A cold-active acidophilic endoglucanase of *Paenibacillus* sp. Y2 isolated from soil in an alpine region

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Abstract A cellulolytic strain Y2 was isolated from soil obtained in the Canadian Alpine region. The isolate was identified as *Paenibacillus* sp. Y2 by 16S rRNA sequencing. When grown in LB medium supplemented with carboxymethyl-cellulose (CMC), CMCase production increased to 122.0% of that observed in LB without CMC. Culture supernatant was concentrated by ultrafiltration and 80% ammonium sulfate precipitates were separated by Hi-Trap Q and CHT-II chromatography. The purified enzyme (EG-PY2) showed a homogeneous single band and the molecular mass was estimated to be 38 kDa by SDS-PAGE. Optimum pH and temperature of the enzyme were 4.5 and 30 °C, respectively. The half-life of enzyme activity at 50 was 140.7 min, but the enzyme was drastically inactivated within 5 min at 55 °C. The enzyme was highly activated to 135.7 and 126.7% by 5.0 mM of Cu²⁺ or Mg²⁺ ions, respectively, and moderately activated by Ba²⁺ and Ca²⁺ ions, whereas it was inhibited to 76.8% by Fe²⁺, and to ≤50% by Mn²⁺, Co²⁺, Zn²⁺, and EDTA. The enzyme was activated to 211.5% in the presence of 0.5 M of NaCl and greatly tolerant to 3.15 M of NaCl. The enzyme showed 2.98 times higher β-glucanase activity than CMCase activity. Based on these results, it can be concluded that EG-PY2 is an acidophilic, cold-active, and halotolerant endoglucanase. The authors suggest it is considered to be useful for various industrial applications, such as, fruit juice clarification, acidic deinking processes, high-salt food processing, textile and pulp industries, and for biofuel production from seaweeds.

Keywords Acidophilic endoglucanase · Alpine region · Cold-active · Halotolerant · Metal ion activation · *Paenibacillus* sp. Y2

Introduction

Lignocellulosic materials mainly present in the cell walls of plants and have recently attracted research attention for industrial applications in the biofuel and fine chemicals sectors (Saini et al. 2015; Álvarez et al. 2016; Kumar and Sharma 2017). Lignocellulosic materials are principally composed of cellulose, hemicellulose, and lignin. Of these, cellulose is the most abundant carbohydrate polymer, and is a homopolysaccharide composed to β-1,4-linked D-glucose polymers. Hemicellulose is the second abundant heteropolysaccharide and is largely composed of xylan, a β-1,4-linked D-xylose, with a complex structure that has D-mannose and D-glucose in its main chains, and D-galactose, L-arabinose, and D-glucuronic acid in its branches (Álvarez et al. 2016).

Lignocellulosic materials can be broken down to fermentable sugars by microbial enzymatic hydrolysis (Mathews et al. 2015; Álvarez et al. 2016). Both fungi and bacteria have been extensively investigated for their abilities to produce novel cellulases and hemicellulases. Because of their merits, such as, high growth rates, greater complexity, multi-enzyme complexes, and their abilities to thrive in different environments, bacteria have been widely exploited for the isolation, purification, and characterization of the novel enzymes (Maki et al. 2009). Cellulolytic bacteria, especially *Bacillus* and *Paenibacillus* strains, have been isolated from various environment, such as, insect gut (Anand et al. 2010; Dantur et al. 2015), agricultural environments (Choe et al. 2008), soils and composts (Lee et al. 1999; Pason et al. 2006; Wang et al. 2008; Amore et al. 2013; Kim et al. 2016), feces (Dong et al. 2016), and forest soils (Liang et al. 2014; Kanchanadumkerng et al. 2017).

Most fungal cellulases show acidic optima, as described in a review (Ben Hmad and Gargouri 2017). However, only a few

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bacterial cellulases have been reported to be acidophilic enzymes, and cellulases from *Bacillus* (Blanco et al. 1998; Li et al. 2006; Zhu et al. 2011; Rawat and Tewari 2012) or *Paenibacillus* species (Dhar et al. 2015; Kanchanadumkerng et al. 2017) are of particular interest. On the other hand, cold-active enzymes have attracted more attention than mesophilic enzymes from the potential application standpoint because of lower limits of protein stability and short of information up to now (Kasana and Gulati 2011). Furthermore, only a few bacterial cold-active cellulases have been described, such as, *Pseudalteromonas* sp., *Pseudomonas* sp., or *Paenibacillus* sp. from marine environments or feces (Zeng et al. 2006; Fu et al. 2010; Yang and Dang 2011; Dong et al. 2016), and *Paenibacillus* sp. from a cold lake sediment (Dhar et al. 2015). In this paper, we describe the isolation of a cellulolytic *Paenibacillus* sp. Y2 from Canadian Alpine soil, and biochemical characteristics of a novel acidophilic and cold-active endoglucanase. Enzyme activity activations by various cations, such as, Na^+ , Cu^{2+} , and Mg^{2+} including halotolerance were also investigated.

