

## Article

# Characterization of a Novel Thermophilic Mannanase and Synergistic Hydrolysis of Galactomannan Combined with Swollenin

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**Abstract:** *Aspergillus fumigatus* HBFH5 is a thermophilic fungus which can efficiently degrade lignocellulose and which produces a variety of glycoside hydrolase. In the present study, a novel  $\beta$ -mannanase gene (AfMan5A) was expressed in *Pichia pastoris* and characterized. AfMan5A is composed of 373 amino acids residues, and has a calculated molecular weight of 40 kDa. It has been observed that the amino acid sequence of AfMan5A showed 74.4% homology with the ManBK from *Aspergillus niger*. In addition, the recombinant AfMan5A exhibited optimal hydrolytic activity at 60 °C and pH 6.0. It had no activity loss after incubation for 1 h at 60 °C, while 65% of the initial activity was observed after 1 h at 70 °C. Additionally, it maintained about 80% of its activity in the pH range from 3.0 to 9.0. When carob bean gum was used as the substrate, the  $K_m$  and  $V_{max}$  values of AfMan5A were  $0.21 \pm 0.05 \text{ mg} \cdot \text{mL}^{-1}$  and  $15.22 \pm 0.33 \text{ U mg}^{-1} \cdot \text{min}^{-1}$ , respectively. AfMan5A and AfSwol showed a strong synergistic interaction on galactomannan degradation, increasing the reduction of the sugars by up to 31%. Therefore, these findings contribute to new strategies for improving the hydrolysis of galactomannan using the enzyme cocktail.

**Keywords:** mannanase; swollenin; synergistic; galactomannan; hydrolysis



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## 1. Introduction

Mannans are one of the significant hemicelluloses, and are an inexpensive and sustainable source of renewable energy, functional food and other uses [1,2]. They are composed of mannose, glucose and galactose by linked  $\beta$ -1, 4 or  $\alpha$ -1, 6 glycosidic bonds [3,4]. Therefore, mannan is divided into four groups: linear mannan, glucomannan, galactoglucomannan, and galactomannan [5]. Galactomannan is the largest group, and has been widely used as a food thickener [6].

Mannanase (Endo- $\beta$ -mannanase, EC 3.2.1.78) is a key enzyme involved in the degradation of mannans and has been widely used in the food, energy and pharmaceutical industries. According to analysis of their amino acid sequence and structure,  $\beta$ -mannanase are classified into four glycoside hydrolase (GH) families: GH5, 26, 113 and 134 [7]. At present, the most widely known  $\beta$ -mannanase belong to the GH5 and GH26 families, and some of them have been cloned and functionally characterized [8]. These enzymes showed optimal temperatures around 40–60 °C and optimal pH around 5.0–8.0, respectively [9]. However, poor tolerance and low catalytic efficiency are serious constraints to the application of  $\beta$ -mannanase.

It is well recognized that a set of glycoside hydrolase is necessary for the degradation of mannans [10], such as  $\beta$ -mannosidase (EC 3.2.1.25),  $\beta$ -glucosidases (EC 3.2.1.21) and  $\alpha$ -galactosidases (EC 3.2.1.22). Apart from the above, a number of studies have attempted to utilize xylanase and endoglucanase to enhance the enzymatic hydrolysis of lignocellulosic

biomasses [11]. Swollenin is an auxiliary protein secreted by various microbes, which is able to alter the cell wall structure of plants [12]. Therefore, swollen shows an ideal degradation effect during the combined use of other enzymes [13,14] (such as swollenin (TgSWO) from *Trichoderma guizhouense* NJAU4742), and could increase cellulase and hemicellulase activity [15]. However, there have been no reports regarding its synergy with  $\beta$ -mannanase. Fortunately, we have obtained a swollenin (AfSwo1) from previous study.

*Aspergillus fumigatus*, a thermophilic fungus, has demonstrated an excellent capability to secrete a wide range of glycoside hydrolase [16]. In the present study, the gene AfMan5A from *A. fumigatus* was efficiently expressed in *Pichia pastoris*, then biochemically characterized. Additionally, the synergistic activities of  $\beta$ -mannanase (AfMan5A) with a swollenin protein (AfSwo1) on the enzymatic hydrolysis of galactomannan were evaluated.

