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Expression of Enhanced Thermostability 42 kDa Chitinase from *Trichoderma asperellum* SH16 in *Escherichia coli*

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ABSTRACT
The 42 kDa chitinase of *T. asperellum* SH16 has been regarded with considerable interest in promising biotechnological applications due to its antifungal activities. However, poor thermostability limits most practical applications. This study investigated the expression of various 42 kDa chitinase mutants in *E. coli*. Findings indicate that the recombinant chitinase I168L mutant demonstrated markedly improved thermostability compared to the native enzyme, retaining substantial activity even at elevated temperatures. Additionally, both *in vitro* and *in vivo* antifungal tests showed that the I168L mutant exhibited a greater inhibitory effect on the growth of *Colletotrichum siamense* strains, which are responsible for anthracnose in chili fruits, when compared to the native chitinase at 35°C. This mutant can prevent fungal infection for up to four days following pretreatment with 80 units of I168L. These findings highlight the potential of the I168L mutant as a biocontrol agent against anthracnose diseases caused by *Colletotrichum* species in fruits.

KEYWORDS
42 kDa chitinase; *E. coli*; antifungal; thermostability; *Trichoderma asperellum*

Introduction

Chitinases (EC 3.2.1.14), a family of glycoside hydrolases, hydrolyze β-1,4-glycosidic bonds in chitin, producing oligosaccharides such as chitobiose and N-acetylglucosamine (Chen, Jiang, and Yang 2020; Dahiya, Tewari, and Hoondal 2006). Chitin is the second most abundant biopolymer after cellulose, serving as a primary component of fungal cell walls, the exoskeletons of insects, and the pharyngeal lumen of nematodes (Moussian 2019; Steinfeld et al. 2019; Vahed et al. 2013).

Due to their ability to degrade chitin, chitinases have been widely studied for their roles in digestion, molting in arthropods, defense mechanisms, and pathogenesis (Bhattacharya, Nagpure, and Gupta 2007; Hamid et al. 2013; Merzendorfer and Zimoch 2003). These enzymes are synthesized by diverse organisms, including bacteria, yeasts, fungi, nematodes, arthropods, and

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vertebrates. Importantly, the catalytic properties of chitinases underpin their extensive potential applications across agriculture, food industries, medicine, and environmental management (Bhattacharya, Nagpure, and Gupta 2007; Hamid et al. 2013). In agriculture, chitinase has been recognized as a promising biocontrol agent to replace harmful chemical solutions for post-harvest preservation (Langner and Göhre 2016; Veliz, Martínez-Hidalgo, and Hirsch 2017).

Our prior studies have successfully identified the 42 kDa chitinase encoded by the *Chi42* gene from the *T. asperellum* SH16 strain (Loc et al. 2011) and expressed this enzyme in various host expression systems, including *Saccharomyces cerevisiae* (Loc et al. 2013), *E. coli* (Luong et al. 2021), *Nicotiana benthamiana* (Tien et al. 2021, 2024), and *Arachis hypogaea* (Hoa et al. 2023). The recombinant 42 kDa chitinases exhibited promising antifungal activities against plant pathogens, highlighting their biocontrol potential.

However, a major challenge in the practical application of native chitinase is its thermostability, particularly in postharvest preservation. This is because fruits and vegetables are often stored and transported under fluctuating environmental temperatures, which can lead to rapid denaturation and loss of enzyme activity. For a biocontrol agent to be effective over the prolonged storage periods required in postharvest management, it must maintain its structural integrity and catalytic efficiency under a wide range of thermal conditions. Enhancing thermostability thus ensures sustained antifungal efficacy, reduces the need for repeated applications, and ultimately contributes to the economic viability and widespread adoption of chitinase as a sustainable alternative to chemical fungicides. Protein engineering techniques using computer-assisted design have been widely employed to enhance the thermostability and catalytic efficiency of chitinases (Emruzi et al. 2018; Tien et al. 2024; Xu et al. 2020). The computer-assisted design, which combines structural and sequence information to introduce targeted mutations, has emerged as a particularly effective approach for improving enzyme performance (Sumbalova et al. 2018; Xiong et al. 2021; Xu et al. 2020). In our recent study, this approach was employed to enhance the thermostability of the 42 kDa chitinase heterologously produced in *N. benthamiana*. The result indicated the chitinase I168L mutant exhibited significantly higher antifungal activity against *Colletotrichum siamense* CH26.1, the causative agent of chili anthracnose, at 35°C compared to the native enzyme (Tien et al. 2024).

Among expression systems, *E. coli* is widely used due to its rapid growth, ease of genetic manipulation, and cost-effectiveness (Baeshen et al. 2015; Chen 2012). The current study focuses on the potential use of thermostable chitinases for protecting post-harvest vegetables and fruits, utilizing *E. coli* as an efficient system for recombinant enzyme production. Advanced protein engineering techniques, particularly semi-rational design, have been employed to improve the thermostability of chitinase and to optimize their expression in

E. coli, opening broader application prospects for this promising enzyme in agriculture. The antifungal efficacy of engineered chitinase against postharvest pathogens was also evaluated in this study.

Materials and methods

Gene and fungus

Six site-directed point mutations (I168L, Q171E, G229N, Y175Q, N223P, and T248S) (Table 1) encoding various 42 kDa chitinase mutants were engineered *in silico* from the *Chi42* gene (HM191683.1) of *T. asperellum* SH16 by Tien et al. (2024). The *s-Chi42–2* gene (also known as the *syncodChi42–2* gene, MT083803.1), encoding the native 42 kDa chitinase, is a codon-optimized version of the *Chi42* gene (Luong et al. 2021). Two fungal strains, *Colletotrichum siamense* AD1.2 isolated from avocado (Thanh et al. 2024) Q4 and *C. siamense* CH26.1 isolated from chili (Tien et al. 2025), were used for *in vitro* and *in vivo* antifungal assays.

