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A Thermophilic GH5 Endoglucanase from *Aspergillus fumigatus* and Its Synergistic Hydrolysis of Mannan-Containing Polysaccharides

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Abstract: In this study, we isolated and identified a thermophilic strain of *Aspergillus fumigatus* from the "Daqu" samples. Transcriptomic analysis of *A. fumigatus* identified 239 carbohydrate-active enzymes (CAZy)-encoding genes, including 167 glycoside hydrolase (GH)-encoding genes, 58 glycosyltransferase (GT)-encoding genes, 2 polysaccharide lyase (PLs)-encoding genes and 12 carbohydrate esterase (CEs)-encoding genes, which indicates that the strain has a strong potential for application for enzyme production. Furthermore, we also identified a novel endoglucanase gene (*AfCel5A*), which was expressed in *Pichia pastoris* and characterized. The novel endoglucanase AfCel5A exhibited the highest hydrolytic activity against CMC-Na and the optimal activity at 80 °C and pH 4.0 and also showed good stability at pH 3.0–11.0 and below 70 °C. The Km and Vmax values of AfCel5 were 0.16 ± 0.05 mg·mL^{-1} and 7.23 ± 0.33 mol mg^{-1}·min^{-1}, respectively, using CMC-Na as a substrate. Further, the endoglucanase exhibited a high tolerance toward NaCl as well as glucose. In addition, the finding that the endoglucanase AfCel5A in combination with β-mannanse (ManBK) clearly increased the release of total reducing sugars of glucomannan by up to 74% is significant.

Keywords: *Aspergillus fumigatus*; transcriptome; thermophilic endoglucanase; expression; synergistic

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1. Introduction

Lignocellulose is the richest renewable source for the industrial production of fuels and chemicals and is comprised of lignin, cellulose and hemicellulose [1–3]. However, large tonnages of unexploited lignocellulosic pose a series of environmental problems [4]. Due to lignocellulose's structural complexity, the complete depolymerization of lignocellulose demands the synergy of a glycoside hydrolase [5]. The endoglucanase EC 3.2.1.4 plays a significant role in lignocellulose hydrolysis, as it can break down lignocellulose into cellobiose and glucose [6]. Commonly, endoglucanases are often known as cellulase, which is widely used in feed additives, pulp and biofuel industries [7,8]. Nowadays, endoglucanases can be isolated from bacteria and fungi, particularly the *Trichoderma* and *Aspergillus* spp. [9,10]. As reported, the optimum pH of endoglucanases from fungi is between pH 3.0 and 6.0, and the optimum temperature is in the range of 40–60 °C [11]. However, circumventing the poor tolerance and the low catalytic efficiency of endoglucanases remains a significant research focus.

Thermophilic enzymes have strong thermal stability and high catalytic efficiency, providing a comprehensive prospect in lignocellulosic biomass utilization [12,13]. Thermophiles have been commonly used as thermophilic enzyme producers and have always been obtained from hot springs, deep-sea vent fields and other thermal environments [14–16]. However, this is particularly unfavorable and demanding for the researchers. Daqu is a unique saccharogenic and fermentative agent and is considered a good source of thermophilic microorganisms [17–19].

As previously mentioned, mannans are the primary hemicellulose components of softwoods [20], and the efficient degradation of mannans is required for many enzymes working synergistically [21]. However, extensive research mainly explores the synergy of β-mannanase, β-mannosidase or α-galactosidase [22]. In addition to the above, other enzymes or proteins should also play a significant role in mannan hydrolysis. Jeon et al. reported that endoglucanase could promote galactomannan degradation [23]. A similar phenomenon has also been found in xylanase and mannanase [24]. However, knowledge about the synergistic effect of thermophilic mannanase and cellulases is scarce.

In this study, AfCel5A, a new thermophilic cellulase from *A. fumigatus*, was efficiently expressed in *P. pastoris* and biochemically characterized. The optimum temperature of AfCel5A is 80 °C, and the optimum pH is 4.0. AfCel5A exhibits excellent stability, including pH stability, thermal stability and salt and glucose tolerance. We applied the endoglucanase AfCel5A to enhance mannan hydrolysis by up to 75% with β-mannanase (ManBK). Thus, this work provides an effective strategy for the utilization of lignocellulose resources.

2. Results

2.1. Isolation and Identification of the Thermophilic Fungi

Daqu is a fermenting starter that has commonly been used in Chinese liquor fermentation for hundreds of years. In this study, HBHF5 strain producing higher endoglucanase activity was successfully isolated from a Daqu sample (Figure 1A).

