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Enzymatic Potential of indigenous Fungal Soil Isolates from Lower Shivalik Ranges of Chandigarh Capital Region (CCR)

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Abstract

The industrial use of fungi has been well-experienced since days past long. Many enzymes produced by fungi have on the point biotechnological applications in several industrial fields. So fungal enzymes have been used for a range of purposes across many different industries. In present study, a total of 35 fungal species belonging to 8 different genera were isolated using soil-dilution technique from soil samples collected from different regions of lower Shivalik ranges of Chandigarh Capital Region with aim to isolate the organisms with maximum number of enzyme producing capability. Identification of the isolates was made with the help of standard manuals/keys of fungi. All the 35 isolates were examined qualitatively and quantitatively for their ability to produce lignocellulolytic enzymes (Cellulase, Xylanase, Laccase, Mannanase, and Pectinase). This study contributes to catalogue soil fungi isolated from lower Shivalik Foothills of Chandigarh Capital Region, India and provides an important and additional information to support future research about the industrial potential of these organisms that may produce enzymes.

Keywords: *Chandigarh, fungal enzymes, isolation*

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INTRODUCTION

Soil is the real hotspot for the isolation of modernly critical enzyme producing fungi having appropriate growth conditions and nutrient composition of the plants tissues that supports the fungal growth. The 95% of the plant tissues are composed of carbon, nitrogen, phosphorous, and sulfur. The role of fungi in the soil is an extremely complex one and is fundamental to the soil ecosystem. Fungi are a critical segment of the soil microbiota regularly constituting a greater amount of the soil biomass than microscopic organisms, which is bacteria, contingent upon soil depth and supplement conditions [1].

The saprobic organisms speak to the biggest extent of fungal species in soil and they play out a critical part in the disintegration of plant basic polymers, for example, cellulose, hemicellulose, and lignin, in this manner adding to the support of the universal carbon cycle. Microorganisms are most prominent source of enzymes. For production of enzymes of industrial use, the isolation and characterization of new encouraging strains from newer biotopes using low-cost carbon and nitrogen source should be a continuous process. The role of many enzymes has been known since time immemorial and their use was related with the historical backdrop of old Greece, where they were utilizing microbial enzymes in bakery, brewing, liquor production, cheddar making, and so on [2]. These days enzymes are utilized extensively in the food (pectinase, protease, cellulose, and oxidoreductase); textile (amylase, cellulose, oxidoreductase); paper (xylanase, oxido-reductase, and lipase); cleansers (protease, lipase, cellulose, amylase, and oxidoreductase); and leather (protease and lipase) industries. The primary classes of enzymes offering quick application are the hydrolytic enzymes [3].

Fungi which are ubiquitous in nature are known for extensive production of extracellular enzymes and have been perceived as a wellspring of newer enzymes with novel attributes [4, 5, 6]. Along these lines, considering the possibilities of utilizing lignocellulolytic enzymes in industries and lignocellulolytic waste management, it

becomes inevitable to acquire fungal isolates that could produce exceptionally dynamic enzymes. Therefore, in present study, the lower Shivalik Ranges of Chandigarh Capital Region (CCR) which embodies a great diversity in terms of topography, climatic conditions, floral, and faunal wealth have been explored extensively for the collection of soil samples from the various diverse habitats of this region for isolation of fungal isolates with lignocellulolytic enzyme potential. The study reports in detail the quantitative and qualitative approaches to screen out the lignocellulolytic potential of successfully isolated fungal cultures.

MATERIALS AND METHODS

Sampling

Soil samples from 6- to 10-inch depth were collected aseptically from different parts of lower Shivalik ranges of CCR (India) using sterile zip-lock covers. Each sample bag was labeled appropriately by indicating the site of collection and date and brought to laboratory for further studies.

