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Short communication

Expression patterns of chitinase produced from *Paenibacillus chitinolyticus* with different two culture media

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ABSTRACT

To investigate the expression patterns of chitinase isozymes on native-PAGE and SDS-PAGE gels *Paenibacillus chitinolyticus* MP-306 was cultured on culture media with and without chitin substrate. *P. chitinolyticus* MP-306 had a strong chitinolytic activity on colloidal chitin medium. Chitinase isozymes of MP-306 were expressed as six bands (CN1–CN6) on native-PAGE gels and thirteen bands (CS1–CS13) on SDS-PAGE gels after incubation in chitin medium. Three bands (CN1, CN2, and CN3) of chitinase isozymes of MP-306 on native-PAGE gels were expressed as nine bands (CS1, CS2, CS3, CS4, CS5, CS6, CS8, CS10, and CS13) of chitinase isozymes on SDS-PAGE gels. Three bands (CN4, CN5, and CN6) of chitinase isozymes of MP-306 were strongly inhibited by metal ions on native-PAGE and SDS-PAGE gels.

1. Introduction

Chitin, the partly deacetylated (1-4)-2-acetamido-2-deoxy-β-D-glucan (Muzzarelli et al., 2012), exists in nature as a principal structural polymer in the integument of insects, cicada in particular. We isolated chitinolytic bacteria from the cast-off shell of a cicada. Pathogenesis-related proteins including chitinase, β-1,3-glucanase, and peroxidase are considered critical in biological control against phytopathogens. Chitinases produced from *Paenibacillus* sp. having an antagonistic activity are capable of inhibiting fungi growth because of disorganization of cell walls of *Phytophthora parasitica* and *Fusarium oxysporum* (Budi et al., 2000) and degradation of cell wall of *Rhizoctonia solani* (Jung et al., 2003). Chitinase produced from *Paenibacillus* sp. D1 has been reported to control Fusarium wilt of *Cajanus cajan* in field (Singh, Ghodke, & Chhatpar, 2009), through the catalytic and thermodynamic properties (Singh & Chhatpar, 2011). Also, *Paenibacillus polymyxa* GBR-1 through plant growth promoting, suppresses root-knot nematode (Khan et al., 2008). Recently chitinase was used in several biotechnological applications such as the extraction of antioxidant [*N*,*N*′-diacetylchitobiose, $(GlcNAc)_2$] from marine waste with *Bacillus* sp. (Nawani, Prakash, & Kapadnis, 2010) and production of chitooligomers by *Serratia marcescens* (Aam et al., 2010).

The genus *Paenibacillus* was first suggested based on phylogenetic data from 16S rDNA sequences by Ash, Priest, and Collins (1993). They demonstrated that *Paenibacillus* sp. is facultatively anaerobic or strictly aerobic, and rod-shaped, producing ellipsoidal spores in swollen sporangia. Based on a phylogenetic analysis of 16S rDNA sequences, some researchers suppose that *Bacillus ehimensis* and *Bacillus chitinolyticus* are closer to the genus *Paenibacillus* than the genus *Bacillus* (Lee, Pyun, & Bae, 2004). Chitinase producing bacteria *B. ehimensis* and *B. chitinolyticus* were first reported as new chitinolytic members of the gene *Bacillus* by Kuroshima, Sakane, Takata, and Yokota (1996).

However, the properties of chitinase isozymes produced *Paenibacillus chitinolyticus* have not been extensively studied. We found the chitinase isozymes from *P. chitinolyticus* were produced on gel electrophoresis after incubationin chitin medium. In this study *P. chitinolyticus* was cultured on with different two culture media (chitin medium and LB medium), and then compared with two media for the production of chitinase isozymes. The objectives of this study were to investigate the expression patterns of chitinase isozymes on native-PAGE and SDS-PAGE gel, and to estimate the effects of metal ions on activity of chitinase from *P. chitinolyticus*. To our knowledge, this is the first report to quantify the high levels of chitinase isozymes in *P. chitinolyticus*.