## 2. Results

### 2.1. Sequence Analysis of AfMan5A

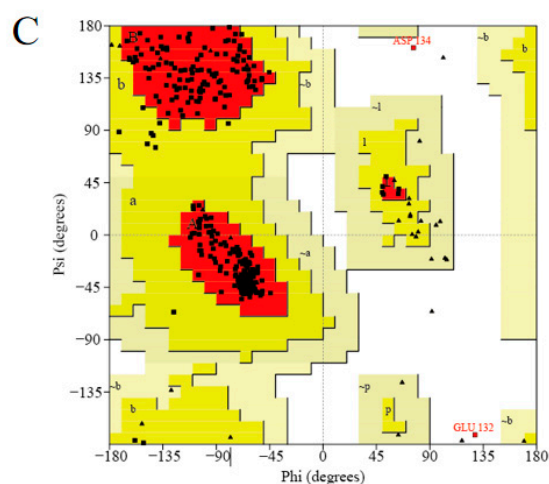
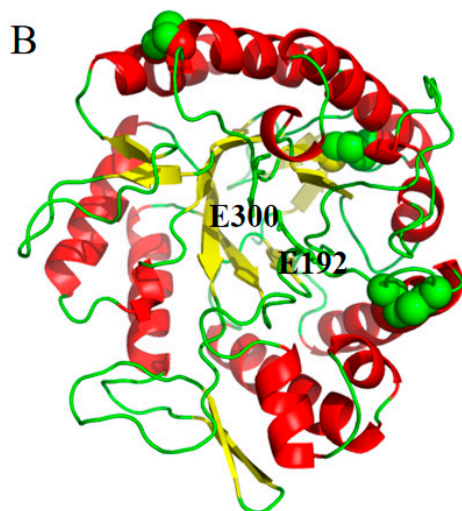
A putative AfMan5A gene (GenBank: XP\_746824.1) was obtained from the *A. fumigatus*. Based on multiple sequence alignment (Figure 1A), AfMan5A shared the highest (74.4%) sequence identity with  $\beta$ -mannanase from *Aspergillus niger* BK01 [17], while sharing 49.9% with Man5A from *Bispora* sp. MEY-1 [18], 58.5% with TrMan5A from *Trichoderma reesei* [19], 60.4% with TlMan5A from *Talaromyces leycettanus* [20], 68.9% with Man5XZ7 A from *Thielavia arenaria* XZ7 [21], and 59.3% with AfMan5 from *A. fumigatus* [22]. Therefore, the alignment results showed that AfMan5A was a new mannanase.

The full-length gene AfMan5A contained 1192 bp, and was interrupted by one intron (683–785, 69 bp). The opening reading frame (ORF) of the AfMan5A gene consisted of 1122 bp, encoding a mature protein of 373 amino acids and a 19 amino acid signal peptide. The theoretical molecular weight and isoelectric point of the mature AfMan5A were 39.25 kDa and 5.42, respectively.

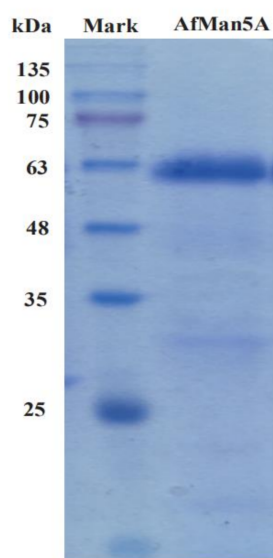
Next, the rationality of the 3-dimensional model of AfMan5A was evaluated using Procheck software. The Ramachandran plot showed that 94.3% of the total residues (344) were in the most favored regions (A, B, L), while 5.1% of the residues were in the additional allowed regions (a, b, l, p) (Figure 1C). Therefore, the model of AfMan5A is shown to be credible and reasonable, and improves our understanding of the structure of AfMan5A. The three-dimensional structure showed that it contains a classical triose phosphate isomerase (TIM) barrel fold, and two catalytic residues (Glu192 and Glu300) stabilized by disulfide bonds Cys195-Cys198, Cys289-Cys296 and Cys308-Cys357 (Figure 1B). These data suggest that AfMan5A is a new member of GH5 mannanase.

