Isolation and screening of thermophilic and thermotolerant fungi for production of hemicellulases from heated environments

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ABSTRACT

Thermostable hemicellulases have potential to improve the quality of products of various industries including pulp and paper, food and feed, textile, etc. This study was aimed to isolate, screen, and identify potential xylanase and mannanase producers from heated environments. Sixty-eight thermophilic and thermotolerant fungi were isolated from various self-heated habitats based on their ability to grow at 45°C. With the aid of cultural and morphological observations, the fungi were identified and grouped into 19 fungal species. The enzyme production was evaluated by cultivating isolated fungi in submerged fermentation using wheat bran as carbon source. Screening of these isolates for hydrolysis of xylan and mannan was confirmed by Congo red plate assay method followed by assessment of xylanase and mannanase activity. Based on experimental analysis, we have found that all the isolates have exhibited xylanase activity, whereas only 22 isolates have found positive for mannanase activity. The highest xylanase and mannanase production was obtained by the cultivation of Malbranchea cinnamomea NFCCI 3724 (242 and 27 nkat/ml) followed by Melanocarpus albomyces (195 and 24 nkat/ml), Aspergillus terreus (165 and 21 nkat/ml), and Myceliophthora thermophila NFCCI 3725 (130 and 18 nkat/ml), respectively.

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Introduction

Thermophilic fungi are ubiquitously occurring in natural habitat. The changing environmental conditions are important from both ecological and industrial point of view (Johri et al. 1999; Singh and Satyanarayana, 2009). The cosmopolitan occurrence of microorganisms in vast variety of extreme physical environments such as temperature, pH, and pressure (Maheshwari et al. 1987; Satyanarayana and Singh 2009). Temperature is one of the most important ecological key factors which affects the survival and growth of microorganisms (Maheshwari et al. 2000). Thermophilic fungi are defined by Cooney and Emerson (1964), which require optimum temperature as 45°C for their growth. The heterogenous group of thermophilic and thermotolerant fungi is distinguished on the basis of their minimum and maximum growth temperature (Cooney & Emerson 1964). Thermophilic fungi have a growth temperature maximum at or above 50°C and a minimum at or above 20°C. On the basis of temperature limit, a truly thermophilic fungus has been considered to show no growth below 20°C but growth well above 50°C. These fungi have the ability to produce novel metabolites or enzymes (Maheshwari et al. 2000). Hemicellulose is the second most abundant heteropolysaccharide after cellulose which is composed of pentoses (xylose and arabinose), hexoses (glucose, mannose, and galactose), and sugar acids. The dominant components of hemicellulose are the xylan and mannan present in higher plants. In nature, xylan content in hardwood is about 25–30% of total hemicellulose, while it is up to 10% in softwood. The mannan is predominant in softwood and forms a minor component of hardwood (Juturu & Wu 2013; Soni & Kango 2013). Synergistic actions of various enzymes are necessary for the complete hydrolysis of hemicelluloses. Among these enzymes, xylanases (β-1,4-endoxylanases EC 3.2.1.8) and mannanases (β-1,4-endomannanase EC 3.2.1.78) are the two most important hemicellulolytic enzymes which cleave off the internal glycosidic bonds present in xylan and mannan. The resulting conversion of complex compounds into simple compounds as xylo-oligosaccharide and manno-oligosaccharide, respectively (Collins et al. 2005; Soni & Kango 2013). Hemicellulolytic enzymes active at extreme conditions have received much
attention due to their potential uses in different industrial processes such as pulp bleaching, in paper and pulp industry, in animal feed stock, in bread, and beverage making (Collins et al. 2005; Berlin et al. 2007). Thermophilic fungi produce enzymes with activity at high temperatures and usually possess a higher thermostability and broad-spectrum tolerance towards pH. One valuable advantage of conducting hydrolysis of lignocellulosic material at elevated temperature is to reduce the risk of contamination by mesophilic fungi. Fungi are important sources of hemicellulases as they produce higher titres as compared to yeasts and bacteria (Krisana et al. 2005). Many thermophilic fungi like Paecilomyces thermophila, Malbranchea cinamomea, Thermomyces lanuginosus, Scytalidium thermophilum, Sporotrichum thermophile, Rhizomucor sp., and Aspergillus sp. (Yang et al. 2006; Maijala et al. 2012; Sadaf & Khare 2014; Robledo et al. 2015) have showed high production of xylanase using agro-industrial wastes. Mannanases have been found in many mesophilic fungi like Aspergillus niger (Lin & Chen 2004), Aspergillus fumigatus (Puchart et al. 2004), and thermophilic fungi such as Thielavia arenaria (Lu et al. 2013), Neosartorya fischeri (Yang et al. 2015), and M. cinamomea (Ahirwar et al. 2016). Hence, in the present study, we have isolated and screened thermophilic and thermo-tolerant fungal strains that could produce xylanase and mannanase with potential biotechnological applications.

