



Research article

Efficient expression and characterization of a cold-active endo-1, 4- β -glucanase from *Citrobacter farmeri* by co-expression of *Myxococcus xanthus* protein S



Xi Bai^{a,b}, Xianjun Yuan^a, Aiyou Wen^c, Junfeng Li^a, Yunfeng Bai^d, Tao Shao^{a,*}

^a Institute of Ensiling and Processing of Grass, Nanjing Agricultural University, Nanjing, Jiangsu, People's Republic of China

^b College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu, People's Republic of China

^c College of Animal Science, University of Science and Technology of Anhui, Fengyang, Anhui, People's Republic of China

^d Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu, People's Republic of China

ARTICLE INFO

Article history:

Received 12 June 2016

Accepted 13 October 2016

Available online 26 October 2016

Keywords:

Cellulose degradation

Cellulose

Cold-active enzyme

Endoglucanases

Enzymatic properties

Escherichia coli

Expression

Novel expression vector

N-terminal fusion

Protein S-tag

Recombinant protein

ABSTRACT

Background: Cold-active endo-1, 4- β -glucanase (EglC) can decrease energy costs and prevent product denaturation in biotechnological processes. However, the nature EglC from *C. farmeri* A1 showed very low activity (800 U/L). In an attempt to increase its expression level, *C. farmeri* EglC was expressed in *Escherichia coli* as an N-terminal fusion to protein S (ProS) from *Myxococcus xanthus*.

Results: A novel expression vector, pET(ProS-EglC), was successfully constructed for the expression of *C. farmeri* EglC in *E. coli*. SDS-PAGE showed that the recombinant protein (ProS-EglC) was approximately 60 kDa. The activity of ProS-EglC was 12,400 U/L, which was considerably higher than that of the nature EglC (800 U/L). ProS-EglC was active at pH 6.5–pH 8.0, with optimum activity at pH 7.0. The recombinant protein was stable at pH 3.5–pH 6.5 for 30 min. The optimal temperature for activity of ProS-EglC was 30°C–40°C. It showed greater than 50% of maximum activity even at 5°C, indicating that the ProS-EglC is a cold-active enzyme. Its activity was increased by Co^{2+} and Fe^{2+} , but decreased by Cd^{2+} , Zn^{2+} , Li^+ , methanol, Triton-X-100, acetonitrile, Tween 80, and SDS.

Conclusions: The ProS-EglC is promising in application of various biotechnological processes because of its cold-active characterizations. This study also suggests a useful strategy for the expression of foreign proteins in *E. coli* using a ProS tag.

© 2016 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V. All rights reserved. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Cellulose (the major component of biomass) is a widely available renewable resource [1,2]. The main component of cellulose is high molecular-weight polysaccharides composed of β -1, 4-linked glucose units. Endo- β -1, 4-glucanase (EglC, EC 3.2.1.4) is an important enzyme in cellulose degradation that randomly hydrolyzes the β -1, 4-glycosidic linkages within cellulose chains and releases smaller fragments of different lengths [3,4,5]. Endoglucanases are attracting increasing attention for their potential applications in the feed, detergent, silage, food, and textile industries [6,7]. Compared with mesophilic proteases, endoglucanases with low temperature activity can decrease energy costs and avoid product denaturation

in biotechnological processes [8,9]. Although many endo- β -1, 4-glucanases from different microorganisms have been isolated and purified, the research on cold-active EglCs is currently lacking [10].

The microbial communities in the intestinal tracts of termites include a number of microbes that efficiently degrade cellulose [11,12,13]. In a previous study, a psychrophilic EglC was isolated from *Citrobacter farmeri* A1 in the wood-inhabiting termite *Reticulitermes labralis* (Unpublished observations). However, the activity of the *C. farmeri* EglC was very low. Therefore, in the present study, we developed an *Escherichia coli* expression system to increase its production level for analyzing enzymatic properties.

Two major roadblocks for expression of heterologous protein in *E. coli* are poor production and the formation of insoluble protein [14,15]. When the EglC gene was cloned into pET-32a and transformed into *E. coli*, it was very poorly expressed (unpublished observations). One approach to overcome this problem is to fuse with another protein, which can enhance the solubility of

* Corresponding author.

E-mail address: taoshaolan@163.com (T. Shao).

