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ISOLATION AND IDENTIFICATION OF XYLANOLYTIC FUNGI FOR THE VALORISATION OF WHEAT STRAW HEMICELLULOSE

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Key words: *Isolation, Wheat straw, Trichoderma asperellum, Aspergillus aculeatus, Xylanase, xylosidase*

Abstract– The capacity of fungi to release extracellular enzymes into their surroundings is well known. Hydrolytic enzymes called xylanases are able to break down Xylan. This study aimed to screen xylanolytic fungi from forest soil and tree bark samples. Using Congo red indicator, 11 fungal isolates out of 45 were chosen for quantitative screening after showing a minimum of 5mm zone of hydrolysis. Quantitative screening revealed that two isolates, ASV10 and TSV9, produced the highest amounts of xylanolytic enzymes that can be further improved by statistical approach and can be used for the release of hemicellulose from lignocellulosic substrates. TSV9 generated 116 U/ml xylanase and 37 U/ml xylosidase, while ASV 10 produced 127 U/ml xylanase and 49 U/ml xylosidase. Molecular identification was done for the two selected fungal isolates by matching the 18 S rRNA sequence with NCBI database and TSV9 showed 99% similarity with *Trichoderma asperellum* and ASV10 showed 99% similarity with *Aspergillus aculeatus*.

INTRODUCTION

Endo-1,4-D-xylanases (EC 3.2.1.8), -D-xylosidases (E.C. 3.2.1.37), -glucuronidase (EC 3.2.1.139), acetylxylan esterase (EC 3.1.1.72), -L-arabinofuranosidases (E.C. 3.2.1.55), p-coumaric esterase (3.1.1.B10), and fer-mannanase: Mannans and xylans, in addition to cellulose and lignin, are the major components of wood. Endo-1,4-mannanases, -mannosidases, -glucosidases, -galactosidases, and acetyl mannan esterases must all work together to completely hydrolyze mannans. *T. reesei* has been shown to produce endo-1,4-mannanase, which hydrolyzes mannan to produce mannotriose and mannobiose. D-xylose and L-arabinose are the two most common pentose sugars in the biosphere. -L-arabinofuranosidase: When *T. reesei* -L-arabinofuranosidase hydrolyzes arabinan, arabinoxylan, and other arabinose-containing polysaccharides, arabinose is released, -galactosidase catalyses the cleavage of terminal -galactose residues from -O-galactosides such as galactomannans and galactoglucomannans. It could help with the digestion of guar gum, which includes around 40% galactoses with -1,6-linkages on a -mannosyl backbone. Because galactomannans and galactoglucomannans are the principal families of hemicelluloses in softwoods, -galactosidase can be utilised to modify wood-derived materials. It may also have medical applications, such as the treatment of Fabry disease (Golubev *et al*., 2004).

Bacteria, fungi, and actinomycetes all produce xylanases. However, xylanases have been found in freshwater mollusks (Yarnura *et al*., 1997) and plant fruits (Yamaki and Kakiuchi, 1979). Though high xylanase-producing cultures have been reported from fungi, there are considerable drawbacks to using fungal xylanases, such as low pH and temperature optimum. Furthermore, most acidophilic fungi are cultivated in medium with an initial pH of less than 7. Cellulase contamination complicates the use of fungal enzymes in the pulp industry since the former limits the amount of cellulose recovered in the pulp. Many publications exist in the field of bacterial xylanases that describe xylanases with high pH or temperature optima, both of which are desirable features in pulp and paper applications (Subramaniyan *et al*., 1997). However, instances of both occurring in the same strain are uncommon or non-existent. As a result, biotechnologists' focus has shifted to isolation of potent organisms producing xylanases with the

aforementioned features. On the basis of physicochemical parameters such as molecular mass and isoelectric point, microbial xylanases are divided into two classes (Wong *et al*., 1988). The first group consists of xylanases with high molecular weight but low pI values. The second kind is characterised by low molecular weight and high pI values (Kuno *et al*., 2000). Over the years, a variety of species have been used to produce xylanase, including *Penicillium sp*. (Gaspar *et al*., 1997), *Trichoderma reesei* (Liu *et al*., 1999), *Aspergillus nidulans* (Ganga *et al*., 1998). However, *Aspergillus niger* is said to be the most powerful xylanase biosynthesising organism (Haq *et al*., 2002). With a few exceptions, bacteria produce larger quantities of xylanases than most fungal enzymes. *Bacillus sp.* members produce significant levels of xylanase activity at alkaline pH and high temperatures (Subramaniyan and Prema, 2000). *Streptomyces sp., S. roseiscleroticus,* and *S. cuspidospororus* are among the filamentous bacteria that generate endoxylanases, xylanase, and polygalacturonase (Maheswari and Chandra, 2000). *Caldocellums accharolyticum* sp., a stringent thermophilic anaerobe, developed xylanases and endoxylanases.

Biopulping, nutritional enhancement of lignocellulosic feedstock, manufacture of ethanol, methane, and other products, and food processing are only a few of the applications for xylanase (Bocchini *et al*., 2005). As explained below, the enzyme is extremely important economically (Motta *et al*., 2013).