Isolation of Fungi From the Samples

The soil dilution method [7] on media such as Potato Dextrose Agar (PDA) used as isolation techniques. Soil dilutions were made by suspending 1 g of soil of each sample in 10 ml of sterile distilled water. Dilutions of 10^{-3}, 10^{-4}, and 10^{-5} were used to isolate fungi in order to avoid overcrowding of the fungal colonies. A 0.1 ml of the suspension of each concentration was added to sterile Petri dishes, in triplicates of each dilution, containing sterile PDA medium, spread with a glass spreader and an antibacterial was added to the medium for preventing bacterial growth, before pouring into Petri plates. The plates were, then, incubated at 28 ± 2^{o}C for 3 to 5 days. Organisms were easily isolated because they formed surface colonies that were well dispersed, particularly at higher dilutions.

Purification of Isolated Fungi

The isolated fungi were purified by point inoculating them on plates containing PDA medium. The fungi were purified by repeated point inoculation. The cultures were subcultured on PDA slants and allowed to grow for a period of 5 to 7 days subsequently stored at 4°C as stock cultures. Usually, working as well as stock cultures are maintained and the working cultures were transferred to fresh PDA slants at regular intervals of 3 months.

Identification of Fungi Using Lactophenol Cotton Blue

Lactophenol cotton blue was used for staining and visualizing the fungal structure by putting a drop on a clean slide of it. With the help of a sterilized needle transfer a small part of the fungus preferably with spores and spores bearing structure. The slide were precisely prepared and seen under low- and high-magnification power of microscope [8, 9].

Enzymatic Screening of Isolated Fungal Samples

Qualitative and quantitative estimation of lignocellulolytic enzymes were done by plate assay method and standard assay protocol, respectively. All 35 fungal isolates were screened for cellulase, xylanase, laccase, mannanase, and pectinase activity.

Cellulase Activity

A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye, Cellulase activity was tested using 0.5% carboxymethylcellulose in PDA agar media, which is a substrate for its activity. The fungal isolates were grown on the above media and incubated at 28^{o}C for 3 to 5 days. The plates were flooded with 0.1% Congo red solution and observed for zone of clearance [10].

Quantitative estimation of cellulase was performed by DNS method [11] with pectin as substrate at 540 nm. The concentration of sugar in the sample was calculated with reference to glucose.

Xylanase Activity

Xylanase activity (qualitatively) was tested using 0.25% xylan in PDA agar media, which is a substrate for its activity. The fungal isolates were grown on the above media and incubated at 28^{o}C for 3 to 5 days. The plates were flooded with iodine solution for observing the zone of clearance.

The xylanase assay was done [12]. For this, 1.80 ml of 0.75% beech wood xylan was

prepared in 0.05 M Na-citrate buffer (pH 5.0) which acted as substrate solution. 200 µl of enzyme was added to it and were incubated at 50°C for 5 minutes. The reaction was terminated by the addition of 3.0 ml of DNSA and the contents were boiled for 15 minutes. After cooling, absorbance was measured at 540 nm. The amount of xylose liberated was quantified using xylose (0.50 mg/ml) as standard. One International unit of xylanase activity (IU) is stated as the measure of µmol of xylose liberated by 1 ml enzyme per minute under standard assay conditions.

Laccase Activity

All the isolated fungal cultures were inoculated on petri plates containing PDA medium with 0.01% v/v guiacol (added after autoclaving) and plates were incubated at 28ºC for 3 to 5 days and observed for development of reddish brown colored zone [13].

Quantitative estimation of laccase activity was done as per standard method in which ABTS (Sigma) was used as the substrate and absorbance was taken at 420 nm using spectrophotometer over 10 min, using $\varepsilon = 3.6 \times 10^4\ cm^{-1}\ M^{-1}$ [14].

Pectinase Activity

For the pectinase activity, all the isolated fungal cultures were inoculated on petriplates containing PDA medium with 0.1% Pectin as a substrate and the plates were then incubated at 28ºC for 3 to 5 days. The fungal isolates were grown on the above media and incubated at 28ºC for 3 to 5 days. After the colonies reached around 3 to 4 mm iodine solution was added to detect the clear zone.

Pectinase was quantified with DNSA method [11] with pectin as substrate at 540 nm using glucose as a standard for the releasing sugar.