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

heterologous proteins expressed in *E. coli*. The protein S (ProS) tag from *M. xanthus* was reported to increase the solubility of target proteins, and importantly, the fusion did not affect the properties of the target protein [16,17]. For this strategy, the Trx tag in pET-32a was replaced with the ProS tag using cloning technique. Then, the *EglC* gene from *C. farmeri* A1 was ligated into the novel vector pET-ProS and expressed in *E. coli*. The recombinant ProS-EglC protein was also fully characterized.

2. Materials and methods

2.1. Strains and plasmids

In our previous study, the *EglC* gene was amplified from the *C. farmeri* A1 genome and deposited into the GenBank database (accession no. KT313000). The pMD20-T cloning vector was purchased from TaKaRa (China). *E. coli* DH5 α and BL21 cells were obtained from our laboratory stocks. The pET-32a expression vector was purchased from Novagen (Germany).

2.2. Construction of pET-ProS vector

The pET-32a plasmid was used as the backbone to construct an expression vector containing a ProS tag (Fig. 1a). The sequence of *ProS* gene (the N-terminal domain of protein S; amino acid residues 1–92; GenBank accession no. J01745.1) from *M. xanthus* was obtained according to the previous paper [16,17,18,19,20]. The *ProS* gene was synthesized by Shanghai Geneary Biotech Co., Ltd. (China) with *NdeI* sites at both terminus. The Trx tag in pET-32a was removed by digestion with *NdeI*. Then, the purified *ProS* gene and pET-32a (without the Trx-tag) were ligated with T4 DNA ligase (TaKaRa, China) to generate the novel expression vector pET-ProS (Fig. 1a).

2.3. Construction and transformation of pET(ProS-EglC) vector

The mature DNA fragment of the *EglC* gene (without the signal peptide) was amplified by PCR with primers *EglC*-F (CTCCATGGGCCTGTACCTGGCCCGCAT) and *EglC*-R (CGCTCGAGATTTGAAGCTTGCGCATTCCTG), which contain the *NcoI* and *XhoI* sites, respectively. The primers were designed based on the nucleotide sequence of *C. farmeri* A1 *EglC* (GenBank accession no. KT313000). The PCR program was as follows: 6 min at 95°C, 35 cycles of 40 s at 95°C, 55 s at 59°C, and 55 s at 72°C, and a final extension step for 8 min at 72°C. The purified PCR fragment was cloned into pMD20-T. Then, pMD20-EglC was double-digested with *NcoI* and *XhoI*, and the isolated *EglC* fragment was ligated into the pET-ProS vector. The obtained plasmid,

pET(ProS-EglC), was transformed into *E. coli* BL21 to express recombinant ProS-EglC (Fig. 1a).

2.4. Expression and purification of the recombinant ProS-EglC

Recombinant *E. coli* pET(ProS-EglC) was grown at 37°C in 250-mL shaker flasks (at 200 rpm) containing LB medium. When the culture density reached an OD₆₀₀ of ~0.6, expression of the fusion protein was induced by isopropyl-1-thio- β -D-galactopyranoside (final concentration of 1 mM). After the cells were grown for 4 h, the culture cells were collected by centrifugation. The pellets were resuspended in 10 mL of Na₂HPO₄-citric-acid buffer (pH 7.0), and the cells were lysed by ultrasonication on ice.

The recombinant fusion protein was purified by 6 \times His-Tagged Protein Purification Kit (CWBI, China). Briefly, the cell lysates containing His-tagged ProS-EglC was passed through the Ni²⁺ affinity chromatography and washed by Soluble Binding Buffer (20 mM Tris-HCl with pH 7.9, 10 mM imidazole and 0.5 M NaCl). The bound proteins were eluted by Soluble Elution Buffer (20 mM Tris-HCl with pH 7.9, 500 mM imidazole and 0.5 M NaCl). The expressed proteins were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Assay of ProS-EglC activity

ProS-EglC activity was measured by the DNS method. The standard reaction mixture, which consisted of 1 mL CMC-Na buffer (substrate) and diluted enzyme solution (1 mL), was heated at 40°C for 30 min in a thermostatic water bath. DNS (3 mL) was added to stop the reaction. The reaction was boiled for 5 min, and the amount of reducing sugars was assessed by measuring the absorbance at 540 nm using a spectrophotometer (Shanghai Precision & Scientific Instruments Co., Ltd., China). One unit of endoglucanase activity was defined as the amount of enzyme that produced 1 μ mol of glucose per min under the assay conditions.

