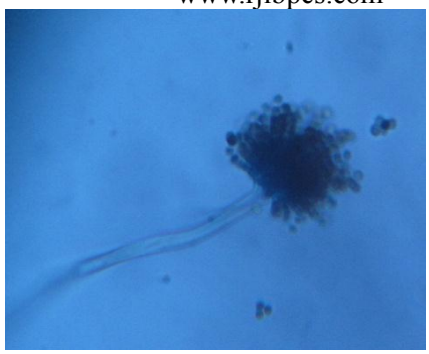

Fig. 8a. Lactophenol cotton blue mount of SJ6 = *Aspergillus niger*

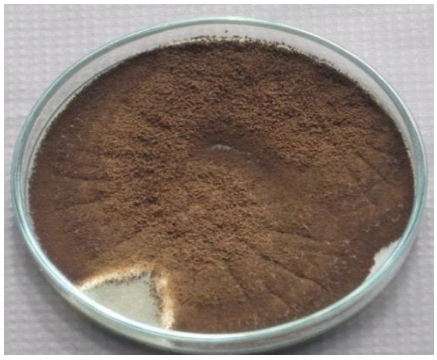

Fig. 8b. SJ6 = *Aspergillus niger* on Sabouraud's Dextrose Agar (SDA) plate

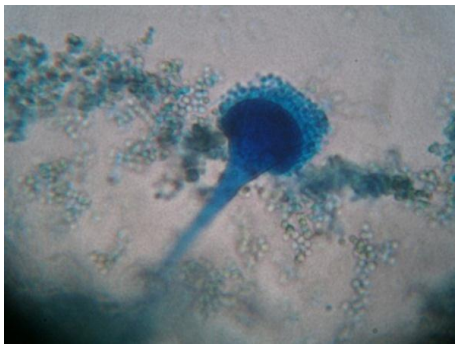

Fig.9a. Lactophenol cotton blue mount of SJ7 = *Aspergills flavus*

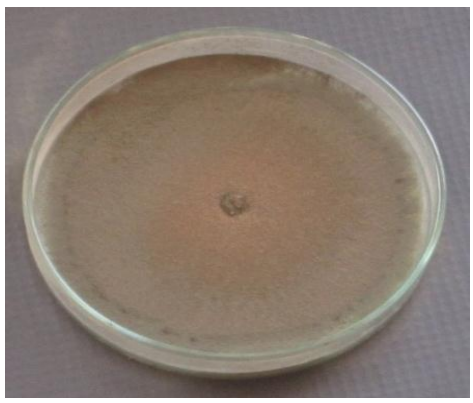

Fig. 9b. SJ7= *Aspergillus flavus* on Sabouraud's Dextrose Agar (SDA) plate

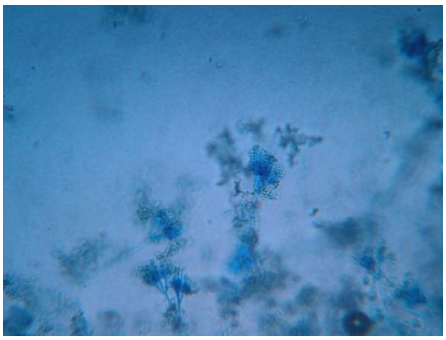

Fig. 10a. Lactophenol cotton blue mount of SJ8 = *Penicillium* sp

Fig. 10b. SJ8 = *Penicillium* s p on Sabouraud's Dextrose agar (SDA) plate

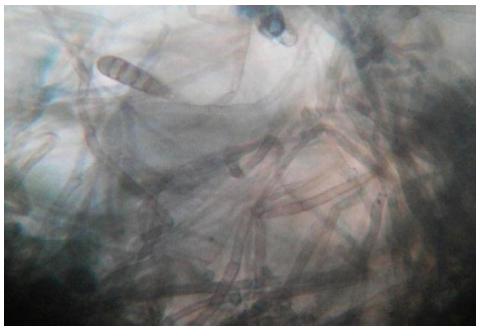

Fig. 11a. Lactophenol cotton blue mount of SJ9 = *Altarnaria* sp.

