Electronic Journal of Biotechnology 24 (2016) 56–62

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

A novel pH-stable, endoglucanase (JqCel5A) isolated from a salt-lake microorganism, *Jonesia quinghaiensis*

Ling Lin [a,*], Xiaozhou Liu [a], Yating Zhou [a], Linyan Guan [a], Jiajia He [a], Weiqian Huang [b]

[a] Provincial Key Laboratory of the Conservation and Exploitation Research of Biological Resources, College of Life Sciences, Anhui Normal University, Wuhu, Anhui, People's Republic of China
[b] Guangdong Rongda Bioengineering Company Limited, Qingyuan, Guangdong, People's Republic of China

ARTICLE INFO

Article history:
Received 21 June 2016
Accepted 7 September 2016
Available online 3 October 2016

Keywords:
Protein modeling
Site-directed mutagenesis
Cellulases
Recombinant endoglucanase gene
Catalytic domain of glycoside hydrolase
Carbohydrate-binding module
High pH stability
Tolerance to deleterious chemicals
Tolerance to heavy metals
Tolerance to detergents

ABSTRACT

Background: Endoglucanase, one of three type cellulases, can randomly cleave internal β-1,4-linkages in cellulose polymers. Thus, it could be applied in agricultural and industrial processes.
Results: A novel endoglucanase gene (*JqCel5A*) was cloned from *Jonesia quinghaiensis* and functionally expressed in *Escherichia coli* Rosetta (DE3). It contained 1722 bp and encoded a 573-residue polypeptide consisting of a catalytic domain of glycoside hydrolase family 5 (GH5) and a type 2 carbohydrate-binding module (CBM2), together with a predicted molecular mass of 61.79 kD. The purified JqCel5A displayed maximum activity at 55°C and pH 7.0, with 21.7 U/mg, 26.19 U/mg and 4.81 U/mg towards the substrate carboxymethyl cellulose, barley glucan and filter paper, respectively. Interestingly, JqCel5A exhibited high pH stability over a broad pH range of pH (3–11), and had good tolerance to a wide variety of deleterious chemicals including heavy metals and detergent. The catalytic mechanism of JqCel5A was also investigated by site mutagenesis and homology-modeling in this study.
Conclusions: It was believed that these properties might make JqCel5A to be potentially used in the suitable industrial catalytic condition, which has a broad pH fluctuation and/or chemical disturbance.

1. Introduction

Cellulases, including three types of cellulases, endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21), can completely hydrolyze cellulose to glucose by synergistic action. Thus, they could be applied in agricultural and waste treatment processes, and be employed as an environmentally bioenergy to replace depleting fossil fuels [1].

Currently, as a large volume industrial enzyme worldwide, endoglucanase possesses a great potential application in the textile industry, paper recycling, detergent industry, juice extraction, animal feed additives and renewable energy. However, the stability for specific processes and hydrolysis efficiency of endoglucanase seemed to be usually insufficient and also an important and difficult challenge in industrial process [2,3]. Besides, organic agents and divalent cations, which were used to solubilize the hydrophobic substrates and eliminate microbial contamination, led to a remarkable decrease in enzyme catalysis activity [4]. Therefore, high pH stability and resistance to metal ions and chemical reagents would make it a strong candidate for commercialization of endoglucanase in biofuel and detergent industry [5].

Jonesia quinghaiensis is a Gram-positive, non-acid-fast coryneform bacteria, which was isolated from mud of a soda lake in Qinghai, western province of China and grows optimally at pH 7–9 and salt concentration of 2.0–7.5% NaCl [6]. It is known that hypersaline habitat conditions might offer hyperproducers desirable properties meeting industrial demands. We have cloned and sequenced a novel endoglucanase gene (designated as *JqCel5A*) from *J. quinghaiensis*. The gene was heterogeneously expressed in *Escherichia coli* Rosetta (DE3), subsequently the recombinant enzyme was purified and characterized. The properties of organic solvent-tolerance and pH-resistance would make this enzyme's potentials in industrial applications. Moreover, computer modeling of JqCel5A's structure and the site-directed mutagenesis were constructed to demonstrate the importance of catalytic residues, Glu193 and Glu334, as providing evidence for double displacement mechanism originally suggested by Koshland [7].

* Corresponding author.
E-mail address: linling8@mail.ahnu.edu.cn (L. Lin).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2016.09.004

2. Materials and method

2.1. Bacterial strains and plasmids

J. quinghaiensis conserved by our laboratory was routinely cultured in Luria-Bertani (LB) broth at 30°C. The genomic DNA was prepared using TIANamp Bacterial DNA Kit (TIANGEN BIOTECH). *E. coli* DH5α and *E. coli* Rosetta (DE3) were used as hosts for cloning and gene expression with pET28a vector (Novagen).

2.2. Gene cloning and site-directed mutagenesis construction

The predicted endoglucanase gene designated as *JqCel5A* was amplified from the genomic DNA of *J. quinghaiensis* DSM 15701 strain by PCR with PrimeSTAR DNA Polymerase (TaKaRa, Japan) and the primers (JQ-F GGAATTCCATATGTGGGGCATCGTGCTCG; JQ-R: CCGCTCGAGTCCGTTCTGGCAGCTTG; The *Nde*I and *Xho*I sites are underlined), which designed using nucleotide sequence (GenBank accession no. NZ_AUHN01000006). PCR program was preformed as follow: 5 min 94°C followed by 30 cycles of 30 s 94°C, 30 s 56°C, 110 s 72°C, and finally 8 min 72°C. The obtained PCR products were purified with the AxyPrep DNA purification kit (Axygen) and cloned into the expression vector, pET-28a, which was further transformed into the competent cells of *E. coli* DH5α and submitted to Genscript (Nanjing, China) for sequencing. Subsequently, the recombinant plasmid was transformed into *E. coli* Rosetta (DE3) host strain for gene expression.