Mannanase Activity

All the isolated fungal cultures were inoculated on specific substrate (0.5% Guar gum) containing petriplates containing PDA and then incubated at 28ºC for 3 to 5 days for visualizing the mannanase activity.

The fungal isolates were grown on the above media and incubated at 28ºC for 3 to 5 days. The plates were flooded with Congo red for observing the zone of clearance.

Mannanase activitiy were assayed by using LBG as substrate. For reducing sugars determination a mannose standard curve was used. One unit of enzymatic activity is defined as the amount of enzyme releasing 1 µmol of corresponding reducing sugar per milliliter per minute under the described assay conditions [11].

RESULTS AND DISCUSSION

Isolation and Identification of Fungal Organisms

There are over 1.5 million fungal species distributed widely throughout the globe [15]. Fungal diversity of soil depends on a large number of factors of the soil such as pH, organic content, and moisture [16, 17]. A total of 10 different soil samples of the most diverse regions of lower Shivalik ranges of CCR (Table 1) were collected.

Over the span of study, Deuteromycotina discovered as prevailing group. An aggregate of 35 diverse fungal species belonging to 8 distinct genera were isolated from collected soil samples from various areas as said above on PDA medium (Table 2).

Table 1: *Biodiverse Soil Sampling Sites of Lower Shivalik Ranges of CCR.*

Sr. No.	Soil sample	Biodiverse sites
1.	Sample 1	Rose Garden, Sector 16
2.	Sample 2	Panjab University Rose Garden, Sector 14
3.	Sample 3	Campus Department of Microbiology, Sector 25
4.	Sample 4	Sukhna Lake Backside
5.	Sample 5	Patiali Rao
6.	Sample 6	Morni Hills
7.	Sample 7	Sukhna wild life Sanctuary
8.	Sample 8	Dhanas Forest
9.	Sample 9	Termite Soil (Leisure Valley Sector 10)
10.	Sample 10	Kansal Forest
11.	Sample 11	Saketri forest
12.	Sample 12	Near Nada Sahib Gurudwara Sahib
13.	Sample 13	Pinjore Garden, Panchkula
14.	Sample 14	Mansa Devi Forest
15.	Sample 15	Kaimbwala Forest
16.	Sample 16	Nepli Forest

Table 2: *Fungal Cultures Isolated From Different Soil Samples.*

Sr. No.	Isolate name	Tentative identification
1.	MG1	*Aspergillus*
2.	MG2	*Fusarium*
3.	MG3	Unidentified
4.	MU2	*Mucor*
5.	MD1	*Aspergillus*
6.	MD2	*Penicillium*
7.	MD3	*Aspergillus*
8.	MD4	*Penicillium*
9.	MS1	*Alternaria*
10.	MS2	*Rhizopus*
11.	MS3	*Mucor*
12.	MS4	*Penicillium*
13.	MCR1	*Aspergillus*
14.	MCR2	*Penicillium*
15.	MCR3	*Aspergillus*
16.	MCR4	*Mucor*
17.	MCR5	Unidentified
18.	MCR6	*Fusarium*
19.	MLBR1	*Aspergillus*
20.	MLBR2	*Rhizopus*
21.	MLBR3	*Aspergillus*
22.	MC1	*Aspergillus*
23.	MC2	Unidentified
24.	MC3	*Penicillium*
25.	RDF1	*Curvularia*
26.	RDF2	*Trichoderma*
27.	RDF3	*Aspergillus*
28.	RDF4	*Rhizopus*
29.	RT1	*Aspergillus*
30.	RT2	Unidentified
31.	RT3	*Alternaria*
32.	RT4	*Penicillium*
33.	RK1	*Trichoderma*
34.	RK2	*Fusarium*
35.	RK3	*Aspergillus*

Pure cultures of all the obtained fungal isolates were maintained on PDA medium and preserved in glycerol at 4ºC. The tentative identification was done. Among the fungal isolates major were belonging to Aspergillus species (11) followed by Penicillium sp. (6) Figure 1. The class Aspergillus is the most considered genra. Among researchers taking a shot at Aspergillus, there is a great interest in their biotechnological potential. Besides producing various valuable extracellular enzymes and natural acids, these molds additionally deliver auxiliary metabolites of significance in biotechnology. Likewise, Pencillium species are of great significance in biotechnology for their potential to secrete extracellular enzymes (e.g., xylanases and cellulases) and pigments, which are utilized as natural colorants.