2. Experimental

2.1. Isolation and identification of chitinolytic bacterium

The cast-off shells of cicadas were collected in arboretum of Chonnam National University, Korea. The sample was grained and inoculated on the 0.5% colloidal chitin (pH 6.0) agar medium at

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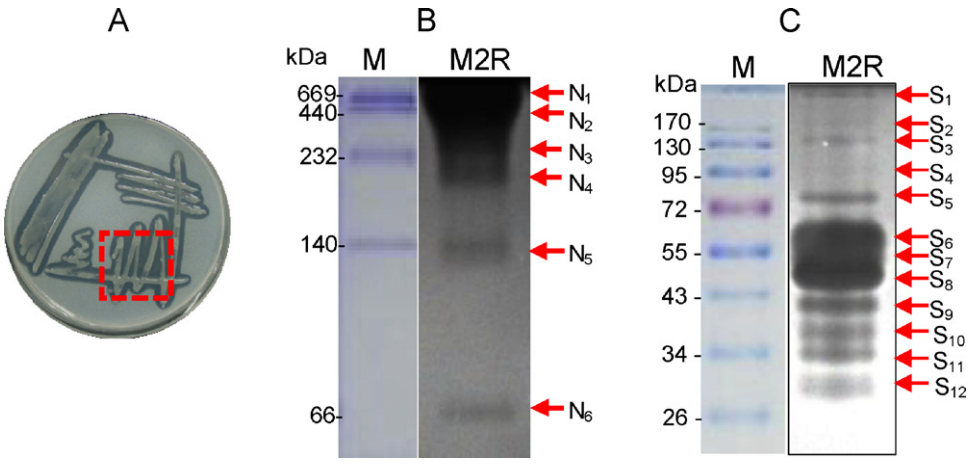

Fig. 1. *Paenibacillus chitinolyticus* MP-306 showed halo formation around colonies on 0.5% colloidal chitin agar at 30 °C after 2 days of incubation (A). Sample fragments (square dotted line) showing halo formation around colonies were collected and dissolved with buffer. Twenty microliters of extracts were loaded on native-PAGE (B) and SDS-PAGE (C) gel.

30 °C for 3 days. One bacterium having strong chitinolytic activity was selected for further characterization.

To identify the bacterium, polymerase chain reaction (PCR), (GeneAmp 9700, Applied Biosystems, USA) was performed to amplify a part of the 16S rRNA gene of the bacterium. Primer used the forward primer 9F (5′-GAG TTT GAT CCT GGC TCA G-3′) and the reverse primer 1412R (5′-ACG GCT ACC TTG TTA CGA CTT-3′). The nucleotide sequence of the 16S rRNA gene of the MP-306 was determined by an ABI PRISM Big Dye™ Terminator Cycle Sequencing Kits (Applied Biosystems, USA) and ABI PRISM 3730xl Analyzer (Applied Biosystems) in Geno Tech Co. (Daejeon, Korea). The nucleotide sequence of the 16S rRNA gene of the MP-306 compared with published 16S rRNA sequences using a blast search at NCBI (Jung et al., 2002).

2.2. Determination of chitinase activity

Chitinase assay mixture consisted of 50 μL of sample, 500 μL of 0.5% colloidal chitin and 450 μL of 50 mM sodium acetate buffer (pH 5.0) (Monreal & Reese, 1969). Following incubation at 37 °C for 1 h, 200 μL of 1 N NaOH was added. Next, the sample was briefly centrifuged (10,000 × *g*, 5 min), after which 500 μL of supernatant was mixed with 1 ml of Schales' reagent and then heated in boiling water for 15 min. The absorbance was then immediately measured at 420 nm using a spectrophotometer (Mecasys, Optizen 3220UV, Korea). The amount of reducing sugar was calculated based on comparison with a standard curve generated from known concentrations of GlcNAc (0–100 μg). One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per hour. The protein concentration was determined using the method described by Bradford (1976).

2.3. Gel electrophoresis and activity staining of chitinase

P. chitinolyticus MP-306 was incubated on 0.5% colloidal chitin and LB medium (without chitin) at 30 °C for 6 days. The two culture media were loaded on native-PAGE and SDS-PAGE gels to investigate the expression patterns of chitinase isozymes, according to the method described by Laemmli (1970).

