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Research Article

Characterization of Extracellular Chitinase from *Bacillus cereus* SAHA 12.13 and Its Potency as a Biocontrol of *Curvularia affinis*

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Abstract
Bacillus cereus SAHA 12.13 can produce chitinase, an enzyme that digests chitin in the main compounds of cell walls, mycelia, and spores in pathogenic fungi that cause leaf spots on oil palm plants such as *Curvularia affinis*. This study aims to determine the properties of the chitinase enzyme *B. cereus* SAHA 12.13 that can inhibit the growth of *C. affinis*. Chitinase enzyme production and characterization were measured using the Spindler method. Antagonism test against pathogenic fungi using dual culture method by testing cell culture and enzyme crude extract. This result showed that the isolate produced a high level of specific chitinase activity at 37 °C for 45 h of incubation with 8.45 U mg^{-1} proteins with a growth rate (k) of 0.25 generation/h, and the generation time was 3.96 h/generation. The optimum chitinase activity was achieved at pH 7.0 and 45 °C and was stable for 3 h with a half-life ($t_{1/2}$) of 770 min. The crude enzyme and cell culture of strain can inhibit the growth of *C. affinis* by 36.27±0.043% and 34.25±0.041%, respectively. These characteristics indicate that *B. cereus* strain SAHA12.13 can be used to inhibit *C. affinis*, which causes leaf blight of oil palm, under varying pH and temperature conditions.

Keywords: *Bacillus cereus*, characterization, chitinase, *Curvularia affinis*, environmental factors, natural resource

1. INTRODUCTION

Chitin is an insoluble polysaccharide in the form of a linear polymer composed of β-1,4-*N*-acetylglucosamine (GlcNac) connected by hydrogen bonds [1]. Chitin is a very abundant polysaccharide after cellulose [2]. Chitin is widely distributed in structural components of organisms such as crustaceans and shells of molluscs, exoskeletons of arthropods, and cell walls of fungi [3]. The chitinase enzyme produced by microorganisms can degrade the presence of chitin in nature. Chitinase (EC 3.2.1.14) is a group of enzymes capable of degrading chitin into small molecular weight products [4] in the form of *N*-acetylglucosamine (GlcNac) monomers [5]. Chitinase can be isolated from various organisms, such as bacteria, fungi, plants, and animals. The existence and number of diverse bacteria that could produce chitinase make bacteria one of the most widely used organisms to produce chitinase.

Some bacteria such as *Bacillus cereus* in the environment reported having chitinase activity [6]-[10]. Chitinase produced by bacteria plays an essential role in the decomposition of chitin. It can potentially be used as a biocontrol agent for pathogenic fungi containing chitin. They have become an alternative to reduce the use of synthetic chemical fertilizers because they are harmful and environmentally friendly [11][12]. Chitinase can lysis pathogenic fungal cell walls, make a change in morphological forms of fungal (sporulation, spore germination, and hyphae growth), have an antagonistic effect on other microorganisms [13], and parasitic mechanisms that can enter host cells [14] thereby reducing the occurrence of pathogenic infections.

Oil palm (*Elaeis guineensis* Jacq.) are plants with the highest level of pathogen attack, *Ganoderma boninense*. Several chitinolytic bacteria that have been used to inhibit *G. boninense* are *Enterobacter* sp., *Bacillus* sp. [15], and *Burkholderia cepacia* [16]. However, oil palm plants are not only attacked by *G. boninense* but also other types of pathogenic fungi such as *Curvularia affinis* [17][18] which attacked during the nursery stage. The quality of oil palm at the nursery stage will be reduced due to this fungus attack [19][20]. The incidence of disease caused by *Curvularia* reaches 61.01% in Thailand [21]. This leaf blight was first reported in Indonesia in the

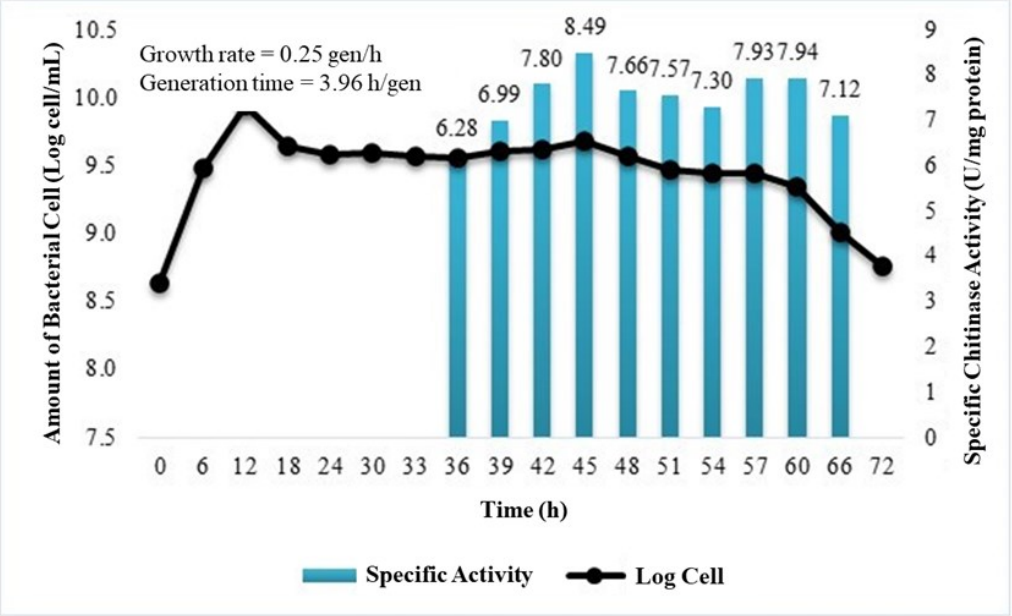

Figure 1. Growth curve and chitinase activity of *B. cereus* SAHA 12.13 on medium.

