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Expression, Purification, Biochemical Characterization and Structural Modeling of an endo-β-1,4-glucanase from *Stachybotrys chartarum* in *Pichia pastoris*

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In this study, we analyzed the physicochemical characteristics and the structure of one glucanase and hoped it will be useful for future studies on the possible synergistic action of cellulases and it will provide guidance for the industrial utilization. A recombinant glucanase gene (*r-ScEG12*) from *Stachybotrys chartarum* was synthesized and expressed in *Pichia pastoris*, then was purified and characterized. The r-ScEG12 belongs to glycosyl hydrolase family 12 (GH12) by phylogenetic analysis. The Asp100, Glu117, and Glu201 residues were proposed to be present at the active site. The apparent molecular weight of r-ScEG12 was approximate 27 kDa, and the optimum temperature and pH was 40°C and 5.0, respectively. The Km and Vmax for CMC were 26.08 g/L, and 1.88 mg $L^{-1}min^{-1}$, respectively. The r-ScEG12 activity was inhibited 71.64 % and 50.97% by Cu^{2+} and Mn^{2+} respectively, while the r-ScEG12 activity was enhanced by Na^{+} and Ca^{2+} mildly.

Key words: Endo-β-1,4-glucanase; Biochemical characterization; Structural Modeling; *Pichia pastoris; Stachybotrys chartarum*.

Cellulose is one of the most abundant form of carbohydrate in nature, composed of repeating glucose units linked by β-1,4-glycosidic bonds. The degradation of cellulose need mutiple enzyme complexs: endoglucanase (endo-β-1,4-glucanase, EC 3.2.1.4,), cellobiohydrolase (exo-1,4-β-D-glucanase, EC 3.2.1.91) and β-glucosidase (1,4-β-D-glucosidase, EC3.2.1.21). These enzymes act synergistically on cellulose and convert it into glucose. Gycosyl hydrolases (GHs) have been classiûed into related families by their evolution and structure, the number of GHs families has grown to 133 in recent years (http:// www.cazy.Org/). Cellulases (Cels) belong to GHs found in at least 12 families[1] and they are applied in various fields[2-5].

Endo-β-1,4-glucanase (3.2.1.4) is one key enzyme of the hydrolysis celluloses. Endoglucanases of GH12 family have been classified into four subfamilies: 12-1 and 12-2 subfamilies are only secreted by fungal enzymes, both of them devoid of carbohydrate binding modules (CBM), subfamily 12-3 contains a CBM and mainly is secreted by *Streptomyces* enzymes and the fourth subfamily 12-4, is secreted by enzymes from thermophiles[6].

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Researchs about three-dimensional structure of GH enzymes have proved that members of GH family share the same overall protein fold, the same reaction mechanism (retaining or inverting) and a catalytic site motif. The structure of *Hypocrea jecorina* Cel6A was the first three-dimensional X-ray crystallography structure of a cellulase[7]. In addition, the structural studies about the GH12 members showed a compact-sandwich structure that was curved to create an extensive cellulose-binding site on the concave of the β–sheet. Structures from both fungal[8, 9] and bacterial[10, 11] sources have been designed, they provided the structural framework for the GH family. In order to discover cellulases with improved properties, more and more novel GH12 endoglucanases have been cloned, sequenced, expressed in different hosts[6].

In this study, the gene of the *Stachybotrys chartarum* Cel12A was cloned by Gene-Bank accession number CAL48345.1. The enzyme has a molecular weight of 27 kDa and devoid of CBM. One research about β-glucanase Cel12A from *Stachybotrys atra*[12] has studied some biochemical characterization and expressed in bacterial and fungal hosts, but it did not provide the structure of the enzyme. Because the devoid of CBM in engineered enzymes can interact the enzymatic process[13], thus, it is important and necessary to understand the function and the catalytic mechanism of cellulases that lack of CBM. In this study, we contrasted the biochemical characterization and showed the structural modeling of this enzyme aim to be a footstone for the further deeper insight.

In all, this study aimed to produce, purify, and characterize biochemical of the endoglucanase from *S. chartarum*. This is the first to report the enduglucanase system of the *S. chartarum* expressed in *Pichia pastoris* and the results will be useful for future studies on the possible synergistic action of cellulases.