### 2.2. Expression and Purification of AfMan5A

The gene AfMan5A was successfully expressed in the *P. pastoris* GS115 host, and the transformants with the highest activity were selected for production with the recombinant protein. It was observed that the mannanase activity was achieved at  $37.3 \text{ U mL}^{-1}$  after induction for 72 h. The crude enzymes were collected, concentrated, and purified to apparent homogeneity. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) illustrated that the apparent molecular mass of AfMan5A was approximately 60 kDa (Figure 2), which was larger than the inferred 40 kDa. In our analysis, the modification sites of N-glycosylation were not found in the AfMan5A protein. However, this protein contains three O-glycosylation sites (176S, 181S and 270S), which may be the main reason for the increase in its molecular weight.



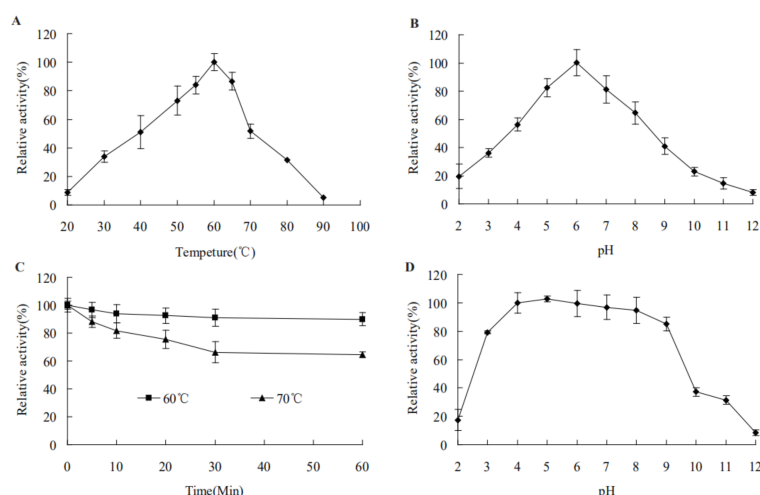
**Figure 1.** Sequence analysis of Mannanase AfMan5A from *A. fumigatus*. **(A)** Alignment of the amino acid sequence with that of other identified GH5 mannanase: ManBK01 (AEY76082.1), AfMan5A (ACH58410.1), TlMan5A (AJF11663.1), TrMan5A (AAA34208.1), Man5XZ7 (AGG69667.1), and MAN5A (EU919724). **(B)** The 3D modeling of AfMan5A, based on mannanase from *A. niger* BK01 belonging to the GH5 family (PDB:3WH9), which is used as a template. **(C)** Ramachandran plot analysis of the prediction structure of AfMan5A.



**Figure 2.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the recombinant AfMan5A.

### 2.3. Characteristics of Recombinant AfMan5A

To examine the enzymatic properties of recombinant AfMan5A,  $\beta$ -mannanase activity was measured under various conditions. The mannanase AfMan5A displayed activity in a broad temperature range of 20–90 °C (Figure 3A). The optimal temperature of AfMan5A was 60 °C, and it showed about 50% of its initial activity at 70 °C. Meanwhile, it was found that AfMan5A was highly stable at 60 °C, as it retained 94.0% of its activity after 60 min. With the processing temperature increase, the loss of mannanase activity became increasingly apparent (Figure 3C). However, it still showed good thermal stability compared to other mannanases. AfMan5A could maintain 65% activity at 70 °C after 60 min of incubation. AfMan5A showed maximum activity at pH 6.0, and maintained greater than 60% activity in the pH range of 4.0–8.5 (Figure 3B). The pH stability test for recombinant AfMan5A showed that it was more stable in the pH range of pH 3.0–9.5, where it retained 80% of its mannanase activity, as illustrated in Figure 3D.



**Figure 3.** Enzyme characteristics of mannanase AfMan5A. (A) The optimum temperature of AfMan5A; (B) the optimum pH of AfMan5A; (C) the thermostability of AfMan5A; (D) the pH stability of AfMan5A.