1. *Pulp and paper industries:* Xylanase treatment of pulps, particularly chemical pulps, has been shown to provide a bio bleaching process capable of increasing both the brightness and viscosity of the treated pulp while reducing chlorine and other bleaching agents' consumption in subsequent bleaching stages. When compared to standard pulp bleaching techniques, treatment with xylanase has been found to improve pulp characteristics and minimize the environmental impact of bleaching process. The impact of pollutants from the paper and pulp industry on the environment has drawn attention to the use of xylanases in this business, which requires independent consideration. When paper pulp is bleached with chlorine, effluents comprising chloroaromatic chemicals such chlorophenols, chlorobiphenyls, and chlorolignin derivatives, as well as carcinogenic pollutants like dioxin, are discharged into the environment. Cooking wood chips with Na;S / NaOH at l60-180 °C is used in the kraft-pulping process. This causes inadequate lignin breakdown, resulting in pulp browning. The reprecipitated and repositioned xylan impedes lignin breakdown even further. Thus, the loss of this xylan veil may affect lignin breakdown by making the lignin more susceptible to less severe oxidatives. The hydrolysis of xylan allows oxidative chemicals to be used in the bleaching process with ease. For xylanases to be used in biotechnology-assisted paper manufacture, they must have a few essential properties. The use of alkaline thermostable xylanases for the pre-treatment procedure before the bleaching stage is required due to the high pH and temperature of the generated pulp. Another major consideration is the cost of enzyme manufacturing, which the businesses can achieve by utilizing cultures that produce higher amounts of xylanases.
2. *Biofuel production:* Xylanase, along with other hydrolytic enzymes, can be utilized to produce biological fuels from lignocellulosic biomass, such as ethanol.
3. *Juice industries:* Xylanase, in combination with pectinase, carboxy methyl cellulase, and amylase, can be used to clarify juices since turbidity is caused by both pectic and non-pectic components suspended in a stable colloidal system. Coffee, plant oils, and starch extraction may all benefit from xylanase. The xylose produced by xylan depolymerization can also be turned to xylitol, a useful sweetener with uses in the pharmaceutical and food industries.
4. *Bakery industries:* Xylanase improves bread quality by raising the specific volume of the loaf.
5. *Enhancement of animal feed nutritional values:* Xylanase enhances the nutritional characteristics of agricultural silage and grain feed. The addition of xylanase from *Trichoderma longibrachiatum* to a rye-based diet for broiler hens reduced intestinal viscosity, resulting in increased weight gain and feed conversion efficiency.
6. Xylanase can also be used to pre-treat arabinoxylan-containing substrates in the brewing industry. Arabinoxylans are a partially water soluble and very viscous aqueous solution that causes brewing issues and the creation of haze in beer.
7. *Value-added product development for the*

pharmaceutical, agricultural, and feed industries: Enzymatic hydrolysis of xylan yields oligomers called xylo oligosaccharides, which are used in medicinal, agricultural, and feed industries. Xylo oligosaccharides have prebiotic properties, meaning they are not hydrolyzed or absorbed in the upper gastrointestinal tract, and they affect the host by encouraging the growth or activity of one or more bacteria in the colon, boosting health. The lowering of cholesterol, the maintenance of gastrointestinal health, and the increase of calcium biological availability are just a few of their significant physiological benefits. They also prevent starch from retrograding, which improves food nutritional and sensory qualities.

8. *Production of environmentally friendly gasoline:* As energy sources become scarce, fuel production is becoming increasingly important. There have been reports of ethanol being produced from garbage by integrating xylanase treatment (Ahring *et al*., 1999).

MATERIALS AND METHODS

Chemicals

The chemicals, medium components and other raw materials used in this study were obtained from different sources. All media ingredients like, yeast extract, agar, urea, peptone, xylose, dextrose, sucrose and ruthenium red, oat spelts xylanetc were obtained from Himedia, Mumbai, India. Birchwood xylan and DNS were obtained from SRL chemicals and other general chemicals were obtained from both SRL chemicals and Qualigens fine chemicals, Mumbai, India. P-Nitro phenyl xyloside (PNPX) from Sigma Aldrich Pvt. Ltd. Lignocellulosic substrates were collected from farms in Medak, Telangana.

Isolation and Primary screening

For isolation and primary screening of xylanolytic microorganisms, different samples like forest soil, agriculture soil, decaying wood barks, lake water and river water were collected. Forest soil was collected from Nallamala forest, Andhra Pradesh, India (16.0131° N, 78.9717° E**6**). The samples were serially diluted in sterile distilled water and plated on SDA agar, which contained (g/L) Dextrose, 40; Mycological peptone, 10; Birchwood xylan, 10; and Agar, 20 (pH-5.5). After 3 days of incubation, clear zones were visualized using Gram's iodine solution (Soares *et al.,* 1999) and also by Congo red. Colonies showing zone of clearance were selected and maintained on potato dextrose agar slants at 4 °C.

Secondary screening

The strains were cultured in Erlenmeyer flasks (250 ml) containing 10g of wheat bran moistened with 18 ml of the basal salt solution (BSS: substrate to moisture ratio 1:1.8). The composition of the basal salt solution was (g/L) NaCl, 30.00; KCl, 0.75; $MgSO_4$, 7.00; NH_4Cl, 0.5; K_2HPO_4, 2.5; KH_2PO_4 0.5; trace metal solution, 1.00 ml; distilled water, 1.0. pH of the medium was adjusted to 5.5. Composition of the trace metal was H_3BO_3, 2.85; $MnCl_2.7H_2O$, 1.80; $FeSO_4.7H_2O$, 2.49; Na–Tartarate, 1.77; $CuCl_2$, 0.03; $ZnCl_2$, 0.02; $COCl_2$, 0.04; $Na_2MoO_4.2H_2O$, 0.02; distilled water, 1.0 L. The media were then autoclaved at 121°C (15 lbs) for 20 min, cooled and inoculated with fungal spores. Seven days old fungal spores were harvested by aseptically adding sterile saline water containing 0.01% (v/v) Tween 80 so as to get the final concentration 1×10^6 spores ml^{-1}. 1 ml spore suspension was used as inoculum. After mixing, the flasks were incubated at 28 ± 2 °C temperature for 7 to 14 days under static conditions. After incubation, at 12 h interval, samples were removed over a period of seven to 14 days. At each time interval 3 g of spent solid substrate was removed and suspended in 30 ml of 50 mM acetate buffer (pH 5.0), vortexed thoroughly to extract the xylanase. The sample was centrifuged at 5000 rpm for 10 min at 4 °C (centrifugation will remove xylanase from substrate). Supernatant was filtered through Whatman no. 1 filter paper and the clear filtrate was used as crude xylanase preparation. The samples were plated on 1% birchwood xylan agar plates. Subsequently after 5 days of incubation, the plates were flooded with 0.01% of 10-15 ml congo red solution (Teatherandwood, 1982) and permitted to stand for 10 to 15 min and destained with 1% NaCl and the zones of hydrolysis around fungal colonies were measured in mm. Among 45 fungi 11 showed minimum of 5 mm zone of hydrolysis were further selected for quantitative screening. The pure cultures of the 11 strains were maintained on potato dextrose agar slants at 4 °C. During primary screening using 1% birch wood agar plates, 45fungi which showed varying levels of hydrolysis zone were isolated were selected for secondary screening. And they were subjected to submerged fermentation (SmF). Spore suspension was prepared in a sterile saline. The cultures were grown in 50 ml