MATERIALS AND METHODS

Strains, plasmids, growth conditions and chemicals

P. pastoris strain GS115 (His-Mut$^+$) was used as a host strain for secretion of endo-β-1,4-glucanase. *Escherichia.coli* DH5α was used for maintenance and manipulation of the plasmids stored at our laboratory . The *E. coli* cells were grown in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) at 37 °C, and ampicillin (50–100 μg ml^{-1}) was added, if necessary. Vector pPIC9K were purchased from Invitrogen (San Diego, CA). T4 deoxyribonucleic acid (DNA) ligase and restriction endonucleases were purchased from Takara (Dalian). Endoglycosidase H (Endo H) was purchased from New England Biolabs (Beverly, MA) carboxy methyl cellulose (CMC) was purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Gene cloning and Construction of expression vector

Based on GenBank accession number CAL 48345.1, the endo-β-1,4-glucanase gene, with an additional of a C-terminal 6× his tag sequence at 3'-end, was optimized using codon usage bias and synthesized by successive polymerase chain reaction (PCR) method[14]. In this study the G+C content was adjusted to about 47.39 %, and AT-rich stretches were eliminated to avoid premature termination. The amplified fragment was digested with *Bam*HI and *Sac*I and then inserted into pYPX88 vector, which contains a 357-bp fragment of the α-factor prepro-leader MF4I (GenBank accession no.: AY145833) with the preferred codon usage for *P. pastoris*, which substitutes the wild-type α-signal sequence to enhance the expression level[15]. Routine DNA manipulations were performed by standard recombinant method.

Transtormation and screening of transformants

The recombinant plasmid was linearized with *Bgl*II and transformed into *P. pastoris* by electroporation method. The cells were plated on histidine-deficient SD medium and incubated at 30°C for 3 days. The transformants were screened for their ability. Small-scale expression experiments were performed to detect expression of the recombinant protein[16].

Purification and SDS-PAGE

All purification steps were carried out at 4 °C. After centrifugation and removed of the cells, the supernatant was concentrated by ammonium sulfate precipitation. The recombinant protein was purified using Ni^{2+}-NTA agarose affinity column. The purified production was checked using 12 % (w/v) sodium dodecyl sulphate polyacrylamide gel electrophoresis **(**SDS-PAGE). The spots of proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Enzyme assays

The enzymatic activity of endo-β-1,4-glucanase was measured using CMC as a substrate, and the reduced sugar was measured by the 3,5-dinitrosalicylic acid (DNS) method[17]. The enzyme activity assay was followed: 70 µl of enzyme solution was incubated with 140 µl of 1 % (w/v) CMC solution and 70 µl citric acid–Na_2HPO_4 (pH 5.0) buffer at 37 °C for 2 h after initiating the reaction by the addition of enzyme; The reaction was terminated by the addition of 280 µl DNS reagent and boiled for exactly 10 min, and the absorption was measured at 540 nm after it cooled down to room temperature. Each glucanase activity determinations were performed in triplicate.

Biochemical characterization of the enzyme

The effect of temperature on the activity of r-ScEG12 was determined by incubating crude enzyme mixture in citric acid-disodium hydrogen phosphate (McIlvaine) buffer (pH 5.0) at temperatures between 20 to 80 °C with a regular interval of 10 °C. Enzyme activity was assayed by the DNS method at different temperatures (as described above). One unit of enzymatic activity was defined as the amount of enzyme that released 1 mmol of reducing sugar equivalent per min under the assay conditions. Thermostability studies of the enzyme were conducted by preincubating the enzyme solution at 20, 30, 40, 50, 60, 70 and 80 °C for 10, 20, 30, 40, 50, 60 min, and all of them begin with the same activity which without any treatment. After incubation, the enzyme activity was checked by the DNS method.

The optimum pH of the enzyme was determined by incubating the enzyme with substrate (1 % CMC), prepared in a 0.05 McIlvaine buffer. To check the stability at different pH, the enzyme was placed in different pH buffers (pH 2, 3, 4, 5, 6, 7 and 8) at the temperature of 37 °C for 24 h. Then, the enzyme activity was measured using a standard assay procedure.

Various metal ions, including Na^+, Ca^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+} ,Li^+ and Cr^{3+} at 10mM concentration, were applied to obtain the optimum activity of the enzyme (PH 5.0, 37 °C) . The enzyme was assayed in McIlvaine buffer (pH 5.0) containing 1 % CMC.

