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Full Length Article

Purification and Expression of a Novel Alkali-Stable Endoxylanase Xynh31

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Abstract

The endoxylanase Xynh31 was isolated and purified from the fermentation broth of *Streptomyces* sp. H31 strain by cation exchange chromatography. The optimal reaction pH of the xylanase was 7–9 and the maximum activity was determined at about 50°C. It showed higher enzyme activity under alkaline conditions. Primers were designed using the conserved region of the glycoside hydrolase family 10, and the endoxylanase gene *xynh31* was successfully cloned. The gene (1380 bp) encodes 459 amino acids. The results of BLAST analysis showed that the amino acid sequence was most similar to Xylanase A from the 10th family of *Streptomyces Halstedii* Jm8, but the highest identity was only 82%. *E. coli* BL21 (DE3) was used to express the gene, the maximum enzyme activity was 186.3 U/mL, and the specific activity was 628.4 U/mg. The enzyme meets the requirements of the paper industry and has good application prospects.

Keywords: Alkali-stable; Endoxylanase; Wide-pH-range; Industrial applications

Introduction

Endo-1, 4-β-D-xylanohydrolase (EC3.2.1.8), referred to as endoxylanase, is the most critical enzyme in the xylan degrading enzyme system (Collins *et al*. 2005). Endoxylanases are widely used in the paper industry (Basu *et al*. 2018). During the pretreatment of raw materials, the endoxylanase can effectively degrade the xylan re-adsorbed or deposited on the fiber surface during the cooking process, while loosening the paper fiber structure and making it easier to separate (Ashwani *et al*. 2009; Weerachavangkul *et al*. 2012). During paper bleaching and fiber modification, xylanase can act on hemicellulose in pulp connected with residual lignin, thus swelling and loosening the fibers (Sandrim *et al.* 2005; Nagar *et al.* 2013). Xylanase removes part of the hemicellulose and refine fibers on the surface of straw pulp fibers, which can be used to improve the performance of straw pulp and increase production efficiency (Buzała *et al.* 2016; Sridevi *et al*. 2017). Because of the characteristics of the paper industry process, xylanases are required to be alkali-resistant and have high thermal stability (Kumar *et al*. 2018).

Endoxylanase-producing microorganisms including bacteria, molds, actinomyces, and yeasts were widely distributed in natural environments (Juturu and Wu 2012). Fungi often produce acid xylanase which has low thermal stability (Liu *et al*. 2013). Alkaline xylanase was usually derived from bacteria and actinomycetes (Chakdar *et al*. 2016). Alkaline xylanase was first isolated and purified from alkalophiles in 1973 (Horikoshi 1973). Sanjivkumar *et al*. (2017) screened a xylanase-producing strain *Streptomyces olivaceus* MSU3 from mangroves, the optimal reaction pH of the endoxylanase produced was 8.0. Bagewadi *et al.* (2016) isolated a thermostable GH-10 xylanase from *Penicillium citrinum* HZN13. The endoxylanase gene *xynB* was cloned using a pair of degenerate primers designed according to the conservative amino acid sequences of xylanases. The gene was expressed in *E. coli*, the molecular weight of the recombinant endoxylanase is about 66 kDa.

Endoxylanase produced by Actinomycete is similar to that produced by Bacterium, both have a wide range of pH tolerance, better thermal stability, etc., and therefore have attracted more and more attention from researchers (Beg *et al*. 2001).

An endoglucanase-producing strain *Streptomyces* spp. H31 was isolated from soil in the early stage. In this paper, an alkali-resistant endoxylanase xynh31 was isolated and purified from the fermentation broth of this strain, and its gene was cloned and expressed in *E. coli.* This enzyme shown excellent properties such as alkali resistance and thermal stability, and shows good industrial application prospects. This research describes the isolation and purification of the enzyme, the cloning and expression of the enzyme gene, and its enzymatic properties.

