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Short Communication

Heterologous Expression of *chi42* Gene from *Trichoderma asperellum* in *Bacillus subtilis*

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Abstract

Chitinase is the enzyme that hydrolyzes chitin, a major component of fungal cell walls. This enzyme has the potential to be applied against certain phytopathogenic fungi for fruit preservation. Therefore, this study aimed to produce the extracellular 42 kDa chitinase of *T. asperellum* SH16 in *B. subtilis* BD170 (rCHI42) and evaluate preliminary its antifungal activity as the basis for further applications. The results showed that the chitinase activity of rCHI42 peaked at 27 U/mL after 8 h of *Bacillus* induction with 4 m*M* IPTG. The investigation revealed that rCHI42 had the optimum pH and temperature of 7 and 45°C, the pH and thermal stability were in the range of 6–8 and 25–35°C, respectively. Some metal ions (Fe^{2+}, Al^{3+}, Ca^{2+}, and Mn^{2+}) increased the relative activity of rCHI42 from 109 to 148%, while the enzyme was inhibited by most of the tested reagents (SDS, EDTA, urea, Triton X-100, and DMSO). rCHI42 also exhibited antifungal ability against phytopathogenic fungus *Aspergillus niger* which contains chitin in its cell wall.

Keywords: 42 kDa chitinase; *Bacillus subtilis*; *chi42*, heterologous expression; *Trichoderma asperellum*

Introduction

Chitinase (EC 3.2.1.14), whose molecular weight ranges from 20 kDa to about 90 kDa, is an enzyme group that hydrolyzes glycosidic linkages in chitin molecule to form oligosaccharides which will be further degraded by β-N-acetylhexosaminidase (EC 3.2.1.52) to N-acetylglucosamine (GlcNAc) (Hamid *et al*. 2013). Chitin is a primary component of cell walls in fungi, the exoskeletons of arthropods and the radula of mollusks (Jones *et al*. 2020). Chitinases were found in fungi, yeasts, actinomycetes, bacteria, plants, arthropods and mammals (Kumar *et al*. 2018). Many have been characterized, mostly from plants and bacteria and in minor proportion from fungi because of the important applications of chitinases in the fields of agriculture, pharmacy, food industry and pollution abatement (Nagpure *et al*. 2014; Rathore and Gupta 2015).

Most chitinase genes were cloned and heterologously expressed in *E. coli* hosts such as *Chi58* from *Sanguibacter* spp. C4 (Tao *et al*. 2006), endochitinase gene from *B. cereus* (Chen *et al*. 2009), *ChiA* from *B. licheniformis* DSM8785 (Songsiriritthigul *et al*. 2010), *Ifu-chit2* from *Isaria fumosorosea* (Meng *et al*. 2015), *ChiKJ406136* from *Streptomyces sampsonii* (Li *et al*. 2018) and *Chi* from *Paenibacillus chitinolyticus* UMBR 0002 (Liu *et al.* 2020). However, some other microorganisms have also been used as suitable hosts for this enzyme, *e.g.*, *B. thuringiensis* for *chiA* gene of *Serratia marcescens* (Okay *et al.* 2008), yeast *Saccharomyces cerevisiae* for chitinase gene of *Thermomyces lanuginosus* (Prasad and Palanivelu 2012), yeast *Pichia pastoris* for *ScCTS1* gene of *S. cerevisiae* (Youxi *et al.* 2015).

Although *B. subtilis* has become an increasingly popular host for recombinant protein expression due to its ability to directly secrete protein into culture media, it is amenable to medium- and large-scale fermentation, lack of codon bias and designation as a safe organism (FDA 2018). To date, only a few studies produced foreign chitinase in *B. subtilis* such as the chitinase derived from *B. pumilus* (Ahmadian *et al*. 2012) and chitinase A from *S. marcescens* (Okay and Alshehri 2020). This study reports on the expression of the 42 kDa chitinase (GH family 18) from *T. asperellum* SH16 in *B. subtilis* BD170 and its characterizations and antifungal activity.

