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ORIGINAL PAPER

Cellulase production from *Pseudoalteromonas* sp. NO3 isolated from the sea squirt *Halocynthia rorentzi*

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Abstract *Pseudoalteromonas* sp. NO3 was isolated from the hemolymph of diseased sea squirts (*Halocynthia rorentzi*) with symptoms of soft tunic syndrome. The strain was found to produce an extracellular cellulase (CelY) that consisted of a 1,476 bp open reading frame encoding 491 amino acid residues with an approximate molecular mass of 52 kDa. Homologies of the deduced amino acid sequence of *celY* with the products of the *celA*, *celX*, *celG* and *cel5Z* genes were 92.6, 93.3, 92.6, and 59.1%, respectively. Additionally, CelY had 50–80% remnant catalytic activity at temperatures of 10–20°C. Highest carboxymethyl cellulose (CMC) hydrolysis was observed at pH 8.0 and 40°C. CMC activity was determined by zymogram active staining and different degraded product profiles for CelY were obtained when cellotetraose, cellopentaose, and CMC were used as substrates. This study identified a transglycosylation activity in CelY that allows the enzyme to digest G4 to G2 and G3 without the production of G1.

Keywords Sea squirts · *Halocynthia* · *Pseudoalteromonas* · Cellulase · Carboxymethyl cellulose · Transglycosylation activity

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Introduction

Cellulose consists of a linear polymer chain of β-linked glucose molecules and is the most abundant biopolymer worldwide [1]. Previous studies have revealed the existence of many different bacterial and fungal cellulases that can be classified into three types: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) [2, 3]. Endoglucanases cleave intramolecular β-1,4-glucosidic linkages at random, whereas exoglucanases hydrolyze long chains from the ends in a progressive process [4]. β-Glucosidases cleave cellobiose into glucose [4]. Endoglucanase activities can be measured based on an increase in reducing ends determined by a reducing sugar assay [5]. The general technique for measurement of endoglucanase activities can also be performed easily on agar plates by staining the residual long chains of polysaccharides with Remazol brilliant blue R dye [6]. The family 113 glycoside hydrolases (GHs) includes a group of glycosidases, transglycosidases and GH-encoding genes that are estimated to contain genomes corresponding to about 47% of the enzymes classified in the carbohydrate-active enzymes databases (CAZy) [3]. Recently, screening of novel enzymes from prokaryotic organisms has increased due to the identification of *Fibrobacter succinogenes* S85, *Cellulomonas pachnodae*, *Bacillus* sp., and various extremophiles as potential sources for the industrial production of cellulases [7–12]. In addition, cold-active enzymes have high enzymatic activity and protein stability at low temperature; therefore, they have generated considerable interest because they have the potential to improve the energy efficiency of industrial applications [12]. The cold-active enzyme CelG is produced by *Pseudoalteromonas haloplanktis*, which was isolated from the deep ocean and the Antarctic [13]. In addition, CelA and CelX,

which both encode cellulase, were isolated from *Pseudoalteromonas* sp. MB-1 and DY3, respectively [14].

We isolated a cellulolytic bacterial strain, designated NO3, from the hemolymph of diseased ascidians (sea squirts, *Halocynthia roretzi*). The diseased sea squirts have thin and soft tunic tissues due to the low density of cellulose microfibrils on their surfaces, whereas normal sea squirts have a thick and hard tunic. Here, we characterized a cellulase (CelY) produced by *Pseudoalteromonas* sp. NO3, which was highly active toward carboxymethylcellulose (CMC).

Materials and methods

Strain and cultivation conditions

Using standard dilution plating techniques, *Pseudoalteromonas* sp. NO3 was isolated from the hemolymph of diseased *H. rorentzi* samples collected from aquaculture sites in Tongyoung, Korea, in June 2008. Isolation was achieved using modified ZoBell's agar (5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate and 15 g Bacto agar in 1 L distilled water) supplemented with 2% artificial sea salts (Sigma, St. Louis, MO) and 1% sodium CMC (Sigma) [21]. The isolate was then cultured routinely on marine agar 2216 (MA; Difco, Detroit, MI) at 25°C and maintained as a glycerol suspension (20%, w/v) at −80°C.

Bacterial identification

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were conducted as previously described [15]. The resultant sequence of strain NO3 (1,444 nt) was then aligned manually against sequences obtained from the GenBank database. Phylogenetic trees were inferred from the regions available in all sequences (positions 24–1440; *Escherichia coli* numbering system) using the neighbour-joining (NJ) method [16]. Evolutionary distance matrices were generated using the method described by Jukes and Cantor [17]. The resultant NJ tree topology was evaluated by means of bootstrap analyses [18] based on 1,000 resamplings. Alignment and phylogenetic analyses were conducted using the jPHYDIT program (available at http://plaza.snu.ac.kr/~jchun/jphydit/) and PAUP 4.0 [19], as described previously [20].