1920s and has spread massively throughout the country [22]. This report demonstrates the growing importance of leaf spot disease and the need for effective disease management using biocontrols such as chitinolytic bacteria at the nursery stage. However, there have not been too many reports about using chitinolytic bacteria to inhibit fungal growth that attacks the oil palm nursery. The use of chitinolytic bacteria in reducing fungal attacks in oil palm nurseries has been reported from *B. thuringiensis* SAHA 12.08, which has resulted in the growth of *C. affinis* and *Colletotrichum gloeosporioides* [17].

Previous research has isolated *B. cereus* SAHA 12.13 from the soil of Hutan Harapan, Jambi Province, Indonesia, which has qualitative chitinase activity [23]. However, quantitative measurement of chitinase enzyme, characterization of chitinase enzyme and its antagonistic ability against *C. affinis* have not been tested. This study aims to produce chitinase enzyme from *B. cereus* SAHA 12.13, characterize it based on temperature, pH, and stability, and test the chitinase enzyme activity of the isolate in inhibiting the growth of *C. affinis*.

2. MATERIALS AND METHODS

2.1. Materials

The materials used in this study were *B. cereus* SAHA 12.13, a chitinolytic bacterial isolate from Harapan Forest soil, Jambi, Indonesia. Isolates were grown in a Nutrient Agar medium (Merck, Germany) at 37 °C for 24 h of incubation. The pathogenic fungi isolated from oil palm plants, namely *Curvularia affinis*, were grown on Potato Dextrose Agar medium (Merck, Germany) at 28 °C for 48 h.

2.2. Methods

2.2.1. Determination of The Growth Curve and Production of an Enzyme

Two single colonies of *B. cereus* SAHA 12.13 incubated for 24 h on NA solid medium + 0.3% colloidal chitin were grown in nutrient broth (NB) enriched with 0.3% colloidal chitin. The starter inoculum was incubated for 15 h at 120 rpm and 37 °C. Furthermore, 1% (10^8 cells/mL) of the starter inoculum was inoculated into the production medium containing 100 mL of NB medium + 0.3% colloidal chitin. The production culture was incubated at 37 °C, 120 rpm. Measurements were carried out every 3 h by taking cell culture to measure cell density at a wavelength of 600 nm, which lasted for 72 h. Calculating the growth rate (number of generations (doublings) per hour) using the Eq. (1) and (2) [24]:

$$k = \frac{\log 10[Xt] - log10[X0]}{0.301\ x\ t} \quad \textbf{(1)}$$

where, Xt is higher CFU/ml in the logarithmic phase, X0 is lower CFU/ml in the logarithmic phase, and t is time interval between the 2 points (X_t and X_0) (in h).

Meanwhile, to find out the generation time, which is the time required for the population to double, using the Eq. (2):

$$t\ gen = \frac{1}{k} \quad \textbf{(2)}$$

The same cell culture was centrifuged for 20 min at 6,000 rpm (Centrifuge Hermle with rotor 220.97) at 4 °C. The supernatant obtained was a crude extract enzyme, which was then measured for its chitinase activity.

2.2.2. Measurement of Chitinase Activity and Protein Concentration

The chitinase activity of *B. cereus* isolate SAHA 12.13 was measured every 3 h using the Spindler method [25]. The enzyme activity was determined by measuring the absorbance at a wavelength of 420 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol of *N*-acetylglucosamine per minute. Meanwhile, protein concentration was measured using bovine serum albumin as standard and Bradford as reagent by Bradford method [26]. Specific activity of enzyme was performed by comparison of chitinase enzyme activity with a protein concentration.

2.2.3. Determination of Optimum pH, Temperature, and Stability of Chitinase

The optimum pH of the chitinase enzyme was determined by testing the supernatant obtained at the highest production time. Measurement of enzyme activity was tested on chitin 0.3% colloidal substrate in buffers with a pH range of 4.0–10.0 (1 unit interval) using different buffers, namely 0.1 M citrate buffer (pH 4.0–6.0), 0.1 M phosphate buffer (pH 7.0–8.0) and 0.1 M glycine-NaOH buffer (pH 9.0–10.0). The optimum temperature of chitinase activity was determined by testing the supernatant at an optimum pH with a temperature range of 25–60 °C (5 °C interval). The enzyme stability test was carried out by testing the supernatant at the optimum temperature and pH obtained in the previous test. Temperature stability measurements were also used to determine the half-life ($t_{1/2}$) of the chitinase enzyme activity. The $t_{1/2}$ was determined using the first order of the rate-law equation through a linear equation between relative activity and incubation time (Eq.(3)).

$$\text{rate} = k\,[A],$$
$$\ln [A] = -kt + \ln[A]0$$
$$t_{1/2} = 0.693/k \quad \textbf{(3)}$$

2.2.4. Antagonistic Test of Pathogenic Fungi

Using the dual culture method, an antagonistic activity test was carried out by testing cell culture and the crude enzyme of *B. cereus* SAHA 12.13

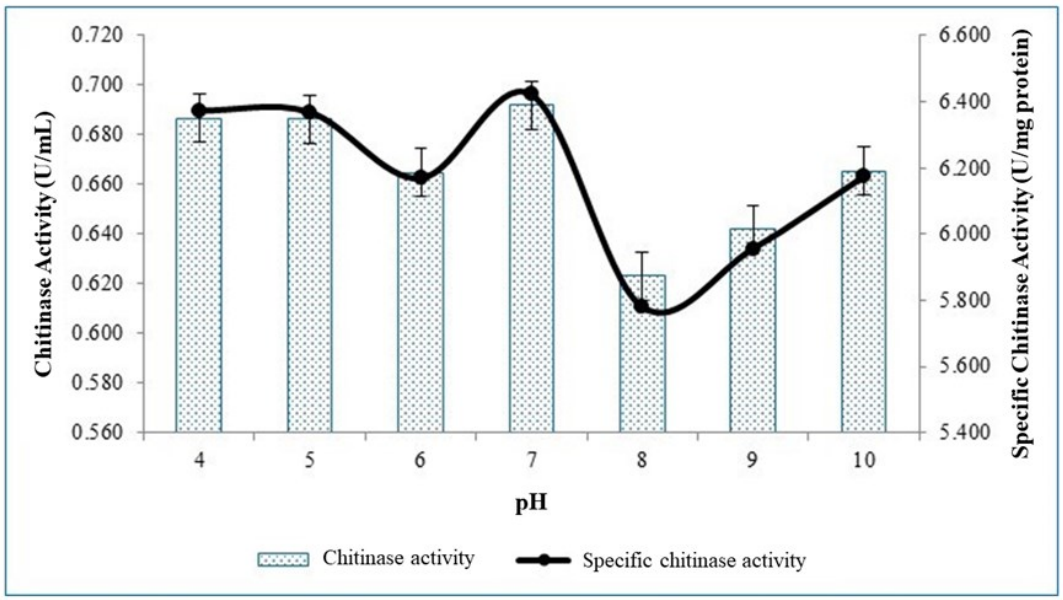

Figure 2. Effect of pH on chitinase activity and specific chitinase activity of *B. cereus* SAHA 12.13.

