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假密环菌 *Armillariella tabescens* EJLY2098 β-甘露聚糖酶的克隆、表达及性质分析

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摘　要: 本研究利用 RT-PCR 和 RACE 技术从 *Armillariella tabescens* EJLY2098(一种食用真菌)中克隆出了 β-甘露聚糖酶的全长 cDNA, 构建到 pPICZaA 载体上, 并在毕赤酵母 GS115 中表达了含 His 标签的 β-甘露聚糖酶(re-atMAN47)。该酶的全长 cDNA 共 1481 bp, 编码 445 个氨基酸, 序列分析表明该序列除含有 β-甘露聚糖酶结构域外, 还含有 CBD 和 GHF5 的结构域, 因此可被归为糖苷水解酶家族 5 的一个新成员。诱导培养 72 h 时重组酶活可达到 1.067 U/mL, 蛋白含量为 440 mg/L。重组酶的最适反应温度为 60°C, 在 30°C~65°C 比较稳定; 酶促最适 pH 为 5.5、4.5~7.0 之间比较稳定。这是首次关于 *Armillariella. tabescens* EJLY2098 产 β-甘露聚糖酶的报道, 得到了一个有较好热稳定性、pH 稳定性和生物安全性的糖苷水解酶, 将在饲料、食品、药物生产等方面有广泛的应用。

关键词: β-1,4-甘露聚糖酶, *Armillariella tabescens* EJLY2098, 酶学性质, GHF5

Cloning, expression and characterization of mannanase from *Armillariella tabescens* EJLY2098 in *Pichia pastoris*

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Abstract: We used reverse transcriptase polymerase chain reaction (RT-PCR) and rapid amplification of cDNA end (RACE) techniques to obtain the full-length cDNA of β-mannanase (EC 3.2.1.78) from *Armillariella tabescens* EJLY2098 (an edible fungus). Sequence analysis of the 1481 bp full-length cDNA encoding 445 amino acid residues indicated that the gene contained two structural domains, cellulose-binding domains (CBD) and glycoside hydrolase family 5 (GHF5) domains, other than the conserved β-mannanase domain. Thus, we classified this gene as a member of glycoside hydrolase family 5. Next, we cloned a 1308 bp fragment encoding the β-mannanase mature peptide (re-*at*MAN47) into the expression vector pPICZαA and expressed it in *Pichia pastoris*. The yield was 440 mg/L. Enzyme activity reached a maximum of 1.067 IU/mL after 72 h of methanol induction. The re-*at*MAN47 had an optimal temperature of 60°C and an optimal pH of 5.5. It manifested broad thermostability from 30°C–65°C, and was stable between pH 4.5–7.0. This study represents the first record of a β-mannanase from *Armillariella tabescens* EJLY2098 and provides a new source of carbohydrate hydrolysis enzyme with good biosafety, thermostability and wide pH stability. It is a good approach for the industrial needs of feed, food and pharmaceutical manufacturers.

Keywords: β-mannanase, *Armillariella tabescens* EJLY2098, characterization, GHF5

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Introduction

Mannan is the major polysaccharide of softwood hemicellulose, legume seeds, coconut kernel and tubers of konjac[1]. Mannanase (β-1, 4-D-mannanase; EC 3.2.1.78) randomly hydrolyzes unsubstituted mannans as well as glucomannans and galactomannans, yielding mainly mannotriose and mannobiose[2]. Mannanases are useful in many fields including biobleaching of pulp in paper industry[3], bioconversion of biomass wastes to fermentable sugars[4], upgrading of animal feed stuff, and reducing the viscosity of coffee extracts[5]. They also have potential application in the production of manno-oligosaccharides, which are utilized selectively by intestinal *Bifidobacterium* species and used as a valuable feed additive for monogastric animals.

