Purification and enzymatic properties of a new thermostable endoglucanase from Aspergillus oryzae HML366

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Abstract

Aspergillus oryzae HML366 is a newly screened cellulase-producing strain. The endoglucanase HML ED1 from *A. oryzae* HML366 was quickly purified by two-step method ammonium sulfate precipitation and strong anion exchange column. SDS-PAGE electrophoresis indicated that the molecular weight of the enzyme was 68 kDa. The optimum temperature of the purified endoglucanase was 60 ℃ and the enzyme activity was stable below 70 ℃. The optimum pH was 6.5, and the enzyme activity was stable at pH between 4.5 to 9.0. The analysis indicated that additional Na^+, K^+, Ca^{2+}, and Zn^{2+} reduced the catalytic ability of enzyme to the substrate, but Mn^{2+} enhanced its catalytic ability to the substrate.The *Km* and *Vmax* of the purified endoglucanase was 8.75 mg/mL and 60.24 μmol/min·mg, respectively. In this study, we for the first time reported that *A. oryzae* HML366 can produce a heat-resistant and wide pH tolerant endoglucanase HML ED1, which has potential industrial application value in bioethanol, paper, food, textile, detergent and pharmaceutical industries.

Introduction

Plant lignocellulose accounts for 50% of total biomass in the world and is an important renewable biomass energy material (Lynd et al 2002; Mikulski and Klosowski 2020; Xu et al 2019). Cellulose is a linear homopolysaccharide of 100–1000 D-glucose units, which are linked together by β-(1,4)-Glycosidic bond (Himmel et al 2007; Yang et al 2021; Zhou et al 2021).

Cellulases are enzymes involved in the hydrolysis of cellulose. Three types of enzymes, including cellobiohydrolase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4) and β -Glucosidase (EC 3.2.1.21), play an essential role in effectively degrading cellulose into glucose (Béguin and Aubert 1994; Qin et al 2021; Zhao et al 2021).

Cellulose can be fermented to produce ethanol and different types of energy and chemicals. Concerns about global climate change, increased demand for energy, and reduced oil supply have prompted people to develop renewable energy to replace fossil fuels. The production of ethanol by cellulose hydrolysis has become a subject of great interest (Chandrasekhar et al 2021; Srivastava et al 2021).

The endoglucanase (EC 3.2.1.4) acts on the non-crystalline areas inside the cellulose molecule to randomly hydrolyze the β-1,4-glycosidic bond in the cellulose molecule to produce short cellulose chains, thus playing an important role at the initation step of reaction. Endoglucanases provide raw materials that can be used in industrial applications. Endoglucanases is used to remove fluff fibers on the surface of cellulose to enhance the softness and brightness of cotton. Endoglucanases are also used to promote the soil removal from fabrics. It can be added to detergent products to increase color and soften fabrics. As a feed additive, it can improve the absorption rate of starch and vegetable oil by animals. In food industry, endoglucanases can increase the juice yield of vegetable juices. It can also increase the strength of pulp. Thus, endoglucanases have been widely used in pulp, textiles, bioethanol, washing, wine and beer, food processing, animal feed, agriculture, biomolecular chemical products and the pharmaceutical

industry (Araújo et al 2021; Haq et al 2021; Aich and Datta 2020; Li et al 2021; Pereira et al 2021; Ibrahim et al 2021; Petrova et al 2009; Zhang et al 2021).

Our group previously collected samples from the National Nature Reserve of Huanjiang County, Guangxi, and screened a cellulase-producing fungus HML366, which was identified as *Aspergillus oryzae* by phenotype and ITS-rDNA sequence analysis (Qin et al 2011). We have completed the purification experiment of partial cellulases from *A. oryzae* HML366 and tandem time-of-flight mass spectrometry detection. Relying on the full-length sequencing data of *A. oryzae* chromosome genes performed by Machida et al. (2005). We first report of a two-step rapid purification of two novel valuable βglucosidases from *A. oryzae* HML366, we quickly isolated and purified a new type of β-glucosidase with high transglycosidase activity and a predicted β-glucosidase from *A. oryzae* HML366 (He et al 2013). *A. oryzae* HML366 can simultaneously produce a new 33.6 kDa xylanase (He et al 2015). An extracellular enzyme HML CBH1 with a molecular weight of 48 kDa can also be isolated from the enzyme solution of *A. oryzae* HML366. This enzyme belongs to glycoside hydrolase family 7, and has both exoglucanase and endoglucanase activities (Qin et al 2020) .

A. oryzae HML366 contains abundant new enzyme resources. Our group aims to complete the purification of endoglucanase from this strain, and provide experimental basis for the comprehensive application of cellulase.

Materials And Methods

Materials and Methods

Microorganism and culture conditions

A. oryzae HML366 used in this study was stored on potato dextrose agar (PDA) slants and stored at 4°C in Guangxi Colleges Universities Key Laboratary of Exploitation and Utilization of Microbial and Botanical Resources. *A. oryzae* HML366 was originally isolated from the soil beneath the rotten wood in Mulun Forestry Center, Huanjiang County, Guangxi, China (Qin et al. 2011) and deposited in the Chinese Center for Type Culture Collection (Accession No. CCTCC AF 2021152).

Cellulase re-screening solid medium: 10 g bagasse and 6 g bran were mixed well with 30 mL Mandels nutrient salt solution in a 500 mL Erlenmeyer flask Eveleigh et al 2009. The cultures were turned twice a day and incubate at 30 °C for 5 days. 200 mL sterile ddH_2O was added to the culture, and extracted in a constant temperature water bath at 40 °C for 1h before filtering with four layers of gauze. The solution was centrifuged at 6000 r/min for 10 min to obtain a crude enzyme solution. The supernatant was collected and stored at 4 °C for future use (He et al 2013).

Endoglucanase rapid identification plate

1% sodium carboxymethyl cellulose made with sodium acetate buffer (pH5.0) was added to the plate with 1.5% agarose, followed by dropping 100 μL enzyme solution to the plate, and reacted at 30 ℃ for 30 min. The plate was stained for 30 min with 0.2 % Congo red, and then decolorized with three times volume of 1 mol/L NaCl. The presence of transparent circle on the plate indicated that there was ndoglucanase activity in the sample (He et al 2013, Sugimura et al 2006).

Determination of enzyme activity and protein concentration

1% (w/v) carboxymethyl cellulose (CMCNa, Fluka) dissolved in 2% (w/v) sodium citrate (50 mM/pH 4.8) was used as a substrate, and the 3,5-di Nitrosalicylic acid (DNS) method was used. Enzyme activity (U) is defined as the amount of enzyme needed to catalyze the production of 1 μmol glucose per minute (Miller 1959). The protein concentration was measured at 595 nm according to the method of Bradford (1976) by using the Bradford Protein Assay Kit (Beyotime Institute of Biotechnology (China). All assays were performed in triplicate.

Purification of *A. oryzae* HML366 endoglucanase

All purification steps were performed at 4 °C.