Electrophoresis with using a Bio-Rad Mini-PROTEAN (80 × 73 × 1.5 mm), the 12% SDS-PAGE gel was stained with 0.12% Coomassie brilliant blue R-250 and silver staining (Blum, Beier, & Gross, 1987). To evaluate the active staining of chitinase, 12% SDS-PAGE containing 0.01% glycol chitin was conducted according to the method described by Trudel and Asselin (1989).

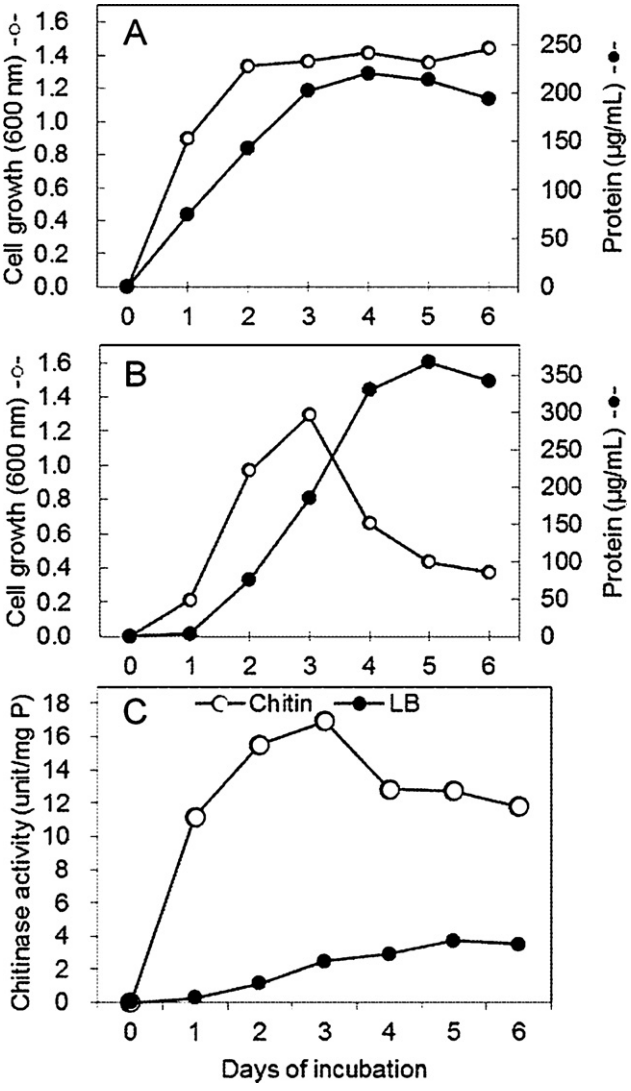

Fig. 2. Time course of cell growth (–○–), protein contents (–●–), and chitinase activity of *P. chitinolyticus* MP-306. The bacterium was grown in LB medium (A) and 0.5% colloidal chitin medium (B) at 30 °C for6 days. Chitinase activity in LB and chitin medium (C).

The gel was incubated in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 and 1% skim milk at 37 °C for 2 h with reciprocal shaking. A subsequent incubation was then conducted overnight under the same conditions, but without skim milk in buffer solution. The gel was then immersed in 500 mM Tris–HCl (pH 8.9) solution containing 0.01% calcofluor white M2R (Sigma F3397). The lysed zones were visualized and photographed using a UV transilluminator (Daihan Sci. Co., WGD-30, Korea). The 10% native-PAGE was conducted according to the method described by Ornstein (1964).

To identify the correct molecular of chitinase isozymes, the band of native-PAGE gel which was loaded from 0.5% colloidal chitin (pH 6.0) agar medium was cut off and separated with 1 × 1 cm gels. Then each fraction gel was extracted with 50 mM sodium acetate buffer (pH 5.0) and loaded on native-PAGE and SDS-PAGE gels. Two-D native/native-PAGE and 2-D native/SDS PAGE were used method modified by Wittig, Braun, and Schägger (2006) using blue-native PAGE technique.