In brief, 3D homology models of native chitinase from *T. asperellum* SH16 were constructed using AlphaFold-2.0, Phyre2, and I-TASSER, based on the crystal structure of 42 kDa chitinase (PDB: 6EPB) from *Trichoderma harzianum*. The quality of these models was evaluated by RMSD analysis and further assessed using tools such as ERRAT, VERIFY 3D, and PROCHECK. The best model was then used as input for HotSpot Wizard 3.1 to predict potential thermostability-related mutation sites. The resulting 20 sites were further analyzed by mCSM, DynaMut2, DDMut, Strum, and DUET. Among these, six mutation sites consistently exhibiting the lowest predicted ΔΔG values across all tools were selected as the most promising candidates for experimental validation of improved thermostability (Tien et al. 2024).

Cloning and expression of mutant chitinases

Six mutant chitinase genes and the *s-Chi42–2* gene were cloned into the pQE30 expression vector (Qiagen, Germany) using the *Bam*HI and *Sac*I recognition sites. The wild-type *Chi42* gene was used as a control. Transformed *E. coli* M15 cells harboring recombinant pQE30 vectors were cultured in Luria-Bertani (LB) medium supplemented with 50 µg/mL ampicillin and 50 µg/mL

Table 1. Mutants in 42 kDa chitinase from *T. asperellum* SH16.

Mutants	Mutation site	Amino acid replacement
I168L	168	Isoleucine (I) is replaced by Leucine (L)
Q171E	171	Glutamine (Q) → Glutamic acid (E)
Y175Q	175	Tyrosine (Y) → Glutamine (Q)
N223P	223	Asparagine (N) → Proline (P)
G229N	229	Glycine (G) → Asparagine (N)
T248S	248	Threonine (T) → Serine (S)

kanamycin overnight at 37°C with a shaking speed of 190 rpm. Five milliliters of overnight culture was transferred to 50 mL of fresh LB medium and grown under the same conditions until an OD_{600} value of 0.6 was reached. The culture was then added with 0.5 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) and incubated at 20°C for 6 h for chitinase expression. Cell biomass was harvested, flash-frozen in liquid nitrogen, and lysed using grinding. The lysate was resuspended in phosphate-buffered saline (PBS) buffer (pH 7), and insoluble debris was removed by centrifugation at 13,000 rpm for 15 min at 4°C. To recover proteins from inclusion bodies, the insoluble pellet was solubilized with 8 M urea. Total protein concentration in the soluble fraction was partially purified by acetone precipitation (Tien et al. 2021) and quantified using the Bradford assay (Bradford 1976) with bovine serum albumin used as a standard.

PCR amplification was performed to identify mutant chitinase genes with specificprimers(forward:5'-TCTAGAAAAACTAAAAGTAGAAGAAAAAT GAAGAACAC-3,'reverse:5-GAGCTCTTAATCAAACCAGATCTGATATT ATC-3'). The composition of the PCR mixture consisted of 1 ng of total DNA, 1 pmol of each primer, 10 mM dNTPs, 0.3 µL of ProFi Taq DNA Polymerase (Bioneer), 5 µL of 10x RXN buffer, and nuclease-free water to a final volume of 50 µL. PCR conditions were set as follows: initial denaturation at 98°C for 30 sec; followed by 35 cycles of 98°C for 10 sec, 58°C for 30 sec and 72°C for 1 min; and a final extension at 72°C for 10 min.

SDS-PAGE and western blot

Fifty micrograms of total soluble protein (TSP) were boiled for 2 min before being loaded onto a 12% polyacrylamide gel for sodium dodecyl sulfate electrophoresis (SDS-PAGE). The separation was performed at 60 V for the first 30 min, followed by an increase to 80 V for 100 min. After electrophoresis, one of the double gels was stained with bromophenol blue for visualization, while the second gel was transferred to a Hybond-C extra nitrocellulose membrane (Amersham Biosciences) for Western blot analysis. The blot was then incubated in 5% skim milk dissolved in TBST buffer (20 mM Tris.HCl pH 7.5, 150 mM NaCl, and 1% Tween 20) for 2 h at room temperature (RT) to block nonspecific bindings. The blot was exposed to the primary antibody (mouse polyclonal anti-Ta-CHI42 antibody, produced in-lab by our team) (Luong et al. 2021) for 2 h at RT with gentle shaking. After three times washed in TBST buffer, the blot was incubated with the secondary antibody (goat anti-mouse antibody conjugated to alkaline phosphatase, AbDserotec, USA) under the same conditions. Following another series of washes with TBST buffer and TMN (100 mM Tris pH 9.5, 5 mM $MgCl_2$, and 100 mM NaCl), the blot was color developed in 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) solution

(Millipore Sigma, Cat No. B6404) for 3 min in the dark to visualize the antigen-antibody complex.

Chitinase activity assay

Chitinase activity was determined spectrophotometrically at 420 nm using ρ-nitrophenyl N-acetyl-β-D-glucosaminide (ρNP-β-GlcNAc) (Sigma-Aldrich, Germany) as a substrate (Tsujibo et al. 1998). Fifty micrograms of TSP was added to 15 μL of 2.5 mM ρNP-β-GlcNAc, and the hydrolysis reaction was carried out at 45°C for 10 min and then stopped by adding 1 mL of 0.2 M sodium carbonate. A 10-minute linear timeframe, with data points taken at 0.2-minute intervals starting from 0 minutes, was established to ensure accurate and reliable measurements. One unit of chitinase was defined as the amount of enzyme required to release 1 μmol of ρ-nitrophenol from ρNP-β-GlcNAc in 1 min. ρ-nitrophenol (Sigma-Aldrich, Germany) was used to build a calibration curve.