Seven aliquots of crude enzyme solution were prepared and 100 mL for each aliquot. The proteins in each sample were precipitated with 30%-90% relative saturation (10% concentration gradient increase) of ammonium sulfate, respectively. After precipitation, the samples were incubated for 12 h at 4 ℃. The enzyme solutions were centrifuged for 20 min at 4 ℃ with a speed of 8000 r/min. The precipitates were collected after centrifugation and dissolved with citric acid buffer solution (pH 4.8), and made up to 1/5 of the original volume. Sephadex G-25 gel column was used to quickly desalt from enzyme solution, and the enzyme activity of endoglucanase was identified with endoglucanase quick identification plate to determine the best conditions for ammonium sulfate precipitation and salting-out. The crude enzyme products were stored at 4 °C for further purification.

The crude enzymes were further purified by using MonoQ10/100GL (Amersham Biosciences, Sweden) strong anion exchange chromatography column (Petrova et al 2009; Zhang et al 2021; Qin et al 2011) with a BioLogic Duo-Flow protein purifier (Bio-Rad). (pressure 530 psi). 0.01 mol/L Tris-HCl (pH 8.3) solution was used as the starting buffer, and 1 mol/L NaCl in 0.01 mol/L Tris-HCl (pH 8.3) was used as the elution buffer, and flow rate was 1 mL/min. The enzyme was eluted using 60 mL of 0 to 0.3 M linear gradient of NaCl, and collected 0.5 mL per tube. The purified enzyme was stored at 4 °C. The enzyme activity of endoglucanase was determined, and the protein purity was detected by SDS-PAGE.

SDS-polyacrylamide gel electrophoresis analysis

According to Laemmli (1970) method, the enzyme products were subjected to separation with 12% SDS-PAGE gel in Tris-glycine buffer (pH 8.3) at a voltage of 120V. The gel was stained with coomassie brilliant Blue R 250, and the molecular weight of enzymes was evaluated by comparing the relative mobility of purified protein with low molecular weight protein standard (Beyotime Institute of Biotechnology).

zymogram analysis

The purified enzyme was subjected to non-denaturing protein gel electrophoresis with pH 8.3 electrophoresis buffer at 4 ℃ by using 50 V constant voltage. The separation gel and stacking gel was made by 8% acrylamide and 4% acrylamide, respectively, and 0.1% (w/v) sodium carboxymethyl cellulose (CMCNa, Fluka) was added in the separation gel. After the electrophoresis, the separation gel was stained for 20 min with 0.2% Congo red and then decolored with 1 mol/L NaCl to perform zymogram analysis (He et al 2013).

The effect of temperature and pH on enzyme activity

The definition of relative enzyme activity: the highest enzyme activity under a certain condition of the experimental project was set to 100%, and the ratio of enzyme activity under other conditions to the highest enzyme activity was defined as relative enzyme activity.

To determine the optimal temperature of endoglucanase, the enzyme activity was measured under the conditions of 30 ℃-90 ℃ in 50 mM acetate buffer. To determine the effect of temperature on the stability of enzyme, the enzyme was incubated for 60 min in a water bath at temperatures between 40 ℃ and 90 ℃, and the residual enzyme activity was then measured at 60 ℃.

To determine the optimal pH of enzyme, the enzyme was stored in following buffers with a concentration of 50 mM：disodium hydrogen phosphate-citric acid buffer, pH 3.0-7.5; Tris-HCl buffer：pH 7.5-pH 9.0；and glycine-NaOH buffer：pH 9.0-11.0. The solutions were first stored for 24h at 4 ℃, followed by 3 h at 30 ℃. The relative enzyme activity was determined at the optimal temperature. Relative enzymatic activity was defined as follows. The maximum enzymatic activity under specific control conditions were defined as 100 %, and the measured activities under varying conditions in the same experiment were normalized to derive the relative enzymatic activity in percentage. Three parallel tests were performed.

Effect of metal ions on enzyme activity

Different metal ions were added to the purified enzyme solution with a final concentration of 2 mM, and the enzyme activity was then tested. The enzyme activity was calculated according to the average value of data from three parallel experiments.

Kinetics analysis of the purified enzyme

To determine the kinetic parameters of the enzymatic reaction of endoglucanase, CMC-Na (0.2–3 mM) was used as the substrate and the reaction was performed in pH 5.0 sodium acetate buffer at optimal temperature. The initial reaction rate was calculated, and the *Km* value and *Vmax* of the purified enzyme was calculated by using Lineweaver-Burk plot (Horovitz and Levitzki 1987).

Thin-layer chromatography analysis of enzymatic hydrolysis products

The CMC-Na containing 50 µL purified enzyme was dissolved in 50 mmol sodium acetate buffer (pH5.0) to make 1% cellobiose, cellotriose and cellotetraose substrates. 1mL substrate was transferred and kept it at 30 ℃ for 12 hours. The hydrolyzed product was detected with silica thin plate chromatography. The extender was made by mixing of n-butanol, ethyl acetate, ammonia, and water with a ratio of 6:3:3:1 (v/v). Color developer A was made by mixing 1g aniline with 25 mL acetone, and developer B was made by mixing 1 mL diphenylamine with 25 mL acetone. Developer A and B was mixed, followed by adding 5 mL 85% phosphoric acid and mixed well. The plate was dried off after chromatography was finished, and the developer was sprayed. The plate was dried at 120°C for 10 min to develop the color (Jo et al 2003).

Results

Purification and identification of endoglucanase produced by solid-state fermentation of *A. oryzae* HML366

The endoglucanase activity and protein amount were increased greatly when the saturation of ammonium sulfate was between 50% and 80%, and the maximum value appeared when the saturation ws 80%. The proteins were precipitated under 80% of the ammonium sulfate saturation, and redissolved in 0.1 mol/L citric acid-sodium citrate buffer solution (pH 4.8). Sephadex G-25 gel column was used to quickly desalt, and the recovered enzyme product was stored at 4 °C for further purification.

linear elution analysis of enzyme purification

Tris-HCl elution buffer that contains 1 mol/L NaCl was used for elution at a flow rate of 1 mL/min. Enzyme was separated and purified with a continuous salt gradient from 0 to 0.5 M NaCl. It was found that the purified HML ED1 was mainly located in the No. 60 collection tube corresponding to peak 1. It was shown that 8% of elution buffer can purify endoglucanase very well (Fig. 1).

The enzyme solution in the No. 60 collection tube was concentrated and subjected to SDS-PAGE electrophoresis, and the molecular weight standard was Prestained Color Protein Molecular Weight Marker P0071 (Beyotime Biotechnology, China). HML ED1 protein was shown as a single band on the gel with the molecular weight of 68 kDa (Fig. 2).

After a two-step purification, the endoglucanase HML ED1 from *A. oryzae* HML366 was isolated (Fig. 2), and the purification yields were 17.6% and the purification folds were 4.8 (Tab. 1).

