Characterization of a Xyloglucananse in Biodegradation of Woody Plant Xyloglucan from *Caldicellulosiruptor kronotskyensis*

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The 2,835-bp open reading frame of *ckxgl74A* (Locus_tag CALKRO_RS04315) with a natural carbohydrate module (CBM3b) from thermophilic anaerobic microorganism *Caldicellulosiruptor kronotskyensis* encodes a calculated 104-kDa of GH74 xyloglucanase Ckxgl74A. The purified recombinant Ckxgl74A expressed in *Escherichia coli* BL21 (DE3) revealed its optimal pH of 4.5 and temperature of 80 °C. The Ckxgl74A was stable over a temperature no more than 70 °C and a pH range of 4.5 to 5.0. Kinetic experiments with xyloglucan as a substrate gave a K_m of 2.29 ± 0.04 mg mL^{-1}, V_{max} of 22.98 ± 0.02 μmol mg^{-1} min^{-1}, and k_{cat} of 66.98 ± 0.01 s^{-1}. Its activity could be activated by Ca^{2+} approximately two folds, while being significantly inhibited by Cu^{2+}. These results showed that Ckxgl74A could be utilized in acid condition and possessed a good thermostability.

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Keywords: Acid resistance; Hemicellulose; Thermal stability; Xyloglucanase

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INTRODUCTION

Hemicellulose is a kind of component commonly found in the plant cell wall (Gupta *et al.* 2020). It is a polysaccharide composed of a variety of monosaccharides, mainly including xylan, mannan, arabinan, xyloglucan, *etc*., and they are able to be hydrolyzed into five- and six- fermentable carbon sugars, such as xylose, mannose, arabinose, and galactose. The hydrolysis application of hemicellulose polysaccharides is very extensive in energy and food industries (Balat 2011; Limayem and Ricke 2012; Keshwani and Cheng 2019).

Xyloglucan consists of highly recalcitrant and substituted polysaccharides found in the primary walls of vascular plants (Vieira *et al*. 2021). It is tightly connected to the rest of the hemicellulose through hydrogen bonding to form a network structure, supporting the stability of plant cell walls (Park and Cosgrove 2015). Xyloglucan is composed of β-1, 4-glucosidic bonds to form the main chain and is further substituted with xylosyl residues on the branch chain (Pauly and Keegstra 2016; Ray *et al*. 2004). It consists of a β-1,4-linked glucan backbone that is further substituted with xylosyl residues. In addition to the strength of the plant cell wall, xyloglucan can assist in gastroenteritis and acute diarrhea (Gnessi *et al*. 2015; Condratovici *et al*. 2016). Xyloglucanase, as one of the hydrolases, hydrolyzes β-1,4-glucosidic bonds in the main chain of xyloglucan to release oligosaccharides. Its

activity can be found among enzymes that are members of glycoside hydrolase 5, 12, 16, 44, and 74, mainly from microbial and vegetative sources (Attia and Brumer 2016; Gloster *et al.* 2007). Among microorganisms, xyloglucanase is mainly produced by certain bacteria and fungi (Arnal *et al*. 2019; Berezina *et al*. 2021). In the existing reports on xyloglucanase, the optimal reaction conditions for xyloglucanase produced by *Paenibacillus xylanilyticus* were at 50 °C (Ishida *et al*. 2007), pH 7.0, and the enzyme activity decreased faster when the pH was lower than 5.0 and the temperature was higher than 50 °C, similar to the xyloglucanase from *Myceliophthora thermophila* (Berezina *et al*. 2021). The study of xyloglucanase produced from *Streptomyces lividans* showed that the optimal temperature was 50 to 55 °C with a pH of 7.5 to 9.0, while the optimal reaction conditions for xyloglucanase from *Phanerochaete chrysosporium* were a temperature of 55 °C and a pH of 6.0 (Wang *et al*. 2016). The reported optimal pH for those xyloglucanases was mostly neutral and alkaline and had almost no ability and tendency to hydrolyze hemicellulose substrates under higher temperature and lower acidic conditions.