of the fermentation medium in 250 ml conical flasks containing (g/L) Dextrose, 40; Mycological peptone, 10; Birchwood xylan, 10 (pH-5.5). The inoculated flasks were incubated at 28 °C in a rotary shaker at 150 rpm for 72 hr. Samples were collected after 72 hr and centrifuged at 10,000 rpm for 15 min at 4°C. Endo xylanase (XYL) and xylosidase (BX) activity of the supernatant was measured.

Enzyme assays

Endo 1,4 β-xylanase assay

Xylanase activity was determined spectrophotometrically by measuring the increase in absorbance at 540 nm (Bailey *et al.*, 1992). The reaction mixture (1.8 ml) containing 50 mM Acetate buffer and 0.2 ml of appropriately diluted crude enzyme was incubated at 50°C for 10 minutes. The reaction was terminated by adding 3 ml of DNS to the mixtures and incubated at 100 °C for 5 min. 6 ml of distilled water was added to the mixture and absorbance was measured at 540 nm against the control (DNS method, Miller, 1959). The tube with 2 ml of only acetate buffer was used as control in the reaction.

One unit (IU) of xylanase corresponds to the amount of enzyme which liberates 1µmol of xylose under assay conditions (Bailey, 1992).

Xylosidase assay

The reaction mixture containing 200 µl of appropriately diluted enzyme and 50 µl of 0.92 mM P-nitrophenyl β-D-xyloside (Sigma Aldrich Pvt. Ltd) and 150 µl 0.15mM acetate buffer (pH 4.5) was incubated at 65 °C for 10 min. The reaction was stopped by adding 1 ml of sodium carbonate. The absorbance was measured at 405 nm against the control (Michele Michelin *et al*., 2012). *One unit* (IU) of β-*xylosidase* activity was *defined as* the amount of enzyme that liberates 1 µmol PNP per minute in the reaction mixture under the assay conditions.

Determination of optimum pH and temperature for XYL and BX activity

Optimum pH and temperature for the maximum XYL and BX activity was determined by carrying the enzyme assay using different buffers (Acetate – 3.8 to 8, tris-HCl – 8 to 9) with pH ranges 4-9 with increment of 0.4 and by incubating the reaction mixtures at 30 °C to 80 °C with the increment of 5 °C at their optimum pH, respectively.

Identification of fungal isolates - TSV 9 and ASV10

Identification using morphological characteristics

Based on colony characters and microscopic observation fungal mycelium by simple staining, and scanning electron micrography, identification of the genus of the isolated strains was done according to Barnette *et al.,* 1972.

Molecular characterization by 16 S rRNA sequencing

Selected isolates were characterized to the species level by 18 S rRNA sequencing, which was carried out at Macrogen international corporation, Korea. Total genomic DNA for PCR amplification of 18 S rRNA was extracted from the isolates TSV9, ASV10 following the method described in the literature (Sambrook *et al*., 1982). The 18 S rRNA was amplified by PCR with universal primers such as

ITS1 Forward primer (5 - TCCGTAGGTGAACCTGCGG - 3)

ITS4 Reverse primer (5 - TCCTCCGCTTATTGATATGC - 3)

Identification was done by matching the isolate 18 S rDNA sequence in database, EZ taxon server 2.1, and the phylogenic tree was constructed using Mega 8.0.6 software (Kumar *et al.,* 2017) by performing by neighbor joining method (Felsenstein, 1985).

RESULTS

Isolation of xylanolytic fungi and qualitative screening

Xylanolytic microbes were isolated and screened utilising a variety of samples including forest soil, agriculture soil, decaying wood barks, lake water, and river water. 45 fungi with varied degrees of hydrolysis zone were isolated after initial screening using 1% birch wood agar plates and were chosen for subsequent screening. 11 fungi out of 45 were chosen for quantitative screening because they

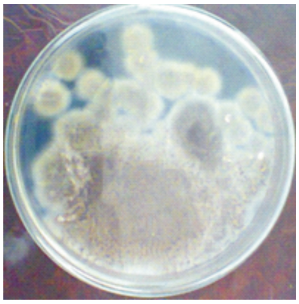

Fig. 1. Fungal colonies in primary screening

Fig. 2. Fungal isolates on PDA

Table 1. Zone of hydrolysis of the isolates and enzyme production in secondary screening

S. No.	Isolate	Zone of hydrolysis in primary screening (mm)	Xylanase activity in secondary screening (U/ml)	Xylosidase activity secondary screening (U/ml)	Source of isolation
1	FSV1	9	86	23	Agricultural soil
2	FSV2	4	51	14	Lake water
3	FSV3	4	45	11	Lake water
4	FSV4	4	58	12	Wood bark
5	FSV5	4	61	13	Agricultural soil
6	FSV6	5	62	15	Agricultural soil
7	FSV7	9	75	27	Forest soil (Nallamala)
8	FSV8	3		10.6	Lake soil
9	TSV9	16	116	37	Agriculture soil
10	ASV10	19	127	49	Forest soil (Nallamala)
11	FSV11	11	95	29	Godavari River water

