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Original Article

Acanthophysium sp. KMF001, a New Strain with High Cellulase Activity[1]

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ABSTRACT

Cellulase is an eco-friendly biocatalyst, and its demand is growing in many industrial applications such as food, textile, paper, and bioenergy. Strains with a high cellulase activities are the starting point for the economic production of cellulase. In a previous study, *Acanthophysium* sp. KMF001 with high cellulase production ability was selected among 54 wood-rotting fungi. In this study, we evaluated the cellulase productivity of *Acanthophysium* sp. KMF001 quantitatively and analyzed its taxonomic location using a genetic method. *Acanthophysium* sp. KMF001 showed high cellulase productivity similar to that of *Acanthophysium bisporum* and was much better than *A. bisporum* in specific enzyme activity. The 28S rRNA sequence of *Acanthophysium* sp. KMF001 was similar to that of *Acanthophysium lividocaeruleum* MB1825, with 98.40% homology. Phylogenetic analysis suggested that *Acanthophysium* sp. KMF001 is a new strain. In this study, we propose a new strain with high cellulase productivity.

Keywords: cellulase, *Acanthophysium*, new strain, 28S rRNA, phylogenetic

1. INTRODUCTION

Cellulose is a biopolymer made of glucose, and it is used as a structural biopolymer that is difficult to break down in ecosystems. Cellulase is a collective enzyme that converts cellulose to glucose with three kinds of cellulase: endo-β-1,4-glucanase (EG; EC 3.2.1.4), cellobiohydrolase (CBH; EC 3.2.1.74), and β-glucosidase (BGL; EC 3.2.1.21) (Goyal *et al.*, 1991; Hong *et al.*, 2001; Li *et al.*, 2006; Kim *et al.*, 2015a). For efficient cellulose degradation, EG, CBH, and BGL degrade cellulose enzymatically in sequence (Ali *et al.*, 2013; Biswas *et al.*, 2011; Gao *et al.*, 2014; Haigler and Weimer, 1991; Howard *et al.*, 2002; Schülein, 1988; Zhu *et al.*, 2009). EG randomly hydrolyzes the amorphous regions of cellulose to produce oligosaccharides of various lengths. CBH hydrolyzes the end of cellulose oligosaccharide chains produced by EG and produces cellobiose units. Cellobioses produced by CBH are converted into two glucoses by BGL. The enzymatic efficiency of cellulose is enhanced by the activity and complementary action of enzymes of each type. Many studies showed that wood-rotting fungi degrade cellulose efficiently by the complementary action of cellulase. Therefore, many commercial cellulases are produced from wood-rotting fungi

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such as *Trichoderma viride*, *Trichoderma koningii*, *Penicillium pinophilum*, *Sporotrichum pulverulentum*, *Fusarium solani*, *Aspergillus niger*, *Trichoderma reesei*, and *Penicillium funiculosum* (Castellanos *et al*., 1995; Fang *et al*., 2010; Gao *et al*., 2008; Howard *et al*., 2002; Jamshidian *et al*., 2016; Leathers *et al*., 2015; Lee *et al*., 2010; Pandey *et al*., 2006; Smits *et al*., 1996; Sutivisedsak *et al*., 2013; Zhang *et al*., 2012).

Among fungi, *T. reesei* has already been studied and used extensively in the production of commercial enzymes (Goldbeck *et al*., 2013; Howard *et al*., 2002; Jørgensen *et al*., 2003; Kurabi *et al*., 2005). *Trichoderma* has been reported to be suitable for the production of industrial cellulase because its ability to produce extracellular enzymes is greater than that of bacteria (Rosgaard *et al*., 2006). *T. reesei*, however, cannot break down lignin (Howard *et al*., 2002) and the BGL of *T. reesei* is partially bound to the mycelium, so BGL is not efficiently recovered during enzyme production (Delabona *et al*., 2012). In the case of *Schizophyllum*, it was reported that the extracellular polysaccharide schizophyllan is produced during cellulase production (Esterbauer *et al*., 1991; Jamshidian *et al*., 2016; Leathers *et al*., 2015). Schizophyllan increased the viscosity of culture with cellulase production by forming a jelly-like structure. It is difficult to produce highly purified extracellular cellulase because of the high viscosity of enzyme solution by schizophyllan. In general, good microbial strains for economic enzyme production have a high enzymatic activity and a high specific activity without production of impurities in culture medium. In this study, we report *Acanthophysium* sp. KMF001, which produced cellulase with high activity and high specific activity.