Physiological and biochemical tests

Growth tests were conducted on nutrient agar (NA, Difco), trypticase soy agar (TSA, Difco), R2A agar and ZoBell's agar (5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate and 15 g Bacto agar in 1 L distilled water) [21]. All media used in physiological tests were supplemented with 2% sea salt except for MA and marine broth 2216 (MB; Difco). The optimum growth temperature (4–50°C) was determined based on the level of growth on MA after incubation for 1 week. Hydrolysis of casein (2% skim milk, w/v), Tween 20 (1%, w/v) and Tween 80 (1%, w/v) was evaluated using the method described Smibert and Krieg [22], with MA being used as the basal medium. DNase activity was determined using DNase test agar (Difco). Other biochemical tests and enzyme activities were performed using API 20E and API ZYM kits (bioMérieux, Marcy l'Etoile, France) and a GN2 MicroPlate (Biolog, Hayward, CA) according to the manufacturer's instructions, with the exception that the bacteria were suspended in artificial sea water (ASW) [23]. Antibiotic resistance was determined using the disc diffusion method by incubating the samples in the presence of commercial antibiotic-impregnated discs (BBL Becton Dickinson, Franklin Lakes, NJ) for 4 days at 25°C in MA. The results were interpreted according to the guidelines set forth by the Clinical Laboratory Standards Institute (CLSI) [24].

Cellulase purification

Pseudoalteromonas sp. NO3 was grown at 25°C for 24 h in a 7 L fermenter (KoBioTech, Incheon, Korea) that contained 4 L marine broth. After centrifugation at 7,000 *g* for 30 min, the bacterial cell pellet was removed and the supernatant concentrated using an Amicon concentrator (10 kDa cut-off filter) against 50 mM Tris buffer (pH 8) at 4°C. Fractionation of bacterial proteins by gel filtration chromatography (GFC) was then conducted using HPLC (Shimadzu system, LC-6AD pump, RID-10A detector, SPD-M20A detector, Kyoto, Japan) with an Asahipak GS-310 column (20 mm ID × 500 mm, Showa Denko Kogyo, Tokyo, Japan). The samples were eluted with water at a flow rate of 5 mL/min. Protein elution was determined using both a refractive index detector (RID-10A detector) and a photodiode array detector (SPD-M20A detector). Aliquots (2 mL) of protein solutions were injected, and fractions were collected.

Cellulase activity assays

The optimal enzyme temperature and pH were estimated within the ranges of 10–50°C and 6.0–10.0, respectively, using a Nelson–Somogyi assay for the measurement of reducing sugars [5]. A standard curve was prepared by measuring the absorbance of glucose at concentrations ranging from 31.5 to 1,000 μg/mL at an absorbance of 525 nm using a spectrophotometer (Mecasys) [5].

Protein fractions obtained by HPLC fractionation were treated with sample buffer without 2-mercaptoethanol and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels according to the method described by Laemmli [25]. After SDS-PAGE, zymogram active staining was performed using the proteins in the gel, which was renatured by incubation in renaturation buffer (50 mM Tris–HCl buffer, pH 8.0, 10 mg/mL casein, 2 mM EDTA, 0.01% NaN_3) containing 25% methanol three times for 30 min each. The polyacrylamide gel was then washed with 100 mM Tris–HCl buffer (pH 7.2) and overlaid on a 2% agarose gel containing 1 mg/mL CMC, 10 mg/mL NaCl, and 100 mM Tris–HCl buffer (pH 7.2). Finally, the gel was incubated at 30°C for 20 h, followed by flooding with an excess of 0.1% aqueous Congo red for 20 min, after which it was washed with excess 1 M NaCl solution.

Analysis of reaction products by thin-layer chromatography

Cellooligosaccharides (cellobiose, cellotriose, cellotetraose and cellopentaose) and CMC (molecular weight 250,000 g/mol) were purchased from Sigma-Aldrich (St. Louis, MO). The degree of substitution of CMC was 1.2. To determine if CelY had cellulase activity, 0.1% (w/v) CMC and 10 mM cellooligosaccharides (cellobiose, cellotriose, cellotetraose and cellopentaose) were digested with 25 µL CelY (2.29 mg/mL) in 50 mM Tris buffer (pH 8) at 40°C. Subsequently, the reaction products were developed in a mixture of 1-propanol, nitromethane, and water (5:3:2, v/v/v) for 2 h, after which the hydrolysis products were separated by thin-layer chromatography (TLC) on a silica gel plate (Analtech, Newark, DE). After separation, the sugars were visualized by spraying the plates with a mixture of 1 mL phosphoric acid and 10 mL stock solution (1 g diphenylamine, 1 mL aniline, 100 mL acetone) [26].

Cloning and sequence analysis

The gene of interest was PCR-amplified from the chromosomal DNA of *Pseudoalteromonas* sp. NO3 using the following modified primers derived from conserved regions of known cellulases [14]: 5′-AGA AGG AGA TAA ACA ATG AAT AAC AGT TCA AAT AAT CAC AAA AG-3′ (CelYF) and 5′-GGA TGA GAC CAG GCA GAT TAA TTA CAA GTA TAA AGA AGC GTC CAC-3′ (CelYR). PCR amplification was as follows: 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 2 min and final extension at 72°C for 5 min. The PCR product was then cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced.

Nucleotide sequence accession numbers

The nucleotide sequence of the 16S rRNA gene and the amino acid sequence of cellulase evaluated in this study were deposited in GenBank. The accession number of the 16S rRNA gene sequence is FJ600315, while that of the amino acid sequence of the cellulase gene (*celY*) is FJ648763.