Materials and Methods

Chemicals

Carboxymethyl-cellulose (CMC), barley β -glucan, laminarin, *p*-nitrophenyl- β -D-glucopyranoside (pNPG), *p*-nitrophenyl- β -D-cellobioside (pNPC), birchwood xylan, 3,5-dinitrosalicylic acid (DNS), Congo red, and other chemicals were purchased from Sigma (St. Louis, MO, USA). Lichenan (Icelandic moss) was obtained from Megazyme (Wicklow, Ireland).

Isolation of cellulolytic bacteria from soil

The soil was obtained near Takakkaw Falls in the Canadian Alpine region (Na et al. 2015). After serial dilutions of soil, supernatants were spread on Luria-Bertani (LB) agar plates. Colonies were tooth-picked onto LBC (LB containing 0.5% CMC) agar plates and were grown at 37 °C for 24 h. A positive colony with the largest zone of hydrolysis on plates was selected for further studies. The isolated strain Y2 was designated *Paenibacillus* sp. Y2 and a sample was deposited in the Korean Collection for Type Cultures (KCTC) as KCTC 33893.

Analysis of 16S rRNA

16S rRNA was analyzed by SolGent Co. (Daejeon, Korea) and the sequence was deposited in GenBank under accession number KY744454. Sequence similarities were determined by 16S rRNA gene sequence comparisons at NCBI, i.e. <http://www.ncbi.nlm.nih.gov>.

Production of cellulolytic enzymes

The strain was cultivated with shaking at 200 rpm in 200 mL of LBC in a 1 L flask for 24 h at 37 °C, and sampled every 3 h to observe cell growth and enzyme activity in culture supernatant.

The strain was also cultured in LB broth as a control.

Enzyme purification

After cultivation in 600 mL broth (200 mL×3 flasks) for 24 h, cells were harvested and supernatants were collected by centrifugation at 12,000×g for 10 min. The supernatant obtained was concentrated to 40 mL by ultrafiltration using a YM10 membrane (Amicon, Beverly, MA, USA) and proteins were precipitated using 80% ammonium sulfate. After dialysis against 20 mM Tris-HCl (pH 8.0) the crude enzyme was loaded onto a Hi-Trap Q column (5 mL, GE Healthcare, Uppsala, Sweden) and eluted with 0–1.0 M NaCl in 20 mM Tris-HCl (pH 8.0) at a flow rate of 1.0 mL/min (Baek et al. 2017). Active fractions were collected, dialyzed against 10 mM sodium phosphate buffer (pH 6.8), and further purified by CHT-II column (5 mL, Bio-Rad, Hercules, CA, USA) chromatography with a gradient using 10–500 mM sodium phosphate (pH 6.8) at a flow rate of 0.5 mL/min. Protein concentrations were determined using the Bradford method (1976) and were monitored at A_{280} nm during purification. SDS-PAGE was carried out using 11.5% polyacrylamide gels (Laemmli 1970), and gels were stained with Coomassie Brilliant Blue R-250.

Enzyme assay

Cellulase activity was assayed using a reaction mixture (1.0 mL) containing 0.5% CMC and an appropriate enzyme in 50 mM sodium citrate (pH 5.0). The mixture was then heated at 50 for 30 min, and the amount of reducing sugar released was determined using the DNS method as a standard condition (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per min under these conditions.