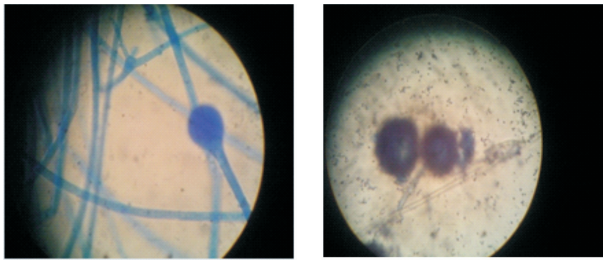

Fig. 3. Microscopic observation of fungal isolates

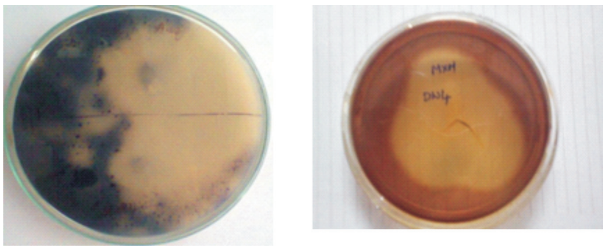

Fig. 4. Zones of xylan hydrolysis by fungal isolates

displayed a minimum of 5mm zone of hydrolysis (Table 1). Pure cultures of 11 strains were maintained at 4 °C on potato dextrose agar slants.

Quantitative Screening of micro-organisms

Eleven strains of xylanolytic fungi with a minimum hydrolytic zone of 4 mm were chosen and submitted to quantitative screening using 1% xylan medium in submerged fermentation. Along with the standard organism *Aspergillus foetidus* MTCC 4898, all 11 selected fungal species were introduced to a fermentation medium containing 1 % birch wood xylan to measure xylanolytic enzyme production. The eleven strains produced large levels of both xylanase and xylosidase. TSV9, ASV10, FSV11, and FSV1 are four strains that have produced considerable amounts of both the enzymes. The xylan hydrolysis index is statistically distinct (Table 1).

Determination of optimum pH and temperature for 1,4 β-endoxylanase and β-xylosidase activity in enzyme assay

When the activity of the two enzymes XYL and BX was measured in the crude enzyme mixture of two selected strains, both enzymes showed maximum activity in the acidic range (pH 4-6), which was determined at 50 °C. The optimal pH for maximum xylanase activity in ASV10 was 5.6, and in TSV9 it was 5.2, with activities of 119 and 108 U/ml, respectively. Xylosidase activity peaked at pH 4 in ASV10 (41 U/ml) and 4.4 in TSV9 (32 U/ml) (Figs. 4.1. and 4.2.).

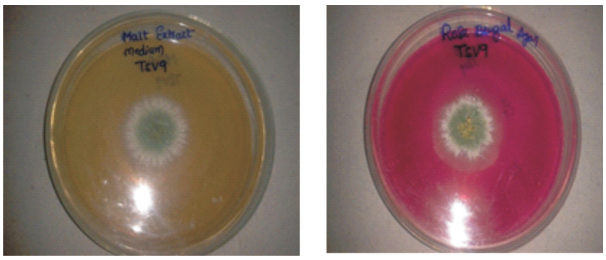

Fig. 5. Colony morphology of TSV9 on MEA and RBA

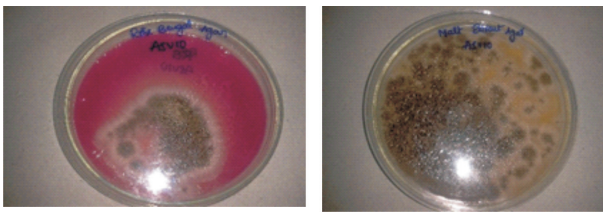

Fig. 6. Colonies of ASV10 on MEA and RBA

At their optimum pH, the optimum temperature for XYL and BX activity was found to be 30 to 80 °C. In strain ASV10, maximum XYL (152 U/ml) activity was observed at 60 °C, whereas maximum BX (56.1 U/ml) activity was found at 65°C. Xylanase from TSV9 (134 U/ml) showed its temperature optimum at 55 °C and xylosidase (45 U/ml) at 60 °C (Fig. 4).

Identification of fungal isolates - TSV 9 and ASV10

Identification using morphological characteristics and microscopic observation

The morphological and microscopic examination of TSV9 and ASV10 was done using Illustrated genera of fungi (Barnette *et al*., 1972) and identified that they belong to Genus *Trichoderma* and *Aspergillus* respectively. The colonies of TSV9 were olive green, the mycelium was septate and 4 µm in diameter. Conidiophores arose from specialized thick-walled enlarged mycelia. It was identified primarily as *Trichoderma sps*. Vegetative mycelium of ASV10 was black, septate and 4 µm in diameter. Conidiophores terminated in a broom like whorl of branches and was Ter verticillate. The conidia were globose. It was identified as *Aspergillus* sp. (Plate VII).

