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Research Note

Isolation and Analysis of the Enzymatic Properties of Thermophilic Fungi from Compost

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Abstract To the best of our knowledge, this is the first report on thermophilic fungi isolated in Korea. Three species of thermophiles were isolated from compost and were identified as *Myriococcum thermophilum*, *Thermoascus aurantiacus*, and *Thermomyces lanuginosus*. They can grow at temperatures above 50°C and produce high levels of cellulolytic and xylanolytic enzymes at high temperatures. Notably, the considerable thermostability of the endo-glucanase produced by *T. aurantiacus* has made the fungus an attractive source of industrial enzymes.

Keywords Cellulase, Compost, Thermophilic fungi, Xylanase

Thermophilic fungi can grow at temperatures ranging from a minimum of 20°C to a maximum of 50°C or at even higher temperatures. Thermophilic species are present in the natural environment in composts, aquatic sediments, piles of hay, stored grains, wood chip piles, and other accumulations of organic matter wherein the conditions are warm, humid, and aerobic [1]. A number of thermophilic fungi can survive in harsh conditions such as those with increased water pressure, an absence of oxygen, and under desiccation [2]. In a previous study, most thermophiles were isolated from composts [3]; their prevalence in composts can be explained by the high temperatures, humidity, and aerobic conditions within the compost. Moreover, the supply of carbohydrates and nitrogen in this mass of organic matter favors the development of thermophilic microflora [1].

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During the composting process, various organic materials are converted into simpler units of organic carbon and nitrogen. The overall efficiency of organic material break down depends on the microbes and their activities [4]. Thermophilic fungi promote the degradation of organic materials by secreting various types of cellulolytic and xylanolytic enzymes. These fungi might have enzymes that maintain their activities at high temperatures. Enzymes from thermophilic fungi are often more stable at higher temperatures than the enzymes from mesophilic fungi, and some even show stability at 70~80°C [5, 6]. Furthermore, biomass-degrading enzymes from thermophilic fungi consistently demonstrate higher hydrolytic capacity, despite the fact that the enzyme titers are lower than those of the enzymes from more conventionally used species [7].

The aim of this study was to isolate thermophilic fungi from compost and to evaluate the potential of thermostable enzymes from the isolated thermophilic fungi for use in industrial applications requiring high reaction temperatures.

Compost was sampled from four different composting processes (1, 3, 8, and 12 wk) at Sam-Hyup Compost Factory (Dangjin, Korea). The compost consisted of bark, sawdust, livestock waste, and bean-curd dregs at a ratio of 50 : 20 : 15 : 15, respectively. The temperature was measured at a depth of 20 cm from the surface and it ranged from 36°C to 70°C. The samples were spread onto yeast starch agar (4.0 g of yeast extract, 1.0 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 15.0 g of soluble starch, and 20.0 g of agar per liter of distilled water) for ten replicates of each process. The fungi were grown at 50°C and sub-cultured until pure cultures were obtained. In order to identify the isolated

fungi, DNA extractions were performed using the *Accuprep* Genomic DNA extraction kit (Bioneer, Daejeon, Korea). The phylogenetically informative portion of the nuclear ribosomal large subunit rDNA was amplified using primers LR0R (5′-ACCCGCTGAACTTAAGC-3′) and LR3 (5′-CCGTGTTTCAAGACGGG-3′). The generated nucleotide sequences were proofread and compared to those in the GenBank database using a BLAST search. They were aligned using Muscle 3.8.31 [8]. Character based Bayesian Markov Chain Monte Carlo analysis was performed with MrBayes 3.1.2 [9].

Isolated fungi were cultivated at 15~65°C at 5°C intervals for 24~96 hr, so as to determine the temperature range for growth. Fungal growth rate was measured at the optimal growth temperature at 24-hr intervals for 72 hr. For fungal enzyme preparation, the fungi were cultivated in 250 mL Erlenmeyer flasks containing 50 mL Mandels' medium (0.3 g of urea, 1.4 g of KH_2PO_4, 2.0 g of $(NH_4)_2SO_4$, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4$, 0.25 g of yeast extract, 0.75 g of peptone, 5 mg of $FeSO_4 \cdot 7H_2O$, 36 mg of $COCl_2 \cdot 6H_2O$, 1.8 mg of $MnSO_4 \cdot H_2O$, and 2.5 mg of $ZnSO_4 \cdot 7H_2O$ per liter of distilled water), with 1% rice straw as the sole carbon source. Fungal cultures were incubated for a week at 50°C on a rotary shaker at 150 rpm. After cultivation, the samples were centrifuged, and the supernatant was then extracted to determine the enzymatic activities.