Protease resistance was tested by incubating 50 µl r-ScEG12 with 2 ml of 0.2 mg/ml pepsin (80 mM glycine-HCl, pH2.5) and 2 ml of 0.2 mg/ml trypsin (80 mM $NH_4H_2CO_4$, pH 7.5), respectively. The r-ScEG12 resistance was measured by assessing residual enzyme activity using McIlvaine buffer under optimum conditions (pH 5.0, 37 °C for 1 h, 3 h, 5h and 7 h).

The Kinetic parameters, Km and Vmax were estimated by the method of Lineweaver and Burk.

Structural modeling of the enzyme

We analyzed the phylogenetic tree by using softwares ClustalX and MEGA6, in addition, the homology modeling project submitted to SWISS-MODEL workspace, with the aid of DSViewerPro6.0, we get the three-dimensional structure of endo-β-1,4-glucanase.

RESULTS

Gene cloning and construction of expression vector

By a BLAST anlysis, the synthesized r-ScEG12 gene and the wild-type showed 83 % homology. The codon-optimized gene was subcloned into pYPX8 expression vector. The inducible promoter from *AOX*1 gene, the chemical synthesized signal MF4I, the native transcription termination and polyadenylation signal from *AOX*1 gene consist pYPX8[16]. The sequencing data proved the expression plasmid was constructed correctly.

Expression and purification of r-ScEG12

Multiple plasmid integration occurred spontaneously in *P. pastoris* at a frequency between 1 % and 10 % of all His^+ transformants.

Table 1. Effect of metal ions on recombinant endo-β-1, 4-glucanase activity

Metal ions	r-Egase relative activity(%)
Control	100.00
Na^+	107.52
Ca^{2+}	109.29
Cu^{2+}	71.64
Zn^{2+}	101.73
Mn^{2+}	50.97
Co^{2+}	96.57
Mg^{2+}	102.92
Fe^{3+}	102.99
Fe^{2+}	103.40
Li^+	96.42
Cr^{3+}	82.94

Table 2. Comparison of Optimum pH and Temperature of the recombinant endoglucanases from Various Fungal Species

Fungus	Opt.pH	Opt.tem.(°C)	Reference
Bacillus circulans F-2	4.5	50	Yusung,Taejon (1995)
Thermoascus aurantiacus IFO9748	5.0	70	Jiong Hong (2007)
Penicillium funiculosum	4.0	65	Karboune *et al*.(2008)
Daldinia eschscholzii(Ehrenb.:Fr.)Rehm	6.0	70	Karnchanatat *et al*.(2008)
Aspergillus Oryzae VTCC-F045	5.5	55	Nguyen and Quyen(2010)
Trichoderma sp.	5.0	50	El-Zawahry *et al*.(2010)
Trichoderma viride	6.5	55	Iqbal *et al*.(2011)
Aspergillus niger ANL301	5.5	50	Chinedu *et al*.(2011)
Trichoderma sp.IS-05	3.0	60	Andrade *et al*.(2011)
Chaetomium cellulolyticum NRRL 18756	5.5	50	Fawzi and Hamdy(2011)
Aspergillus glaucus XC9	4.0	50	Tao *et al*.(2011)
Aspergillus niger VTCC-F021	5.0	55	Pham *et al*.(2012)
Penicillium simplicissimum H-11	3.2	60	Hongzhi Bai (2013)
*Alicyclobacillus sp.*A4	3.4	60	Yingguo Bai (2013)
Sclerotinia sclerotiorum	5.0	50	Haifa Chahed (2014)
Talaromyces emersonii CBS394.64	4.5	90	Kun Wang (2014)

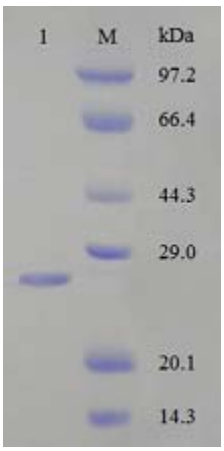


Fig. 1. SDS-PAGE patterns of the purified and recombinant endo-β-1,4-glucanase. M was molecular mass standard, lane 1 wa purified recombinant phytase endo-β-1,4-glucanase by Ni^{2+}-NTA agarose affinity.

