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Analysis and Characterization of Chitinase in *Bacillus salmalaya* Strain 139SI

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ABSTRACT

Introduction: Chitinases are enzymes that hydrolyze the internal β-1,4 glycosidic linkages of chitin, a significant structural component of arthropod exoskeletons and fungus cell walls.

Objective: Main objective of current study was to determine the protein and to check the antimicrobial, antifungal activity of chitinase in *Bacillus salmalaya* strain 139SI.

Methodology: Purified and estimation enzymes were quantified by the method of Lowry method. Antimicrobial action of chitinase was done using standard Disc Diffusion method while the hyphal extension inhibition assay was used to test the antifungal activity of pure chitinase strains 139SI, 140SI, and 141SI.

Results: In this study, *Bacillus salmalaya* 139SIexhibited strong haemolytic activity and their protein concentration was measured as 56.43 mg/mL. In addition, strain 139SI had strong antifungal activity against phytopathogenic fungus including *Fusarium* sp., *R. solani* and *Phytophthora* sp. Strain 139SI had capability in degrading the peptidoglycan component of cell walls gram-negative bacteria such as *Escherichia coli* but not against the gram positive, *Staphylococcus aureus*. Chitinase activity was observed when 200µl crude extract of 139SI able to degrade 0.09 g chitin of shrimp shell by breaking down shrimp shell structure and bonds of chitin effectively as early as 2 days or up to 7 days.

Conclusion: Hence, based on the results, *B. salmalaya* 139SI has potential to be a novel biofunctional chitinase that could use as biological agent in degrading the chitin component of fungal cell walls and shells waste of many kind of insect and crustacean for solving future problem in agricultural sector and fishery industry.

Key Words: Chitinases, *B. Salmalaya*, Biofunctional, Peptidoglycan, Antimicrobial, Antifungal activity

INTRODUCTION

Many diseases, affect agricultural crops, resulting in considerable productivity losses including fungal infections.[1]The cell wall of the fungus is the first structure that comes in contact with the host. Chitin is a homopolymer of *N-acetyl-glucosamine* (GlcNAc) units that makes up the major component of the fungal cell wall.[2] As chitin is a major cell wall component of most pathogenic fungus, chitinase-producing bacteria can break down chitin, which can be employed as a biological control of fungal infection in crops. Chitinase also has been proved in as biocontrol agents in degrading chitin of exoskeleton of white fly.[3]

Chitin is a crucial part in the construction of fungal cell walls, as well as other vertebrates (fish scales) and invertebrates (mollusk, nematode, worm, arthropod, cephalopod

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etc.).[4] Chitinase is a hydrolytic enzyme and belongs to the glycosyl-hydrolase family that hydrolyzes glycosidic links in chitin and converts polymeric chitin to chitooligosaccharides.[5]

Furthermore, seafood processing businesses, notably those in the processing foods industry, exploit marine resources to meet humanity's needs, resulting in massive amounts of chitin-containing waste in coastal areas.[6] Despite of its gigantic abundance, utilization of chitinous waste such as crab, shrimp,lobster is still in its primitive stage due to the crystallinity and insolubility of chitin itself. Chitinase capable in breakage of bonds typical to that of chitin in shrimp therefore, degradation of chitin crustacean waste by chitinase enzyme is one of the industrial interest because it can be use as bioremediation of seafood waste at large scale and has green characteristics, not harm the environments.[6] The main objective of this study was to analyse and characterize chitinase activities in *B. salmalaya* strain 139SI. To analyse chitinase activities in *B. salmalaya* 139SI, 140SI and 141SI strains and their relationship with chitinase enzyme purification and observe potential of the strains against various type of fungus and bacteria.To test capability of the strains in degrading chitin composed material such as shrimp shell.

