ANTIFUNGAL ACTIVITY AND CHARACTERIZATION OF B-1,3-GLUCANASE FROM *BACILLUS TEQUILENSIS* ML6

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Abstract. This study aims to investigate the antifungal efficacy and enzymatic attributes of extracellular β-1,3-glucanase produced by *Bacillus tequilensis* ML6, isolated in Thua Thien Hue Province, Vietnam. After growing the ML6 strain for 21 h, we found that the extracellular β-1,3-glucanase reached its highest activity level at around 2.059 U/mL. In an *in vitro* experiment, β-1,3-glucanase applied at a concentration of 0.400 U/mL significantly inhibited the growth of four *Colletotrichum* pathogens (*C. scovillei* HUD1, *C. fructicola* HUCL5, *C. siamense* HUCL3, and *C. scovillei* HUCL1), with inhibition rates of 72%, 90%, 95%, and 99%, respectively. In addition, β-1,3-glucanase from *B. tequilensis* ML6 demonstrated a notable ability to mitigate anthracnose development induced by *Colletotrichum* spp. on chilli fruits for an extended duration of up to 144 h following enzyme pretreatment at 2 U/mL. The optimal activity conditions for the enzyme were determined to be 40°C and pH 8.0. Thermal and pH stability ranged from 30°C to 50°C and pH 7.0 to 8.0, respectively. The presence of Fe^{3+} at 5 mM significantly increased β-1,3-glucanase activity up to 121%. Ions (Cu^{2+}, Mn^{2+}, Al^{3+}, Zn^{2+}, and Ca^{2+}) and surfactants (urea, EDTA, Triton X-100, and SDS) had inhibition effects on the enzyme, relative activity ranged from 73-91%.
Keywords: *anthracnose, Bacillus tequilensis, β-1,3-glucanase, chilli, Thua Thien Hue*

Introduction

The chilli (*Capsicum annuum* L.) is popular worldwide for its culinary, industrial and medicinal purposes. In Vietnam, chilli has been cultivated for generations and plays a crucial role in both local consumption and international trade, particularly in Central Vietnam, including Ninh Thuan, Quang Nam, Thua Thien Hue, Quang Tri, and Quang Binh. However, plant diseases reduce crop yields, to typically only 7-10 tons per hectare (Truong and Van, 2022). Anthracnose and *Phytophthora* are economically important diseases affecting *Capsicum* crops (Truong and Van, 2022). *Colletotrichum* spp. is the main pathogen causing anthracnose in chilli, in a recent study, our team identified the presence of *Colletotrichum brevisporum, C. siamense, C. fructicola,* and *C. scovillei* in anthracnose-infected chilli samples were collected from North-Central Vietnam (Vu et al., 2023).

Therefore, preventing and treating chilli anthracnose caused by *Colletotrichum* is critical. Currently, various methods are employed to control plant diseases. Chemical control, though effective, poses environmental threats and risks to human health.

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Biocontrol, involving microbial antagonists or antimicrobial production, offers a promising option to fungicides, reducing reliance on harmful chemicals. Hence, microbial antagonists are increasingly favored as biocontrol agents (Wonglom et al., 2019).

Biocontrol of plant pathogens has garnered global attention as a safer and more environment-friendly alternative to traditional crop protection chemicals (Nguyen et al., 2019). Various microbial genera have been identified as potential biocontrol agents against phytopathogens. Among these, the genus *Bacillus*, has emerged as a significant source of these agents. This is attributed to their unique capability to produce resilient endospores, allowing them to survive in extreme environmental conditions, and synthesizing diverse antimicrobial compounds (Dame et al., 2021). In a recent study, a bacterial strain (*Bacillus tequilensis* ML6) was isolated, which exhibited strong β-1,3-glucanase activity and antagonistic activity against the anthracnose fungal *Colletotrichum* spp. (Tram et al., 2023).