Many microorganisms were reported to be capable of secreting mannanase[6]. With the increased demand for mannanase, production of mannanase by conventional methods could not satisfy the market; while by gene engineering we could obtain larger amounts of mannanase at a lower cost. *Pichia pastoris* expression system is a common eukaryotic cell expression system which was developed in 1980s by which many active mannanases were expressed successfully. For instance, the genes of mannanases from *Agaricus bisporus* strain C54-carb8 and *Mytilus edulis* were expressed by Tang *et al*[7] and Xu *et al*[8]. Man1 gene of *Trichoderma reesei* was expressed in *P. pastoris* by Wei *et al*[9].

Preliminary screening of mannanase-producing microorganisms in our study revealed that *A. tabescens* EJLY2098, a type of edible fungus, is capable of secreting two mannanases (*at*MAN47 and *at*MAN97), of which one with molecular weight of 97 kD (*at*MAN97) was purified[10]. However, the secretion of various extracellular enzymes gave challenges to purification. Thus, this problem was circumvented by inserting a C-terminal His6 tag while cloning the *A. tabescens* EJLY2098 mannanase gene into pPICαA vector to enhance protein purification. Moreover, the recombinant mannanase was inducibly expressed using the *P. pastoris* expression system. Biochemical characterization of the purified recombinant EJLY2098 mannanase was performed. Furthermore, of the numerous fungal mannanases found in the database, this is the first report of cloning, expression and characterization of mannanase from *A. tabescens*.

1 Materials and methods

1.1 Strains, plasmids and culturing conditions

A. tabescens EJLY2098 is a stored strain in our laboratory. It was activated on seed medium (1% konjac, 1% [*W*/*V*] peptone, 2% [*V*/*V*] potato juice, 2% [*W*/*V*] glucose, and 1.5% agar). After two days, it was inoculated into the induction medium (2% [*W*/*V*] konjac, 1% [*W*/*V*] peptone, and 25% [*V*/*V*] potato juice). It was then cultured under 27°C with increased shaking from 160 r/min to 200 r/min on a tertiary enlargement scale.

Escherichia coli TOP10F' (Tet^r), was used as host for plasmid propagation. It was cultured in LB/tet medium. Transformation was achieved by $CaCl_2$ method. The *E. coli* TOP10F' transformant was cultured in LLB/zeocin medium (1% [*W*/*V*] Tryptone; 0.5% [*W*/*V*] yeast extract; 0.5% [*W*/*V*] NaCl; pH 7.0) and selected on zeocin plates.

Pichia pastoris GS115 (His4, Mut$^+$; Invitrogen, USA) was used as the host for expression of EJLY 2098 mannanase. It was grown on YPD medium. After electroporation into *Pichia pastoris* GS115, the positive transformants were screened on YPDS medium containing 100 μg/mL, 500 μg/mL or 1000 μg/mL zeocin.

BMGY: 1% yeast extract; 2% peptone; 100 mmol/L potassium phosphate, pH 6.0; 1.34% YNB; 4× 10^{-5}% biotin; 1% glycerol or 0.5% methanol; For BMMY, add 100 mL 10× Methanol instead of glycerol.

pMD18-T vector (TaKaRa, Japan) was used in cloning of PCR fragments.

pPICZαA vector (Invitrogen, USA) was used in *P. pastoris* expression.

1.2 Isolation of the full-length cDNA of mannanase gene from *A. tabescens* EJLY2098

1.2.1 *Total RNA isolation and cDNA synthesis*

EJLY2098 mycelia were frozen in liquid nitrogen before grounded up in an ice-cold mortar until powdery consistency was achieved. Trizol (Invitrogen, USA) was used for total RNA extraction from powdered mycelia, following the manufacturer's instruction. The quality and integrity of RNA was determined by gel electrophoresis in 1.1% agarose.