Materials and Methods

Cloning *chi42* gene

The *chi42* gene (NCBI: HM191683) was isolated from genomic DNA of *T. asperellum* SH16 by PCR amplification with specific primers as described in our previous report (Loc *et al.* 2011) containing two overhangs of *Xma*I and *Bam*HI and then cloned in pGEM-T Easy vector (Promega). The recombinant cloning vector was cut at *Xma*I and *Bam*HI sites to insert the *chi42* gene into the same site downstream of the P*grac* promoter and the SP amyQ signal peptide of pHT43 *Bacillus* expression vector (MoBiTec). Finally, the pHT43/*chi42* vector was transformed into *B. subtilis* BD170 cells by the chemical method according to Vojcic *et al.* (2012). Restriction digestion and PCR amplification were performed to determine the presence of the *chi42* gene in transformed cells.

Expression of *chi42* gene

BD170 cells containing vector pHT43/*chi42* were grown in Luria-Bertani (LB) broth at a shaking speed of 180 rpm at 37°C overnight. After dilution to an OD_{600} of 0.15 using LB broth, the culture was continued until OD_{600} reaching a value of 0.7 to 0.8. Four millimoles of isopropyl *β*-d-1-thiogalactopyranoside (IPTG) were then added to the culture and the mixture was incubated at 37°C for 2–10 h to induce *chi42* expression. The supernatant from induction culture containing the recombinant 42 kDa chitinase (rCHI42) was harvested every 2 h. The crude enzyme from the supernatant was partially purified by precipitation of neutral salt to use for further studies. The precipitation was carried out at 4°C for 2 h in stirring conditions using 70% saturation ammonium sulfate. Centrifugation at 15,000 rpm at 4°C for 10 min was performed to recover the pellet which was then re-suspended in 0.1 *M* sodium acetate buffer (pH 5) and dialyzed with 12 kDa molecular weight cut-off membrane overnight against the same buffer. The expression of the *chi42* gene was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue R-250.

Zymography of rCHI42 was carried out according to Berini *et al*. (2017) with a slight modification using 12% (*w/v*) polyacrylamide gels containing 0.7 mg/mL of carboxymethyl-chitin-remazol brilliant violet. The mixture of enzyme and loading buffer was incubated at room temperature (RT) for 10 min. Electrophoresis was then performed at 4°C in the Tris-glycine-SDS buffer. The gel was washed twice in 2.5% (*v/v*) Triton X-100 for 30 min at RT to remove SDS and incubated in 50 m*M* acetate buffer (pH 6) at 37°C until the clear zones of the chitinolytic activity were observed.

Assay for chitinase activity

The agar plate method with 1.2% (*w/v*) colloidal chitin as the substrate was used for the assay of the chitinolytic activity of rCHI42. 10 U enzyme was loaded into pre-punched holes in agar plate and kept at 28°C for 6 h, then stained with 0.1% Lugol solution to detect substrate hydrolysis. Chitinase activity of rCHI42 was determined by the measurement of the absorbance of the hydrolyzed product at 420 nm with *p*NP-GlcNAc as a substrate (Tsujibo *et al.* 1998). 70 μL rCHI42 was added to 140 μL of substrate solution at a concentration of 2.5 m*M* (pH 6) and left at 50°C for 10 min. The reaction was then stopped with 0.2 M sodium carbonate. The chitinase activity of rCHI42 is defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol from the substrate within one minute. The *p*-nitrophenol standard was purchased from Sigma-Aldrich.

Characterization of rCHI42

The optimum temperature and pH of rCHI42 were investigated in the ranges of 30–70°C and 4–10, respectively. Buffers such as 20 m*M* citrate solution (pH 4–6), 20 m*M* phosphate solution (pH 7–8) and 20 m*M* glycine-sodium hydroxide solution (pH 9–10) were used to find the optimum pH. The enzyme was incubated at 25–70°C and pH 4–10 without substrate for 30 min to determine their thermal and pH stability.

5 m*M* of metal ions (Na^+, Al^{3+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+} and Fe^{3+}) or different concentrations of reagents (1 M urea, 1% sodium dodecyl sulfate (SDS), 1 m*M* ethylenediaminetetraacetic acid (EDTA), 5% dimethyl sulfoxide (DMSO), and 1% Triton X-100) was added to rCHI42 solution and kept at 35°C and pH 7 for 30 min to evaluate their effect on the enzyme activity. The relative activity (%) of rCHI42 is the percentage ratio of the enzyme activity with treatment and without treatment (control).