To our knowledge, the *Bacillus tequilensis* has been previously used as a fungal antagonist caused by *Colletotrichum* spp. For instance, *B. tequilensis* GYUN-300 strain has revealed antagonistic effects against pathogens *C. acutatum* (KACC42403, ANBPC and ANYPC) under *in vitro* conditions (Kwon et al., 2022) while *B. tequilensis* A3, isolated from avocado rhizospheric soil, was found to reduce the mycelial growth of *C. gloeosporioides* mycelial by 25% after three days post-inoculation (Guerrero-Barajas et al., 2020). The anthrace pathogen fungal antagonism activity depends on the β-glucanase production ability of *B. tequilensis* (Kwon et al., 2022; Wang et al., 2014). β-glucanase enzyme facilitates the hydrolysis of β-glucans which are the sugars located in the cell walls of microorganisms. This enzyme specifically hydrolyzes the 1,4-/1,3-glycosidic bonds in mixed-links glucans, consequently compromising the structural integrity of the endospermic cell wall (Kaushal et al., 2022). The first research on 1,3-1,4-β-glucanase from *B. tequilensis* was conducted by Wang et al. (2014).

In this study, the characteristics and antifungal activity of β-1,3-glucanase from *B. tequilensis* ML6 were investigated for potential applications in the protection of crops and post-harvest storage.

Materials and methods

Strains

Bacillus tequilensis ML6 strain produces β-1,3-glucanase was isolated from the topsoil layer beneath chilli cultivation in Thua Thien Hue province, Vietnam (Tram et al., 2023).

Anthracnose fungal strains (*Colletotrichum siamense* HUCL3, *C. fructicola* HUCL5, *C. scovillei* HUD1, and HUCL1) were obtained from the Laboratory of Gene Technology, Institute of Biotechnology, Hue University, Vietnam.

In vitro antifungal activity of β-1,3-glucanase

The β-1,3-glucanase production was performed according to the method of Dewi et al. (2016). The ML6 bacterial strain was sub-cultured for β-1,3-glucanase production on 1% laminarin medium. The cell-free supernatants were collected every 3 h (from 12 to 27 h, at 30°C and 180 rpm on a shaker) to measure β-1,3-glucanase activity. And an

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activity assay was conducted as described in Wu et al. (2018). The assay based on inhibiting hyphal growth of *Colletotrichum* spp., which have 1,3-glucan in their cell wall, was used to estimate *in vitro* antifungal activity of β-1,3-glucanase. Enzyme incorporation into a petri dish (Φ = 9 cm) in a medium containing 1/2 potato dextrose agar (PDA) was supplemented with 0.100-0.400 U/mL. After incubating the culture at 28°C for five days. The mycelium cells were collected by centrifugation at 4.000 rpm for five min, followed by rinsing with distilled water and drying at 65°C until reaching a constant weight for dry biomass determination (Loc et al., 2020).

Antifungal activity of β-1,3-glucanase in chilli fruit

Healthy chilli fruits from 5 plants were collected and washed with tap water, followed by treatment with 70% ethanol and rinsed with sterile distilled water (Loc et al., 2020).

Fungal prevention. Chilli fruits were wounded, sprayed with glucanase at different concentrations ranging from 0.020 to 2.000 U/mL (0.020 U/mL, 0.200 U/mL, and 2.000 U/mL), left dry naturally, and then inoculated artificially with fungal phytopathogen (*Colletotrichum* spp.), three lesions per fruit six days after enzyme treatment. The diameters of the anthracnose lesions (cm) on fruits stored at room temperature (26 ± 2°C) were measured six days after inoculation to evaluate the progress of the disease over time.

Fungal treatment. Chilli fruits were wounded and inoculated artificially with *Colletotrichum* spp. After anthracnose lesions appear, the fruits will be treated with β-1,3-glucanase at different concentrations of 0.020-2.000 U/mL (0.020 U/mL, 0.200 U/mL, and 2.000 U/mL) and stored at room temperature (26 ± 2°C). The diameters of the anthracnose lesions (cm) on fruits were measured six days after enzyme treatment to evaluate disease progress over time.