Results and discussion

Isolation and description of *Pseudoalteromonas* sp. NO3

Bacterial colonies (strain NO3) grown on an agar plate that developed a clear zone around them were isolated (Fig. 1a). Agarolytic activity was confirmed when the colony of the isolated bacterium formed a clear zone and depression on commercial agar medium. Cellulase activity of the isolate was confirmed by a Congo red overlay assay, which detects hydrolysis of CMC based on Congo red staining of the carbohydrate reducing ends (Fig. 1b) [6].

Several studies have been conducted to evaluate the infection of ascidians by viruses and tributyltin [27]. Artificially infected normal *H. rorentzi* with *Pseudoalteromonas* sp. NO3 (10^5 CFU/one sea squirt) reared at 10°C for 20 days induced 50% mortality of *H. rorentzi* at 12 days post-challenge, which suggested that the strain is responsible for the disease symptoms resulting from the degradation of cellulose microfibrils on the tunic tissues of sea squirts [S.J. Jung, interview transcript, 15 June 2009]. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain NO3 belonged to the family *Pseudoalteromonadaceae* and was related to the genus *Pseudoalteromonas*; its closest neighbours were *P. nigrificans* NCIMB 8614^T (99.86% sequence similarity), *P. haloplanktis* ATCC 14393^T (99.79%), and *P. undida* NCIMB 2128^T (99.71%). Based on this information, the strain was designated as *Pseudoalteromonas* sp. NO3. A neighbour-joining tree based on 16S rRNA gene sequences showed that strain NO3 formed a monophyletic clade with *P. haloplanktis* ATCC 14393^T (X67024) with 64% bootstrap support (Fig. 2).

A number of physiological and chemotaxonomic characteristics [28–32] clearly distinguished our isolate from the physiologically related species *P. haloplanktis* ATCC 14393^T (Table 1). For example, strain NO3 can grow at 4°C (optimum 20–25°C) and does not hydrolyze DNA and starch, whereas *P. haloplanktis* ATCC 14393^T is not able to grow at 4°C and is capable of hydrolyzing DNA and starch. Strain NO3 digests CMC as well as agar extracted from *Gelidium*. Additionally, strain NO3 can utilize the following substrates: D-lactose, D-melibiose, D-trehalose, cellobiose,

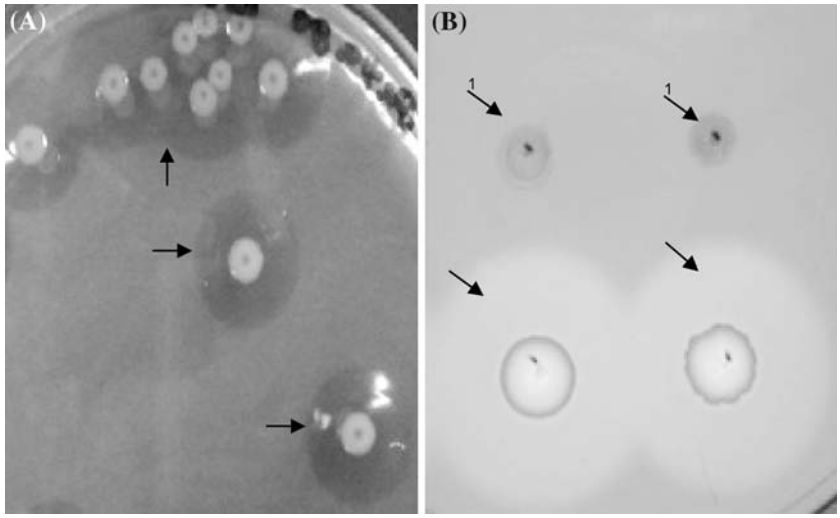

Fig. 1 **a** Isolation of clear-zone-forming bacteria from diseased sea squirt *Halocynthia rorentzi* on modified Zobell's agar. **b** Plate screening of carboxy-methylcellulose (CMC)-hydrolyzing activity using a Congo red assay. *Arrows* indicate agarase (**a**) and cellulase (**b**) positive colonies. *Arrows*^{1} indicate cellulase-negative colonies in the Congo red assay