2.6. Biochemical characterization of ProS-EglC

To determine the optimal pH for ProS-EglC, the protein was incubated in buffers with various pH values (Na₂HPO₄-citric acid buffer, pH 3.5–pH 7.0 and sodium phosphate buffer, pH 8.0) at 40°C for 30 min. To measure pH stability, ProS-EglC was pre-incubated in buffers with different pH values (pH 3.5, 4.5, 5.5, and 6.5) at 40°C for 30 min. Then, residual activity was measured under optimal conditions (40°C, pH 7.0).

The optimum temperature for ProS-EglC activity was determined by incubating the reaction mixture at different temperatures (5°C–70°C)

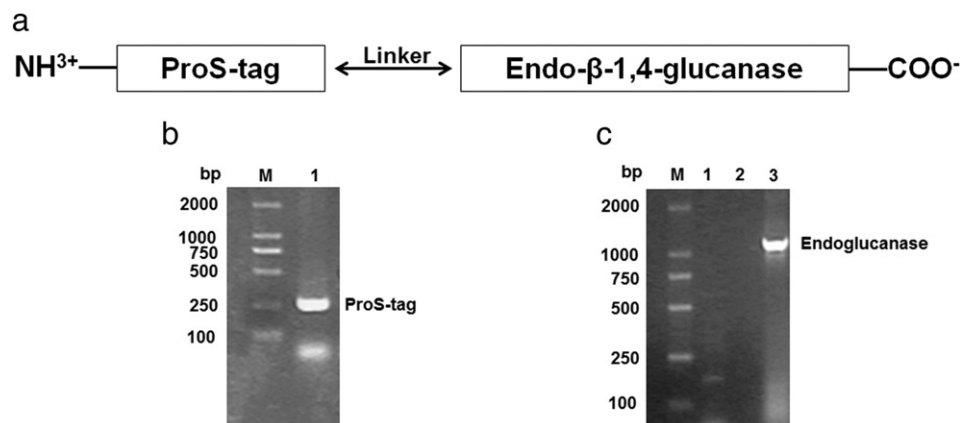


Fig. 1. Construction of expression vector pET(ProS-EglC). (a) Schematic presentation of a protein S fusion protein. (b) Amplification of protein S. Lane M: DNA markers (100–2000 bp); Lane 1: Protein S fragment. (c) Amplification of *EglC* gene. Lane M: DNA markers (100–2000 bp); Lanes 1–2: Negative control; Lane 3: *EglC* gene.

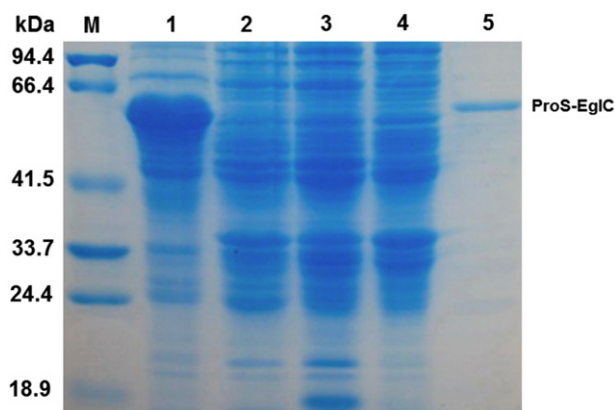


Fig. 2. SDS-PAGE analysis. Lane M: protein markers; Lane 1: IPTG induced *E. coli* pET(ProS-EglC); Lane 2: *E. coli* pET(ProS-EglC); Lane 3: IPTG induced *E. coli* pET-ProS; Lane 4: *E. coli* pET-ProS; Lane 5: Purified ProS-EglC.

at the optimal pH value (7.0). Thermal stability was evaluated by pre-incubation of samples without substrate at various temperatures (40°C–60°C) for 30 min, and then the enzyme samples were assayed for residual activity under standard assay conditions.