Purified HML ED1 rapid identification plate

Sodium carboxymethyl cellulose is a specific substrate for endoglucanase, which can produce a transparent circle after being hydrolyzed (He et al 2013). The transparent circle in Figure 3 confirmed that the enzyme solution produced by solid fermentation of *A. oryzae* HML366 had endoglucanase activity. After active staining with 1% Congo red stain solution and decolorizing with 1 M NaCl, a clear band was shown, indicating that HML366 can produce an endoglucanase (Fig. 4, lane 1).

The influence of temperature and pH on the activity of purified endoglucanase HML ED1

The enzyme was reacted with sodium carboxymethyl cellulose substrate at 25 ℃-90 ℃, and the optimal reaction temperature of HML ED1 was determined by measuring the enzyme activity. The enzyme showed highest activity at 60 ℃, indicating that the optimal enzymatic reaction temperature of the enzyme was 60 ℃ (Fig. 5A). The enzyme activity of the purified enzyme was on the rise between 25 ℃ and 60 ℃, and the relative enzyme activity was 100% at 25 ℃ for 0.5 h and 30 ℃ for 1 h, and there was almost no loss of enzyme activity. The enzyme activity at 60 ℃ for 0.5 h and 1 h still remained 97% and 96.8%, respectively. While the loss of enzyme activity was gradually increased when the enzyme solution was incubated at 70 ℃-90 ℃. These data indicated that the enzyme activity was relatively stable between 25 °C and 70 °C (Fig. 5B).

The enzyme activity of HML ED1 was gradually increased at pH between 3.0 to 6.5, and the highest enzyme activity was shown at pH 6.5 (Fig. 5C), indicating that the enzyme was acid cellulase. The enzyme activity of HML ED1 was greatly influenced at pH between 3.0 to 4.5, and the relative enzyme activity was 62.14%, 70.08%, 86.54% and 90.56% for pH value at 3, 3.5, 4, and 4.5, respectively. The relative enzyme activity remained between 95.46%-100% at pH 5.0 to 7.0, indicating that the enzyme activity was relatively stable in this interval. The enzyme activity loss was gradually increased over pH 9.0, and the enzyme activity was 94.46% and 64.26% at pH 9.0 and 11.0, respectively (Fig. 5D). Thus, HML ED1 was stable in the range of pH 4.5-9.0. These characteristics make this isolated enzyme suitable for industrial saccharification processes for bioethanol production and other applications.

Analysis of purified endoglucanase HML ED1 hydrolysate

The CMC Na, cellobiose, cellotriose, and cellotetraose hydrolysates were analyzed by TLC (Fig. 6). Four spots were found for CMC Na hydrolyses, they were glucose, cellobiose, cellotriose, and cellotetraose; cellotriose was hydrolyzed to obtain cellobiose; cellotetraose was hydrolyzed to obtain cellobiose and cellotriose. Cellotriose and cellotetraose cannot be hydrolyzed to produce glucose. No spots were found in cellobiose (Fig. 6). It was speculated that the randomly cleavage of internal β-1,4-glycosidic bonds by HML ED1 mainly produced cellobiose and cellotriose, but not glucose. These results indicated that HML ED1 has endoglucanase activity.

Enzymatic reaction kinetics

Based on Lineweaver-Burk plot, it was shown that *Km* and *Vmax* were 8.75 mg/mL and 60.24 μmol/min·mg, respectively.

The influence of metal ions on enzyme activity

Metal ions are often used as activators or inhibitors in the catalytic reaction of enzymes. Therefore, adding appropriate metal ions to the enzyme reaction system can improve the catalytic efficiency of the enzyme.

Ag^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+} had a strong inhibitory effect on the purified endoglucanase HML ED1 of *A. oryzae* HML366, while Mn^{2+}, Ca^{2+} and Mg^{2+} had obvious activation effects on HML ED1, and Na^{+} and K^{+} had no significant effect (Tab. 2). Hg^{2+} interacts with cysteine residues in the sulfhydryl bond, and it reacts with cysteine residues, and can change the tertiary structure of the protein. The active site may contain sulfhydryl groups. These sulfhydryl groups participate in catalysis and are essential for maintaining the structure of the enzyme. The divalent cobalt ion forms a complex with a variety of amino acids, and the enzyme active site bound by the cobalt ion is irreversible, completely denaturing the enzyme's activity. Other ions are the same as Mg^{2+}, Mn^{2+}, Ca^{2+}, Na^{+}, Cu^{2+} and Fe^{3+}, and these metals also tend to form metal complexes with proteins, which ultimately affect enzyme activity by changing their structures (Amisha and Amita 2021; Wang et al 2020).

Discussion

Endoglucanases can be produced by various filamentous fungi, such as *Penicillium*, *Fusarium*, *Trichoderma* and *Aspergillus* (Hirasawa et al 2019). Among them, *Trichoderma* is widely used as a cellulase producer, and *Aspergillus* has received more attention due to its powerful ability to secrete cellulase (Hirasawa et al 2019; Liu et al 2011; Tian et al 2018).

The endoglucanase of *Clostridium thermocellum* has an optimal pH of 6.6 and an optimal temperature of 70°C. It hydrolyzes carboxymethyl cellulose and hydrolyzes cellodextrin, cellotetraose and cellopentose at a higher rate, but does not hydrolyze crystalline cellulose (Fauth et al 1991).

Koga et al. extracted the endoglucanase STCE1 from *Staphylotrichum coccosporum* NBRC 31817. The optimum temperature of STCE1 is 60 ℃. STCE1 has high resistance to anionic surfactants and oxidants, indicating that STCE1 is a universal enzyme used for laundry (Koga et al 2008). Chaabouni et al. purified two endoglucanase EG A and EG B from *Penicillium occitanis*. The optimal temperature for the enzyme activity of EG A is 60°C, and for enzyme activity of EG B is 50°C. Both endoglucanases can hydrolyze carboxymethyl cellulose, but cannot hydrolyze microcrystalline cellulose (Avicel), but it is inhibited by the divalent cations Hg^{2+},

Co^{2+} and $Mn2^{+}$ (Chaabouni et al 2005). The highest activity of thermophilic fungal endoglucanase is usually at 50°C -80°C. As catalysts, heat-resistant enzymes have obvious advantages. Heat-resistant enzymes have obvious advantages as catalysts. In these processes, high temperatures often promote enzymes to penetrate cell wall materials and destroy cellulose raw materials, resulting in better hydrolysis. Thermophilic fungi are now considered to be a promising source of enzymes. The thermostable cellulase used for cellulose degradation can increase the rate of hydrolysis and saccharification (Fontes et al 1997; Lee et al 2010; Li et al 2006; Ghio et al 2020; Saqib et al 2010).

Thermophilic fungi can produce heat-resistant enzymes. In the process of cellulose degradation, cellulose swells at a higher temperature and converts to a form that can be more easily broken down. The screening of thermophilic fungi and the application of heat-resistant enzymes are important directions for

comprehensive applications of cellulose. Thermal stability is an important feature of industrial applications. These thermal enzymes have great application potential in the food, chemical, pharmaceutical industry and environmental biotechnology (Araújo et al 2021).

