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Isolation and Identification of Thermophilic and Thermotolerant Cellulase Producing Fungi from Compost and Soil

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A B S T R A C T

Cellulases produced by thermophilic and thermotolerant fungi are usually thermostable and can be applied to industrial processes that occur at high temperature. Considering this, present study was aimed to isolate and identify the thermophilic and thermotolerant cellulase producing fungi from compost and soil samples. For that samples were plated on Potato Dextrose Agar (PDA)-Chloramphenicol plate and incubated at 45℃ up to five days. Total twenty-nine fungal strains were isolated belonging to three genera *Aspergillus*, *Rhizomucor* and *Rhizopus* based on their morphological characterization. According to result of thermal stability test performed at two different temperatures of 18 and 45 ℃ from 29 fungal isolates 15 were thermotolerant while 14 were found to be thermophilic in nature. Screening of these isolates for hydrolysis of cellulose was confirmed by formation of clear halo zone surrounding fungal growth on Carboxymethyl Cellulose (CMC) after flooding with grams iodine. All the isolates found positive for cellulase production in which member of genus *Rhizomucor* shows highest cellulolytic index values (1.51 to 2.32), followed by genus *Aspergillus* (1.17 to 1.59), and then *Rhizopus* (1.9). Four morphologically different potential isolates were further identified by ITS sequencing, among these two belongs to Zygomycotina (*Rhizomucor pusillus* FWC-B, *Rhizopus microspores* NADEP-W) and other two belong to Ascomycota (*Aspergills fumigatus* FWC-B, *Aspergillus quadrilineatus* PC-W).

***Key words*:** Cellulase, Compost, Fungi, Molecular identification, Thermophilic

Lignocellulosic waste from agriculture and forestry has potential to be used as economical and renewable substrate for large-scale production of fuels and chemicals [1]. Biodegradation of cellulose in to fermentable sugars requires collaborative action of cellulases endoglucanase: EC 3.2.1.4, exoglucanase /cellobiohydrolase : EC 3.2.1.91and β-glucosidase :EC 3.2.1.21 [2]. Cellulases are the third largest industrial enzyme because of their wide range of industrial applications in pulp and paper industry, laundry and detergent, bio fuel production, textile industry, food and feed industry, medical application as well as in agricultural industry worldwide [3].

Fungi are the major contributor of cellulases among all microbes and responsible for approximately 80% of the cellulose hydrolysis on the Earth [4] and they are the only described eukaryotes in a position to grow at temperatures above 45°C [5]. Recently thermophilic fungi are gaining interest of researchers as the enzymes produced by these fungi show activity at elevated temperatures and they usually possess a greater thermostability, longer shelf life and greater resistance to denaturing agents and metal ions and broad tolerance to pH variation [6]. The growth of thermophlic fungi is promoted in warm, moist and aerobic environment that commonly develops in piles of agricultural and forestry products, heaped masses of plant matter, and other cumulations of organic matter. Around thirty species of thermophilic fungi are currently known, among them majority of were primarily isolated from composts [7]. Considering potential applications of thermostable cellulases, it is necessary to isolate and characterize novel cellulase producing native thermophilic fungal strains. Several researchers reported on isolation of thermophilic and thermotolerant cellulase producing fungi, like [8] isolated 27 thermophilic and thermotolerant fungal strains belonging to genera *Aspergillus*, *Thermomucor*, *Thermomyces*, *Myceliophthora* and *Candida* from sugarcane pile, soil and decaying organic matter. Similarly, [9] isolated *A. terreus* from the compost containing cellulose and evaluated production of cellulases using different lignocellulose waste.

Thermophilic fungi can survive at high temperatures, in which compost and soil are of their habitats. The increase in temperature during composting process creates selective environment that eliminates many mesophilic and favours growth of thermophilic and thermotolerant organisms

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particularly acclimatized to high temperatures [10]. In this study twenty-nine thermophilic and thermotolerant fungal isolates belonging to ascomycetes and zygomycetes families were isolated from various sample of compost and soil and characterized for their cellulolytic ability on CMC plate. From which four morphologically different fungal isolates showing significant cellulolytic index were identified by sequencing of ITS1/ITS4 region and further bioinformatics analysis.

