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Antagonistic *Bacillus* sp. strains against *Phytophthora* sp. in jackfruit: Isolation, identification, and enzyme production

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ABSTRACT

Jackfruit (Artocarpus heterophyllus) is an important economic crop in tropical and subtropical regions, especially in South and Southeast Asia. In recent years, trunk canker disease caused by Phytophthora palmivora has emerged as a major threat to jackfruit production. To explore sustainable alternatives to chemical control, this study investigated the antagonistic potential of Bacillus rhizobacteria. Four Bacillus sp. strains (CT1, CT2, CT3, and CT4) were isolated from 12 jackfruit rhizosphere soil samples from jackfruit fields in Chau Thanh commune, An Giang province, Viet Nam. Morphological characterization was performed, followed by in vitro antagonism assays against Phytophthora sp. One strain exhibiting the highest inhibition activity was selected for ITS gene sequencing and enzymatic profiling. The results showed that all were gram-positive, catalase-positive, KOH-negative, and showed antifungal activity. Among them, the strain identified as Bacillus velezensis CT4 exhibited the most stable antagonistic effect (17.8–73.7%) and the strongest production of cellulase (33.4–64.2 mm), β-glucanase (40.7–68.8 mm), and protease (45.5–65.7 mm) from 3 to 9 days after the test. Bacillus velezensis CT4 was considered a promising biocontrol agent for managing trunk canker disease, with the potential for commercial applications or scalability in jackfruit.

1. INTRODUCTION

Jackfruit (*Artocarpus heterophyllus* Lam.) is a tropical fruit tree that is easy to cultivate and early-bearing, widely grown across Southeast Asia (Asia-Pacific Association of Agricultural Research Institutions, 2012). It is currently a high-value crop, and its cultivation area is rapidly expanding in the Mekong Delta. According to the Department of Crop Production, the total area of jackfruit in Southern Viet Nam reached approximately 43,200 hectares, with the Mekong Delta alone accounting for about 30,600 hectares in 2021(Ministry of Agriculture and Rural Development, 2022). Borines et al. (2013) reported previous jackfruit decline *(Phytophthora palmivora)* in the Philippines; in Viet Nam (2012–2013) Tri et al. (2015) recorded the disease in four southern provinces (Ba Ria - Vung Tau, Binh Duong, Binh Phuoc, Dong Nai) with a rate of 2–60% in each planting and symptoms including root rot, ulcers, stem gummosis, yellowing, wilting, leaf fall, brown rot of fruit and plant death.

The abuse of pesticides pollutes the environment, affected human health and the quality of agricultural products (Nguyen et al., 2020). In that context, the application of antagonistic microorganisms, especially *Bacillus* strains, was of interest because many *Bacillus* species produced antibiotics and enzymes — more than 60 antibiotics have been identified (Nguyen, 2022; Compant et al., 2005). *Bacillus* species including *B. velezensis*, *B. subtilis*, *B. amyloliquefaciens*, *B. pasteurii*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* could induce systemic resistance in plants, thereby reducing disease incidence (Kloepper et al., 2024). In addition, *Bacillus* sp. excrete extracellular metabolites, including antibiotics, lytic enzymes, and siderophores, and demonstrate antagonistic activity against phytopathogens (Aimen et al., 2022). This study aimed to isolate, screen, and evaluate the *in vitro* antagonistic activity of *Bacillus* strains against *Phytophthora palmivora*, the causal agent of trunk canker disease in jackfruit, thereby providing preliminary scientific evidence for further studies on the application of biological approaches in disease management.

2. MATERIALS AND METHOD

2.1. Materials

The *Phytophthora palmivora* fungus, causing trunk canker disease in jackfruit, was identified and provided by the Plant Pathology Laboratory, Faculty of Agriculture and Natural Resources, An Giang University. *Bacillus* strains (CT1, CT2, CT3, and CT4) were isolated from *jackfruit* orchards in Chau Thanh district, An Giang province on luria bertani (lb) agar, characterized following Logan and De Vos (2009), Lawrence (2012), and Abdulkadir and Waliyu (2012), then purified and stored at the same laboratory.