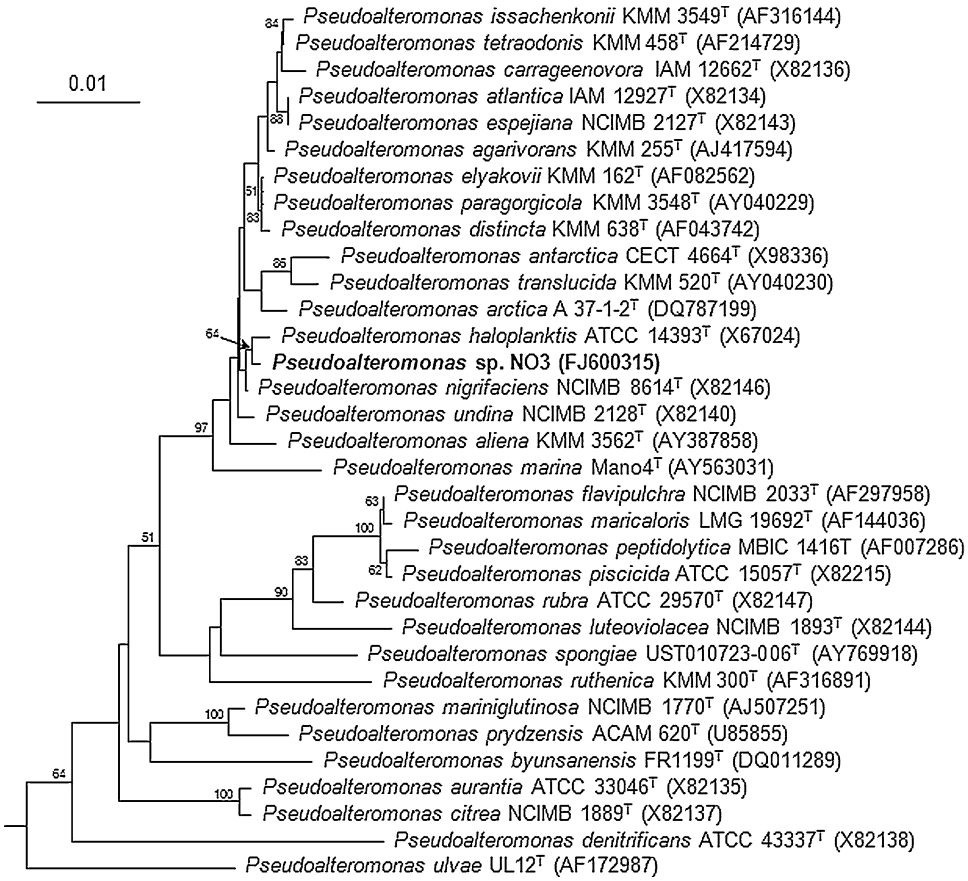

Fig. 2 Neighbor-joining (NJ) tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain NO3 and other species belonging to the genus *Pseudoalteromonas*. *Alteromonas marina* SW-47^{T} (AF529060) was used as an outgroup (not shown). The percentage numbers at the nodes indicate the levels of bootstrap support >50% based on NJ analyses of 1,000 resampled data sets. *Bar* 0.01 nucleotide substitutions per position. *T* type strain

α-cyclodextrin, dextrin, D-galactose, D-galacturonic acid, gentiobiose, D-glucose, L-glutamic acid, glycogen, glycyl L-glutamic acid, inosine, α-ketoglutaric acid, maltose, D-mannitol, methylpyruvate, L-proline, succinic acid, sucrose, Tween 40, Tween 80, uridine, alaninamide, L-alanine, L-alanyl glycine, and L-asparagine. In contrast to strain NO3, *P. haloplanktis* ATCC 14393^{T} is positive for the utilization of myo-inositol, but negative for the utilization of D-lactose, D-melibiose and D-trehalose. The strain is also resistant to the following antibiotics (micrograms per disc): amikacin (30), ampicillin (10), gentamicin (10), kanamycin (30), streptomycin (10), tetracycline (30) and vancomycin (30), but sensitive to chloramphenicol (30), erythromycin (15), and nalidixic acid (30), whereas *P. haloplanktis* ATCC 14393^{T} is sensitive to kanamycin (30), streptomycin (10), ampicillin (10) and tetracycline (30).

Table 1 Differential characteristics between strain NO3 and *Pseudoalteromonas haloplanktis* ATCC 14393[T]. Data from Gauthier et al. [28], Isnansetyo and Kamei [29], Nam et al. [30], Romanenko et al. [31] and this study

Characteristic	Strain NO3	*P. haloplanktis*
Growth at 4°C	+	−
Pigmentation	+	−
Hydrolysis of		
Agar	+	−
DNA	−	+
Starch	−	+
Utilization of		
myo-Inositol	−	+
D-Lactose	+	−
D-Melibiose	+	−
D-Trehalose	+	−
Susceptibility to (μg/disc)		
Kanamycin 30	R	S
Streptomycin 10	R	S
Ampicillin 10	R	S
Tetracycline 30	R	S