2.4. Effect of metal ions on chitinase activity

To measure the effect of various metal ions on chitinase activity and gel electrophoresis, *P. chitinolyticus* MP-306 was incubated on 0.5% colloidal chitin at 30 °C for 6 days. The enzyme activity of *P. chitinolyticus* MP-306 culture medium was measured under standard conditions in the presence of 10 mM metal ions (Zhao, Jo, Ju, Jung, & Park, 2011). Chitinase activity was analyzed by method of Monreal and Reese (1969). One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per hour.

3. Results and discussion

3.1. Identification of chitinolytic bacterium

The isolate MP-306 had a strong chitinolytic ability on agar medium containing 0.5% (w/v) colloidal chitin, showing clear zone around colonies after 2 days of incubation at 30 °C (Fig. 1A). This bacterium was a gram-positive, rod-shaped. On the basis of the nucleotide sequence of a conserved segment of 16S rRNA gene, the bacterium was identified as *P. chitinolyticus* and named as *P. chitinolyticus* MP-306. Within a 1450 bp sequence, there were only eight base differences between MP-306 (ID JN816404) and *P. chitinolyticus* NBRC 15660 16S rRNA sequences (ID AB680938). Fragments showing clear zone around colonies were collected on colloidal chitin agar medium and then twenty microliter of the extracts sample loaded on native-PAGE (Fig. 1B) and SDS-PAGE gel (Fig. 1C). Several chitinase isozymes having chitinase activity was detected on native-PAGE (N_1, N_2, N_3, N_4, N_5, and N_6) and SDS-PAGE (S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, S_9, S_{10}, S_{11}, and S_{12}) gels containing 0.01% (w/v) glycol chitin as a substrate.

3.2. Cell growth and chitinase activity

Cell growth and protein contents of *P. chitinolyticus* MP-306 were determined in LB medium (Fig. 2A) and 0.5% colloidal chitin