Therefore, both fungi are of great importance, not only in terms of biotechnological applications but also for economic reasons, due to their metabolic properties [18]. Similar work has been reported by other scientists that Aspergillus spp. and Penicillum spp. were the two common soil fungi in three different districts at Allahabad, Mirzapur, and Varanasi, of Uttar Pradesh in India [19]. In Edrine, the flora of Penicillium and Aspergillus in different habitat soils was studies and it was found that 23 species and 2 varieties were belonging to Aspergillus and 16 species belonging to Penicillium [20].

Screening for Various Enzymes

All the samples were screened qualitatively and quantitatively for various lignocellulolytic enzyme productions on their particular substrates. Each isolate was able to produce one or the other extracellular enzymes. The results shows out of 35 isolates 13 were able to produce mannanase qualitatively as well as quantitatively more among all the other tested enzymes. This was followed by pectinase which was produced by 12 isolates. Indeed, 10 fungal isolates were able to produce cellulases qualitatively; however, 18 were able to produce cellulase quantitatively that's why secondary screening is a reliable method as primary screening is only the preliminary one. Furthermore, 8 isolates were found producing xylanase qualitatively, while 13 quantitatively (Table 3).

Cellulase Activity

In qualitative assays, cellulase activity was shown by 10 isolates as they were giving halo zone. Gram iodine plate assay was the most widely used method for the screening of cellulase activities [21]. However, plate-screening methods using dyes are not quantitative method for the poor correlation between enzyme activity and halo size [22]. Therefore, we go for quantitative screening which resulted in 18 isolates were cellulase postitive viz. MG1, MD1, MD2, MD4, MS1, MS3, MCR1, MCR5, MLBR1, RDF1, RDF2, RDF3, RT1, RT2, RT3, RT4, RK3 (Figure 2).

The MCR5 (Unidentified) isolate was found producing the maximum quantity of cellulase enzyme 4.5 ± 0.26 U/ml followed by RT3 (*Alternaria* sp.) with the activity of 1.14 ± 0.04 U/ml.

Xylanase Activity

All the 35 fungal isolates were screened for Xylanolytic activity out of which 8 were found xylanase positive qualitatively, while 13 were found quantitatively (Figure 3). *Rhizopus* sp. (MLBR2) was found to possess maximum activity, that is, 5.6 ± 0.67 U/ml followed by *Aspergillus* sp. (3.4 ± 020 U/ml). Xylanase positive colonies by using 0.1% Congo red, followed by washing with 1M NaCl [23].

Mannanase Activity

The fungal isolates were evaluated for their ability to produce mannanase. Mannanase is a group of hydrolytic enzymes acting together in degrading hemicellulose. After xylanases, mannanases are the second most important enzymes for the hydrolysis of hemicelluloses. This enzyme actually plays a very important role in natural biodegradation process. Fungi are well-known agents of decomposition of organic matter [24] and are very good source of enzymes capable of degrading natural polymeric compounds such as cellulose. Based on the results of present investigation, 13 fungal species were capable of producing mannanase qualitatively as well quantitatively with the maximum activity of 5.56 ± 1.30 U/ml (Figure 4).

Pectinase Assay

Out of 35 isolates tested for their abilities of producing pectinase on soild medium as well as thorough standard assay, only 12 isolates were found producing pectinase with MS2 (Rhizopus) giving the maximum activity, that is, 4.5 ± 0.22 U/ml (Figure 5). Pectinase is produced by number of fungi including *Aspergillus* sp., *Botrytis cinerea*, *Fusarium moniliforme*, *Rhizoctonia solani*, *Rhizopus stolonifer*, *Trichoderma* sp., *Neurospora crassa* but Aspergillus is the major producer [25].