Antifungal activity of rCHI42

The antifungal activity of rCHI42 was preliminarily tested using fungus *A. niger*, a type of black mold that causes disease in plants. A test based on inhibition of mycelial growth of *A. niger* was carried out to determine *in vitro* antifungal activity of rCHI42. 60 U/mL rCHI42 and 10 μL of fungal spore suspension (about 10^6 spores/mL) were added to a Petri dish containing 1/2 potato dextrose agar medium and incubated at 28°C for 48 h to evaluate the inhibitory effect of the enzyme.

Healthy mango fruits were washed under running water, followed by treatment with 70% ethanol, finally washed again with sterile deionized water. Each mango fruit was sprayed with 1 mL rCHI42 (60 U), then allowed to dry naturally and artificially inoculated with about 10^4 fungal spores/lesion, 6 lesions/fruit. After treatment, mangoes were left in boxes and kept at RT to track the disease progression.

Statistics

All treatments were repeated three times. Data on the activity of rCHI42 were expressed as the means ± SEs, followed by an analysis of variance with Duncan's test (*P* at 0.05 level).

Results

Expression of *chi42* gene

The *chi42* gene from *T. asperellum* SH16 was isolated by PCR amplification, then inserted into the pHT43 vector and finally transformed into BD170 cells. The presence of the pHT43/*chi42* vector was determined by digestion of *Bam*HI and PCR amplification to produce a linearized DNA fragment of about 9.5 kb (pHT43 vector of 8 kb long and *chi42* gene of 1.5 kb long) and a PCR product of about 1.5 kb (*chi42* gene), respectively, as expected. A DNA band of uncut pHT43/*chi42* vector as control located at a lower site of about 7 kb on the agarose gel compared to that digested by *Bam*HI (Fig. 1).

Expression of rCHI42 was determined by SDS-PAGE. A protein band predicted as rCHI42 with estimated molecular weight of approximately 42 kDa (mature protein) was found on the gel (Fig. 2A). A signal peptide of about 4 kDa of the full-length chitinase molecule of ~46 kDa, corresponding to *chi42* gen of about 1.5 kb, could have been cleaved from the rCHI42 after this enzyme was secreted outside the cell (Carsolio *et al*. 1994). The zymogram also showed a clear zone on the gel that has the same size as the target protein band in SDS-PAGE (Fig. 2B). Chitinase activity of partially purified rCHI42 from bacterial culture peaked at about 27 U/mL after 8 h of induction with 4 m*M* IPTG (Fig. 2C). However, in another observation, chitinase activity from parental *B. subtilis* BD170 used as control was not found.

Five transformed BD170 cell colonies (clones) selected to test the chitinolytic activity of their rCHI42 on colloidal chitin-containing agar plates. The largest *D-d* of about 1.5 cm was found in the C-1 clone, while the hydrolysis was not present in the control (Fig. 2D). In which, *D* and *d* are the diameters of the hydrolysis zone and the pre-punched hole, respectively.

Characterization of rCHI42

Fig. 3A and B show that the relative activity of rCHI42 peaked at 143% (~38 U/mL) at the optimum temperature and pH of 45°C and 7, respectively. rCHI42 has thermal and pH stability in the range of 25–35°C and 6–8 with the relative activity being from 83–86% and 90–93%, respectively.

In the tested metal ions, Fe^{2+}, Al^{3}+, Ca^{2+} and Mn^{2+} increased chitinolytic activity of rCHI42, among them Mn^{2+} had the highest effect, the relative activity of the enzyme reached 148%. While other ions such as Fe^{3+}, Zn^{2+}, Co^{2+}, Mg^{2+}, Cu^{2+} and Na^{+} were the opposite, of which Zn^{2+} had the strongest inhibitory effect, the enzyme has only 25% relative activity. The chitinolytic activity of rCHI42 was inhibited by most reagents such as SDS, Triton X-100, EDTA, urea and DMSO. The relative activity of the enzyme reached the lowest value of approximately 11% when was treated with 1% SDS (Fig. 3C).

Antifungal activity assay

The study showed that rCHI42 at a concentration of 60 U/mL inhibited *in vitro* growth of fungus (Fig. 4A and 4B).