First-strand cDNA from EJLY2098 was synthesized using TaKaRa LA PCR^{TM} Kit (TaKaRa, Japan) according to the supplier. First-strand cDNAs were then used as template for further PCR amplification with *Taq* polymerase (Dingguo, China) in a total volume of 20 μL containing 4.0 μL cDNA, 10× PCR buffer (Dingguo, China), and 0.4 μL primers P1: 5′-GGTCCGGTGCTAGGCCTACNATHAAYAC-3′, and P2: 5′-CGCGGTTCATTTGCCARYTCCCANGC-3′ (Invitrogen, USA), wherein N denotes A, T, C, G; H denotes A, T; Y denotes C, T; and R denotes A, G. The PCR condition was: 1 cycle of 2 min at 94°C, 5 cycles of 30 s at 94°C, 30 s at 69°C, 1 min at 72°C, 30 cycles

of 30 s at 94°C, 30 s at 64°C and 1 min at 72°C, and finally 5 min at 72°C. PCR products were analyzed by 1.5% agarose gel electrophoresis. The amplicon of the expected size was gel purified using Wizard® SV Gel and PCR Clean-up Kit (Promega, USA) and cloned into pMD18-T vector (TaKaRa, Japan). Sequencing was performed by TaKaRa (Japan) using the Sanger Method.

1.2.2 *Isolation of the full-length EJLY2098 mannanase gene by 5'RACE and 3'RACE*

For 5′RACE, first-strand cDNAs were generated from 1 μL of total RNA from EJLY2098 with primer P3: 5′-TCCACCATAGTCGGACCAGTTGTTCG-3′ (Invitrogen, USA) using SMART RACE cDNA Amplification Kit (Clontech). PCR was performed on this condition: 1 cycle of 3 min at 94°C, 5 cycles of 45 s at 94°C, 45 s at 71°C and 3 min at 72°C, 30 cycles of 45 s at 94°C, 45 s at 66°C and 3 min at 72°C, and finally 10 min at 72°C. RACE products were analyzed, purified and sequenced as described above.

For 3′RACE, first-strand cDNA were generated from 1 μL of total RNA from EJLY2098 with primer P4: 5′-ACAGAGTTCCGCCTCAACGGTGCCAA-3′ (Invitrogen, USA) using SMART RACE cDNA Amplification Kit (Clontech). PCR was performed on this condition: 1 cycle of 3 min at 94°C, 45 s at 94°C and 5 min at 72°C, 4 cycles of 45 s at 94°C, 45 s at 71°C and 3 min at 72°C, 30 cycles of 45 s at 94°C, 45 s at 68°C and 3 min at 72°C, and finally 5 min at 72°C. RACE products were analyzed, purified and sequenced as described above.

The full-length sequence of EJLY2098 mannanase gene was deduced by the software DNAMAN (Lynnon Biosoft, USA). DNA sequence was translated into amino acid sequence by the Bioedit Sequence Alignment Editor program. The nucleotide and deduced amino acid sequences were searched through GenBank database and compared to other related sequences using Clustal X program. The nucleotide sequence of the full-length EJLY2098 mannanase cDNA was submitted to GenBank (Accession No. DQ286392).

1.3 Cloning and expression of the recombinant mannanase in *Pichia pastoris*

The mature mannanase gene was amplified by PFU DNA Polymerase (Promega, USA) using primers P5: 5′-CCGGAATTCGCTGTTCCTGAGTGGGGCCAATG-3′, and P6: 5′-CGGGGTACC**CTA***GTGGTGGTGGTGGTGGTG*CGCCCGCGCTTTCAATGTAAC-3′ (Invitrogen, USA), wherein the underlined font denotes the cleavage sites of *Eco*R I and *Kpn* I; the bolded font denotes the stop codon; And the italicized font denotes the His6 tag. The His6 tag codons were inserted before the stop codon (TAG) to enhance purification. The PCR condition was: 1 cycle of 4 min at 94°C, 5 cycles of 45 s at 94°C, 45 s at 70°C and 3 min at 72°C, 25 cycles of 45 s at 94°C, 45 s at 65°C and 3 min at 72°C, and finally 5 min at 72°C. The PCR product was gel purified as described above and digested with *Eco*R I and *Kpn* I (TaKaRa, Japan) before subcloning into pPICZαA (Invitrogen, USA). Recombinant pPICZαA plasmid was propagated in *E. coli* TOP10F' by $CaCl_2$-mediated protoplast transformation, and sequenced at Shanghai Boya Company (China) using the Sanger method. Electro-transformation was used to transform the recombinant pPICZαA plasmid into *P. pastoris*.