Characterization of the enzyme

To analyze optimum temperature, enzyme activity was measured at different temperatures, 20, 30, 40, 50, 55, and 60 °C, and for thermostability analysis, enzyme activity was measured by preincubating the enzyme in the absence of substrate for 5, 10, 30, 60, 90, 120, and 150 min at predetermined temperatures. To analyze optimum pH, the enzyme was assayed between pH 3.5 and 8.0 using 50 mM buffers: sodium-citrate (pH 3.5–6.0) and sodium-phosphate (pH 6.0–8.0). The influences of various cations and other entities on enzyme activity were investigated at a concentration of 5.0 mM for Na^+ , K^+ , Mg^{2+} , Mn^{2+} , Ca^{2+} , Ba^{2+} , Cu^{2+} , Co^{2+} , Fe^{2+} , Zn^{2+} , and ethylenediaminetetraacetic acid (EDTA), and at 0.5% and 1.0% for SDS, Triton X-100, and Tween 80. For investigating the possibility of changes of thermostability in the presence of activating metal ions, the enzyme was preincubated for 60 min at 50 °C in the presence of each effective metal ion, and then residual activity was assayed in the standard reaction mixture and compared to residual activity of a metal ion free control. To analyze halotolerance, the enzyme was preincubated with 2.25 M (13.2%) and 3.15 M (18.4%) of NaCl at 4 °C for 24 h, the residual activity was measured in the standard

condition. Polysaccharides (0.5% lichenan, barley β -glucan, laminarin, and birchwood xylan) were analyzed using the standard method described above. Modified oligosaccharides (2 mM pNPG and pNPC) were assayed as described previously (Yang et al. 2017). Reaction mixtures containing xylan and lichenan were clarified by centrifugation prior to measuring absorbances.

Results and Discussion

Isolation of strain and analysis of 16S rRNA

After tooth-picked colonies were grown on LBC, a colony with the largest halo was selected and named Y2 (data not shown). The 16S rRNA sequence of the isolate Y2 showed highest similarity (99%) with *Paenibacillus amylolyticus* strain NRRL NRS-290 (GenBank accession number NR_025882), *P. amylolyticus* strain JCM 9906 (NR_112163), *Paenibacillus tundae* strain A10b (NR_044525), *Paenibacillus xylanexedens* strain B22a (NR_044524), and *Paenibacillus tylopili* strain MK2 (NR_115990) in descending order by BLAST analysis at the NCBI. Phylogenetic tree analysis showed isolate Y2 belongs to a group containing *P. amylolyticus* NRRL NRS-290 and *P. amylolyticus* JCM 9906 (Fig. 1). The isolate was named *Paenibacillus* sp. Y2.

Cell growth and enzyme production

Paenibacillus sp. Y2 showed stable CMC-hydrolyzing activity after repeated transfer (data not shown). When it was cultured in 200 mL of LB or LBC in a 1 L flask, it grew up to 18 h and showed similar cell growths (Fig. 2). Cellulolytic enzyme productions in LB and LBC were also similar; enzyme activity appeared after culture for 6 h and peaked at 21 h. However, the maximum

Fig. 2 Growth curves and cellulase activities of the isolate *Paenibacillus* sp. Y2 in LB and LBC broths. Symbols: ▲, growth in LB; ×, growth in LBC; ◆, activity in LB; ■, activity in LBC

amount of the enzyme produced in LBC was 22.0% greater than produced in LB (Fig. 2). The result suggests that CMC substrate moderately induce the productions of extracellular cellulolytic enzymes.

Purification of a major extracellular cellulase

After growing *Paenibacillus* sp. Y2 for 24 h in LBC, culture supernatant was collected by centrifugation, and a major extracellular cellulase was isolated by ultrafiltration, 80% ammonium sulfate precipitation, and Hi-Trap Q and CHT-II column chromatography. Using these methods, the cellulase was purified 9.9-fold with an enzyme activity yield of 18.9 % (Table 1). SDS-PAGE showed the purified protein produced a homogeneous band and that its estimated molecular mass was 38 kDa (Fig. 3). The enzyme was named EG-PY2. The molecular mass of EG1-PY2 was close to that of endoglucanase PgluE8 (40.4 kDa) (Dong et al. 2016), larger than that of Cel5A (33 kDa) (Cho et al. 2008), and smaller

Fig. 1 Phylogenetic tree of *Paenibacillus* sp. Y2

Table 1 Purification of the extracellular cellulase isolated from *Paenibacillus* sp. Y2

Procedure	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Supernatant	600.0	43.4	28.6	0.66	1.0	-
Ultrafiltration	40.0	14.8	12.0	0.81	1.2	42.0
(NH ₄) ₂ SO ₄ precipitation	9.0	8.5	8.8	1.04	1.6	30.8
Hi-Trap Q chromatography	7.0	2.7	6.6	2.44	3.7	23.1
CHT-II chromatography	8.0	0.83	5.4	6.52	9.9	18.9

Fig. 3 SDS-PAGE result of EG-PY2. Lanes: M, size markers; 1, ammonium sulfate precipitate; 2, pool of active fractions obtained by Hi-Trap Q chromatography; 3, pool of active fractions obtained by CHT-II chromatography. Numbers represent the molecular masses of proteins in kilodaltons (kDa)

than those of EG5C (63.5 kDa) (Dhar et al. 2015), Cel9P (60 kDa) (Fu et al. 2010), Cel5A (64 kDa) (Park et al. 2012), and Cel9P (60 kDa) (Kanchanadumkerng et al. 2017) from *Paenibacillus* sp. These results showed EG-PY2 is a low-molecular weight cellulase.