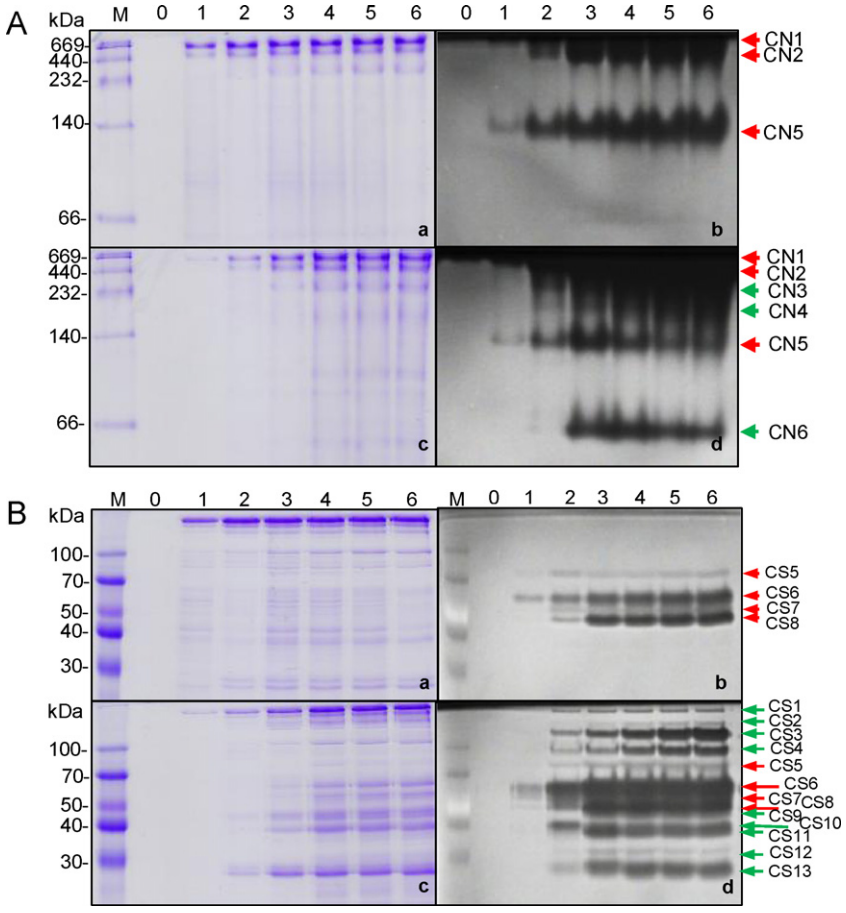

Fig. 3. Changes of chitinase activity of *P. chitinolyticus* MP-306 on native-PAGE (A) and SDS-PAGE (B) gel after Coomassie brilliant blue R-250 staining (CB) and M2R staining (M2R). The bacterium was grown in LB medium and 0.5% colloidal chitin medium at 30 °C for 6 days. Native-PAGE [LB medium; A-a (CB) and A-b (M2R), and chitin medium; A-c (CB) and A-d (M2R)] and SDS-PAGE [LB medium; B-a (CB) and B-b (M2R), and chitin medium; B-c (CB) and B-d (M2R)]. Lane 0 (Day 0), 1 (Day 1), 2 (Day 3), 4 (Day 4), 5 (Day 5), and 6 (Day 6) after incubation. M: marker of proteins.

medium (Fig. 2B). Also, chitinase activity of *P. chitinolyticus* MP-306 was determined in LB medium and 0.5% colloidal chitin (pH 5.0) medium (Fig. 2C). Cell growth increased rapidly for 2 days and then maintained continuously in LB medium. In the same medium, protein content increased for 4 days and then decreased for incubation (Fig. 2A). Cell growth of MP-306 showed gradually increases for 3 days and then rapidly decreased for incubation in 0.5% colloidal chitin liquid medium. In the same medium, protein content increased rapidly for 5 days and then decreased for incubation (Fig. 2B). Chitinase activity increased rapidly with maximum level (16.9 units/mg protein) at 3 days of incubation in colloidal chitin medium. In LB medium, chitinase activity increased slightly for 5 days incubation with maximum level (3.7 units/mg protein) (Fig. 2C). *Paenibacillus* sp. D1 chitinase showed the highest activity in the pH 5.0 at 50 °C (Singh & Chhatpar, 2011).

3.3. Properties of chitinase isozymes

P. chitinolyticus MP-306 was incubated in LB medium and 0.5% colloidal chitin medium for 6 days. Chitinase activity staining of *P. chitinolyticus*MP-306 was detected on native-PAGE and SDS-PAGE gel after M2R staining (Fig. 3). Chitinase isozymes in LB medium showed as CN1, CN2, and CN5 on native-PAGE gel (Fig. 3A-b). Chitinase isozymes in chitin medium showed as CN1, CN2, CN3, CN4, CN5, and CN6 on native-PAGE gel (Fig. 3A-d). Three bands (CN1, CN2, and CN5) of chitinase isozymes showed on native-PAGE gel regardless of LB medium or chitin medium. Chitinase isozymes (CN3, CN4, and CN6) showed only on native-PAGE gel after incubation in chitin medium. Chitinase isozymes in LB medium showed as CS5, CS6, CS7, and CS8 on SDS-PAGE gel (Fig. 3B-b). Chitinase isozymes in chitin medium showed as CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS8, CS9, CS10, CS11, CS12, and CS13 on SDS-PAGE gel (Fig. 3B-d). Six major bands (CS3, CS4, CS6, CS8, CS10, and CS13) of chitinase isozymes showed in SDS-PAGE gel after incubation 0.5% colloidal chitin medium for 6 days. Four bands (CS5, CS6, CS7, and CS8) of chitinase isozymes showed in SDS-PAGE gel regardless of LB medium or chitin medium. *Paenibacillus azotofixans* YUPP-5 showed only one enzyme 70 kDa band in degrading polysaccharides with β-1,4 linkage, chitin by using zymogram (Zhou et al., 2012). Also, *Paenibacillus* sp. D1 showed single chitinase 56.56 kDa band on 10% SDS-PAGE gel by zymogram analysis with 1% ethylene glycol chitin (Singh & Chhatpar, 2011). *Paenibacillus illinoisensis* KJA-424 having an antifungal activity showed three chitinase isozymes (63, 54, and 38 kDa) on SDS-PAGE gel (Jung, Kuk, Kim, Kim, & Park, 2005).