To induce mannanase production in *P. pastoris*, the cells were grown at 28°C–30°C, with 250–300 r/min shaking, in 25 mL BMGY medium until the culture reached an OD_{600} of 2–6. Next, the cell pellets were harvested and resuspended in 100 mL BMMY medium in a 1.0 L conical flask. Absolute methanol was added every 24 h to a final concentration at 0.5% [*V*/*V*] to maintain induction. The culture supernatant was collected at 24 h, 48 h, 72 h, and 84 h, respectively. Secreted proteins were analyzed by Bradford assay using BSA standard, SDS-PAGE and mannanase activity assay.

1.4 Enzyme activity and protein analysis

Positive clones were inoculated on BMGY agar plate, and cultured under 28°C for 24 h. Then they are transferred to BMMY plate containing 1% konjac and 0.3% trypan blue, and cultured under 28°C. Absolute methanol was added every 12 h to induce enzyme production. After 96 h, the plates are put under 4°C for visualization of circular white clones.

The mannanase activity was assayed by mixing 80 μL enzyme solution with 0.92 mL pH 5.5 0.2 mol/L Na_2HPO_4-0.1 mol/L citric acid buffer containing 0.5% konjac at 60°C for 15 min. The reducing sugar released was determined by 3,5-dinitro salicylic acid (DNS) method using mannose as standard. One unit (IU) of enzyme activity was defined as the amount of enzyme producing 1 μmol of mannose per minute under the assay conditions. To determine the optimal pH and temperature profiles, the enzymatic reaction was performed at different pH and temperatures as indicated in the figure legends. Thermostability was assessed by measuring residual enzyme activity after incubation at pH 5.5 for 30 min at different temperatures (30°C–80°C); while pH stability was measured after incubation at 60°C for 30 min at pH 3.0–8.0.

1.5 Purification of the re-*at*MAN47 from *Pichia pastoris*

Chelating Sepharose™ Fast Flow (Amersham Biosciences, USA) was chelated with 0.1 mol/L $NiSO_4$ to make an affinity column (1.0 cm × 9.5 cm). The cell-free medium was loaded in this column and eluted with pH 7.4, 500 mmol/L imidazole-20 mmol/L Na_2HPO_4 buffer. The enzyme fraction was collected and assayed.

2 Results

2.1 Isolation of the full-length cDNA of *at*MAN47 gene from *A. tabescens* EJLY2098

Based on the conserved sequence of mannanase from various mannanase-producing species, degenerate primers (P1, P2, P3, and P4) were designed, respectively, for RT-PCR, and 5′RACE and 3′RACE of the *at*MAN47 gene from *A. tabescens* EJLY2098.

The first PCR amplification yielded a 298 bp fragment while the 5′RACE and 3′RACE yielded a 676 bp and a 1266 bp (where 322 bp overlapped with the 5′RACE product) DNA fragments, respectively. After sequence assembly, the putative 1481 bp full-length *at*MAN47 cDNA was obtained.

Comparison analysis using blastx reveals that it shows 65% and 64% identity with CEL4a and CEL4b of *Agaricus bisporus* respectively, and 76% similarity towards the two. With ORF Finder, the open reading frame was determined to be the sequence between 14 bp and 1351 bp in the full-length cDNA, encoding for 445 amino acids. This sequence contains a start codon (ATG) and a stop codon (TAG). Furthermore, identification of signal peptide cleavage site with Signal P V2.0 program suggested that the first 18 amino acid residues are the signal sequence. A His6 tag was inserted upstream of the stop codon. Consequently, the mature mannanase was predicted to have molecular weight of approximately 47 kD. After rpsblast analysis we know that re-*at*MAN besides mannanase domains, it also contains domains of CBD and GHF5. And it contains 8 conservative sites of GHF5 (Arg-144, His-189, Asn-258, Glu-259, His-339, Tyr-341, Glu-377, Trp-406), these illustrate *at*MAN47 is a new member of GHF5.