To determine the effect of metal ions and chemical reagents on ProS-EglC activity, various metal ions (CoCl₂, FeCl₂, CdCl₂, ZnCl₂, and Li₂SO₄) and chemicals (methanol, Triton-X-100, acetonitrile, Tween 80, and SDS) were added to the reaction mixture. Then, the activities of ProS-EglC were measured under standard assay conditions. The reaction mixture without any ions or chemical reagents was considered as the control.

All the above measurements were carried out in duplicate. The statistical analyses of the values were performed by Microsoft Excel 2010. Data were presented as means with standard deviation (SD).

3. Results and discussion

3.1. Construction of the novel expression plasmid pET(ProS-EglC)

To improve its expression level, a ProS tag was fused to the N-terminus of *EglC* in an *E. coli* expression system (Fig. 1a). The novel expression plasmid pET(ProS-EglC) was obtained using vector pET-32a as the backbone. PCR analysis of pET(ProS-EglC) is shown in Fig. 1b and Fig. 1c. The sizes of the DNA fragments were similar to the

expected sizes of *ProS* (276 bp) and *EglC* (1056 bp), suggesting that *ProS* and *EglC* genes were successfully cloned into the expression vector.

3.2. Expression and purification of ProS-EglC

SDS-PAGE was performed to analyze the expression products from *E. coli* pET(ProS-EglC). As shown in Fig. 2, crude protein extracts from the recombinant *E. coli* strain showed a strong band with a molecular weight (MW) of ~60 kDa, which was higher than the predicted MW of full-length *EglC* because of the fusion tag. However, this band was not present in the supernatant of the cell lysates from an uninduced recombinant strain (Fig. 2). Recombinant ProS-EglC was purified by affinity chromatography, since the SDS-PAGE analysis showed only one band (Fig. 2). The specific activity of purified ProS-EglC was 15.6 U/mg.

When the *EglC* gene from *C. farmeri* A1 was cloned into the pET-32a expression vector and transformed into *E. coli*, it was poorly expressed (data not shown). Therefore, the novel expression vector pET(ProS-EglC) was developed to increase its production level. In the present study, the cellular extract from *E. coli* pET(ProS-EglC) showed activity of 12,400 U/L, which was higher than that of the original endoglucanase from *C. farmeri* A1 (800 U/L). In addition, the ProS-EglC activity was also higher than that of the recombinant endoglucanase expressed in *E. coli* EF-EG2 (1000 U/L), *E. coli* Cel5D (1.44 U/mg), and *E. coli* Cel8H (4.9 U/mg) [21,22,23]. These results suggested that the presence of ProS tag in the N-terminus would make the foreign protein in a soluble fraction and dramatically increase expression level [17,18]. Furthermore, the recombinant protein fused with ProS tag maintains their functions [19,20].

3.3. Effect of pH on the recombinant ProS-EglC activity

The pH characteristics of recombinant ProS-EglC were similar to those of other neutral endoglucanases [24,25]. ProS-EglC was active at pH 6.5–pH 8.0, with optimum activity at pH 7.0 (Fig. 3a). At the pH values below 4.5, the activity of ProS-EglC rapidly decreased (Fig. 3a). ProS-EglC was stable and maintained greater than 70% of maximum activity after incubation at pH 3.5–pH 6.5 for 30 min (Fig. 3b).

3.4. Effect of temperature on the recombinant ProS-EglC activity

Many endo- β -glucanases from various microorganisms (*i.e.*, bacteria, fungi, and actinomycetes) have been studied [26]. However, only a few cold-active endoglucanases have been cloned and expressed