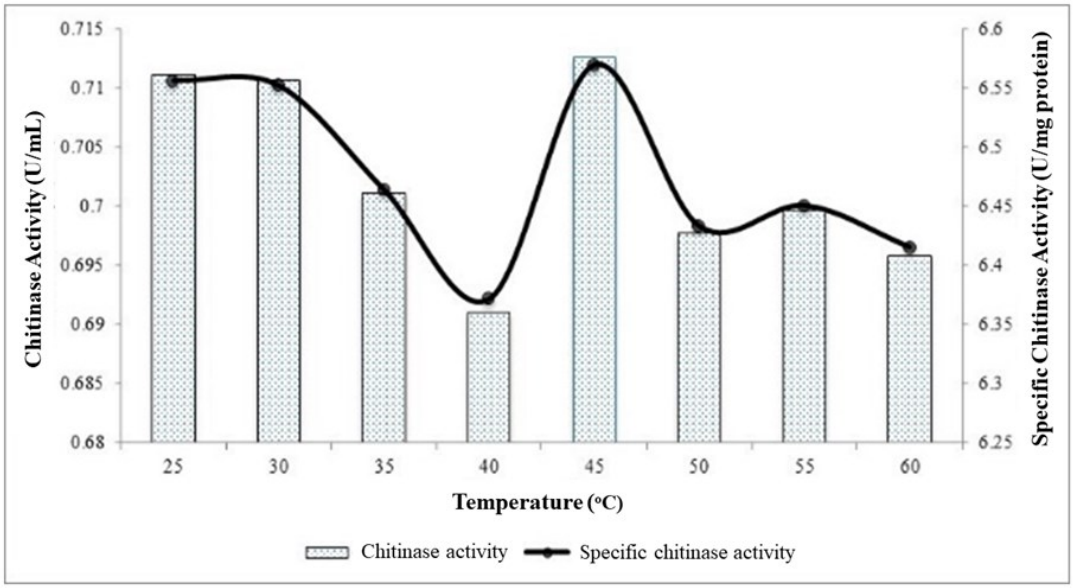

Figure 3. Effect of temperature on chitinase activity and specific chitinase activity of *B. cereus* SAHA 12.13.

against *C. affinis*. The cell culture and crude extract enzyme chitinase of *B. cereus* SAHA 12.13 was taken at the incubation time, with the highest enzyme activity. Inhibition control using sterile distilled water. The inhibition of pathogenic fungal mycelium elongation leading to discs containing cell culture or enzymes and aquadest (control) was observed for 9 days at 28 °C. The percentage of inhibition of pathogenic fungi can be measured using the formula [27]. The chitinase enzyme antagonist test was determined based on the inhibition of pathogenic fungal mycelium during contact with the chitinase enzyme:

$$\% \text{ Inhibition} = [100\% \times (r_1 - r_2) / r_1] \quad \textbf{(4)}$$

where, r_1 is the length of mycelium growth towards the edge of the Petri dish (3 cm), and r_2 is the length of the mycelium towards the well.

3. RESULTS AND DISCUSSIONS

3.1. Growth Curve and Chitinase Activity of Bacillus Cereus SAHA 12.13

B. cereus isolate SAHA 12.13 can grow on Nutrient Broth + 0.3% colloidal chitin media. The isolate can hydrolyze colloidal chitin as a substrate and is used as a source of carbon and nitrogen by bacteria [28]. Chitinase is an inducible enzyme, so the presence of colloidal chitin is an important factor in inducing chitinase production by bacteria [1]. Colloidal chitin is a substrate that is more rapidly hydrolyzed by chitinase produced by bacteria than crude chitin or chitin from fungal cell walls [29]. Bacterial cells began to grow at 6 h of incubation and reached the logarithmic phase at 12 h of incubation (Fig. 1). Cell growth begins to decrease stably at 18-45 h of incubation. A significant decrease in the death phase was detected at 66 h of incubation. This decrease is due to the reduced amount of nutrients contained in the production medium. Based on the growth curve, the *B. cereus* had a growth rate of 0.25 generation/h, so the generation time of this isolate was 3.96 h/ generation. The generation time of bacteria of the genus *Bacillus* tends to be slow. Previous research reported that the *B. cereus* strain BP14 grown in a medium richer in carbon and nitrogen sources such as casein, yeast extract, glucose, and skim milk only had a generation time of 9.45 h [30].

Chitinase production *by B. cereus* SAHA 12.13 began to be detected at the 36th h of the incubation period. The highest chitinase activity was obtained at 45 h of incubation with a specific activity of 8.4897 ± 0.208 U/mg proteins or chitinase activity of 0.721 ± 0.0004 U/mL. The previously reported chitinase activity of *B. cereus* was 0.73 – 0.813 U/ mL [30][31] using 1% colloidal chitin. The chitinase activity of this isolate was better than that of *B. cereus* RBLG1 isolated from *Oryctes*

rhinoceros larvae gut which only had a specific activity from the crude enzyme of only 0.36 U/mg, although the chitinase activity that had been purified with gel filtration with a purity level of 1.90 only increased to 0.69 U/mg [32]. The highest chitinase activity was produced at the stationary phase of cell growth, 9.7 log cells. The highest chitinase activity was found in the stationary phase because the medium used was NB + colloidal chitin. Bacteria will use NB for their growth. The chitinase enzyme will work when the NB nutrients have run out, so it will use colloidal chitin as its carbon source. When chitin appears after other carbon sources are exhausted, the enzyme work will be optimal so that NAG will be easily released [33]. Most bacterial chitinases have optimum activity at an incubation period of 30–96 h [17][34].