MATERIALS AND METHODS

Soil and compost samples

Three soil samples collected from agricultural field were mixed with different agricultural waste like saw dust; rice straw and wheat straw in equal proportion, as three separated treatment and then kept for a period of one month in warm and humid environment to allow enrichment of cellulolytic microbial population, a method adopted from [11]. Nine compost samples collected from various sites of South Gujarat region were also analyzed for presence of thermophilic and thermotolerant cellulase producing fungi. The details of samples are provided in table 1. The compost samples were collected in to sterile polythene bags and proceed on the same day for isolation of fungi and in case of delay stored in refrigerator at 4°C.

Table 1 Compost samples used for isolation of thermophilic and thermotolerant fungi

S. No.	Sample	Collection site	Description
1	Precompost	Vermicomposting plant, Surat Municipal Corporation (SMC)	Mixture of lignocellulosic waste (60%) like flowers, leaves, thin branches and cow dung (40%) kept under covered condition for 30 to 35 days. Temperature rise up to 70 to 75°C.
2	Vermicompost	Vermicomposting plant, Surat Municipal Corporation (SMC)	The precomposted material stated above composted with help of earthworms for 30-35 days. Temperature controlled to 25 to 30°C.
3	NADEP	Vermicomposting plant, Surat Municipal Corporation (SMC)	Made from flowers, leaves layered with cow dung in tank made of clay bricks and composted for 90-100 days. Temperature rise up to 60°C.
4	Flower waste compost	Solid Waste Laboratory, Sardar Vallabhbhai National Institute of Technology (SVNIT), Surat	Made from flower waste mixed with saw dust and cow dung composted in rotary drum composter. Temperature rise up to 50-60°C.
5	Garden waste compost	Solid Waste Laboratory, Sardar Vallabhbhai National Institute of Technology (SVNIT), Surat	Made from leaves, grass cuttings, flowers, small twigs and branches mixed with food waste and composted by rotary drum method. Temperature rise up to 74°C.
6	Compost H	Farm site of village Hansot	Made of agricultural waste mixed with cow dung.
7	Local compost 1	Farm site, Surat	Made of grass, leaves, and other agricultural waste mixed with cow dung.
8	Local compost 2	From local farmer, Surat	Made of waste of rice straw, wheat star, sugarcane straw mixed with cow dung.
9	Bapalal compost	Composting site, Veer Narmad South Gujarat University (VNSGU)	Made of campus green cellulosic waste mixed with cow dung.

Isolation and morphological characterization of thermotolerant/thermophilic fungi

Serial dilution plating method was used for isolation of thermophilic and thermotolerant fungi from samples of soil and compost. For that samples were serially diluted and dilutions (10^{-2}, 10^{-4} and 10^{-6}) were plated on Potato Dextrose Agar (PDA- Hi Media) supplemented with chloramphenicol 100 µg/ml, pH 5.5 by spread plate method. Plates were incubated at 45°C under humidity condition for 4 to 5 days in incubator. Growing fungal colonies were picked up and purified by repeated streaking on the PDA plate. The purified fungal isolates were preserved on PDA slant in refrigerator at 4°C till further use. Morphological characterization of isolated fungal strains was made by observing growth characteristics including color of the mycelia and spores on PDA plate, as well as microscopic analysis of their vegetative and reproductive structures under the low power compound microscope using Lacto phenol cotton blue staining [12].

Thermal stability test

Thermophilic and therotolerant forms of fungi can be differentiated based on their minimum and maximum limit of temperature for their growth, as thermophilic fungi have a growth temperature maximum at or above 50°C and a minimum at or above 20°C. So, a truly thermophilic fungus is one which show no growth below 20°C but grow well above 50°C while the thermotolerant forms have a growth temperature range of < 20 to 55°C [13]. So, to differentiate thermophilic and thermotolerant forms, fungal isolate was point inoculated in middle of PDA plate and incubated at two selected temperatures of 18°C and 45°C and fungal radial growth pattern was observed and rated after 48h.