After hydrolysis of cellulose to produce glucose, it can be further fermentated to ethanol. The thermostable endoglucanase can improve the hydrolysis of cellulose and promote high-efficiency saccharification, generating more glucose than previous reports. Thus, thermostable endoglucanase can enhance the hydrolysis efficiency and catalyze the conversion of cellulosic biomass into fermentable sugars, thus it can be used in the production of cellulosic ethanol. In addition, the thermostable endoglucanase may also be used as a robust hydrolase that can be integrated into the industrial fermentation process (Fauth et al 1991; Koga et al 2008; Lee et al 2010; Tian et al 2018).

Recent studies have also shown that the addition of purified endoglucanases to commercial cellulases can cause stimulating effects on the hydrolysis of lignocellulosic biomass (Sujit et al 2014).

A. oryzae HML366 is a cellulase-producing strain newly screened by our group from the original forest sampling. 68 kDa endoglucanase HML ED1 was isolated by the two-step rapid purification method. Javed et al. Isolated a 25 kDa endoglucanase (Javed et al 2009), and Kitamoto et al. purified and obtained 31 kDa and 53 kDa endoglucanase (Kitamoto et al 1996), but 68 kDa endoglucanase has not been reported so far. We showed that the enzyme activity was stable below 70 ℃, and it was also stable at pH 4.5 to 9.0. Our analysis indicated that *Km* and *Vmax* of the enzyme was 8.75 mg/mL and 60.24 μmol/min·mg, respectively. This endoglucanase has many useful features, including a wide range of pH stability, thermal stability. These characteristics make the enzyme very suitable for hydrolysis involved in saccharification processes, including the production of bioethanol, fabrics, food and animal feed. In this study, we for the first time reported *A. oryzae* HML366 can produce heat-resistant and wide pH tolerance endoglucanase HML ED1, which has potential industrial value in bioethanol, papermaking, feed, food, textile, detergent and pharmaceutical industries.

Declarations

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

This work did not involve the direct study of humans. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and all. This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

The authors confirm that the work described has not been published before, that it is not under consideration for publication elsewhere, that its publication has been approved by all co-authors. The authors agree to publication in the ***International Microbiology*** Express.

Competing interest

The authors declare that they have no conflict of interest.

References

1. Aich S, Datta S (2020) Engineering of a highly thermostable endoglucanase from the GH7 family of *Bipolaris sorokiniana* for higher catalytic efficiency. Appl Microbiol Biotechnol 104(9): 3935-3945. https://doi.org/10.1007/s00253-020-10515-0
2. Amisha Patel, Amita Shah (2021) Purification and characterization of novel, thermostable and non-processive GH5 family endoglucanase from Fomitopsis meliae CFA 2. Int J Biol Macromol 182:1161-1169. https://doi.org/10.1016/j.ijbiomac.2021.04.110
3. Araújo EA, Dias AHS, Kadowaki MAS, Piyadov V, Pellegrini VOA, Urio MB, Ramos LP, Skaf MS, Polikarpov I (2021) Impact of cellulose properties on enzymatic degradation by bacterial GH48 enzymes: Structural and mechanistic insights from processive *Bacillus licheniformis* Cel48B cellulase. Carbohydr Polym 264: 118059. https://doi.org/10.1016/j.carbpol.2021.118059
4. Béguin P, Aubert JP (1994) The biological degradation of cellulose. FEMS Microbiol Rev 13(1):25-58. https://doi.org/10.1111/j.1574-6976.1994.tb00033.x
5. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254. https://doi.org/10.1016/0003-2697(76)90527-3
6. Chaabouni SE, Mechichi T, Limam F, Marzouki N (2005) Purification and Characterization of Two Low Molecular Weight endoglucanases Produced by *Penicillium occitanis* Mutant Pol 6. Appl Biochem Biotechnol 125(2): 99-112. https://doi.org/10.1385/ABAB:125:2:099

7. Chandrasekhar K, Kumar AN, Kumar G, Kim DH, Song YC, Kim SH (2021)
8. Electro-fermentation for biofuels and biochemicals production: Current status and future directions. Bioresour Technol 323: 124598. https://doi.org/10.1016/j.biortech.2020.124598
9. *Eveleigh DE, Mandels M, Andreotti R, Roche C (2009) Measurement of Saccharifying cellulase. Biotechnol Biofuels 2:21.* https://doi.org/10.1186/1754-6834-2-21
10. Fauth U, Romaniec MP, Kobayashi T, Demain AL (1991) Purification and characterization of endoglucanase Ss from *Clostridium thermocellu*. Biochem J 279: 67-73. https://doi.org/10.1016/0307-4412(91)90111-K
11. Fontes CM, Clarke JH, Hazlewood GP, Fernandes TH, Gilbert HJ, Ferreira LM (1997) Possible roles for a non-modular, thermostable and proteinase-resistant cellulase from the mesophilic aerobic soil bacterium *Cellvibrio mixtus*. Appl Microbiol Biotechnol 48(4): 473-479. https://doi.org/10.1007/s002530051082
12. Ghio S, Bradanini MB, Garrido MM, Ontanon OM, Piccinni FE, Marrero Diaz de Villegas R, Talia PM, Campos E (2020) Synergic activity of Cel8Pa beta-1,4 endoglucanase and Bg1Pa beta-glucosidase from *Paenibacillus xylanivorans* A59 in beta-glucan conversion. Biotechnol Rep (Amst) 28:e00526. https://doi.org/10.1016/j.btre.2020.e00526
13. Haq IU, Nawaz A, Liaqat B, Arshad Y, Fan XL, Sun MT, Zhou X, Xu Y, Akram F, Jiang K (2021) Pilot Scale Elimination of Phenolic Cellulase Inhibitors From Alkali Pretreated Wheat Straw for Improved Cellulolytic Digestibility to Fermentable Saccharides. Front Bioeng Biotechnol 9: 658159. https://doi.org/10.3389/fbioe.2021.658159
14. He HY, Qin YL, Chen GG, Li N, Liang ZQ (2013) Two-Step Purification of a Novel β-Glucosidase with High Transglycosylation Activity and Another Hypothetical β-Glucosidase in *Aspergillus oryzae* HML366 and Enzymatic Characterization. Applied Biochemistry and Biotechnology 169(3): 870-884. https://doi.org/10.1007/s12010-012-9936-9.
15. He HY, Qin YL, Li N, Chen GG, Liang ZQ (2015) Purification and Characterization of a Thermostable Hypothetical Xylanase from *Aspergillus oryzae* HML366. Applied Biochemistry and Biotechnology 175(6): 3148–3161. https://doi.org/10.1007/s12010-014-1352-x.
16. Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315(5813): 804–807. https://doi.org/10.1126/ science.1137016.
17. Hirasawa H, Shioya K, Mori K, Tashiro K, Aburatani S, Shida Y, Kuhara S, Ogasawara W (2019) Cellulase productivity of *Trichoderma reesei* mutants developed in Japan varies with varying pH conditions. J Biosci Bioeng 128(3): 264–273. https://doi.org/10.1016/j.jbiosc.2019.03.005.
18. Horovitz A, Levitzki A (1987) An accurate method for determination of receptor-ligand and enzyme-inhibitor dissociation constants from displacement curves. Proc Natl Acad Sci U S A 84(19):6654-6658. https://doi.org/10.1073/pnas.84.19.6654.
19. Ibrahim AM, Hamouda RA, El-Naggar NE, Al-Shakankery FM (2021) Bioprocess development for enhanced endoglucanase production by newly isolated bacteria, purification, characterization and in-

vitro efficacy as anti-biofilm of *Pseudomonas aeruginosa*. Sci Rep 11(1):9754. https://doi.org/10.1038/s41598-021-87901-9