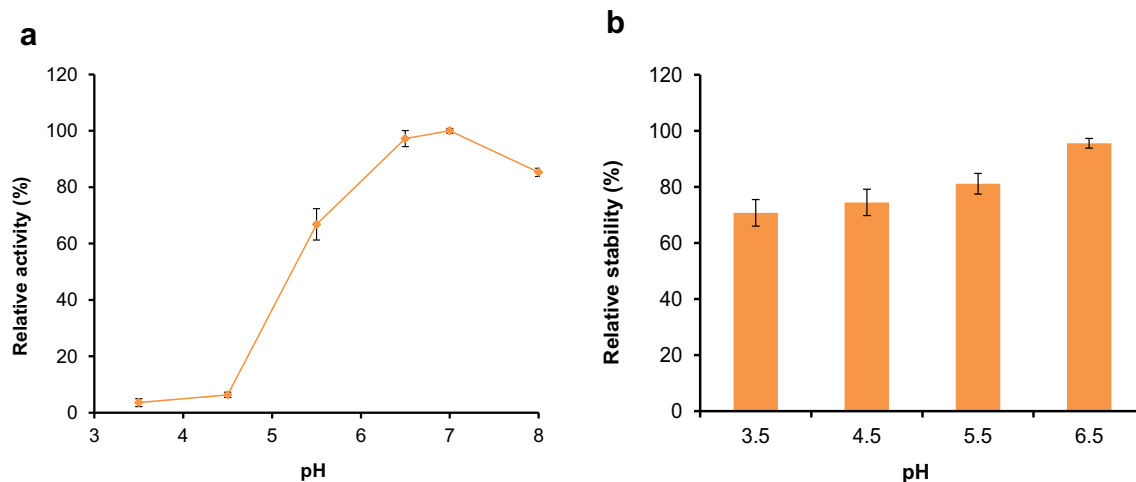


Fig. 3. Effect of pH on ProS-EglC activity and stability. (a) Effect of pH on the ProS-EglC activity. (b) The pH stability of ProS-EglC. The ProS-EglC activity determined at the optimal pH (7.0) and temperature (40°C) was considered as the control. Data were presented as means with SD.

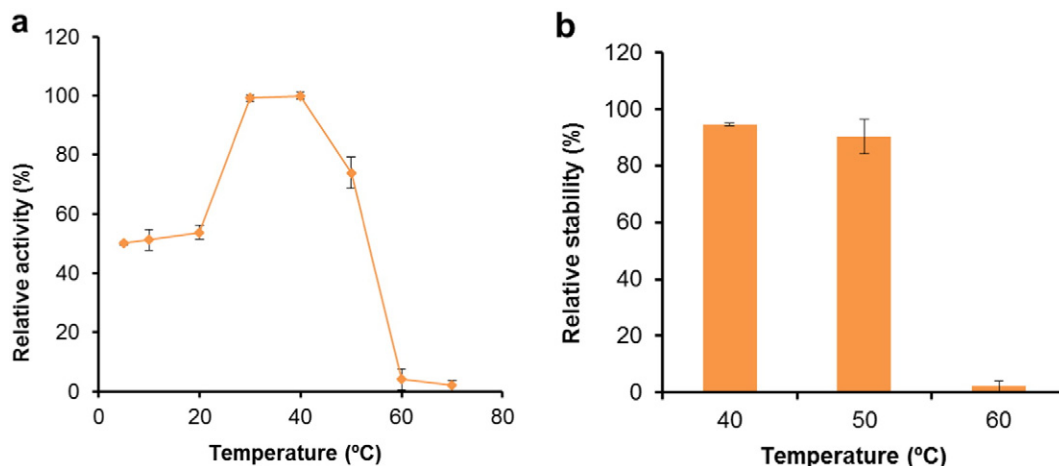


Fig. 4. Effect of temperature on ProS-EglC activity and stability. (a) Effect of temperature on the ProS-EglC activity. (b) The temperature stability of ProS-EglC. The ProS-EglC activity determined at the optimal pH (7.0) and temperature (40°C) was considered as the control. Data were presented as means with SD.

for specific applications [27]. Compared with mesophilic enzymes, cold-active endoglucanases have the advantage of avoiding alteration or denaturation of the product in biotechnological processes [21]. The typical properties of cold-active enzymes are relatively high activity at low temperatures and poor thermostability [28,29].

Fig. 4a showed that the optimal temperature of ProS-EglC was 30°C–40°C, and the enzyme showed greater than 50% of maximum activity even at 5°C (Fig. 4a). In contrast, the activity of ProS-EglC decreased dramatically at the temperatures above 60°C (Fig. 4a). The results of the temperature stability assay showed that approximately 92% of ProS-EglC activity was lost after incubation at 60°C for 30 min (Fig. 4b). Based on these properties, the recombinant ProS-EglC protein expressed in *E. coli* pET(ProS-EglC) cells was a cold-active endoglucanase. Similar results have been reported for other low-temperature glucanases and cellulases from *Paenibacillus* sp. IHB B 3084 and *Eisenia fetida* [21,29]. However, the activities of general and thermostable endoglucanases are rapidly lost at the temperatures below 20°C [26,30].