Characterization of β-1,3-glucanase

The optimum factors for β-1,3-glucanase activity were determined by evaluating temperature ranges of 30-70°C and pH levels of 4-10. For pH optimization, 20 mM citrate buffer was used for pH 4-6, 20 mM phosphate buffer for pH 7-8, and 20 mM glycine-NaOH buffer for pH 9-10. The thermal and pH stability of the enzyme was examined by incubating it without substrate at temperatures of 30-70°C and pH levels from 4-10 for 30 min, followed by immediately cooling to 4°C (Loc et al., 2020).

The influence of various metal ions and surfactants on enzyme activity was assessed by incubating enzyme with 5 mM metal ion (Na^+, Al^{3+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, or Fe^{3+}) or reagents such as 1% SDS (sodium dodecyl sulfate), 1 mM EDTA (ethylenediaminetetraacetic acid), 1 M urea, 5% DMSO (dimethyl sulphoxide) or 1% Triton X-100. This incubation occurred at a suitable temperature and pH levels of 30 min. The relative activity of β-1,3-glucanase was determined as described in the "β-1,3-glucanase activity assay" section, using a boiled enzyme solution as the blank (Loc et al., 2020).

Statistical analysis

The experiments were replicated three times per treatment. Data are represented as the mean of these replicates, and statistical comparisons were conducted using ANOVA with Duncan's test at $p < 0.05$.

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Results

Glucanase production

The crude of β-1,3-glucanase obtained after the culture was measured for its activity. Results reveal that β-1,3-glucanase activity was detectable in the culture medium after 12 h of growth. The activity then increased steadily over time, reaching a maximum total activity of 2.059 U/mL after 21 h of growth (specific activity of 11.461 U/mg) (*Fig. 1*).