2.2. Methods

2.2.1. Morphological identification of Bacillus sp. strains

Isolation of bacteria from jackfruit rhizosphere soil samples using procedure as described by Kumar et al. (2016). Bacterial morphology was observed under the oil-immersion objective. Gram staining was performed following Lawrence (2012) using crystal violet, Lugol's iodine, alcohol, and safranin. Catalase activity was tested using 3% H_2O_2, oxidase activity with reagent paper, and KOH reaction with 3% KOH to distinguish Gram (+) and Gram (-) (Nguyen, 2015; Pham et al., 2021).

2.2.2. Antagonistic ability of bacterial strains against pathogenic fungi

Four *Bacillus* strains (CT1, CT2, CT3, and CT4) were isolated and purified on LB medium, while the pathogen was cultured on PDA. A 5 mm fungal disc was placed 1 cm from the plate edge, opposite a bacterial streak 2 cm from the edge; the control had only the fungus (Shouan et al., 2010). Antagonistic efficiency ($I = [(C–T)/C] \times 100$) in which I: antagonistic efficiency of bacteria; C: diameter of fungal colony growing in the control formula; T: diameter of fungal colony growing towards bacteria, was evaluated after 2, 4, 6, and 8 days. The experiment followed a completely randomized design with 5 replicates (3 plates/strain) and was analyzed using Excel and SAS software.

2.2.3. Identification of antagonistic bacterial strains by molecular methods

The most effective antagonistic *Bacillus* strain was selected and sent for DNA sequencing to DNA Sequencing Company Limited (Can Tho province, Viet Nam). The 16S rDNA gene was amplified by PCR from extracted DNA following Heiko Packeiser et al. (2013) using primers 27F and 1492R. The obtained sequence was compared with GenBank data via BLAST (NCBI) and phylogenetic relationships were analyzed using MEGA 11 software.

2.2.4. Evaluation of the ability to produce antagonistic enzymes of Bacillus sp.

**Cellulase activity test (CMC):* A completely randomized design with 5 replicates was used, with each bacterial strain as one treatment. Filter paper samples were dipped in bacterial suspension (10^8 cfu/ml) and inoculated at 3 points per plate, then incubated at room temperature. After incubation, plates were stained with Lugol's solution for 5 min; clear zones indicated cellulose degradation (Saroj et al., 2018). The diameter of the degradation zone was measured on days 3, 5, 7, and 9.

**β-glucanase activity test:* The experiment followed a completely randomized design with 5 replicates, each bacterial strain as one treatment. Following Renwick et al. (1991), *Bacillus* spores were cultured, collected, and adjusted to a concentration of 10^8 cfu/ml. Discs (r = 5 mm) soaked in the suspension were placed on β-glucan medium (3 spots/plate) and incubated at room temperature. Plates were stained with 0.6% Congo red, rinsed, and clear zone diameters were measured on days 11, 13, and 15.

**Protease activity* test: The experiment was arranged in a completely randomized design with four replications, each treatment being an actinomycete strain. According to Mitra and Chakrabartty (2005), after 6 days of incubation on MS medium, actinomycete suspensions (10^8 CFU/mL) were impregnated onto 5 mm paper discs and placed on casein-containing medium. Protease activity was determined by measuring the clear zone diameter after treatment with 10% TCA, and the diameter was recorded at 3, 5, and 7 days of incubation.

**Data analysis:* Experimental data were analyzed by ANOVA test using SAS software based on the Duncan test method.

3. RESULTS AND DISCUSSION

3.1. Isolation and morphological identification of antagonistic bacterial strains

From 12 jackfruit rhizosphere soil samples from jackfruit fields in Chau Thanh commune, An Giang province, Viet Nam, four *Bacillus* sp. strains were successfully isolated: CT1, CT2, CT3, and CT4. These isolates exhibited distinct colony characteristics on LB medium (Figure 1), as observed under an optical microscope and identified based on the criteria described by Logan and De Vos (2009).