20. Javed MR, Rashid MH, Nadeem H, Riaz M, Perveen R (2009) Catalytic and thermodynamic characterization of endoglucanase (CMCase) from *Aspergillus oryzae* cmc-1. Appl Biochem Biotechnol 157(3) 483-497. https://doi.org/10.1007/s12010-008-8331-z
21. Jo YY, Jo KJ, Jin YL, Kim KY, Shim JH, Kim YW, Park RD (2003) Characterization and kinetics of 45 kDa chitosanase from *Bacillus* sp P16. Biosci Biotechnol Biochem 67(9): 1875-1882. https://doi.org/10.1271/bbb.67.1875
22. Kitamoto N, Go M, Shibayama T, Kimura T, Kito Y, Ohmiya K, Tsukagoshi N (1996)
23. "Molecular cloning, purification and characterization of two endo-1,4-beta-glucanases from *Aspergillus oryzae* KBN616.". Appl Microbiol Biotechnol 46(5-6): 538-544. https://doi.org/10.1007/s002530050857
24. Koga J, Baba Y, Shimonaka A, Nishimura T, Hanamura S, Kono T (2008) Purification and Characterization of a New Family 45 Endoglucanase, STCE1, from *Staphylotrichum coccosporum* and Its Overproduction in Humicola insolens. Appl Environ Microbiol 74(13): 4210–4217. https://doi.org/10.1128/AEM.02747-07
25. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259): 680-685. https://doi.org/ 10.1038/227680a0
26. Lee KM, Jeya M, Joo AR, Singh R, Kim IW, Lee JK (2010) Purification and characterization of a thermostable endo-beta-1,4-glucanase from a novel strain of *Penicillium purpurogenum*. EnzymeMicrob Tech 46: 206-211. https://doi.org/10.1016/j.enzmictec.2009.11.002
27. Liu DY, Zhang RF, Yang XM, Wu HS, Xu DB, Tang Z, Shen QR (2011) Thermostable cellulose production of *Aspergillus fumigatus* Z5 under solid-state fermentation and its application in degradation of agricultural wastes. Int Biodeterior Biodegrad 65: 717–725. https://doi.org/10.1016/j.ibiod.2011.04.005
28. Li Y, Gao N, Zhang XY, Zhao GQ, Song X (2021) Sophorolipid Production Using Lignocellulosic Biomass by Co-culture of Several Recombinant Strains of Starmerella bombicola with Different Heterologous Cellulase Genes from *Penicillum oxalicum*. Appl Biochem Biotechnol 193(2): 377-388. https://doi.org/10.1007/s12010-020-03433-4
29. Li YH, Ding M, Wang J, Xu GJ, Zhao FK (2006) A novel thermoacidophilic endoglucanase, Ba-EGA, from a new cellulose-degrading bacterium, *Bacillus* sp. AC-1. Appl Microbiol Biotechnol 70(4): 430-436. https://doi.org/10.1007/ s00253-005-0075-x
30. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol Mol Biol Rev 66: 506-577. https://doi.org/10.1128/MMBR.66.3.506-577.2002
31. Machida M, Asai K, Sano M, Tanaka T, Kumagai T, Terai G, Kusumoto K, Arima T, Akita O, Kashiwagi Y, Abe K, Gomi K, Horiuchi H, Kitamoto K, Kobayashi T, Takeuchi M, Denning D W, Galagan JE, Nierman WC, Yu J, Archer DB, Bennett JW, Bhatnagar D, Cleveland TE, Fedorova ND, Gotoh O,

Horikawa H, Hosoyama A, Ichinomiya M, Igarashi R, Iwashita K, Juvvadi PR, Kato M, Kato Y, Kin T, Kokubun A, Maeda H, Maeyama N, Maruyama J, Nagasaki H, Nakajima T, Oda K, Okada K, Paulsen I, Sakamoto K, Sawano T, Takahashi M, Takase K, Terabayashi Y, Wortman JR, Yamada O, Yamagata Y, Anazawa H, Hata Y, Koide Y, Komori T, Koyama Y, Minetoki T, Suharnan S, Tanaka A, Isono K, Kuhara S, Ogasawara N, Kikuchi H (2005) Genome sequencing and analysis of *Aspergillus oryzae*. Nature 438(7071): 1157-1161. https://doi.org/10.1038/nature04300

32. Mikulski D, Klosowski G (2020) Hydrotropic pretreatment on distillery stillage for efficient cellulosic ethanol production. Bioresour Technol 300: 122661. https://doi.org/10.1016/j.biortech.2019.122661
33. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31, 426-428. https://doi.org/10.1021/ac60147a030
34. Pereira CS, Silveira RL, Skaf MS (2021) QM/MM Simulations of Enzymatic Hydrolysis of Cellulose: Probing the Viability of an Endocyclic Mechanism for an Inverting Cellulase. J Chem Inf Model 61(4): 1902-1912. https://doi.org/ 10.1021/acs.jcim.0c01380
35. Petrova SD, Bakalova NG, Kolev DN (2009) Properties of Two endoglucanases from a Mutant Strain *Trichoderma* sp M7 with Potential Application in the Paper Industry. Applied Biochemistry and Microbiology 45(2): 171–175. https://doi.org/10.1134/S0003683809020069
36. Qin XY, Duan C, Feng XM, Zhang YL, Dai L, Xu YJ, Ni YH (2021) Integrating phosphotungstic acid-assisted prerefining with cellulase treatment for enhancing the reactivity of kraft-based dissolving pulp. Bioresour Technol 320: 124283. https://doi.org/ 10.1016/ j.biortech. 2020.124283
37. Qin YL, Fu Y, Li QQ, Luo FF, He HY (2020) Purification and Enzymatic Properties of a Difunctional Glycoside Hydrolase from *Aspergillus oryzae* HML366. Indian Journal of Microbiology 60(4): 475-484. https://doi.org/ 10.1007/s12088-020-00892-5
38. Qin YL, Zhang YK, He HY, Zhu J, Chen GG, Li W, Liang ZQ (2011) Screening and Identification of a Fungal β-Glucosidase and the Enzymatic Synthesis of Gentiooligosaccharide. Applied Biochemistry and Biotechnology 163: 1012–1019. https://doi.org/10.1007/s12010-010-9105-y
39. Saqib AAN, Hassan M, Khan NF, Baig S (2010) Thermostability of crude endoglucanase from *Aspergillus fumigatus* grown under solid state fermentation (SSF) and submerged fermentation (SmF). Process Biochem 45: 641-646. https://doi.org/10.1016/j.procbio.2009.12.011
40. Srivastava RK, Shetti NP, Reddy KR, Kwon EE, Nadagouda MN, Aminabhavi TM (2021) Biomass utilization and production of biofuels from carbon neutral materials. Environ Pollut 276: 116731. https://doi.org/10.1016/j.envpol. 2021.116731
41. Sugimura M, Watanabe H, Lo N, Saito H (*2003*) *Purification, characterization, cDNA cloning and nucleotide sequencing of a cellulase from the yellow-spotted longicorn beetle, Psacothea Hilaris. Eur J Biochem 270(16): 3455–3460. https://doi.org/10.1046/j.1432-1033.2003.03735.x*
42. Sujit SJ, Saurabh SD, Tae-SU K, In-Won K, Jung-Kul L (2014) Characterization of a novel endo-β-1,4-glucanase from *Armillaria gemina* and its application in biomass hydrolysis. Appl Microbiol Biotechnol 98(2): 661-669. https://doi.org/10.1007/s00253-013-4894-x