Acanthophysium is a fungus in the Stereaceae family and Basidiomycota phylum and is known to be widespread in nature, but the ecological role of *Acanthophysium* is still unclear.

2. MATERIALS and METHODS

2.1. Strains used in this study

Acanthophysium sp. KMF001 (KCTC 18282P) was deposited at Korea Collection for Type Culture (Jeongeup-si, Korea). *Acanthophysium bisporum* (MUCL 32213), *Acanthophysium cerussatum* (MUCL 32645), and *Acanthophysium lividocaeruleum* (MUCL 33688) were purchased from the Belgian Co-ordinated Collections of Micro-organisms (Ottignies-Louvain-la-Neuve, Belgium) as comparative strains for cellulase activity.

2.2. Culture conditions for the production of crude cellulase

Acanthophysium sp. KMF001 was activated on potato dextrose agar (catalog no. 7349834; BD Biosciences Korea, Seoul, Korea) plates at 30°C for 5 to 7 days. *A. bisporum*, *A. cerussatum*, and *A. lividocaeruleum* were activated on malt extract agar (20 g/L malt extract, 20 g/L glucose, 1 g/L peptone, and 20 g/L agar) plates at 25°C for 5 to 7 days.

The activated *Acanthophysium* sp. KMF001 was inoculated in 100 mL of potato dextrose broth (catalog no. 8129687; BD Biosciences Korea) and incubated at 30°C for 5 days under the shaking condition at 150 rpm to make a pre-culture. For *A. bisporum*, *A. cerussatum*, and *A. lividocaeruleum*, the activated cells were inoculated in 50 mL of malt extract broth (catalog no. 218630; BD Biosciences Korea) and incubated at 25°C for 5 days under the shaking condition at 150 rpm to make a pre-culture.

In the main culture, all four strains were inoculated with 5% (v/v) pre-culture in 200 mL modified TYE medium with cellulose [7 g/L tryptone, 3 g/L yeast extract, 5 g/L K_2HPO_4, 5 g/L KH_2PO_4, 3 g/L $MgSO_4 \cdot 7H_2O$, and 20 g/L cellulose (20–100 mm; catalog no.25384405; Daejung, Seoul, Korea) at pH 6.0] in

a 1 L baffled flask and incubated for 3 weeks under the shaking condition at 150 rpm at 30°C for *Acanthophysium* sp. KMF001 and at 25°C for *A. bisporum*, *A. cerussatum*, and *A. lividocaeruleum*.

2.3. Cellulase production

The crude supernatant from each culture was collected, cells were removed by filtration using Whatman filter paper No. 1 (catalog no. 1001-110; GE Healthcare Life Sciences, Seoul, Korea). The cell free supernatant of each was analyzed on the cellulase activity.

2.4. Measuring enzymatic activity of cellulase

For the EG activity assay, 5 μL of crude enzyme solution was mixed with 45 μL of substrate solution made with 2% (w/v) carboxylmethylcellulose sodium salt (catalog no. MKBW6753V; Sigma-Aldrich Korea LLC, Yongin, Korea) in 0.1 M sodium citrate buffer at pH 4.5. The mixture was incubated at 50°C for 30 minutes. The reaction was stopped by adding 50 μL of copper solution and heating at 100°C for 10 minutes. The amount of produced reducing end by EG was measured using the Somogyi–Nelson method (Nelson, 1944). One unit of EG was defined as the amount of enzyme producing 1 μmol glucose for 30 minutes under the described reaction condition.

For the CBH activity assay, 100 μL of crude enzyme solution was mixed with 100 μL of 10 mM *p*-nitrophenyl-β-D-cellobioside (catalog no. 077M4023V; Sigma-Aldrich Korea LLC) and 800 μL of 0.1 M sodium citrate buffer at pH 4.5. The mixture was incubated at 50°C for 15 minutes. The reaction was stopped by adding 100 μL of 2 M Na_2CO_3. The amount of produced *p*-nitrophenol by CBH was measured at 405 nm using a Opsys MR microplate reader (Hynex Technologies, Chantilly, VA, USA). One unit of CBH was defined as the amount of enzyme producing 1 μmol *p*-nitrophenol for 15 minutes under the described reaction condition.