Point-mutant genes were prepared by site-directed mutagenesis using the overlap extension PCR method with some modification [8]. Initial PCRs, performed with mutagenic primers (E193A-F CGCTGACTTAAAAAATGCTCCCCACGGCAGTGC; E193A-R GCACTGCCGTGGGGAGCATTTTTTAAGTCAGCG; E334A-F CCCCTTCTCATTGGAGCTTGGGGCGGGTTCCTCG; E334A-R CGAGGAACCCGCCCAGCTCTCCAATGAGAAGGGG; the mutated sites are underlined), generated overlapping gene segments that were then used as template DNA for a subsequent PCR to create a full-length product. All mutant genes were inserted into the expression vector pET28a (Novagen). The constructed plasmids were introduced into *E. coli* Rosetta (DE3) for recombinant protein expression.

2.3. Protein expression and purification

Expression and purification of JqCel5A and its variants E193A and E334A were carried out following a method described previously by the protocol of WorkBeads™ 40Ni (TIANGEN) with slight modification. Respectively, *E. coli* Rosetta (DE3) cells harboring wild and mutant genes were grown at 20°C to an OD_{600} of 2.0 in LB medium containing 20 μg/mL kanamycin. Subsequently, IPTG was added to a final concentration of 0.1 mM. After 10 h induction at 20°C, cells were harvested and resuspended in lysis buffer (20 mM NaH_2PO_4, 0.5 M

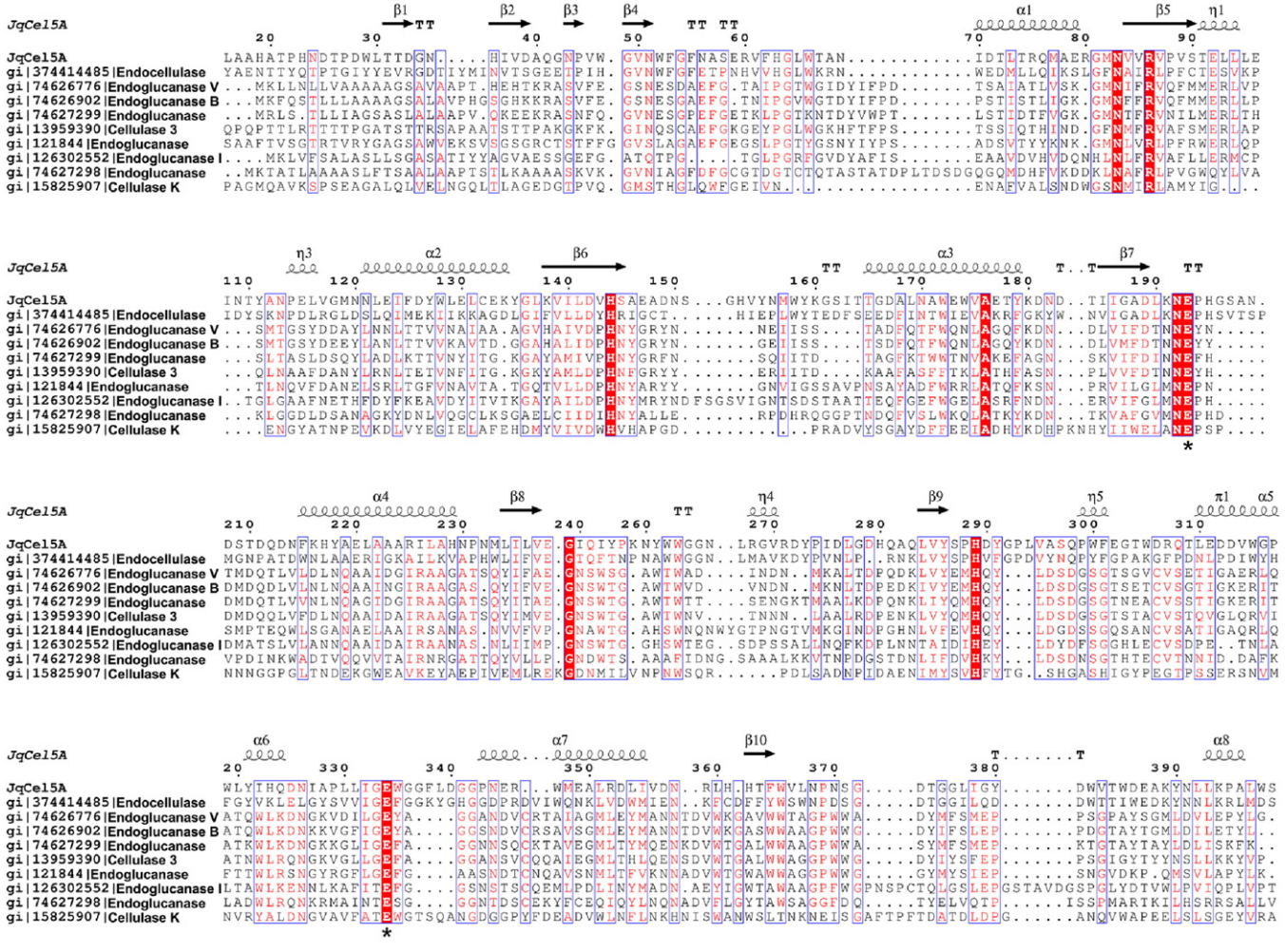

Fig. 1. Multiple sequence alignment between glycoside hydrolase domain of JqCel5A and similar family 5 endoglucanases. The multiple sequence alignment was performed using ClustalX program and ESPript 3.0. Framed areas indicate high amino acid similarity, in which the catalytic residues, Glu193 and Glu334, are marked with asterisks. The multiple endoglucanase sequences, Endocellulase (gi 374414485), Endoglucanase V (gi 74626776), Endoglucanase B (gi 74626902), Endoglucanase (gi 74627299), Cellulase 3 (gi 13959390), Endoglucanase (gi 121844), Endoglucanase I (gi 126302552), Endoglucanase (gi 74627298) and Cellulase K (gi 15825907) belonging to GH5 were from *P. horikoshii*, *A. aculeatus*, *A. niger*, *M. phaseolina*, *H. insolens*, *R. solanacearum*, *Robillarda* sp. Y-20, *M. phaseolina* and *Bacillus* sp. KSM-635, respectively.