Table 1 shows that various metal ions and chemical reagents exhibited various effects on the enzyme activity. Among these,  $\text{Cu}^{2+}$  showed a promoting effect on mannanase activity, and this effect became more apparent as the concentration increased (increasing mannanase activity by 37% in 5 mM). On the contrary, the mannanase activity of AfMan5A was strongly inhibited by SDS. Aside from this, AfMan5A exhibited a tolerance to other reagents, such as  $\text{Li}^+$ ,  $\text{Na}^+$  and  $\text{Zn}^{2+}$ . When carob bean gum, konjac, pectin, and CMC-Na were used as substrates, the AfMan5A showed higher degradation activity for the former substrate. Examples include LBG (100%), KMG (46%), pectin (21%) and CMC-Na (0%). The kinetic parameters of AfMan5A were also determined using the Lineweaver–Burk method. The apparent  $K_m$  and  $V_{\max}$  values were  $0.21 \pm 0.05 \text{ mg} \cdot \text{mL}^{-1}$  and  $15.22 \pm 0.33 \text{ mol mg}^{-1} \cdot \text{min}^{-1}$ , respectively.

**Table 1.** The effects of metal ions and chemical reagents on the mannanase activity of recombinant AfMan5A.

Metal Ions and Chemicals	Relative Activity (%) <sup>a</sup>	
	1 mM	5 mM
None	100.0 ± 2.4	100.0 ± 1.9
$\text{Fe}^{3+}$	83.8 ± 4.2	97.9 ± 3.7
$\text{Cu}^{2+}$	119.7 ± 1.5	137.6 ± 4.8
$\text{K}^+$	41.2 ± 2.0	109.6 ± 3.1
$\text{Li}^+$	99.7 ± 3.1	90.0 ± 4.9
$\text{Na}^+$	96.8 ± 3.2	106.5 ± 0.3
$\text{Zn}^{2+}$	108.5 ± 7.1	109.1 ± 2.0
$\text{Mn}^{2+}$	79.3 ± 4.7	13.0 ± 5.0
$\text{Mg}^{2+}$	75.5 ± 0.8	112.1 ± 1.2
$\text{Ca}^{2+}$	35.8 ± 7.9	82.6 ± 0.7
EDTA	49.4 ± 5.8	106.7 ± 0.4
SDS	0 ± 7.4	63.6 ± 5.8

<sup>a</sup> Values represent means ±SD ( $n = 3$ ) relative to the untreated control samples.

#### 2.4. Synergistic Action of AfMan5A and AfSwo1 on Galactomannan Degradation

As shown in Table 2, the simultaneous addition of AfMan5A and AfSwo1 showed a worse performance in galactomannan degradation than did sequential reaction ( $\text{AfMan5A} \rightarrow \text{AfSwo1}$ , or  $\text{AfSwo1} \rightarrow \text{AfMan5A}$ ). The simultaneous reaction only increased the hydrolysis efficiency of galactomannan 1.1-fold. At the same time, we also found that the sequential addition of AfMan5A and AfSwo1 had a slight influence on galactomannan degradation efficiency. When galactomannan was treated with AfMan5A or AfSwo1 and then hydrolyzed by AfSwo1 or AfMan5A, the reducing sugar content increased by 1.31-fold and 1.26-fold compared to treatment with AfMan5A alone.

**Table 2.** The synergistic hydrolysis of locust bean gum (LBG) by AfMan5A and AfSwo1.

Enzyme Added		Reducing Sugar Content ( $\mu \text{mol}$ )	Synergistic Effect (Degree of Synergy (DS))
First Enzyme	Second Enzyme		
AfMan5A	None	0.458	None
AfSwo1	None	None	None
AfMan5A	AfSwo1	0.600	1.31
AfSwo1	AfMan5A	0.581	1.26
AfMan5A + AfSwo1	None	0.516	1.13

### 3. Discussion

*A. fumigatus*, which is widely distributed in nature, has received attention as an excellent producer of glycoside hydrolase, including amylase, tannase and cellulase. Furthermore, some excellent  $\beta$ -mannanase obtained from *A. fumigatus* has been reported. Puchar et al. (2004) purified two mannanases from *A. fumigatus* IMI 385708 (formerly

known as *Thermomyces lanuginosus* IMI 158749), and held that these mannanases were transcribed by the same gene and only showed diversity in post-translational modification [23]. Subsequently, Duruksu et al. (2010) cloned and characterized the gene above (afman, AfuA\_8G07030) [24]. However, our study found another mannanase gene (AfMan5A) in the *A. fumigatus* genome, which shared 59.3% amino acid sequence identity. It was also found that the 3D structure of AfMan5A displayed the classic ( $\beta/\alpha$ )<sub>8</sub>-TIM barrel, which is similar to other GH5 mannanase [20]. In addition, AfMan5A does not possess the same CBM model as afman. Due to these differences, these genes likely have different properties.