According to Krishnapillaim and Wijeratnam (2013), *A. niger* fungus has caused significant economic damage to mangoes in some regions in Sri Lanka and India. Our result also revealed that rCHI42 could inhibit the growth of *A. niger* in mango fruits. The fungus still did not appear on the mango after 96 h of enzyme treatment, while they grew quite strongly in the control (Fig. 4C and 4D).

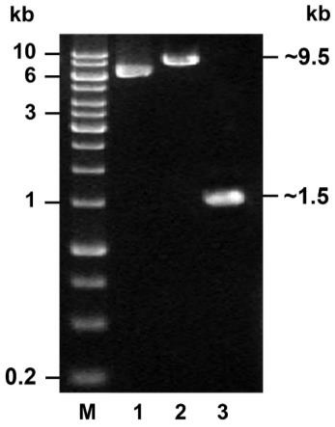

Fig. 1: Identification of pHT43/*chi42* vector in transformed *B. subtilis* BD170 by *Bam*HI digestion and PCR amplification. 1: uncut pHT43/*chi42* vector as control, 2: pHT43/*chi42* vector was linearized by *Bam*HI, 3: PCR amplification of *chi42* gene. M: DNA size marker (Thermo Scientific™ O'GeneRuler 1 kb DNA Ladder)

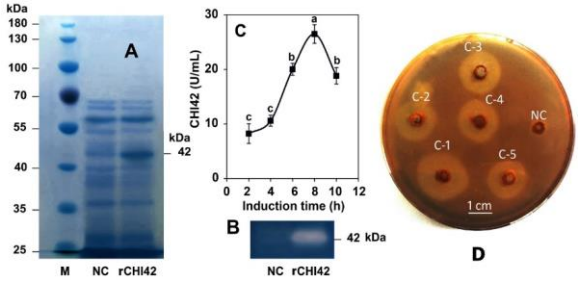

Fig. 2: (**A**) Expression analysis of rCHI42 in *B. subtilis* BD170 by SDS-PAGE, NC: parental *B. subtilis* BD170 as control, rCHI42: transformed *B. subtilis* BD170, M: protein weight marker (PageRuler™ Prestained Protein Ladder, Thermo Fisher Scientific). (**B**) Zymogram of rCHI42. (**C**) A profile of chitinase activity in transformed *B. subtilis* BD170 after different induction times with 4 m*M* IPTG, different letters represent statistically significant differences based on Duncan's test ($P < 0.05$). (**D**) Chitinolytic activity of rCHI42 from various transformed BD170 cell clones (C-1 to C-5) on the agar plate containing colloidal chitin, NC: parental BD170 as control

Discussion

To date, only a few microbial chitinase genes have been successfully expressed on *Bacillus* hosts such as the *chiA* gene from *S. marcescens* (Okay *et al.* 2008; Okay and Alshehri 2020) or the chitinase gene from *B. pumilus*

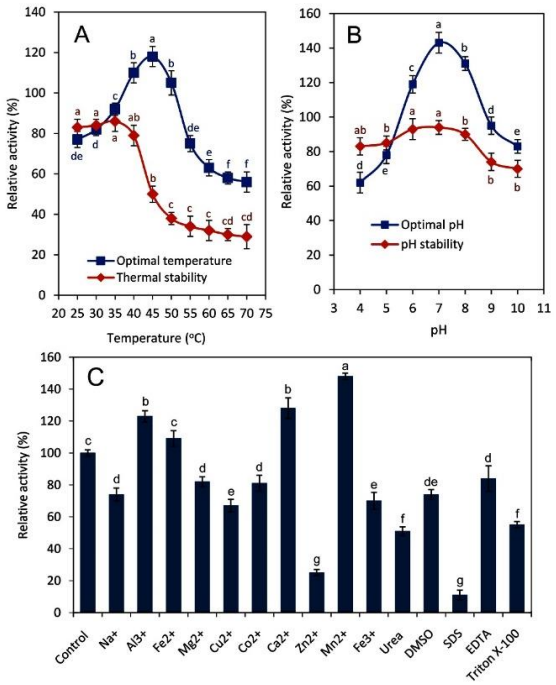

Fig. 3: Characterization of rCHI42 from transformed BD170 cells (C-1 clone). **A**, **B** and **C**: effect of temperature, pH, and metal ions and reagents on enzyme activity. Different letters on a curve or columns represent statistically significant differences based on Duncan's test ($P < 0.05$)