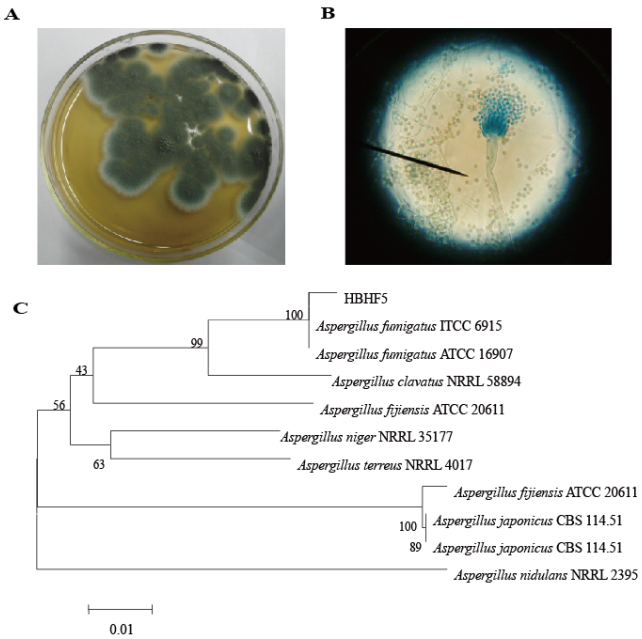

Figure 1. Identification of isolated *A. fumigatus* HBHF5 strain. (**A**) Strain HBHF5 cultivated on PDA plate at 45 °C. (**B**) Morphology of strain HBHF5 stained with lactophenol cotton blue. (**C**) Phylogenetic tree analysis of strain HBHF5.

Colonies of strain HBHF grew rapidly at 45 °C as dark blue-green colonies with a floccose-like surface but did not grow at temperatures lower than 19 °C (Figure 1A). Microscopic observation showed a typical broom flask-shaped conidiophore like other Aspergillus species (Figure 1B). The ITS rDNA sequence of the HBHF5 strain showed that its sequence is most closely related to *A. fumigatus* rDNA sequence with 99% similarity (Figure 1C). In conclusion, strain HBHF5 was identified as *A. fumigatus* HBHF.

2.2. Transcriptome Analysis of A. fumigatus HBHF

To further explore the gene expression of *A. fumigatus* HBHF under wheat bran conditions, the transcriptome was analyzed by RNA-Seq and is listed in Table 1. A total of

239 genes could be assigned to different CAZy families based on the CAZy database (http://www.cazy.org, accessed on 26 May 2021). Glycoside hydrolases (GHs) were the largest category in the expressed CAZy genes, accounting for 69.8%, followed by glycosyltransferases (GTs, 24.3%) and carbohydrate esterases (CEs, 5.0%). Finally, polysaccharide lyases (PLs) only accounted for ~0.8% and played a less critical role in the degradation of wheat bran. Further analyses identified these GH genes, including chitinase, cellulase, amylase and mannanase. In summary, this finding further demonstrated that *A. fumigatus* HBHF5 is a good producer of glycoside hydrolases and is worthy of further evaluation.

Table 1. CAZy-encoding genes identified from the transcriptome of *A. fumigatus* HBHF5.

CAZy Family	Gene Number	Gene ID
CE1	1	AFUA_5G09860
CE4	5	AFUA_4G12330, AFUA_1G10540, AFUA_2G05900, AFUA_3G00520, AFUA_4G12120
CE8	3	AFUA_3G07650, AFUA_8G06880, AFUA_3G07650
CE9	1	AFUA_8G04100
CE12	1	AFUA_1G03890
PL1	2	AFUA_2G00760, AFUA_7G06400
GH1	2	AFUA_2G05580, AFUA_3G06060
GH5	2	AFUA_6G08840, AFUA_7G01320
GH13	6	AFUA_1G15150, AFUA_2G13460, AFUA_3G07380, AFUA_4G10130, AFUA_5G10540, AFUA_2G00710
GH15	3	AFUA_2G00690, AFUA_3G00610, AFUA_4G10140
GH18	12	AFUA_1G02800, AFUA_3G07110, AFUA_3G07160, AFUA_3G11280, AFUA_5G01400, AFUA_5G03760, AFUA_5G03850, AFUA_7G05140, AFUA_8G01410 AFUA_5G03960, AFUA_5G06840, AFUA_8G00700,
GH20	4	AFUA_2G00640, AFUA_8G05020, AFUA_3G11780, AFUA_8G04060
GH28	6	AFUA_1G17220, AFUA_4G13920, AFUA_1G17220, AFUA_8G01970, AFUA_8G02630, AFUA_8G06890
GH30	13	AFUA_1G05770, AFUA_1G14710, AFUA_1G16400, AFUA_1G17410, AFUA_5G07080, AFUA_5G07190, AFUA_6G03570, AFUA_6G08700, AFUA_6G12010, AFUA_7G06140, AFUA_3G00230, AFUA_5G07080, AFUA_8G07120
GH32	3	AFUA_2G01240, AFUA_5G00480, AFUA_6G05000
GH33	1	AFUA_4G13800
GH35	7	AFUA_1G14170, AFUA_3G00380, AFUA_5G14090, AFUA_5G14550, AFUA_6G06660, AFUA_4G00390, AFUA_1G16700
GH36	5	AFUA_1G01200, AFUA_5G02130, AFUA_5G13830, AFUA_8G01100, AFUA_8G01130
GH54	2	AFUA_1G16920, AFUA_3G02090
GH55	6	AFUA_1G03600, AFUA_1G14450, AFUA_6G13270, AFUA_1G11460, AFUA_3G07520, AFUA_6G09250
GH62	2	AFUA_1G09900, AFUA_2G15160
GH63	5	AFUA_1G16250, AFUA_4G10150, AFUA_8G07070, AFUA_5G03500, AFUA_6G04210
GH65	1	AFUA_4G13530
GH74	8	AFUA_2G09520, AFUA_3G03870, AFUA_5G01830, AFUA_6G01800, AFUA_6G07480, AFUA_6G11600, AFUA_7G01540, AFUA_7G06740
GH75	4	AFUA_3G14980, AFUA_4G01290, AFUA_6G00500, AFUA_8G00930
GH92	5	AFUA_1G14560, AFUA_4G10070, AFUA_6G06790, AFUA_6G12360, AFUA_3G08200