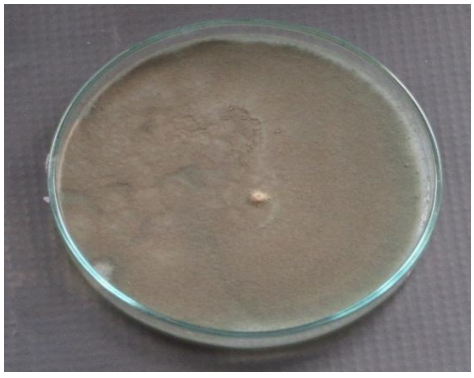

Fig. 11b. SJ9 = *Alternaria* s p. on Sabouraud's Dextrose Agar (SDA) plate

Fig. 12a. Lactophenol cotton blue mount of SJ10 =*Curvularia* sp

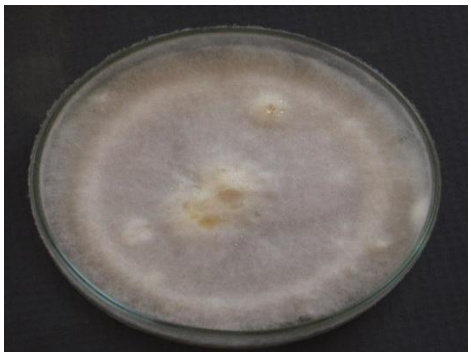

Fig. 12b. SJ10 = *Curvularia* sp. on Sabouraud's Dextrose Agar (SDA) plate

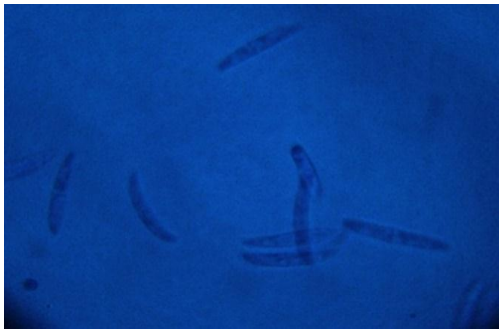

Fig. 13a. Lactophenol cotton blue mount of SJ11 = *Helminthosporium* sp.

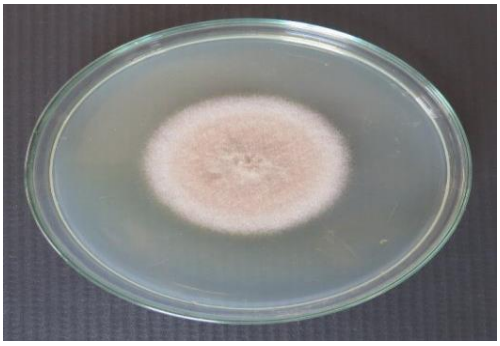

Fig. 13b. SJ11= *Helminthosporium* s p. on Sabouraud's Dextrose Agar

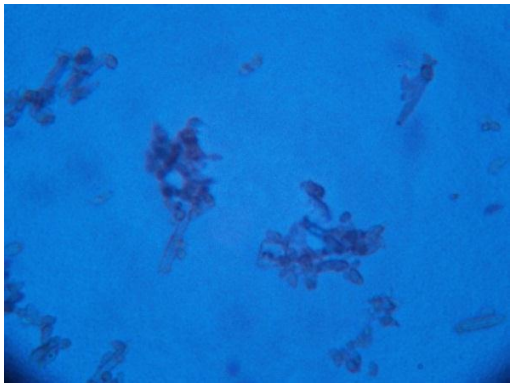

Fig.14a. Lactophenol cotton blue mount of SJ12=*Cladosporium* sp.

Fig. 14b. SJ12 = *Cladosporium* sp. on Sabouraud's Dextrose Agar (SDA) plate

Identification of strains based on 18S rDNA sequencing

The 18S rDNA gene of the *Fusarium sporotrichoides* was amplified using Polymerase Chain Reaction (PCR) with the help of 18S rDNA Universal primers. The sequences were compared against 18S rDNA sequences available in the RDP database (http: 11rdp (me. msu. edu/). The sequence analysis revealed that the strains were phylogenetically closely related to the genus *Fusarium* and the genus *sporotrichoides*. BLAST analysis of 18S rDNA sequence of isolate revealed that the selected isolates showed the maximum similarity of 98% with *Fusarium sporotrichoides*. The phylogenetic relationship was obtained using neighbor joining by pairwise comparison among the 18S rDNA gene sequence of selected isolates with species. The dendrogram was constructed for their phylogenetic relationship and it revealed that the isolate *Fusarium sporotrichoides*was distinctly placed under separate clusters. The gene sequence of *Fusarium sporotrichoides* was submitted in the NCBI with accession number KX092008 (Fig. 15). The results of the soil samples taken from different locations in Munnar Hills (Kerala) are presented here. While Table 1 shows the results of the Total Fungal Counts in the soil samples of the different locations. The percentage frequencies of occurrence of fungal isolates are presented in Table 2, while the results of the cultural characteristics of isolates are presented in Table 3. The results of the xylanase levels produced by the fungal isolates are presented in Table 4. Nayamakadu, Ikkanagar and Moolakadai had the highest amount of fungal counts of 2.0×10^{-4}, Kannimalai, Kannan devan hills; and Vatanappally had a similar fungal count of 1.9×10^{-4}, Mattupatty, Nallatanni had a fungal count as 1.8×10^{-4}. Whereas, Podimettu and Pattanile had the least amount of fungal count of about $1.6\times10^{-4.}$ *Rhizopus*