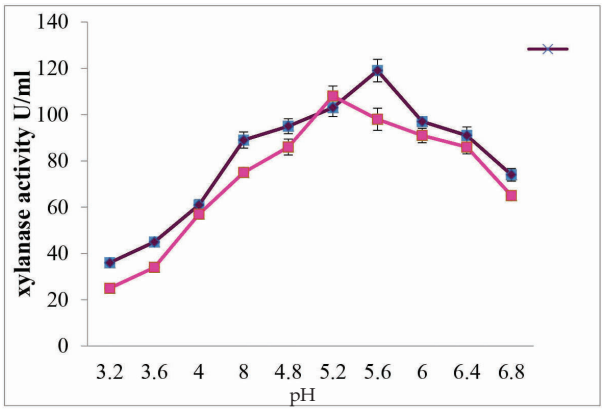

Fig. 7. Determination of optimum pH for XYL

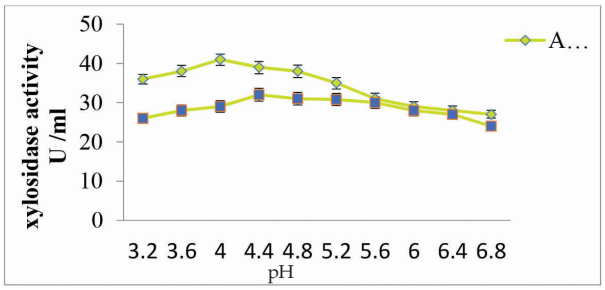

Fig. 8. Determination of optimum pH for BX

Identification using ITS sequence and phylogenetic tree construction

Selected isolates were further characterized to the species level by 18 S rRNA sequencing, which was carried out at Macrogen international corporation,

Table 2. Colony characters of the isolates

S.No	Isolate	Size	Shape	Elevation	Colour
1	FSV1	Small	circular	Flat	White
2	FSV2	Small	irregular	Raised	Yellow to green
3	FSV3	Medium	Circular	convex	White
4	FSV4	Large	Circular	Flat	White
5	FSV5	Medium	Filamentous	Raised	Orange
6	FSV6	Small	rhizoid	Raised	White
7	FSV7	Small	Irregular	flat	Yellow to green
8	FSV8	Medium	Circular	Raised	White
9	TSV9	Medium	Filamentous	Flat	White to green
10	ASV10	Large	Filamentous	Raised	White
11	FSV11	Medium	Irregular	Flat	Green

Table 3. Growth of two selected isolates on different media

Isolate	Media	Colony diameter (mm)	Colony characters		Sporulation
			Surface Colour	Reverse Colour	
TSV9	CDA	36.02±1.07	White	White to green	Moderate
	RBA	28.13±0.86	White to green	Light green	Poor
	MEA	31.4±0.94	white	Yellow to green	moderate
ASV10	CDA	47.00±1.15	White	Light yellow	Heavy
	RBA	47.2±0.98	white	white	Moderate
	MEA	54.6±2.12	White	Cream to yellow	heavy

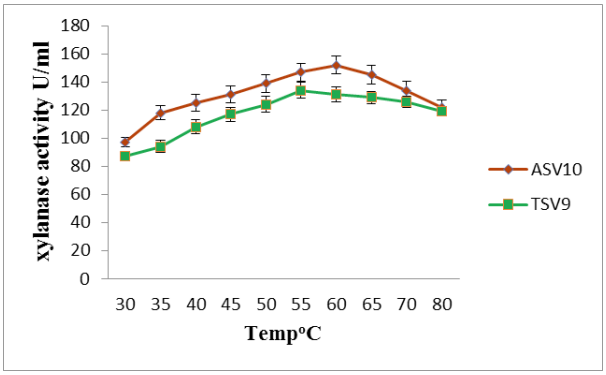

Fig. 9. Determination of optimum temperature for XYL

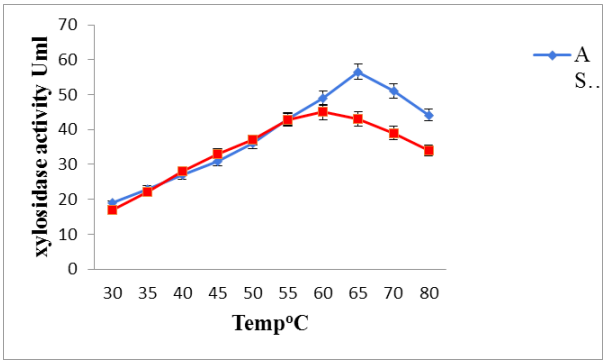

Fig. 10. Determination of optimum temperature for BX

Korea. The 18 S rRNA was amplified by PCR with universal primers
ITS1 Forward primer (5 - TCCGTAGGTGAACCTG CGG - 3)
ITS4 Reverse primer (5 - TCCTCCGCTTATTGA TATGC - 3)

Identification was done by matching the isolate 18 S rRNA sequence with NCBI database and TSV9 showed 99% similarity with *Trichoderma asperellum* and ASV10 showed 99% similarity with *Aspergillus aculeatus*. The phylogenic tree was constructed using MEGA 8.06 software (Kumar *et al.*, 2004) by

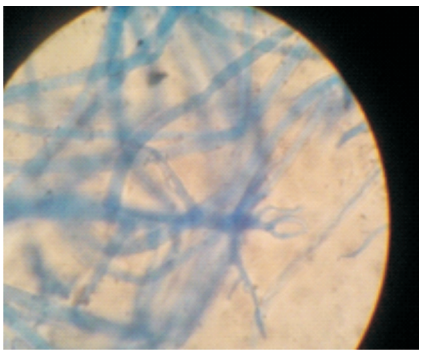

Fig. 11. Compound microscopic image of TSV9

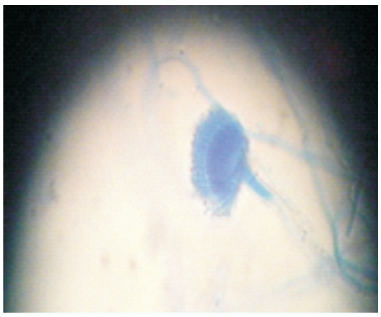

Fig. 12. Compound microscopic image of ASV10

neighbor joining method (Felsenstein, 1985). The sequences of FSV9 and FSV10 were bp 1056 bp length respectively and deposited in the NCBI, Genbank with the accession numbers of KP965728 and KP965729 respectively.