Table 1. *Cont.*

CAZy Family	Gene Number	Gene ID
GT2	10	AFUA_3G10400, AFUA_5G08210, AFUA_1G12600, AFUA_2G01870, AFUA_2G13430, AFUA_2G13440, AFUA_3G14420, AFUA_4G04180, AFUA_5G00760, AFUA_8G05630
GT4	3	AFUA_4G13400, AFUA_5G13210, AFUA_6G06940
GT5	1	AFUA_5G02480
GT15	1	AFUA_1G06890
GT20	6	AFUA_6G12950, AFUA_2G04010, AFUA_3G05650, AFUA_3G07370AFUA_7G03940, AFUA_2G04020,
GT21	1	AFUA_5G09550
GT22	1	AFUA_1G13870
GT33	1	AFUA_6G14180
GT35	1	AFUA_1G12920
GT39	3	AFUA_1G07690, AFUA_3G06450, AFUA_8G04500
GT48	1	AFUA_6G12400
GT58	1	AFUA_5G11990
GT59	1	AFUA_2G11080
GT66	1	AFUA_8G04430
GT90	16	AFUA_2G02360, AFUA_5G14780, AFUA_6G14480, AFUA_2G01450, AFUA_2G14910, AFUA_2G15910, AFUA_4G10750, AFUA_5G13090, AFUA_6G04450, AFUA_6G14040, AFUA_7G01300, AFUA_5G10760, AFUA_4G09130, AFUA_4G11280, AFUA_5G06050, AFUA_1G01380

2.3. Sequence Analysis, Expression and Purification of AfCel5A

A putative endoglucanase *AfCel5A* gene (accession No. XP_751043.1) was found in *A. fumigatus* HBHF5. The full-length gene *AfCel5* contained 1397 bp and was interrupted by four introns (84–131, 343–388, 454–512, 611–660) (Figure 2A). The AfCel5 gene contains an open reading frame (ORF) of 1194 bp, encoding a protein of 397 amino acids in length and a signal peptide of 16 amino acids at the *N*-terminus. The mature protein contains three functional domains: a catalytic domain belonging to the GH5 family (35–327 aa), a CBM1 domain (365–397 aa) and a linker region (328–364 aa) (Figure 2B). The theoretical molecular weight of the mature AfCel5 was 42.6 kDa, and the isoelectric point was 4.89.

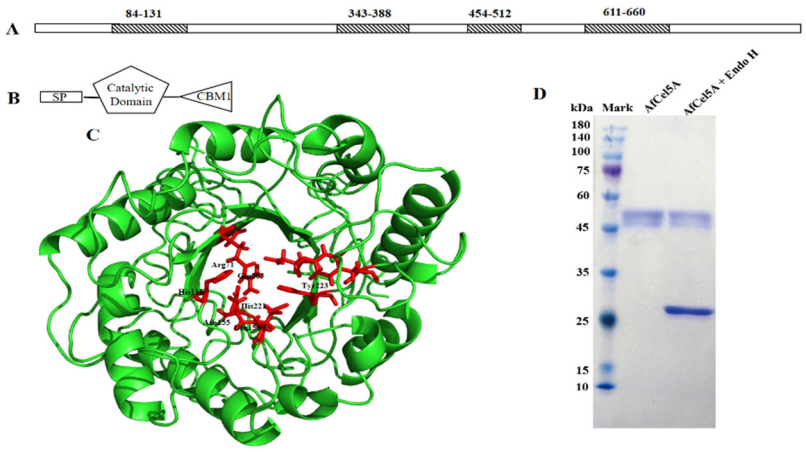

Figure 2. Structure analysis of endoglucanase AfCel5A. (**A**) The exon/intron structure analysis of gene AfCel5A. (**B**) The domain analysis of the AfCel5A protein. (**C**) Three-dimensional structure of the AfCel5A protein. (**D**) SDS-PAGE analysis of the AfCel5A protein.