Properties of EG-PY2

The purified enzyme was optimally active at pH 4.5 in sodium citrate buffer (Fig. 4A), and showed 66% and 63% of maximum activity at pH 4.0 and 5.5, respectively. The optimum temperature of EG-PY2 was 30 °C (Fig. 4B). At 40 and 50 °C, only about 10% of enzyme activity was decreased compared to the maximum activity. At 20 °C enzyme activity was 79% of maximum activity. EG-PY2 activity decreased to 59 and 47 % of its original activity after incubation for 150 min at 40 or 50 °C, respectively (Fig. 4C), and its half-life at 50 °C was 140.7 min. However, it was drastically inactivated after 5 min at 55 °C. These results suggest that EG-PY2 is an acidophilic, cold-active cellulase.

The optimum pH of EG-PY2, that is, 4.5, was slightly higher than those, pH 4.0, of the endoglucanases Cel1A from *Bacillus* sp., and Egl173 and CMCase from *Bacillus subtilis* (Blanco et al. 1998; Zhu et al. 2011; Rawat and Tewari 2012), and slightly lower than those of EG5C and CelP from *Paenibacillus* sp., which showed a pH optimum at 5.0 and a sharp decrease at pH 6.0 (Dhar et al. 2015; Kanchanadumkerng et al. 2017), and that of Ba-EGA

Fig. 4 Effects of pH (A) and temperature (B) on the activity of EG-PY2, and (C) thermal stability (C) of EG-PY2. In the thermal stability experiments, the enzyme was preincubated at temperatures for the indicated times in the absence of substrate (CMC), and then residual activity was measured in the presence of 0.5% substrate as the standard assay

(pH 4.5–6.5) from *Bacillus* sp. (Li et al. 2006). Acidic endoglucanases have been suggested to be practically suitable in the animal feed industry, for fruit juice clarification, paper manufacture, and non-ionic-surfactant-assisted acidic deinking of old news print or old magazines (Blanco et al. 1998; Rawat and Tewari 2012).

The optimum temperature of EG-PY2 (30 °C) was the same as those of endoglucanase Cel5M from a deep-sea bacterium

Table 2 Effect of cations and effectors on the activity of purified EG-PY2

Cation/effector	Concentration	Relative activity (%)
Control	-	100*
	5 mM	111.3±3.4
NaCl	50 mM	144.4±4.54
	500 mM	211.5±1.89
KCl	5 mM	103.3±10.5
MgCl ₂	5 mM	126.7±6.5
MnCl ₂	5 mM	60.4±9.2
CaCl ₂	5 mM	110.9±6.5
CuCl ₂	5 mM	135.7±1.8
CoCl ₂	5 mM	43.5±6.4
BaCl ₂	5 mM	111.9±8.1
FeCl ₂	5 mM	76.8±1.2
ZnCl ₂	5 mM	22.6±9.3
EDTA	5 mM	63.1±2.31
SDS	1.0%	11.7±1.1
	2.0%	14.9±6.9
Triton X-100	1.0%	27.8±4.4
	2.0%	35.0±5.7
Tween 80	1.0%	57.7±6.6
	2.0%	41.4±7.2

*The value of control had a specific activity of 6.0 U/mg of protein
EDTA=ethylenediaminetetraacetic acid

Pseudomonas sp. and CEL8M from a soil metagenome, however, Cel5M and CEL8M showed steep decreases in activity at either side of their optimum temperatures (Yang and Dang 2011; Bhat et al. 2013). The optimum temperature of EG-PY2 was lower than that of CelX isolated from a deep sea-bacterium *Pseudalteromonas* sp. and of EglC isolated from a symbiotic bacterium *Citrobacter farmeri* (Zeng et al. 2006; Bai et al. 2016). The cold activity pattern of EG-PY2 did not change markedly between 20 and 50 °C, which is similar to those reported for reasons of the endoglucanases Cel9P, EG5C and PgluE8 from *Paenibacillus* sp. obtained from the sea, a cold lake sediment, and feces, respectively. However, optimum temperature of the EG-PY2 was lower than those of these three endoglucanases (35, 40 and 50 °C, respectively) (Fu et al. 2010; Dhar et al. 2015). Cold-active and acidophilic enzymes are being exploited in various fields (Kasana and Gulati 2011; Dong et al. 2016). Recently, a cold-active and acidic endoglucanase was isolated from an animal source and used to for the hydrolysis of seaweeds to produce biofuels (Song et al. 2017).