+ Positive, − negative, *R* resistant, *S* sensitive

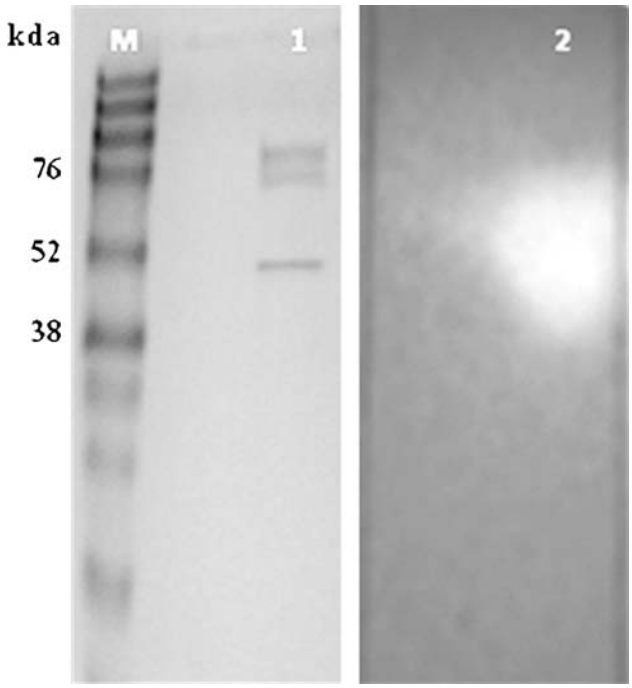

Fig. 3 Nondenaturing SDS-PAGE and zymogram activity staining of cellulase (*celY*-NO3). The *left panel* shows a polyacrylamide gel stained with Coomassie Brilliant Blue R-250 after SDS-PAGE without 2-mercaptoethanol. The *right panel* shows an agarose gel containing CMC after zymogram activity staining. Lanes: *M* Molecular weight marker proteins, *1* cellulase obtained from the HPLC fraction, *2* cellulase on an agarose gel after zymogram activity staining

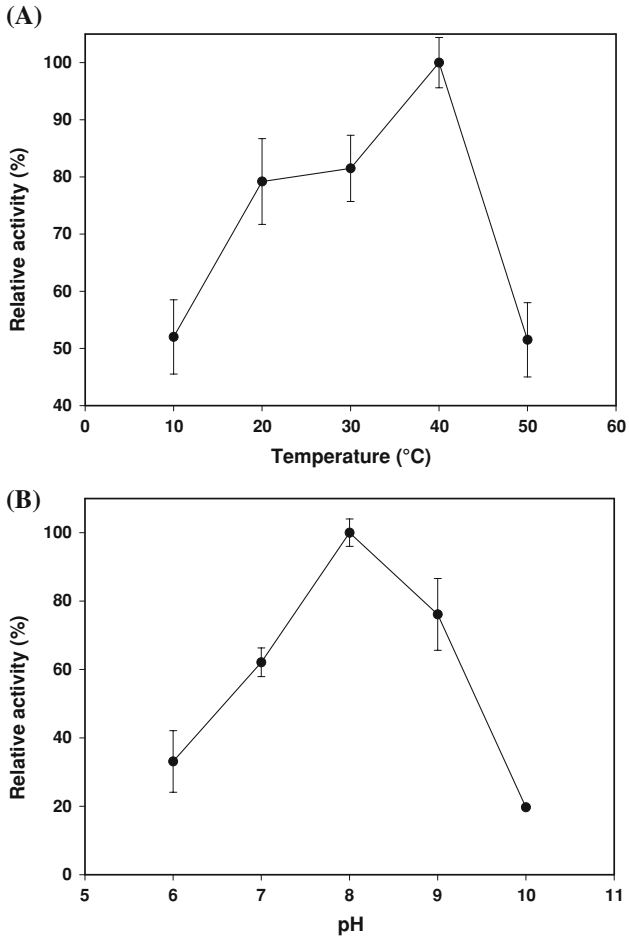

Fig. 4 The effect of temperature (**a**) and pH (**b**) on the cellulase activity of bacterial cell supernatant (above 10 kDa) of *Pseudoalteromonas* sp. NO3 in a reducing sugar assay

Characterization of cellulase CelY

CMCase was detected in cell-free supernatant by a reducing sugar assay. The bacterial cell supernatant was concentrated using an Amicon concentrator, after which the extraneous proteins were fractionated by GFC using a recycling preparative column (GS-310), which enables purification of both the organic gel permeation chromatography and aqueous gel filtration chromatography products [33]. The active fraction with the highest cellulolytic activity produced three bands in 10% SDS-PAGE with estimated molecular masses of approximately 52, 76 and 90 kDa (Fig. 3a). After SDS-PAGE, the proteins in the gel were analyzed by SDS-PAGE under renaturation conditions and then subjected to activity staining. The cellulase activity of *Pseudoalteromonas* sp. NO3 was detected in a clear band with a molecular weight of approximately 52 kDa, which is in accordance with the theoretical molecular mass of 52.7 kDa (Fig. 3b).