3.2. Characterization of Chitinase Activity

The optimum chitinase activity of the crude enzyme of *B. cereus* SAHA 12.13 at pH 7.0 with a chitinase activity value of 0.692 ± 0.294 U/mL and specific chitinase activity of 6,422 ± 0.021 U/mg protein (Fig. 2). The crude enzyme of *B. cereus* SAHA 12.13 shows chitinase activity in a fairly wide pH range. These are indicated by the chitinase activity seen at pH 4.0 to 10.00. This suggests that *B. cereus* SAHA 12.13 bacteria can produce chitinase enzymes and they are active under various environmental pH conditions. Various reports regarding the chitinase enzyme's character are generally at a neutral pH of 6.0 – 7.0, such as *B. cereus* [7][8][35] and *B. thuringiensis* [1] which had optimum activity at pH 7.0.

In addition to pH, temperature plays an essential role in characterizing enzyme activity. The crude extract enzyme *B. cereus* SAHA 12.13 shows optimum activity at 45 °C with chitinase activity of 0.712 ± 0.004 U/mL and specific activity of 6.569 ± 0.034 U/mg protein (Fig. 3). In testing the enzyme activity in the temperature range 25 – 60 °C, the enzyme from *B. cereus* SAHA 12.13 had activity in the mesophylic to the thermophilic environment. In general, chitinase enzymes produced by bacteria have optimum activity at quite varied temperatures, namely at temperatures of 30 – 75 °C [17][36]-[39].

Another characteristic that needs to be studied is the stability of the chitinase enzyme produced by bacteria. The stability of chitinase *B. cereus* SAHA 12.13 enzyme at optimum pH and temperature shows stable activity. The stability of an enzyme at its optimum temperature and pH is influenced by the ionic amino acid residues found in the active site of the protein. These conditions are very important to maintain protein conformation [40]. If the protein conformation is not disturbed by ionic amino acid residues, the enzyme will be stable for a certain time. This is indicated by the absence of a half chitinase activity from the maximum activity for 3 h of incubation (Fig. 4). The $t_{1/2}$ value of chitinase *B. cereus* SAHA 12.13 using first orders rate-law was 770 min or 12.83 h (Fig. 5). Chitinase

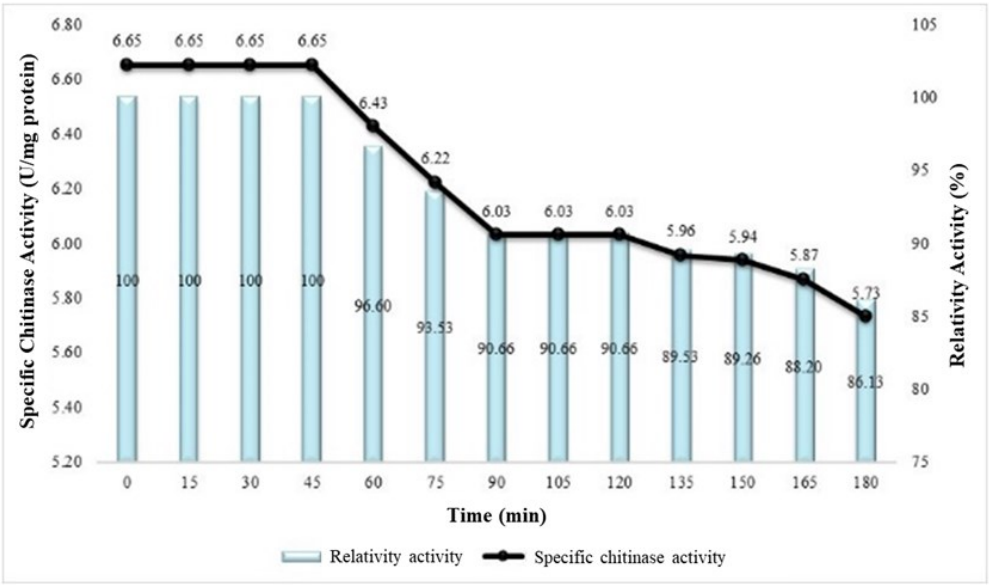

Figure 4. Stability of crude chitinase of *B. cereus* SAHA 12.13 at optimum pH and temperature.

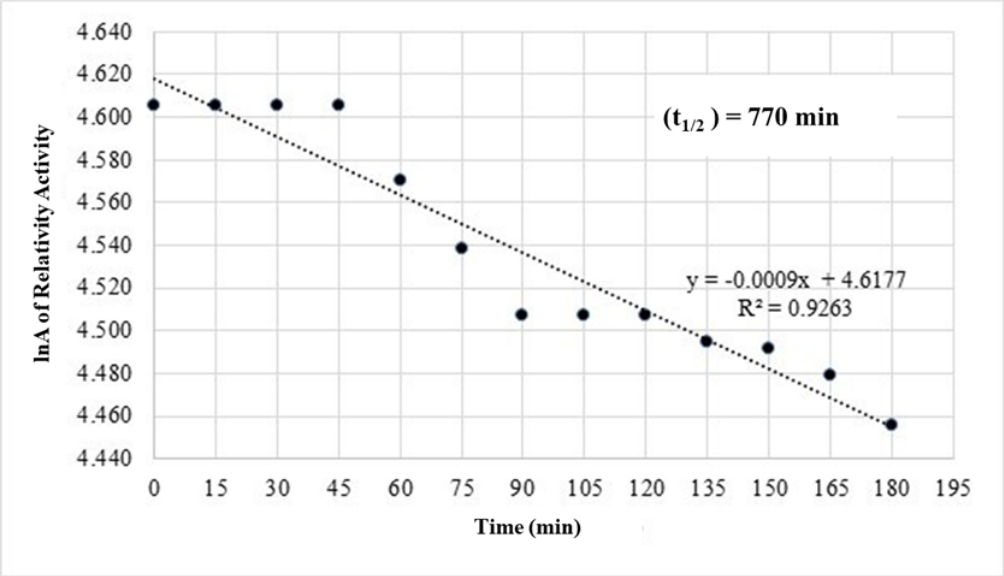

Figure 5. First-order rate-law with linear equations of concentration and time to calculate the half-life of chitinase enzyme stability.