MATERIALS AND METHODOLOGY

Isolation and screening of the strain

Bacillus salmalaya 139SI was first discovered in soil from a private farm in Selangor, Malaysia.[7]

Growth condition and Chitinase production

Single colony of 139SI, 140SI and 141SI from Brain Heart infusion(BHI) agar plate was inoculated and grown in 1 litre of Difco™, USA.BHI medium containing potassium chloride (5 g/L), dextrose (3 g/L), disodium hydrogen phosphate (2.5 g/L), gelatin (14.5 g/L), BHI (6 g/L), and peptic digest of animal tissue (6 g/L) plus 1 percent (w/v) chitin powder from shrimp shell as inducer was shaken in a shaking incubator at 150 rpm for 72 hours at 35 °C. The 72-hour culture was centrifuged (8000×g for 15 minutes) and filtered through a Whatman no.1 filter using a SORVALL ST 16R centrifuge. The cell-free broth or supernatant was concentrated by freeze-drying (at Microbiology laboratory, near PASUM) and was stored at −20 °C.

Protein determination

Purified and estimation enzymes were quantified by the method of Lowry method.[8] Bovine serum albumin was used as standard. Firstly, Lowry reagent or alkaline copper sulfate solution was prepared by adding and mixing 50 ml solution A (2% sodium carbonate was added into 0.1 M NaOH) with 1 ml of solution B (0.5% copper sulfate was added into 1% sodium potassium tartrate solution). Then, Standard Protein Solution (BSA) was prepared by dissolving 200 mg of BSA into 100 ml distilled water and diluted this stock BSA into 100 ml of distilled water. Finally, the solution was prepared and its absorbance measured at 700 nm using the NV203 spectrophotometer.

In the test tubes, different dilutions of BSA solutions were prepared by mixing stock BSA solution (1 mg/ ml) and water as shown in table 1. The BSA concentration ranges from 0.05 to 1 mg/ml. 5 mL alkaline copper sulphate reagent was added to these various dilutions. After incubating for 10 minutes at room temperature, added 0.5 ml of reagent FolinCiocalteau solution (reagent solutions) to each tube and incubated again for 30 minutes or until the solution becomes blue. Each of the test tubes has a final volume of 6.5 ml. A standard calibration curve was created by plotting the absorbance against protein concentration graph.

Assay of purified chitinase for antifungal

The hyphal extension inhibition assay was used to test the antifungal activity of pure chitinase strains 139SI, 140SI, and 141SI.[9]

Antimicrobial action of chitinase

Antimicrobial action of Chintinase was done using standard Disc Diffusion method.[10]

100ug/ml original extract of chitinase crust 139SI, 140SI and 141SI were dropped onto disc (6.0/mm). Then, they were place 25mm perimeter from each other onto bacteria culture along with positive and negative control dick.The inoculated plates were cooled to allow the bioactive compounds to diffuse into the agar, then incubated at 37°C and checked for inhibitory zones around the wells after 24, 48, and 72 hours. A ruler was used to measure inhibition zones in millimetres under and around discs.

Effect of crude chitinase prawn shell

Two prawn shell were prepared and treated with different concentration of purified chitinase of strain 139. Prawn shell 1 was treated with 200ug/ml of strain 139SI, while prawn shell 2 was treated with 400ug/ml strain 139SI. Then, shell structure was observed in day 1, 3,7,14,21,28 and 41 days to identify the ability of chitinase in degradation action under 1000x magnification compound microscope (NIKON 261871).

RESULTS

Chitinase production

Chitinase crust (in powder form) produced and extracted from 25ml of bacterial supernatant of each strain of *Bacillussalmalaya* after being freeze-dried. Strain 141SI produced large

amount which is 1.19g compared with strain 1140SI (1.06g) and 139SI (0.89g), respectively.

Enzyme purification

Concentration of chitinase of each strain plotted against absorbance at 700nm on BSA standard graph are highlighted in figure 1. Concentration purified protein of strain 139SI was 87.86 Mg ml $^{-1}$ and its absorbance was 0.464 O.D. While, concentration purified protein of strain 140SI and 141SI were 82.70 Mg ml $^{-1}$ and 56.43 Mg ml $^{-1}$ and their absorbance were 0.435 O.D and 0.288 O.D, respectively. Based on the result, regularity of purified enzyme chitinase in each sample can be simplified as shown; 139SI>141SI>140SI.