43. Tian M, Wai A, Guha TK, Hausner G, Yuan Q (2018) Production of endoglucanase and xylanase using food waste by solid-state fermentation. Waste Biomass Valorization 9: 2391–2398. https://doi.org/10.1007/s12649-017-0192-7
44. Wang SS, Lin R, Ren YY, Zhang T, Lu HZ, Wang L, Fan DD (2020) Discussion Non-chromatographic purification of thermostable endoglucanase from Thermotoga maritima by fusion with a hydrophobic elastin-like polypeptide. Protein Expr Purif 173:105634. https://doi.org/10.1016/j.pep.2020.105634
45. Xu YH, Zhou Q, Li MF, Bian J, Peng F (2019) Tetrahydro-2-furanmethanol pretreatment of eucalyptus to enhance cellulose enzymatic hydrolysis and to produce high-quality lignin. Bioresour Technol 280: 489-492. https://doi.org/10.1016/j.biortech.2019.02.088
46. Yang G, Yang D, Wang X, Cao W (2021) A novel thermostable cellulase-producing *Bacillus licheniformis* A5 acts synergistically with Bacillus subtilis B2 to improve degradation of Chinese distillers' grains. Bioresour Technol 325:124729. https://doi.org/ 10.1016/j.biortech.2021.124729
47. Zhang PQ, Cui M, Huang RL, Qi W, Thielemans W, He ZM, Su RX (2021) Enhanced enzymatic hydrolysis of cellulose by endoglucanase via expansin pretreatment and the addition of zinc ions. Bioresour Technol 333: 125139. https://doi.org/10.1016/j.biortech.2021.125139
48. Zhao QQ, Liu Q,Wang Q, Qin YQ, Zhong YH, Gao LW, Liu GD, Qu YB (2021) Disruption of the *Trichoderma reesei* gul1 gene stimulates hyphal branching and reduces broth viscosity in cellulase production. J Ind Microbiol Biotechnol 48:1-2. https://doi.org/10.1093/jimb/kuab012
49. Zhou Z, Ju X, Chen J, Wang R, Zhong Y, Li L (2021) Charge-oriented strategies of tunable substrate affinity based on cellulase and biomass for improving in situ saccharification: A review. Bioresour Technol 319:124159. https://doi.org/ 10.1016/j.biortech.2020.124159

Tables

Table 1 Summary of purification of HML ED1 produced by *A. oryzae* HML366

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification Yield (%)	Purification fold
Crude enzyme	636.6	426.0	1.5	100.0	1.0
$(NH_4)_2SO_4$	432.6	162.4	2.7	68.0	1.8
Mono Q 10/100	112.2	15.6	7.2	17.6	4.8

Table 2 Effect of various metal ions on activity of purified endoglucanase

Metal ions (1 mM)	Relative activity (%)
Control (Crude enzyme solution)	100
Na^+	105
K^+	108
Mg^{2+}	152
Mn^{2+}	156
Ca^{2+}	136
Zn^{2+}	62
Cu^{2+}	52
Ag^+	38
Co^{2+}	36
Hg^{2+}	26

Values represent the means of values from three independent experiments, with a standard deviation.