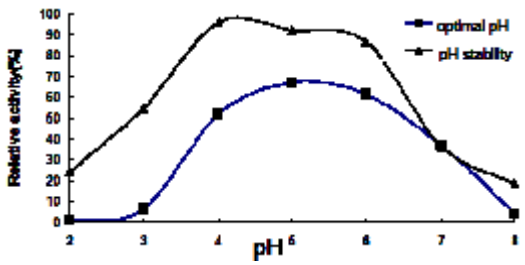


Fig. 2. Optimal pH and pH stability of the recombinant endo-β-1,4-glucanase

Small-scale strain will be used for expression of recombinant glucanase.

After induction for 8 h in 96-well plates, 21 % of the tested transformants showed glucanase activity. Some single colonies with higher activity were selected for further induction with methanol in shake flasks to improve r-ScEG12 yield. After 72 h, The maximal glucanase activity in the culture supernatant reached 0.114 U/mg. Ni^{2+}–NTA agarose affinity column (Sigma) was used to purify the protein with an additional six histidine residues at the C-terminal. The protein concentration of the purified glucanase was 10 µg/ml detected by the Bradford assay, bovine serum albumin was standard. By using SDS-PAGE, the apparent molecular mass of the recombinent protein was 27 kDa (Fig. 1).

Biochemical characterization of r-ScEG12

The r-ScEG12 activity was assayed in the mixture contained Na_2HPO_4 and citrate buffer under different pH values (2 to 8) at 37 °C for 4 h. The results showed the r-ScEG12 exhibited maximum activity around 40 °C and pH 5.0, respectively (Fig. 2 and Fig. 3). The activity of r-ScEG12 remained more than 80 % after incubated under weakly acidic condition (pH 4.0–6.0) at 37 °C for 4 h (Fig. 2). As the temperature increases from 40 °C to 80 °C, the enzyme activity sharply declined and the enzyme denaturation until the temperature was raised to 80 °C (Fig. 3).

As shown in Tab1, analysis of the effect of metallic ions at 10 mM concentration on r-ScEG12 activity showed that Mn^{2+}, Cu^{2+} inhibited the enzyme, Na^{+}, Ca^{2+} and Fe^{2+} enhanced the activity. Most studies[12,18] have suggested that glucanases activity was usually inhibited by Mn^{2+}, which might be due to its interaction with sulfhydryl groups present on the enzyme.

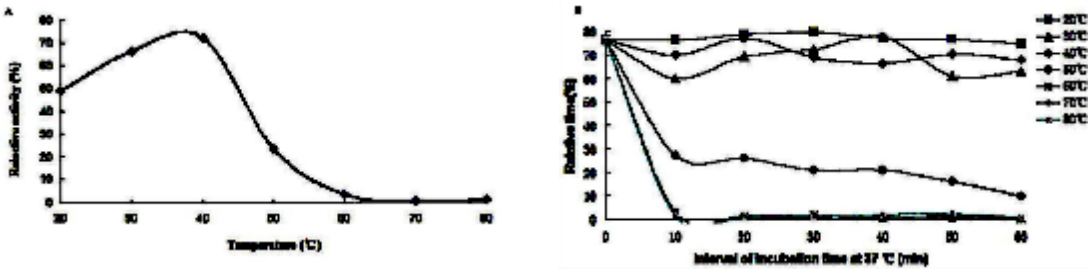


Fig. 3. Optimal temperature and thermostability of the recombinant endo-β-1,4-glucanase