For the BGL activity assay, all measuring procedure was similar with the above CBH activity assay with switching the substrate to 10 mM p-nitrophenyl-β-D-glycopyranoside (catalog no. BCBP2339V; Sigma-Aldrich Korea LLC). Enzymatic activity was defined as the enzyme activity of the crude solution based on the volume and expressed in U/mL.

2.5. Measuring specific activity of cellulase

To calculate a specific activity of each cellulase, the protein concentration was measured using the Bradford's methods with bovine serum albumin as a standard (Bradford, 1976). Enzyme with 400 μL and protein assay reagent with 100 μL (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were mixed violently in tubes for 10 seconds. The absorbance was measured at 595 nm after 5-minute incubation in room temperature. The specific activity of enzymes was defined as the cellulase activity per milligram of total protein and expressed in U/mg.

2.6. Genetic analysis of *Acanthophysium* sp. KMF001

Cells of *Acanthophysium* sp. KMF001 from the main culture were disrupted with TissueLyser LT (Qiagen Korea Ltd., Seoul, Korea), and the genomic DNA was purified using the DNeasy Plant Mini kit (Qiagen Korea Ltd.). The internal transcribed spacer region of 28S rRNA was amplified using polymerase chain reaction (PCR) with two primers: ITS4 (5′-TCCTCCGCTTA TTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGT CGTAACAAGG-3′). The PCR condition with 35 cycles was 95°C for 20 seconds for denaturation, 55°C for 4 seconds for annealing, and 72°C for 60 seconds for

extension. The amplified DNA was sequenced by Biofact Co., Ltd. (Daejeon, Korea). The analyzed sequence was registered at GenBank at the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) under MM680758.

The homologous sequences in the nucleotide database were identified using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). They were aligned, and phylogenetic trees were analyzed using MEGA4 software (Tamura *et al*., 2007). The corresponding ribosomal DNA region of each strain was used for making phylogenetic trees with the distance methods based on the similarity.

3. RESULTS and DISCUSSION

3.1. Cellulase activity of *Acanthophysium* spp.

Acanthophysium sp. KMF001 was selected among 54 wood-rotting fungi based on the cellulase activity (Kim *et al*., 2015b). To evaluate the cellulase activity of *Acanthophysium* sp. KMF001, we purchased three *Acanthophysium* strains, namely, *A. bisporum*, *A. cerussatum*, and *A. lividocaeruleum*, and the cellulase activities of all four strains, including *Acanthophysium* sp. KMF001, were analyzed (Fig. 1). Cellulase production was analyzed on the basis of culture medium volume (Fig. 1A, 1C, and 1E) and protein weight for the specific activity (Fig 1B, 1D, and 1F). As shown in Fig. 1, *A. cerussatum* and *A. lividocaeruleum* showed low enzymatic and specific activity of cellulase, and *Acanthophysium* sp. KMF001 and *A. bisporum* showed strong cellulase activity among the four tested strains.

The EG activity of *Acanthophysium* sp. KMF001 showed the highest enzymatic activity at 94.54 U/mL on 15 days of cultivation and similar enzyme activity until 21 day. On the other hand, EG enzyme activity of *A. bisporum* showed a similar level of EG activity to *Acanthophysium* sp. KMF001 at 15 days of cultivation, but the enzyme activity decreased with further incubation time after that (Fig. 1A). However, the specific activities of EG in *Acanthophysium* sp. KMF001 and *A. bisporum* showed 245 U/mg and 165 U/mg, respectively, at the 15 days of cultivation (Fig. 1B). These results indicated that the enzyme productivity per unit volume of the culture was similar in both strains, but the cellulase content based in the protein released to the outside of the cell in *Acanthophysium* sp. KMF001 was 1.5 times higher than that in *A. bisporum*. The other two cellulase enzyme activities, namely, CBH (Fig. 1D) and BGL (Fig. 1F), showed similar differences in specific enzyme activities. The production of such high-purity cellulase is advantageous for enzyme production and application.