Optimal temperature and thermostability

The optimum temperature of the mutant chitinases was determined by measuring enzyme activity over a temperature range of 25°C to 70°C, with 5°C increments. Thermostability was assessed by incubating the enzyme in pH 7 buffer at 40°C for 7 h, 50°C and 60°C for 2 h, in the absence of substrate. After each treatment, the enzyme was rapidly cooled to 4°C to prevent any further thermal degradation. Chitinase activity was then measured at the optimum temperature and pH 7, using the same assay method described previously. A boiled enzyme solution was used as a negative control to account for nonspecific activities.

In vitro antifungal activity of mutant chitinase

The antifungal activity of mutant chitinases was determined based on their ability to inhibit the growth of *C. siamense* AD1.2 mycelium. 6-cm Petri dishes containing 1/2 potato dextrose agar (PDA) medium were supplemented with 60 units of the enzyme and 10 μL of a fungal conidial suspension (10^6 conidia/mL). The Petri dishes were incubated at 28°C for 72 h to observe fungal growth. The extent of mycelial growth was monitored to determine the antifungal efficacy of the enzyme. A PDA dish without chitinase was used as a negative control. A PDA dish containing wild type chitinase from *T. asperellum* SH16 (produced in *E. coli*) served as a positive control. Native chitinase (s-Chi42–2) was used for comparison of its antifungal efficacy to six mutant chitinases.

Antifungal ability of mutant chitinases on chili fruits

Healthy chili fruits of similar size were collected and washed with running tap water. The samples were then surface sterilized with 2% sodium hypochlorite and rinsed again three times with sterile distilled water. To assess the *in vivo* antifungal activity, mutant chitinases were used to treat fungal causing chili anthracnose following two methods.

Fungal prevention: Mutant enzymes of different concentrations (20–80 units, increment of 20 units) were sprayed on the chili fruits, then let them dry naturally. The chili fruits were inoculated artificially with *C. siamense* CH26.1 using wound methods by applying conidial suspension (10^5 conidia/mL) to the wounds. Subsequently, the fruits were incubated at 35°C in plastic boxes with moistened paper towels for 7 days. The infected fruits without enzyme treatment incubated at 28°C and 35°C were used as controls. The appearance of anthracnose was observed, and the lesion diameter was measured each day with a vernier caliper.

Fungal treatment: Chili samples were infected with *C. siamense* CH26.1 using the same method described above. After the anthracnose symptoms appeared on the fruits (2–3 days), recombinant chitinase from 20 to 80 units (increment of 20 units) was used to treat the infected fruits. To evaluate the disease progression level, the lesion diameter of each wound on the infected fruits was measured daily after 4 days of incubation at 35°C in boxes. Similarly, the controls were fruits without chitinase at 28°C and 35°C.

Statistical analysis

Experimental data were collected from at least three replicates and analyzed using the SPSS program (v. 20.0). Results were expressed as mean ± standard error. Analysis of variance (ANOVA) was used to evaluate the significant difference among means, applying Duncan's test at a significance level of $p < 0.05$.

Results

Expression of mutant chitinases in E. coli

Six chitinase mutants (I168L, Q171E, G229N, Y175Q, N223P, and T248S) and the *s-Chi42–2* gene were successfully cloned into the pQE30 expression vector and transformed into *E. coli* M15 cells. To confirm their integrity, two randomly selected colonies from each construct were verified by PCR amplification (Fig. S1). Subsequently, the transformed *E. coli* cells were induced with IPTG for chitinase expression. SDS-PAGE and Western blot analyses confirmed the overexpression of all seven chitinase-encoding genes in *E. coli* M15 cells (Figure 1AB). The recombinant chitinases have

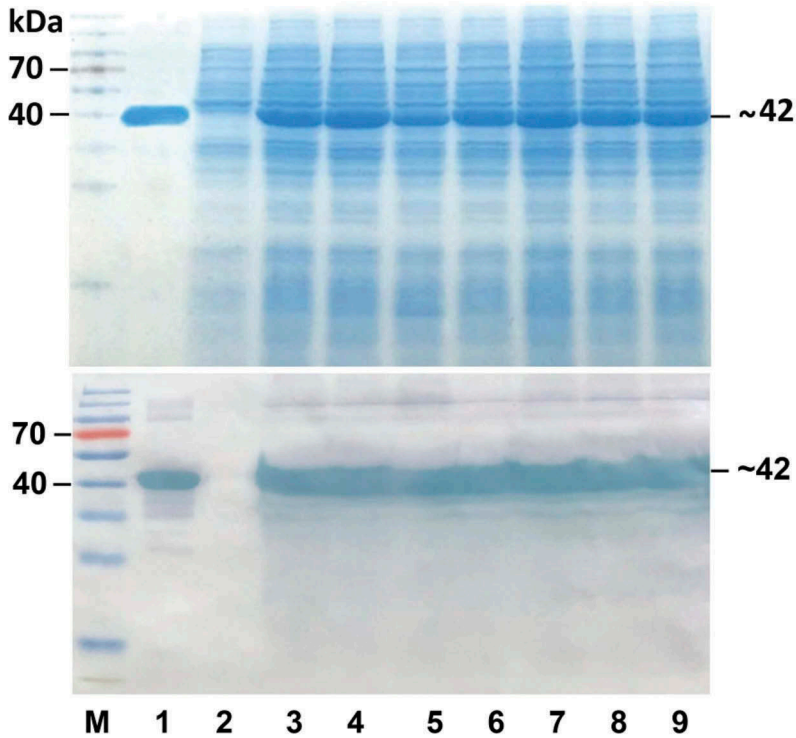

Figure 1. Expression of chitinases in transformed *E. coli* M15 cells. (A). SDS-PAGE of TSP, (B). Western blot. M: PageRuler pre-stained protein ladder (thermo fisher scientific), 1: wild-type *Chi42* as a positive control, 2: *E. coli* cells without IPTG induction as a negative control, 3–9: *E. coli* transformed with *s-Chi42–2* and six mutant *Chi42* genes (I168L, Q171E, G229N, N223P, Y175Q, and T248S) and induced with IPTG.

a molecular weight of about 42 kDa as expected. The expression level of native chitinase and six mutant chitinases in *E. coli* seems to have no significant difference (Figure 1B). The chitinolytic activity of these enzymes was also determined. The control *s-Chi42–2* and the four mutants I168L, T248S, Y175Q, and Q171N exhibited the highest activity from 76.66 units/mL to 79.03 units/mL with no statistically significant difference ($p > 0.05$). The mutants N223P and G229N showed the lowest activity of 58.71 units/mL and 62.73 units/mL, respectively (Figure 2). The linearity of chitinase activity over the 10-minute time frame ensured that the measurement was reliable (data not shown).