Endo-β-1,4-glucanase (EG) and endo-β-1,4-xylanase (XYL) activities were assayed according to standard procedures [10, 11]. The activities of β-glucosidase, cellobiohydrolase (CBH), and exo-β-1,4-xylosidase were determined according to the method of Valásková and Baldrian [12]. The protein content was determined using the Bradford method [13].

Three thermophilic fungi were identified from the compost according to the phylogenetic analysis: *Myriococcum thermophilum*, *Thermoascus aurantiacus*, and *Thermomyces lanuginosus* (Table 1, Fig. 1). They are well-known thermophiles. *T. aurantiacus* and *T. lanuginosus* belong to Eurotiales, Ascomycota and *M. thermophilum* belongs to Sordariomycetes, Ascomycota [14, 15]. The thermophiles grew at temperatures ranging from 25°C to 60°C, thus satisfying the characteristic of being a thermophile (Table 1). The optimal temperature for *M. thermophilum* growth is 45°C and for *T. aurantiacus* and *T. lanuginosus* is 50°C. At the optimal temperature, the growth rate of *T. aurantiacus*

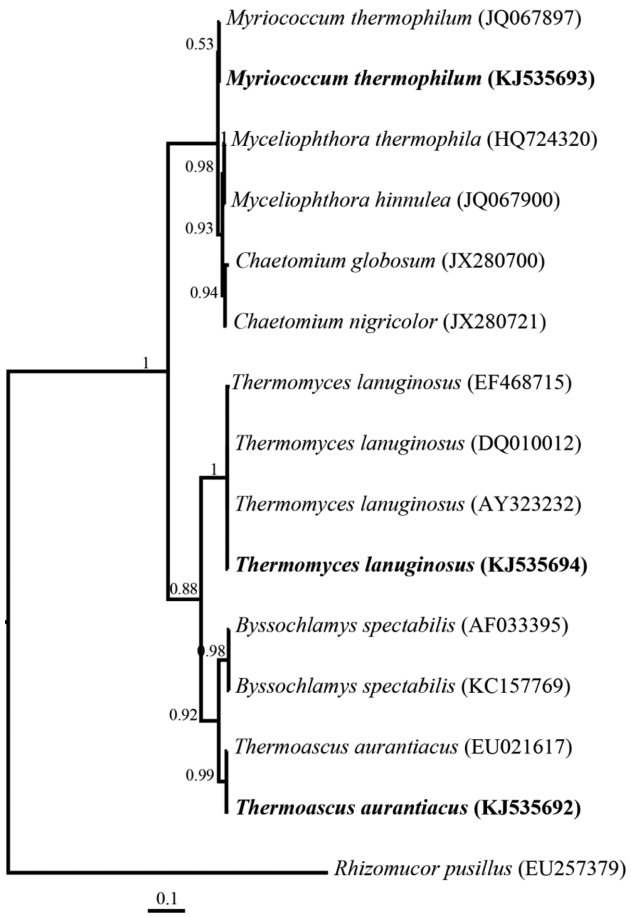

Fig. 1. Consensus phylogram of 1,800 trees resulting from a Bayesian analysis of 15 large subunit rDNA region sequence alignment. The tree was rooted to the sequence of *Rhizomucor pusillus* strain. Fungi found in this study are in bold. GenBank accession numbers are in parentheses.

reached to 8.91 ± 0.72 cm/day. The fungus fully covered the plate within two days. This rapid growth rate could be advantageous if used in industrial processes.

Each thermophile was isolated at particular composting periods. *T. aurantiacus* and *M. thermophilum* were only found in composts after 8 and 12 wk, respectively. On the other hand, *T. lanuginosus* was frequently found in the composts at all time points. *T. lanuginosus* was the only fungus isolated in the first week of composting. This can

Table 1. Thermophilic fungi isolated from compost

Species	Closest fungal match (accession No.)[a]	Similarity (%)[b]	Genbank No.	Temperature range (°C)	Optimum temperature (°C)	Growth rate (cm/day)	Occurrence[c] 1/3/8/12 (wk)
Myriococcum thermophilum	*M. thermophilum* (JQ067897)	556/557 (99)	KJ535693	25~55	45	2.45 ± 0.16	-/-/-/3
Thermoascus aurantiacus	*T. aurantiacus* (EU021617)	558/561 (99)	KJ535692	30~60	50	8.91 ± 0.72	-/-/3/-
Thermomyces lanuginosus	*T. lanuginosus* (AY323232)	570/570 (100)	KJ535694	35~60	50	1.33 ± 0.35	1/10/10/10

[a]Accession numbers of large subunit (LSU) sequences.
[b]Similarity of LSU sequence.
[c]Thermophilic fungi isolated from composts of 1/3/8/12 wk periods with ten replicates.