3.5. Effects of chemical reagents and metal ions on the activity of ProS-EglC

Martin et al. [31] and Rawat et al. [32] reported that Co^{2+} and Fe^{2+} enhanced the activity of endoglucanases from *E. coli* Rosetta 2 and *Aspergillus niger*, respectively. As shown in Fig. 5, the activity of ProS-EglC was increased by Co^{2+} and Fe^{2+} . However, its activity was decreased by Cd^{2+} , Zn^{2+} , Li^+ , methanol, Triton-X-100, acetonitrile, Tween 80, and SDS (Fig. 5). These inhibitive effects may be caused by denaturation of the protein structure [31].

Tween 80, and SDS (Fig. 5). These inhibitive effects may be caused by denaturation of the protein structure [31].

4. Conclusions

The main *EglC* gene from *C. farmeri* was successfully expressed in *E. coli* as a fusion with ProS tag. Based on characterizations, ProS-EglC is a typical cold-active enzyme. It has potential applications in numerous biotechnological processes. This study also suggests a useful strategy for improving heterologous protein expression in *E. coli*.

Financial support

This work was supported by the Independent Innovation of Agricultural Sciences in Jiang Su Province [CX(15)1003], National key Research and Development Program (2016YFC0502005) and Network and Technology Served of Chinese Academy of Sciences Program (STS) “Grassland agricultural system construction and industrialization demonstration of typical village (Jina village) in Tibet” (KFJ-EW-STS-071).

Conflict of interest

The authors confirm that this article content has no conflict of interest.

References

- [1] Collard FX, Blin J. A review on pyrolysis of biomass constituents: Mechanisms and composition of the products obtained from the conversion of cellulose, hemicelluloses and lignin. *Renew Sustain Energy Rev* 2014;38:594–608. <http://dx.doi.org/10.1016/j.rser.2014.06.013>.
- [2] Ho DP, Ngo HH, Guo W. A mini review on renewable sources for biofuel. *Bioresour Technol* 2014;169:742–9. <http://dx.doi.org/10.1016/j.biortech.2014.07.022>.
- [3] Tang Y, Yang S, Yan Q, Zhou P, Cui J, Jiang Z. Purification and characterization of a novel β -1, 3-1, 4-glucanase (lichenase) from thermophilic *Rhizomucor miehei* with high specific activity and its gene sequence. *J Agric Food Chem* 2012;60:2354–61. <http://dx.doi.org/10.1021/jf2049799>.
- [4] Jagtap SS, Dhiman SS, Kim TS, Kim IW, Lee JK. Characterization of a novel endo- β -1, 4-glucanase from *Armillaria gemina* and its application in biomass hydrolysis. *Appl Microbiol Biotechnol* 2014;98:661–9. <http://dx.doi.org/10.1007/s00253-013-4894-x>.
- [5] Lin L, Meng X, Liu P, Hong Y, Wu G, Huang X, 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>.
- [6] Li Y, Yin Q, Ding M, Zhao F. Purification, characterization and molecular cloning of a novel endo- β -1, 4-glucanase AC-EG65 from the mollusc *Ampullariacrossean*. *Comp Biochem Physiol B Biochem Mol Biol* 2009;153:149–56. <http://dx.doi.org/10.1016/j.cbpb.2009.02.011>.

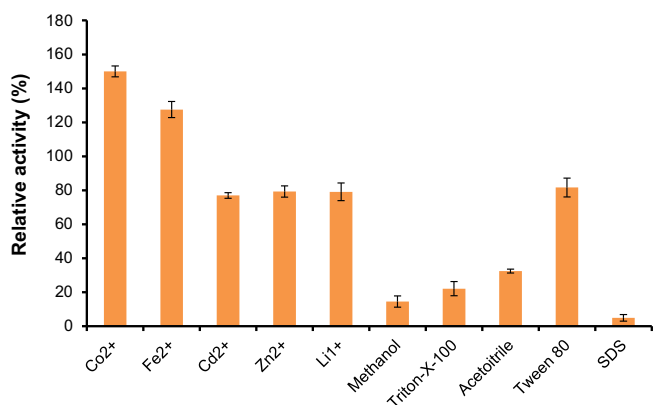


Fig. 5. Effects of chemical reagents and metal ions on the activity of ProS-EglC. The reaction mixture without any ions or chemical reagents was considered as the control. Data were presented as means with SD.

