Appl Biochem Biotechnol (2010) 160:1627–1636
DOI 10.1007/s12010-009-8648-2

A Novel Endoglucanase (Cel9P) From a Marine Bacterium *Paenibacillus* sp. BME-14

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Received: 7 February 2009 / Accepted: 12 April 2009 /
Published online: 16 May 2009

Abstract By constructing a genomic library, an endoglucanase gene (*cel9P*) was cloned from *Paenibacillus* sp. BME-14 which was isolated from the sea. It had an open-reading frame of 1,629 bp, encoding a peptide of 542-amino acid residue with a calculated molecular mass of 60 kDa. The enzyme showed the highest amino acid identity of 52% with other known endoglucanases and had a C-terminal catalytic domain belonging to the glycosyl hydrolases family 9. The optimum pH and temperature for enzymatic activity was pH 6.5 and 35 °C. The metal ions of Ca^{2+}, Mg^{2+}, and Mn^{2+} had a positive effect on the activity while Hg^{2+}, Cu^{2+}, and EDTA had a negative effect. Notably, Cel9P had 65% of the maximal activity at 5 °C. Based on the special characteristic of Cel9P, it had a potential significance for study of cold-active mechanism and industry applications.

Keywords *Paenibacillus* sp. · Sea bacteria · Endoglucanase · Glycoside hydrolase family 9 · Alkali tolerant

Introduction

Cellulose, the major component of the plant biomass, is an important renewable resource existing on earth enormously and composed of glucose units linked by β-1,4-glucosidic bonds. Energy crisis prompted people to pay more and more attention on utilization of plant biomass. Cellulase including endo-β-1,4-glucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) can degrade the cellulose to utilizable glucoses by the synergistic action and played a key role in bioconversion of cellulose.

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Endoglucanase is a glycoside hydrolases endohydrolyzing 1,4-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans, and is widely detected in various organisms. To date, a large number of endoglucanase genes have been cloned from bacteria, such as *Ruminococcus flavefaciens* [1], *Bacillus* [2], *Clostridium thermocellum* [3], *Fibrobacter succinogenes* [4] and *Erwinia carotovora* [5]. Cellulases are being introduced in paper manufacture to improve the drainability of pulp from recovered paper and some reports indicate that endoglucanases are more effective than exoglucanases in modifying the properties of fibers [6].

Organisms are able to produce proteins with particular characteristics that enable themselves to survive under extreme conditions. Thus some distinct enzymes were isolated from the extreme conditions. For example, thermophilic cellulases have been isolated from an Egyptian hot spring [7]; salt-activated endoglucanase has been isolated from seawater [8]. Sea is the largest region on earth and possesses abundant resource to be discovered and utilized. However, there are few endoglucanases from sea organisms; thus, searching for valuable resources from it will be attractive and we focus our attention on exploring rare resources from it.

Here, we report the cloning, purification and characteristic of an endoglucanase Cel9P from a sea bacterium *Paenibacillus* sp. This endoglucanase is alkali-tolerant besides being cold-active, which make it have more potential applications.

Materials and Methods

Strains and Vectors

Paenibacillus sp. BME-14 was isolated from the Xiamen shallow sea and analyzed by 16 S rDNA. *E. coli* DH10B and Plasmid pUC18 was used for construction of the genomic library. *E. coli* BL21 (DE3) and Plasmid pGEX-6P-1 was used for expression and purification of Cel9P.

Materials and Chemicals

Sodium carboxymethycellulose (CMC-Na), barley glucan, oat-spelt xylan, birchwood xylan, locust bean gum, and laminarin from *Laminaria digitata* were purchased from Sigma (St. Louis, MO, USA). GST-Bind Purification Kit was purchased from Novagen. AxyPrep DNA purification kit was purchased from Axygen. DNA purification Kit and Protein Quantification Kit was purchased from Sangon (Shanghai, China). All other chemicals were of analytical grade.

Construction of the Genomic Library

The genomic DNA was extracted from *Paenibacillus* sp. BME-14 by CTAB method [9] and partially digested with *Eco*RI. Then DNA fragments of 4 to 9 kb were recovered and inserted into *Eco*RI-digested and dephosphorized pUC18. Positive clones were screened for activity on the CMC-Na medium by using Congo red stain [10].