enzyme stability up to 3 h at temperature was also shown by chitinase from *Streptomyces* sp. The chitinase of this isolate was stable for 180 minutes at a temperature of 20 – 40 °C [41]. The stability of chitinase at 45 °C for 8 h was shown by *Paenibacillus* sp. [42]. However, this result was better when compared to the purified chitinase produced by *Bacillus* sp. R2. This enzyme is stable for 1 h at a temperature of 40 °C and a pH of 7 – 8 [43].

Chitinase with good stability at temperature and optimum pH is often associated with the synergism of the enzyme with its ability as a biocontrol of disease [1]. This is because bacteria with stable chitinase activity will perform chitin-degrading activities within a specified period with the best ability without decreased activity if applied to pathogens such as pathogenic fungi.

3.3. Antagonistic Activity of B. cereus SAHA 12.13 Isolates Against C. affinis

The growth inhibition of the pathogenic fungi C. affinis was carried out in vitro using 45-h cell culture and crude enzyme chitinase of B. cereus SAHA 12.13. Crude enzyme extracts had better inhibition than cell culture. Crude enzyme inhibited C. affinis by 36.27 ± 0.043% and 45-h cell culture by 34.25 ± 0.041% on the 6th day after inoculation (Table 1). The 9-d observation showed that cell culture and crude enzyme inhibited C. affinis. However, the crude enzyme extract showed cell growth around the well, and the observation was carried out until 9 d, thus allowing the cells to grow. The inhibition of cell culture and crude enzyme extract showed quite different inhibitions. Given that chitinase is able to degrade chitin in the fungal cell wall [44], the amount of chitinase in the form of crude enzyme is higher because it has been released to the outside of the cell compared to cell culture, which causes better inhibitory activity. As reported by Lee et al. [45], the activity of supernatant or crude extract chitinase from Streptomyces cavourensis is able to inhibit spore germination and form malformations in hyphae *Colletotrichum gloeosporioides*. This is indicated by the presence of inhibition in the spores of *C. affinis* (black spores disappear) in this study.

The inhibition shown by cell culture and enzyme crude extract was in the form of spore inhibition and slight mycelial inhibition. The mycelium manifestations around the inhibition zone indicated that the chitinase produced by *B. cereus* SAHA

Table 1. Percentage of inhibition of B. cereus SAHA 12.13 against C. affinis after nine days of incubation.

Treatments	% Inhibition
45-hour cell culture	34.25 ± 0.041
Crude chitinase enzyme	36.27 ± 0.043

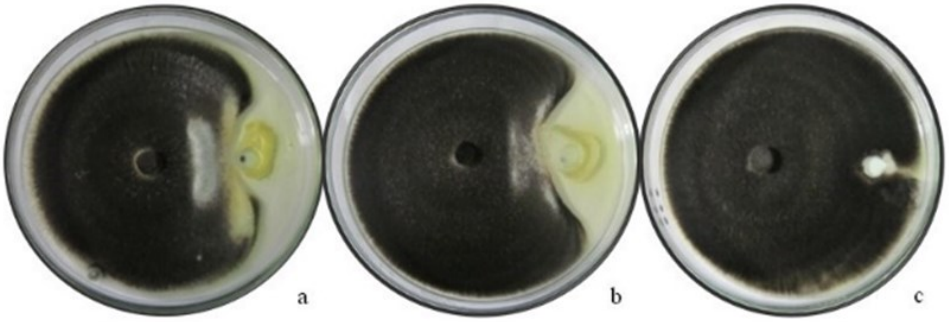

Figure 6. Antagonistic activity of a) cell culture 45-hours and b) crude extract chitinase of *B. cereus* SAHA 12.13 and c) control against *C. affinis* after 9 days of incubation at 28 °C.

12.13 could only inhibit or degrade chitin in spores but not be too active in inhibiting chitin in the mycelium (Fig. 6). Chitinase plays a key role in hyphal/mycelium development and branching, spore production and germination, fungal cell wall degradation or morphogenesis of fungal cell wall structure [46]. This key role defines the role of *B. cereus* SAHA 12.13 chitinase enzyme in inhibiting pathogenic fungi, namely inhibiting spore production and germination. The same report showed that chitinase from *B. cereus sensu lato* B25 directly inhibited *Fusarium verticillioides* by inhibiting fungal conidia germination [6].

Black spores in *C. affinis* are black pigments in the form of DHN-melanin pigments found in mycelium [47]. DHN-melanin in fungal pathogens is not an essential factor in cell growth and development, but it can make fungi survive and compete in extreme environments. Additionally, melanin protects against sclerotia and conidia from lysis and turgor pressure for appressoria to penetrate the leaves of plant leaves [48]-[50]. Inhibition of black spores by crude chitinase of *B. cereus* SAHA 12.13 also developed that crude chitinase isolates reduced the black pigment of *C. affinis*. Melanin is localized in the cell wall of pathogenic fungi and crosslinks with polysaccharides [48][49]. Chitin is a kind of polysaccharide in fungal cell walls. This condition is expected to indirectly degrade DHN-melanin on the mycelium or conidia walls so that it can reduce the mycelium pigmentation of *C. affinis* [18].

In general, bacteria can inhibit pathogenic fungi in various ways, including the production of antibiotics such as iturin, bacillomycin, surfactin, fengycin, and bacteriocin [51], antifungal, lytic enzymes such as chitinase [52], β-1,3-glucanase [18] and several other hydrolysis enzymes. Chitinase produced by *B. cereus* was able to inhibit the growth of *F. verticillioides* [6], *F. oxysporum, Rhizoctonia solani* and *C. gloeosporioides* [8]. The differences in the effects of chitinase produced by bacteria on the morphology of fungi have been widely reported. The main effects produced are inhibition of spore germination, spore rupture, shortening of hyphae, and germ tube elongation [29].