Screening for cellulase production

To determine the cellulolytic ability, isolates were subjected to screening on solid media. For that selective media containing cellulose as only carbon source was used for growth of fungi and then stained by grams iodine solution. The composition of Cellulose agar medium is (g L^{-1}): $NaNO_3$-2.0, KH_2PO_4-1.0, $MgSO_4 \cdot 7H_2O$-0.5, KCl-0.5, CMC sodium salt-10.0, peptone-0.2 and agar-17.0 in distilled water pH 6.0 [14]. The 15 µl of fungal spore suspension in sterile normal saline is point inoculated in middle of cellulose agar pate, the drop of spore suspension is allowed to dry. Then plates were incubated at 45 °C for 2-3 days depending upon the growth rate of fungi. After incubation the plates were flooded with grams iodine solution and observed for the zone of cellulose degradation and the cellulolytic index was calculated using following equation[15-16]. All the assays were done in triplicates and mean and SD was calculated in Excel. The differences between

the cellulolytic indices of isolates were evaluated using statistical analysis one way ANOVA in excel. P value < 0.05 was considered as significant.

$$\text{Cellulolytic index} = \frac{\text{Diameter of cellulose degradation (cm)}}{\text{Diameter of fungal colony (cm)}}$$

Molecular characterization and phylogenetic study of potential isolates

Four morphologically different potential cellulase producing fungal isolates FWC-G, FWC-B, NADEP-W and PC-W were identified by ITS sequencing. For that genomic DNA from fungi was isolated using genomic DNA extraction kit (mini) by SLS research Pvt Ltd. The ITS region was amplified using universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR amplification was checked by agarose gel electrophoresis and after performing column purification of PCR amplicon to remove contaminants, DNA sequencing reaction was done with ITS1/ITS4 primer using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

The obtained gene sequence was used to carry out BLAST with the database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs (Mega 11). Dendrograms were created using neighbour joining (NJ) plot and the boot strapping was carried using 1000 replications. The sequences have been submitted to the GenBank database.

RESULTS AND DISCUSSION

Isolation and morphological characterization of thermophilic and thermotolerant fungi

Among total 29 of obtained fungal isolates 23 were isolated from compost while 6 were isolated from soil. According to their morphological (microscopic and macroscopic) characterization isolates were found to belong to three fungal genera *Aspergillus, Rhizomucor* and *Rhizopus*.

Aspergillus

Based on morphological characters 16 isolates were identified as *Aspergillus*, there were two types of colonies with different characteristics. The microscopic and macroscopic characteristics of the both types are as follows: (i) Macroscopic: flat spreading bluish green colonies with white margins and dense felt of conidiophores. Reverse of colony is crème. The results were similar with a study by [17] who observed white colonies turned to green with a smooth colony surface and identified as *A. fumigatus*. Microscopic: Conidiophore stipes are short and possesses conical shaped terminal vesicles with single row of phialides. There are numerous spherical conidia. Total 14 isolates have shown these characteristics and identified as *Aspergillus sp., a* are RS-G, WS-G,SD-G, FWC-G, GWC-G, CH-G, BC-G, PC-G, PC-LG, VC-G, VC- LG, NADEP-LG, NADEP-G, LC 1 –G. (ii) Macroscopic : Buff and white colonies which are moderately deep, slightly sulcate with entire margins the texture is floccose; with low sporulation. Reverse of colony is yellowish brown. Microscopic: Numerous globose hulle cells are found to present which is characteristics of nidulans section of *Aspergillus*. Ascospores are smooth, globose. Conidiophores with smooth stipes, and possess conidia on upper half to two thirds. Only two isolates PC-W, VC-W have shown these characteristics and this type was identified as *Aspergillus sp.b.*

Rhizomucor

There were 13 isolates identified as *Rhizomucor*.

Macroscopic: Colonies characterized by flat, floccose, low aerial growth, and grey to greyish brown-colored mycelium.