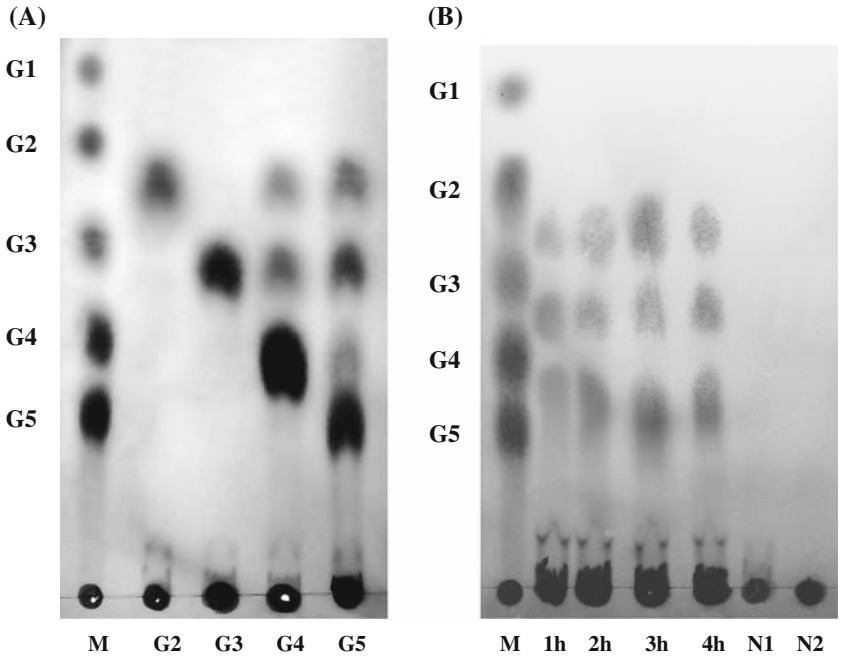

Fig. 5 Thin layer chromatography analysis of degradation products released by CelY. **a** Hydrolysis of cellooligosaccharides (*G2–G5*) after 30 min of incubation at 40°C. **b** Time course degradation of carboxymethyl cellulose (CMC) sampled at the times indicated. *M* Standard mixture of *G1* glucose, *G2* cellobiose, *G3* cellotriose, *G4* cellotetraose and *G5* cellopentaose. *N1* without enzyme and *N2* without CMC are negative controls

The endoglucanase activity of the enzyme was determined by a reducing sugar assay in which a greater number of reducing sugars leads to increased color intensity in response to enzymatic hydrolysis of the glycosidic bonds of the carbohydrates [33]. The reducing sugar assay revealed that the cellulolytic activity was highest at 20–40°C. Furthermore, the results revealed that the activity was greater than 80% throughout that temperature range, with the highest value being observed at 40°C. We also found that the activity of the enzyme at low temperatures (10–20°C) was only 50% that of the activity at 40°C (Fig. 4). Furthermore, the activity decreased quickly at temperatures greater than 40°C. Taken together, these findings are characteristic of a cold-active enzyme. The maximum cellulolytic activity was obtained at pH 8, while 60 and 78% residual activity was detected at pH 7 and pH 9, respectively (Fig. 4).

The end products of the hydrolysis of cellulooligosaccharides (cellobiose, cellotriose, cellotetraose and cellopentaose) and CMC by CelY were determined by TLC after 30 min and 1–4 h of incubation, respectively (Fig. 5). Different product profiles for the cellulase CelY were obtained when cellotetraose, cellopentaose, and CMC were used as the substrates. It is well known that certain β-glucosidases not only have cellulose hydrolysis activity but also transglycosylation activity. Recently interest in the transglycosylation activity of β-glucosidases has gained momentum due to their ability to synthesize novel oligosaccharides linked with the β-1,2, β-1,3, β-1,6 and β-1,4 bonds [34]. When cellooligosaccharides are hydrolyzed by cellulase, the cellooligosaccharides serve as an acceptor for glucosyl units and place on a subsite positioned at the reducing end of the catalytic site in transglycosylation [35]. CelY hydrolyzed G4 to G2 and G3 without the production of G1, whereas the enzyme digested G5 to G2, G3 and G4. However, CelY was not able to cleave G2 and G3. These results suggested that CelY has transglycosylation activity, which allows the enzyme to digest G4 to G1 and then use G1 as a glycosyl donor to synthesize G5 from G4. The enzyme degraded G5 efficiently to cellobiose, cellotriose, and cellotetraose as the main final hydrolysis products. Hydrolysis of CMC yielded oligosaccharides such as cellobiose, cellotriose, and cellotetraose. Both cellulase-catalyzed hydrolysis of CMC and cellooligosaccharides and neo-cellooligosaccharides production occurred via the transglycosylation activity, which indicates that the enzyme may be capable of synthesizing various β-linked cellooligosaccharides.

Amino acid sequencing analysis

CelY contains three domains with distinct structures and functions, including a catalytic domain at the N-terminal end, a C-terminal cellulose-binding domain belonging to GH family 5 (EC 3.2.1.4) of the carbohydrate-binding modules [3], and a longer linker between the two domains than in Cel5Z. The homologies of the deduced amino acid sequence of *celY* (*Pseudoalteromonas* sp. NO3) with those of *celA* (*Pseudoalteromonas* sp. MB-1), *celX* (*Pseudoalteromonas* sp. DY3), *celG* (*P. haloplanktis* ATCC 14393^T) and *cel5Z* (*Erwinia chrysanthemi*) genes were 92.6, 93.3, 92.6, and 59.1%, respectively (Fig. 6). The characteristics of *Pseudoalteromonas* sp. NO3 that distinguish it from other *Pseudoalteromonas* species are its ability to grow at 4°C and its production of extracellular cellulase that has a