Utilizing endoglucanase (PDB:1H1N) as a structural template, the three-dimensional model(3D) of AfCel5A protein was constructed by homology modeling approach (Figure 2C). After validation using Procheck, the final model was found to be of good quality as 91.1% of the total residues fall in the most favored regions. The 3D structure of AfCel5A displays a (βα)8 TIM-barrel fold structure, similar to another endoglucanase of the GH5 family (Figure 2C). Based on the multiple sequence alignment, the result indicated that deduced AfCel5A contained seven highly conserved residues of the GH5 family: Arg72, His116, Asn155, Glu156, His221, Tyr223 and Glu263. Meantime, AfCel5A showed the highest sequence homology of 89.2% to endoglucanase from *Aspergillus udagawae* (GFF23991.1), 69.9% to that from *Talaromyces leycettanus* (AYW35863.1) and 58.5% to that from *Aspergillus terreus* (AAW68436.2). AfCel5A gene was successfully heterologously expressed in *P. pastoris* GS115 host. We found that the endoglucanase activity can reach 3.74 U·mL^{-1} after 72 h induction. The culture supernatant was collected, purified and concentrated to apparent homogeneity. AfCel5A has an apparent molecular mass on SDS-PAGE of around 50 kDa (Figure 2D), larger than the inferred 42.6 kDa. *N*-glycosylation prediction showed that AfCel5A had one unique *N*-glycosylation site at residues 32–35. However, its treatment with EndoH did not alter the molecular weight to the theoretical value. Further analyses identified many O-glycosylation sites in the Thr/Ser-rich linker domain, which is likely why the molecular weight was higher.

2.4. Characteristics of Recombinant AfCel5A

In general, an enzyme derived from thermophilic microorganisms has a high reaction temperature and excellent stability, which are essential parameters in determining its potential commercial applications. AfCel5A displayed an optimal reaction temperature at 80 °C and still retained more than 30% activity at 90 °C (Figure 3A). Meanwhile, AfCel5 showed a highly stable activity after incubating at 70 °C for 1 h, whereas after 1 h incubation at 75 °C, 70% of the activity remained (Figure 3C). The highest enzyme activity of AfCel5A was observed at pH 4.0 (Figure 3B), while the enzyme was utterly inactive at pH less than 2.0 or higher than 10.0. AfCel5A was also remarkably stable in a wide pH range (pH 2.0 to 12.0) and maintained approximately 90–100% of activity (Figure 3D).

As shown in Table 2, most metal ions had a little inhibitory effect on the AfCel5 activity, with Mn^{2+} and Ag^{+} being the exception. Mn^{2+} showed a promoting effect on endoglucanase activity at low concentrations, while it had the opposite effect at a high concentration. Likewise, the chemical reagent SDS showed a strong inhibitory effect on AfCel5 activity. In addition to this, AfCel5 exhibited an individual tolerance to surfactants and other organic reagents, such as urea, EDTA and acetone.

AfCel5A exhibited distinct features in terms of substrate specificity. The enzyme activity was set at 100% for reactions with 1% CMC-Na as the substrate; AfCel5A displayed higher activity for KGM (129%), followed by guar gum (57%), pectin (31%) and Avicel (27%), and no activity was detected for LBG. The apparent Km and Vmax values of AfCel5A were observed as 0.16 ± 0.05 mg·mL^{-1} and 7.23 ± 0.33 mol mg^{-1}·min^{-1}, respectively, using CMC-Na as substrate.

2.5. Activity of AfCel5A in a High Concentration of NaCl and Glucose

It is known that cellobiose and glucose are released from cellulose degradation, which is one of the key factors that inhibit the enzymatic hydrolysis of endoglucanase. Therefore, the effect of varying glucose concentrations on AfCel5A activity was assessed (Figure 4A). It was found that AfCel5A well tolerated up to 35 mM glucose without any significant effect on activity. Further, in the presence of more than 35 mM glucose, relative enzyme activity gradually decreases with increasing concentration. On the other hand, AfCel5A displayed an excellent salt tolerance, as more than 80% endoglucanase activity was observed at 3M NaCl (Figure 4B).

Table 2. Effect of various metal ions or chemical reagents on the activity of AfCel5A.

Metal Ions and Chemicals	Relative Activity (%) [a]	
-	1 mM	5 mM
None	100.0 ± 1.5	100.0 ± 0.7
Ag^{+}	56.9 ± 2.7	13.1 ± 3.3
Mn^{2+}	133.4 ± 2.6	13.9 ± 6.9
Zn^{2+}	96.7 ± 1.3	56.3 ± 5.2
Al^{3+}	90.6 ± 2.3	79.3 ± 1.3
Mg^{2+}	94.8 ± 1.4	89.1 ± 1.4
Fe^{3+}	84.6 ± 1.4	91.6 ± 1.8
Li^{+}	84.75 ± 1.4	95.7 ± 4.1
Ca^{2+}	80.1 ± 1.5	87.2 ± 2.9
K^{+}	89.5 ± 3.6	102.7 ± 1.2
Acetone	77.5 ± 1.8	70.3 ± 0.6
Triton X-100	75.5 ± 3.0	58.8 ± 4.6
SDS	20.9 ± 8.9	0.5 ± 3.4
CTAB	82.9 ± 7.5	65.9 ± 4.1
EDTA	90.4 ± 3.6	84.3 ± 6.6
Urea	97.3 ± 4.3	96.8 ± 3.5

[a] Values represent mean ± SD (n = 3) relative to the untreated control samples.