The CBH activities on the 15th day of cultivation with *Acanthophysium* sp. KMF001 and *A. bisporum* showed 6.83 U/mL and 7.19 U/mL, respectively (Fig. 1C). The BGL activities on the 18th day of cultivation in *Acanthophysium* sp. KMF001 and *A. bisporum* showed 63.20 U/mL and 57.46 U/mL, respectively (Fig. 1E). The specific activity of CBH and BGL in *Acanthophysium* sp. KMF001 was higher, with 17% and 28% more, respectively, than those in *A. bisporum* (Fig. 1D and 1F, respectively). In particular, on the 15th day of cultivation, the specific CBH activity in *Acanthophysium* sp. KMF001 was about double of that in *A. bisporum* (Fig. 1D), and the specific BGL activity in *Acanthophysium* sp. KMF001 on the 12th day of cultivation was also about double of that in *A. bisporum* (Fig. 1F). In the case of *Acanthophysium* sp. KMF001, the decrease in CBH activity was moderate after the 15th day of cultivation, but the CBH activity of *A. bisporum* rapidly decreased during the same incubation time.

On the basis of the above results, all enzymatic cellulase activities of *Acanthophysium* sp. KMF001 were similar to that of *A. bisporum*, but the specific

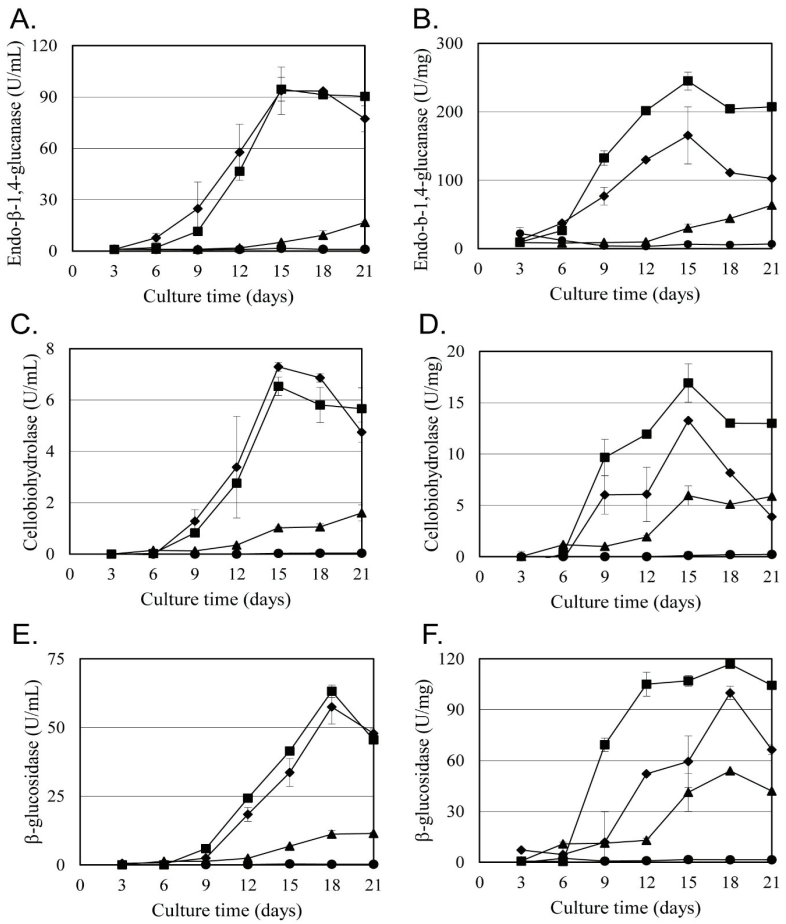

Fig. 1. Cellulase activity of four strains: *Acanthophysium* sp. KMF001 (■), *A. bisporum* (◆), *A. cerussatum* (▲), and *A. lividocaeruleum* (●). (A and B) EG activity, (C and D) CBH activity, and (E and F) BGL activity. (A, C, and E) Enzymatic activity in the crude solution based on the culture volume and (B, D, and F) specific activity of the enzyme based on the protein weight. The values are the average of three replicates. The standard deviations are shown.