CONCLUSION

Soil is considered as the wealthiest and the most diverse hotspot for an assortment of microorganisms which produce never-ending metabolites and enzymes critical for applications in different industrial processes. Present study carried out for an effort to understand the soil fungal diversity of lower Shivalik Region of CCR and for evaluation their various enzymatic potential to be used in various biotechnological and industrial purposes. This study strives to unravel the immense cellulose and lignin-degrading potential of fungal species from indigenous flora of Chandigarh region and also to make this data available to promote future research. Thirty five indigenous fungal species were isolated and screened for extracellular oxidative and hydrolytic enzymes. The most promising results obtained with these lignocellulolytic fungal strains led to exploring the hidden enzymatic potentials of various fungal species.

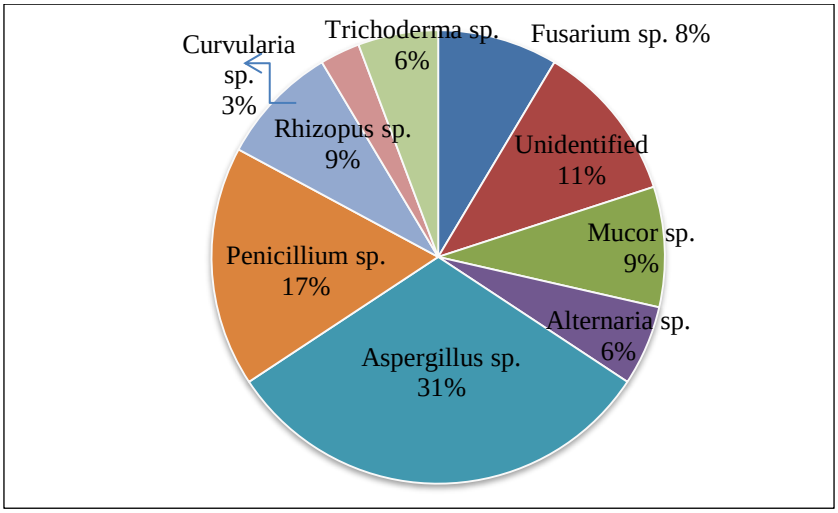

Fig. 1: *Pie chart representing the percentage occurrence of the diversity of different fungal flora in the soil of Lower Shivalik Region of CCR.*

Table 3: *Primary and Secondary Screening for Various Enzyme Isolated by the Fungal Cultures.*