4. CONCLUSIONS

The highest chitinase activity from *B. cereus* SAHA 12.13 was obtained at 45 h of incubation with a growth rate of 0.25 generation/h and a generation time of 3.96 h/generation. Crude enzymes have optimum activity at pH 7.0, 45 °C and they are stable at optimum pH and temperature. Crude extract enzymes and cell culture could inhibit *C. affinis* by inhibiting spores. The chitinase character and the potential shown by *B. cereus* SAHA 12.13 is expected to be applied as a biocontrol agent against leaf blight attacks in oil palm nurseries with diverse environmental conditions.

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Conceptualization, M.A.; Methodology, M.A.; Validation, M.A., D.S; Analysis and Interpretation of Data, M.A. and D.S.; Data Curation, X.X.; Writing – Original Draft Preparation, M.A. and D.S.; Writing – Review & Editing, M.A. and D.S. Both authors have read and approved to the final version of the manuscript.

Conflicts of Interest

The author(s) declare no conflict of interest.

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REFERENCES

[1] E. Z. Gomaa. (2012). "Chitinase production by Bacillus thuringiensis and Bacillus licheniformis: their potential in antifungal biocontrol". *Journal of Microbiology.* **50** (1): 103-11. https://doi.org/10.1007/s12275-012-1343-y.

[2] S. Banerjee and N. C. Mandal. (2019). In: "Microbial Diversity in Ecosystem Sustainability and Biotechnological Applications, ch. Chapter 18". 457-491. https://doi.org/10.1007/978-981-13-8487-5_18.

[3] M. Ikeda, K. Miyauchi, A. Mochizuki, and M. Matsumiya. (2009). "Purification and characterization of chitinase from the stomach of silver croaker Pennahia argentatus". *Protein Expression and Purification.* **65** (2): 214-22. https://doi.org/10.1016/j.pep.2009.01.015.

[4] N. Jayanthi, M. G. M. Purwanto, R. Chrisnasari, T. Pantjajani, A. Wahjudi, and M. Sugiarto. (2019). "Characterization of thermostable chitinase from Bacillus licheniformis B2". *IOP Conference Series: Earth and Environmental Science.* **293** (1). https://doi.org/10.1088/1755-1315/293/1/012030.

[5] M. Mubarik. (2010). "Chitinolytic Bacteria Isolated from Chili Rhizosphere: Chitinase Characterization and Its Application as Biocontrol for Whitefly (Bemisia tabaci Genn.)". *American Journal of Agricultural and Biological Sciences.* **5** (4): 430-435. https://doi.org/10.3844/ajabssp.2010.430.435.

[6] E. Morales-Ruiz, R. Priego-Rivera, A. M. Figueroa-Lopez, J. E. Cazares-Alvarez, and I. E. Maldonado-Mendoza. (2021). "Biochemical characterization of two chitinases from Bacillus cereus sensu lato B25 with antifungal activity against Fusarium verticillioides P03". *FEMS Microbiology Letters.* **368** (2). https://doi.org/10.1093/femsle/fnaa218.

[7] A. Kumar and G. Dukariya. (2020). "Chitinase Production from Locally Isolated Bacillus cereus GS02 from Chitinous Waste Enriched Soil". *Journal of Advances in Biology & Biotechnology.* 39-48. https://doi.org/10.9734/jabb/2020/v23i130137.

[8] N. Thakur, A. K. Nath, A. Chauhan, and R. Gupta. (2022). "Purification, characterization, and antifungal activity of Bacillus cereus strain NK91 chitinase from rhizospheric soil samples of Himachal Pradesh, India". *Biotechnology and Applied Biochemistry.* **69** (5): 1830-1842. https://doi.org/10.1002/bab.2250.

[9] A. M. Figueroa-Lopez, K. Y. Leyva-Madrigal, R. G. Cervantes-Gamez, L. I. Beltran-Arredondo, N. R. Douriet-Gamez, C. Castro-Martinez, and I. E. Maldonado-Mendoza. (2017). "Induction of Bacillus cereus chitinases as a response to lysates of Fusarium verticillioides". *Romanian Biotechnological Letters.* **22** (4): 12722-31.

[10] C. S. Devi, V. M. Srinivasan, B. Archana, S. S. Roy, and S. J. Naine. (2015). "Production and partial purification of antifungal chitinase from Bacillus cereus VITSD3". *Bioscience*

Journal. **31** (3): 960-968. https://doi.org/10.14393/BJ-v31n3a2015-26263.

[11] E. Kurniawan, S. Panphon, and M. Leelakriangsak. (2019). "Potential of marine chitinolyticBacillusisolates as biocontrol agents of phytopathogenic fungi". *IOP Conference Series: Earth and Environmental Science.* **217**. https://doi.org/10.1088/1755-1315/217/1/012044.

[12] R. Marlin and M. Asril. (2022). "Inhibition of Bilimbi Leaf Extract (Averrhoa bilimbi Linn.) Against the Growth of Candida albicans ATCC 10231". *Jurnal Biota.* **8** (1): 39-46. https://doi.org/10.19109/Biota.v8i1.9554.

[13] A. Rostami, K. Hinc, F. Goshadrou, A. Shali, M. Bayat, M. Hassanzadeh, M. Amanlou, N. Eslahi, and G. Ahmadian. (2017). "Display of B. pumilus chitinase on the surface of B. subtilis spore as a potential biopesticide". *Pesticide Biochemistry and Physiology.* **140** : 17-23. https://doi.org/10.1016/j.pestbp.2017.05.008.

[14] N. Dahiya, R. Tewari, and G. S. Hoondal. (2006). "Biotechnological aspects of chitinolytic enzymes: a review". *Applied Microbiology and Biotechnology.* **71** (6): 773-82. https://doi.org/10.1007/s00253-005-0183-7.