After native-PAGE of enzyme cultured from chitin medium, the piece of gel cut by 5 mm was dissolved and loaded on native-PAGE and SDS-PAGE gel again (Fig. 4). Three chitinase isozymes

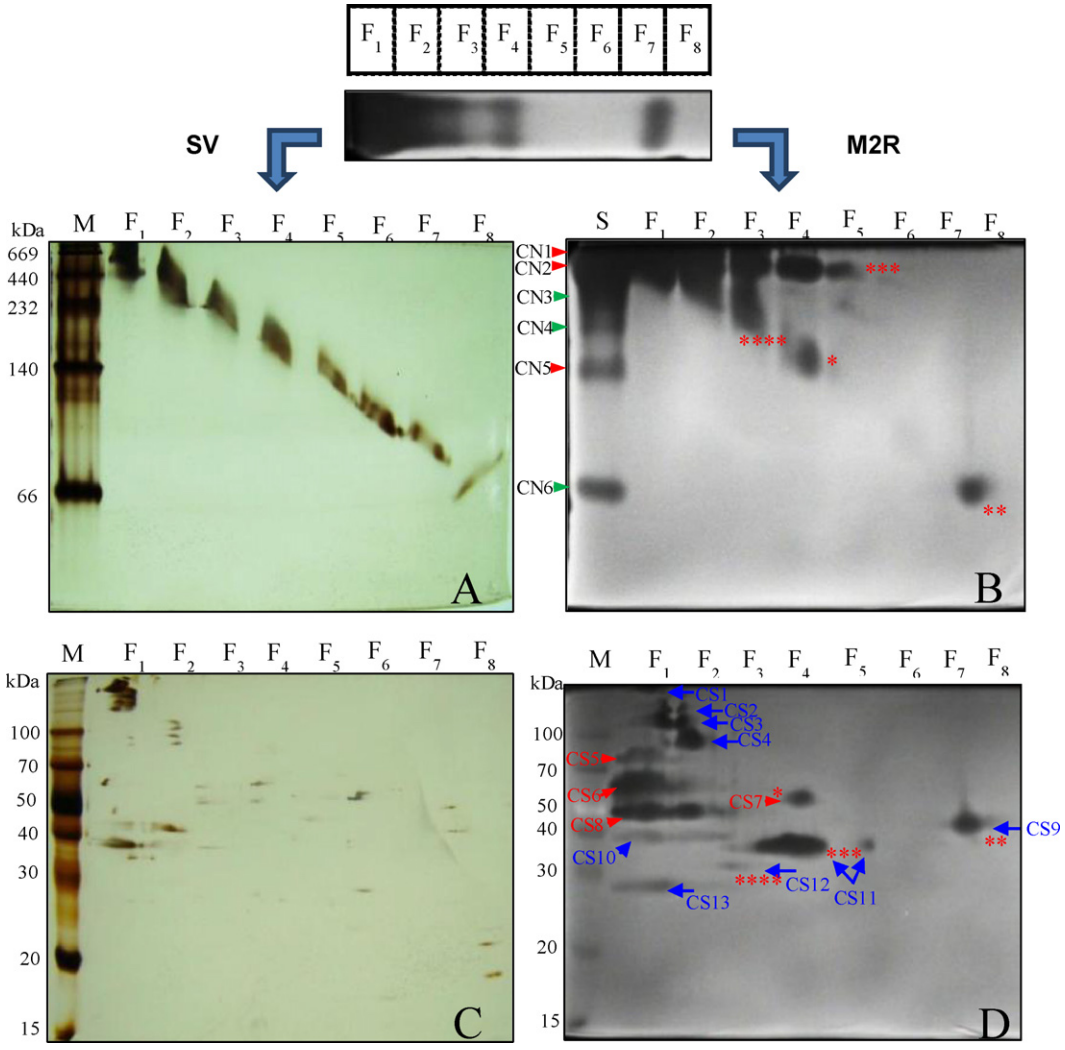

Fig. 4. Chitinase activity of *P. chitinolyticus* MP-306 on 2-D native/native-PAGE gel [A (SV) and B (M2R)] and 2-D native/SDS-PAGE gel [C (CV) and D (M2R)]. Fractions (F1–F8) of native-PAGE gel loaded on native-PAGE and SDS-PAGE gel. Silver staining (SV) and M2R staining (M2R). S: sample of native-PAGE gel.