Optimal temperature and thermostability of mutant chitinases

The effect of temperature on the chitinase activity of six mutant chitinases was investigated at a range of 25°C to 70°C. Figure 3A revealed that only 2 mutants, Y175Q and I168L, have the optimal temperature at 50°C, higher than that of native chitinase (Luong et al. 2021) and other mutants (45°C). The chitinolytic

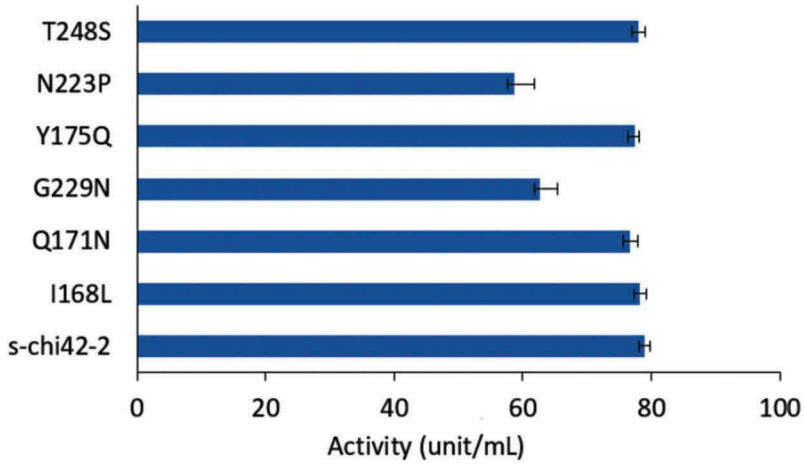

Figure 2. The activity of native chitinase (s-Chi42–2) and mutant chitinases (T248S, N223P, Y175Q, G229N, Q171N, and I168L) produced in *E. coli*.

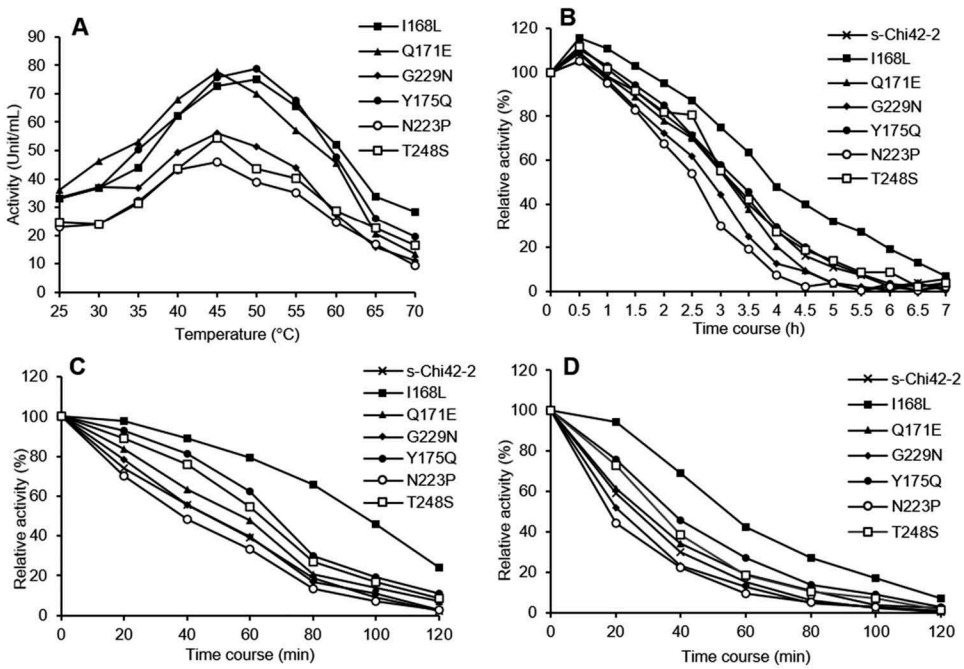

Figure 3. Effect of temperatures on the activity of native chitinase (s-Chi42–2) and mutant chitinases (T248S, N223P, Y175Q, G229N, Q171N, and I168L) produced in *E. coli*. (A) the optimal temperature of chitinases. (B-D) the thermostability of chitinases at 40°C, 50°C and 60°C. s-Chi42–2: native chitinase as the control.

activity of these 2 mutants was also the highest, with 78.63 units/mL for Y175Q and 74.98 units/mL for I168L.

The thermostability of partially purified chitinases was determined by incubating enzymes at 40°C for 7 h and at 50°C and 60°C for 120 min, with the findings illustrated in Figures 3BC and D. Notably, the mutant I168L exhibited the highest thermostability at all investigated temperatures. This enzyme exhibited considerable stability at 40°C, retaining approximately 75% of its relative activity after 3 h of incubation, surpassing the native chitinase, which maintained only 55.4%. The other five mutants displayed reduced thermostability, with relative activities ranging from 44% to 57% for G229N, Q171E, T248S, and Y175Q, while N223P showed a significantly lower relative activity of about 29% (Figure 3B). Furthermore, the I168L mutant preserved a high relative activity of around 79% and 42% at 50°C and 60°C, respectively, after 60 minutes of exposure. In contrast, the native chitinase exhibited relative activities of approximately 38% and 15% under the same conditions. The remaining mutants did not show any notable enhancement in thermostability at 50°C and 60°C, with their relative activities being comparable to or even lower than that of the native chitinase (Figure 3C and D).