microsporus, Aspergillus niger and *Cuvularia* sp had the highest percentage frequency of occurrence of 90%, followed by *Apergillus flavus, Fusarium sporotrichoides, Penicillium* sp. and *Cladosporium* sp and *Fusarium sporotrichoides* had 80% and *Syncephalastrum racemosum, Aspergillus tamari, Fusarium oxysporum* and *Helminthosporium* sp had 70% frequency of occurrence respectively. *Altrenaria* sp had the least percentage of frequency of occurrence of 50% (Table 2).

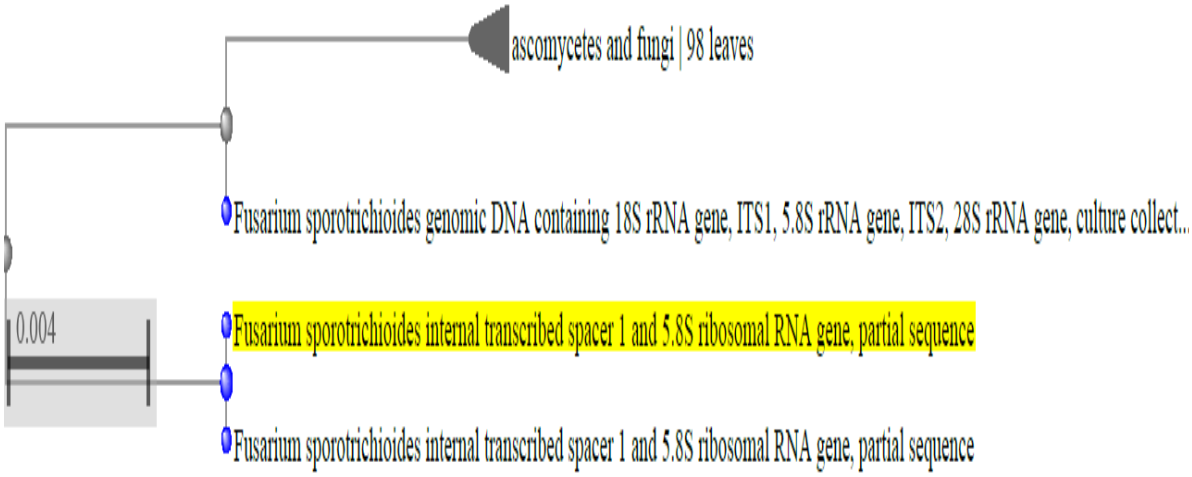

Fig.15.Phylogenetic tree view of *Fusarium sporotrichoides*

Table 1. Total Fungal Count (TFC/g) of soil samples from different location of Munnar Hills Name TFC/g

NAME	TFC/g
A	1.8×10-4 ± 0.8
B	2.0×10-4 ± 0.5
C	1.9×10-4 ± 0.7
D	1.6×10-4 ± 0.5
E	1.9×10-4± 0.8
F	1.8×10-4± 0.9
G	2.0×10-4 ± 0.8
H	1.6×10-4 ± 0.6
I	1.9×10-4 ± 0.9
J	2.0×10-4 ± 0.8

Key: A = Mattupatty; B= Nayamakadu; C= Kannimalai; D= Podimettu; E= Kannan Devan hills; F= Nullatanni; G= Ikkanagar; H= Pattaline; I= Vatanappally; J= Moolakadai

Table 2. Percentage frequency of occurrence of fungal isolates

Fungal isolates	A	B	C	D	E	F	G	H	I	J	% of Occurrence
Fusarium sporotrichoides	+	-	+	+	+	+	+	+	+	+	80
Rhizopus microspores	+	+	+	-	+	+	+	+	+	+	90
Aspergillus tamari	+	-	+	+	+	-	+	+	-	+	70
Fusarium oxysporum	-	+	+	+	+	+	+	+	-	-	70
Syncephalastrum racemosum	+	-	+	+	-	+	+	+	+	+	70
Aspergillus niger	+	-	+	+	+	+	+	+	+	+	90
Aspergillus flavus	+	+	+	+	-	+	+	+	-	+	80
Penicillium sp	+	-	-	+	+	+	+	+	+	+	80
Alternaria sp	-	+	+	+	-	-	-	+	+	-	50
Curvularia sp.	-	+	+	+	+	+	+	+	+	+	90
Helminthosporium sp.	+	+	+	+	+	+	-	-	-	+	70
Cladosporium sp.	+	+	+	+	+	+	-	-	-	+	80