Sequencing and Analysis

The insert of possible positive clone was sequenced and the homologies of the DNA and protein sequence were analyzed with the blastn and blastx programs (http://www.ncbi.nlm.

nih.gov/BLAST/). Multiple sequence alignment was performed with the program ClustalW. The signal peptide was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/). Protein functional analysis was performed with InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/).

Gene Cloning, Expression, and Protein Purification

For cloning of the gene *cel9P*, PCR was carried out using the primers BCF (5′-CAAGAATTCATGCGTATTTCGAATCGTAA, containing an *Eco*RI site with the underline), and BCR (3′-GACCTCGAGTCAAGCAAATTCATTCCAGT, containing an *Xho*I site with the underline). The PCR products were cloned into the pGEX-6p-1 vector and positive transformants were screened on the medium containing CMC-Na by using Congo red stain. The recombinant plasmid pGEX-cel was transformed into the expression host *E. coli* BL21 (DE3) and the protein was purified with GST-Bind Purification Kit according to the manufacturer's instructions.

Protein Measurement

SDS-PAGE (10%) was carried out to detect the purity of protein collected by the method of Laemmli [11]. Protein concentration was determined using the Protein Quantification Kit with bovine serum albumin as the standard.

Characteristic of Enzyme

The activity of enzyme was quantified by measuring the amount of reducing sugars produced in the reaction according to the dinitrosalicylic reagent method [12]. One unit was defined as the amount of enzyme releasing 1 μmol reducing sugars in glucose equivalents per minute. Proper diluted enzyme was added into a serials of buffers pH ranging between 4.5 to10 containing 0.5% (*w*/*v*) CMC-Na as substrate, incubating for 30 min at 37 °C to determine the optimum pH. The buffers used were citrate- phosphate (pH 4.5 to 8) and glycine–NaOH (pH 8 to10). The optimum temperature was determined by incubating the enzyme mixed with substrate at various temperatures ranging from 0–45 °C at 5 °C intervals for 30 min. The pH and thermal stability were assayed by pretreating the enzyme respectively in various pH ranges (pH 4 to 10) for 1 h and temperatures ranging from 10–60 °C for 30 min without substrate and measuring the remaining activity under the optimum condition.

Effects of the metal ions and chemical compounds on the enzyme activity were determined at 35 °C for 30 min with a substrate buffer solution (pH 6.5) containing 1 mmol l^{-1} additional reagent.

Substrate specificity was determined by using a set of polysaccharides as substrate, including 0.5% (*w*/*v*) CMC-Na, 0.5% (*w*/*v*) barley glucan, 0.5% (*w*/*v*) avicel, 0.5% (*w*/*v*) oat-spelt xylan, 0.5% (*w*/*v*) birch wood xylan, 0.5% (*w*/*v*) locus bean gum, 0.5% (*w*/*v*) laminarin, 0.5% (*w*/*v*) chitin and 0.5% (*w*/*v*) starch.

To determine the kinetic parameters, substrates were used in the following concentration ranges: CMC-Na, 2.5–25 mg mL^{-1} and barley glucan, 0.5–5 mg mL^{-1}.

Thin-layer chromatography (TLC) was performed using silica gel 60 F254 (0.20–0.25 mm) with *n*-butanol–acetic acid–water (3:2:2) as the running solvent. The plates were visualized by spraying with a 9:1 (*v*/*v*) mixture of methanol and sulfuric acid containing 0.2% methanolic orcinol.

Nucleotide Sequence Accession Number

The DNA sequence of *cel9P* had been submitted to the NCBI database under accession number FJ458447.

Results

Cloning and Sequencing of an Endoglucanase Gene

A genomic library of *Paenibacillus* sp. was constructed and a clone with a 5-kb insert was found to have cellulase activity. The fraction contained a complete ORF of 1,629 bp that started with an ATG codon and terminated in a TGA codon, encoding a putative endoglucanase of 542 amino acids. The calculated molecular mass of the predicted protein was 60 kDa. Upstream of the gene, a predicted promoter with −10 (TATCAT) and −35 (AGGCCG) regions locate at 59 bp and 80 bp from the start codon, respectively. No signal peptide was detected by program SignalP and PSORT Prediction.