The production and utilization of hemicellulose resources generally have been carried out under high temperature and acidic conditions, one of the benefits being to prevent the contamination of microorganisms such as bacteria. The application of xyloglucanase with excellent heat and acid resistance in bioenergy and other industrial fields has become one of the main research directions. Therefore, this study reports the expression and enzymatic properties of the thermostable and acidic xyloglucanase derived from *Caldicellulosiruptor kronotskyensis* including its kinetic parameters, optimum temperature and pH, and substrate specificity.

EXPERIMENTAL

Materials

Bacterial strains, plasmids, and general growth conditions

Caldicellulosiruptor kronotskyensis 18902 was purchased from DSMZ (Braunschweig, Germany). Plasmid pET-28a was used for cloning and expression. *Escherichia coli* DH5α from Sigma-Aldrich (St. Louis, MO, USA) was selected as the cloning host, while *E. coli* BL21 (DE3) was utilized as the expression host. The components of the Luria-Bertani (LB) medium used for culture were from Oxoid Ltd. (Basingstoke, England).

Primers were synthesized by Generay Biotech (Shanghai, China). The DNA polymerase was purchased from Takara (Dalian, China). The DNA endonucleases, and ligase were purchased from New England Biolabs (Beijing, China). Substrate xyloglucan (from tamarind seed) was purchased from Neogen Bio-scientific Technology Co., Ltd. (Shanghai, China). β-D-Glucan (from barley) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Methods

DNA manipulation

Genomic DNA of *C. kronotskyensis* and plasmid pET-28a were used as templates, and the following primers (Table 1) were used for gene cloning. Primers F1 and R1 were used to *ckXgl74A* gene amplification, while F2 and R2 for pET-28a amplification. Then, primers F2 and R1 were used to amplify pET-28a-*ckXgl74A* fused gene using mixed *ckXgl74A* and pET28a products above with same concentration as templates using DNA

polymerase. The polymerase chain reaction (PCR) was conducted as follows: pre-denaturation at 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min; then extensions at 72 °C for 10 min. A Biomiga Gel/PCR Kit and a Biomiga Plaminiprep Kit (Shanghai, China) were used for the purification of PCR fragments and recombinant vectors respectively. T4-ligase was used to construct the circular pET-28a-*ckXgl74A*, and then the recombinant plasmid was confirmed by sequencing by Sangon Biotech (Shanghai, China). The DNA transformation was conducted *via* electroporation by using GenePulser Xcell (Bio-Rad, Hercules, CA, USA).

Table 1. Nucleotide Sequences of Used Primers

Primers	Nucleotide Sequences
F1	5'-CATCATCACCGAAGGGGTATAGCGTTATTTATTGCA-3'
F2	5'-ACCATCACCACTAACAAAGCCCGAAAGGAAGC-3'
R1	5'-TTGTTAGTGGTGATGGTTCCCCATACCAATACTCCATCTATATACGCAGT-3'
R2	5'-CCCTTCGGTGATGATGATGATGATGGCTGC -3'

Protein expression and purification

Relevant recombinant *E. coli* BL21 was cultured on a kanamycin plate at 37 °C for 12 h. Then, the single colony was selected and cultured overnight in a 20 mL LB medium flask containing kanamycin of 50 μg mL^{-1}, and then it was inoculated in the 1000 mL flask with 200 mL LB medium. After about 2 to 3 h incubation, the cells were grown to an OD_{600} of about 0.6, and then 0.5 mM isopropyl β-D-thiogalactoside (IPTG) (Sangon Biotech, Shanghai, China) was added to induce the protein expression. The cells were collected by centrifugation at 8000 rpm, 4 °C for 20 min. After that, cell pellets were resuspended and centrifuged again. Then, the crude enzyme solution was crushed for 25 min by sonication for 4 s, intermittently for 2s, and at 45% power. The crude mixture was centrifuged at 11000 rpm for 20 min at 4 °C.