DISCUSSION

Primary screening of xylanolytic fungi was conducted using a variety of samples including

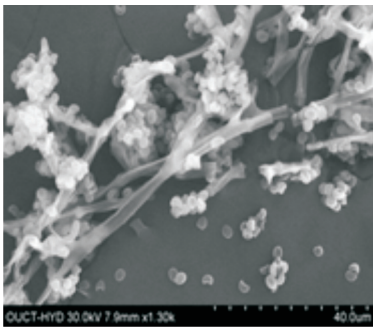

Fig. 13. SEM image of TSV9

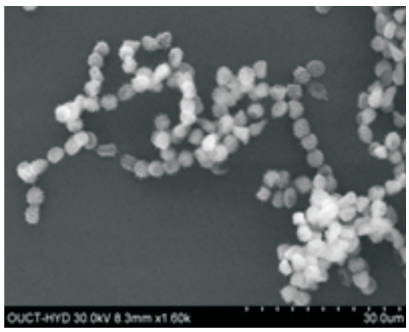

Fig. 14. SEM image of ASV10

Fig. 15. Gel image of the isolates TSV9 and ASV10

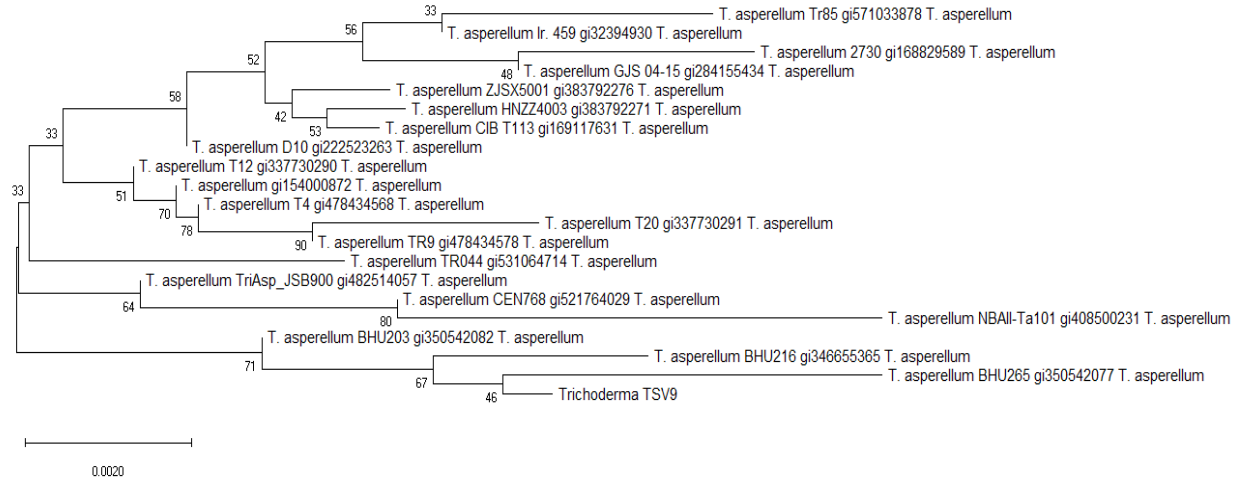

Fig. 16. Phylogenetic tree of nucleotide sequence analysis of rRNA-ITS of fungi TSV9

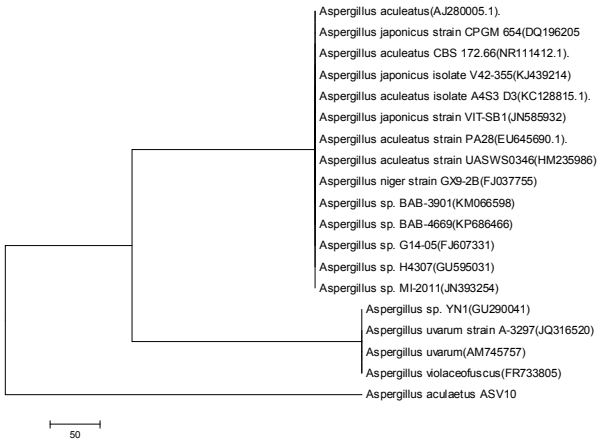

Fig. 17. Phylogenetic tree of nucleotide sequence analysis of rRNA-ITS of fungi ASV10

forest soil (Nallamala forest), agriculture soil, decaying wood barks, lake water, and river water (Palaniswamy *et al.*, 2008). The samples were diluted with sterile saline and inoculated on Sabouraud dextrose *agar* medium containing 1 % birchwood xylan using the serial dilution procedure. The fungal isolates were sub cultured on fresh Sabouraud dextrose *agar* plates containing 1% (w/v) birch wood xylan after incubation. The fungi were sub cultured on Sabouraud dextrose *agar* slants and kept at 4°C. The ratio of the diameter of the clearance zone (CZ) to the diameter of the colony's size was used to determine the xylanolytic activity of each colony. On the basis of hydrolysis zone, eleven fungal strains were chosen and preserved for further studies.

Among 45 xylanolytic fungi, eleven strains have got a minimum of 4 mm hydrolytic zone and further selected and subjected to quantitative screening by submerged fermentation using 1% xylan medium. All the 11 selected fungal organisms were transferred to fermentation medium containing 1% birchwood xylan to determine the xylanolytic enzyme production along with the standard organism *Aspergillus foetidus* MTCC 4898. Altogether the eleven strains produced both xylanase and xylosidase in significant amounts. Four strains (TSV9, ASV10, FSV11and FSV1) have produced significant quantities of both the enzymes. The results showed that the xylan hydrolysis index is statistically different.