Sequence Analysis

The amino acid sequence analysis with InterProScan revealed that the Cel9P contained two domains. The N-terminal immunoglobulin-like (Ig-like) domain of 91 amino acids distributed the gene into the group B1 of family 9, and the C-terminal catalytic domain from position 94 to 540 belonged to glycosyl hydrolase family 9 (GH9), so the gene was designated *cel9P* [13], coding the protein Cel9P. The Ig-like domain showed 38% sequence identity with Ig-like domain protein from *Geobacillus* sp. Y412MC10 (GenBank accession no. EDV78524) and 34% identity with EngO from *Clostridium cellulovorans* [14]. The catalytic domain (CD) showed 45% identity with cellulase (CelA) from *Alicyclobacillus acidocaldarius* [15], 44% identity with EngO from *C. cellulovorans* [14] and 40% identity with cellodextrinase (Ced1) from *Butyrivibrio fibrisolvens* [16]. A multiple sequence alignment was carried out by ClustalW (Fig. 1). Cel9P was conserved in Asp151, Asp154, Asp513 and Glu522, which were strictly conserved in all cellulases of family 9. These carboxylate residues might stabilize the positively charged state of substrates and Glu522 was the most likely catalytic site similar with Glu 555 in CelD [17]. The 3D structure of CelD from *C. thermocellum* had been solved by X-ray crystallography [18].

Purification of the Endoglucanase Cel9P

SDS-PAGE showed that a large amount of the target protein expressed in the supernatant. The protein was then purified to homogeneity. The specific activity of the final preparation using 0.5% CMC-Na as substrate was 6.99 U mg^{-1} under optimum conditions.

Enzymatic Properties of the Cel9P

Cel9P showed the maximal activity at pH 6.5 for degradation of CMC-Na (Fig. 2a). It had a narrow activity pH range near neutrality. However, it was stable after pretreated in buffers with pH 5–10 at 4 °C for 1 h, with little loss of activity under alkaline condition (Fig. 2b).