Microscopic: sporangiophores shows typical sympodial branching, Sporangia are globose, and there are short rhizoids at base. Isolates showing these characteristics and identified as *Rhizomucor sp*., are RS-B, WS-B, SD-B, FWC-B, GWC-B, CH-B, BC-B, PC-B, VC-B, VC-B, NADEP-B, LC 1-B and LC 2-B.

Rhizopus

Macroscopic: Surface texture deeply cottony; with abundant radial growth touching to lid of the petri plates, the white color of colony become grey brown on surface on prolong incubation. The Reverse of colony is white.

Microscopic: Small sporangia present on short stalks arising from branched rhizoids. Columellae are slightly elongated. *Rhizopus* shows presence of well-developed rhizoids. Only singe isolate was found with these characters NADEP-W and identified as *Rhizopus sp.*

Thermal stability and screening for cellulase production

By observation of fungal growth at 18 and 45°C, from 29 fungal isolates 14 are thermophilic while 15 are found to be thermotolerant in nature. All fungal isolates were found to produce cellulase on CMC agar plate; however, they show widely different cellulolytic index as determined through statistical analysis of cellulolytic index data by one way ANOVA in excel where p value was found to be < 0.01, indicating significant difference in cellulolytic index of fungal isolate. (Table 2) present information regarding distribution of thermophilic and thermotolerant fungi along with their cellulolytic index isolated from compost and soil. (Fig 1) shows degradation of cellulose polymer surrounding growing fungal colonies, which confirms the production and extracellular secretion of hydrolytic enzymes by fungi. Highest cellulolytic index values were obtained for the members of genus *Rhizomucor*, among which maximum value of 2.32 ± 0.20 was obtained for isolate FWC-B. However, plate assay only gives qualitative information on cellulase production ability, further quantitative experiment by measuring cellulase activity during fermentation is needed to find potential producers among isolates.

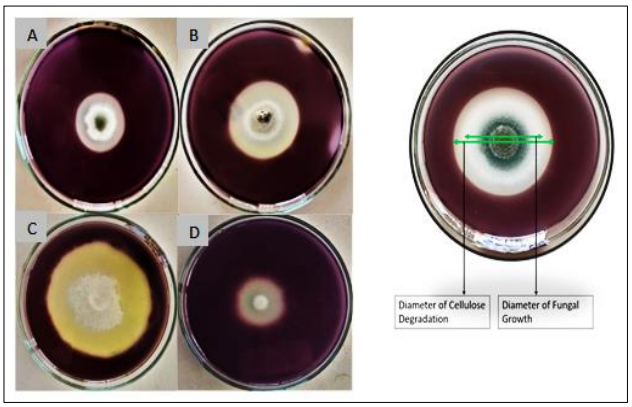

Fig 1 Clear halo zone of cellulose degradation on CMC agar plate after flooding with grams iodine solution (A) *A. fumigatus* FWC-G, (B) *A. quadrilineatus* PC-W, (C) R. *pusillus* FWC-B, (D) *R. microspores* NADEP-W

Table 2 Temperature nature and cellulolytic indices of fungal isolates obtained from various samples