The colonies were generally round, with colors ranging from white to ivory white. Most showed concentric ring patterns (slightly raised or concave), slightly smooth-to-rough surfaces with serrated or occasionally lobed margins.

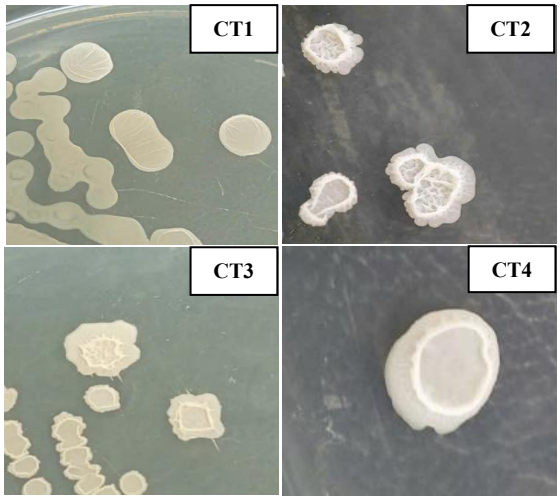

Figure 1. Colony morphology of *Bacillus* spp. isolates (CT1–CT4)

Observation of cell morphology and Gram staining: After purification, the bacterial isolates were cultured in LB medium at room temperature for 72 hours, followed by Gram staining to determine cell wall type and morphology under a microscope. The staining results (Table 1) indicated that all isolates appeared purple, confirming they are Gram-positive bacteria with thick peptidoglycan layers in their cell walls. The cells were rod-shaped with rounded ends, typical of the *Bacillus* genus, measuring 3.2–4.4 µm in length. In terms of cell arrangement, strains CT2 and CT4 were mostly single cells, whereas CT1 and CT3 tended to form short chains. These findings align with the morphological descriptions of *Bacillus* provided by Holt et al. (1994).

Table 1. Biochemical and colony traits of four *Bacillus* strains from Chau Thanh commune, An Giang province, Viet Nam

Bacterial strain	Catalase	Gram stain	Colony characteristics				
			Shape	Color	Edge	Buoyancy	Cell size (µm)
CT1	+	+	Rod	Ivory white	Slightly smooth	slightly raised	4,2-4,3
CT2	+	+	Round	White	Serrated	slightly concave	3,3-4,3
CT3	+	+	Round	Ivory white	Serrated	slightly concave	3,2-4,1
CT4	+	+	Round	White	Slightly smooth	slightly concave	3,8-4,4

Positive: (+).

Catalase reaction: Catalase is an enzyme that decomposes hydrogen peroxide (H_2O_2) into water and oxygen, thereby protecting bacterial cells from oxidative damage. Most Gram-positive bacteria, including those of the genus *Bacillus*, were typically catalase-positive (Madigan et al., 2018). In this study, catalase activity was assessed by adding a drop of 3% H_2O_2 solution to bacterial colonies on a glass slide. All four isolates (CT1, CT2, CT3, and CT4) produced visible gas bubbles upon contact, confirming a positive catalase reaction (Table 1).

KOH reaction (3%): The 3% KOH test was a rapid method used to support the identification of bacterial Gram type. In this reaction, Gram-negative bacteria (with thin cell walls) were lysed by KOH, releasing DNA and forming a viscous, stringy solution that can be pulled out. In contrast, Gram-

positive bacteria, which have thicker cell walls, do not produce such slime (Do, 2008). The experimental results showed that all four isolates (CT1, CT2, CT3, and CT4) did not generate any slimy substance when exposed to 3% KOH, indicating they were Gram-positive (Figure 2). This result was confirmed by comparison with a Gram-negative control, which produced a clear viscous string under identical test conditions.