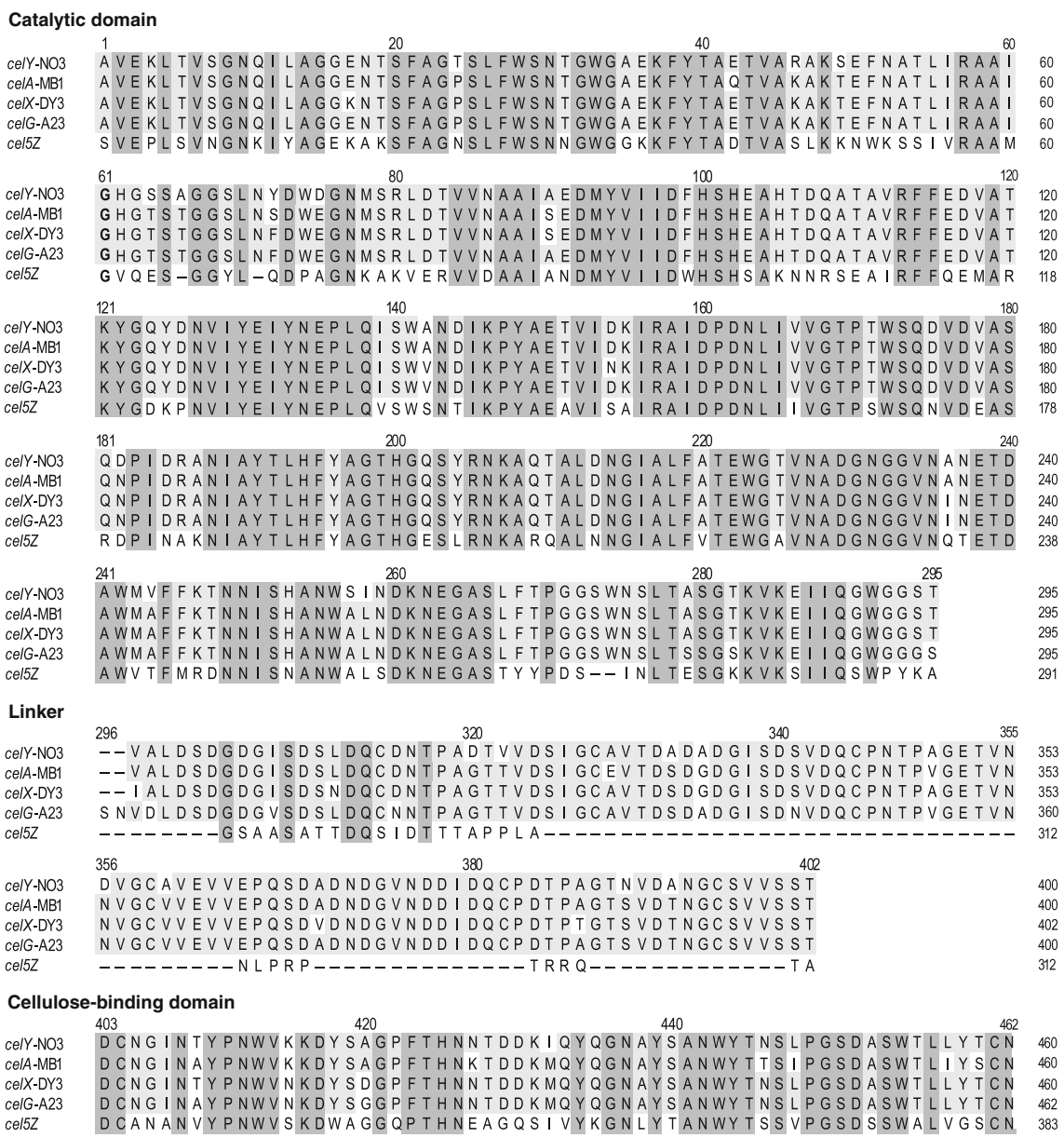

Fig. 6 Amino acid sequence alignment of psychrophilic (*celY*-NO3, *celA*-MB1, *celX*-DY3, *celG*-A23) and mesophilic (*cel5Z*) cellulases. The GenBank accession numbers of *celY*, *celA*, *celX*, *celG* and *cel5Z* genes are FJ648763, AY551322, AY250997, Y17552 and AF208495, respectively

50–80% remnant catalytic activity at temperatures ranging from 10 to 20°C. Conversely, mesophilic cellulase (Cel5Z) produced by *E. chrysanthemi* was found to be totally inactive at this temperature but had a higher thermostability than CelY at temperatures greater than 40°C. When primary structure alignment was conducted, the linker sequences of CelY, CelA and CelX had the same motifs (DxDxDGxxDxxD). It has been suggested that this linker sequence may play an important role in the enzymatic stability of typical psychrophilic cellulases [14].

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References

1. Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583
2. Cho KM, Hong SY, Lee SM, Kim YH, Kahng GG, Kim H, Yun HD (2006) A cel44C-man26A gene of endophytic *Paenibacillus*