*Pichia pastoris* is an excellent expression host, and more than 1000 genes were successfully expressed [25]. In this study, the gene AfMan5A was also efficiently expressed in *P. pastoris*. AfMan5A showed maximal hydrolysis activity at pH 6.0, similar to other reported mannanase [26]. It also showed relatively high activity (>65%) under alkaline conditions (pH 8.0), which is a clear difference from other fungal mannanases such as Lrman5B from *Lichtheimia ramosa* (0% at pH 8.0), ManAK from *Aspergillus kawachii* IFO 4308 (0% at pH 8.0) and mRmMan5A from *Rhizomucor miehei* (0% at pH 8.0). We speculate that the main reason for this difference is in isoelectric points. AfMan5A had an isoelectric point of about 5.53, which was higher than that of the mannanase mentioned above ( $I_p < 4.5$ ). It is worth noting that AfMan5A also showed good stability by retaining 80% of its activity in the pH range of 3.0 to 9.0. This feature made the mannanase AfMan5A more advantageous to apply in the kraft pulp industry.

Indeed, the optimal temperature of AfMan5A was 60 °C, putting it in the range of a thermophilic enzyme. There is still a considerable gap between the optimal reaction temperature of other thermophilic mannanases and that of AfMan5A. For instance, Man5A from *T. leycettanus* JCM12802 exhibited the highest activity at 85–90 °C. ManBK from *A. niger* BK01 also exhibited optimal hydrolytic activity at 80 °C. However, the thermostability of AfMan5A shows similarly excellent characteristics, such as the fact that it remained stable at 60 °C for 1 h without loss of activity. This feature enhances catalytic efficiency, prevents contamination, and reduces costs compared to other mesophilic enzymes [27]. In general, the amount of mannanase activity was inhibited in the presence of Cu<sup>2+</sup> ions. However, it was observed that Cu<sup>2+</sup> ions could significantly enhance the mannanase activity of AfMan5A. A similar phenomenon was also observed in other studies regarding enzymes, such as amylase [28], pectate lyase [29] and laccase [30]. We speculate that this is likely caused by the structure changes of AfMan5A in the presence of Cu<sup>2+</sup> ions. Nevertheless, the above viewpoints require further study.

Meanwhile, we found that mannanase AfMan5A and swollenin AfSwol had a synergistic effect on galactomannan hydrolysis, and hydrolytic efficiency increased about 1.3-fold. Similar results were obtained from Malgas's study, and they found that the synergistic effect between mannanase and galactosidase improved efficiency 1.21-fold [31]. However, these mechanisms of synergy are likely different. Galactosidase could remove the side-chain substituent of galactomannan, which is favorable for the degradation of the galactomannan main chain by mannanase [5]. Therefore, it is likely that a novel synergistic mechanism of swollenin and AfMan5A is effective in combination against galactomannan. We speculate that swollenin could disrupt the galactomannan structure, which enhances galactomannan hydrolysis by mannanase. A similar observation was presented in Zhang et al. (2002) in the soybean straw hydrolysis caused by swollenin and xylanase [14]. Apart from this, swollenin showed slight glycoside hydrolase activity in the study of Andberg, M et al. It could also have accelerated galactomannan's degradation rate [12]. Thus, further studies are still required. We found that the effect of sequential action was superior compared to the simultaneous action of AfMan5A and AfSwol. Similar observations have also been reported for expansin and cellulase [31], xylosidase, and xylanase [32]. The reason for this might be that both enzymes' simultaneous action led to a competition for binding sites, creating a less obvious synergistic effect. In recent years, research on enzyme immobilization technology has been receiving more attention [33–35]. For example, Dal et al. [36] and Tasgin et al. [37] reported that the immobilized pectin lyase

displayed excellent thermal stability and pH stability, increasing the enzyme operation range. Thus, it can be concluded that immobilized AfMan5A may also be more beneficial for use in applications than it is in its free form.