NaCl, pH 7.4) and disrupted by ultrasonication on ice. The cell lysate was centrifuged (12,000 *g*, 30 min, 4°C) and the supernatant was collected and applied to a Ni^{+}-NTA agarose gel column for purification. After washing with three times column wash buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 20 mM imidazole, pH 7.4), the recombinant protein was eluted with 20 mM Na_2HPO_4, 0.5 M NaCl, 300 mM imidazole, pH 7.4. The purified protein was visualized and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the concentration was determined by Bradford method, using bovine serum albumin as a standard [9].

2.4. Characterization of enzyme activity

The standard enzyme activity of JqCel5A, E193A and E334A was determined using 3, 5-Dinitrosalicylic acid (DNS) method. Fifty microliters of diluted enzymes was applied into 100 μL reaction mixture containing 0.5% CMC and 0.2 M Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0). After incubation at 55°C for 30 min, 100 μL DNS reagent was added, and the reaction mixture was further heated in a boiling water bath for 5 min. Later, 800 μL H_2O was added into the mixture and the absorbance of the mixture was measured at 540 nm. The activity of the enzyme was further determined by using the glucose as the standard. One activity unit was defined as the amount of enzyme capable of releasing 1 μmol of reducing sugars per minute at 55°C [10].

The optimal working pH of JqCel5A was determined by evaluating their maximal activities in the buffer conditions ranging from pH 3.0–11.0 (0.2 M Na_2HPO_4-citric acid (pH 3.0–8.0) and 0.05 M Glycine-NaOH (pH 9.0–11.0)). The optimal working temperature was evaluated at the temperatures ranging from 10°C to 90°C. Thermal stability of JqCel5A was determined by assessing residual activities after incubation at the temperature from 20°C to 90°C for 1 h. For pH stability, relative activity was determined after the enzyme had been incubated with different pH buffers (pH 3–11) at 4°C for 1 h.

For the determination of the K_m and V_{max} values, the enzymatic activities towards various concentrations (ranging from 0.25–10 mg/mL) of CMC were measured. The data was fitted into the Michaelis–Menten equation using GraphPad Prism software (v. 5.1, GraphPad Software, Inc.), and the K_m and V_{max} values were calculated.

To investigate the substrate specificity of the enzymes, the activities were determined under the optimal conditions by replacing CMC with following substrates: 0.5% barley glucan (Sigma), 1% avicel (Sigma), 1% filter paper (Whatman) and 1% laminarin (Sigma) [11].

2.5. Molecular modeling of protein

Structural modeling of JqCel5A was performed by "Swiss-model Workspace" [12,13] using the reported crystal structure of EGPh from *Pyrococcus horikoshii* (Protein Data Bank code 3QHO) and type 2 carbohydrate-binding module from *Cellulomonas fimi* (Protein Data Bank code 1EXG) as the templates [14,15]. The hypothetical cartoon diagrams were predicted and illustrated by PyMoL v 1.2.1.

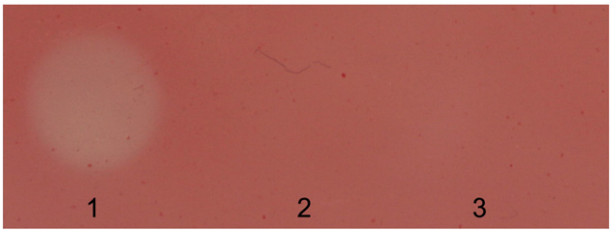

Fig. 2. Halo-forming activities of colonies detected by Congo red staining. (1): *E. coli* Rosetta (DE3) harboring the plasmid pET-*JqCel5A*; (2): *E. coli* Rosetta (DE3) harboring the plasmid pET-*E193A*; (3): *E. coli* Rosetta (DE3) harboring the plasmid pET-*E334A*. The colonies were cultured on CLB plate for 24 h at 37°C and followed by Congo red staining.

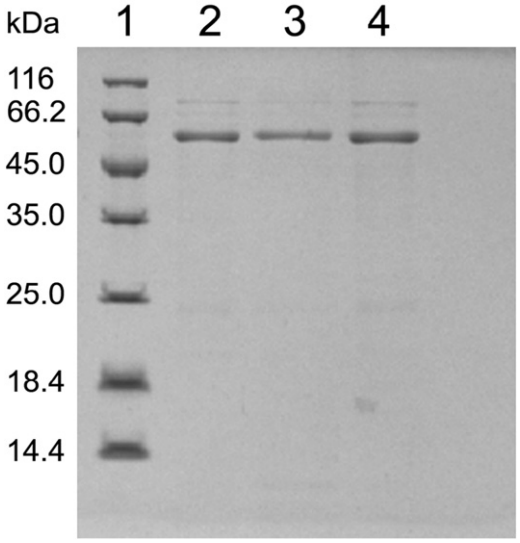

Fig. 3. SDS-PAGE of the purified proteins. Lane 1: the standard protein markers; Lane 2: JqCel5A (wild type); Lane 3: Mutant E193A; Lane 4: Mutant E334A.