Sr. No.	Isolate	Cellulase	Cellulase activity (U/ml)	Xylanase	Xylanse activity (U/ml)	Laccase	Laccase activity (U/ml)	Mannanase	Mannase activity (U/ml)	Pectinase	Pectinase activity (U/ml)
1.	MG1	–	0.23±0.05	+	3.4 ± 020	–	Nil	–	Nil	+	2.45 ± 0.13
2.	MG2	–	Nil	–	Nil	–	Nil	+	1.23 ± 0.30	–	Nil
3.	MG3	–	Nil	–	Nil	–	Nil	+	3.6 ± 0.67	–	Nil
4.	MU2	–	0.12 ± 0.06	–	Nil	–	Nil	–	Nil	–	Nil
5.	MD1	–	0.45 ± 0.05	–	0.34 ± 0.12	–	Nil	–	Nil	–	Nil
6.	MD2	–	0.03 ± 0.01	–	Nil	–	Nil	–	Nil	–	Nil
7.	MD3	–	Nil	–	Nil	–	Nil	–	Nil	–	Nil
8.	MD4	–	0.90 ± 0.40	–	Nil	–	Nil	+	0.45 ± 0.01	–	Nil
9.	MS1	–	0.12 ± 0.03	–	Nil	–	Nil	+	2.6 ± 0.09	+	1.34 ± 0.09
10.	MS2	–	Nil	–	0.45 ± 0.04	–	Nil	–	Nil	+	4.5 ± 0.22
11.	MS3	+	1.23 ± 0.45	–	Nil	–	Nil	–	Nil	+	2.56 ± 0.56
12.	MS4	–	Nil	–	0.23 ± 0.02	–	Nil	+	1.24 ± 0.13	–	Nil
13.	MCR1	–	0.34 ± 0.03	–	Nil	–	Nil	–	Nil	+	0.69 ± 0.05
14.	MCR2	–	Nil	–	0.03 ± 0.01	–	Nil	–	Nil	+	1.98 ± 0.34
15.	MCR3	–	Nil	+	1.23 ± 0.54	–	Nil	–	Nil	+	2.34 ± 0.23
16.	MCR4	+	Nil	–	Nil	–	Nil	–	Nil	–	Nil
17.	MCR5	+	4.5 ± 0.26	–	0.45 ± 0.09	–	Nil	–	Nil	–	Nil
18.	MCR6	–	0	–	Nil	–	Nil	–	Nil	–	Nil
19.	MLBR1	–	0.13 ± 0.06	–	Nil	–	Nil	+	1.34 ± 0.45	–	Nil
20.	MLBR2	–	Nil	+	5.6 ± 0.67	–	Nil	–	Nil	+	3.4 ± 1.2
21.	MLBR3	–	Nil	–	Nil	–	Nil	–	Nil	–	Nil
22.	MC1	–	Nil	–	Nil	–	Nil	–	Nil	+	0.23 ± 0.01
23.	MC2	–	Nil	–	Nil	–	Nil	–	Nil	–	Nil
24.	MC3	–	Nil	–	Nil	–	Nil	–	Nil	–	Nil
25.	RDF1	+	1.23 ± 0.09	+	2.45 ± 0.43	–	Nil	+	1.23 ± 0.04	–	Nil
26.	RDF2	+	2.67 ± 0.45	–	Nil	–	Nil	+	5.56 ± 1.30	–	Nil
27.	RDF3	+	1.21 ± 0.23	+	0.34 ± 0.03	–	Nil	+	2.3 ± 0.90	–	Nil
28.	RDF4	–	Nil	–	Nil	–	Nil	–	Nil	–	Nil
29.	RT1	+	0.89 ± 0.03	+	1.02 ± 0.04	–	Nil	+	1.21 ± 0.08	+	1.23 ± 0.05
30.	RT2	+	0.16 ± 0.01	–	Nil	–	Nil	+	3.8 ± 0.76	+	2.1 ± 0.23
31.	RT3	–	2.8 ± 0.34	–	Nil	–	Nil	–	Nil	–	Nil
32.	RT4	+	1.34 ± 0.07	+	2.7 ± 0.07	–	Nil	+	1.80 ± 0.04	+	2.9 ± 0.38
33.	RK1	–	Nil	+	2.1 ± 0.10	–	Nil	–	Nil	–	Nil
34.	RK2	–	Nil	–	Nil	–	Nil	–	Nil	–	Nil
35.	RK3	+	1.14 ± 0.04	–	Nil	–	Nil	+	3.2 ± 0.05	–	Nil

Note. The symbol (+) denotes results are positive and showing enzyme activity. The symbol (–) denotes results are negative and showing no enzyme activity. Values are M ± SD of triplicates. P < .05.