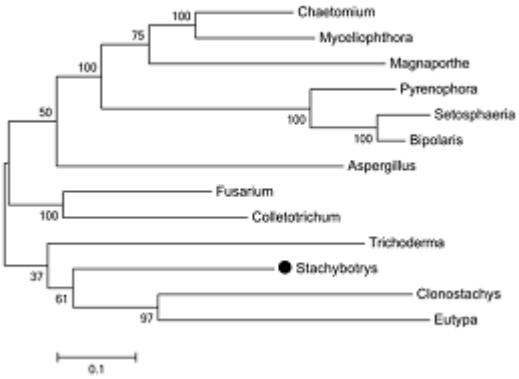
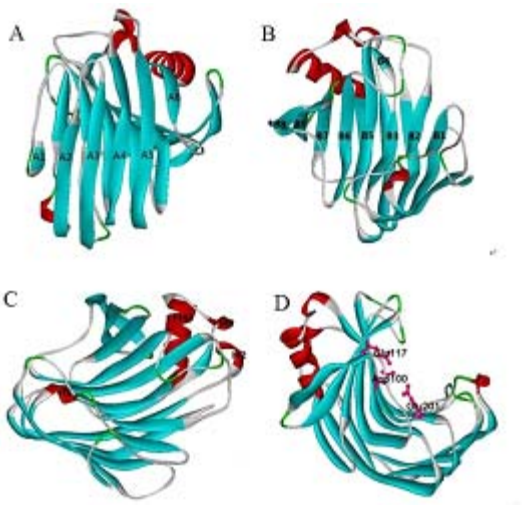


Fig. 4. The phylogenetic tree of the recombinant endo-β-1,4-glucanase



(A) The two β-sheets in the structure are labeled A and B. (B) Individual strands are labeled (A1-A6 or B1-B6) according to their positions in the two β-sheets. (C)The β-jelly roll structure of the r- ScEG12. (D) Closer view of the catalytic site

Fig. 5. The three-dimensional structure of the recombinant endo-β-1,4-glucanase.

The r-ScEG12 had no resistance to pepsin, it almost lost activity after incubation at 37 °C for 30 min, but showed a good resistance effect to trypsin, it retained 73.01 % activity under the same conditions and activity survived 23.54 % after further incubation for 1.5 h. The Km and Vmax for CMC were 26.08 mg/ml and 1.88 mg $L^{-1}min^{-1}$, respectively.

Structural modeling and phylogeny of the enzyme

Using the softwares (ClustalX and MEGA6), The phylogenetic tree (Fig. 4) showed the relationship with other different microorganisms based on amino acid sequence. The tree showed that *S. chartrum* endoglucanases was more closely related to *Clonostachys* than other endoglucanases. The homology between them is approximately 58 %.

The three-dimensional structure of r-ScEG12 (Fig.5) was determined by swiss-model work-page and DSViewerPro6.0 software with the Endoglucanase 3 (ThEG3) from *Trichoderma harzianum* as a template. The structure of r-ScEG12 was composed by two leaflets of anti-parallel β-sheets, the convex six strands (A1–A6) and concave nine strands (B1–B9), as depicted in Fig.5A and B. The β-strands were connected via four á-helices (H1, H2, H3 and H4; Fig. 5C) and several loops. The catalytic cleft which binds to the cellulose chains during the hydrolysis presents in the concave part of the leaflets. Fig. 5D showed the three catalytic residues Asp100, Glu117, and Glu201, strictly conserved in the glycosyl hydrolase family 12 [8],