activity of *Acanthophysium* sp. KMF001 showed significantly higher levels of all tested enzyme activities than *A. bisporum*. This observation suggested that *Acanthophysium* sp. KMF001 was better than the others in *Acanthophysium* spp. tested in this study for high cellulase production. In a previous study, the CBH and BGL activities of *Acanthophysium* sp. KMF001 were higher than it of *Trichoderma reesei*, *Fomitopsis palustris*, and *Aspergillus niger* (Kim *et al.*, 2015b). Considering the enzyme activities in the culture solution and specific activities in this study, the 15th day of cultivation is ideal for cellulase production of *Acanthophysium* sp. KMF001. In a previous study, the crude cellulase of *Acanthophysium* sp. KMF001 improved the quality of fabric effectively (Shin *et al.*, 2016). In addition to effective cellulase production, *Acanthophysium* sp. KMF001 produced a novel endo-β -1,4-xylanase, which was an enzyme for hemicellulose

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  1 AGCGGAGGAA AAGAAACTAA CAAGGATTCC CCTAGTAACT GCGAGTGAAG CGGGAAAAGC 60
 61 TCAAATTTAA AATCTGGCGG CCTCTGGTCG TCCGAGTTGT AGTCTGGAGA AGCGTTTTCC 120
121 GCGTTGGACC GTGTACAAGT TTCCTGGAAC GGAGCGTCAT AGAGGGTGAG AATCCCGTCT 180
181 TTGACACGGA TCCCAATGCT TTGTGATGCG CTCTCAAAGA GTCGAGTTGT TTGGGAATGC 240
241 AGCTCAAAAT GGGTGGTGAA TTCCATCTAA AGCTAAATAT TGGCGAGAGA CCGATAGCGA 300
301 ACAAGTACCG TGAGGGAAAG ATGAAAAGCA CTTTGGAAAG AGAGTTAAAC AGTACGTGAA 360
361 ATTGTTGAAA GGGAAACGCT TGAAGTCAGT CGCGTCGGCC GGGACTCAGC CTTGCATTCG 420
421 CTTGGTGTCA TTTCCGGTCG ACGGGCCAGC ATCAGTTTTG ATCGCGGGAT AAAGGCGGAG 480
481 GGAATGTGGC TCTTTCGGGA GTGTTATAGC CCTCTGTCGG ATGCCGTGGT TGGGACTGAG 540
541 GAACTCAGCA CGCCTTTATG GCCGGGGTTC GCCCACGTAC CTGCTTAGGA TGCTGGCGTA 600
601 ATGGCTTTAA ACGACCCGTC TTGA
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Fig. 2. Base sequence of 28S rRNA in *Acanthophysium* sp. KMF001. This sequence was registered at GenBank at the NCBI under MM680758.