The target protein was purified using Novagen's His Bind Purifucation Kit (Beijing, China), eluted with different concentrations of imidazole (50 mM, 200 mM, and 400 mM) in 20 mM Tris-HCl buffer (0.5 M NaCl and pH 7.9). The molecular mass of protein and purity were determined by 12% SDS-PAGE gel using a protein marker from Thermo Fisher Scientific Inc. (Shanghai, China). After this, the obtained recombinant enzymes were finally stored in 30% glycerol with 1 mM of Dithiothreitol (DTT) in -20 °C freezer.

Enzyme activity determination

For the enzyme activity determination, 100 μL of the corresponding pH buffer, 90 μl of 0.5% (w/v) xyloglucan (in water), and 10 μL of the appropriately substituted enzyme solution were added to the 200 μL reaction system, and then incubated at 80 °C for 10 min. Subsequently, 300 μL of 3, 5-dinitrosalicylic acid (DNS) (Miller 1959) was added to stop the reaction, and then the mixture was boiled for 5 min. The reducing sugar absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme to release 1 μmol of reducing sugar per min. All assays were conducted in triplicate.

Enzyme properties

The optimum temperature was measured by setting a reaction temperature of 50 to 90 °C (5 °C intervals) in 200 μL system containing 50 mM citrate buffer at pH 4.5 for 10 min. The optimum pH measurement conditions were set at an optimum reaction

temperature of 80 °C at 3.0 to 8.0 (0.5 intervals in 50 mM citrate buffer). The highest enzyme activity at each temperature or pH was set to 100%.

The pH stability was determined by adding 10 μL of enzyme and 90 μL of a buffer of different pHs to a 200 μL reaction system, incubating in a water bath at 40 °C for 2 h; then 100 μL of 0.5% xyloglucan was added to start the reaction by incubating at 80 °C for 10 min. Thermal stability was investigated by adding 10 μL of enzyme and 90 μL (pH 4.5) buffer to a 200 μL reaction system, incubating at 50 to 90 °C (10 °C intervals) for 0.5 to 2 h (0.5 h intervals).

Kinetic parameters

The 100 μL 0.1% to 0.5% substrate (0.1% intervals), 90 μL of a pH 4.5 citrate-sodium citrate buffer, and 10 μL of an appropriately substituted enzyme solution were added to the reaction system, and the process was carried out at 80 °C water bath for 10 min. Kinetic parameters, K_m, V_{max}, and k_{cat}, were deduced form double reciprocal Lineweaver-Burk mapping.

RESULTS AND DISCUSSION

Protein Expression, Purification, and Sequences Alignment

In this study, the *ckxgl74A* gene was successfully constructed and expressed in *E. coli* BL21 (DE3) with unique characteristics. The theoretical protein of *ckXgl74A* was 104 kDa, which was consistent with the results of the protein electropherogram (Fig. 1). The purification process was conducted as shown in Table 2, and the purified fraction of lane 4 (Fig. 1) was used to characterize the enzyme activity. Multiple sequence alignment of xyloglucanases obtained from CAZy (http://www.cazy.org/) was processed using Clustal X2 (data not shown). Based on amino acid similarity, CkXgl74A was judged to be a typical member of GH74 xyloglucanases relative to other *Caldicellulosiruptor* homologs (Arnal *et al*. 2019; Conway *et al*. 2018).