In vitro *antifungal activity of mutant chitinases*

The antifungal activity of mutant chitinases was evaluated in vitro against *C. siamense* AD1.2, the pathogen responsible for anthracnose in avocados in Vietnam. In general, all mutant chitinases demonstrated the ability to inhibit the mycelial growth of this fungus compared to the control (Figure 4). These findings were consistent with the results obtained from Western blot and

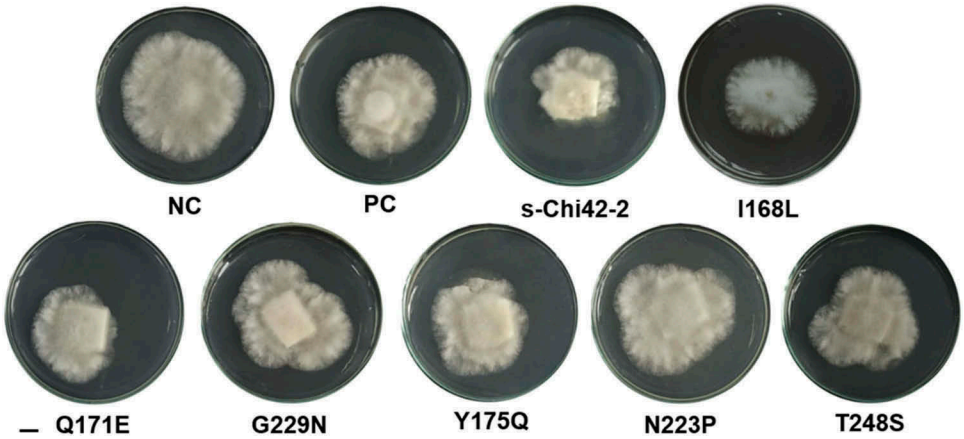

Figure 4. *In vitro* antifungal activity of native chitinase (s-Chi42–2) and mutant chitinases (T248S, N223P, Y175Q, G229N, Q171N, and I168L) produced in *E. coli* against *C. siamense* AD1.2 after 72 h of incubation at 28°C. NC: without chitinase, PC: wild-type chitinase from *T. asperellum* SH16 produced in *E. coli*, s-Chi42–2: native chitinase as the control. Scale bar 1 cm.

chitinolytic activity assessments of mutant enzymes. Among the six chitinase mutants examined, I168L and Q171E demonstrated the highest efficacy in inhibiting the growth of *C. siamense* AD1.2 hyphae. After 72 h of incubation at 28°C, the colony diameter of *C. siamense* AD1.2 when treated with these mutants reached approximately 4.5 cm, comparable to that observed with the native chitinase. In contrast, the fungus *C. siamense* AD1.2 exhibited a colony diameter of around 7 cm when grown on a medium without enzyme treatment (NC) over the same period. The three other mutants (Y175Q, G229N, and T248S) displayed slightly reduced resistance to *C. siamense AD1.2*, with colony diameters ranging from 5.1 to 5.5 cm, like that of the wild-type chitinase (PC). The mutant N223P exhibited the least fungistatic effect, with a colony diameter reaching 6.1 cm (Figure 4). Considering the chitinolytic activity, thermostability, and *in vitro* antifungal properties, the I168L mutant was selected for further investigation into its potential to prevent or treat anthracnose in chili plants.

In vivo antifungal activity of mutant chitinases

Anthracnose prevention: *C. siamense* CH26.1, which induces anthracnose disease in chili fruits, was utilized for artificial inoculation to evaluate the antifungal activity of mutant chitinases produced in *E. coli*. This fungus demonstrated growth across a temperature ranging from 25°C to 35°C, with 28°C identified as the optimal temperature for mycelial development (unpublished data). To assess the thermostability of the mutant I168L, the antifungal efficacy of this enzyme was tested at 35°C, representing the typical summer temperature in Vietnam, alongside 28°C as a control.

Data presented in Table 2 and Figure 5A indicate that the mutant I168L effectively inhibited the onset of anthracnose in chili fruits at a temperature of 35°C. An increase in enzyme concentration corresponded with enhanced antifungal activity against *C. siamense* CH26.1. Specifically, signs of fungal infection emerged three days post-inoculation when utilizing 20–60 units of I168L. The anthracnose lesions varied in size, measuring between 0.48 and 1.25 cm in diameter after 7 days. The treatment with 80 units of I168L exhibited the most significant antifungal effect, with lesions appearing on day 5 and measuring only 0.28 cm in diameter by day 7. In contrast, symptoms were observed earlier in chili treated with native chitinase, with lesions developing after 2, 3, and 4 days of inoculation at enzyme concentrations of 20, 40–60, and 80 units, respectively; this resulted in faster fungal growth and lesion diameters ranging from 0.7 to 1.63 cm. Generally, the lesion diameter resulting from I168L treatment was significantly smaller ($p < 0.05$) compared to that observed with the native chitinase. The control groups exhibited a more severe progression of the disease compared to the treated groups, with lesion diameters of 2.4 cm

Table 2. Prevention effect of chitinase on Chili anthracnose disease caused by *C. siamense* CH26.1.