Seeing from the cladogram of β-mannanase (Fig. 1), *A. tabescens* EJLY2098 and *Agaricus. bisporus* β-mannanase had the highest homology. Firstly, both of them belonged to *Basidiomycota Agaricales*, and they were categorized in the same class with other five fungi which also secrete mannanase (GHF5). These five fungi belonged to *Ascomycota*.

2.2 Cloning and expression of the recombinant *at*MAN47 in *P. pastoris*

RT-PCR was performed to obtain the mature mannanase gene, fused with a His6 tag in its C terminus with primers P5 and P6. A fragment of 1308 bp was obtained and cloned into the yeast expression vector, pPICZαA. *P. pastoris* transformants were screened on YPDS medium containing 100 μg/mL, 500 μg/mL or 1000 μg/mL Zeocin. Mannanase activity of positive clones was monitored by the agar plate diffusion assay.

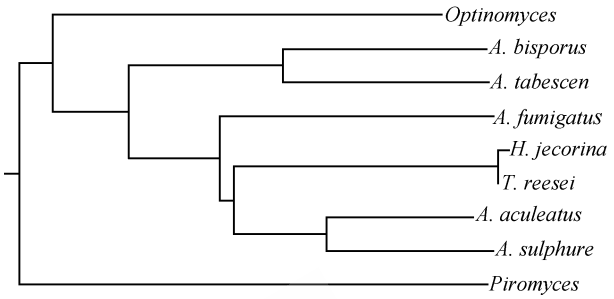

Fig. 1 Cladogram of β-mannanase from 9 fungi. *A. bisporus*: *Agaricus bisporus* (CAB76904); *A. fumigatus*: *Aspergillus fumigatus* Af293 (EAL85463). *A. sulphureus*: *Aspergillus sulphureus* (ABC59553); *A. aculeatus*: *Aspergillus aculeatus* (AAA67426). *H. jecorina*: *Hypocrea jecorina* (AAA34208); *T. reesei*: *Trichoderma Reesei* (1QNS); *Orpinomyce*: *Orpinomyces* sp. PC-2 (AAL01213); *Promyces*: *Piromyces* sp. (CAA62968).

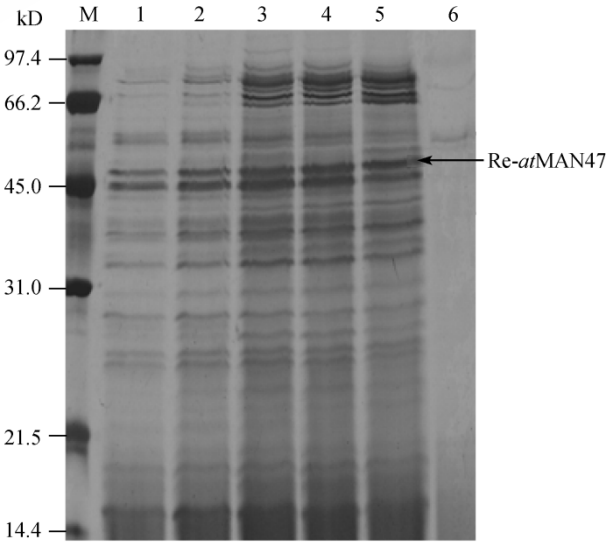

Fig. 2 SDS-PAGE of the supernatant of *P. pastoris* expressed re-*at*MAN47. Transformed *P. pastoris* was grown in BMGY and induced with 1.0% methanol [*V*/*V*] in BMMY. The culture supernatant was collected at 12 h, 24 h, 48 h, 72 h, and 84 h, respectively (lanes 1–5). Lane 6 represents GS115/pPICZαA. Twenty microlitres of the supernatant were loaded onto each lane. Lane M represents protein marker (Bio-Rad, USA). The gel was stained with Coomassie brilliant blue R250. We could see from Fig. 3 that the mannanase production increased with time and reached a maximum after 72 h. No target protein was detected without methanol induction.