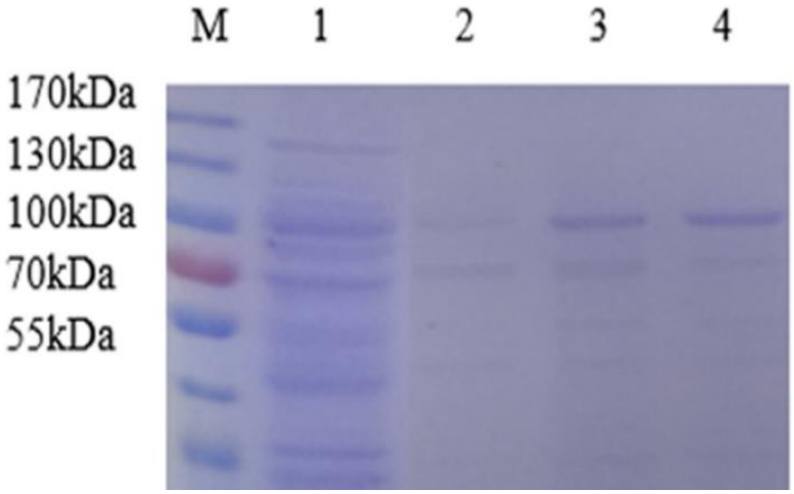

Fig. 1. SDS-PAGE of *ckXgl74A*. Lane M: marker; lane 1: the extract of recombinant *E. coli* BL21 (DE3) (10 μL); lane 2: the fractions purified *via* nickel affinity column chromatography (50 mM imidazole) (10 μL); lane 3: the purified proteins *via* nickel affinity column chromatography (200 mM imidazole) (10 μL); lane 4: the purified proteins *via* nickel affinity column chromatography (400 mM imidazole) (10 μL)

Table 2. Purification Process of CkXgl74A

Purification Step	Total Capacity (mL)	Total Activity (μmol/min)	Total Protein (mg)	Specific Enzyme Activity (μmoL/mg^{-1} min^{-1})	Recovery Rate	Purification Factor
Crude enzyme solution	200	230.2 ± 3.2	150.0 ± 0.5	1.55 ± 0.1	100%	1
Affinity nickel column	1	51.5 ± 0.1	2.6 ± 0.2	19.8 ± 0.2	22.4%	12.8

Enzymatic Properties

The optimum temperature for CkXgl74A was 80 °C (Fig. 2a). The results showed that the activity was relatively stable below 80 °C (Fig. 2b). At 50 and 60 °C, the relative activity was maintained for more than 90% within 1 h; however, it decreased rapidly when incubating for 1 to 2 h, maintaining only about 40% activity. The recombinant CkXgl74A also showed that it was very unstable above 80 °C. The optimum pH value of the recombinant enzyme was 4.5 (Fig. 2c), and it showed good pH stability at pH 4.5 to 5.5 (Fig. 2d), remaining above 70% activity after incubation for 2 h. This establishes that the xyloglucanase has good acid resistance under pH 4.5. These results showed that recombinase CkXgl74A possessed good high-temperature catalysis and acidic resistance.