Table 3. Cultural characteristics of fungal isolates on Sabouraud's dextrose agar

Isolates	Surface	Reverse
Fusarium sporotrichoides	White	White
Rhizopus microspores	White	White
Aspergillus tamari	Green	White
Fusarium oxysporum	White	Pinkish
Syncephalastrum racemosum	Black	White
Cladosporium sp.	Black	Black
Aspergillus niger	Greenish-Black	Blackish- White
Penicillium sp.	Brownish	Whitish-Red
Alternaria sp.	Brownish	Greenish
Curvularia sp.	Whitish	Whitish
Helminthosporium sp.	Greenish-Black	Brownish
Aspergillus flavus	Greenish	Black

Table 4. Xylanase production by fungal isolates

Fungal isolates	Xylanase (IU/ml)
Fusarium sporotrichoides	3.341 ± 0.008
Rhizopus microspores	3.059 ± 0.005
Aspergillus tamari	2.412 ± 0.005
Fusarium oxysporum	2.227 ± 0.006
Syncephalastrum racemosum	1.897 ± 0.002
Aspergillus niger	1.677 ± 0.005
Aspergillus flavus	1.117 ± 0.005
Penicillium sp.	1.475 ± 0.005
Alternaria sp.	1.345 ± 0.004
*Curvularia*sp.	1.562 ± 0.005
Helminthosporium sp.	1.315 ± 0.006
Cladosporium sp.	1.677 ± 0.004

Screening, Isolation and Identification of Xylanase Producing Fungi

In the present study leaf litter soil samples were collected from ten different sites of Munnar hill, Kerala. The soil samples were serially diluted and the potential fungal strains were isolated from the soil. These fungal strains were designated as SJ1 to SJ12. Among the twelve strains xylanase producing fungi were confirmed by Congo red method. The potential xylanase producing fungal strain was conformed as *Fusarium* sp. by morphology on Sabouraud‟s Dextrose agar plate and Lacto Phenol Cotton Blue staining. The 18S rRNA sequence of *Fusarium sporotrichoides* was submitted in the NCBI with accession number KX092008. In another study [21] reported that soil was collected from five places in and around Davangere, mainly soil from rice mill, soil under decaying wood, soil near sugar industry, soil dumped with agro waste and soil from saw mill. The garden soil samples were collected using pre-sterilized sample bottles and sterile spatula from Bangalore south of India. Precautionary measures were taken to minimize the contamination. The soil was collected from 5 to 6 places in and around Bangalore and the collected soil samples were pooled. A total of 7 fungal strains were isolated from the pooled soil sample from Bangalore south of India, they are *Aspergillus niger, Trichoderma, Cladosporium, Rhizopus, Fusarium, Aspergillus sp, Mucor. Aspergillus niger* being the most predominant was used for xylanase production [24]. There exists a close resemblance with the earlier researchers who reported that the soil samples collected from ten different locations of Sathuragiri Hills of Western Ghats. A potent fungal isolate was obtained *Aspergillus niger* through screening based on cultural and 18S rRNA sequencing. Among the isolates *Aspergillus niger* found to produce the highest quantity of citric acid (2.5 ± 0.01 g/L) and it

is selected for further investigation. [22]. In addition to that a potent cellulase producing fungi *Aspergillus niger* obtained through screening based on cultural and 18S rDNA sequencing among the twelve isolates this strain was capable of producing cellulase which is similar to our current investigation [25]. In a study soil bacterium *Paenibacillus macquariensis* was isolated in pure form from petroleum containing soil collected from the vicinity of a petrol pump at Indore [23]. In another study a total of 150 soil samples approximately 100 g were collected by sterile forceps and tweezers, packed individually in sterile plastic bags. All samples analyzed for isolation of xylanase producer *H. insolens*. Xylanase activity of *H. insolens* was detected by Congo red plate assay [8]**.** Twelve isolates were obtained from soil samples of different areas in the Rajshahi University campus and studied for detection of xylanase activity. One of the strains was identified as *Bacillus cereus* on the basis of the nucleotide sequence of the 16S rRNA gene which produces xylanase extracellularly [18].

4. CONCLUSION

Biodiversity of xylanase producing fungi *Fusarium sporotrichoides* can be harnessed for further industrial applications.

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