3. Result and discussion

3.1. Cloning and sequence analysis of JqCel5A

The *JqCel5A* gene was PCR-cloned from *J. quinghaiensis* using the genomic DNA as the template. The sequencing result showed that the open-reading frame of the gene contained 1722 bp, which encoded a 573-residue polypeptide with a predicted molecular mass of 61.79 kD and a calculated pI of 4.18. Conserved domains analysis showed that JqCel5A harbors two completely distinct structures, the glycoside hydrolase family 5 (GH5, 36-418 residue) and the type 2 carbohydrate-binding module (CBM2, 465-573 residue) separated by short Gly/Ser/Asp-rich linker region [16,17].

Multiple sequence alignments were performed with ClustalX and ESPript 3.0 (Fig. 1). The result showed that GH5 module of JqCel5A shared low identities with Endocellulase (30.6%, gi 374414485), Endoglucanase V (14.8%, gi 74626776), Endoglucanase B (14.8%, gi 74626902), Endoglucanase (7.2%, gi 74627299), Cellulase 3 (8.0%, gi 13959390), Endoglucanase (10.0%, gi 121,844), Endoglucanase I (5.7%, gi 126302552), Endoglucanase (8.7%, gi 74627298) and Cellulase K (16.8%, gi 15825907) belonging to GH5 were from *P. horikoshii*, *Aspergillus aculeatus*, *Aspergillus niger*, *Macrophomina phaseolina*, *Humicola insolens*, *Ralstonia solanacearum*, *Robillarda* sp. Y-20, *M. phaseolina* and *Bacillus* sp. KSM-635, respectively. However, they all shared high similarities at some amino acid sites (marked with frame), especially for the predicted catalytic residues, Glu193 and Glu334 labeled with asterisks, which were strictly conserved in all endoglucanases of glycosyl hydrolase family 5. This nucleotide sequence of JqCel5A data also had been deposited in GenBank database. (GenBank accession no. KX058198).

Table 1
Activity of JqCel5A towards different substrates.[a, b]

Substrate	Specific activity (U/mg)		
	JqCel5A	E193A	E334A
CMC	21.72 ± 1.32	<0.01	<0.01
Barley glucan	26.19 ± 1.36	<0.01	<0.01
Filter paper	4.81 ± 0.21	<0.01	<0.01
Avicel	<0.01	<0.01	<0.01
Laminarin	<0.01	<0.01	<0.01

[a] Assay was performed at the optimum condition.
[b] Standard deviations were shown behind the specific activities.