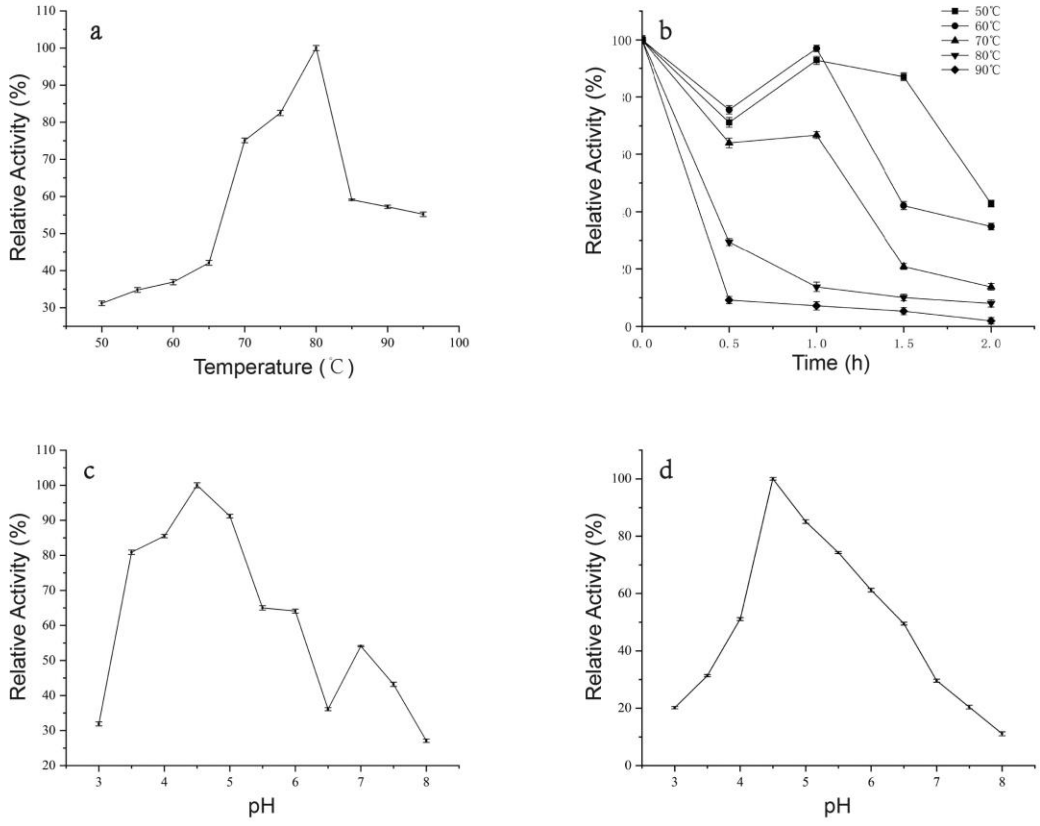

Fig. 2. Temperature and pH on CkXgl74A activity. a) Temperature optimum (pH 4.5, 50 °C to 95 °C for 10 min); b) Thermal stability (pH 4.5, 50 °C to 90 °C for 0, 30, 60, 90, and 120 min); c) pH optimum (pH 3 to 8, 80 °C for 10 min); and d) pH stability (pH 4.5 to 9, 40 °C for 2 h)

Compared to the xyloglucanases from *P. xylanilyticus* and *Streptomyces lividans* (Ishida *et al*. 2007; Wang *et al*. 2016), CkXgl74A from *C. kronotskyensis* in this study showed higher temperature optima and stability at an acidic condition. Generally, fermentation processes such as beer are usually carried out under acidic condition and saccharification at relatively high temperature (65 °C for 1 h) (Tokpohozin *et al*. 2019). Therefore, this recombinant xyloglucanase could be used in the fermentation processes of bioethanol, beer, and cereals, generating more fermentable sugars.

Effect of cations on CkXgl74A *activity*

When the concentration of the metal ion was 1 or 10 mM, it was found that Fe^{2+}, Fe^{3+}, and Ca^{2+} could obviously activate the enzyme activity, while Mg^{2+}, Mn^{2+}, and Cu^{2+} could significantly inhibit the enzyme activity. Besides, the enzyme activity could be significantly increased to 180% by adding 10 mM of Ca^{2+} (Fig. 3), which was similar with other studies (Wong *et al*. 2010).