Materials and methods

Chemicals
Locust bean gum (LBG), oat spelt xylan, glucose, mannose, 3,5-dinitrosalicylic acid (DNS), and other chemicals were sourced from Sigma-Aldrich, St. Louis, MO, USA. Other analytical grade chemicals and media ingredients were purchased from HiMedia, Mumbai, India. Wheat bran was purchased from local market.

Surveyed areas and collection of samples
Eight different habitats, namely compost (CM), wheat straw (WS), wood chips (WC), storage seeds (SS), bird nesting material (BNM), decaying organic material (DOM), litter (L), and soil (SL), were targeted for sample collection and further for isolation of thermophilic fungi from Sagar district, Madhya Pradesh, India. During collection of samples, probable prevalence of thermophilic fungal strains in self-heated habitats like CM, storage, and geothermal soils was kept in mind. After collection, the samples were brought to the laboratory for isolation of thermophilic fungi. All the samples were air-dried and subjected to pretreatment at a higher temperature of 45°C for 2–7 days to enhance the population of thermophilic fungi.

Isolation and identification of thermophilic fungi
Isolation of fungi from collected samples of soils, stored seeds, and decomposing organic matter was carried out by direct plate method as suggested by Warcup (1950). Yeast extract soluble starch agar (YpSs) medium was used throughout isolation studies. The medium was having the following compositions: starch, 15.0 g/l; magnesium sulphate, 1.0 g/l; dipotassium hydrogen phosphate, 1.0 g/l; and yeast extract 4.0 g/l. Samples have possessed the different nature; hence, preliminary treatment was required before their plating. After plating, plates were incubated at 45°C and monitored at regular time interval for monitoring the appearance of any fungal growth for 10 days. These were checked for purity, and impure fungi were purified by dilution plating technique. The purity of the isolated fungus was confirmed by microscopic examination of the culture at 40× magnification using light microscope. After ensuring purity, fungi were subcultured on YpSs agar slants and allowed to grow for 5–7 days at 45°C and subsequently stored at 4°C as stock cultures. Fungi were identified on the basis of their colonial and morphological characteristics (Cooney & Emerson 1964).

Growth–temperature relationship
Generally, thermophilic and thermostolerant fungi optimally grow well at 45°C, whereas thermostolerant fungi show well growth below 20°C. Therefore, in order to understand the growth–temperature relationship of fungal isolates, we subjected them to grow at two different
temperatures, i.e. 18°C and 45°C, on YpSs agar plates. Further, these isolated fungi were inoculated at the centre of petri plate, and radial growth pattern was monitored for a period of 5 days. On the basis of growth patterns at two different temperatures, the nature of isolates as thermotolerant or thermophilic was determined.

Production of hemicellulase under submerged condition

All the test fungi were screened for production of hemicellulase on broth medium; the broth medium was used throughout the screening studies. The Czapek mineral medium has the following compositions: solution A: NaNO$_3$, 40 g/l; KCl, 10 g/l; MgSO$_4$, 10 g/l; and FeSO$_4$, 0.2 g/l and solution B: K$_2$HPO$_4$, 20 g/l. For preparation of 1 l of Czapek mineral medium, 50 ml each of solutions A and B was mixed and final volume was made up to 1 l by adding 900 ml of distilled water. To this further, 1 ml each of ZnSO$_4$ (1% w/v) and CuSO$_4$ (0.5% w/v) was added. Fifty millilitres of this solution were added to 150 ml Erlenmeyer flasks containing 2% (1 g) of wheat bran. The flasks were plugged and autoclaved for 20 min at 120°C. Each flask was inoculated with four mycelial agar discs of 4–6-day-old cultures under aseptic conditions. The flasks were incubated at 45°C under stationary condition for 7 days.

Enzyme extraction

After 7 days of incubation, the culture broth was filtered through Whatman NO. 1 filter paper, and the obtained filtrate was centrifuged (9000 g, 4°C) for 15 min. Thus, the obtained clear culture filtrate was used as a source of hemicellulase.

Qualitative and quantitative assay of hemicellulases

Agar plate assay

Hemicellulolytic (xylanase and mannanase) activity was detected by well diffusion method of culture filtrate on 1% xylan and 0.5% mannan (LBG) agar medium. Medium was poured into Petri plates and were allowed to solidify, well of equal diameter 5 mm and filled with 75 µl of culture filtrate. These plates were incubated at 50°C for 15–20 h, stained by 0.1% solution of Congo red for 30 min (Kango et al. 2003), destained with 1% NaCl solution to remove unbound dye, and then observed zone of hemicellulose hydrolysis and taken the diameter of zone of hydrolysis. The hydrolytic activities were recorded by measuring zone of hydrolysis in millimeters.