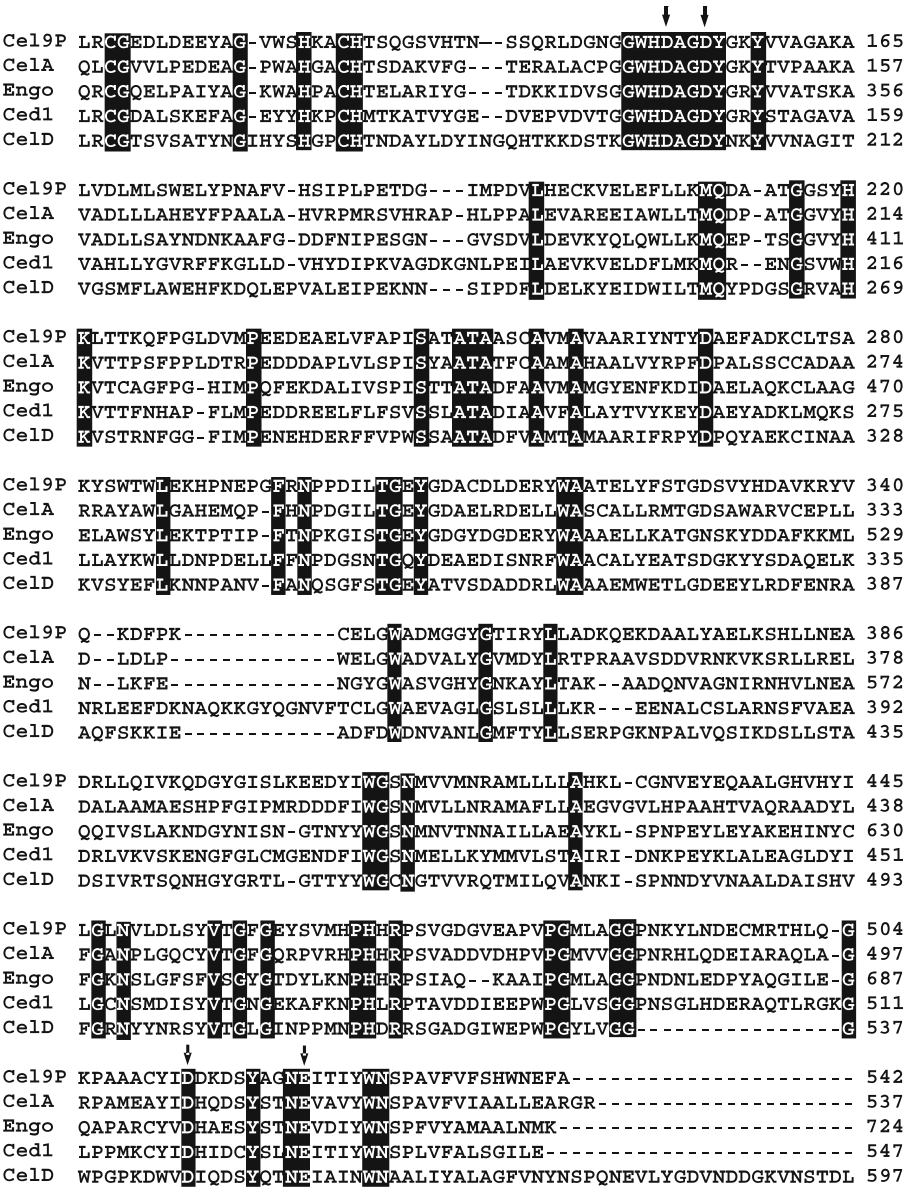

Fig. 1 Multiple sequence alignment was performed by ClustalW. Identical amino acids were *shaded*. The conserved sites were labeled with *arrow*. CelA (CAC34051), EngO (AAT66046), Ced1 (CAA39264) and CelD (1CLC_A) belonging to GH9 were from *Alicyclobacillus acidocaldarius*, *C. cellulovorans*, *Butyrivibrio fibrisolvens*, and *C. thermocellum*, respectively

At pH 6.5, the optimal temperature was determined to be 35 °C, and the activity decreased sharply above 35 °C. However, it was adapted to the low temperature retaining around 65% of the maximal activity at 5 °C (Fig. 2c). The enzyme retained 70% of the maximal activity after incubating 30 min at 50 °C but nearly no activity after incubating 30 min at 60 °C (Fig. 2d).

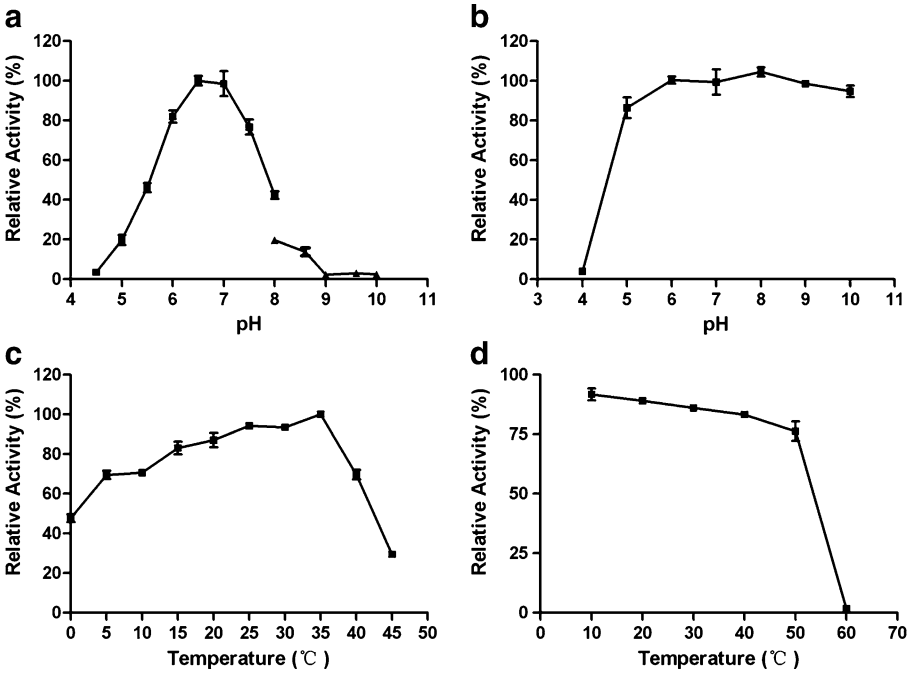

Fig. 2 **a** Effect of pH on the activity of Cel9P. Assay was performed in buffers containing 0.5% CMC-Na, pH ranging from 4.5 to 10 at 37 °C for 30 min. The maximal activity was taken as 100%. **b** Effect of pH on the stability of Cel9P. The enzyme was pretreated in various buffers ranging from 4 to 10 for 1 h at 4 °C, and then measured the activity under the optimum conditions. The activity without treatment was taken as 100%. Buffers used were citrate–phosphate (pH 4.5–8), glycine–NaOH (pH 8–10). **c** Effect of temperature on the activity of Cel9P. Assay was performed in 0.2 M citrate–phosphate buffer containing 0.5% CMC-Na, pH 6.5, at various temperatures. The maximal activity was taken as 100%. **d** Effect of temperature on the stability of Cel9P. The enzyme was pretreated in various temperatures for 30 min, and then measured the activity under the optimum conditions. The activity without treatment was taken as 100%

Table 1 Effect of the metal ions and reagents on the activity of Cel9P.