- [7] Jeya M, Joo AR, Lee KM, Sim WI, Oh DK, Kim YS, et al. Characterization of endo- β -1,4-glucanase from a novel strain of *Penicillium pinophilum* KMJ601. *Appl Microbiol Biotechnol* 2010;85:1005–14. <http://dx.doi.org/10.1007/s00253-009-2070-0>.
- [8] Wang N, Zang J, Ming K, Liu Y, Wu Z, Ding H. Production of cold-adapted cellulase by *Verticillium* sp. isolated from Antarctic soils. *Electron J Biotechnol* 2013;16:1–10. <http://dx.doi.org/10.2225/vol16-issue4-fulltext-12>.
- [9] Iyo AH, Forsberg CW. A cold-active glucanase from the ruminal bacterium *Fibrobacter succinogenes* S85. *Appl Environ Microbiol* 1999;65:995–8.
- [10] Goñi O, Sanchez-Ballesta MT, Merodio C, Escribano MI. A cryoprotective and cold-adapted 1,3- β -endoglucanase from cherimoya (*Annona cherimola*) fruit. *Phytochemistry* 2011;72:844–54. <http://dx.doi.org/10.1016/j.phytochem.2011.03.017>.
- [11] Zhou X, Smith JA, Oi FM, Koehler PG, Bennett GW, Scharf ME. Correlation of cellulase gene expression and cellulolytic activity throughout the gut of the termite *Reticulitermes flavipes*. *Gene* 2007;395:29–39. <http://dx.doi.org/10.1016/j.gene.2007.01.004>.
- [12] Breznak JA, Brune A. Role of microorganisms in the digestion of lignocellulose by termites. *Annu Rev Entomol* 1994;39:453–87. <http://dx.doi.org/10.1146/annurev.en.39.010194.002321>.
- [13] Scharf ME, Tartar A. Termite digestomes as sources for novel lignocellulases. *Biofuels Bioprod Biorefin* 2008;2:540–52. <http://dx.doi.org/10.1002/bbb.107>.
- [14] Huang J, Cao L, Guo W, Yuan R, Jia Z, Huang K. Enhanced soluble expression of recombinant *Flavobacterium heparinum* heparinase I in *Escherichia coli* by fusing it with various soluble partners. *Protein Expr Purif* 2012;83:169–76. <http://dx.doi.org/10.1016/j.pep.2012.03.016>.
- [15] Costa SJ, Almeida A, Castro A, Domingues L, Besir H. The novel Fh8 and H fusion partners for soluble protein expression in *Escherichia coli*: A comparison with the traditional gene fusion technology. *Appl Microbiol Biotechnol* 2013;97:6779–91. <http://dx.doi.org/10.1007/s00253-012-4559-1>.
- [16] Kobayashi H, Swapna GVT, Wu KP, Afinogenova Y, Conover K, Mao B, et al. Segmental isotope labeling of proteins for NMR structural study using a protein S tag for higher expression and solubility. *J Biomol NMR* 2012;52:303–13. <http://dx.doi.org/10.1007/s10858-012-9610-0>.
- [17] Kishii R, Falzon L, Yoshida T, Kobayashi H, Inouye M. Structural and functional studies of the HAMP domain of EnvZ, an osmosensing transmembrane histidine kinase in *Escherichia coli*. *J Biol Chem* 2007;282:26401–8. <http://dx.doi.org/10.1074/jbc.M701342200>.
- [18] Kudou M, Okazaki F, Asai-Nakashima N, Ogino C, Kondo A. Expression of cold-adapted β -1,3-xylanase as a fusion protein with a ProS2 tag and purification using immobilized metal affinity chromatography with a high concentration of ArgHCl. *Biotechnol Lett* 2015;37:89–94. <http://dx.doi.org/10.1007/s10529-014-1666-3>.
- [19] Yoshida T, Qin L, Egger LA, Inouye M. Transcription regulation of *ompF* and *ompC* by a single transcription factor, OmpR. *J Biol Chem* 2006;281:17114–23. <http://dx.doi.org/10.1074/jbc.M602112200>.