Xylanase and mannanase activity

Xylanase and mannanase activity was measured using xylan (1% w/v) and LBG (0.5% w/v) as substrates. Xylan and LBG was dissolved in 0.05 M sodium citrate buffer (pH 5.0) by stirring constantly for 8 h at 60°C (xylan) and for 1 h at 60°C (LBG). After stirring, clear solution was obtained by centrifugation at 10,000 g for 10 min. One hundred microlitres of enzyme sample were added to 900 µl of each substrate and incubated at 50°C for 5 min in case of xylanase activity and 10 min in case of mannanase activity. The reaction was stopped by adding 1.5 ml DNS reagent and boiling at 100°C for 5 min. Reducing sugar was measured at 540 nm against the blank (Miller 1959). One nanokatal/millilitre (nkat/ml) of enzyme activity was defined as the amount of enzyme required to produce 1 nm of sugar (xylose/mannose) per second under experimental conditions.

Results and discussion

Isolation of thermophilic and thermotolerant fungi

In this work, Table 1 has information about the distribution of thermophilic and thermotolerant fungi of different habitats. A total of 79 collected samples have been examined for the presence of thermophilic fungi from various ecological niches. Out of these 79 samples, 57 samples are showing the presence of thermophilic fungi. In the isolation method, the following samples have been used, viz. storage samples (17), soil samples (14), CM (12) WC samples (12), WS samples (11), litter samples (6), and few samples from bird nest and decaying materials respectively, to know the presence of thermophilic fungi. A total number of 68 thermophilic and thermotolerant fungi were isolated from 57 positive samples. In addition, the isolated fungi included 15 fungal
isolates from stored seeds, 12 from soils, 11 from CMs, 10 from WS, 9 from WC, 5 from litter, and 3 from decaying organic matter and bird nests, respectively. From collected samples, overall 72.1% of samples have showed presence of thermophilic fungi. The maximum percentage of thermophilic mycoflora was found in soil samples (78.6%), followed by the second highest in CM samples (75%) and wood chip samples (75%), and lowest was found in BNM samples (50%). Soil is an excellent habitat for development and growth of thermophilic fungi. The high percentage of thermophilic fungi in soil samples is attributed to sun heating of soils. The work is supported by previous findings of Hassouni et al. (2006). Rajavaram et al. (2010) have isolated thermophilic fungi from coal mine soil samples where there is a higher ambient temperature. Several thermophilic fungi were isolated from CM soils by Chadha et al. (2004) and were identified as Rhizomucor pusillus, S. thermophilum, Melanocarpus albomyces, Chaetomium thermophile, and T. lanuginosus. Salar and Aneja (2006) have isolated 19 species of thermophilic and thermotolerant fungi from temperate soils of northern India and identified as thermophilic (10 species) and thermotolerant (6 species). The occurrence of large quantity of thermophilic microorganisms was observed in mature CM where the peak heat hours of daily temperature reaches maximum 65–70°C (Bru-Adan et al. 2009; Rajavaram et al. 2010; Langarica-Fuentes et al. 2014; Sebok et al. 2015). Rajavaram et al. (2010) isolated 46 thermophilic fungi from various substrates such as underground coal mine soil, bird nest materials, vermin CM, cow dung, poultry litter, and decomposing organic materials collected from different places of Andhra Pradesh. They concluded that thermophilic Humicola lanuginosa was present in all substrates, and A. fumigatus was found prevalence in the decomposing litter materials. Figure 1 highlights microscopic image of newly isolated fungi, and these isolates have identified as on the basis of their morphology. A total of 68 thermophilic fungal forms belonging to 15 genera and 19 species were isolated during the course of the present study. The results of isolation are given in Table 2. Thermomyces lanuginosus is one of the most common thermophilic fungi occurring in soil (Maheshwari et al. 1987). Three species of thermophilic moulds were isolated from CM and were identified as Myriococcum thermophilum, Thermoascus aurantiacus, and T. lanuginosus (Lee et al. 2014). The growth of fungal culture on Yps agar medium in different temperatures clearly indicated that 26 of the species were thermophilic, whereas 42 were thermotolerant and showed lower temperature regime compared to the other investigated species (Table 3) (Cooney & Emerson 1964).