polymyxa GS01 has multi-glycosyl hydrolases in two catalytic domains. Appl Microbiol Biotechnol 73:618–630
3. Cantarel BL, Countinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37:D233–D238. doi:10.1093/nar/gkn663
4. Zhang YHP, Himmel ME, Mielenz JR (2006) Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 24:452–481. doi:10.1016/j.biotechadv.2006.03.003
5. Green F, Clausen CA, Highley TL (1989) Adaptation of the Nelson–Somogyi reducing-sugar assay to a microassay using microtiter plates. Anal Biochem 182:197–199. doi:10.1016/0003-2697(89)90578-2
6. Peralta-Yahay P, Carter BT, Lin H, Tao H, Cornish VW (2008) High-throughput selection for cellulase catalysts using chemical complementation. J Am Chem 130:17446–17452. doi:10.1021/ja8055744
7. Iyo AH, Forsberg CW (1999) A cold-active glucanase from the ruminal bacterium *Fibrobacter succinogenes* S85. Appl Environ Microbiol 65:995–998. doi:10.1128/AEM.68.1.430-433.2002
8. Hilden L, Johansson G (2004) Recent developments on cellulases and carbohydrate-binding modules with cellulose affinity. Biotechnol Lett 26:1683–1694
9. Ando S, Ishida H, Kosugi Y, Ishikawa K (2002) Hyperthermostable endoglucanase from *Pyrococcus horikoshii*. Appl Environ Microbiol 68:430–433
10. Gomes J, Steiner W (2004) The biocatalytic potential of extremophiles and extremozymes. Food Technol Biotechnol 42:223–235
11. van Soligen P, Meijer D, van der Kleij W, Barnet C, Bolle R, Power SD, Jones BE (2001) Cloning and expression of an endocellulase gene from a novel *Streptomyces* isolated from an East African soda lake. Extremophiles 5:333–341. doi:10.1007/s007920100198
12. Feller G, Gerday C (1997) Psychrophilic enzymes: molecular basis of cold adaptation. Cell Mol Life Sci 53:830–841
13. Violot S, Haser R, Sonan G, Georlette D, Feller G, Aghajari N (2003) Expression, purification, crystallization and preliminary X-ray crystallographic studies of a psychrophilic cellulase from *Pseudoalteromonas haloplanktis*. Acta Cryst D59:1256–1258. doi:10.1107/S0907444903008849
14. Zeng R, Xiong P, Wen J (2006) Characterization and gene cloning of a cold-active cellulase from a deep-sea psychrotrophic bacterium *Pseudoalteromonas* sp. DY3. Extremophiles 10(79):82. doi:10.1007/s00792-005-0475-y
15. Chun J, Goodfellow M (1995) A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. Int J Syst Bacteriol 45:240–245
16. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
17. Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic, New York, pp 21–132
18. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
19. Swofford DL (1998) Phylogenetic analysis using parsimony (PAUP). Sinauer, Sunderland
20. Chun J, Bae KS, Moon EY, Jung SO, Lee HK, Kim SJ (2000) *Nocardiopsis kunsanensis* sp. nov., a moderately halophilic actinomycete isolated from a saltern. Int J Syst Evol Microbiol 50:1909–1913
21. ZoBell CE (1941) Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. J Mar Res 4:42–75
22. Smibert RM, Krieg NR (1994) General characterization. In: Gebhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington DC, pp 607–654
23. Lyman J, Fleming RH (1940) Composition of sea water. J Mar Res 3:134–146
24. CLSI (2003) Performance standards for antimicrobial disk susceptibility tests. Clinical Laboratory Standards Institute, Wayne
25. Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685. doi:10.1038/227680a0
26. Voget S, Steele HL, Streit WR (2006) Characterization of a metagenome-derived halotolerant cellulase. J Biotechnol 126:26–36. doi:10.1016/j.jbiotec.2006.02.011
27. Azumi K, Nakamura S, Kitamura S, Jung SJ, Kanehira K, Iwata H, Tanabe S, Suzuki S (2007) Accumulation of organotin compounds and marine birnavirus detection in Korean ascidians. Fish Sci 73:263–269
28. Gauthier G, Gauthier M, Christen R (1995) Phylogenetic analysis of the genera *Alteromonas*, *Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. Int J Syst Bacteriol 45:755–761
29. Isnansetyo A, Kamei Y (2003) *Pseudoalteromonas phenolica* sp. nov., a novel marine bacterium that produces phenolic antimethicillin-resistant *Staphylococcus aureus* substances. Int J Syst Evol Microbiol 53:583–588
30. Nam YD, Chang HW, Park JR, Kwon HY, Quan ZX, Park YH, Lee JS, Yoon JH, Bae JW (2007) *Alteromonas marina* sp. nov., a marine bacterium isolated from tidal flats of the Yellow Sea, and reclassification of *Pseudoalteromonas sagamiensis* as *Algicola sagamiensis* comb. nov. Int J Syst Evol Microbiol 57:12–18
31. Romanenko LA, Zhukova NV, Lysenko AM, Mikhailov VV, Stackebrandt E (2003) Assignment of '*Alteromonas marinoglutinosa*' NCIMB 1770 to *Pseudoalteromonas mariniglutinosa* sp. nov., nom. rev., comb. nov. Int J Syst Evol Microbiol 53:1105–1109
32. Chi H, Kim S, Jeong E, Park N, Son J (2007) Isolation and identification of bioactive compounds from soybean and fermented soybean paste chungkukjang. Treat Crop Res 8:918–924
33. Leemhuis H, Euverink GW, Dijkhuizen L (2003) High-throughput screening for gene libraries expressing carbohydrate hydrolase activity. Biotechnol Lett 25:1643–1645
34. Nozaki N, Kano A, Amano Y, Murata T, Usui T, Ito K, Kanda T (2004) Transglycosylation reaction and acceptor specificity of exo- and endo-type cellulase. J Appl Glycosci 51:87–92
35. Kanda T, Noda I, Wakabayashi K, Nishizawa K (1983) Transglycosylation activities of exo- and endo-type cellulases from *Irpex lacteus* (*Polyporus tulipiferae*). J Biochem 93:787–794