Chitinase (units)		Lesion diameter (cm) by day						
		1	2	3	4	5	6	7
I168L	20	–	–	$0.18 \pm 0.03^{a,AB}$	$0.45 \pm 0.05^{b,CD}$	$0.75 \pm 0.05^{c,BC}$	$0.98 \pm 0.03^{d,DE}$	$1.25 \pm 0.1^{e,E}$
	40	–	–	$0.1 \pm 0.1^{a,AB}$	$0.23 \pm 0.08^{ab,AB}$	$0.33 \pm 0.08^{b,A}$	$0.58 \pm 0.03^{c,BC}$	$0.8 \pm 0.05^{d,C}$
	60	–	–	$0.08 \pm 0.08^{a,AB}$	$0.15 \pm 0.05^{ab,A}$	$0.3 \pm 0.1^{b,A}$	$0.4 \pm 0.05^{b,AB}$	$0.48 \pm 0.08^{b,AB}$
	80	–	–	–	–	$0.1 \pm 0.05^{ab,A}$	$0.2 \pm 0.05^{b,A}$	$0.28 \pm 0.08^{c,A}$
s-Chi42–2	20	–	$0.1 \pm 0.05^{a,A}$	$0.25 \pm 0.05^{b,B}$	$0.48 \pm 0.03^{c,D}$	$0.83 \pm 0.5^{d,BC}$	$1.2 \pm 0.08^{e,EF}$	$1.63 \pm 0.13^{f,F}$
	40	–	–	$0.18 \pm 0.03^{a,AB}$	$0.33 \pm 0.03^{a,BC}$	$0.63 \pm 0.08^{b,B}$	$0.8 \pm 0.1^{c,CD}$	$1.15 \pm 0.05^{d,DE}$
	60	–	–	$0.08 \pm 0.03^{ab,AB}$	$0.2 \pm 0.00^{bc,AB}$	$0.3 \pm 0.05^{c,A}$	$0.6 \pm 0.1^{d,BC}$	$0.85 \pm 0.05^{e,CD}$
	80	–	–	–	$0.18 \pm 0.03^{a,A}$	$0.28 \pm 0.5^{a,A}$	$0.45 \pm 0.1^{b,AB}$	$0.7 \pm 0.13^{c,BC}$
C1	–	–	$0.1 \pm 0.05^{a,A}$	$0.25 \pm 0.05^{a,B}$	$0.78 \pm 0.08^{b,E}$	$1.18 \pm 0.13^{c,D}$	$1.83 \pm 0.1^{d,G}$	$2.4 \pm 0.2^{e,H}$
C2	–	–	$0.25 \pm 0.03^{a,B}$	$0.28 \pm 0.08^{a,B}$	$0.53 \pm 0.03^{b,D}$	$0.93 \pm 0.1^{c,C}$	$1.45 \pm 0.15^{d,F}$	$2.05 \pm 0.13^{e,G}$

Q9 C1 (control 1): incubation at 28°C without chitinase, C2 (control 2): incubation at 35°C without chitinase.
Data are expressed as the mean of three replicates ± standard error.
Different uppercase letters in a column represent a statistically significant difference (Duncan's test, $p < 0.05$).
Different lowercase letter row represent a statistically significant difference (Duncan's test, $p < 0.05$).

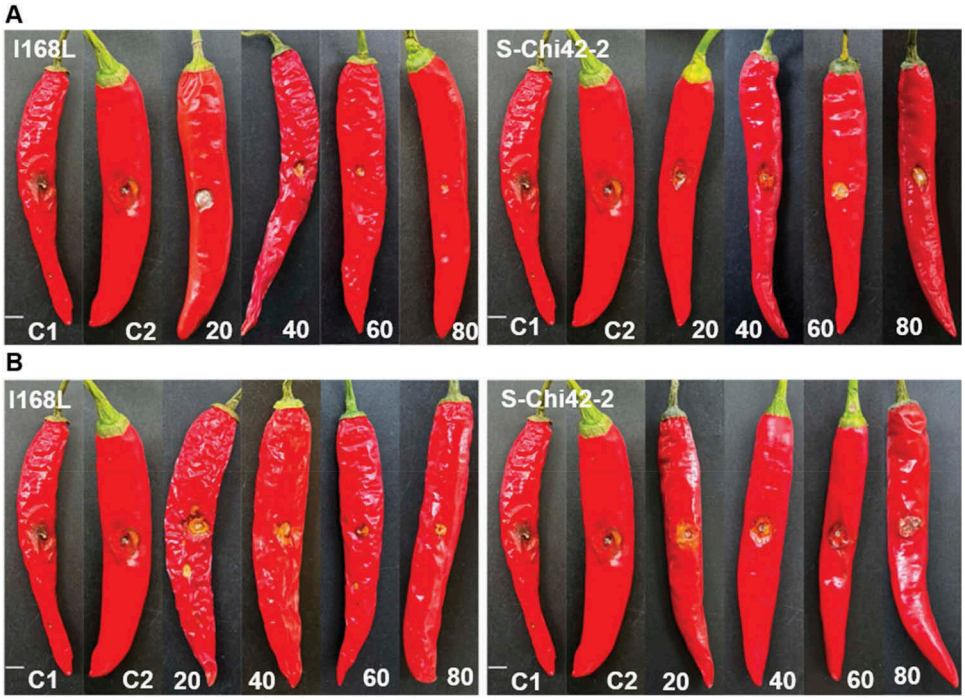

Figure 5. *In vivo* antifungal activity of mutant chitinase (I168L) and native chitinase (s-Chi42–2) against *C. siamense* CH26.1 that causes Chili anthracnose. A. Fungal prevention after 7 days of inoculation at 35°C. B. Fungal treatment after 4 days of incubation when treated with chitinase at 35°C. C1: without chitinase and incubated at 28°C. C2: without chitinase and incubated at 35°C. 20–80: mutant I168L and native chitinase at 20, 40, 60, 80 units, respectively. Scale bar of 1 cm.

and 2.05 cm at 28°C and 35°C, respectively, after 7 days of incubation. These findings suggest that the mutant I168L at a concentration of 80 units can effectively prevent anthracnose caused by *C. siamense* on chili fruits for up to 4 days at 35°C.