Reagent	Concentration (mmol l^{-1})	Relative activity[a] (%)
Control	1	100
Ca^{2+}	1	137
Mg^{2+}	1	156
Cu^{2+}	1	2.9
Mn^{2+}	1	177
Co^{2+}	1	112
Pb^{2+}	1	167
Hg^{2+}	1	3.8
Triton X-100	1%	58
Dithiothreitol	1	164
β-Mercaptoethanol	1%	158
EDTA	1	8

[a] Assay was performed at the optimum condition

Table 2 Substrate specificity of Cel9P.

Substrate	Specific activity[a] (U mg^{-1})
CMC-Na	6.99±0.02[b]
Barley glucan	17.46±0.17
Avicel	<0.01
Oat-spelt xylan	<0.01
Birchwood xylan	<0.01
Chitin	<0.01
Starch	<0.01
Locust bean gum	<0.01
Laminarin	<0.01

[a] Assay was performed at the optimum condition

[b] Standard deviations were shown behind the specific activities

The effect of metal ions and chemical compounds on the Cel9P was shown in Table 1. The activity measured with no additional reagent was taken as 100%. The activity was strongly inhibited by Cu^{2+} (2.9%), Hg^{2+} (3.8%) and was activated by Ca^{2+} (137%), Mg^{2+} (156%), Mn^{2+} (177%), Co^{2+} (112%), Pb^{2+} (167%). The chemical reagent dithiothreitol and β-Mercaptoethanol activated the enzymatic activity by 164% and 158%, respectively. While Triton X-100 and EDTA inhibited the enzymatic activity by 58% and 8%, respectively.

The specificity activity of the enzyme on the various substrates was shown in Table 2. The enzyme exhibited higher activity on barley glucan and CMC-Na and lower activity on

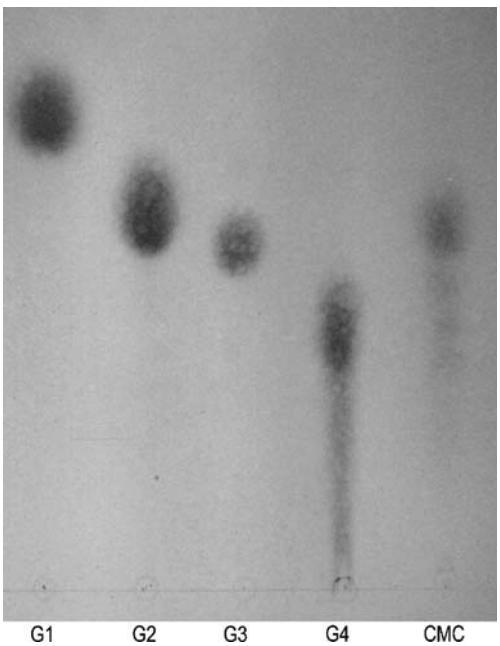

Fig. 3 Thin-layer chromatography analysis of degradation products of CMC-Na. *G1* glucose, *G2* cellobiose, *G3* cellotriose, *G4* cellotetraose, *CMC* the degradation products of CMC-Na

insoluble Avicel. It showed nearly no activity on xylan, mannan, and laminarin. This certified that the enzyme was an endo-β-1,4-glucanase.

The V_m and K_m toward CMC-Na and barley glucan were 36.82 μmol min^{-1} mg^{-1} protein, 13.24 mg ml^{-1}, and 18.11 μmol min^{-1} mg^{-1} protein, 1.65 mg ml^{-1}.

TLC analysis of the soluble sugars released from CMC indicated that the main product was cellobiose with small amounts of cellotriose and cellotetraose (Fig. 3).

Discussion

Paenibacillus sp. isolated from the root of plants [19] and soil [20] had been detected to produce cellulase. In the present study, we first cloned a novel endoglucanase from *Paenibacillus* sp. isolated from the sea.