The effects of metal ions and a chelating agent on enzyme activity were investigated at a concentration 5.0 mM. EG-PY2 was highly activated to 135.7 and 126.7% by Cu²⁺ and Mg²⁺ and moderately activated by Ba²⁺ and Ca²⁺ ions, but inhibited to 76.8% by Fe²⁺, and to ≤50% by Mn²⁺, Co²⁺, and Zn²⁺ (Table 2), and EDTA reduced its activity to 63.1%. EG-PY2 was activated to 211.5% in the presence of 0.5 M NaCl, indicating halo-stimulating properties. EG-PY2 was inhibited by surfactants, although it was less sensitive to nonionic surfactants (Triton X-

Fig. 5 Effect of metal ions on thermal stability of EG-PY2 and halotolerance by NaCl. The enzyme was preincubated for 60 min at 50 °C in the presence of each metal ion, and then residual activity was assayed in the standard condition. For analyzing halotolerance, the enzyme was preincubated with NaCl for 24 h at 4 °C, prior to the measurement of residual activity. C, Control EG-PY2 was preincubated reaction in the absence of metal ions or NaCl

100 or Tween 80) than to ionic surfactant SDS (Table 2).

The effects of metal ions on endoglucanases vary considerably. Most studies on the effects of the Cu²⁺ ion have reported that it moderately or significantly inhibits purified endoglucanases (Li et al. 2006; Fu et al. 2010; Cheng et al. 2016; Gupta et al. 2017; Kanchanadumkerng et al. 2017; Pimentel et al. 2017; Segato et al. 2017), but a few have been described enzyme activation by Cu²⁺ ion, mostly in *Paenibacillus* species (Wang et al. 2008; Park et al. 2012; Dhar et al. 2015).

The change for the enzyme thermostability by Ca²⁺ was not observed, and those by Na⁺, Mg²⁺, Cu²⁺ ions were not significant, i.e., ranged between 105–116%. However, the change by Ba²⁺ ion was effective to be 135% compared to residual activity of a metal ion free control (Fig. 5). When the enzyme was preincubated with 2.25 M (13.2%) and 3.15 M (18.4%) of NaCl, it showed 130 and 141%, respectively. This indicate EG-PY2 is highly halotolerant and moreover activated. It can be used in specific processes, for example, high-salt food processing (Zhu et al. 2011; Dong et al. 2016).

The β-glucanase activity of EG-PY2 was 2.98 times greater than its CMCase activity, which suggests it hydrolyzes β-1,3-1,4 linked polymers (Table 3), similarly to endoglucanases from *Paenibacillus* sp. and a soil metagenome [Kanchanadumkerng et al. 2017; Pimentel et al. 2017]. But its lichenase activity was 59.0% lower than its CMCase activity. Furthermore, EG-PY2 showed little activities for laminarin, which has β-1,3-1,6 linkages, and for xylan and pNPC hydrolyzing activity. These results suggest EG-PY2 is an endo-β-1,4-glucanase and that the ratio of 1,3- to 1,4 linkages importantly affects its ability to hydrolyze the mixed-linked polysaccharides.

Based on the results obtained, we conclude EG-PY2 is an acidophilic, cold-active, and highly halotolerant endoglucanase.

Table 3 Substrate specificity of EG-PY2

Substrate	Main linkage/Monomer	Relative activity (%)
CMC	β -1,4/Glucose	100
Barley β -glucan	β -1,3-1,4/Glucose	298.0 $\pm$ 7.2
Lichenan	β -1,3-1,4/Glucose	59.0 $\pm$ 1.4
Laminarin	β -1,3-1,6/Glucose	10.0 $\pm$ 2.4
Xylan	β -1,4/Xylose	8.08 $\pm$ 2.4
pNPC		2.0 $\pm$ 0.8
pNPG		1.0 $\pm$ 0.5

We suggest it has potential uses in acidic and low-temperature processes, such as, for fruit juice clarification, acidic deinking, high-salt food processing, textiles, pulp, and biofuel production from seaweeds.

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