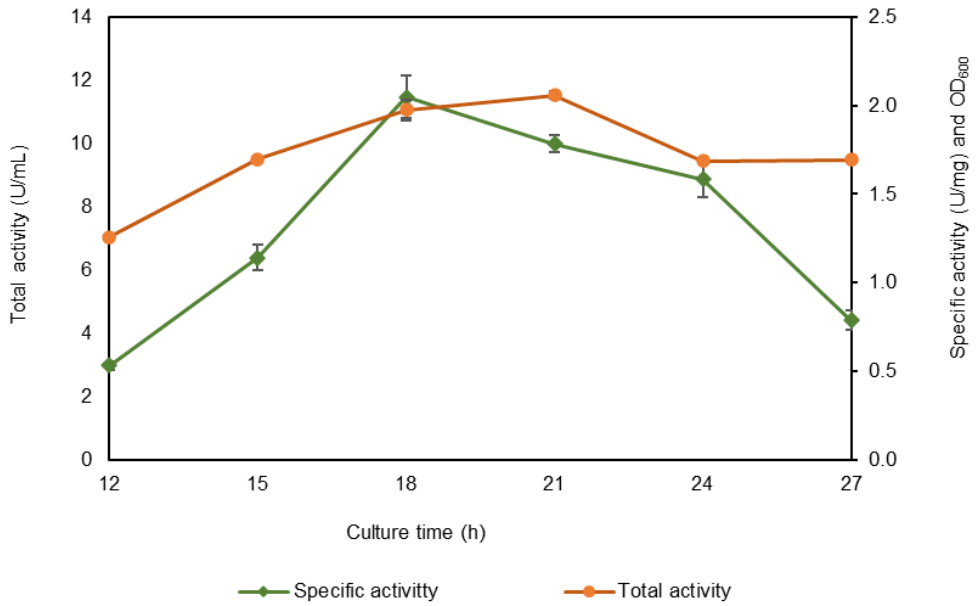

Figure 1. *Extracellular 1,3-glucanase production from B. tequilensis ML6*

In vitro antifungal activity of β-1,3-glucanase

The our outcomes demonstrate that β-1,3-glucanase produced by *B. tequilensis* ML6 had a significant inhibitory effect on the growth of *Colletotrichum* spp. (*Table 1*). There was a notable reduction in the biomass of *Colletotrichum* strains at a concentration of 0.100 U/mL of β-1,3-glucanase treatment, ranging from approximately 42% (HUD1, 0.237 g fresh weight (FW) and 0.022 g dry weight (DW)) to 91% (HUCL1, 0.037 g FW and 0.040 g DW), respectively. With a higher concentration of 0.400 U/mL β-1,3-glucanase, the growth of *Colletotrichum* strains was completely inhibited, from 90% (HUCL5), 95% (HUCL3), and 99% (HUCL1) (except for HUD1 with 72%). *Figure 2* exhibits the growth of *Colletotrichum* strains in the medium containing various concentrations of β-1,3-glucanase from 0.100 to 0.400 U/mL.

Chilli fruit anthracnose prevention and treatment

The antifungal capability of β-1,3-glucanase from *B. tequilensis* ML6 strain in chilli fruits was investigated after 144 h of enzyme treatment. In general, the *Colletotrichum* prevention of β-1,3-glucanase was found in *C. scovillei* HUD1 and HUCL1 at various enzyme concentrations, but unaffected for *C. siamense* HUCL3 and *C. fructicola* HUCL5 at the low enzyme concentration (*Fig. 3*).

The enzyme also had effects on anthracnose treatment, the growth of *C. scovillei* HUD1 and HUCL1 and *C. fructicola* HUCL5 on chilli fruits were inhibited by β-1,3-

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glucanase. Following a 144 h treatment with 2.000 U/mL of the enzyme, the growth of the fungus was completely inhibited, except in the case of HUCL3. Wounded chilli fruits may be suitable for the growth of *C. siamense* HUCL3 and the effects of enzymes for this strain are not too high.

Table 1. *In vitro antifungal activity of β-1,3-glucanase from B. tequilensis ML6*

Enzyme treatment (U/mL)	HUD1		HUCL1		HUCL3		HUCL5	
	FW (g)	DW (g)	FW (g)	DW (g)	FW (g)	DW (g)	FW (g)	DW (g)
0	0.565^{a}	0.053^{a}	0.420^{a}	0.057^{a}	0.490^{a}	0.039^{a}	1.131^{a}	0.134^{a}
0.100	0.237^{b}	0.022^{b}	0.037^{b}	0.004^{b}	0.157^{b}	0.012^{b}	0.251^{b}	0.031^{b}
0.200	0.190^{bc}	0.018^{bc}	0.019^{b}	0.004^{b}	0.123^{b}	0.009^{b}	0.134^{b}	0.017^{b}
0.400	0.156^{c}	0.014^{c}	0.005^{b}	0.001^{b}	0.025^{c}	0.002^{c}	0.107^{b}	0.014^{b}

Different letters (a, b, c, etc.) in each column indicate significantly different means (Duncan's test, $p < 0.05$); FW: fresh weight, DW: dry weight