[15] S. Dwi. (2012). "A possibility of chitinolytic bacteria utilization to control basal stems disease caused by Ganoderma boninense in oil palm seedling". *African Journal of Microbiology Research.* **6** (9). https://doi.org/10.5897/ajmr11.1343.

[16] B. F. Azadeh, M. Sariah, and M. Y. Wong. (2012). "Characterization of Burkholderia cepacia genomovar I as a potential biocontrol agent of Ganoderma boninense in oil palm". *African Journal of Biotechnology.* **9** (24): 3542-3548. https://doi.org/10.4314/ajb.v9i24.

[17] N. R. Mubarik, M. Asril, and A. T. Wahyudi. (2014). "Partial Purification of Bacterial Chitinase as Biocontrol of Leaf Blight Disease on Oil Palm". *Research Journal of Microbiology.* **9** (6): 265-277. https://doi.org/10.3923/jm.2014.265.277.

[18] R. Dewi, N. Mubarik, and M. Suhartono. (2016). "Medium optimization of beta-glucanase production by Bacillus subtilis SAHA 32.6 used as biological control of oil palm pathogen". *Emirates Journal of Food and Agriculture.* **28** (2). https://doi.org/10.9755/ejfa.2015-05-195.

[19] Y. S. Kurniawan, T. Indriani, H. Amrulloh, L. C. Adi, A. C. Imawan, K. T. A. Priyangga, and E. Yudha. (2023). "Journey of Natural Products: From Isolation Stage to Drug's Approval in Clinical Trials". *Bioactivities.* **1** (2): 43-60. https://doi.org/10.47352/bioactivities.2963-654X.190.

[20] K. Sak. (2023). "Role of Flavonoids as Potential Plant Fungicides in Preventing Human Carcinogenesis: A Short Communication". *Bioactivities.* **1** (2): 39-42. https://doi.org/10.47352/bioactivities.2963-654X.187.

[21] J. Kittimorak, C. Pornsuriya, A. Sunpapao, and V. Petcharat. (2013). "Survey and Incidence of Leaf Blight and Leaf Spot Diseases of Oil Palm Seedlings in Southern Thailand". *Plant Pathology Journal.* **12** (3): 149-153. https://doi.org/10.3923/ppj.2013.149.153.

[22] Suwandi, S. Akino, and N. Kondo. (2012). "Common Spear Rot of Oil Palm in Indonesia". *Plant Disease.* **96** (4): 537-543. https://doi.org/10.1094/PDIS-08-10-0569.

[23] A. Haryanto, N. R. Mubarik, and S. Listiyowati. (2013). "Isolation of Chitinolytic Bacteria Used as Biological Control of Suspected Pathogenic Fungi on Oil palm Seedlings". *Undergraduate Thesis*. IPB University, Bogor.

[24] F. Widdel. (2010). "Theory and Measurement of Bacterial Growth". Grundpraktikum Mikrobiologie.

[25] K. Spindler. (1997). In: " P. Jollès and R. A. A. Muzzarelli (Eds) Chitin Handbook". Alda Tecnografica, Boston.

[26] M. M. Bradford. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding". *Analytical Biochemistry.* **72** : 248-54. https://

doi.org/10.1006/abio.1976.9999.

[27] N. J. Fokkema. (1973). "The rôle of saprophytic fungi in antagonism against Drechslera sorokiniana (Helminthosporium sativum) on agar plates and on rye leaves with pollen". *Physiological Plant Pathology.* **3** (2): 195-205. https://doi.org/10.1016/0048-4059(73)90082-9.

[28] H. Natsir, A. R. Patong, M. T. Suhartono, and A. Ahmad. (2010). "Oduction And Characterization Of Chitinase Enzymes From Sulili Hot Spring In South Sulawesi, Bacillus sp. HSA,3-1a". *Indonesian Journal of Chemistry.* **10** (2): 256-260. https://doi.org/10.22146/ijc.21470.

[29] T. Taechowisan, J. Peberdy, and S. Lumyong. (2003). "Chitinase production by endophytic Streptomyces aureofaciens CMUAc130 and its antagonism against phytopathogenic fungi". *Annals of Microbiology.* **53** : 447-461.

[30] M. Dliyauddin, T. Ardyati, and S. Suharjono. (2020). "Evaluation of Proteolytic and Chitinolytic Activities of Indigenous Bacillus Speciesfrom Crab Shell Waste". *The Journal of Experimental Life Sciences.* **10** (1): 1-5. https://doi.org/10.21776/ub.jels.2019.010.01.01.

[31] N. Herdyastuti, F. Fauziah, R. Widodo, Y. Prabowo, I. Apriliana, and S. Cahyaningrum. (2021). "Diversity of Chitinolytic Bacteria from Shrimp Farms and Their Antifungal Activity". *Journal of Natural Science, Biology and Medicine.* **12** (3): 317-324.

[32] A. N. Idris, S. S. Tang, and A. F. Mohd Adnan. (2021). "Study of Thermostable Chitinase Isolated and Purified from Oryctes rhinoceros Larvae Gut". *Sains Malaysiana.* **50** (2): 339-349. https://doi.org/10.17576/jsm-2021-5002-06.

[33] A. Alhasawi and V. D. Appanna. (2017). "Enhanced extracellular chitinase production in Pseudomonas fluorescens: biotechnological implications". *AIMS Bioengineering.* **4** (3): 366-375. https://doi.org/10.3934/bioeng.2017.3.366.

[34] N. Nurdebyandaru, N. Rachmania Mubarik, and T. Sri Prawasti. (2010). "Chitinolytic Bacteria Isolated from Chili Rhizosphere: Chitinase Characterization and Application as Biocontrol for Aphis gossypii". *Microbiology Indonesia.* **4** (3): 103-107. https://doi.org/10.5454/mi.4.3.1.

[35] S. Sudha, P. Sharon, K. Yadav, and R. Priyanka. (2020). "Optimization of Chitinase Production from Lake Sediment Inhabitant Bacillus thuringiensis Strain LS1 and Bacillus cereus Strain LS2". *Asian Journal of Pharmaceutics.* **14** (2): 175-182. https://doi.org/10.22377/AJP.V14I2.3611.