Fungal xylanases have a wide variety of commercial applications in various arenas, such as bio bleaching, paper and pulp, (Nisha Sharma, 2017) and also in the lignocellulosic bioconversion to sugars, bio ethanol and other allied chemicals. Xylo oligosaccharides are the only nutraceutical that can be obtained from lignocellulose biomass. Their ingestion selectively induces positive gut microbiota i.e., prebiotic activity and also offers beneficial influence on lowering blood glucose, cholesterol and decrease of pro-carcinogenic enzymes, improvement of mineral absorption and stimulus of immune system (Samanta *et al.*, 2015). Other important applications of xylanases include fruit juice clarification, enhancement of animal feed and processing of fabrics (Dhiman, 2008; Deutschmann, 2012, Juturu, 2014).

According to Barnette *et al.* (1972), colony parameters such as coly size, colony shape, elevation, and colony colour of eleven fungal isolates (FSV1, FSV2, FSV3, FSV4, FSV5, FSV6, FSV7, FSV8, TSV9, ASV10, and FSV11) were investigated. Of all the eleven fungal isolates showed medium

and large colonies except FSV1, FSV2, FSV6 and FSV7. They showed different colony shapes and margins, FSV2, FSV9 and ASV10 showed filamentous type colony whereas FSV1, FSV3, FSV4 showed circular colonies. FSV6 showed rhizoid type colony. The colony colour and elevation were also diverse for all the eleven isolates, colonies of FSV1, FSV4, FSV7, TSV9 and FSV11were flat and rest of other colonies were raised. The colour of the mycelium was white for all the isolates but FSV2 was yellow, FSV7 was orange and FSV11 was green in colour.

As TSV9 and ASV10 among the eleven isolates produced highest yields of both endo xylanase and xylosidase in quantitative screening, they are selected for further studies. Czapek's Dox Agar (CDA), Malt Extract Agar (MEA), and Rose Bengal Agar media were used to study their colony features (RBA). The colony diameter of the fungal isolate TSV9 was 36.02±1.07mm, 28.13±0.86mm, 31.4±0.94mm on CDA, MEA and RBA respectively. And fungal isolate ASV10 showed 47.00±1.15mm, 47.2±0.98mm, 54.6±2.12mm on CDA, MEA and RBA respectively.

The activity of the two enzymes XYL and BX in a crude enzyme combination of two selected strains was evaluated at 50 °C, and both enzymes showed maximum activity in the acidic range (pH 4 to 6). Optimum pH for the maximum xylanase activity was found at pH 5.6 in ASV10, 5.2 in TSV9 with the activities of 119 and 108 U/ml respectively. Maximum activity of Xylosidase was found at pH 4 in ASV10 (41 U/ml), 4.4 in TSV9 (32 U/ml).

The optimum temperature for the XYL and BX activity was determined at 30 to 80°C at their optimum pH. In strain ASV10, maximum XYL (152 U/ml) activity was observed at 60 °C, whereas maximum BX (56.1 U/ml) activity was found at 65 °C. Xylanase from TSV9 (134 U/ml) showed its temperature optimum at 55 °C and xylosidase (45 U/ml) at 60 °C. The optimum pH and temperature values are in accordance with other reports stating fungal xylanase production (César, 2013).

The morphological and microscopic examination of TSV9 and ASV10 was done using Illustrated genera of fungi (Barnette *et al.*, 1972) and identified that they belong to Genus *Trichoderma* and *Aspergillus* respectively. The colonies of TSV9 were olive green, the mycelium was septate and 4 μm in diameter. Conidiophores arose from specialized thick-walled enlarged mycelia. It was identified primarily as *Trichoderma sps.* Vegetative mycelium of ASV10 was black, septate and 4 μm in diameter. Conidiophores terminated in a broom like whorl of branches and was Ter verticillate. The conidia were globose. It was identified as *Aspergillus sp.* (Plate VII).

Selected isolates TSV9 and ASV 10 were further characterized by 18 S rRNA sequencing which was carried out at Macrogen international corporation, Korea. Then the 18 S rRNA sequence was matched with NCBI database and FSV9 showed 99% similarity with *Trichoderma asperellum* and FSV10 showed 99% similarity with *Aspergillus aculeatus*. TSV9 had a 584-bp sequence that was deposited in the NCBI's Genbank with the accession number KP965728. C.J. Wijesinghe *et al*. (2010) also identified 3 fungal isolates from soil sample as *T. asperellum* by 18 S r RNA gene sequence analysis that are in the range of 570-600bp length followed by phylogenetic tree construction. ASV10 had a 1056-bp sequence that was deposited in the NCBI Gene Bank under the accession number KP965729. Daiani *et al.* (2011) identified a fungus from soil as *A. aculeatus* 01201 by 18s rRNA analysis. The phylogenic trees of the TSV9 and ASV10 were constructed using MEGA 8.06 software (Kumar *et al.,* 2004) by neighbor joining method (Felsenstein, 1985).

CONCLUSION

Two important xylanolytic fungi were screened from various fungal isolates and were identified as *Aspergillus aculeatus* ASV10and *Trichoderma asperellum* TSV9 *using* molecular characterization. The two selected fungal isolates produced significant amounts of two important xylanolytic enzymes xylanase and xylosidase and further can be improved by statistical optimization techniques. The efficient inhouse produced Xylanolytic enzyme mixture can be utilized for the effective utilization of biomass for the production of bioethanol.

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Competing interests

The authors declare that they have no competing interests.

REFERENCES

Ahring, B.K., Licht, D., Schmidt, A.S., Sommer, P. and

Thomsen, A.B. 1999. Production of ethanol from wet oxidised wheat straw by *Thermoanaero bactermathranii*. *Bioresource Technology.* 68(1): 3-9.

Bailey, M. J., Biely, P. and Bailey Poutanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology.* 23(3): 257-270.

Barnett, J. A., Delaney, M. A., Jones, E., Magson, A. B. and Winch, B. 1972. The numbers of yeasts associated with wine grapes of Bordeaux. *Archiv für Mikrobiologie.* 83(1) : 52-55.