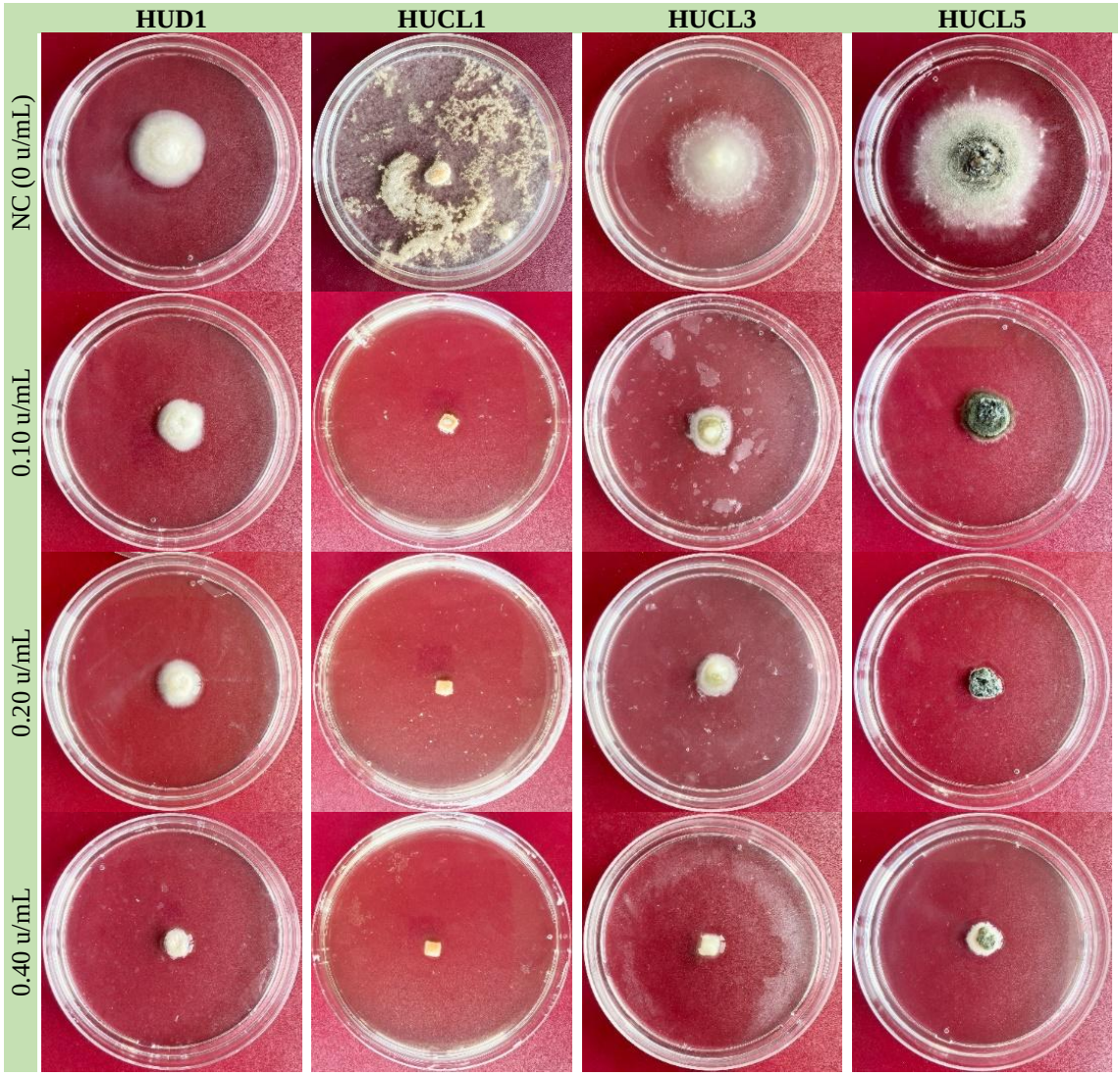

Figure 2. *Effect of β-1,3-glucanase from B. tequilensis ML6 strain on in vitro growth of Colletotrichum spp. NC: control without enzyme*