### Table 1. Distribution of thermophilic and thermotolerant fungi in different habitats.

| S. No. | Habitat                        | No. of samples | No. of fungi isolated | No. of samples found positive (%) |
|--------|--------------------------------|----------------|-----------------------|-----------------------------------|
| 1.     | Storage seed                   | 17             | 15                    | 12 (70.6)                         |
| 2.     | Litter                         | 6              | 5                     | 4 (66.6)                          |
| 3.     | Wheat straw                    | 11             | 10                    | 8 (72.7)                          |
| 4.     | Compost                        | 12             | 11                    | 9 (75)                            |
| 5.     | Soils                          | 14             | 12                    | 11 (78.6)                         |
| 6.     | Wood chips                      | 12             | 9                     | 9 (75)                            |
| 7.     | Decaying organic material       | 3              | 3                     | 2 (66.6)                          |
| 8.     | Bird nesting material           | 4              | 3                     | 2 (50)                            |
| Total  |                                | 79             | 68                    | 57 (72.1)                         |

Xylanase and mannanase production in submerged fermentation

Since the cost of the substrate plays an important role in the economics of enzyme production, low-value crude raw materials may be used as viable substrates for hemicellulase production. In the present study, wheat bran was used as carbon source and was employed as substrate for hemicellulase production. Kango and Jain (2005) suggested that the use of cheaper hemicellulosic substrates in media for xylanase production from thermophilic and thermotolerant fungi. All the fungal strains were subjected to enzyme diffusion technique for qualitative analysis of extracellular xylanase and mannanase under submerged fermentation. Cell-free culture filtrate on incubation has produced clear zones visible against opaque xylan and mannan agar medium. Zones of clearance were recorded before and after Congo red staining. Figure 2 illustrates zone of hydrolysis around the well on xylan and mannan agar plate. After screening, all the fungi were found positive for xylanase activity.
Figure 1. Microscopic image projection system (MIPS) photograph showing morphological characteristics of some fungi: Thermotolerant fungi: (a) Aspergillus terreus (b) Absidia sp. (c) Mucor sp.; Thermophilic fungi: (d) Chaetomium thermophile (e) Malbranchea cinnamomea (f) Melanocarpus albomyces (g) Scytalidium thermophile (h) Myceliophthora thermophila (i) Sporotrichum thermophile.

Table 2. Results of isolation of fungi from different habitats.

| S. No. | Isolate                     | SS | L | WS | CM | SL | WC | DOM | BNM | Total | % Occurrence |
|--------|-----------------------------|----|---|----|----|----|----|-----|-----|-------|--------------|
| 1.     | *Absidia corymbifera*       | 2  | – | –  | 1  | –  | –  | –   | –   | 3     | 4.4          |
| 2.     | *Absidia* sp.               | –  | – | –  | –  | 2  | 1  | –   | –   | 3     | 4.4          |
| 3.     | *Aspergillus fumigatus*     | 3  | – | 1  | –  | –  | –  | –   | –   | 4     | 5.8          |
| 4.     | *Aspergillus* sp.           | 2  | 1 | –  | 2  | 1  | –  | –   | 6   | 8.8          |
| 5.     | *Aspergillus terreus*       | 3  | – | 2  | 1  | 1  | –  | 1   | 8   | 11.7         |
| 6.     | *Chaetomium thermophile*    | –  | 1 | 2  | 1  | –  | –  | –   | 4   | 5.8          |
| 7.     | *Emericella nidulans*       | –  | 1 | –  | 1  | –  | –  | –   | 3   | 4.4          |
| 8.     | *Humicola insolens*         | 1  | 1 | 1  | –  | –  | –  | –   | 3   | 4.4          |
| 9.     | *Malbranchea cinnamomea*    | 1  | – | –  | –  | –  | –  | 1   | 1   | 1.4          |
| 10.    | *Melanocarpus albomyces*    | –  | 1 | –  | 1  | –  | –  | –   | 2   | 2.9          |
| 11.    | *Mucor miehei*              | –  | 1 | –  | 1  | –  | –  | –   | 2   | 2.9          |
| 12.    | *Mucor* sp.                 | 1  | 2 | 2  | 1  | –  | –  | –   | 6   | 8.8          |
| 13.    | *Myceliophthora thermophila*| –  | – | –  | 2  | 1  | 1  | –   | 4   | 5.8          |
| 14.    | *Paeilomyces* sp.           | 1  | 1 | –  | –  | 1  | –  | –   | 3   | 4.4          |
| 15.    | *Rhizopus* sp.              | –  | 1 | 2  | 1  | –  | –  | –   | 4   | 5.8          |
| 16.    | *Scytalidium thermophile*   | –  | – | –  | 2  | 1  | –  | –   | 3   | 4.4          |
| 17.    | *Sporotrichum thermophile*  | –  | – | –  | –  | –  | 1  | 1   | 1   | 1.4          |
| 18.    | *Thermoascus aurantiacus*   | –  | 1 | –  | –  | –  | 1  | –   | 2   | 2.9          |
| 19.    | *Thermomyces lanuginosus*   | 2  | 1 | 1  | 3  | 1  | –  | –   | 7   | 10.2         |
| Total  |                             | 15 | 5 | 11 | 12 | 3  | 9  | 3   | 68  | 100             |