Figures

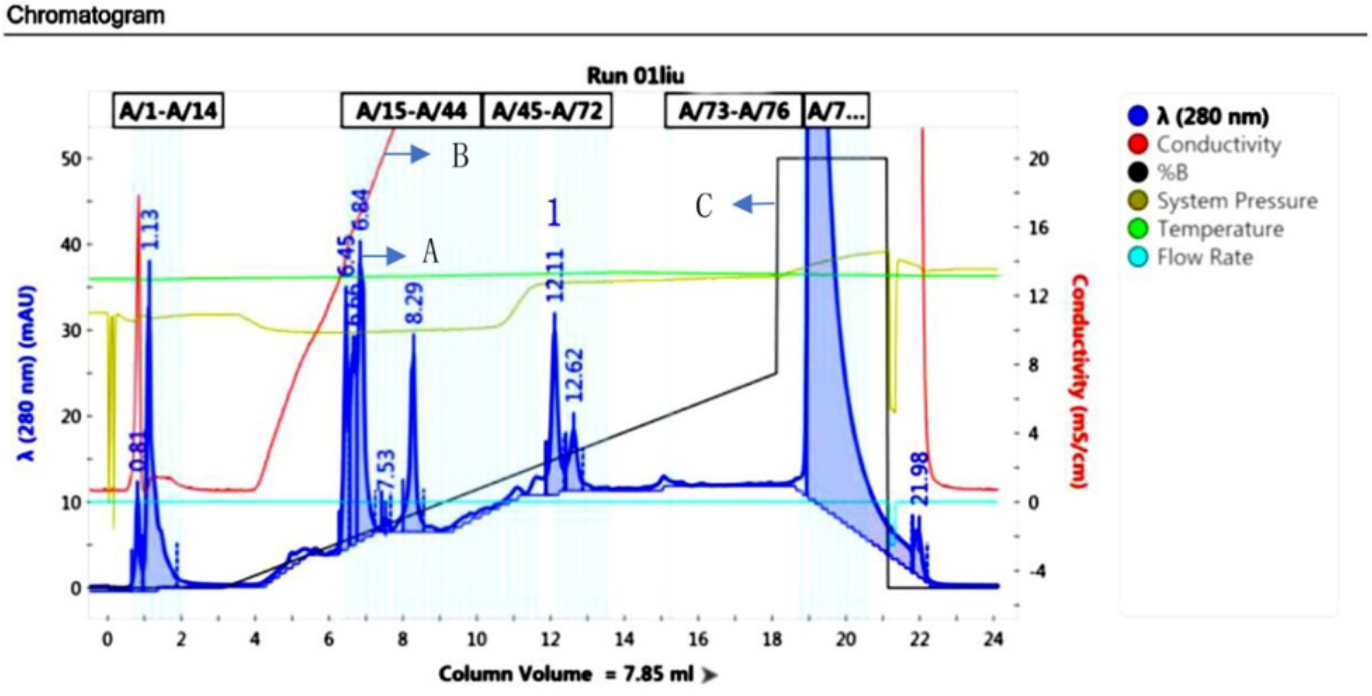

Figure 1

Protein purification map of anion exchange chromatography. 1, the peak of HML ED1 protein; A the ultraviolet absorption curve of protein; B the conductivity curve. C the elution buffer concentration (%). The abscissa showed the elution time, and the left ordinate was the protein UV absorbance (AU) at 280 nm and the elution buffer concentration (%), and the right ordinate was the conductance (mS/cm).

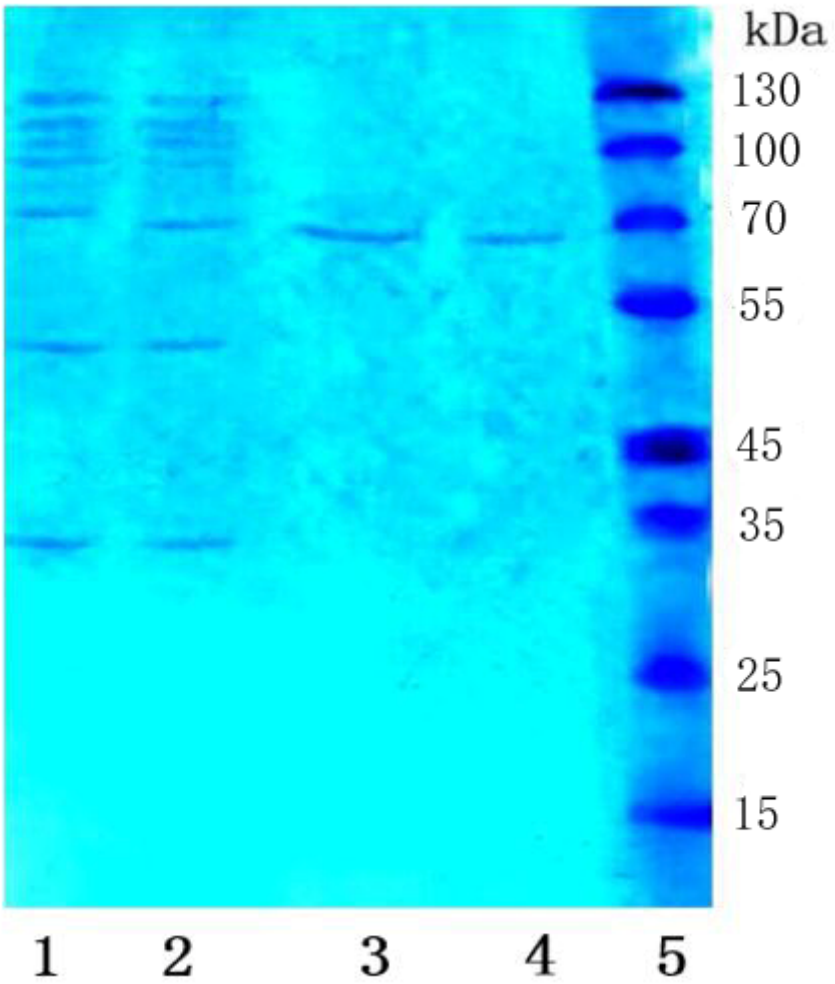

Figure 2

SDS-PAGE of the purified enzyme HML ED1. 1, 2 original fermented solution; 3, 4 HML ED1; 5 Prestained Color Protein Molecular Weight Marker P0071(Beyotime Biotechnology，China).

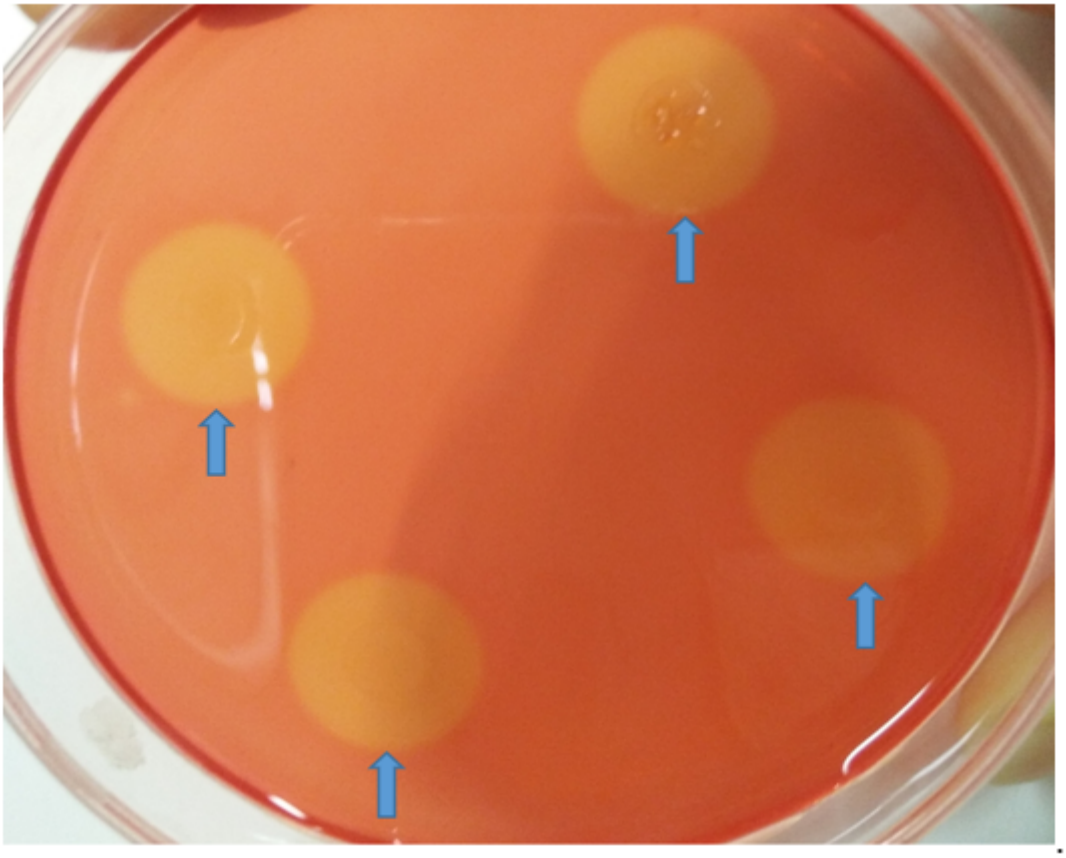

Figure 3

Detection of endoglucanase activity of purified HML CBH1 enzyme. The arrow points were the clear circles produced by the hydrolysis of sodium carboxymethyl cellulose substrate by endoglucanase enzyme.