Fig. 4: Effect of rCHI42 on *in vitro* growth of *A. niger* - (**A**): medium without chitinase as control and (**B**): medium containing 60 U/mL of rCHI42. Effect of rCHI42 pre-treatment on mango after 96 h of *A. niger* infection - (**C**): control without rCHI42, and (**D**): fruit with 60 U/mL rCHI42

(Ahmadian *et al.* 2012; Rostami *et al.* 2017). And most of them are promising sources for agricultural and biotechnological applications.

Several studies found different optimal temperatures of chitinase from *Trichoderma* species. The optimum temperature of *T. viride* chitinase was 50°C (Ekundayo *et al.* 2016) while Rao *et al.* (2016) found that the optimal temperature of chitinase from *Trichoderma* isolates was 30°C. CHIT42 chitinase from *T. harzianum* displayed maximum activity at 35°C when expressed in yeast *Pichia pastoris* (Kidibule *et al.* 2018). Kapat and Panda (1997) showed that chitinase from *T. harzianum* has an optimum temperature of 24°C with thermal stability in the range of 50–60°C. *T. asperellum* UTP-16 revealed the highest chitinase activity at 35°C (Kumar *et al.* 2012).

Generally, *Trichoderma* sp. can grow in a wide range of pH, however, the optimum range was reported to be between 4.6 and 6.8 (Singh *et al.* 2014). Chitinase of *T. viride* had the highest activity at pH 5 (Ekundayo *et al.* 2016). A study by Kapat and Panda (1997) showed that optimal pH of chitinase from *T. harzianum* was 5.4 whereas the strain *T. asperellum* UTP-16 has the optimal pH at 6 (Kumar *et al.* 2012).

The inhibitory effect of metal ions such as Fe^{3+}, Zn^{2+} and Mg^{2+} at a concentration of 5 m*M* was observed for chitinase of *T. viride* (Omumasaba *et al.* 2001). However, unlike rCHI42 derived from *T. asperellum* SH16 in this study, *T. viride* chitinase was inhibited by Ca^{2+} and Mn^{2+}. Another study showed that the chitinase of *T. viride* was reduced in activity when was treated with Zn^{2+}, Mn^{2+} and EDTA whereas Ca^{2+} maximized the enzyme activity (Ekundayo *et al.* 2016). However, the concentration to which the ions were used was not mentioned in this study.

Filamentous fungi *Trichoderma* spp. were considered as biocontrol agents against plant pathogenic fungi because they can produce chitinolytic enzymes. A study by Harighi *et al.* (2007) indicated that chitinase 42 from *Trichoderma atroviride* PTCC5220 inhibited the growth of *Rhizoctonia solani*, a plant pathogenic fungus. Mazrou *et al.* (2020) found the relationship between antagonistic activity of six *Trichoderma harzianum* strains against some plant pathogenic fungi such as *Colletotrichum gossypii*, *Fusarium oxysporum*, *Fusarium fujikuroi*, *R. solani*, *Aspergillus calidoustus* and *Alternaria brassicicola* and their chitinolytic enzyme production.

To date, many studies have reported the antifungal activity of *Trichoderma* spp. (Loc *et al.* 2020). However, it is difficult to find relevant studies in *T. asperellum*, especially their recombinant chitinase, except for the previous reports by Cruz-quiroz *et al.* (2018), Loc *et al.* (2020), Tien *et al.* (2021), Luong *et al.* (2021).

Conclusion

In conclusion, *chi42* gene coding 42 kDa chitinase of *T. asperellum* SH16 was successfully expressed in *B. subtilis* BD170. 42 kDa chitinase is a neutral enzyme that was highly active at 45°C and inhibited the growth of *A. niger*.

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Author Contributions

NH Tue: literature search, data collection, data analysis and interpretation. TTM Uyen, HA Thi, NH Minh, TGC Tuong, NTM Thu and ND Chung: data collection and data analysis. NH Loc: design of the work, performing the analysis, drafting the article, critical revision. All authors final approval of the version to be published.

Conflicts of Interest

All authors declare no conflicts of interest

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper.

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