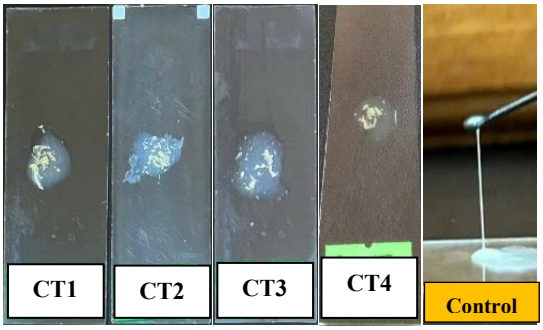

Figure 2. KOH reaction results

In summary, all four isolated strains exhibited typical *Bacillus* characteristics: round colonies with white to ivory color, dry and wrinkled surfaces, raised centers, and serrated or lobed edges. The cells were rod-shaped with rounded ends, Gram-positive (purple), and arranged singly or in short chains. They showed positive catalase activity (bubble formation) and negative KOH reaction (no slime), confirming their Gram-positive nature. These morphological and biochemical traits were consistent with the description of Bacillus reported by Tran (2024).

3.2. Antagonistic activity of *Bacillus* spp. against *Phytophthora* sp. *in vitro*

The antagonistic ability was evaluated using the direct dual-culture method on PDA medium. Results showed that inhibition efficiency (I) of *Bacillus* strains ranged from 10.2 to 73.7%, with significant differences ($p < 0.01$) across 2 to 8 days (Table 2, Figure 3). Specifically, CT4 exhibited the highest antagonistic effect throughout (from 17.8 to 73.7%), followed by CT1 (from 11.4 to 67.8%), while CT2 and CT3 were consistently the lowest (from 10.2 to 58.4%). The CT4 strain demonstrated the strongest and most stable inhibition across all periods, aligning with previous studies reporting high antagonistic potential of *Bacillus* spp. such as *B. siamensis* (Nguyen et al., 2023), *B. sonorensis* (Nguyen et al., 2018), and *B. velezensis* (Rabbee et al., 2019) against *Phytophthora* spp.

Table 2. Inhibition efficiency (%) of bacterial strains against pathogens

Treatments	Inhibition efficiency (%) of bacterial strains			
	2 DAT	4 DAT	6 DAT	8 DAT
CT1	11.4 ± 3.8^{b}	45.0 ± 2.4^{b}	67.8 ± 1.2^{b}	67.3 ± 1.3^{b}
CT2	10.2 ± 2.1^{c}	28.4 ± 1.3^{c}	56.0 ± 0.6^{c}	55.0 ± 1.4^{c}
CT3	11.4 ± 2.4^{b}	35.1 ± 1.7^{c}	58.1 ± 2.2^{c}	55.0 ± 1.6^{c}
CT4	17.8 ± 3.7^{a}	53.6 ± 2.3^{a}	73.7 ± 0.6^{a}	72.2 ± 1.7^{a}
CV (%)	**7.7**	**10.6**	**3.6**	**3.6**
Significance	**	**	**	**

Values followed by different superscript letters are significantly different within a column, according to Duncan's test ($p<0.01$); ±: SE; DAT: Days after test.

3.3. Molecular Analyses of Antagonistic *Bacillus*

The most representative isolate was selected for species identification using 16S rRNA gene sequencing. The amplified fragment of strain CT4 was 1,429 bp in length. Sequence analysis and comparison with reference sequences in the NCBI BLAST database revealed 99.72–99.79% similarity with seven *Bacillus* species: *B. velezensis* strain FZB42, *B. valismortis* strain DSM 11031, *B. subtilis subsp. subtilis strain 168*, *B. siamensis* KCTC 13613 strain PD-A10, *B. nakamurai* strain NRRLB-41091 and *B. amyloliquefaciens* (Figure 3).