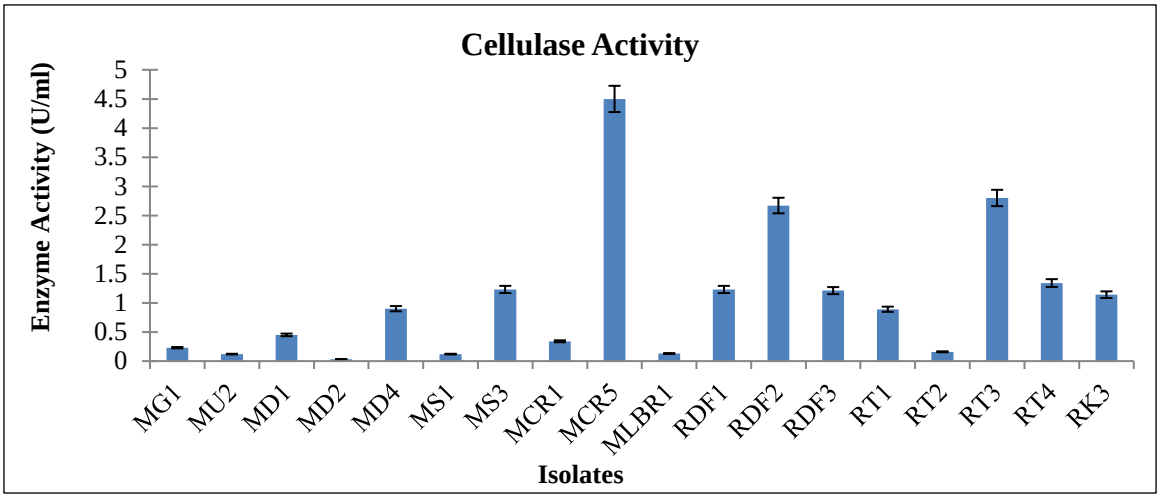

Fig. 2: *Cellulase Activity (U/ml) by the Various Isolated Fungal Cultures.*
Note. Values are M ± SD of triplicates. Error bars shows the deviation from mean value.

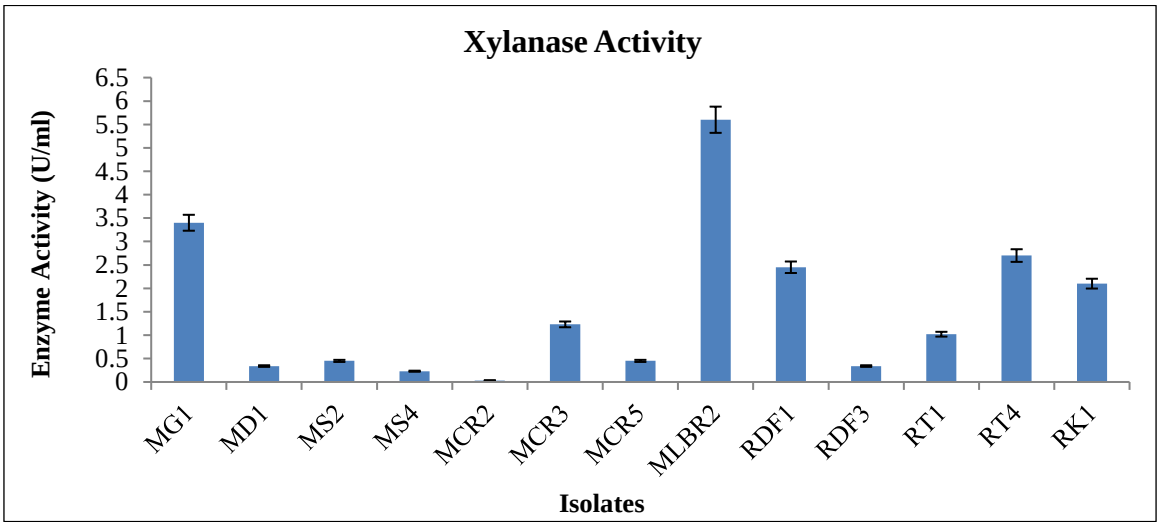

Fig. 3: *Xylanase Activity by Different Fungal Isolates.*
Note. Values are M ± SD of triplicates. Error bars shows the deviation from mean value.