[36] W. M. Haggag and E. Abdallh. (2012). "Purification and Characterization of Chitinase Produced by Endophytic Streptomyceshygroscopicus Against Some Phytopathogens". *Journal of Microbiology Research.* **2** (5): 145-151. https://doi.org/10.5923/j.microbiology.20120205.06.

[37] S. Margino, C. Behar, and W. Asmara. (2012). "Isolation and Purification of Chitinase Bacillus sp. D2 Isolated from Potato Rhizosfer". *Indonesian Journal of Biotechnology.* **17** (1): 69-78. https://doi.org/10.22146/IJBIOTECH.7851.

[38] M. S. Brzezinska, U. Jankiewicz, and M. Walczak. (2013). "Biodegradation of chitinous substances and chitinase production by the soil actinomycete Streptomyces rimosus". *International Biodeterioration & Biodegradation.* **84** : 104-110. https://doi.org/10.1016/j.ibiod.2012.05.038.

[39] N. Harini, Y. X. Han, and J. Sukweenadhi. (2021). "The Role of Calcium Ions to Improve Activity of Chitinase Isolated from Vibrio sp". *Jordan Journal of Biological Sciences.* **14** (05): 925-931. https://doi.org/10.54319/jjbs/140507.

[40] K. Subramanian, B. Sadaiappan, W. Aruni, A. Kumarappan, R. Thirunavukarasu, G. P. Srinivasan, S. Bharathi, P. Nainangu, P. S. Renuga, A. Elamaran, D. Balaraman, and M. Subramanian. (2020). "Bioconversion of chitin and concomitant production of chitinase and N-acetylglucosamine by novel Achromobacter xylosoxidans isolated from shrimp waste disposal area". *Scientific Reports.* **10** (1): 11898. https://doi.org/10.1038/s41598-020-68772-y.

[41] N. Karthik, P. Binod, and A. Pandey. (2015). "Purification and characterisation of an acidic and antifungal chitinase produced by a Streptomyces sp". *Bioresource Technology.* **188** : 195-201. https://doi.org/10.1016/j.biortech.2015.03.006.

[42] J. Du, S. Duan, J. Miao, M. Zhai, and Y. Cao. (2021). "Purification and characterization of chitinase from Paenibacillus sp". *Biotechnology and Applied Biochemistry.* **68** (1): 30-40. https://doi.org/10.1002/bab.1889.

[43]` B. A. Cheba, T. I. Zaghloul, A. R. El-Mahdy, and M. H. El-Massry. (2016). "Effect of pH and Temperature on Bacillus sp. R2 Chitinase Activity and Stability". *Procedia Technology.* **22** : 471-477. https://doi.org/10.1016/j.protcy.2016.01.092.

[44] K. Liu, H. Ding, Y. Yu, and B. Chen. (2019). "A Cold-Adapted Chitinase-Producing Bacterium from Antarctica and Its Potential in Biocontrol of Plant Pathogenic Fungi". *Marine Drugs.* **17** (12). https://doi.org/10.3390/md17120695.

[45]` S. Y. Lee, H. Tindwa, Y. S. Lee, K. W. Naing, S. H. Hong, Y. Nam, and K. Y. Kim. (2012). "Biocontrol of anthracnose in pepper using chitinase, beta-1,3 glucanase, and 2-furancarboxaldehyde produced by Streptomyces cavourensis SY224". *Journal of Microbiology and Biotechnology.* **22** (10): 1359-66. https://doi.org/10.4014/jmb.1203.02056.

[46] N. D. Putri, L. Sulistyowati, L. Q. Aini, A. Muhibuddin, and I. Trianti. (2022). "Screening of endophytic fungi as potential antagonistic agents of Pyricularia oryzae and evaluation of their ability in producing hydrolytic enzymes". *Biodiversitas Journal of Biological Diversity.* **23** (2). https://doi.org/10.13057/biodiv/d230248.

[47] T. Lanisnik Rizner and M. H. Wheeler. (2003). "Melanin biosynthesis in the fungus Curvularia lunata (teleomorph: Cochliobolus lunatus)". *Canadian Journal of Microbiology.* **49** (2): 110-9. https://doi.org/10.1139/w03-016.

[48] M. J. Beltran-Garcia, F. M. Prado, M. S. Oliveira, D. Ortiz-Mendoza, A. C. Scalfo, A. Pessoa, Jr., M. H. Medeiros, J. F. White, and P. Di Mascio. (2014). "Singlet molecular oxygen generation by light-activated DHN-melanin of the fungal pathogen Mycosphaerella fijiensis in black Sigatoka disease of bananas". *PLoS One.* **9** (3): e91616. https://doi.org/10.1371/journal.pone.0091616.

[49] I. Geoghegan, G. Steinberg, and S. Gurr. (2017). "The Role of the Fungal Cell Wall in the Infection of Plants". *Trends in Microbiology.* **25** (12): 957-967. https://doi.org/10.1016/j.tim.2017.05.015.

[50] N. Ludwig, M. Lohrer, M. Hempel, S. Mathea, I. Schliebner, M. Menzel, A. Kiesow, U. Schaffrath, H. B. Deising, and R. Horbach. (2014). "Melanin is not required for turgor generation but enhances cell-wall rigidity in appressoria of the corn pathogen Colletotrichum graminicola". *Molecular Plant-Microbe Interactions.* **27** (4): 315-27. https://doi.org/10.1094/MPMI-09-13-0267-R.

[51] R. Ramachandran, A. G. Chalasani, R. Lal, and U. Roy. (2014). "A broad-spectrum antimicrobial activity of Bacillus subtilis RLID 12.1". *Scientific World Journal.* **2014** : 968487. https://doi.org/10.1155/2014/968487.

[52] S. Atalla, N. E. Gamal, and H. Awad. (2020). "Chitinase of Marine Penicillium chrysogenum MH745129: Isolation, Identification, Production and Characterization as Controller for Citrus Fruits Postharvest Pathogens". *Jordan Journal of Biological Sciences.* **13** (1): 19-28.