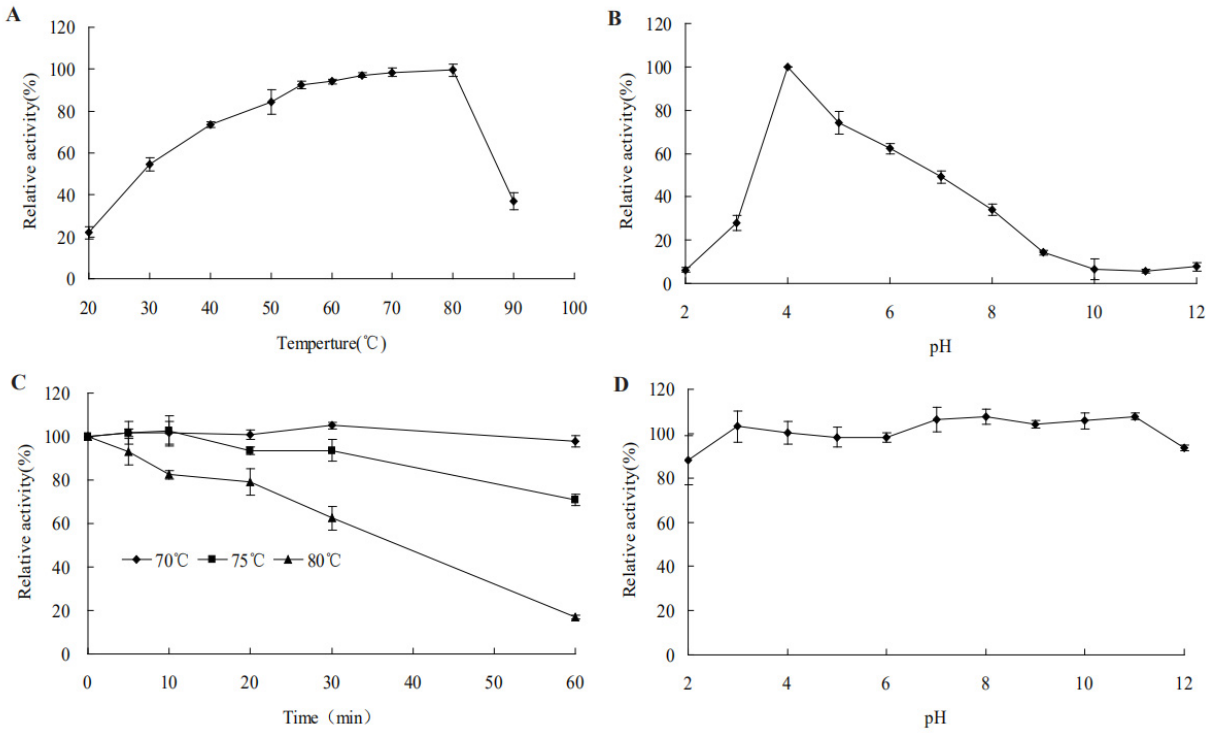

Figure 3. Characteristics of the endoglucanase AfCel5A. (**A**) The optimum temperature for endoglucanase activities. (**B**) The optimum pH for endoglucanase activities. (**C**) The thermostability of AfCel5A at 70, 75 and 80 °C. (**D**) The pH stability of AfCel5A at different pH values.

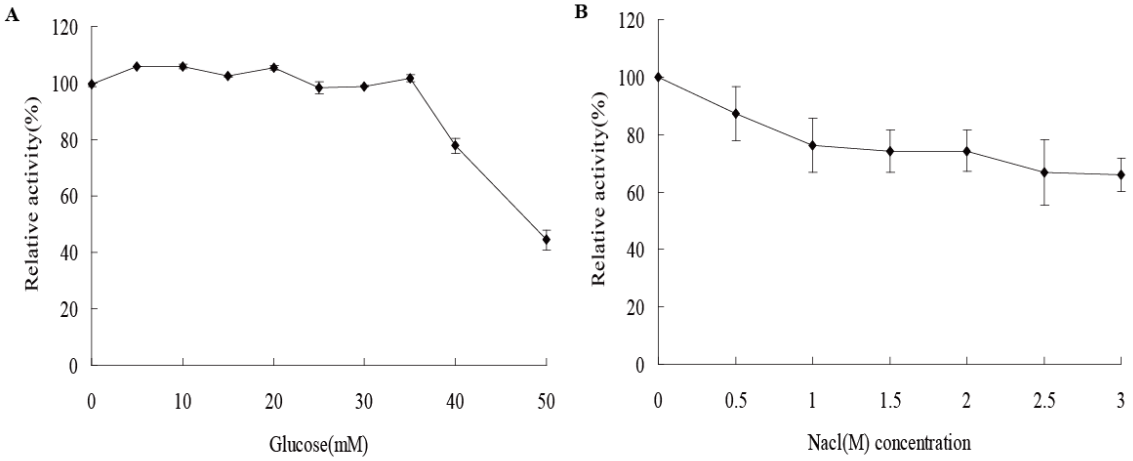

Figure 4. Effect of different (**A**) glucose and (**B**) NaCl concentrations on AfCel5A activity.

2.6. Synergistic Action of AfCel5A and ManBK on Mannan Degradation

It was noted (Table 3) that the addition of AfCel5A significantly enhanced the overall conversion by up to 74%, compared to ManBK alone. AfCel5A had very weak capacities of degrading LBG alone. Sequential and simultaneous enzyme combinations showed a synergistic effect on LBG degradation, increasing the reducing sugar release by up to 1.57-fold.