Table 2. Extracellular protein concentration and the secreted enzyme activities from the thermophilic fungi

Species	Protein concentration (mg/mL)	Enzyme activity (U/mL)				
		EG	CBH	BGL	XYL	BXL
Myriococcum thermophilum	0.21 ± 0.02^{ab}	0.21 ± 0.05^{b}	-	0.54 ± 0.12^{a}	21.3 ± 3.43^{c}	0.07 ± 0.02^{b}
Thermoascus aurantiacus	0.25 ± 0.01^{a}	0.64 ± 0.07^{a}	0.08 ± 0.02^{a}	0.75 ± 0.09^{a}	430.5 ± 33.24^{a}	0.15 ± 0.04^{a}
Thermomyces lanuginosus	0.15 ± 0.05^{b}	0.15 ± 0.03^{b}	-	0.23 ± 0.05^{b}	94.8 ± 21.6^{b}	0.11 ± 0.02^{ab}

Values are presented as mean ± SD.
Numbers followed by the same letter in each row are not significantly different (α = 0.05) according to Tukey's test.
EG, endo-β-1,4-glucanase; CBH, cellobiohydrolase; BGL, β-glucosidase; XYL, endo-β-1,4-xylanase; BXL, exo-β-1,4-xylosidase.

be explained by the relationship between mesophiles and thermophiles. Mesophiles flourish at the premature stage in the composting process. In contrast, thermophiles appear and maintain their growth at later stages, after the compost reaches the highest temperature [16]. Fungal occurrence and diversity might therefore be lower during the first week in the present study because the temperature was not increased sufficiently.

Enzyme activities derived from the three fungal species are shown in Table 2. Except for CBH, all the cellulolytic and xylanolytic enzymes were detected in this study. CBH activity was only detected from *T. aurantiacus*. *T. aurantiacus* showed a considerable ability to produce cellulolytic and xylanolytic enzymes. According to statistical tests, EG and XYL activities from *T. aurantiacus* were significantly higher than those from the other tested thermophilic fungi. In particular, *T. aurantiacus* showed remarkable XYL activity, 430.5 U/mL. EG and XYL secreted by *T. aurantiacus* was stable at a temperature range of 40°C to 80°C. As shown in Fig. 2, XYL activity was stable and slightly increased from 40°C to 60°C, after which it rapidly decreased until 80°C. EG activity gradually increased from 40°C to 70°C; at 80°C, the activity decreased immediately. However, the relative activity of EG at 80°C was still greater than 57% of the highest activity measured at 70°C. These results correlate with those of the previous reports in which thermophilic fungi are regarded as a significant source of thermostable enzymes [14, 16].

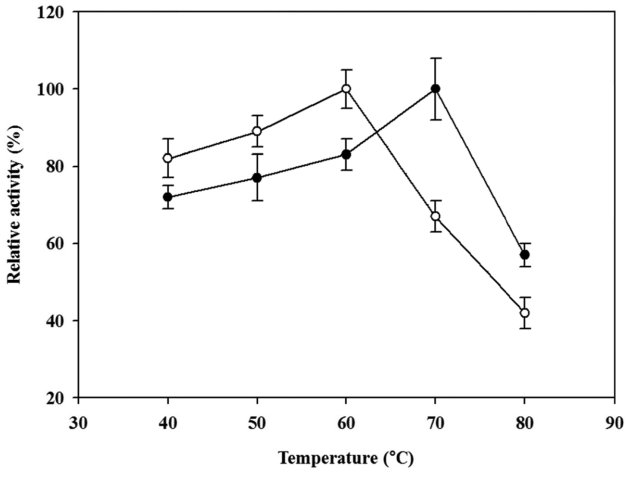

Fig. 2. Effect of temperature on endo-β-1,4-glucanase (●) and endo-β-1,4-xylanase (○) activities by *Thermoascus aurantiacus*.

In this study, three thermophilic fungi were isolated from compost and identified as *M. thermophilum*, *T. aurantiacus*, and *T. lanuginosus*. The fungi showed growth at high temperatures up to 60°C and have optimal growth temperature near 50°C, which satisfy the criteria to be a thermophilic species. In addition, the isolated fungi produced cellulolytic and xylanolytic enzymes. EG and XYL from *T. aurantiacus* had remarkable thermostable properties at a wide range of temperatures. Since the use of enzymes is necessary in some industrial processes, which are carried out at high temperatures, the use of thermophilic fungi in such cases would be advantageous.

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