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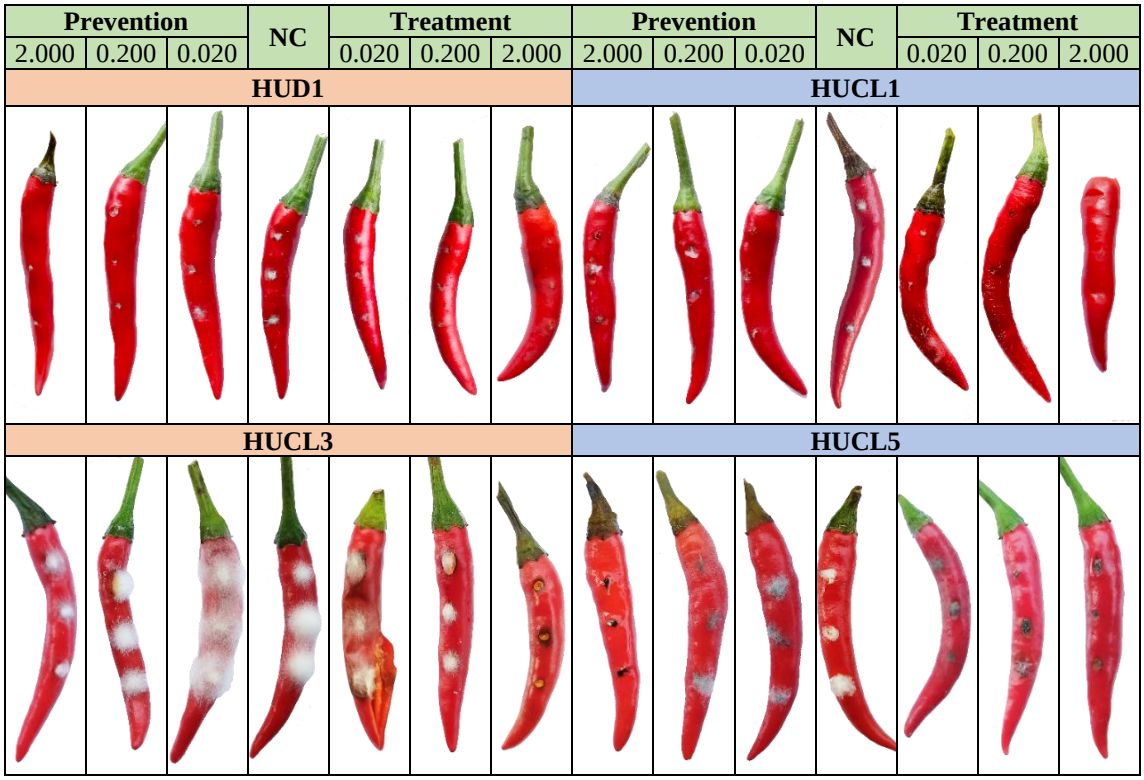

Figure 3. *Effects of β-1,3-glucanase from B. tequilensis ML6 strain on chilli fruits after Colletotrichum inoculum. NC: non-enzyme treatment*

Characterizations of β-1,3-glucanase

Enzyme activity relies on protein folding and is therefore sensitive to environmental factors such as temperature, pH, and salt concentration (Moran, 2018). For *B. tequilensis* ML6, the optimum temperature for β-1,3-glucanase activity was 40°C, with a significant decrease in activity observed at temperatures above 45°C. Enzyme activity remained relatively unaffected within the temperature range of 30-50°C, and 92-93% of its activity was retained after being exposed to temperatures between 30-45°C for 30 min (*Fig. 4*).

Enzyme activities are affected by pH, the optimal pH for total activity was 8.0 (3.44 U/mL) and the enzyme was stable in the pH condition ranging from 7.0 to 8.0. In the strong acid and base medium (pH < 7.0 and pH > 9.0), the activity of β-1,3-glucanase was remarkably decreased (*Fig. 5*).

Enzyme activity showed variation with different surfactants and ions (*Fig. 6*). Among the tested ions (Cu^{2+}, Mn^{2+}, Al^{3+}, Zn^{2+}, and Ca^{2+}) exhibited inhibitory effects on the enzyme, resulting in relative activity ranging from 73% to 91%. Only Fe^{3+} significantly increased β-1,3-glucanase activity by approximately 121%, while Co^{2+} showed no effect on enzyme activity. However, the surfactants tested inhibited enzyme activity, with activity levels remaining at about 71 to 86% in the presence of urea, EDTA, Triton X-100, and SDS.

Discussion

The bacterial genus *Bacillus*, renowned for its phytopathogen suppression capabilities, has been broadly utilized in agriculture. However, variations in probiotic

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efficiency and mechanisms among *Bacillus* species highlight the importance of investigating the biocontrol potential of new *Bacillus* isolates (Wang et al., 2023). *Bacillus* spp. was commonly used for antagonization on anthracnose fungus caused by *Colletotrichum* spp.