Rhizosphere *Bacillus* species such as *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. pumilus*, *B. mycoides*, *B. pasteurii*, and *B. sphaericus* were known to produce diverse bioactive compounds and effectively suppress various plant pathogens (Gnanamanickam, 2009; Govindasamy et al., 2010; Narayanasamy, 2013). Nguyen et al. (2023) found that *Bacillus siamensis* CT7 strongly inhibited *P. palmivora* (36.5–59.7%). Similarly, Rabbee et al. (2019) noted *B. velezensis* effectively controlled chili root rot caused by *P. capsici*. Although there are limitations in resolving the *Bacillus subtilis* complex and we currently lack data for *gyrA* or *rpoB* markers, our identification remains presumptive, based on the available molecular and biochemical data. Given the high sequence similarity (99.79%) of strain CT4 to

Bacillus velezensis strain FZB42, it was identified as *Bacillus velezensis* CT4.

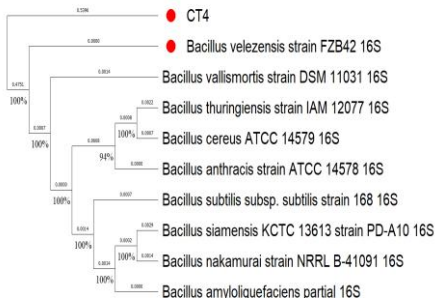

Figure 3. Phylogenetic tree of *Bacillus velezensis* CT4

3.4. Investigation of degrading enzyme secretion by *Bacillus* strains

3.4.1. Cellulase secretion ability

Cellulase degrades fungal cell walls, helping antagonistic bacteria inhibit pathogens and support plant growth (Tran, 2024). In this study (Table 3, Figures 4), the decomposition zone (DZ) at 3 days ranged from 30.1 to 33.4 mm, with CT1 and CT4 highest (from 32.6 to 33.4 mm), followed by CT3 (31.6 mm) and CT2 lowest (30.1 mm). At 5 days, DZ increased from 40.6 to 46.9 mm, CT4 remaining highest (46.9 mm), while CT1, CT2 and CT3 fluctuated lowest from 40.6 to 43.4 mm. From 7 to 9 days, CT1, CT3, and CT4 reached from 55.7 to 64.2 mm, and CT2 reached the lowest (62.3 mm). These results show CT4 has the strongest, most stable cellulase activity, correlating with its antagonistic effect. This is consistent with the study by Trinh et al. (2017), which indicated that *B. velezensis* synthesizes various extracellular enzymes, such as cellulase, chitinase, protease, glucanase, lipase, amylase, and cyclase. These enzymes can degrade fungal cell walls. Furthermore, this species has been shown to inhibit *Phytophthora* sp., the causal agent of late blight in tomatoes (Le et al., 2021).

Table 3. Cellulase degradation zone diameter (mm) of bacterial strains

Treatments	Cellulase degradation zone diameter (mm) of strains over time			
	3 DAT	5 DAT	7 DAT	9 DAT
CT1	32.6 ± 0.2^{ab}	43.4 ± 0.6^{b}	57.6 ± 0.7^{a}	63.9 ± 0.3^{a}
CT2	30.1 ± 0.3^{d}	40.6 ± 0.4^{c}	54.7 ± 0.6^{b}	62.3 ± 0.4^{b}
CT3	31.6 ± 0.3^{c}	41.6 ± 0.5^{bc}	55.7 ± 0.6^{b}	63.4 ± 0.4^{a}
CT4	33.4 ± 0.1^{a}	46.9 ± 1.9^{a}	57.8 ± 0.8^{a}	64.2 ± 0.2^{a}
CV (%)	**2.5**	**4.4**	**2.4**	**2.9**
Significance	**	**	**	**

Values followed by different superscript letters are significantly different within a column, according to Duncan's test ($p<0.01$); ±: SE; DAT: Days after test.