SS: storage seeds; L: litter; WS: wheat straw; CM: compost; SL: soil; WC: wood chips; DOM: decaying organic material; BNM: bird nesting material.
### Table 3. Temperature relationships of thermophilic and thermotolerant fungi.

| S. No. | Fungal species          | Sample type         | 18°C | 45°C |
|--------|-------------------------|---------------------|------|------|
| 1.     | Absidia corymbifera CM-4| Compost             | +    | +++  |
| 2.     | Absidia corymbifera SS-19| Stored seeds         | -    | ++   |
| 3.     | Absidia corymbifera SS-37| Stored seeds         | -    | +++  |
| 4.     | Absidia sp. DOM-38       | Decaying organic material | +    | ++   |
| 5.     | Absidia sp. WC-31        | Wood chips           | +    | +++  |
| 6.     | Absidia sp. WC-59        | Wood chips           | +    | +++  |
| 7.     | Aspergillus fumigatus SS-1| Stored seeds         | -    | ++   |
| 8.     | Aspergillus fumigatus SS-3| Stored seeds         | +    | ++   |
| 9.     | Aspergillus fumigatus SS-30| Stored seeds      | +    | +++  |
| 10.    | Aspergillus fumigatus WS-13| Wheat straw          | +    | +++  |
| 11.    | Aspergillus sp. SL-22    | Soil                | –    | +++  |
| 12.    | Aspergillus sp. SL-28    | Soil                | –    | ++   |
| 13.    | Aspergillus sp. SS-39    | Stored seeds         | +    | ++   |
| 14.    | Aspergillus sp. SS-44    | Stored seeds         | +    | +++  |
| 15.    | Aspergillus sp. WC-26    | Wood chips           | –    | ++   |
| 16.    | Aspergillus sp. WS-41    | Wheat straw          | +    | ++   |
| 17.    | Aspergillus terreus BNM-49| Bird nesting material | –   | +++  |
| 18.    | Aspergillus terreus CM-17| Compost             | –    | ++   |
| 19.    | Aspergillus terreus CM-35| Compost             | +    | ++   |
| 20.    | Aspergillus terreus SL-8 | Soil                | +    | ++   |
| 21.    | Aspergillus terreus SS-43| Stored seeds         | +    | +++  |
| 22.    | Aspergillus terreus SS-53| Stored seeds         | +    | +++  |
| 23.    | Aspergillus terreus SS-6  | Stored seeds         | +    | ++   |
| 24.    | Aspergillus terreus WC-57| Wood chips           | +    |+++  |
| 25.    | Chaetomium thermophile CM-56| Compost             | –    |++++ |
| 26.    | Chaetomium thermophile CM-60| Compost             | –    |+++  |
| 27.    | Chaetomium thermophile L-61| Litter             | –    |+++  |
| 28.    | Chaetomium thermophile SL-66| Soil             | –    |+++  |
| 29.    | Emericella nidulans BNM-54| Bird nesting material | –   |+++  |
| 30.    | Emericella nidulans WC-23| Wood chips           | +    |++   |
| 31.    | Emericella nidulans WS-14| Wheat straw         | –    |+++  |
| 32.    | Humicola insolens SL-12  | Soil                | –    |++++ |
| 33.    | Humicola insolens SL-48  | Stored seeds         | –    |+++  |
| 34.    | Humicola insolens WS-20  | Wheat straw          | –    |+++  |
| 35.    | Malbranchea cinnamomea L-64| Litter             | –    |+++  |
| 36.    | Melanocarpus albomyces DOM-65| Decaying organic material | – |+++  |
| 37.    | Mucor miehei WC-47       | Wood chips           | +    | ++   |
| 38.    | Mucor miehei WS-29       | Wheat straw          | –    |+++  |
| 39.    | Mucor sp. CM-15          | Compost             | –    |+++  |
| 40.    | Mucor sp. CM-16          | Compost             | –    |+++  |
| 41.    | Mucor sp. SS-34          | Stored seeds         | +    |++   |
| 42.    | Mucor sp. WC-2           | Wood chips           | –    |+++  |
| 43.    | Mucor sp. WS-10          | Wheat straw          | –    |+++  |
| 44.    | Mucor sp. WS-52          | Wheat straw          | +    |+++  |
| 45.    | Myceliophthora thermophila CM-24| Compost             | –    |+++  |
| 46.    | Myceliophthora thermophila CM-5| Compost             | –    |+++  |
| 47.    | Myceliophthora thermophila SL-51| Soil             | –    |+++  |
| 48.    | Myceliophthora thermophila WC-67| Wood chips           | –    |+++  |
| 49.    | Paecilomyces sp. L-27    | Litter              | +    | ++   |
| 50.    | Paecilomyces sp. SL-25   | Soil                | –    | ++   |
| 51.    | Paecilomyces sp. SS-42   | Stored seeds         | –    |+++  |
| 52.    | Rhizopus sp. L-21        | Litter              | –    |+++  |
| 53.    | Rhizopus sp. SL-11       | Soil                | +    |+++  |
| 54.    | Rhizopus sp. WS-18       | Wheat straw          | –    |+++  |
| 55.    | Rhizopus sp. WS-46       | Wheat straw          | +    |++   |
| 56.    | Scytalidium thermophile CM-58| Compost             | –    |+++  |
| 57.    | Scytalidium thermophile CM-62| Compost             | –    |+++  |
| 58.    | Scytalidium thermophile SL-36| Soil             | –    |++   |
| 59.    | Sporotrichum thermophile BNM-55| Bird nesting material | –  |+++  |
| 60.    | Thermoascus aurantiacus DOM-33| Decaying organic material | – |+++  |
| 61.    | Thermoascus aurantiacus L-68| Litter             | –    |+++  |
| 62.    | Thermomyces lanuginosus SL-50| Soil             | –    |+++  |
| 63.    | Thermomyces lanuginosus SL-63| Soil             | –    |+++  |
| 64.    | Thermomyces lanuginosus SL-9  | Soil             | –    |+++  |
showing clear zone after hydrolysing xylan as a substrate. While only 22 test fungi were found positive for mannanase activity showing clear zone by hydrolysing mannan as a substrate (Table 4). The diameter of zones ranged from a minimum of 4 mm on xylan agar plates (Aspergillus sp. SL-28, Scytalidium thermophile CM-62, and Myceliophthora thermophila WC-67) and 5 mm on mannan agar plates (Mucor sp. WS-52 and M. thermophila WC-67) to a maximum of 29 mm on xylan medium (A. fumigatus SS-30 and Emericella nidulans WC-23) and