Through genomic library constructed, we obtained an endoglucanase gene belonging to group B1 of GH9. Cellulases of family 9 were classified into four thematic modular arrangements [21]. Group A had a family IIIc CBD downstream from its GH9 CD. Group B including group B1 and group B2 had an Ig-like domain and a GH9 CD, but groupB2 had an additional family IV CBD compared to group B1. Group C was the simplest cellulase, consisting of CD alone. Previously studied endoglucanase belonging to family 9 from *Paenibacillus* sp. BP-23 had a family IIIc CBD, which was able to bind to Avicel, and the CD showed low identity (21%) with the Cel9P [20]. Besides family 9 and 44 [19], most of the endoglucanase from *Paenibacillus* species belonged to family 5 [22, 23]. Cel9P hydrolyzed β-1,4-glucosidic linkages in substrates such as CMC-Na and barley glucan, and had nearly no activity on the substrates lack of β-1,4-glucosidic linkages. Because of the lack of the cellulose-binding domain, Cel9P had no ability to degrade the crystalline Avicel [20, 24].

According to the assays of enzymatic properties, the endoglucanase was showed as a neutral enzyme. However, after being pretreated in a broad pH range (pH 6–10) conditions for 1 h, the activity was nearly no loss, and about 80% of maximum activity was retained after overnight treatment in high alkaline condition (pH 10). Accordingly, the endoglucanase was able to reserve its activity when processed by some chemicals in industrial application. Like CelD and CelA, the activity of Cel9P was stimulated by Ca^{2+}, indicating the presence of Ca^{2+}-binding sites that stabilize the conformation of Cel9P with an increased affinity for the substrate [15, 17]. Moreover, it was strongly inhibited by EDTA, which might be the result of the removal of divalent cations. Hg^{2+} strongly inhibited the activity of Cel9P, which may be ascribed to interactions with thiol groups of Cys residue(s), try residue(s), or carboxyl group(s) of amino acid(s) [25].

Up to now, few endoglucanases could catalyze at low temperature, and most of them belonged to family 5, such as CelX from *Pseudoalteromonas* sp. DY3 [26], Cel5G from *Pseudoalteromonas haloplanktis* [27] and CelG from *F. succinogenes* S85 [28]. CelX and Cel5G were 96% in identity with each other. Cel9P had no similarity with them. The optimum temperature of CelX was 40 °C and it kept 55% remnant activity at 15 °C. The adaptation of the low temperature may be attributed to the long linker between the CD and CBD, which made the structure less compact and increased the flexibility of the structure [29]. However, the mechanism of cold adaptation was poorly understood. Cel9P did not have the long linker, and the flexibility may due to the amino acid residues near the active site. It reported that the catalytic residues were conserved, so substitutions around the active site to be small amino acids reduced the steric hindrance at the entrance of the active site, which could facilitate the substrate binding at low energy cost [30]. At the basis of the multiple sequence alignment, E522 was an active site corresponding with E555 in CelD

that had been studied [17]. Near this site, there were three consecutive three Ala residues and two small amino acids distinguished to other sequences. Such as CelG, the active site was flanked with three small amino acids and one small amino acid, respectively. Although it was putative, Cel9P was to be a useful material to seek out the cold-active mechanism. The endoglucanase belonging to family 9 was first detected to have high activity at low temperature, and it may be a consequence of evolution to adapt to the cold circumstance the strain *Paenibacillus* sp. BME-14 grown.

Endoglucanase as one type of cellulase was applied in a wide range of industrial processes by the synergistic action with other types of cellulases, such as bio-polishing in textile industry and detergent additives in detergent industry [31]. Catalyzing by the enzyme could decrease the energy consumption and the clothing abrasion at room temperature [32]. In food and animal feeds process, catalysis could reduce contamination and preserve the quality of the nutrition at low temperature [33, 34]. Moreover, as a key enzyme in bioconversion of plant biomass [35], exploiting these endoglucanases would reduce the waste of the extra energy.

In conclusion, Cel9P was a novel endoglucanase from *Paenibacillus* sp. BME-14, which was cold-active and alkaline-tolerant. It would be valuable in molecular research on cold-active mechanism and the special industry applications.

Acknowledgments This work was supported by grants from the National Natural Sciences Foundation of China (30770021 and 30570057) and the 111 project (B07041).

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