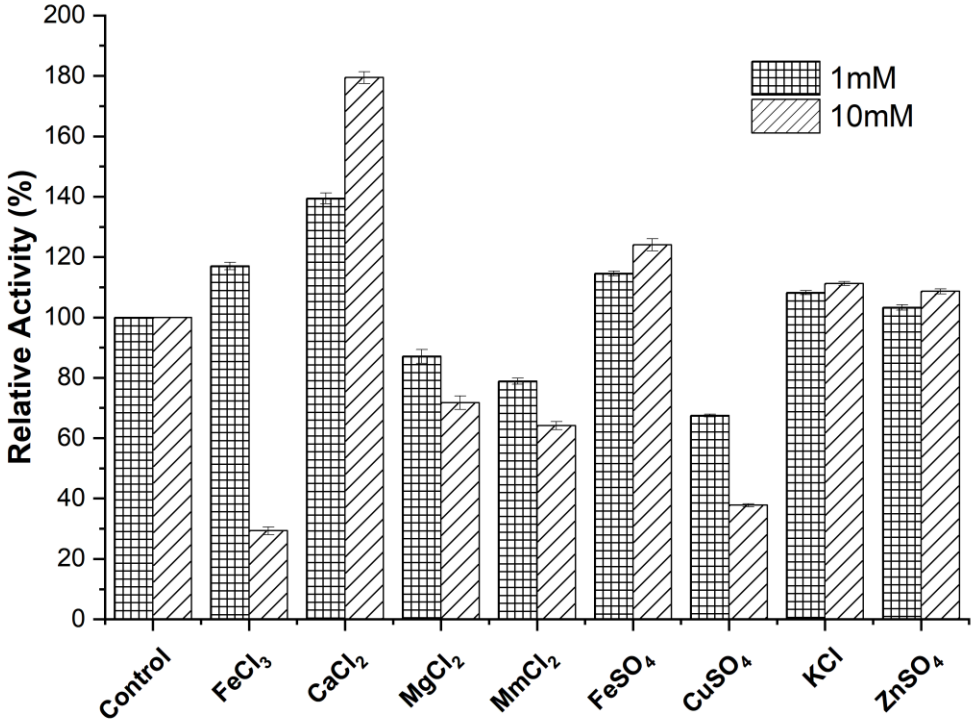

Fig. 3. Effects of cations on CkXgl74A activity. The rection was conducted using 0.5% xyloglucan and 4 μg of purified enzyme in 50 mM citrate buffer at pH 4.5 for 10 min. Values shown were the mean of triplicate experiments.

Substrate specificity and enzyme kinetics

The recombinant enzyme had no substrate activity on CMC-Na, filter paper, xylan, and β-D-glucan (Table 3). The enzyme was found to have a K_m of 2.29 ± 0.04 mg mL^{-1}, k_{cat} was 6.69 ± 0.01 s^{-1}, and V_{max} was 22.98 ± 0.02 μmol mg^{-1} min^{-1} (Table 4). These results showed that the K_m value was smaller than xyloglucanase XEG5 (2.0 mg mL^{-1}) (Yaoi *et al.* 2005).

Table 3. Substrate Specificity

Substrates	Specific activity (μmol min-1 mg-1)
CMC-Na	ND
Filter Paper	ND
Xylan	ND
β-D-Glucan	ND
Xyloglucan	115.07 ± 0.02

ND: Not detected

Table 4. Kinetic Parameters

Parameters	Recombinant xyloglucanase
k_m	2.29 ± 0.04 mg mL^{-1}
k_{cat}	6.69 ± 0.01s^{-1}
V_{max}	22.98 ± 0.02 μmol mg^{-1} min^{-1}
k_{cat}/k_m	29.25 ± 0.02 mL mg^{-1} s^{-1}

Application of Xyloglucanase

Xyloglucanase is very useful in biodegradation of xyloglucan. It hydrolyzes the xyloglucan, one component of hemicellulose in plant cell, to liberate fermentable sugars (Pauly and Keegstra 2016; Arnal *et al*. 2019; Berezina *et al*. 2021). Furthermore, it can be used to cooperate with cellulase, xylanase, and glucanase in degradation of cellulose, hemicellulose, and starch. CkXgl74A was only active to xyloglucan, and with its temperature and acidic stability, it should be a potential candidate in process of saccharification in industrial application.

CONCLUSIONS

1. The recombinant pET-28a harboring the CkXgl74A gene (2835 bp) was introduced into *E. coli* BL21 (DE3) for expression. After sonication and centrifugation of cell debris, cell extracts were purified *via* a nickel column. The molecular weight was 104 kDa, which was consistent with theoretical molecular weight.
2. The temperature and pH optima of CkXgl74A were 80 °C and 4.5, respectively. The CkXgl74A showed good thermal and acidic stability, and it could remain above 60% activity after a 1 h incubation at 50, 60, and 70 °C. Besides, it could maintain over 70% activity after incubation for 2 h at pH 4.5, 5.0, and 5.5.
3. CkXgl74A was only active toward xyloglucan, and it was significantly activated by Fe^{2+}, Fe^{3+}, and Ca^{2+}, and strongly inhibited by Mg^{2+}, Mn^{2+}, and Cu^{2+}. The kinetic parameters of α-glucosidase were a K_m of 2.29 ± 0.04 mg mL^{-1}, V_{max} of 22.98 ± 0.02 μmol mg^{-1} min^{-1}, and a k_{cat} of 6.69 ± 0.01 s^{-1}.
4. These characteristics of *ckXgl74A* revealed that it may possess desirable industrial applications in the fermentation process of bioethanol, beer, and other bioproducts.

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