| S. No. | Fungal species            | Sample type | 18°C | 45°C |
|--------|---------------------------|-------------|------|------|
| 65.    | Thermomyces lanuginosus SS-32 | Stored seeds | –    | +++  |
| 66.    | Thermomyces lanuginosus SS-7  | Stored seeds | –    | +++  |
| 67.    | Thermomyces lanuginosus WC-45 | Wood chips  | –    |+++++ |
| 68.    | Thermomyces lanuginosus WS-40 | Wheat straw | –    |+++  |

The observations were recorded in terms of no growth (–), very poor growth, colonies attaining 6 mm diameter (+); poor growth, colonies attaining 7–20 mm diameter (++); good growth, colonies attaining 21–50 mm growth (+++); excellent growth, colonies attaining more than 50 mm diameter (++++).

Figure 2. Plates showing zone of hydrolysis of crude enzyme: plate (a) and (b) showing xylan hydrolysis; (c) and (d) showing mannan hydrolysis.

(C) Control (1) Malbranchea cinnamomea (2) Melanocarpus albomyces (3) Chaetomium thermophile (4) Sporotrichum thermophile (5) Mucor sp. (6) Aspergillus terreus (7) Absidia sp. (8) Aspergillus fumigatus (9) Myceliophthora thermophila (10) Humicola insolens (11) Absidia corymbifera (12) Aspergillus terreus (13) Myceliophthora thermophila.
Table 4. Qualitative and quantitative profiles of the extracellular hemicellulase produced by fungi.