SDS-PAGE was performed to determine the size of the recombinant *at*MAN47 from the culture supernatant of positive clones at various times of induction (12 h, 24 h, 48 h, 72 h, and 84 h) (Fig. 2). The result showed a major band of approximately 47 kD, which was close to the predicted size of the deduced amino acid sequence fused with a His6 tag. The optimal induction time was determined to be 72 h (Fig. 3).

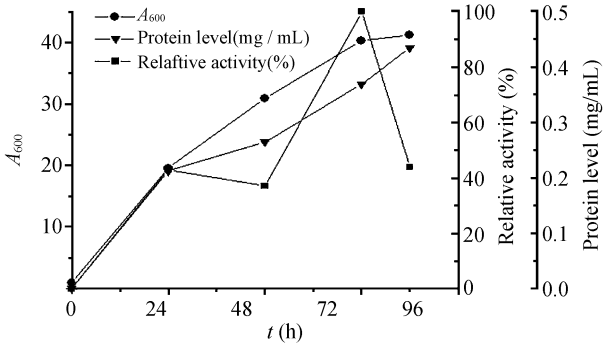

Fig. 3 Expression of transformed *P. pastoris* GS115/pPICZαA-man'. Cultured under 28ºC, induced with 1% methanol, re-*at*MAN47 was highly active after induction with methanol for 72 h and has activity of 1.067 U/mL, and the yield was 440 mg/L.

2.3 Purification of the re-*at*MAN47 from *Pichia pastoris*

*Re-at*MAN47 was produced at a larger scale in a volume of 100 mL BMMY culture. A yield of 440 mg/L was obtained after 72 h of induction. Protein purification was carried out using a single step affinity chromatography resulting in a yield of single band. A Coomassie-stained gel showed a band of 47 kD (Fig. 4).

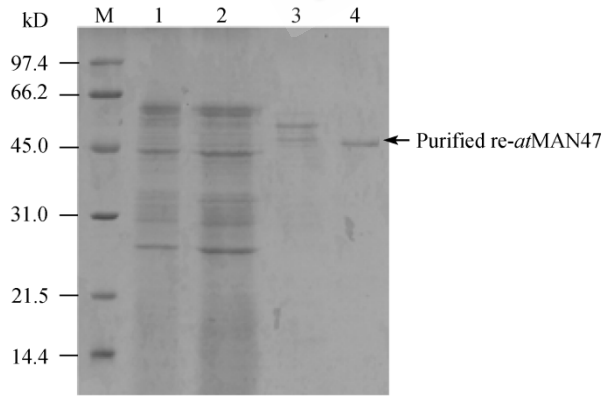

Fig. 4 SDS-PAGE of the purified re-*at*MAN47. The sample preciped by $(NH_4)_2SO_4$ was dissolved in pH 5.5 0.2 mol/L Na_2HPO_4-0.1 mol/L citric acid buffer and purified through an affinity column using Ni^{2+}-Chelating Sepharose™ Fast Flow. Lane M: marker (Bio-Rad, USA); 1: elution peak; 2: $(NH_4)_2SO_4$ sample peak; 3: elution peak I (60 mmol/L imidazole); 4: elution peak II (500 mmol/L imidazole). The gel was stained with Coomassie brilliant blue R250.

2.4 Enzyme activity and protein analysis

Clones of GS115/pPICZαA-man' and the empty vector GS115/pPICZαA were inoculated on BMGY agar plate and induced by methanol for 96 h, the test clones appeared as hydrolysis ring, while control clones without(Fig. 5).

Next, to determine the optimal temperature of the purified re-*at*MAN47, the enzyme was incubated at pH 5.5 for 30 min at different temperatures (30ºC–80ºC).