Bocchini, D.A., Oliveira, O.M.M.F., Gomes, E. and Da Silva, R. 2005. Use of sugar cane bagasse and grass hydrolysates as carbon sources for xylanase production by *Bacillus circulans* D1 in submerged fermentation. *Process Biochemistry.* 40: 3653–3659.

Cesar, C. R. F., Temer, B., Sarto, C., Silva Júnior, F. G. and Carmona, E. C. 2013. Xylanase and ß-xylosidase from Penicillium janczewskii: Production, physico-chemical properties, and application of the crude extract to pulp biobleaching. *BioRes.* 8(1): 1292-1305.

Deutschmann, R. and Dekker, R. F. 2012. From plant biomass to bio-based chemicals: latest developments in xylan research. *Biotechnology Advances.* 30(6): 1627-1640.

Dhiman, S. S., Sharma, J. and Battan, B. 2008. Industrial applications and future prospects of microbial xylanases: a review. *Bio Resources.* 3(4): 1377-1402.

Gaspar, A., Cosson, T., Roques, C. and Thonart, P. 1997. Study on the production of a xylanolytic complex from *Penicillium canescens* 10-10c. *Applied Biochemistry and Biotechnology.* 67: 57-70.

Golubev, Alexander. 2004. Crystal Structure of alpha; -Galactosidase from Trichoderma reesei and its Complex with Galactose: Implications for Catalytic Mechanism. *Journal of Molecular Biology.* 339: 413-422.

Haq, Ikram, Ayesha, Khan, Butt, Waseem, Ali, Sikander, and Qadeer, M. 2002. Effect of Carbon and Nitrogen Sources on Xylanase Production by Mutant Strain of *Aspergillus niger* GCBMX-45". *Online Journal of Biological Sciences* (Pakistan).

Juturu, V. and Wu, J. C. 2012. Microbial xylanases: engineering, production and industrial applications. *Biotechnology Advances.* 30(6): 1219-1227.

Kumar, A. K. and Sharma, S. 2017. Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. *Bioresour Bioprocess.* 4: 7.

Kuno, A., Kaneko, S., Ohtsuki, H., Ito, S., Fujimoto, Z., Mizuno, H. and Hayashi, K. 2000. Novel sugar-binding specificity of the type XIII xylan-binding domain of a family F/10 xylanase from *Streptomyces olivaceoviridis* E-86". *FEBS Letters.* 482(3): 231-236.

Liu, JH., Selinger, L.B. and Cheng, KJ. 1997. Plant seed oil-bodies as an immobilization matrix for a recombinant xylanase from the rumen fungus Neocallimastixpatriciarum. *Molecular Breeding.* 3: 463–470.

Maheswari, M. and Chandra, T.S. 2000. Production and potential applications of a xylanase from a new strain of *Streptomyces cuspidosporus*. *World Journal of Microbiology and Biotechnology.* 16: 257-263.

Michelin, M., Polizeli, M. D. L., Ruzene, D. S., Silva, D. P., Ruiz, H. A., Vicente, A. A. and Teixeira, J. A. 2012. Production of xylanase and b-xylosidase from autohydrolysis liquor of corncob using two fungal strains. *Bioprocess and Biosystems Engineering.* 35(7): 1185-1192.

Motta, Fernanda, C. P. Andrade, Cristiane, and Santana, Maria Helena, 2013. A Review of Xylanase Production by the Fermentation of Xylan: Classification, Characterization and Applications. *Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications, and Commercialization.* 1: 251-275.

Palaniswamy, M., Pradeep, B. V., Sathya, R. and Angayarkanni, J. Isolation, identification and screening of potential xylanolytic enzyme from litter degrading fungi. *African Journal of Biotechnology.* 7: 12.

Samanta, A. K., Jayapal, N., Jayaram, C., Roy, S., Kolte, A. P., Senani, S. and Sridhar, M. 2015. Xylooligosaccharides as prebiotics from agricultural by-products: Production and applications". Bioactive *Carbohydrates and Dietary Fibre.* 5(1): 62-71.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1982. *Molecular Cloning: A Laboratory Manual*". Cold Spring Harbor Laboratory Press.

Silva, D.M., Batista, L.R., Rezende, E.F., Fungaro, M.H., Sartori, D. and Alves, E. Identification of fungi of the genus Aspergillus section nigri using polyphasic taxonomy. *Brazilian Journal of Microbiology.* 42.

Siv, Subramaniyan and Prema, P. 2000. Cellulase-free xylanases from Bacillus and other microorganisms. *FEMS Microbiology Letters.* 183, 1-7.

Soares, H.C.G. 1999. Xylanase screening by iodine staining: an artifact. *Enzym Microb Technol.* 9:214–216.

Subramaniyan, S., Prema, P. and Ramakrishna, V. 1997. Isolation and screening for alkaline thermostable Xylanases. *J. Basic Microb.* 37: 431–437.

Wijesinghe, C. J., Wijeratnam, R. W., Samarasekara, J. K. R. R. and Wijesundera, R. L. C. 2010. Biological control of Thielaviopsis paradoxa on pineapple by an isolate of *Trichoderma asperellum*. *Biological Control.* 53(3): 285-290.

Wong, Ken & Tan, L. and Saddler, Jack. 1988. Multiplicity of beta-1,4-xylanase in microorganisms: functions and applications. *Microbiological Reviews.* 52: 305-17.

Yamaki, S. and Kakiuchi, S. 1979. Changes in hemicellulose degrading enzymes during devlopment and ripening of Japanese pear fruit. *Plant Cell Physio.* 20: 301-306.

Yarnura, 1., Koga, T., Matsumoto, T. and Kato, T. 1997. Purification and some properties of endo-1,4-D-xylanse from a fresh water mollusc, Pomacea insularus (de Ordigny). *Biosc. Biotech. Biochem.* 61 (4): z 615-620.