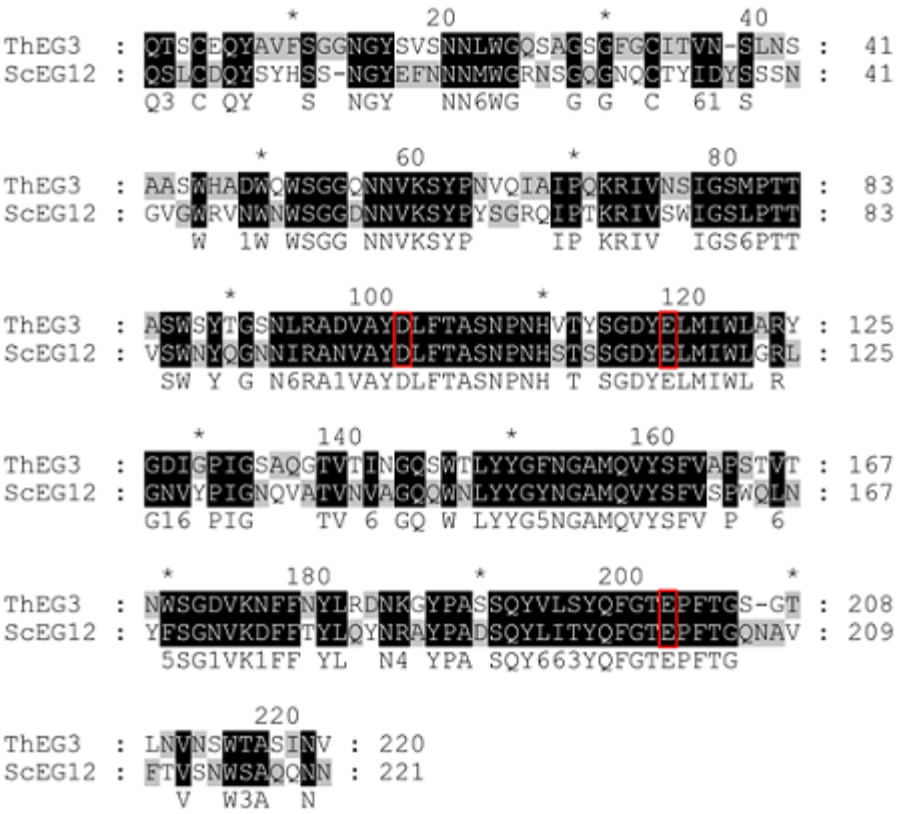


Fig. 7. Substrate binding cleft of the ScEG12. Some important residues were drawn explicitly. The highlighted residues differ from ThEG3. Residues Leu93, Asp96, Val193, Leu194 and Ser195in ThEG3 are replaced by Ile93, Asn96, Leu193, Ile194 and Thr195 in ScEG12.

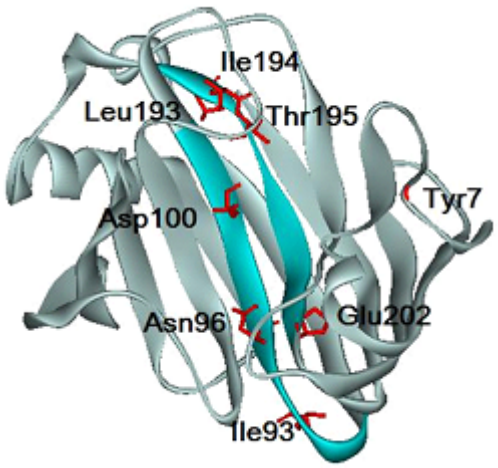


Fig. 6. Comparison of the primary structure of the r-ScEG12 and ThEG3. Alignment of the primary sequence of ScEG12 and ThEG3 with the catalytic triad residues marked in red border

At the same time, the Fig. 5D also highlighted the three aromatic residues located in the loops that for the 'thumb' and 'fingers' of the catalytic cleft of enzyme, the three residues should play an essential and important role in the efficiency of the hydrolytic catalysis.

DISCUSSION

In this study, we expressed an *S. chartarum* gene in *P. pastoris* and secreted the enzyme successfully, so the constructed yeast strain may be widely applied in cellulose degradation system. The results showed the *P. pastoris* is an excellent host for EG expression. To date, this gene has been expressed in *Escherichia coli*, *Aspergillus niger*[12], *P.pastoris* and showed a prominent protein band of 27, 24 and 27 kDa, respectively. Yeast recombinant proteins often

show glycosylation which increases the molecular weight and thermal stability of the proteins[19]. In this study, r-ScEG12 show this characterization, our previous study[20] have indicated that the endoglucanase produced in *P. pastoris*. So this gene showed different molecular weight expressed different strains. In addition, generally speaking, for protein expression in *P. pastoris*, 30 °C is usually used[21].

We find most endo-β-1,4-glucanases with different sources are acidic from table 2 (the lowest optimal pH is 3.2), becaouse cellulase used in textile industry have to be active in acidic environment[22]. In this study, the optimal ph is 5.0, it is lower than Cel12A expressed in *E. coli* (6.5) and *A. niger* (5.0)[12]. Different acidic endoglucanases have been previously expressed in *P. pastoris*[23-27], Some expression of endoglucanases in yeast *Saccharomyces cerevisiae* showed the same pH range[19, 28] with our study. From this study, we know *S. chartarum* is capable of secreting some amounts endoglucanases, and expressed in *P. pastoris* showed the lowest optimal pH.