| S. No. | Isolate                        | Xylanase activity | Mannanase activity |
|--------|--------------------------------|-------------------|--------------------|
|        |                                | Zone of hydrolysis (mm) | nkat/ml | Zone of hydrolysis (mm) | nkat/ml |
| 1      | Aspergillus fumiugatus SS-1    | 15                | 121 ± 0.21         | 13 | 6 ± 0.03 |
| 2      | Mucor sp. WC-2                | 21                | 46 ± 0.47          | 11 | 5 ± 0.02 |
| 3      | Aspergillus fumiugatus SS-3    | 8                 | 65 ± 0.91          | Nil | BDL   |
| 4      | Absidia corymbifera CM-4      | 19                | 119 ± 0.64         | 24 | 8 ± 0.05 |
| 5      | Myceliophthora thermophila CM-5| 22                | 54 ± 0.75          | 19 | 9 ± 0.03 |
| 6      | Aspergillus terreus SS-6      | 15                | 32 ± 0.53          | 7  | 4 ± 0.02 |
| 7      | Thermomyces lanuginosus SS-7  | 18                | 2130 ± 0.49        | Nil | BDL   |
| 8      | Aspergillus terreus SL-8      | 16                | 25 ± 0.69          | 22 | 8 ± 0.05 |
| 9      | Thermomyces lanuginosus SL-9  | 9                 | 2480 ± 0.52        | Nil | BDL   |
| 10     | Mucor sp. WS-10               | 11                | 45 ± 0.58          | Nil | BDL   |
| 11     | Rhizopus sp. SL-11            | 8                 | 23 ± 0.096         | Nil | BDL   |
| 12     | Humicola insolens SL-12       | 13                | 186 ± 0.36         | 21 | 6 ± 0.03 |
| 13     | Aspergillus fumiugatus WS-13  | 18                | 118 ± 0.05         | Nil | BDL   |
| 14     | Emericella nidulans WS-14     | 23                | 120 ± 0.68         | Nil | BDL   |
| 15     | Mucor sp. CM-5                | 258               | 37 ± 0.57          | Nil | BDL   |
| 16     | Mucor sp. CM-16               | 7                 | 55 ± 0.49          | Nil | BDL   |
| 17     | Aspergillus terreus CM-17     | 16                | 71 ± 0.38          | Nil | BDL   |
| 18     | Rhizopus sp. WS-18            | 26                | 83 ± 0.38          | Nil | BDL   |
| 19     | Absidia corymbifera SS-19     | 9                 | 56 ± 0.58          | Nil | BDL   |
| 20     | Humicola insolens WS-20       | 6                 | 192 ± 0.93         | Nil | BDL   |
| 21     | Rhizopus sp. L-21             | 18                | 66 ± 0.68          | Nil | BDL   |
| 22     | Aspergillus sp. SL-22         | 27                | 86 ± 0.06          | 28 | 7 ± 0.09 |
| 23     | Emericella nidulans WC-23     | 28                | 222 ± 0.57         | Nil | BDL   |
| 24     | Myceliophthora thermophila CM-24| 16              | 130 ± 0.89         | 12 | 18 ± 0.13 |
| 25     | Paecilomyces sp. SL-25        | 18                | 61 ± 0.47          | Nil | BDL   |
| 26     | Aspergillus sp. WC-26         | 8                 | 450 ± 0.57         | 18 | 38 ± 0.03 |
| 27     | Paecilomyces sp. L-27         | 6                 | 75 ± 0.78          | Nil | BDL   |
| 28     | Aspergillus sp. SL-28         | 4                 | 920 ± 0.67         | Nil | BDL   |
| 29     | Mucor miehei WS-29            | 23                | 86 ± 0.59          | Nil | BDL   |
| 30     | Aspergillus fumiugatus SS-30  | 29                | 106 ± 0.43         | 18 | 7 ± 0.01 |
| 31     | Absidia sp. WC-31             | 17                | 59 ± 0.86          | 11 | 6 ± 0.02 |
| 32     | Thermomyces lanuginosus SS-32 | 14                | 1950 ± 0.04        | Nil | BDL   |
| 33     | Thermoascus aurantiacus DOM-33 | 24               | 83 ± 0.55          | Nil | BDL   |
| 34     | Mucor sp. SS-34               | 7                 | 34 ± 0.76          | 8  | 5 ± 0.01 |
| 35     | Aspergillus terreus CM-35      | 16                | 142 ± 0.48         | Nil | BDL   |
| 36     | Scytalidium thermophile SL-36  | 5                 | 118 ± 0.69         | Nil | BDL   |
| 37     | Absidia corymbifera SS-37     | 29                | 89 ± 0.45          | Nil | BDL   |
| 38     | Absidia sp. DOM-38            | 24                | 55 ± 0.79          | Nil | BDL   |
| 39     | Aspergillus sp. SS-39         | 8                 | 103 ± 0.45         | Nil | BDL   |
| 40     | Thermomyces lanuginosus WS-40 | 5                 | 1280 ± 0.87        | Nil | BDL   |
| 41     | Aspergillus sp. WS-41         | 14                | 132 ± 0.45         | Nil | BDL   |
| 42     | Paecilomyces sp. SS-42        | 16                | 82 ± 0.98          | Nil | BDL   |
| 43     | Aspergillus sp. SS-43         | 23                | 165 ± 0.67         | 22 | 21 ± 0.06 |
| 44     | Aspergillus sp. SS-44         | 24                | 66 ± 0.34          | 20 | 9 ± 0.05 |
| 45     | Thermomyces lanuginosus WC-45 | 15                | 730 ± 0.45         | Nil | BDL   |
| 46     | Rhizopus sp. WS-46            | 14                | 85 ± 0.45          | Nil | BDL   |
| 47     | Mucor miehei WC-47            | 22                | 91 ± 0.38          | Nil | BDL   |
| 48     | Humicola insolens SS-48       | 8                 | 67 ± 0.55          | Nil | BDL   |
| 49     | Aspergillus terreus BNM-49     | 26                | 42 ± 0.56          | Nil | BDL   |
| 50     | Thermomyces lanuginosus SL-50 | 14                | 620 ± 0.86         | Nil | BDL   |
| 51     | Myceliophthora thermophila SL-51| 18               | 59 ± 0.35          | Nil | BDL   |
| 52     | Mucor sp. WS-52               | 8                 | 138 ± 0.98         | 5  | 13 ± 0.12 |
| 53     | Aspergillus terreus SS-53     | 7                 | 44 ± 0.46          | Nil | BDL   |
| 54     | Emericella nidulans BNM-54    | 27                | 108 ± 0.65         | Nil | BDL   |
| 55     | Sporotrichum thermophile BNM-55| 24               | 49 ± 0.59          | 9  | 4 ± 0.02 |
| 56     | Chaetomium thermophile CM-56  | 9                 | 89 ± 0.23          | Nil | BDL   |
| 57     | Aspergillus terreus WC-57     | 11                | 49 ± 0.08          | Nil | BDL   |
| 58     | Scytalidium thermophile CM-58 | 17                | 52 ± 0.04          | Nil | BDL   |
| 59     | Absidia sp. WC-59             | 13                | 32 ± 0.08          | Nil | BDL   |
| 60     | Chaetomium thermophile CM-60  | 14                | 81 ± 0.56          | Nil | BDL   |
| 61     | Chaetomium thermophile L-61   | 7                 | 138 ± 0.44         | 15 | 6 ± 0.02 |
| 62     | Scytalidium thermophile CM-62 | 5                 | 72 ± 0.87          | 14 | 12 ± 0.07 |
| 63     | Thermomyces lanuginosus SL-63 | 16                | 1220 ± 0.56        | Nil | BDL   |
| 64     | Malbranchea cinnamomea L-64   | 26                | 242 ± 0.34         | 13 | 27 ± 0.4 |