Materials and Methods

SP-sepharose Fast Flow column and HisTrap™ column was purchased from GE Healthcare (General Electric Co., U.S.A.). Vector pET-28a (+) and *E. coli* BL21 (DE3) were used for the expression of endoxylanase. Vector pMD19-T and *E. coli* top10F´were used for plasmid construction and propagation. Xylan (Birchwood) and other reagents were all of analytical grade.

Isolation and purification of natural endoxylanase Xynh31

The culture broth of *Streptomyces* sp. H31 was centrifuged at 12000 r/min and the supernatant was loaded onto an SP-Sepharose Fast Flow column with PBS buffer pH 6.0 at a flow rate of 1 mL/min. Gradient elution was performed using NaCl-containing PBS buffer (pH 6.0), and the elution peaks were collected to determine the enzyme activity. The enzyme activity peak was dialyzed and concentrated, and then analyzed by SDS-PAGE electrophoresis (Brunelle and Green 2014).

Cloning and plasmid construction

Extraction protocols of Genomic DNA and plasmid were used as described (Sambrook *et al*. 1989). Endoxylanases mostly belong to the glycoside hydrolase (GH) family 10/11. The Pfam 26.0 (Sanger institute) platform (Finn *et al*. 2016) was used to analyze the conserved sequences of glycoside hydrolase families 10 and 11. Degenerate primers that were based on the most highly conserved amino acid residues are then designed to amplify the conserved sequence of endoxylanase gene (Staheli *et al*. 2011). The upstream and downstream sequences were amplified by TAIL-PCR as described (Liu and Whittier 1995). The cloned endoxylanase gene *xynh31* was ligated into the vector pET-28a (+) and then transformed into *E. coli BL21* (DE3).

Expression and purification of the recombinant enzyme

The selected recombinant was induced by IPTG and the cultures were harvested by centrifugation. The bacteria deposit was suspended in Tris-HCl buffer (50 m*M*, pH 8.0) and lysis by ultrasonic for 20 min. The cell-free extract was dialyzed and then was loaded onto a HisTrap™ column with Equilibration buffer (Tris-HCl buffer, 50 m*M*; NaCl, 0.2 *M*, pH 8.0). A linear gradient (*i.e*., 0.02, 0.05, 0.1, 0.2 and 0.5 *M*) of imidazole containing 0.2 M NaCl (flow rate: 1 mL/min) was used for the elution of adsorbed proteins. Fraction with endoxylanase activity were collected and tested for hydrolysis of 1% xylan at 45°C for 20 min. The Recombinant rXynh31 was subsequently examined by SDS-PAGE.

Biochemical characterization of endoxylanase

Xylan (Birchwood) was used as the substrate for the assay of the endoxylanases activity in different pH values and temperatures. The amount of enzyme required to produce 1.0 mg of xylose per hour is defined as one enzyme activity unit (U/mL). The method was used as described previously (Costa-Ferreira *et al*. 1994).

Results

Purification of natural endoxylanase Xynh31

The strain *Streptomyces* spp. H31 was inoculated in liquid culture at 37°C, 200 r/min for 5 days and the fermentation broth was centrifuged at 4°C, 12000 r/min for 10 min.

The SP-Sepharose Fast Flow column was equilibrated to baseline using pH 6.0 PBS buffer. Then apply the crude enzyme solution at a flow rate of 1 mL/min. Gradient elution was performed with PBS solutions containing NaCl (0.1 *M*/0.2 *M*/1 *M*) and the eluate is collected and measured for enzyme activity. The purified fraction which can be detected enzyme activity was further dialyzed and concentrated and analyzed by SDS-PAGE protein electrophoresis (Fig. 1). A single band was visible, which proved that the endoxylanase *xynh31* was successfully separated and purified by one-step cation exchange chromatography. Protein electrophoresis showed that the molecular weight of the enzyme was about 42 kDa. The specific activity of the enzyme was calculated to be 671.63 U/mg.

Cloning and sequence analysis of gene *xynh31*

The conservative sequence of the *xyn31* gene was amplified by conservative primers. TAIL-PCR was used to clone the full-length endoxylanases gene from the genome of *Streptomyces* spp. H31. The gene has an open reading frame (ORF) with 1380 bp, which starts with ATG, and TGA as the stop codon. The gene encoded a protein consisting of 459 amino acids with a calculated molecular mass of 42 kDa.