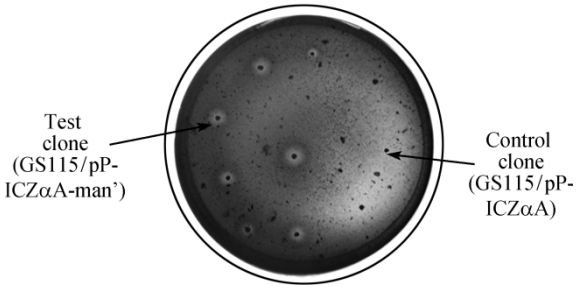

Fig. 5 Activity assay of clones of GS115/pPICZαA-man' and the empty vector GS115/pPICZαA.

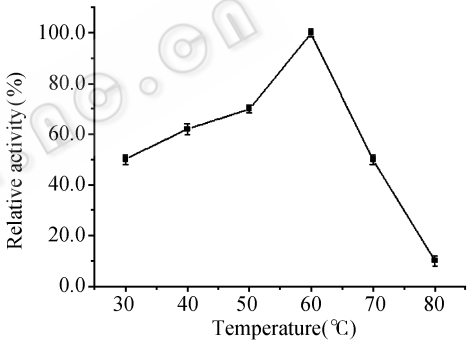

Fig. 6 Effect of temperature on the activity of purified re-*at*MAN47. The optimal temperature was obtained by incubating the enzyme in pH 5.5 0.2 mol/L Na_2HPO_4-0.1 mol/L citric acid buffer for 30 min at different temperatures as indicated in the graph. After the incubation period, the enzyme activity was assayed by the DNS method.

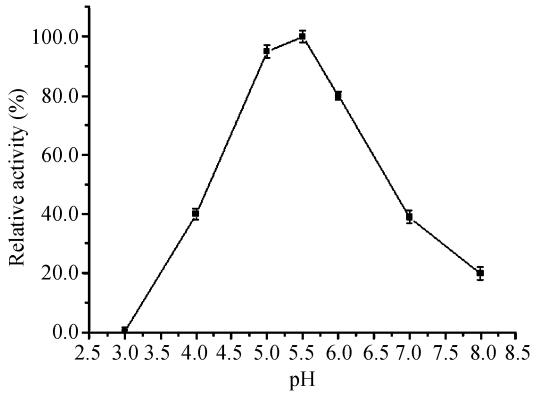

Fig. 7 Effect of pH on the activity of purified re-*at*MAN47. The optimal pH was obtained by incubating the enzyme at 60ºC for 30 min at different pH. To adjust the pH range, 0.2 mol/L Na_2HPO_4-0.1 mol/L citric acid buffer was used for pH 3.0–5.5; 0.2 mol/L Na_2HPO_4–0.2 mol/L NaH_2PO_4 buffer was used for pH 6.0–8.0. After the incubation period, the enzyme activity was assayed by the DNS method.

It was found that the purified enzyme had an optimal temperature at 60°C (Fig. 6). For optimal pH, the enzyme was incubated at 60°C for 30 min at different pH (pH 3.0–8.0), it showed an optimal pH of 5.5 (Fig. 7). Furthermore, the re-*at*MAN47 remained active between 30°C–65°C (Fig. 8). This activity dropped to almost 0% at 70°C. In addition, the operational pH of this enzyme was found to be pH 4.5–7.0 (Fig. 9).

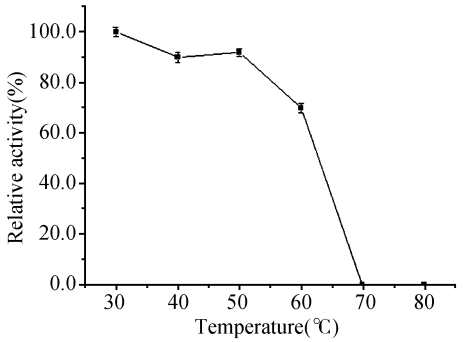

Fig. 8 Thermostability of the purified re-*at*MAN47. The enzyme was incubated in pH 5.5 0.2 mol/L Na_2HPO_4-0.1 mol/L citric acid buffer for 30 min at different temperatures as indicated in the graph. After the incubation period, the enzyme activity was assayed by the DNS method.