(Continued)
28 mm on mannan medium (*M. albomyces* DOM-65). These findings gave only a qualitative result of the hemicellulases producers with the clear zones only able to distinguish producers from non-producers among the isolates. Therefore, there was a need for a more quantitative result that will be able to distinguish the high producers of hemicellulase among the isolates, thus a quantitative screening was done. Cell-free culture filtrate was assayed against DNS and colour change in the mixture was observed at 540 nm. Results of qualitative and quantitative estimation of xylanase and mannanase activity in culture filtrate of test fungi are given in Table 4. Results show that fungal isolates were the best for xylanolytic index, i.e. *A. fumigatus* SS-1 (121 nkat/ml), *Absidia corymbifera* CM-4 (119 nkat/ml), *T. lanuginosus* SL-9 (2480 nkat/ml), *Humicola insolens* WS-20 (192 nkat/ml), *E. nidulans* WS-23 (222 nkat/ml), *M. thermophila* NFCI 3725 (130 nkat/ml), *Aspergillus terreus* SS-43 (165 nkat/ml), *Scytalidium thermophile* SL-36 (118 nkat/ml), *C. thermophile* L-61 (138 nkat/ml), *M. cinnamomea* NFCI 3724 (242 nkat/ml), and *M. albomyces* DOM-65 (195 nkat/ml), respectively. Whereas, among the 60 fungal isolates, *M. cinnamomea* NFCI 3724, *M. albomyces* DOM 65, *A. terreus* SS-43, and *M. thermophila* NFCI 3725 are presenting relatively high mannanase indexes, i.e. 27, 24, 21, and 18 nkat/ml. Soni et al. (2016) screened 88 fungi for mannanase activity and found 28 fungi produced extracellular mannanase when LBG used as carbon source in liquid medium. Moretti et al. (2012) isolated 27 thermophilic and thermotolerant fungal strains from self-heated habitats and screened for xylanase production. Sadaf and Khare (2014) described the production of xylanase by the *S. thermophile*. Hemicellulases from extremophilic sources have tremendous utility in many biotechnological processes. In particular, thermophilic xylanases could be used in applications where high temperatures are required to increase the solubility of substrates or to reduce viscosity.

### Conclusion

New thermophilic fungal strains with the ability to produce hemicellulases were isolated from various self-heated habitats. Fungi were found to produce hemicellulases in submerged fermentation using wheat bran as sole carbon source. The results have suggested that the best producer among 68 thermophilic fungi and 22 thermophilic moulds has showed higher titre of hemicellulases (xylanase and mannanase). Hemicellulases produced by these newly isolated thermophilic fungi may have potential for applications in different areas, including in the pulp and paper, food and feed, and textile industries that require enzyme work on high temperature.

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No potential conflict of interest was reported by the authors.

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