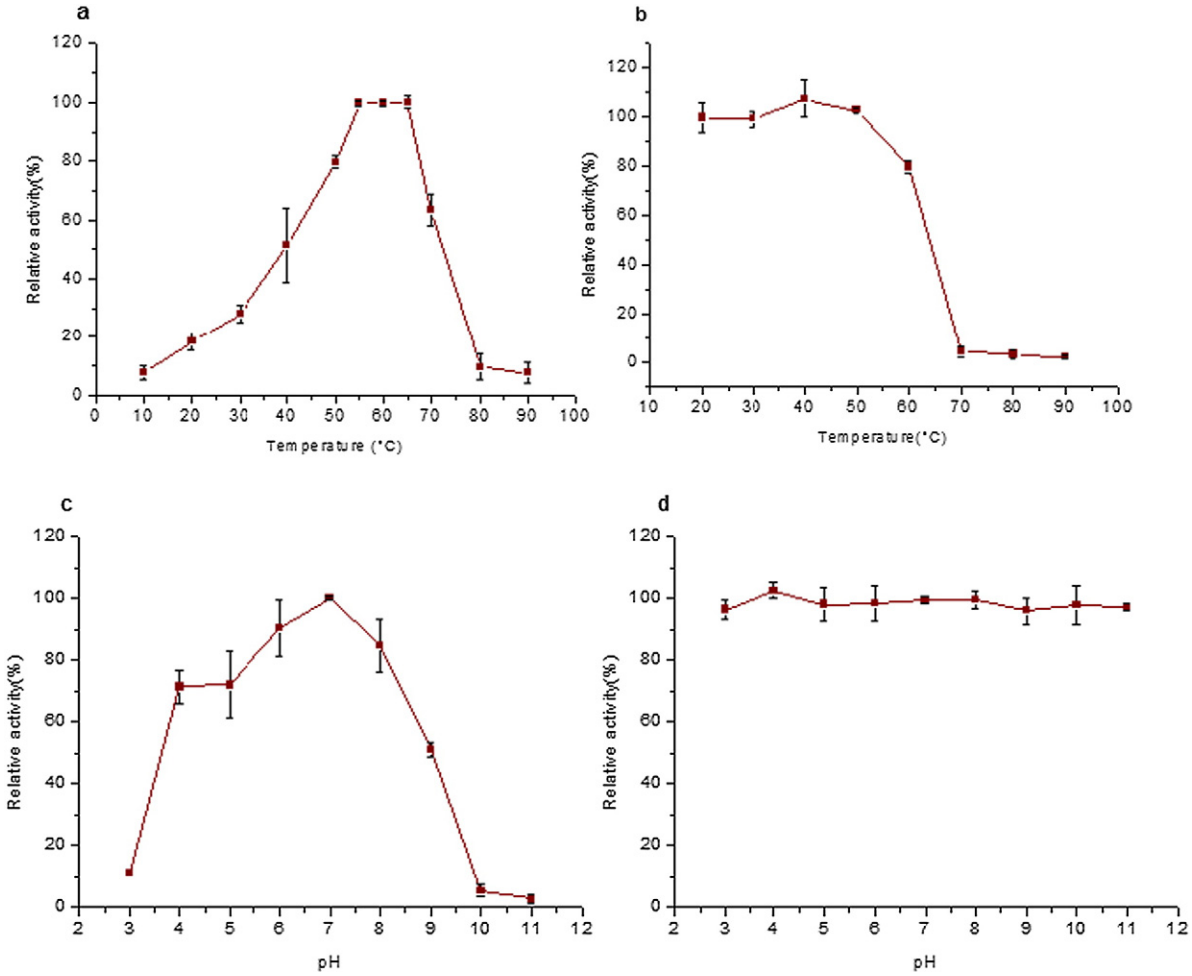

Fig. 4. The effects of pH and temperature on the activity and stability of JqCel5A. (a) Effect of temperature on enzyme activity. The reaction was carried out in 0.2 M Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) containing 0.5% CMC, at indicated temperatures. The maximum activity observed was taken as 100%. (b) Effect of temperature on enzyme stability. Enzymes were incubated for 1 h at indicated temperatures. Then samples were measured under the standard conditions. The activity without treatment was taken as 100%. (c) Effect of pH on enzyme activity. Enzyme activity was measured at 55°C in the different buffers with pH ranging from 3 to 11. The maximum activity observed was taken as 100%. (d) Effect of pH on enzyme stability. Enzymes were incubated at 4°C for 1 h at indicated pHs in various buffers. All the reactions were measured under the same condition of CMC activity assay. The activity without treatment was taken as 100%. Error bars represent the SD of the mean calculated for three replicates. °°.

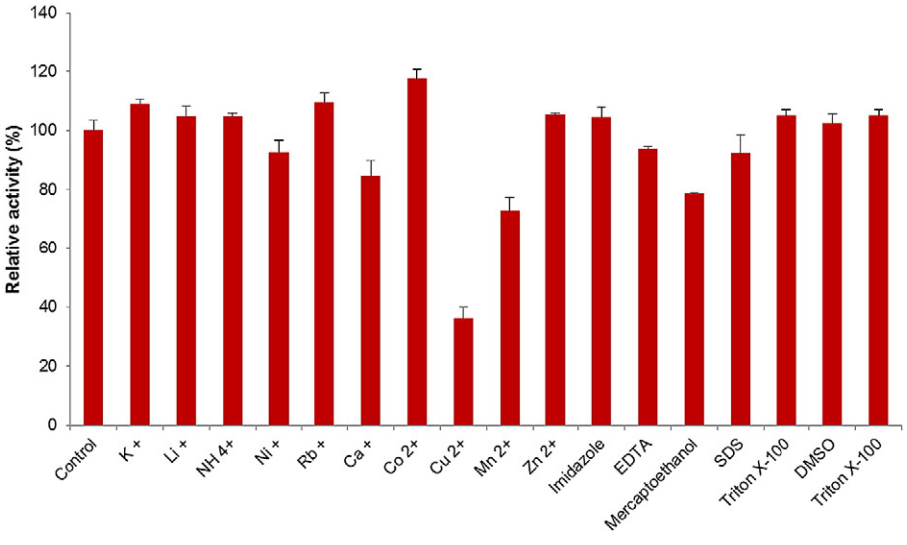

Fig. 5. Effects of metal ions and reagents on the activity of JqCel5A. Error bars represent the SD of the mean calculated for three replicates.

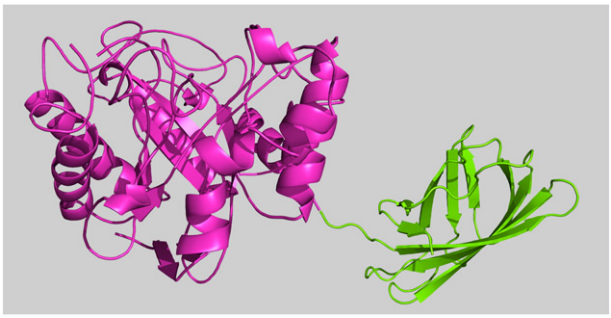

Fig. 6. Modular and structure predicted for JqCel5A. JqCel5A three dimensional structure was predicted by Swiss-model and visualized as cartoon diagrams in PyMOL v1.2.1. The catalytic module, on the left, adopts a $(\beta/\alpha)_8$ barrel fold and the right module represents the CBM3 folded in a β-sandwich fashion.

3.2. Activity identification of colonies by Congo red staining methods

Congo red staining method has long been widely used to identify different activities of endoglucanase mutants by measuring the halo-forming capability on the plates with cellulose substrate [1]. In this study, we cloned the *Jqcel5A* gene into pET28a and obtained the wild type colony with obviously halo-forming activity (Fig. 2 (1)). However, the colonies harboring *E193A* and *E334A*, in which Glu193 and Glu334 were substituted by Ala respectively, exhibited rare catalysis ability towards CMC plate (Fig. 2 (2–3)), suggesting that these two acid residues had been assigned an essential role involved in catalytic process of *Jqcel5A*.

3.3. Expression and characterization of the enzymes

In order to characterize the JqCel5A and mutant enzymes, the gene coding proteins were produced in *E. coli* Rosetta with H-terminal 6His-tag and purified using an affinity chromatography. The purity and molecular weight of the proteins was analyzed and observed with SDS-PAGE (Fig. 3), which showed the same target protein bands of 62 kDa, quite close to the predicated molecular weight (61.79 kDa).

The activities of the purified JqCel5A, E193A and E334A were measured using the DNS method as described above. As shown in Table 1, among the different polysaccharide substrates, the JqCel5A exhibited the hydrolysis activity towards CMC, barley glucan and filter paper, and did not acquire activity against avicel and laminarin. In contrast to the purified wild type enzyme, the purified mutants E193A and E334A showed no detected activities towards all of the substrates. This observation was in agreement with the previous results as described in the study of halo-forming activities.