## 4. Materials and Methods

### 4.1. Strains, Vector and Medium

*A. fumigatus* HBFH5 was separated and stored in our laboratory using potato dextrose agar (Sigma St. Louis, Mo, USA) culture at 4 °C. In order to induce  $\beta$ -mannanase and swollenin protein production, *A. fumigatus* HBFH5 was grown at 45 °C in various enzyme-producing media (1 L): 10.0 g  $\text{KH}_2\text{PO}_4$ , 0.3 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 3.0 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.5 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 5.0 g Locust bean gum (Sigma St. Louis, Mo, USA).

*Escherichia coli* DH5 $\alpha$ , grown in Luria–Bertani (Beijing Land Bridge Technology, Beijing, China) broth at 37 °C, was used only as a sub-cloning host for cloning and plasmid preparation. The strain *P. pastoris* GS115 and vector pPIC9K (Invitrogen, Carlsbad, CA, USA) were used for heterologous protein production. Buffered glycerol-complex media (Beijing Suolaibao Technology, Beijing, China) and buffered methanol-complex media (BMMY) were used to perform the transformed yeast growth and induction, and were prepared following the *P. pastoris* manual (Invitrogen, Carlsbad, CA, USA).

### 4.2. Genes Cloning and Bioinformatic Analysis

The full-length AfMan5A (GenBank accession no. AFUA\_7G01070) genes were determined by Basic Local Alignment Search Tool (BLAST) searches with the genome sequence of *A. fumigatus* Af293. After 3 days of cultivation in an enzyme-producing medium, the mycelia of *A. fumigatus* HBFH5 were harvested for total RNA extraction with the TRIzol method. The gene sequence of AfMan5A was amplified from reverse transcription cDNA by a polymerase chain reaction (PCR) using the specific primers listed in Table 3. The PCR products were subcloned into the *pEASY*-T1 vector and sequenced.

**Table 3.** The polymerase chain reaction (PCR) primers used in this study.

Primers	Sequences (5' → 3') <sup>a</sup>	Size (bp)	Tm
AfMan5A-F	ATGAAGTTCTCCTGGCTCAC	20	56
AfMan5A-R	CTAAACCCACCCCTGCTTC	19	56
AfMan5A-PF	CGGAATTCGCCCCAGCGCAAAG	23	62
AfMan5A-PR	AGAATGCGCCGCAACCCACCCCTGCTTC	29	56

<sup>a</sup> Restriction sites incorporated into primers are underlined.

The isoelectric point (pI) values and molecular weight (Mw) of proteins was determined using the online pI/MW tool ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/), accessed on 11 January 2021). The potential N-glycosylation sites and O-glycosylation sites were performed by the NetNglyc server and the NetOGlyc 4.0 server, respectively. The SignalP 5.0 software (<http://www.cbs.dtu.dk/services/SignalP/>, accessed on 11 January 2021) was applied to predict the signal peptide cleavage site. Multiple alignment and visualization of mannanase sequences were carried out using ESPript 3.0 (<http://esprpt.ibcp.fr/ESPript/ESPript>, accessed on 11 January 2021). The I-TASSER server was used to model the tertiary structure of the AfMan5A, which was calculated via Rampage Ramachandran plot analysis (<http://zhanglab.ccmb.med.umich.edu/I-TASSER>, accessed on 6 January 2021).

### 4.3. Heterologous Expression of AfMan5A

Next, the correct plasmid was digested by EcoR I and Not I, and ligated into vector pPIC9K to yield plasmid pPIC9K–AfMan5A. Finally, the positive plasmids were confirmed by means of colony PCR and DNA sequencing. Immediately afterwards, the confirmed plasmids pPIC9K–AfMan5A were digested with Bg/II for high efficiency transformation into the *P. pastoris* GS115 strain. Transformants were incubated on MD plates at 30 °C until visible colonies formed. Positive colonies were inoculated in 4000  $\mu\text{L}$  of BMGY medium

and grown at 30 °C under 200 rpm for 48 h. The cells were collected by centrifugation and resuspended in 2000 µL of BMMY medium for induction expression. After 72 h of methanol induction, the culture supernatant was harvested, and cell debris was discarded by centrifugation (10 min, 8000× g) for follow-up research.