Antifungal

Based on the Figure 2(a & b), the results showed that 100ug/ml chitinase could inhibit growth of *R. solani.* Mean and standard error of inhibition zone of strain 139SI within 2 dayswas 15 ± 9.90, while strain 140SI and 141 SI were 11.5 ± 4.95 and 13.5 ± 6.36, respectively. The inhibitory indices of all samples have a certain regularity against *R. solani,* which is 139SI> 141SI > 140SI.

Based on the Figure 2(c & d), the results showed that 100µg/ml of all purified chitinase could inhibit mycelial growth of *Phytophthora sp.* Mean and standard error of inhibition zone of strain 139SI within 2 dayswas 19 ± 10.61, while strain 140SI and 141 SI were 16.5 ± 10.61 and 16.5 ± 7.78, respectively. The inhibitory indices of all samples have a certain regularity against *Phytophthora sp.* mycelial growth which is 139SI> 141SI > 140SI.

Based on the Figure 2 (e &f), the results showed that 100µg/ml of each purified chitinase could inhibit growth of *Fusarium sp.*Mean and standard error of inhibition zone of strain 139SI within 2 dayswas 15±11.31 while strain 140SI and 141 SI were 11.5±7.78 and 13.5±9.19,respectively. The inhibitory indices of all samples have a certain regularity against *Fusarium sp.* mycelial growth which is 139SI> 141SI > 140SI.

Overview of mean and standard error of purified chitinase in inhibit the mycelial growth of three different type of pyhtopathogenic fungi; *R.solani*, *Pytophthora sp*., *Fusarium sp*. are shown in Table 2.

Antibacterial

100ug/ml of purified chitinase of each strain 139SI, 140SI and 141SI had been tested against *S. aureus* (gram +ve) and *E. coli* (gram -ve). Based on the observation after 24 hours, all purified chitinase were resistant towards *S. aureus* while, strain 139SI was more sensitive compare to strain 140SI and 141SI against *E. coli* (Figure 3 a & b).

Effect of crude chitinase on prawn waste

Two prawn shell were treated with different concentration of chitinase 139SI. Prawn shell that was treated with 200ug/ml of chitinase was started to degrade at day 7 and their mass. While, prawn shell treated with 400ug/ml of chitinase was degrade as early as day 3 and their shell part obviously shrunken at day 7 (figure 4 a, b,c,d & e).

DISCUSSION

Enzyme purification or protein measurement Lowry assay was used to measure amount of purified chitinase concentration in each strain. The quantity of protein was calculated using a BSA standard curve, and the advantages of this test include its sensitivity and, most significantly, accuracy.[11] Crust chitinase of each sample were diluted twice before measured at 700nm. Based on the result obtained from BSA standard graph (figure1), strain 139SI have highest amount of purified chitinase enzyme concentration which is, 87.86 Mg ml^{-1} at 0.464 O.D. Strain 140SI contained 82.70 Mg ml^{-1} of chitinase enzyme concentration at 0.435 O.D. While, strain 141SI only have 56.43 Mg ml^{-1} of purified chitinase at 0.288 O.D even though, it is produced highest amount of chitinase crust (powder form) in previous assay (freeze-drying assay). Hence, it proved that chitinase crust of 141SI was not pure compared to 139SI. The reason might due to the chitinase crust was dominated by other substance. Increased concentration of crude extracts will be accompanied with the enhancement of cell viability.[7] Meanwhile, highest concentration of chitinase enzyme will lead to increases of performance especially against various type of fungus, bacteria and ability in degradation of chitin-composed material.

Pathogenic fungi can cause disease in human and animal and contamination and severe damage to crops, which lead to huge economic losses.[12] Significant growth retardation in the mycelial growth of *R. solani*, *Fusarium sp.* and *phytophthora* sp.by strain 139SI was observed. 139SI showed strong antifungal activity against all these types of fungi as strong as positive control inhibit fungal growth. While, the zone of inhibition was less around the wells when applied with strain 141SI and followed with strain 140SI. The antifungal activity indices of all samples have a certain regularity, which is 139SI> 141SI > 140SI. Results above demonstrated inhibitory rates and antifungal activity increases with the rise of the concentration of purified chitinase. As the 139SI has highest concentration of chitinase enzyme (87.86 mg/ml), hence it is more efficient in inhibition all chitin-containing fungi. Antifungal activities must be along with hyphae distortion, heavy vacuolization and swelling and lysing hyphae.[13] It can be inferred that over-expression of recombinant chitinase protein or enzyme has a strong and considerable antifungal effect.