Table 3. The synergistic hydrolysis of AfCel5 and ManBK using mannans as the substrate.

Enzyme Added			Reducing Sugar Content (μ mol)	Synergistic Effect (DS)
Mannan	First Enzyme	Second Enzyme		
LBG	AfCel5	None	0.02	-
	ManBK	None	0.30	-
	AfCel5	ManBK	0.55	1.57
	ManBK	AfCel5	0.54	1.53
	AfCel5 + ManBK	None	0.49	1.39
Guar gum	AfCel5	None	0.44	-
	ManBK	None	0.45	-
	AfCel5	ManBK	1.06	1.19
	ManBK	AfCel5	1.06	1.19
	AfCel5 + ManBK	None	1.07	1.20
KGM	AfCel5	None	0.05	-
	ManBK	None	0.29	-
	AfCel5	ManBK	0.59	1.74
	ManBK	AfCel5	0.63	1.61
	AfCel5 + ManBK	None	0.41	1.19

When enzyme combinations depolymerized KGM, similar results were also found. However, sequential reaction (AfCel5A→ManBK, or ManBK→AfCel5A) was more effective than the simultaneous reaction of AfCel5A and ManBK. At the same time, we also found that the synergistic effect changed with different substrate kinds. The synergy degree of AfCel5A and ManBK was approximately 1.2 when using GG as substrate, while the synergy degree could reach about 1.5 with LBG as substrate and 1.6 with KGM as substrate.

3. Discussion

In the present study, a new *A. fumigatus* strain HBHF was obtained from a Daqu sample. We identified a total of 239 CAZy-encoding genes were expressed under wheat bran induction. Among these carbohydrate-active enzymes, the GHs accounted for the highest proportion, 69.8%. Similar results were reported by De Gouvêa et al. [25]. The

above results further confirmed that strain HBHF is a very promising producer of cellulose-degrading enzymes.

With the completion of 24 genome sequences of *A. fumigatus*, several CAZy-encoding genes from *A. fumigatus* have been cloned and characterized, such as β-glucosidase [26], mannanase [27], pectinase and xylanase [28]. In addition to this, endoglucanase is the key enzyme in lignin degradation. There are 25 endoglucanases in the *A. fumigatus* genome that are distributed in eight chromosomes unevenly [29]. We obtained a novel endoglucanase gene from this strain, which exhibits a difference in amino acid sequence from the characterized endoglucanase, suggesting that endoglucanase AfCel5A may have different properties or specificities.

We successfully performed expression and enzymatic characteristic assays on the *AfCel5A* gene. It was determined that the maximal production of endoglucanase AfCel5A (3.74 U/mL) was observed 72 h after induction. The optimal pH value of AfCel5A was 4.0, which was close to the most previously reported value for fungal endoglucanase. Meanwhile, it showed relatively higher activity (>30%) under alkaline conditions (pH 7.0–8.0), which is a clear distinction from other thermophilic endoglucanases. For example, the pH value of 8.0 could cause a complete loss of activity of TeEgl5A from *Talaromyces leycettanus* JCM12802 and caused *TlCel5A* to only keep less than 10% activity [30]. Besides the hydrolysis pH of enzymes, pH stability is another important property for their potential industrial applications [31]. AfCel5A displayed higher stability in a broad pH range (3.0–11.0) than other endoglucanases.

The comparatively high optimal temperature activity of AfCel5A obtained here was surprising. The optimal temperature of AfCel5 was 80 °C, which is significantly higher than that reported for others endoglucanases from the thermophilic fungi *A. fumigatus*. For example, the *A. fumigatus* Af293 endoglucanase Af-EGL7 had maximal activity at 55 °C [32], the *A. fumigatus* ABK9 endoglucanases had maximal activity at 50 °C [33], the *A. fumigatus* Z5 endoglucanases Egl2 and Egl3 had maximal activity at 50 and 60 °C, respectively [34], the *A. fumigatus* MKU1 endoglucanase Eng61 had maximal activity at 60 °C [35] and the *A. fumigatus* DBiUN-1 endoglucanases had maximal activity at 60 °C [36]. As far as we know, this is the highest optimal temperature of endoglucanase AfCel5A so far reported for *A. fumigatus*. Besides, we also found that AfCel5A's optimum temperature is much higher than those of other endoglucanases. For example, the optimum temperature ranges for most cellulases are between 40 and 70 °C generally. TlCel5A from *Talaromyces leycettanus* JCM12802 and MtEG5A from *Myceliophthora thermophila* displayed optimal activity at 75 and 70 °C, respectively. Furthermore, AfCel5A's optimum temperature is lower than that reported for *T. emersonii* CBS394.64 endoglucanase TeEgl5A (90 °C) [37]. Interestingly, AfCel5A had very excellent pH stability in comparison with TeEgl5A. AfCel5A is exceptionally stable at pH 11.0, where TeEgl5A completely loses its activity. It was also found that AfCel5A exhibited better thermal stability compared with some thermophilic endoglucanases, including TlCel5A and TlCel6A from *T. leycettanus* JCM12802, with TlCel5A losing 20% of its activity and TlCel6A losing 60% of its activity at 70 °C after 1 h incubation, as well as *Talaromyces emersonii* CBS394.64 endoglucanase Egl7A, which lost 80% of its activity. The thermal stability of the enzyme was lower than that of *T. emersonii* CBS394.64 endoglucanase. AfCel5A retained 30–60% of its original activity after 1 h of incubation at 80–85 °C.