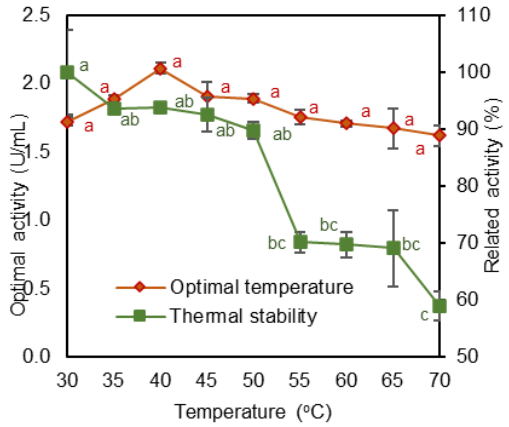

Figure 4. *Optimal temperature and thermal stability of enzyme at pH 7.0*

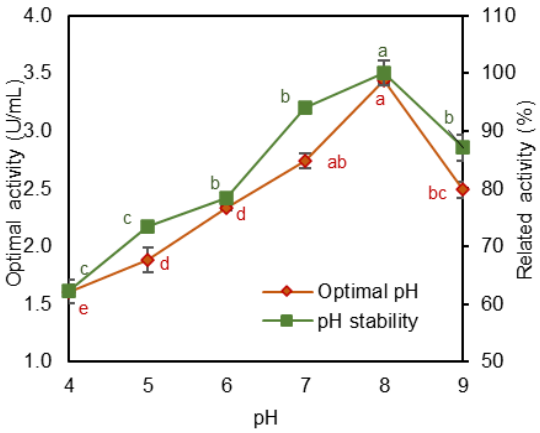

Figure 5. *Optimal pH and pH stability of enzyme at 40°C*

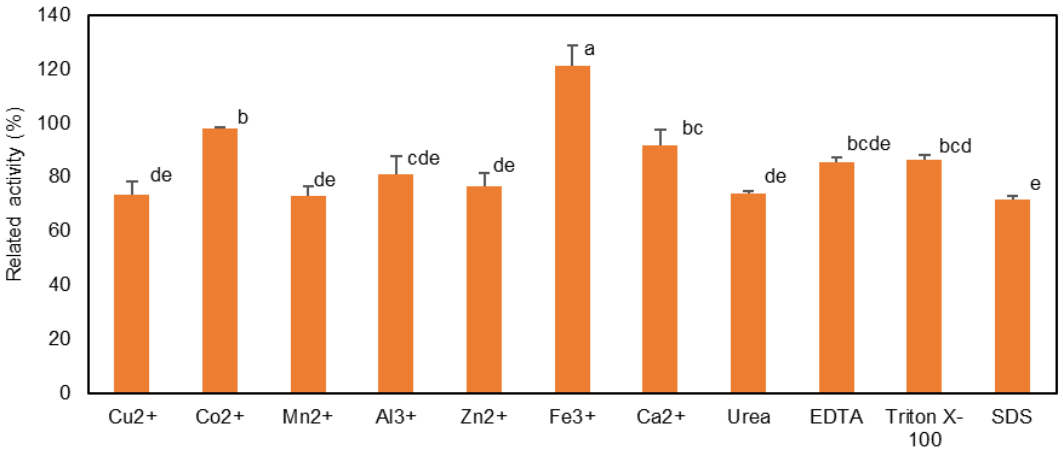

Figure 6. *Effect of metal ions and surfactants on β-1,3-glucanase from B. tequilensis ML6*

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In our study, the *in vitro* antagonistic ability of *B. tequilensis* ML6 was higher than that of other reports (Guerrero-Barajas et al., 2020; Choub et al., 2021), the growth of *Colletotrichum* strains was completely inhibited, from 90% (*C. fructicola* HUCL5), 95% (*C. siamense* HUCL3), and 99% (*C. scovillei* HUCL1), except for *C. scovillei* HUD1 (72%) (*Fig. 2*). The results illustrated in *Figure 3* showed that extracellular enzyme from *B. tequilensis* ML6 can be used for anthracnose prevention and treatment on chilli fruits, especially for *C. scovillei* HUD1, *C. scovillei* HUCL1, and *C. fructicola* HUCL5. The high antifungal ability showed that *B. tequilensis* ML6 could be used for the production of anti-anthracnose bio-product, used for the prevention and treatment of anthracnose disease in chilli in Central Vietnam. This figure is consistent with different research, for instance, isolates of *Bacillus amyloliquefaciens* and *B. velezensis* from pepper leaves demonstrated growth inhibition rates of 79 and 80%, respectively against *C. scovillei* mycelium (Wei et al., 2023). *Bacillus mycoides* (strain A1 and A2) isolated from avocado rhizospheric soil significantly inhibited the mycelial fungal growth of *C. gloeosporioides* by 75% and 70%, respectively (Guerrero-Barajas et al., 2020). *B. tequilensis* CNU082075 strongly inhibition the growth of *C. acutatum* (Paul et al., 2013).