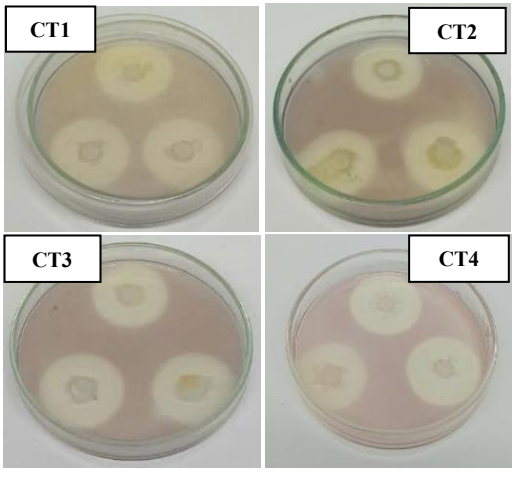

Figure 4. Cellulase activity of bacterial strains at 3 DAT

3.4.2. β-Glucanase secretion ability

β-Glucanase, like cellulase, degrades β-glucan in fungal cell walls, killing pathogens and providing carbon for bacterial growth, enhancing antagonism (Huyen et al., 2024). All four strains (CT1, CT2, CT3 and CT4) secreted β-glucanase, with DZ increasing from 34.2 to 68.8 mm over 3–7 days after test (DAT) and significant differences at 1% (Table 4, Figure 5). CT4 strain showed the highest (from 40.7 to 68.8 mm) and most stable activity, consistent with its antagonistic performance. Previous studies also report strong β-glucanase activity in *Bacillus* spp., effective against pathogens such as *Colletotrichum fructicola* and other fungi (Otsuka et al., 2022; Le, 2023; Nguyen et al., 2024).

Table 4. β-glucanase degradation zone diameter (mm) of bacterial strains

Treatments	β-glucanase degradation zone diameter (mm) of strains over time		
	3 DAT	5 DAT	7 DAT
CT1	36.9 ± 0.3[b]	53.6 ± 0.5[b]	65.4 ± 0.3[b]
CT2	34.9 ± 0.2[c]	49.7 ± 0.3[d]	57.5 ± 0.4[d]
CT3	34.2 ± 0.2[c]	52.0 ± 0.4[c]	60.7 ± 0.4[c]
CT4	40.7 ± 0.5[a]	54.9 ± 0.5[a]	68.8 ± 0.3[a]
CV (%)	**1.7**	**1.8**	**1.2**
Significance	**	**	**

Values followed by different superscript letters are significantly different within a column, according to Duncan's test (p<0.01); ±: SE; DAT: Days after test.