BLAST analysis showed that the amino acid sequence of this gene had the highest homology with the family 10 xylanase from *Streptomyces Halstedii* Jm8 but had only 82% similarity. Analysis of the conserved domain of Xynh31 revealed that it belongs to the glycoside hydrolase 10 family (GH10) and contains a second family carbohydrate-binding domain (CBM_2) at the C-terminal (Fig. 2).

Expression and purification of recombinant endoglucanases rXynh31

The endoxylanase gene *xynh31* was ligated into the expression vector pET-28a (+) and introduced into *E. coli BL21* (DE3). The optimal enzyme activity was up to 186.3 U/mL.

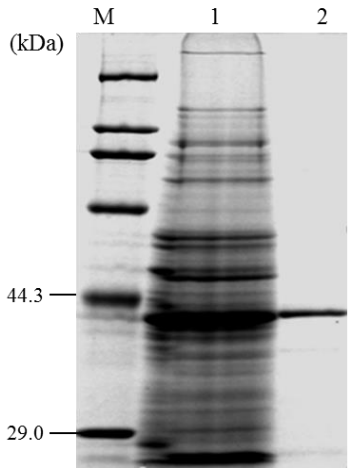

Fig. 1: SDS-PAGE of purified Xynh31
M: Premixed Protein Marker; 1: Crude enzyme; 2: SP-Sepharose Fast Flow Enzyme activity fraction

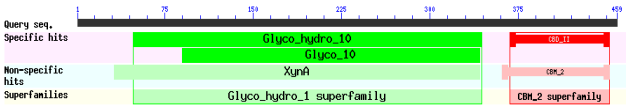

Fig. 2: Conserved domain analysis of Xynh31
The green is the glycosylhydrolase 10 family (GH10) catalytic domain, red is the binding domain CBM_2

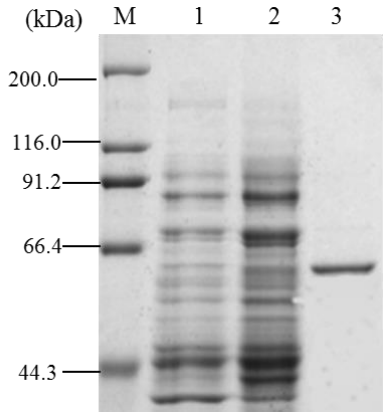

Fig. 3: SDS-PAGE of the expression and purified endoxylanase
M: Premixed Protein Marker (High); 1: Without IPTG; 2: Induction with 0.2 m*M* IPTG; 3: Purified protein

The Ni-column affinity chromatography was used to purify the recombinant enzyme rXynh31. The purified protein was analyzed by SDS-PAGE (Fig. 3). The results were shown in Fig. 3, indicating that the enzyme has been successfully isolated and purified with a molecular weight of approximately 60 kDa. The theoretical value of the recombinant protein is about 55 kDa, which may be related to the basic amino acids (such as His tags at both ends) that affect the SDS-PAGE mobility, resulting in a difference between the apparent molecular weight and the theoretical molecular weight. The specific activity of the recombinant endoxylanase was 628.4 U/mg.

Enzymatic properties of endoxylanase Xynh31 and rXynh31

Effect of temperature on enzyme activity

Analysis of the optimal reaction temperature of the native endoxylanase Xynh31and the recombinant rXynh31, the results can be seen from Fig. 4-A. The optimal reaction temperature range of the endoxylanases is 45–50°C. When the temperature exceeds 60°C, the enzyme activity decreases rapidly. The residual enzyme activity of rXynh31 is slightly higher than that of Xynh31 at a lower temperature (≤ 40°C), and the residual enzyme activity of the Xynh31 is slightly higher than that of the recombinant enzyme rXynh31 at higher temperature (≥ 50°C).