- [20] Kobayashi H, Yoshida T, Inouye M. Significant enhanced expression and solubility of human proteins in *Escherichia coli* by fusion with protein S from *Myxococcus xanthus*. *Appl Environ Microbiol* 2009;75:5356–62. <http://dx.doi.org/10.1128/AEM.00691-09>.
- [21] Ueda M, Ito A, Nakazawa M, Miyatake K, Sakaguchi M, Inouye K. Cloning and expression of the cold-adapted endo-1,4- β -glucanase gene from *Eisenia fetida*. *Carbohydr Polym* 2014;101:511–6. <http://dx.doi.org/10.1016/j.carbpol.2013.09.057>.
- [22] Dong J, Hong Y, Shao Z, Liu Z. Molecular cloning, purification, and characterization of a novel, acidic, pH-stable endoglucanase from *Marteella mediterranea*. *J Microbiol* 2010;48:393–8. <http://dx.doi.org/10.1007/s12275-010-9361-0>.
- [23] 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, heterogenous expression, and biochemical characterization. *J Microbiol* 2010;48:318–24. <http://dx.doi.org/10.1007/s12275-009-0188-5>.
- [24] Li J, Tang C, Shi H, Wu M. Cloning and optimized expression of a neutral endoglucanase gene (*ncel5A*) from *Volvariella volvacea* WX32 in *Pichia pastoris*. *J Biosci Bioeng* 2011;111:537–40. <http://dx.doi.org/10.1016/j.jbiosc.2011.01.002>.
- [25] Sadhu S, Saha P, Sen SK, Mayilraj S, Maiti TK. Production, purification and characterization of a novel thermotolerant endoglucanase (CMCase) from *Bacillus* strain isolated from cow dung. *SpringerPlus* 2013;2:1–10. <http://dx.doi.org/10.1186/2193-1801-2-10>.
- [26] Pandey S, Kushwah J, Tiwari R, Kumar R, Somvanshi VS, Nain L, et al. Cloning and expression of β -1,4-endoglucanase gene from *Bacillus subtilis* isolated from soil long term irrigated with effluents of paper and pulp mill. *Microbiol Res* 2014;169:693–8. <http://dx.doi.org/10.1016/j.micres.2014.02.006>.
- [27] Yang J, Dang H. Cloning and characterization of a novel cold-active endoglucanase establishing a new subfamily of glycosyl hydrolase family 5 from a psychrophilic deep-sea bacterium. *FEMS Microbiol Lett* 2011;325:71–6. <http://dx.doi.org/10.1111/j.1574-6968.2011.02413.x>.
- [28] Marx JC, Collins T, D'Amico S, Feller G, Gerday C. Cold-adapted enzymes from marine Antarctic microorganisms. *Marine Biotechnol* 2007;9:293–304. <http://dx.doi.org/10.1007/s10126-006-6103-8>.
- [29] Dhar H, Kasana RC, Dutt S, Gulati A. Cloning and expression of low temperature active endoglucanase EG5C from *Paenibacillus* sp. IHB B 3084. *Int J Biol Macromol* 2015;1:59–266. <http://dx.doi.org/10.1016/j.ijbiomac.2015.07.060>.
- [30] Boyce A, Walsh G. Characterisation of a novel thermostable endoglucanase from *Alicyclobacillus vulcanalis* of potential application in bioethanol production. *Appl Microbiol Biotechnol* 2015;9:7515–25. <http://dx.doi.org/10.1007/s00253-015-6474-8>.
- [31] Martin M, Biver S, Steels S, Barbeyron T, Jam M, Portetelle D, et al. Identification and characterization of a halotolerant, cold-active marine endo- β -1,4-glucanase by using functional metagenomics of seaweed-associated microbiota. *Appl Environ Microbiol* 2014;80:4958–67. <http://dx.doi.org/10.1128/AEM.01194-14>.
- [32] Rawat R, Kumar S, Chadha BS, Kumar D, Oberoi HS. An acidothermophilic functionally active novel GH12 family endoglucanase from *Aspergillus niger* HO: Purification, characterization and molecular interaction studies. *Antonie Van Leeuwenhoek* 2015;107:103–17. <http://dx.doi.org/10.1007/s10482-014-0308-z>.