The results indicated that AfCel5A showed synergistic interactions with ManBK in the hydrolysis of mannan-containing polysaccharides, and the maximum synergy degree reached 1.74. AfCel5A showed a weak ability to hydrolyze LBG, while had a more potent synergy with ManBK. In contrast to this, AfCel5A showed a strong GG degradation ability, but we observed that the reducing sugar release is much less when AfCel5A and ManBK act on GG than LBG, as the synergy degree was only 1.2. As we know, GG and LBG belong to the galactomannans, which are composed of mannose and galactose. It was reported that GG and LBG have mannose-to-galactose (M/G) ratios of 2 and 4, respectively [38,39]. Thus, we speculated that the synergy degree differences are likely because GG has more galactose

residues, which inhibited the enzymatic hydrolysis. Given the results above, efficient GG hydrolysis always requires the synergy between β-mannanase and α-galactosidase [40–42]. In studies, the synergy degrees of AfCel5A and ManBK displayed similar values and reached 1.6 when using KGM and LBG as substrates. Unlike LBG and GG, KGM is composed of glucose and mannose backbone, lightly modeled and branched by the glucosyl units. Thus, α-galactosidase appeared much less intense in the KGM degradation process.

4. Materials and Methods

4.1. Strain and Culture Medium

Escherichia coli strain DH5α was used as a subcloning host for cloning and plasmid preparation, grown in the Luria–Bertani (LB) broth at 37 °C. Strain *P. pastoris* GS115 and plasmid pPIC9K (Invitrogen, Carlsbad, CA, USA) were utilized for heterologous expression. The clones were cultured in buffered glycerol complex media (BMGY) and then induced in buffered methanol complex media (BMMY).

4.2. Microorganism Isolation and Identification

The Daqu sample was obtained from Hebei Liulingzui Distillery Co., Ltd. (Hebei, China). One gram of Daqu sample was suspended in 9 mL buffered NaCl (0.85%) and subsequently transferred onto 1.0% CMC-Na agar plates at 45 °C for 3–5 days. Strains producing clear zones around their colonies were isolated and maintained on potato dextrose agar (PDA) medium at 45 and 19 °C, respectively.

The fungi morphological analysis was conducted using a light microscope (Olympus CX21, Tokyo, Japan). Molecular identification of the fungal isolates was carried out by PCR amplifying and sequencing the internal transcribed spacer (ITS) fragment, using universal reverse primers (ITS1 and ITS4). Finally, the phylogenetic tree was generated using MEGA6.0 software.

4.3. RNA Isolation and Sequencing Analysis

A. fumigatus HBHF was incubated in enzyme-producing medium (10.0 $g \cdot L^{-1}$ of KH_2PO_4, 0.3 $g \cdot L^{-1}$ of $CaCl_2 \cdot 2H_2O$, 3.0 $g \cdot L^{-1}$ of $MgSO_4 \cdot 7H_2O$, 2.0 $g \cdot L^{-1}$ of $(NH_4)_2SO_4$, 0.5 $g \cdot L^{-1}$ of $FeSO_4 \cdot 7H_2O$ and 5.0 $g \cdot L^{-1}$ of wheat bran) at 45 °C for 3 days. The mycelia were ground with a mortar and pestle under liquid nitrogen. Total RNA was extracted using TRizol Reagent (Invitrogen, Waltham, MA, USA) according to kit instructions. SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) was applied to reversely transcribe RNA to cDNA. RNA sequencing libraries were generated with the TruSeq Stranded mRNA Sample Prep kit (Illumina, San Diego, CA, USA). The libraries were sequenced with HiSeq 2500 system (Illumina, San Diego, CA, USA). For functional annotations, the assembled unigenes were aligned using public databases, including SwissProt databases, CAZy databases and KEGG databases.

4.4. Cloning and Bioinformatic Analysis of AfCel5A Gene

Primers of the *AfCel5* gene (AfCel5A-F: AGGAATTCGCGCCGAATGCGAAG, GATAATAGTGGAAGCCTTCAT, AfCel5A-R: CTACAGGCATTGAGAGTAGTAGTCG TTC) were designed regarding the putative endoglucanase gene (AFUA_6G11600) from the full genome sequence of *A. fumigatus* Af293. RNA was reverse transcribed into cDNA with ReverTra Ace-α-kit (Toyobo, Osaka, Japan). Subsequently, the AfCel5 gene was amplified with primers (AfCel5A-PF: CGGAATTCGCGCCGAATGCGAAG, AfCel5A-PR: AATGCGGCCGCC TAC AGGC ATT GAGAGTAGTAGTC), and then the purified PCR products were cloned into the pMD19-T vector (TaKaRa, Dalian, China) to generate pMD19-T/AfCel5A. The ExPASy server (http://www.expasy.org/tools/, accessed on 16 May 2021) was used to predict the isoelectric point and molecular weight of the AfCel5 protein. The CBS server (http://www.cbs.dtu.dk/services, accessed on 20 May) was used to predict the potential *N*- and O-glycosylation sites in the AfCel5A. Signal peptide cleavage site analyses were conducted using SignalP 5.0 server (http://www.cbs.dtu.dk/services/SignalP/, accessed

on 20 May 2021). The AfCel5A tertiary structure was modeled using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/, accessed on 26 May 2021).