Sample	Fungal isolate	Cellulolytic index	Growth rate		Temperature nature
			18°C	45°C	
Soil treated with rice straw	RS-G	1.32 ± 0.041	+	++	Thermotolerant
	RS-B	2.12 ± 0.10	-	++++	Thermophilic
Soil treated with wheat straw	WS-G	1.17 ± 0.11	+	++	Thermotolerant
	WS-B	1.89 ± 0.11	-	+++	Thermophilic
Soil treated with saw dust	SD-G	1.44 ± 0.05	+	++	Thermotolerant
	SD-B	1.68 ± 0.10	-	++++	Thermophilic
Compost H	CH-G	1.44 ± 0.05	++	+++	Thermotolerant
	CH-B	1.58 ± 0.080	-	++++	Thermophilic
Flower waste compost	FWC-G	1.59 ± 0.08	+	++	Thermotolerant
	FWC-B	2.32 ± 0.20	-	+++	Thermophilic
Garden waste compost	GWC-G	1.35 ± 0.06	+	++	Thermotolerant
	GWC-B	1.89 ± 0.03	-	++++	Thermophilic
Bapalal compost	BC-G	1.24 ± 0.04	+	+++	Thermotolerant
	BC-B	1.62 ± 0.26	-	++++	Thermophilic
Precompost	PC-LG	1.45 ± 0.05	+	+++	Thermotolerant
	PC-G	1.21 ± 0.03	+	++	Thermotolerant
	PC-W	1.30 ± 0.06	-	++	Thermophilic
	PC-B	1.51 ± 0.02	-	++++	Thermophilic
NADEP compost	NADEP-W	1.9 ± 0.1	+++	++++	Thermotolerant
	NADEP-G	1.27 ± 0.08	+	++	Thermotolerant
	NADEP-B	1.98 ± 0.22	-	++++	Thermophilic
	NADEP-LG	1.27 ± 0.02	+	+++	Thermotolerant
Vermicompost	VC-G	1.28 ± 0.04	+	++	Thermotolerant
	VC-W	1.47 ± 0.03	-	++	Thermophilic
	VC-B	1.59 ± 0.04	-	++++	Thermophilic
	VC-LG	1.33 ± 0.03	+	+++	Thermotolerant
Local compost 1	LC1-G	1.25 ± 0.05	+	++	Thermotolerant
	LC1-B	1.34 ± 0.05	-	++++	Thermophilic
Local compost 2	LC2-B	1.32 ± 0.01	-	++++	Thermophilic

Molecular identification

By comparison of ITS rDNA sequences of isolates of this study to those available in the databases using NCBI-BLAST the four morphologically different cellulase producing isolates FWC-G, PC-W, FWC-B and NADEP-W were identified as *Aspergillus fumigatus FWC-G, Aspergillus quadrilineatus PC-W, Rhizomucor pussilus FWC-B* and *Rhizopus microspores NADEP-W*. Sequence of these identified fungi have been submitted genbank database and accession numbers have been received. Phylogenetic tree of identified fungi along with their morphological characteristics are presented in (Fig 2-3).

The finding of this study shows that compost and soil are potential habitat for various cellulase producing thermophilic fungi. The known thermophilic fungi are either ascomycetes members of the orders sordariales, eurotiales, and onygenales or zygomycetes of the order mucorales [5]. During present study16 fungal isolates belonging to ascomycetes order eurotiales and 13 fungal isolates belonging to zygomycetes order mucorale were isolated. From which fungal isolates of genera *Aspergillus* and *Rhizomucor* are found to be very prevalent among analyzed compost and soil samples. The high abundance of these fungi in composting material also noted during other studies like, [18] studied fungal succession during first month of composting of shredded straw of elephant grass added with pig manure and found that initial short period of one to two days is dominated by mesophilic fungi, but as temperature increases to 45°C *A. fumigatus* and *R. pusillus* predominates. One such another study was done by [19] who isolated and identified filamentous fungi from compost during compost maturation period of 21-day of composting plant in Liguria, and found high prevalence of *A. fumigatus* in the mature samples, moreover the study also reported presence of *A. quadrilineatus* and *R. micosporus* during composting process which were also found to be present in precompost and NADEP compost respectively during present study. Dominance of few species of fungi in composting material can be attributed to high temperature during compost maturation that shapes the mycobiota and only few specialized species can remain alive and grow in these particularly adverse conditions [10]. Another reason can be the laboratory conditions used during isolation that favors isolation of fungi which are fast growing and easily obtained from many substrates.