In order to calculate K_m and V_{max} of JqCel5A, the activities towards different concentrations of CMC substrates were measured and the data was plotted according to the Michaelis–Menten equations. The K_m value of JqCel5A was 8.699 mg/mL and the calculated V_{max} was 0.0642 mmol/min∗mg. Comparison of CelB with the K_m values of 6.6 mg/mL [18]; Cel124 with K_m and V_{max} values of 5.63 mg/mL and 0.0397 mmol/min∗mg [19], it indicated that JqCel5A had medium affinity and catalytic efficiency for CMC substrate.

3.4. Effects of temperature and pH on the activity of JqCel5A

As shown in Fig. 4, the JqCel5A was optimally active at 55 to 65°C, and gained less than 60% relative activity at temperature below 40°C, rapidly decreased activity above 70°C, and completely lost activity at 80°C. These observations are compatible with the reports that endoglucanases possess optimum temperatures of 40–70°C such as from *Chrysosporium lucknowense*, *A. niger* and *Bacillus subtilis* [20,21,22].

Thermal stability assay shows that JqCel5A was very stable below 50°C, and retained nearly 80% of its initial activity after 1 h incubation at 60°C for 1 h. However, the activity decreased rapidly to less than 5% after incubation at 70°C. Similar to this, some other endoglucanases also showed stability under the same temperatures, examples include endocellulase from *Chaetomium thermophilum*, EG from *Trichoderma reesei* and cellulose from *A. niger* were thermal stable up to 60°C, but losses its activity after treatment at 70°C [23,24,25].

Maximal activity of the purified enzyme was observed at pH 7.0, showing more than 50% relative at pH range of 4–9, which satisfies the fundamental conditions of the application in food, paper and textile industries. Notably, it was very stable over a wide range of pH, maintaining over 95% activity after incubation in at the pH range of 3–11 for 1 h. These properties were similar to some previously reported endoglucanases, such as EG27 from *Ampullaria crossean* and Cel8H from *Halomonas* sp. both exhibited stability at acidic and alkaline condition, showing nearly full activity after incubation at pH range of 4–11 [5,26]. Moreover, this high pH stability in both acidic and alkaline region would make the neutral JqCel5A a strong candidate for production of bioethanol, detergents, fabrics and feed additives [27].

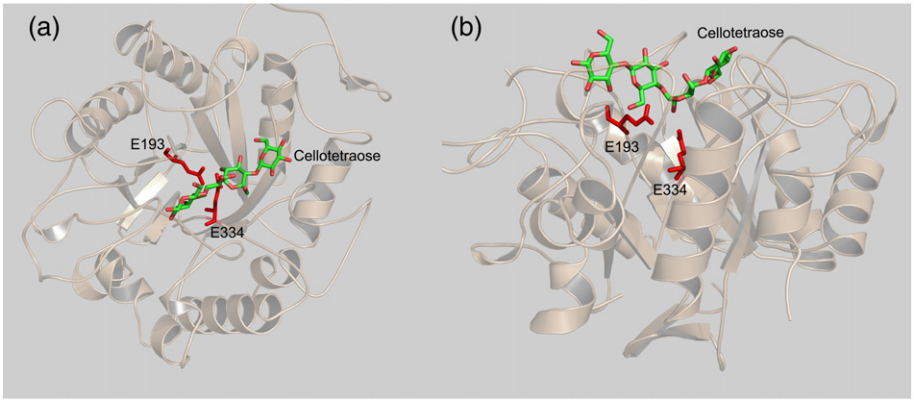

Fig. 7. Interaction of cellopentaose with catalytic center of JqCel5A GH5 module. Catalytic module of JqCel5A is visualized as cartoon diagrams from a top (a) and side (b) view using PyMOL v1.2.1. The α-helices and β-sheets are shown as ribbons, while the catalytic residues (Glu193 and Glu334) and substrate (Cellopentaose) are shown as stick model, respectively.

3.5. Effects of metal ions and reagents on enzyme activity

As shown in Fig. 5, the relative activity of the JqCel5A in the presence of different metal ions and chemical reagents was determined. Rb^{+}, K^{+} and Co^{2+} at 1 mM concentration slightly stimulated enzyme activity, while Ca^{2+}, Mn^{2+} and β-mercaptoethanol reduced the activity, and Cu^{2+} could bind the thiol groups and interact with imidazole or carboxyl groups of amino acids resulting in the dramatically decreased enzyme activity. It must be noted that the anionic surfactant SDS and the chelating agent EDTA showed nearly no effect on the activity of the enzyme. This resistance to the denaturant of protein suggests JqCel5A as a potential candidate for industrial detergents, in which divalent cations, detergents and chelating agent were commonly used [28].

3.6. Protein modeling

In order to more clearly understand the GH5 and CBM2 modules of JqCel5A at the tertiary levels, crystal structures of the enzyme was predicted and determined by SWISS-MODEL workspace and illustrated using PyMOL v1.2.1, based on the reported crystal structure of EGPh from *P. horikoshii* (Protein Data Bank code 3QHO) [14] and type 2 carbohydrate-binding module from *C. fimi* (Protein Data Bank code 1EXG) [15]. As shown in Fig. 6, a typical clan GHA structure with a $(\beta/\alpha)_8$ barrel and a β-sandwich fashion are finally predicted by homology [15,29].

In the structures of wild type enzyme, one cellopentaose unit was observed along the active-site cleft, and glucose subsites interacted with the potential catalytic residues by forming hydrogen-bonding with proton-donor oxygen of Glu193 and oxygen of Glu334, which situated on the opposite side of this cleft, respectively (Fig. 7) [29,30]. Therefore, the supposed catalytic residues (Glu193 and Glu334) could be located at the non-reducing end of the bound G2 in cellopentaose involving the formation of a covalent glycosyl-enzyme intermediate, which had been previously reported in this class of enzymes [14,31,32,33].