#### 4.4. Purification and SDS-PAGE Analysis of Recombinant Protein

The supernatant was concentrated 10-fold by ultrafiltration with a 10 kDa cutoff membrane (Sartorius AG, Göttingen, Germany). Subsequently, the concentrated supernatant was further purified by gel filtration using the AKTA system (GE Health Care Life Sciences, Little Chalfont, UK). The purity and molecular weights of the recombinant protein were analyzed by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The protein content was analyzed using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Deglycosylation was performed using endoglycosidase H (New England Biolabs, Beverly, MA, USA) according to the manufacturer's instructions.

#### 4.5. Enzyme Activity Assay

The mannanase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method [38], which estimated the amount of reducing sugar released from 0.5% (*w/v*) locust bean gum (LBG). The reaction mixture consisted of 100 µL of enzyme sample and 900 µL of LBG solution (citric acid buffer, pH 5.0). Next, the reaction mixture was incubated at 60 °C for 10 min and 1500 µL of DNS reagent was added to the system to cease the reaction, after which the absorbance of the mixture was measured at 540 nm. One unit of mannanase activity was defined as the amount of enzyme required to release 1 µmol of mannose per minute under the standard conditions.

#### 4.6. Biochemical Characterization

The optimal pH of the enzyme activity was detected at 60 °C using the different buffers, namely citrate–phosphate buffer (pH 2.0–8.0), Tris-HCl buffer (pH 8.0–9.0) and glycine NaOH buffer (pH 9.0–12.0). For pH stability, the enzyme was pre-incubated in the buffers described above without substrate at 0 °C for 1 h. Then, the residual mannanase activities were assayed according to the standard method. To determine the optimal reaction temperature, the activity was carried out at temperatures between 0 °C and 80 °C. The thermostability was determined accordingly, as the enzyme was pre-incubated at 60 °C and 70 °C for 0, 5, 10, 20, 30 and 60 min, respectively. Then, the residual activities were determined under the standard conditions.

The  $K_m$  and  $V_{max}$  values of AfMan5A were identified by incubating the enzyme in citrate buffer (pH 5.0) containing 0.25–10.0 mg·mL<sup>−1</sup> LBG at 60 °C for 5 min, and were extracted from Lineweaver–Burk plots.

#### 4.7. Synergistic Hydrolysis of Galactomannan by AfMan5A and AfSwo1

In order to investigate their synergistic hydrolytic capabilities towards LBG, AfMan5A and AfSwo1 were either tested alone, simultaneously or sequentially. The AfSwo1 protein was taken from our laboratory, and the mannanase AfMan5A was obtained in this work. The reaction system (1000 µL) contained 10.0 mg of LBG and 200 µL of the enzymes solution (AfMan5A, AfSwo1 or AfMan5A + AfSwo1) in a 50 mM Na<sub>2</sub>HPO<sub>4</sub>–citric acid buffer (pH 6.0). For simultaneous reactions, both AfMan5A and AfSwo1 were incubated at 60 °C for 10 min, and then terminated at 100 °C for 5 min. Similarly, the sequential reaction was carried out with the sequential addition of AfMan5A and AfSwo1. First, the reaction containing AfMan5A (or AfSwo1) was performed at 60 °C for 10 min, and was terminated in a boiling water bath for 5 min. Then, AfSwo1 or AfMan5A was added into the reactions system, and the result was also incubated at 60 °C for 10 min. Ultimately, the reaction system was ceased at 100 °C for 5 min. The reduced sugar content in the reaction system after hydrolysis was measured by the DNS method, using mannose as a reference standard.

The degree of synergy (DS) was calculated using the ratio between the reducing sugar content from the reactions of enzyme mixture and alone.

#### 4.8. Statistical Analysis

The experiments described above were conducted in triplicate. Experimental data were performed using one-way analysis of variance by the GraphPad Prism 7.0 software and expressed as the mean  $\pm$  standard deviation.

**Author Contributions:** Conceptualization: X.G. and H.L.; methodology: H.L.; validation: W.C.; formal analysis: X.G.; investigation: H.L.; data curation: H.L. and W.C.; writing—original draft preparation: X.G.; writing—review and editing: H.L.; visualization: X.M.; supervision: X.G.; project administration: X.M.; funding acquisition: X.G. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The data that support the findings of this study are available from the corresponding author [X.M.], upon reasonable request.

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**Conflicts of Interest:** The authors declare that they have no competing financial interests or other conflicts of interest.

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