According to qualitative screening for cellulase production on CMC media morphologically different isolates namely FWC-G, PC-W, FWC-B and NADEP-W presents highest cellulolytic indices. Based on morphological characterization isolates FWC-G and PC-W belonged to *Aspergillus*, FWC-B belonged to *Rhizomucor* and NADEP-W belonged to *Rhizopus*. However, identification of fungi based on morphological characters allows classification up to family or genus level only, classification up to species level is not possible solely based on morphology [20]. On the other hand, molecular methods are more objective, yield results that are independent of growth environment, faster compare to phenotypic method and can distinguish among fungi that cannot produce distinctive morphological characters [21]. The ITS region has been used as molecular marker for species level identification from long ago in ecological and taxonomic

studies of fungi [22]. Sharma *et al*. [23] used different molecular approaches like ITS region, 18S rDNA, and D1/D2 hyper variable region for identification of thermophilic fungi and found ITS gene tree as most robust and reliable. Thus, ITS region has been selected in present study for identification of potential fungal isolates. With the help of molecular identification using ITS sequencing FWC-G, PC-W, FWC-B and NADEP-W were closely related to *Aspergillus fumigatus, Aspergillus quadrilineatus, Rhizomucor pusillis* and *Rhizopus microspores* respectively.

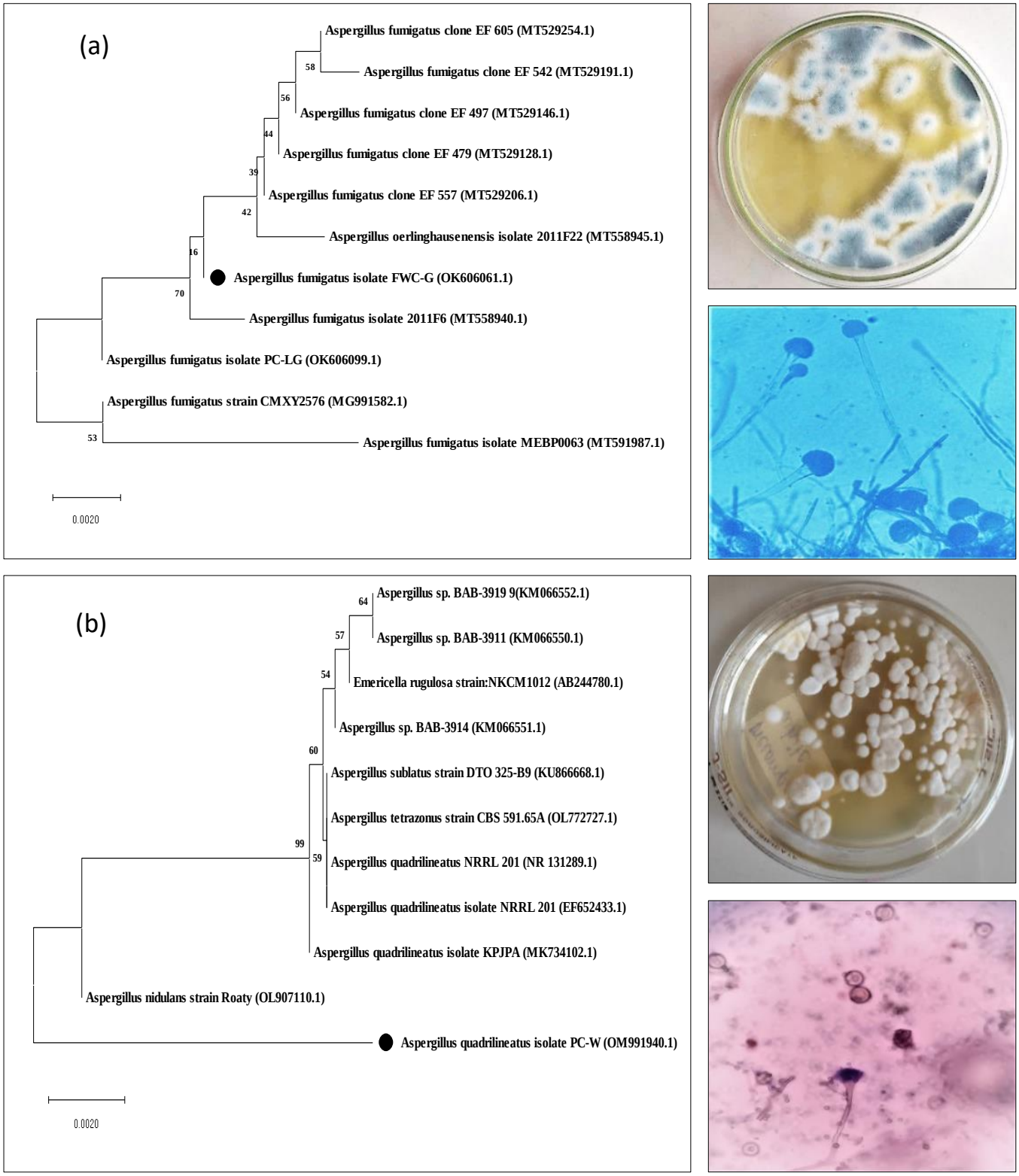

Fig 2 Morphological characterization and Phylogenetic tree of ITS sequences of fungal isolates of ascomycetes (a) *A. fumigatus* FWC-G, (b) *A. quadrilineatus* PC-W. The trees were constructed using neighbor joining (NJ) method with the bootstrap value of 1000

Aspergillus fumigatus is able to grow in temperature range of 12-57°C, and commonly isolated from compost and plant remains particularly when the material is incubated at high temperature [11]. High sporulation capacity of this fungus is responsible for its wide distribution in nature [24]. Production of cellulase has been described for many *Aspergillus* species from which the most promising strains for enzyme production in laboratory screenings have been isolated from sources like compost or agricultural soil, hay, straw or husks from cereal plants [25]. Liu *et al*. [26] used the *A. fumigatus* isolated from

compost and [1] used *A. fumigatus* isolated from sugar cane bagasse for production of cellulases. *Aspergillus* section Nidulantes species are also widely distributed and most commonly found in plant remains, soil, plant and food material [27]. The optimal temperature for their growth is 37°C but can grow up to45°C but not at 50°C [28]. Suryawanshi *et al*. [29] reported production of various ligocellulolytic enzymes like endoxylanase, β-mannanase, β-xylosidase, α-galactosidase, and β-glucosidase through solid state fermentation of copra meal by *A. quadrilineatus*.