Anthracnose treatment: In the anthracnose treatment assay, chitinases in varying amounts (20–80 units) were applied to lesions on fruits 2–3 days after disease symptoms emerged, aiming to inhibit the growth of *C. siamense* CH26.1. Consistent with the fungal prevention assay, the mutant I168L exhibited a more potent antifungal effect at 35°C compared to the native chitinase. The effectiveness of the chitinases in suppressing fungal growth was directly proportional to the quantity of enzyme applied. The highest efficacy was observed with 80 units of I168L, resulting in lesion diameters of approximately 0.35 cm after 4 days, whereas lesions treated with the same amount of native chitinase measured about 1.08 cm, three times larger ($p < 0.05$). Concentrations of 20–60 units provided partial inhibition of mycelial growth, with less pronounced effects; lesion diameters recorded after 4 days

ranged from 0.65–1.47 cm for I168L and 1.15–1.78 cm for native chitinase ($p < 0.05$) (Table 3 and Figure 5B). In the control groups lacking chitinase, lesions on chili fruits infected with *C. siamense* CH26.1 expanded rapidly, leading to severe disease at both 28°C and 35°C.

Discussion

Chitinases are enzymes that facilitate the hydrolysis of chitin, a common polymer present in the cell walls of many fungi. One key physiological role of chitinases is the capacity to safeguard plants from phytopathogenic fungi, whose cell walls contain chitin, making them appealing candidates for the development of biocontrol strategies against fungal pathogens (Oyeleye and Normi 2018). Numerous studies have reported the heterologous expression of chitinases from various organisms in *E. coli*, a highly effective expression system, for diverse applications. These include chitinases derived from bacteria (Churklam and Aunpad 2020; El-Sayed et al. 2024; Guo et al. 2024; Li et al. 2020), fungi (Boer et al. 2007; Liu et al. 2023; Sharma, Sharma, and Srivastava 2014), and plants (Sinelnikov et al. 2021; Singh, Kirubakaran, and Sakthivel 2007; Toufiq et al. 2017). Recently, mutation techniques have been employed to engineer chitinases, aiming to elucidate the structure and function of the enzyme or to enhance its chitinolytic activity, thermostability, affinity properties, and other characteristics suitable for various applications (Oyeleye and Normi 2018). To our knowledge, this is the first report detailing the expression of mutants generated through site-directed mutagenesis of chitinase from *T. asperellum* in *E. coli* to improve their thermostability.

Table 3. Treatment effect of chitinases on Chili anthracnose disease caused by *C. siamense* CH26.1.

		Lesion diameter (cm) by day				
			After treatment			
Chitinase (units)		Before treatment	1	2	3	4
I168L	20	$0.18 \pm 0.13^{a,A}$	$0.33 \pm 0.1^{ab,AB}$	$0.73 \pm 0.13^{bc,BCD}$	$1.13 \pm 0.08^{cd,CDE}$	$1.47 \pm 0.13^{d,BC}$
	40	$0.25 \pm 0.05^{a,A}$	$0.33 \pm 0.03^{a,AB}$	$0.48 \pm 0.08^{a,AB}$	$0.85 \pm 0.05^{b,BCD}$	$1.13 \pm 0.13^{c,B}$
	60	$0.1 \pm 0.08^{a,A}$	$0.18 \pm 0.03^{ab,A}$	$0.25 \pm 0.05^{ab,A}$	$0.4 \pm 0.1^{b,A}$	$0.65 \pm 0.05^{c,A}$
	80	$0.18 \pm 0.03^{a,A}$	$0.2 \pm 0.05^{a,A}$	$0.23 \pm 0.08^{a,A}$	$0.28 \pm 0.08^{a,A}$	$0.35 \pm 0.03^{a,A}$
s-Chi42–2	20	$0.13 \pm 0.08^{a,A}$	$0.4 \pm 0.15^{a,AB}$	$0.83 \pm 0.13^{b,CD}$	$1.35 \pm 0.05^{c,EF}$	$1.78 \pm 0.08^{d,CD}$
	40	$0.23 \pm 0.03^{a,A}$	$0.4 \pm 0.05^{a,AB}$	$0.75 \pm 0.1^{b,BCD}$	$1.15 \pm 0.08^{c,DEF}$	$1.58 \pm 0.13^{d,C}$
	60	$0.18 \pm 0.13^{a,A}$	$0.33 \pm 0.13^{ab,AB}$	$0.58 \pm 0.08^{ab,BC}$	$0.83 \pm 0.13^{bc,BC}$	$1.15 \pm 0.2^{c,B}$
	80	$0.2 \pm 0.00^{a,A}$	$0.33 \pm 0.05^{a,AB}$	$0.45 \pm 0.08^{a,AB}$	$0.8 \pm 0.1^{b,B}$	$1.08 \pm 0.13^{b,B}$
C1	–	$0.25 \pm 0.05^{a,A}$	$0.78 \pm 0.08^{b,C}$	$1.18 \pm 0.13^{c,E}$	$1.83 \pm 0.1^{d,G}$	$2.4 \pm 0.2^{e,E}$
C2	–	$0.28 \pm 0.08^{a,A}$	$0.53 \pm 0.03^{b,BC}$	$0.93 \pm 0.1^{c,DE}$	$1.45 \pm 0.15^{d,F}$	$2.05 \pm 0.13^{e,DE}$

Q10 C1 (control 1): incubation at 28°C without chitinase, C2 (control 2): incubation at 35°C without chitinase.
Data are expressed as the mean of three replicates ± standard error.
Different uppercase letters in a column represent a statistically significant difference (Duncan's test, $p < 0.05$).
Different lowercase letters row represent a statistically significant difference (Duncan's test, $p < 0.05$).

Basically, the properties of chitinases produced in *E. coli* (this study) and in *N. benthamiana* (Tien et al. 2024) were not much different. However, extracting and purifying recombinant proteins from plants (e.g., *N. benthamiana*) is often more complex than from bacteria (e.g., *E. coli*). *E. coli* offers rapid growth, ease of culture, simple genetic manipulation, high expression yields, and a more straightforward purification process. In contrast, *N. benthamiana* typically has lower expressed protein concentrations, a more complex proteome, higher purification costs, requires post-extraction processing, and its protocols are not as widely established as those for *E. coli*. In summary, while plants like *N. benthamiana* offer distinct advantages (e.g., the ability to perform complex post-translational modifications like glycosylation and the absence of bacterial endotoxins), from an extraction and purification standpoint, they usually demand more intricate and costly procedures compared to the *E. coli* system (Burnett and Burnett 2020; İncir and Kaplan 2024).