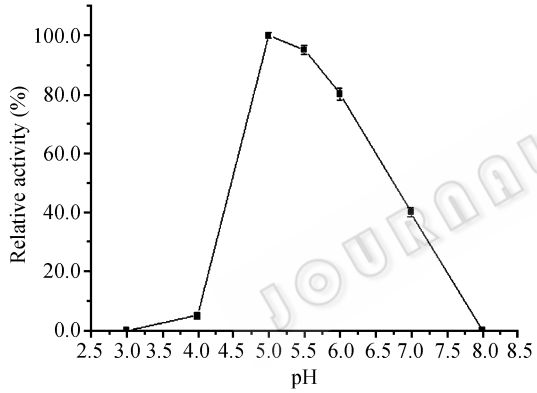

Fig. 9 pH stability of the purified re-*at*MAN47. The enzyme was incubated at 60°C for 30 min at different pH as indicated in the graph. After the incubation period, the enzyme activity was assayed by the DNS method.

3 Discussion

The *A. tabescens* EJLY2098 mannanase (*at*MAN47) reported in this paper is the first cloned from *A. tabescens*. It consists of a 1338 bp nucleotide sequence encoding 445 amino acids. It depicted partial similarity towards *Agaricus bisporus* CEL4a and CEL4b. Bioinformatics study of the deduced amino acid sequence predicts that it has three disulphide bonds (Scratch Protein Predictor), nine possible phosphorylation sites (NetPhos 2.0), and one N-glycosylation site (Predict Protein Server). Moreover, an 18-residue signal peptide at the N terminus (Signal P V2.0), and orientation analyses (TMHMM Server v2.0, and WOLF PSORT) imply that it is an extracellular protein. Therefore, based on the above predictions, we adopted the *P. pastoris* expression system to maintain the enzyme's post-translational modifications and to facilitate inducible extracellular production.

According to rpblast result, the *at*MAN47 belongs to the glycoside hydrolase family 5. And using the software SWISS MODEL, the *at*MAN47 has a $(\beta/\alpha)_8$ structure, which is very similar to the tertiary structures of mannanases found in *Lycopersicon esculentum*[11] and *Trichoderma reesei*[12]. Additionally, it possesses a cellulose-binding domain (CBD) at its N terminus which is very similar to that of *Trichoderma reesei*[13].

The yield of 440 mg/L was obtained after 72 h of induction in *P. pastoris* and had activity (1.067 IU/mL) toward konjac while the wild type mannanase has activity of 1.106 IU/mL[10]. According to data, the mannanase from *Trichoderma reesei* expressed in *P. pastoris* has activity of 12.5 IU/mL[9], this data hint, the possibility that the recombinant *P. pastoris* expressed re-atMAN47 is raise of may be existent. We can raise the *P. pastoris* expression level of *re-at*MAN47, thorough advanced research such as optimization incubation condition.

The recombinant *at*MAN47 has similar thermostability and optimal pH with the purified wild type mannanase. However, its optimal temperature increased by 10°C and its stable pH range is wider (recombinant = 4.5–7.0; wild type = 5.0–6.5). On the other hand, the optimal temperature of the recombinant *at*MAN47 is 60°C, slightly higher than that of *Trichoderma harzianum*[14], *Aspergillus niger*[15] and *Sclerotium rolfsii*[16]. Moreover, it maintains 50% activity at 65°C, which is higher than most other fungal mannanases[14,17–19], although extreme bacteria like *Thermomonospora fusca* mannanase is active even at 80°C and pH 14.0. In addition, it has a fairly broad pH range (4.5–7.0) compared to other mannanases reported[14–21]. Its slightly acidic pH optimum (pH 5.5) and broad operational temperature range (30°C–65°C) privilege its application in various industrial applications including the feed additive industry.

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