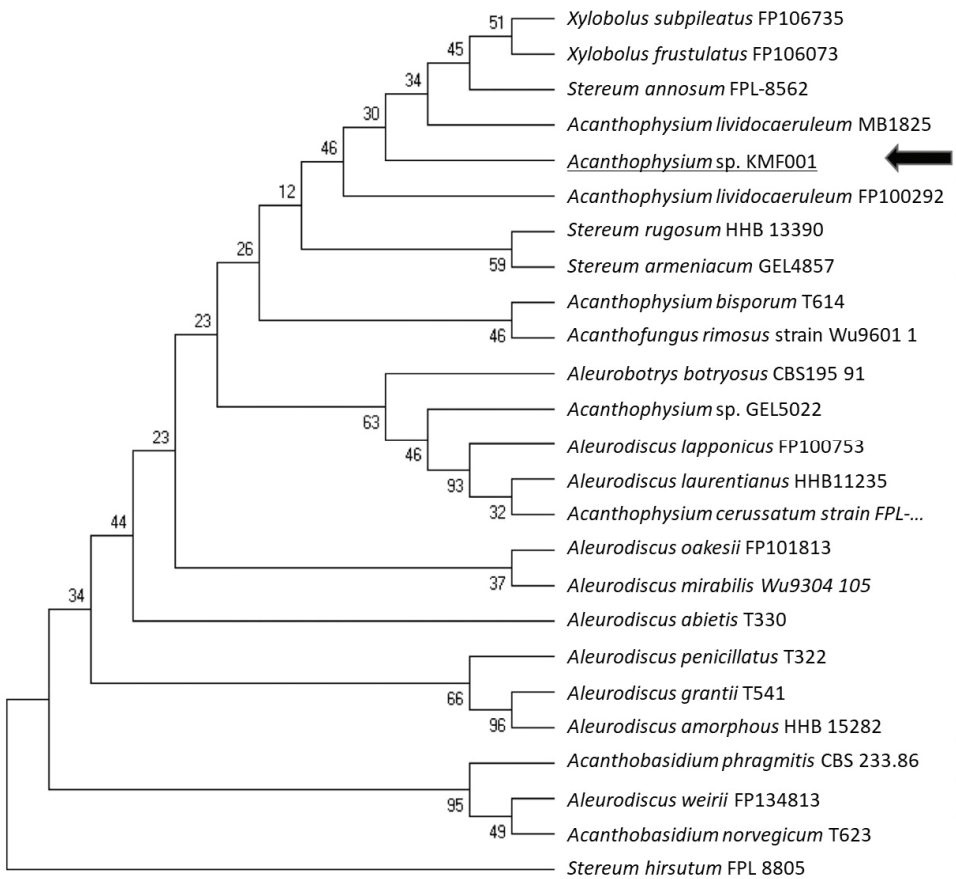

Fig. 3. Phylogenetic trees of *Acanthophysium* sp. KMF001 with other related fungal species.

Table 1. Homology search by the NCBI, *Acanthophysium* sp. KMF001

Accession number	Description	Query coverage (%)	Expect value	Identity (%)
AY039314	*A. lividocaeruleum* MB1825	100	0.0	98.40
AF506400	*A. lividocoeruleum*	100	0.0	98.24
MH877876	*Xylobolus subpileatus* CBS 126032	100	0.0	97.92
MH866478	*Xylobolus frustulatus* CBS 271.28	100	0.0	97.92
KX066002	*X. subpileatus* FP102567	100	0.0	97.92
AF506491	*X. frustulatus* KGN980928	100	0.0	97.92
AY039319	*A. lividocaeruleum* FP100292	100	0.0	97.92
AY039307	*X. frustulatus* FP106073	100	0.0	97.92
MH867100	*X. subpileatus* CBS 415.34	100	0.0	97.76
AY039309	*X. subpileatus* FP106735	99	0.0	97.91
AB733325	*Stereum hirsutum* NBRC 6520	100	0.0	97.60
AY039310	*A. cerussatum* HHB11294	100	0.0	97.60
AF042563	Unidentified basidiomycete	100	0.0	97.60
AY293189	*Hypsizygus tessulatus*	100	0.0	97.60

degradation (Yoon *et al.*, 2018). All studies suggested that *Acanthophysium* sp. KMF001 was a good enzyme-producing fungus for decaying wood.

3.2. Genetic identification of *Acanthophysium* sp. KMF001

To identify *Acanthophysium* sp. KMF001 genetically, the genetic information of its 28S rRNA was analyzed (Fig. 2). The results of the BLAST search with the 28S rRNA nucleotide sequence of *Acanthophysium* sp. KMF001 using the NCBI nucleotide database are shown in Table 1. The most homologous strain was *A. lividocaeruleum* MB1825, with 98.40% identity. The next most homologous strains were *Acanthophysellum lividocoeruleum*, with 98.24% identity, and additional six strains with 97.92% identity (Table 1). Using phylogenetic analysis with strains in Table 1, the taxonomic position of *Acanthophysium* sp. KMF001 was analyzed in Fig. 3. The phylogenetic tree analysis revealed that *Acanthophysium* sp. KMF001 separated into different phylogenetic branches from *A. lividocaeruleum* MB1825, the most similar strain with 99% identity by Nucleotide BLAST search. Although *Acanthophysium* sp. KMF001 was similar to the previously reported *A. lividocaeruleum* MB1825 with an identity of 98.40%, the phylogenetic analysis suggested that *Acanthophysium* sp. KMF001 might be a new strain.

4. CONCLUSION

Comparison of the enzyme activity with other strains of *Acanthophysium* genus showed that *Acanthophysium* sp. KMF001 had similar levels of cellulase activity with *A. bisporum* in all three types, namely, EG, CBH, and BGL, but the specific enzyme activity of *Acanthophysium* sp. KMF001 was higher than cellulase from other tested *Acanthophysium* strains. Although *Acanthophysium* sp. KMF001 showed 98.40% nucleotide identity with the most homologous strain, the phylogenetic tree suggested that *Acanthophysium*

sp. KMF001 is a new strain by separating into a different phylogenetic branch from the most homologous strain. This study suggested that *Acanthophysium* sp. KMF001 is a novel bacterium that produces an effective cellulase for the wood decay.

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