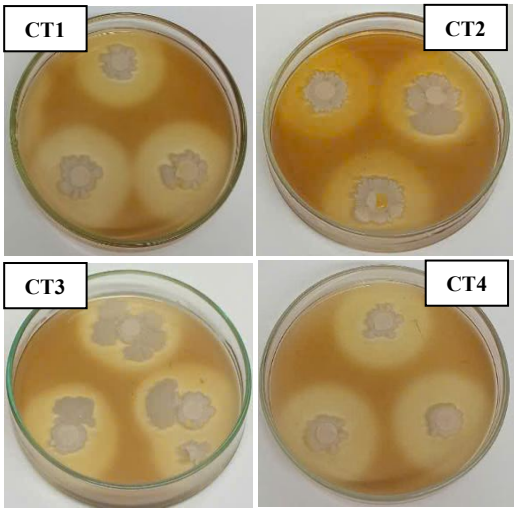

Figure 5. β--glucanase activity of bacterial strains at 3 DAT

3.4.3. Protease secretion ability

Many *Bacillus* strains produce extracellular proteases that degrade proteins, suppress growth, and disrupt *Phytophthora* structures, a key antagonistic mechanism alongside antibiotics, siderophores, and lytic enzymes (Harwood & Kikuchi, 2021). Table 5 and Figure 6 indicated that all four strains (CT1, CT2, CT3, and CT4) produced proteases, with DZ values increasing from 42.0 to 66.7 mm between 3 and 7 DAT, showing significant differences at the 1% level. Among them, strain CT4 exhibited the highest protease activity (from 45.5 to 67.7 mm) and the most consistent protease activity, aligning with its strong antagonistic effect. Moon et al. (2021) reported that *Bacillus velezensis* CE 100 controlled Phytophthora root rot and promoted *C. obtusa* seedling growth by producing β-1,3-glucanase and protease, which degraded oomycete cell-wall components and inhibited *Phytophthora* spp. growth by 54.6–74.3%. Similarly, Admassie et al. (2022) showed that extracellular proteases from Bacillus hydrolyze membrane proteins and structural proteins of hyphae and zoospores in *Phytophthora capsici*, thereby weakening the pathogen's ability to infect and develop. *Bacillus* has been shown to degrade cell wall proteins and effectors of oomycetes. In *Phytophthora* (cell wall composed mainly of glucan and protein), protease combined with glucanase can directly damage the cell wall (Li et al., 2023).

Thus, the high stability of strain CT4 is driven by its strong enzymatic activity, reflecting the synergistic effect of cellulase, β-glucanase, and protease in degrading oomycete cell walls.

Table 5. Protease degradation zone diameter (mm) of bacterial strains

Treatments	Protease degradation zone diameter (mm) of strains over time		
	3 DAT	5 DAT	7 DAT
CT1	42.0 ± 0.8[b]	55.3 ± 0.5[b]	62.5 ± 0.3[c]
CT2	45.9 ± 1.5[a]	54.2 ± 1.5[c]	62.9 ± 2.2[c]
CT3	46.5 ± 0.6[a]	54.9 ± 0.4[c]	63.5 ± 0.9[b]
CT4	45.5 ± 0.6[a]	57.0 ± 0.2[a]	65.7 ± 0.4[a]
CV (%)	**4.1**	**2.6**	**3.5**
Significance	**	**	**

Values followed by different superscript letters are significantly different within a column, according to Duncan's test (p<0.01); ±: SE; DAT: Days after test.

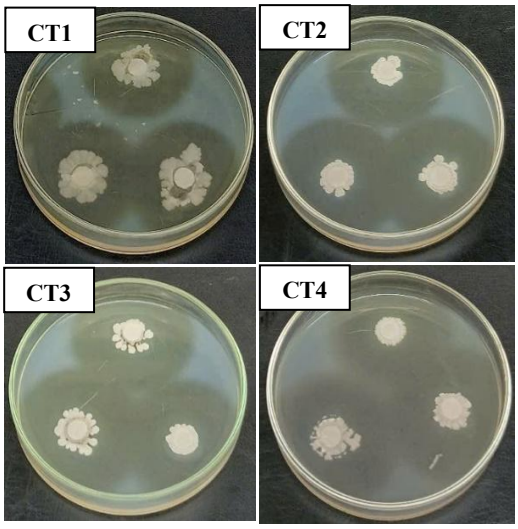

Figure 6. Protease activity of bacterial strains at 3 DAT

4. CONCLUSION

All four isolates exhibited typical characteristics of the genus *Bacillus*, including round, white-to-opaque colonies with dry, wrinkled surfaces and serrated margins; Gram-positive, rod-shaped cells; positive catalase activity and negative KOH reaction; and all demonstrated antagonistic activity against the trunk canker pathogen.

Strain CT4, identified as *Bacillus velezensis CT4*, had the highest antagonistic efficiency (17.8–73.7%) and consistently secreted cellulase (33.4–64.2 mm) β-glucanase (40.7–68.8 mm) and proteases (45.5–65.7 mm) from 3 to 9 days, indicating strong potential as a biological control agent.

Because *in vitro* assays may not reflect field performance, larger, multi-season trials of B. velezensis CT4 are needed to validate its use as a biological agent for the control of trunk canker disease of jackfruit.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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