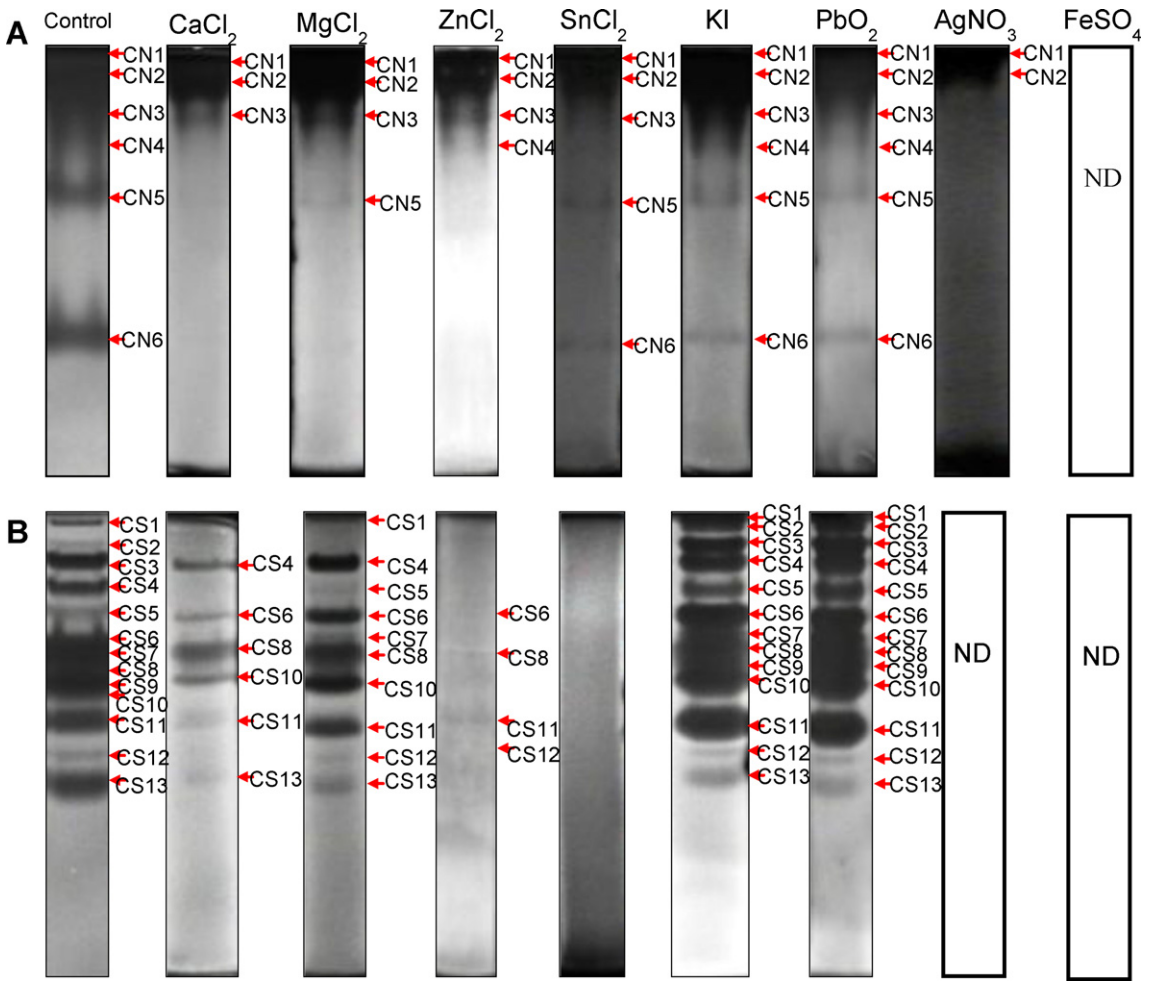

Fig. 5. Chitinase activity of *P. chitinolyticus* MP-306 with different metal ions on native-PAGE (A) and SDS-PAGE gels (B). Not detected (ND).

(CN1, CN2, and CN3) on native-PAGE gel were distributed as nine chitinase isozymes (CS1, CS2, CS3, CS4, CS5, CS6, CS8, CS10, and CS13) on SDS-PAGE gel (Fig. 4B and D). Chitinase (CN5*) on native-PAGE gel corresponded with chitinase (CS7*) 53 kDa on SDS-PAGE gel. Chitinase (CN6**) of about 66 kDa on native-PAGE gel corresponded with chitinase (CS9**) 45 kDa on SDS-PAGE gel. Chitinase (Lane F_4 and F_5***) on native-PAGE gel corresponded with chitinase (CS11***) 37 kDa on SDS-PAGE gel. Chitinase (CN4 in lane F_3****) on native-PAGE gel corresponded with chitinase (CS12****) 32 kDa on SDS-PAGE gel. From these results, we found that expression patterns of chitinase isozymes on native-PAGE gels were distinctly tracked on SDS-PAGE gels with different chitinase isozymes.

Inhibition of metal ions from enzyme of *P. chitinolyticus*MP-306 showed in Table 1 and Fig. 5. Chitinase activity of *P. chitinolyticus* MP-306 was relatively low value at $FeSO_4$ and PbO_2 (Table 1).

Table 1
Effects of metal ions on activity of chitinase from *P. chitinolyticus* MP-306.

Metal ions	Chitinase activity (unit/mg protein)	Relative activity (%)
CH_3COONa	93.4	100
$CaCl_2$	84.6	90.6
$MgCl_2$	84.3	90.3
$ZnCl_2$	ND[a]	ND
$SnCl_2$	ND	ND
KI	93.9	100.5
PbO_2	47.2	50.5
$AgNO_3$	ND	ND
$FeSO_4$	61.1	65.4

[a] ND: not detected.