The diversity of *Bacillus* spp. showed fungus-inhibiting factors. Some key antagonistic traits were observed in *B. tequilensis* GYUN-300, including production of lytic enzymes (cellulase, protease, and amylase), solubilization of insoluble phosphate, and siderophore production (Kwon et al., 2022); meanwhile in *B. mycoides* A1, involving production of proteases, siderophores, and indolacetic acid (Guerrero-Barajas et al., 2020). The crude enzyme extracted from *Bacillus velezensis* CE 100 revealed the activity of β-l,3-glucanase, chitinase, and protease (Choub et al., 2021). The cell-free supernatant of *B. tequilensis* A13 was found to contains six antifungal compounds, eight antifungal compound synthases, and several functional proteins associated with plant stress resistance (Wang et al., 2023). *Bacillus tequilensis* PKDN31 and *B. licheniformis* PKDL10 were noted to produce protease, lipase, amylase and β-1,3 glucanase (Karthika et al., 2022). Previous studies have reported the use of β-glucanase for antagonizing anthracnose fungus. *B. tequilensis* CGX5-1 strain showed remarkable 1,3-1,4-β-glucanase activity. The *bgl5* gene encoding 1,3-1,4-β-glucanase was cloned and expressed in *E. coli* BL21, resulting in the production of a recombinant enzyme weighing 24 kDa. The enzyme was detected in the cell culture supernatant, with an activity level of 2,978.2 U/mL. The enzyme showed stability within the pH range of 5.0-7.5, with the highest activity recorded at pH 6.0 and thermostability within the temperature of 45 to 60°C (Wang et al., 2014). A recent study showed that β-1,3-glucanase derived from *B. tequilensis* GYUN-300 was employed to antagonize the anthracnose fungus *C. acutatum* in red peppers in Korea. Treatment of red pepper fruits with GYUN-300 resulted in preventive effects of 66.6% and curative effects of 38.3% in wounded fruits (Kwon et al., 2022). The characteristics of β-1,3-glucanase from *B. tequilensis* ML6 were investigated, revealing an optimal temperature of 40°C and pH of 8.0. At these conditions, the enzyme exhibited relative activities of 111.67% (at 40°C and pH 7.0) and 125.46 (at 40°C and pH 8.0). Besides, the enzyme demonstrated a thermal range from 30-50°C and pH stability of 7.0-8.0, with a relative activity of about 89-93% and from 94-100%, respectively (*Figs. 4* and *5*). Despite enzymes being stable and showing optimum activity at pH 8.0, increasing the pH of the medium significantly diminishes their activity. Only Fe^{3+} significantly increased the enzyme activity (about 121%). All other ions (Cu^{2+}, Mn^{2+}, Al^{3+}, Zn^{2+} and Ca^{2+}) and surfactants (urea, EDTA, Triton X-100, and SDS) had inhibition effects on the enzyme, relative activity ranged from 73 to 91% (exception for Co^{2+} had no effect).

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Conclusions

The antifungal activity and characteristics of β-1,3-glucanase from *B. tequilensis* ML6 strain were investigated. The enzyme exhibited optimal activity at 40°C and pH 8.0. β-1,3-glucanase of *B. tequilensis* ML6 effectively inhibited the growth of *Colletotrichum* spp., suggesting its potential as an environmentally friendly fungicide in the future.

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