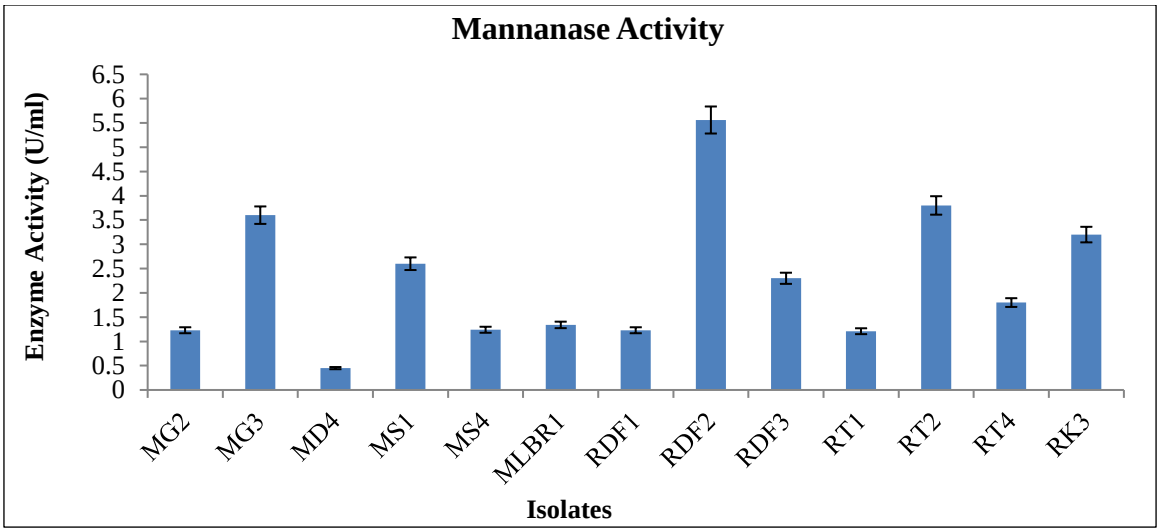

Fig. 4: *Mannanase Activity by Different Fungal Isolates.*
Note. Values are M ± SD of triplicates. Error bars shows the deviation from mean value.

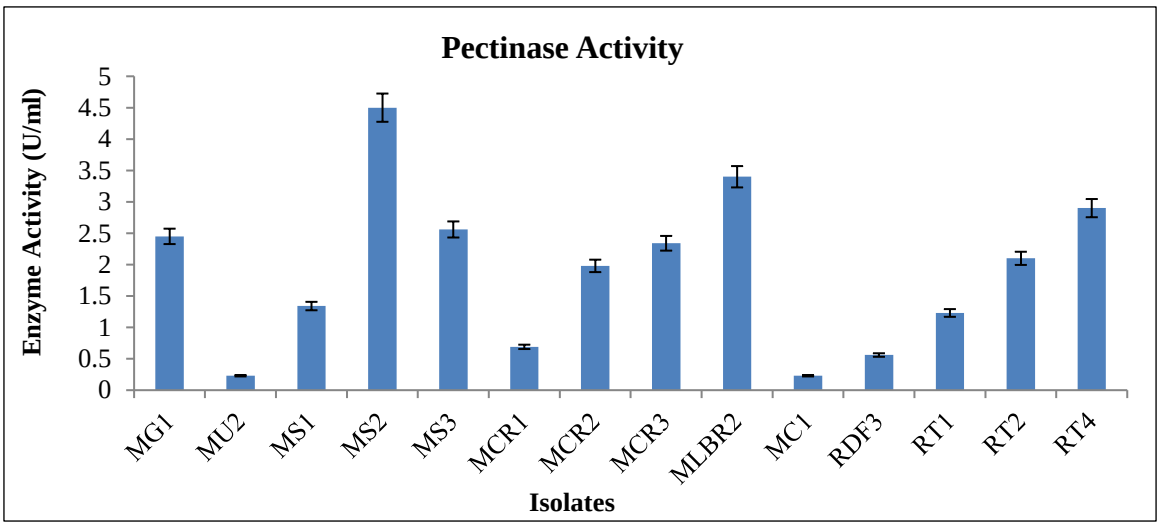

Fig. 5: *Pectinase Activity by Different Fungal Isolates.*
Note. Values are M ± SD of triplicates. Error bars shows the deviation from mean value.

CONFLICTS OF INTEREST

The author(s) declare no conflicts of interest.

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