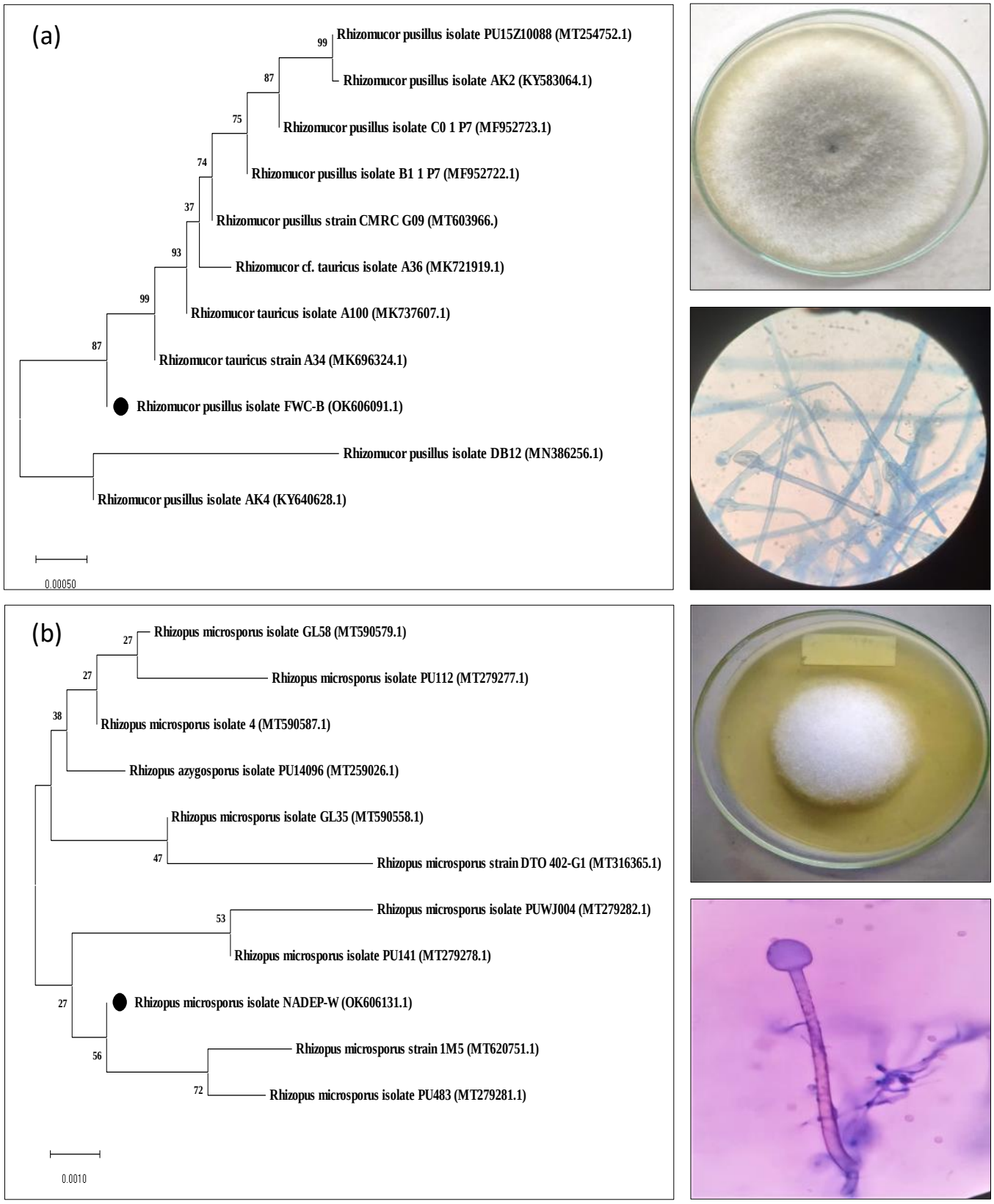

Fig 3 Morphological characterization and Phylogenetic tree of ITS sequences of fungal isolates of zygomycetes (a) *R. pusillus* FWC-B (b) *R. microspores* NADEP-W. The trees were constructed using neighbor joining (NJ) method with the bootstrap value of 1000

Based on the study of Azad *et al*. [30] temperature range for growth of *Rhizomucor pusillus* is 40-50°C and the temperature observed for its maximum growth was 45°C. Same also reported production of enzymes like xylanase, cellulase and polygalacturonase by *Rhizomucor pusillus*. *Rhizopus microsporus* is commonly found in neutral pH and low salinity

soil. Dolatabadi *et al*. [31] found optimum temperature for growth of *Rhizopus micosporus* in the range of 36°C to 40°C, however it also shows well growth at 30°C and 45°C, showing thermotolerance nature. *Rhizopus microspous* have the ability for producing enzymes like cellulase, hemicellulase, pectinase, tannase, phytase, amylase, protease, and lipase [32].

CONCLUSION

The isolation and identification of filamentous fungi from the compost and soil shows the presence and abundance of some potential cellulolytic thermophilic and thermotolerant fungi. Out of total 29 fungal strains 16 were from genus *Aspergillus*, 12 from genus *Rhizomucor* and only 1 isolate from genus *Rhizopus* was isolated. During study it has been realized that while using traditional culture method for isolation of thermophilic and thermotolerant fungi, it favours the growth of some few rapidly growing fungi, which may suppress the growth of other slow growing and less abundant fungal species, this drawback of traditional isolation method can be overcome by use of modern culture independent approaches. However, all the isolated fungi during the study show significant hydrolysis of cellulose on CMC agar plate. Among which the fungal isolate *Rhizomucor pusillus* FWC- B shows highest cellulolytic index of 2.32. Further fermentation studies are required to get quantitative estimation of cellulase activity by different isolated fungi. The cellulolytic thermophilic / themotolerant fungal strains obtained in present study can be used to promote decomposition of cellulosic waste as well as can be used in the design of a production method to obtain extracellular cellulases in large quantities in a bioreactor.

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