To clarify a functional role for Glu193 and Glu334 in the catalytic action of JqCel5A, we constructed the one-site mutants, E193A and E334A, in which Glu193 and Glu334 were replaced by alanine, respectively. As expected, both mutant E193A and E334A exhibited slight hydrolytic activity towards CMC and other polysaccharides. These laboratory studies confirmed previous catalytic mechanism of the family 5 enzymes that Glu193 acted as a proton donor in the double-displacement process, while adjacent Glu334 acted as a nucleophile and appeared to attract a glucose moiety through the interaction in the initial substrate-binding stage [30]. And it also provides experimental evidence of retention mechanism in polysaccharide hydrolysis, correlated well with the previous studies [31,34].

4. Conclusion

In summary, this is the first study to clone and sequence a novel endoglucanase gene (JqCel5A) from *J. quinghaiensis*. The prokaryotic expressed protein was purified and characterized by a series of experiments. It has been shown that recombinant JqCel5A has some distinct properties with high stability in a wide range of pH, as well as active and resistance to divalent cations, detergents and chelating agent, which would make it some promising for industrial application, especially in the detergent industry. In addition, analyses of mutants E193A and E334A with a dramatic loss of polysaccharose hydrolysis activity yielded evidence that supported the Glu193 and Glu334 were the proton donor and nucleophile involved in the catalytic reaction. Knowledge of these two catalytic glutamate residues may give some insights into further investigation on the double displacement mechanism of glycoside hydrolase family 5 enzymes.

Conflict of interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Financial support

This study was supported by grants from the Key Laboratory of Biotic Environment and Ecology Safety in Anhui Province, and Anhui Provincial Natural Science Foundation [grant number 1208085QC56].

References

[1] Lin L, Meng X, Liu PF, Hong YZ, Wu GB, Huang XL, et al. Improved catalytic efficiency of Endo-β-1,4-glucanase from *Bacillus subtilis* BME-15 by directed evolution. Appl Microbiol Biotechnol 2009;82:671–9. http://dx.doi.org/10.1007/s00253-008-1789-3.

[2] Liang C, Xue Y, Fioroni M, Rodriguez-Ropero F, Zhou C, Schwaneberg U, et al. Cloning and characterization of a thermostable and halo-tolerant endoglucanase from *Thermoanaerobacter tengcongensis* MB4. Appl Microbiol Biotechnol 2011;89:315–26. http://dx.doi.org/10.1007/s00253-010-2842-6.

[3] Ng IS, Li CW, Yeh YF, Chen PT, Chir JL, Ma CH, et al. A novel endo-glucanase from the thermophilic bacterium *Geobacillus* sp. 70PC53 with high activity and stability over a broad range of temperatures. Extremophiles 2009;13:425–35. http://dx.doi.org/10.1007/s00792-009-0228-4.

[4] Mai Z, Su H, Yang J, Huang S, Zhang S. Cloning and characterization of a novel GH44 family endoglucanase from mangrove soil metagenomic library. Biotechnol Lett 2014;36:1701–9. http://dx.doi.org/10.1007/s10529-014-1531-4.

[5] Huang X, Shao Z, Hong Y, Lin L, Li C, Huang F, et al. Cel8H, a novel endoglucanase from the halophilic bacterium *Halomonas* sp. S66-4: Molecular cloning, heterogonous expression, and biochemical characterization. J Microbiol 2010;48: 318–24. http://dx.doi.org/10.1007/s12275-009-0188-5.

[6] Schumann P, Cui X, Stackebrandt E, Kroppenstedt RM, Xu LJiang C. *Jonesia quinghaiensis* sp. nov., a new member of the suborder *Micrococcineae*. Int J Syst Evol Microbiol 2004;54:2181–4. http://dx.doi.org/10.1099/ijs.0.63223-0.

[7] Koshland DE. Stereochemistry and the mechanism of enzymatic reactions. Biol Rev 1953;28:416–36. http://dx.doi.org/10.1111/j.1469-185X.1953.tb01386.x.

[8] Heckman KL, Pease LR. Gene splicing and mutagenesis by PCR-driven overlap extension. Nat Protoc 2007;2:924–32. http://dx.doi.org/10.1038/nprot.2007.132.

[9] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54. http://dx.doi.org/10.1016/0003-2697(76)90527-3.

[10] Lin L, Kan XZ, Yan H, Wang DN. Characterization of extracellular cellulose-degrading enzymes from *Bacillus thuringiensis* strains. Electron J Biotechnol 2012;15:3. http://dx.doi.org/10.2225/vol15-issue3-fulltext-1.

[11] Fu XY, Liu PF, Lin L, Hong YZ, Huang XL, Meng X, et al. A novel endoglucanase (Cel9P) from a marine bacterium *Paenibacillus* sp BME-14. Appl Biochem Biotechnol 2010;160:1627–36. http://dx.doi.org/10.1007/s12010-009-8648-2.

[12] Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. Bioinformatics 2006;22: 195–201. http://dx.doi.org/10.1093/bioinformatics/bti770.

[13] Kiefer F, Arnold K, Kunzli M, Bordoli L, Schwede T. The SWISS-MODEL repository and associated resources. Nucleic Acids Res 2009;37:387–92. http://dx.doi.org/10.1093/nar/gkn750.

[14] Kim HW, Ishikawa K. Functional analysis of hyperthermophilic endocellulase from *Pyrococcus horikoshii* by crystallographic snapshots. Biochem J 2011;437:223–30. http://dx.doi.org/10.1042/BJ20110292.