The optimal temperature of recombinant endoglucanases was usually increased from 50 °C to 90 °C (table 2), most endo-β-1,4-glucanases are stable up to 50–55 °C, including the commercial enzyme from *Trichoderma reesei*[29] which have been expressed in *P. pastoris* and *E. coli* [30]. The optimal temperature of r-ScEG12 is 40 °C in this study, Cel12A expressed in *E. coli* and *A. niger* showed optimal temperature 45°C and 50°C, respectively[12], so r-ScEG12 is not thermophilic. However thermophilic cellulases are more favorable for industrial application because of their high activity and stability at high temperatures. A number of thermophilic cellulases have been purified, cloned and characterized in recent years though not all shown in table 2. Some thermophilic cellulases display maximum activities even over 100 °C, such as the GH 12 endoglucanase from *Pyrococcus furiosus*, endoglucanase celB from *Thermotoga neapolitana*[31], thermophilic fungi like *Talaromyces emersonii*, *Thermoascus aurantiacus*, and *Trichoderma koningii* as the main microbial source of thermophilic cellulases have been reported to produce cellulases of GH 3, 5, and 7 families with optimal temperature of 65"80 °C [32-35]. The r-ScEG12 did not show thermostable characterization, it may be related to its structure, so we analyzed the structure of r-ScEG12 in this study.

In previous studies about glucanase, one research has involoved extensive analysis of the enzymes substrates and indicated that the recombinant glucanase had higher affinity for low viscosity CMC substrate[12]. In this study, in order to contrast with ThEG3 [36], we use CMC as substrate and did not address the substrate analysis.

Endoglucanases (with very few exceptions) usually are composed by the large catalytic core domain (CCD) and the small CBM, which are connected by a heavily glycosylated fragment. One characteristic of GH family 12 fungal cellulases was lack of a CBM, resulting these enzymes degrade amorphous, but not crystalline cellulose. The r-ScEG12 contained 221 residues and had high primary sequence identity (56.76 %) with ThEG3. Fig. 6 showed some residues such as Tyr37, Asn207 and Asn221 which localized in the C-terminus of r-ScEG12 and ThEG3 lacked, while r-ScEG12 lacks Gly13. These differences between r-ScEG12 and ThEG3 must be relevant to the function of them such as thermophilicity and so on. The ThEG3 structure has been described in research[36] and most structural features of r-ScEG12 are similar to ThEG3.

The Fig.7 showed residues that were solvent exposed in the substrate binding cleft of r-ScEG12. There were two evident rows of hydrophobic residues in the cleft, one was formed by Ile93, Asn96 and Asp100 and the other was formed by Leu193, Ile194, Thr195 and Glu201. In GH family 12, a tryptophan (Trp) residue was most frequently found instead of tyrosine (Tyr) at the amino acid position #7. One research reported that it was likely because Tyr7 would hold somewhat weaker interactions with the substrate due to the smaller hydrophobic contact area relative to Trp7, so the Km of ThEG3 (21.4 g/L) is different from the endoglucanase 3 of *T. reesei* (TrEG3) (1.5 g/L) [36]. In this study, both ScEG12 and ThEG3 showed tyrosine (Tyr) in the position #7, so there is little difference between the Km of ScEG12 (26.08 g/L) and ThEG3 (21.4 g/L)[37]. This suggested that the substrate CMC binding affinity of r-ScEG12 was nearly equal to ThEG3. Close to the acid catalyst Asp100, r-ScEG12 had Ile93 and Asn96, whereas ThEG3 had Leu93 and Asp96. In addition, some differences found in the other row, Close to the

acid catalyst Glu201, ScEG12 has Leu193, Ile194 and Thr195, whereas ThEG3 has Val193, Leu194 and Ser195. This changes lead to important differences in the biochemical characterization of the two enzymes such as thermostability.

CONCLUSIONS

We successfully recombined the glucanase gene from *stachybotrys chartarum (r-ScEG12)* and synthesized, expressed in *P. pastoris*. Then we detected the biochemical characterization of the enzyme and developed the structure analysis of this gene. We found the apparent molecular weight of *r-ScEG12* was approximate 27 kDa; the optimum temperature and pH was 40°C and 5.0, respectively; the Km and Vmax for CMC were 26.08 g/L, and 1.88 mg $L^{-1}min^{-1}$, respectively; the r-ScEG12 activity was inhibited 71.64 % and 50.97 % by Cu^{2+} and Mn^{2+} respectively, while the r-ScEG12 activity was enhanced by Na^{+} and Ca^{2+} mildly. In addition, the Asp100, Glu117, and Glu201 residues were proposed to be present at the active site.

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