Thermal stability of the endoxylanases

The endoxylanase Xynh31 and rXynh31 were analyzed for thermal stability. It can be seen from Fig. 4-B that the enzyme has a constant activity after incubation for 60 min at a temperature lower than 50°C. However, when the temperature was higher than 55°C, the temperature stability of the enzymes decreased rapidly. At 55°C, the relative enzyme activity was closer to 80%. When the temperature was raised above 70°C, the relative enzyme activity dropped below 20%.

Effect of pH on enzyme activity

From Fig. 5-A, it can be seen that the relative residual enzyme activity of the recombinant enzyme under acidic conditions (pH 3–5) is higher than the native enzyme. The relative enzyme activity is maintained at a very high level between pH 7–9, and the relative enzyme activity is close to 100% at pH 7 and pH 9, which indicates that the enzyme Xynh31 is alkaline resistant endoxylanase, its optimum reaction pH is 7–9. The relative enzyme activity exceeds 50% at a pH of 10, indicating that the enzyme can adapt to the drastic change from weakly acidic to strong alkaline, and can perform better under alkaline conditions than under acidic conditions.

pH stability of the endoxylanases

Endoxylanases Xynh31 and rXynh31 were incubated at different pH for 60 min. The results were shown in Fig. 5-B. At pH 3–4, the relative enzyme activity of the recombinant enzyme is slightly higher than that of the natural enzyme; at pH 5–11, the relative enzyme activity of the enzyme is higher than 60%; at pH 11, it can still exceed 50%. It can be seen that the enzyme can maintain a relatively high relative enzyme activity under the condition of pH 5–11, especially the residual enzyme activity is above 70% in the entire range of pH 6–10. From the enzymatic nature of the enzyme,

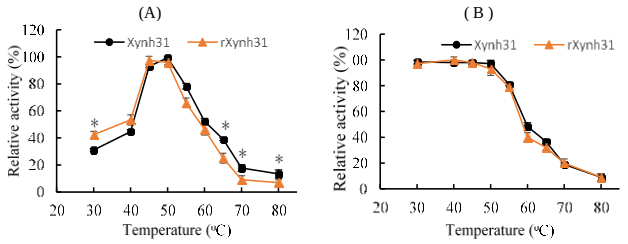

Fig. 4: The effect of temperature on the endoxylanase
(**A**) Optimum reaction temperature of the endoxylanase. (**B**) Thermostability of the endoxylanase
*: $P < 0.05$; **: $P < 0.01$

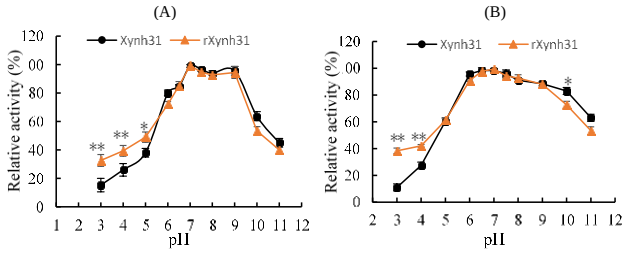

Fig. 5: The effect of pH on the endoxylanase
(**A**) Optimum reaction pH of the endoxylanase. (**B**) The pH stability of the endoxylanase
*: $P < 0.05$; **: $P < 0.01$

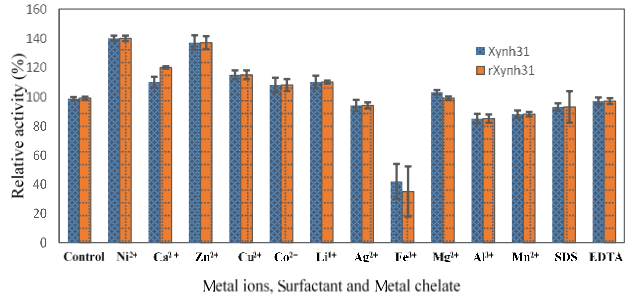

Fig. 6: Effect of metal ions, surfactant and metal chelate on endoxylanase activity
The control group enzyme activity is defined as 100%, Data are expressed as mean ± SD (n = 3) relative to control samples

its alkali resistance is better and its stability is high under neutral and alkaline conditions. This can be reasonably connected with the requirements of the enzyme used in the paper industry and has a good application prospect.