[15] Xu GY, Ong E, Gilkes NR, Kilburn DG, Muhandiram DR, Harris-Brandts M, et al. Solution structure of a cellulose-binding domain from cellulomonasfimi by nuclear-magnetic-resonance spectroscopy. Biochemistry 1995;34:6993–7009. http://dx.doi.org/10.1021/bi00021a011.

[16] Lombard V, Ramulu HG, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 2014;42:490–5. http://dx.doi.org/10.1093/nar/gkt1178.

[17] Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, et al. CDD: NCBI's conserved domain database. Nucleic Acids Res 2015;43:222–6. http://dx.doi.org/10.1093/nar/gku1221.

[18] Zhang GM, Li SY, Xue YF, Mao LW, Ma YH. Effects of salts on activity of halophilic cellulase with glucomannanase activity isolated from alkaliphilic and halophilic *Bacillus* sp BG-CS10. Extremophiles 2012;16:35–43. http://dx.doi.org/10.1007/s00792-011-0403-2.

[19] Xiang L, Li A, Tian C, Zhou Y, Zhang G, Ma Y. Identification and characterization of a new acid-stable endoglucanase from a metagenomic library. Protein Expr Purif 2014;102:20–6. http://dx.doi.org/10.1016/j.pep.2014.07.009.

[20] Bose S, Barnes CA, Petrich JW. Enhanced stability and activity of cellulase in an ionic liquid and the effect of pretreatment on cellulose hydrolysis. Biotechnol Bioeng 2012;109:434–43. http://dx.doi.org/10.1002/bit.23352.

[21] Bukhtojarov FE, Ustinov BB, Salanovich TN, Antonov AI, Gusakov AV, Okunev ON, et al. Cellulase complex of the fungus *Chrysosporium lucknowense*: Isolation and

characterization of endoglucanases and cellobiohydrolases. Biochemistry 2004;69: 542–51. http://dx.doi.org/10.1023/B:BIRY.0000029853.34093.13.

[22] Wang Y, Yuan H, Wang J, Yu Z. Truncation of the cellulose binding domain improved thermal stability of endo-β-1,4-glucanase from *Bacillus subtilis* JA18. Bioresour Technol 2009;100:345–9. http://dx.doi.org/10.1016/j.biortech.2008.06.001.

[23] Li DC, Lu M, Li YL, Lu J. Purification and characterization of an endocellulase from the thermophilic fungus *Chaetomium thermophilum* CT2. Enzyme Microb Technol 2003; 33:932–7. http://dx.doi.org/10.1016/S0141-0229(03)00245-X.

[24] Sprey B, Uelker A. Isolation and properties of a low molecular mass endoglucanase from *Trichoderma reesei*. FEMS Microbiol Lett 1992;92:253–7. http://dx.doi.org/10.1111/j.1574-6968.1992.tb05270.x.

[25] Hurst PL, Nielsen J, Sullivan PA, Shepherd MG. Purification and properties of a cellulase from *Aspergillus niger*. Biochem J 1977;165:33–41. http://dx.doi.org/10.1042/bj1650033.

[26] Li YH, Guo R, Yin QY, Ding M, Zhang SL, Xu GJ, et al. Purification and characterization of two endo-β-1,4-glucanases from Mollusca, *Ampullaria crossean*. Acta Biochim Biophys Sin 2005;37:702–8. http://dx.doi.org/10.1111/j.1745-7270.2005.00099.x.

[27] Gole A, Vyas S, Sainkar SR, Lachke A, Sastry M. Enhanced temperature and pH stability of fatty amine-endoglucanase composites: Fabrication, substrate protection, and biological activity. Langmuir 2001;17:5964–70. http://dx.doi.org/10.1021/la010597b.

[28] Liu JA, Liu WD, Zhao XL, Shen WJ, Cao H, Cui ZL. Cloning and functional characterization of a novel endo-β-1,4-glucanase gene from a soil-derived metagenomic library. Appl Microbiol Biotechnol 2011;89:1083–92. http://dx.doi.org/10.1007/s00253-010-2828-4.

[29] Ducros V, Czjzek M, Belaich A, Gaudin C, Fierobe HP, Belaich JP, et al. Crystal structure of the catalytic domain of a bacterial cellulase belonging to family 5. Structure 1995;3: 939–49. http://dx.doi.org/10.1016/S0969-2126(01)00228-3.

[30] Sakon J, Adney WS, Himmel ME, Thomas SR, Karplus PA. Crystal structure of thermostable family 5 endocellulase E1 from *Acidothermus cellulolyticus* in complex with cellotetraose. Biochemistry 1996;35:10648–60. http://dx.doi.org/10.1021/bi9604439.

[31] White A, Tull D, Johns K, Withers SG, Rose DR. Crystallographic observation of a covalent catalytic intermediate in a β-glycosidase. Nat Struct Biol 1996;3:149–54. http://dx.doi.org/10.1038/nsb0296-149.

[32] Kang HJ, Ishikawa K. Analysis of active center in hyperthermophilic cellulase from *Pyrococcus horikoshii*. J Microbiol Biotechnol 2007;17:1249–53.

[33] Zarafeta D, Kissas D, Sayer C, Gudbergsdottir SR, Ladoukakis E, Isupov MN, et al. Discovery and characterization of a thermostable and highly halotolerant GH5 cellulase from an icelandic hot spring isolate. PLoS One 2016;11:e0146454. http://dx.doi.org/10.1371/journal.pone.0146454.

[34] Notenboom V, Birsan C, Nitz M, Rose DR, Warren RAJ, Withers SG. Insights into transition state stabilization of the β-1,4-glycosidase Cex by covalent intermediate accumulation in active site mutants. Nat Struct Biol 1998;5:812–8. http://dx.doi.org/10.1038/1852.