4.5. Heterologous Expression and Purification of AfCel5

The positive plasmid was digested with restriction enzymes EcoR I /Not I and then inserted into vector pPIC9K. Finally, the confirmed pPIC9K/*AfCel5* plasmids were linearized with *Bgl*II and then transformed into *P. pastoris* GS115 strain. The transformed cultures were coated on minima dextrose (MD) agar plates and cultured at 30 °C until visible clones appeared. The positive clones were cultured in 4 mL BMGY medium at 30 °C with shaking at 200 rpm for 48 h. Cells were harvested by centrifugation and then resuspended with 2 mL BMMY medium for induction. Finally, the culture supernatant was harvested 72 h after induction by centrifugation at 8000× *g*.

The crude enzyme from the culture supernatant was concentrated via ultrafiltration (10 kDa cutoff, Millipore, Sartorius, Germany). For further purification, the concentrated supernatant was treated by the AKTA system. Protein purity and molecular weights were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, 12% gel). The amount of protein was determined by the Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Protein samples were treated by endoglycosidase H (New England Biolabs, Ipswich, MA, USA) according to the operation manual.

4.6. Determination of Enzymatic Activity

The activity of endoglucanase was calculated by DNS method [43]. The reaction solution contained 100 µL enzyme liquid, 450 µL CMC-Na solution (1g·L^{-1}) and 450 µL citric acid buffer (pH 5.0). The above solution was reacted for 10 min at 80 °C, followed by adding 1500 µL of DNS reagent to the system to stop the reaction, and then the absorbance of the mixture at 540 nm was measured. An endoglucanase unit (U) was expressed as the amount of enzyme required to release 1 µmol of glucose per min.

4.7. Biochemical Characterization

The enzyme activity's optimal pH was evaluated using the standard method with the different buffers (pH 2.0–12.0). The pH stability was analyzed after incubation of AfCel5A at 25 °C for 1 h in different buffers (pH 2.0–12.0). Then, the residual activities were measured by the standard method. The optimal reaction temperature was determined under various temperatures (20–90 °C). The thermostability of AfCel5A was determined after the enzyme was preincubated at 70, 75 and 80 °C for different timespans (0–60 min).

Substrate specificity of AfCel5A was evaluated using 0.5% (*w/v*) of five different substrates: carob galactomannan, guar gum, konjac glucomannan, pectin and CMC-Na. The Km and Vmax values of AfCel5 were calculated in different concentrations of CMC-Na (0.25–10.0 mg·mL^{-1}, pH 4.0) at 80 °C for 5 min.

4.8. Effect of NaCl and Glucose on Activity

The tolerance of enzymes to high NaCl and glucose concentrations is essential for their industrial application. To evaluate the effects of glucose or NaCl on activity, AfCel5A was incubated at room temperature with different concentrations of salt (0–3 M) or glucose (0–50 mM) for 1 h, and the relative activity measured in the absence of NaCl/glucose was regarded as 100%.

4.9. Synergistic Hydrolysis of Mannans by AfCel5 and ManBK

To investigate the synergistic mannan hydrolysis capabilities, AfCel5A and ManBK were tested alone, simultaneously or sequentially. ManBK of a thermophilic mannanase was obtained from our laboratory, and AfCel5A was obtained in this work. To calculate the degree of synergy (DS), the sum of sugars released was divided by the sugars released by each enzyme alone. The hydrolysis of GG, KGM and LBG was conducted at 70 °C, pH 4.0.

The reaction system (1.0 mL) was prepared by mixing 10.0 mg of LBG as a substrate and 0.2 mL of the enzyme solution (AfCel5A, ManBK or AfCel5A + ManBK) in $NaHPO_4$–citric acid buffer (pH 6.0, 50 mM). For simultaneous reactions, the AfCel5A and ManBK were coincubated for 10 min at 70 °C and then terminated by boiling for 5 min. The sequential reactions were implemented with AfCel5A and ManBK added sequentially. First, AfCel5A (or ManBK) reactions were conducted for 10 min at 70 °C and terminated by heating in a boiling water bath for 5 min. ManBK or AfCel5A was then added to the reaction mixture and treated for 10 min at 70 °C. Finally, the reaction was terminated by boiling, and the DNS method was used to determine the reducing sugar.

4.10. Statistical Analysis

All experiments were performed as triplicates. All data were analyzed using the software GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

5. Conclusions

A novel endoglucanases gene, AfCel5A, from *A. fumigatus* HBHF was successfully expressed and characterized. AfCel5A showed a temperature optimum at 80 °C and a pH optimum at 4.0. AfCel5A exhibits excellent stability, including pH stability, thermal stability and salt and glucose tolerance. In addition, the AfCel5A protein showed an obvious synergistic effect on enzymatic hydrolysis with mannanase ManBK.

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