In this study, all six mutants of the 42 kDa chitinase were successfully expressed in *E. coli* M15 utilizing the pQE30 vector system. Most of the mutant enzymes demonstrated chitinase activity comparable to that of the native enzyme, approximately 79 units/mL, with some showing enhanced thermostability. Notably, the I168L mutant maintained 75% of its relative activity at 40°C after 3 h of incubation, and at 50°C and 60°C, it retained 79% and 42% of its activity after 60 min, respectively, which is significantly higher than that of the native chitinase. The I168L mutant involves the substitution of the amino acid isoleucine with leucine at position 168 of the chitinase enzyme. Both isoleucine and leucine are hydrophobic amino acids with branched side chains (Kathuria et al. 2015). However, there are slight structural and flexibility differences between them that may explain the improvement in thermal stability: 1) Replacing the bulkier isoleucine with the more flexible leucine at position 168 likely reduces local steric hindrance or improves protein packing, leading to a more stable structure at elevated temperatures. 2) This substitution may optimize hydrophobic interactions within the protein's core, making the protein more resilient at high temperatures. 3) The I168L mutation is located near a crucial region of the enzyme in the predicted 3D structure, suggesting that alterations at this site can influence the enzyme's overall stability.

Some studies also reported positive results in improving the thermostability or catalytic activity of chitinase using a mutagenesis approach. Kozome et al. (2021) determined the crystal structure and subsequently introduced proline residues, disulfide bonds, and salt bridges into the chitinase derived from *Ficus microcarpa*. As a result, all mutants with proline substitutions demonstrated an extended half-life at 60°C and displayed higher melting temperature values compared to the wild type chitinase (GlxChiB). Liu et al. (2025) conducted modifications on BcChiA1, a chitinase derived from *Bacillus circulans* WL-12, utilizing computer-aided

design techniques. The thermostable variant, designated Mu5, was produced through five specific point mutations to BcChiA1 and demonstrated a half-life of 295 minutes at 60°C, which is 59 times greater than that of the wild-type chitinase. In a separate investigation, Tubkanlu et al. (2017) employed 3D structural analysis along with the QuikChange site-directed mutagenesis technique to substitute Ser 390 with Ile in the chitinase from *Serratia marcescens* B4A. The findings indicated that this mutagenesis did not affect the optimal temperature of the native enzyme; however, it enhanced the chitinase's stability at high temperatures, allowing it to withstand conditions up to 90°C. Additionally, the enzyme's activity was found to increase at 50°C and 60°C after 120 min of incubation.

The antifungal activity of recombinant 42 kDa chitinase from *T. asperellum* SH16 against phytopathogenic fungi such as *Aspergillus niger*, *Sclerotium rolfsii*, and *C. siamense* was demonstrated in our previous studies (Hoa et al. 2023; Luong et al. 2021; Tien et al. 2024; Tue et al. 2021). In the current study, the thermostable mutant I168L demonstrated significant antifungal efficacy against *C. siamense*, which causes anthracnose in chili, during both preventive and therapeutic trials conducted at 35°C. When applied at a concentration of 80 units, this mutant was able to avert pathogenic fungal infection for a duration of up to 4 days and hinder mycelial development, resulting in a lesion diameter of merely 0.28 cm following 7 days of artificial inoculation. Furthermore, in chili fruits afflicted by anthracnose, an 80-unit application of the mutant I168L effectively suppressed fungal growth after 4 days of treatment. Similarly, other studies on mutant chitinase have demonstrated enhanced antifungal activity in comparison to wild-type enzymes. Ni et al. (2015) developed mutants of a *Bacillus thuringiensis* chitinase through molecular docking and site-directed mutagenesis. The purified mutant ChiW50A showed significantly stronger inhibitory effects on the hyphal growth of *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Fulvia fulva*, and *Botrytis cinerea* than the wild-type Chi9602. In the study conducted by Vahed et al. (2013), the antifungal efficacy of recombinant mutant chitinases (ChiL and ChiLm) derived from *B. pumilus* SG2 against *F. graminearum*, *Bipolaris* sp., *S. sclerotiorum*, and *Aspergillus parasiticus* was found to be superior to that of the wild-type enzyme.

Studies on the application of chitinase as a biocontrol agent to combat *Colletotrichum* spp., the pathogens responsible for anthracnose in fruits and vegetables, remain limited. Several studies have documented the antifungal properties of cell-free supernatants derived from chitinase-producing bacteria or partially purified chitinase against *Colletotrichum* species (Anees et al. 2019; Delfini et al. 2021; Asril, Mubarik, and Wahyudi 2014; Lee et al. 2012; Nurdin, Mubarik, and Sudirman 2016). However, most of these investigations were conducted *in vitro*, and there is a lack of research

examining the impact of recombinant chitinase on these pathogenic organisms.

In conclusion, our findings collectively suggest that the recombinant mutant chitinase I168L derived from *T. asperellum* SH16, when produced in *E. coli*, demonstrated improved thermostability and antifungal properties. This advancement presents a promising opportunity for utilizing this enzyme in the preservation of post-harvest stages of fruits and vegetables prior to food processing stages from anthracnose infections caused by *Colletotrichum* spp. However, this study only tested antifungal activity against two strains of *C. siamense*. Therefore, future studies are needed to test the enzyme's efficacy against a wider range of fungal pathogens and with the wider context of improving food quality in the post-harvest stages.

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Disclosure statement

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Data availability statement

All data supporting the findings of this study are available within the paper.

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