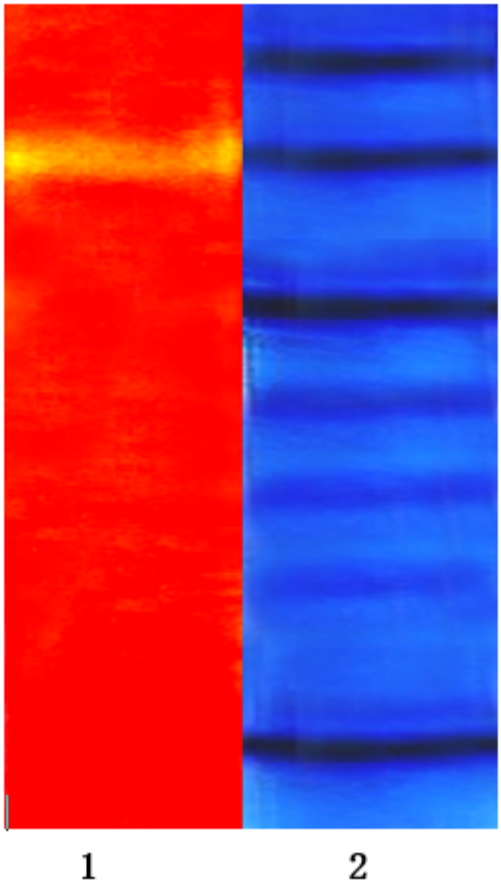

Figure 4

Zymogram analysis of extracellular enzyme solution and purified enzyme produced by *Aspergillus oryzae* HML366. 1 Purified enzyme stained with 1% Congo red, 1 M NaCl cleaning. 2 It is the original fermented solution, stained with Coomassie Brilliant Blue.

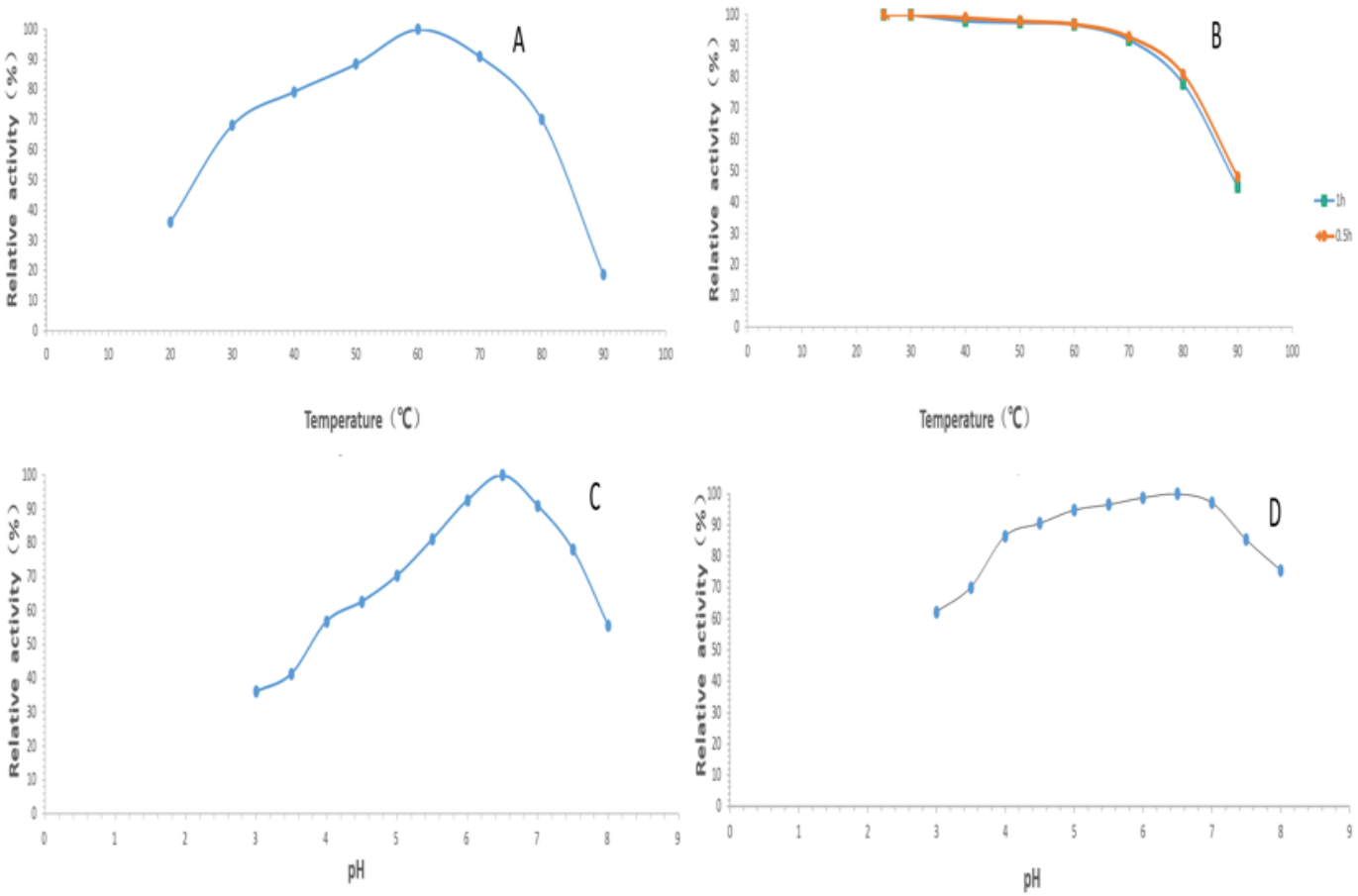

Figure 5

A. Optimum temperature of the purified endoglucanase from *A. oryzae* HML366. B. Thermal stability of the purified endoglucanase from *A. oryzae* HML366. C. Optimum pH on the activity of the purified endoglucanase from *A. oryzae* HML366. D. The pH stability of the purified endoglucanase from *A. oryzae* HML366.

Figure 6

Hydrolytic activity of the purifed endoglucanase from *A. oryzae* HML366 shown by TLC. Lane 1, mixed standards containing G1 Glucose, G2 Cellobiose, G3 Cellotriose, and G4 Cellotetraose; Lane 2, Cellobiose standard; Lane 3, Glucose standard; Lane 4, CMC Na + HML ED1; Lane 5, Cellobiose + HML ED1; 6. Cellotriose + HML ED1; 7. Cellotetraose + HML ED1.