Chitinase isozymes (CN4, CN5, and CN6) on native-PAGE gels were strongly inhibited by metal ions (Fig. 5A) and that isozymes were not clearly expressed on SDS-PAGE gels (Fig. 5B). Chitinase isozyme CN6 on native-PAGE gels has effect on expression of chitinase isozyme CS9 on SDS-PAGE gels. This result coincides with protein molecular in Fig. 4. Also, chitinase isozymes (CN4 and CN5) on native-PAGE gels have effect on expression of chitinase isozymes (CS12 and CS7) on SDS-PAGE gels.

4. Conclusions

We investigated the chitinase isozymes properties of *P. chitinolyticus*MP-306 on native-PAGE and SDS-PAGE gels. Chitinase activity was higher level in colloidal chitin medium than LB medium. Three novel chitinase isozymes (CN3, CN4, and CN6) were expressed on native-PAGE gel after incubation in chitin medium. To date, there are no reports of *Paenibacillus* sp. that produce approximately 13 chitinase isozymes on SDS-PAGE gel. Specially, six major chitinase isozymes (CS3, CS4, CS6, CS8, CS10, and CS13) were expressed on SDS-PAGE gels after incubation in colloidal chitin medium. Activity of chitinase isozymes indicated various response with different metal ions on SDS-PAGE gels. We found that expression patterns of chitinase isozymes on native-PAGE gels did not coincide constantly with chitinase isozymes on SDS-PAGE gels. In the future, specific characterizations of the chitinase isozymes of *P. chitinolyticus* MP-306 could clarify questions on enzyme purification and proteomic works.

Acknowledgement

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