Effect of metal ions, surfactant and metal chelate on endoxylanase activity

Metal ions with a final concentration of 10 m*M* and SDS and EDTA with a final concentration of 0.5% were used to investigate the effects of metal ions and surfactants on the activity of endoxylanase (Fig. 6). In general, metal ions had little effect on the original enzyme and recombinase. Ni^{2+}, Zn^{2+} has an obvious promotion effect on enzyme activity, and Fe^{3+} has a significant inhibitory effect. SDS and EDTA had little effect on enzyme activity.

Discussion

Endoxylanase can be widely used in a variety of industrial production processes (Basit *et al.* 2018). Currently reported endoxylanases generally have deficiency such as narrow optimal pH range and poor thermal stability (Walia *et al.* 2017; Singh 2019) and in industrial applications (especially in the textile and paper industry), However, often face situations such as a large change in the pH of the reaction environment and a drastic change in the reaction temperature. It can be seen that these shortcomings have become constraints to its further application. Therefore, it is of great interest to discover new types of endoxylanases with wide pH adaptability suitable for industrial applications.

In this paper, the endoxylanase gene *xynh31* from *Streptomyces* spp. H31 was successfully cloned. Based on the sequence analysis results and the differences in molecular weight and enzymatic characteristics between this enzyme and the other existing endoxylanase, it showed that the gene xyh31 is a newly discovered endoxylanase gene.

After analysis with signalP (Nielsen 2017), the possible signal peptide sequences were removed. The gene sequence excluding the predicted signal peptide was expressed in *E. coli*, but the activity was found to be extremely low. It is speculated that the signal peptide sequence inferred by SignalP software may be inaccurate, resulting in the N segment of the enzyme being excised, thereby affecting the activity. Therefore, the entire open reading frame of the gene sequence from ATG was expressed and analyzed in this paper.

Through the study of enzymatic properties, it was found that the optimal reaction pH of this enzyme is between 7–9, and the optimal reaction temperature is between 45–50°C. The enzyme has good alkali-resistance, and the pure enzyme still has nearly 70% residual activity after being stored for 60 min at pH 10.0. It is a typical alkali-resistant xylanase. This is related to the natural alkaline environment of the strain *Streptomyces* sp. H31 (Chen *et al.* 2017). In addition, the enzyme also has many excellent properties such as resistance to a variety of metal ions and resistance to SDS and EDTA. The enzymatic characteristic of the endoxylanase determine its application potential and application fields. Generally, it requires high catalytic efficiency, good resistance to stress, a wide range of acid-base and temperature adaptation, etc. In the paper and pulp industry, lignin is often removed by high-temperature cooking under alkaline conditions (Kumar *et al.* 2016). However, endoxylanases currently used in industrial processes is generally not alkali-resistant and has poor thermal stability, which requires temperature and pH to be adjusted before adding enzymes, which is laborious, time-consuming, and extremely uneconomical (Sun and Li 2008). The development of new, high-quality alkaline-tolerant xylanase can not only improve the quality of pulp and finished paper, but also contribute to environmental

protection (Bajaj and Singh 2016). Based on the comprehensive enzymatic properties, it can be judged that the endoxylanase Xynh31 is a high-quality alkaline endoxylanase, which is suitable for industrial applications such as papermaking, waste paper deinking and recycling.

Conclusion

A novel endoxylanase Xynh31 was isolated and purified from *Streptomyces* spp. H31, and the complete open reading frame (ORF) of the enzyme gene was obtained by PCR cloning. The *xynh31* gene was successfully expressed in *E. coli* using the pET-28a (+) expression vector. The natural and recombinant endoxylanases studied in this paper have excellent enzymatic characteristics such as alkali resistance, high temperature resistance, metal ion resistance, surfactant resistance, and metal chelator resistance, which meet the basic requirements of industrial enzymes such as papermaking.

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