Among all those three types of pathogenic fungi, 139SI was more sensitive towards *phtophthora* sp. and less effective against *Fusarium* sp. especially after 24 h incubated. Figure 2 depicts the inhibition zone of all pure chitinases, including 139SI, which ranges from 6 to 7 mm only after 24 hours. Therefore, the degree of inhibition is proportional to the amount of chitin in the cell wall of the target fungus.Chitinolytic enzymes, as well as the genes that code for them, could be used to create transgenic microorganisms with improved biocontrol capabilities and could be used to control fungal plant pathogens.[13] The appearance of unambiguous zones of inhibition confirmed and identified antibacterial activity. Purified chitinase was used against two species of bacteria (*S. aureus* and *E. coli*), and microorganisms were considered positive for bioactive substances if an inhibitory zone of at least 8 mm wide was detected around the disc.[14] Because *B. salmalaya* is a gram-positive bacterium, it showed no inhibition zone or antibacterial activity against *S. aureus* which is also a gram+ve bacteria and causes a wide range of infections ranging from skin infections to life-threatening diseases.[14] Penicillin was employed as a positive control, with a 27mm inhibition zone.

While gram-negative bacteria such as *E. coli* were inhibited by 100ug/ml pure chitinase strain. Strains 139SI, 140SI, and 141SI had 12mm, 11mm, and 10mm inhibition zones against *E. coli* agar plate culture, respectively. As the concentration of pure protein in strain 139SI increased, the highest antibacterial activity was seen. 140SI, on the other hand, was less sensitive to *E. coli* due to a decrease in pure chitinase. Ampicillin was utilised as a positive control; however, it was shown to be resistant to *E. coli*. Resistance to β -lactam antimicrobial drugs in *E. coli* has been proven to be ineffective against pathogenic *E. coli*, which causes diarrhoea, meningitis, and urinary tract infections, among other clinical syndromes.[15]

This study showed the degradation chitin-composed material and action mechanisms of chitinase enzyme was related with volume of treatment given on prawn shell. Highest concentration of purified chitinase was given on prawn shell, hence the enzyme action mechanisms in hydrolyze chitin, a linear polymer of β-(1,4)-linked *N*-acetylglucosamine (NAG),[16] was more effective. This fit the result obtained in this study where 400ug/ml of purified chitinase was more effective in degrading chitin of prawn shell as their cracked can be seen as early at day 3 and obviously shrunken at day 7 compare to the prawn shell that was treated with 200ug/ml chitinase, which their degrading part only appear at day 7 and shrunken not obvious. The chitin found in the peritrophic matrixand the interior layers of exoskeletons of crustaceans such as prawns, shrimp, and crabs provide support for the muscle system as well as growth and development.[17] Chitinase enzyme can directly degrading their chitin-containing structures by breaking down typical bond that bind with chitin and as crustacean shells especially on prawn shell as a major carbon or nitrogen source for the production of chitinase.[18] As a result, it is great for bioremediation and waste management, as well as releasing nutrients and keeping the carbon, nitrogen, and other biogeochemical cycles in check.[19]

CONCLUSION

Chitinase is an enzyme that aids in the breakdown of chitin-based materials. Among all the *Bacillus salmalaya* strains tested, 139SI, which has strong haemolytic activity, showed the best and strongest antifungal activity against three types of phytopathogenic fungi, including *Fusarium* sp., *Phytophthora* sp., and R. *solani,* suggesting that it could be used in the field to combat plant pathogenic fungi. This work revealed that, strain 139SI also has capable in degrading peptidoglycan of gram-negative bacteria such *Escherichia coli* and degrading prawn shell structure at concentration of 400ug/ml of chitinase enzyme. Increased concentration of crude extracts will be accompanied with the enhancement of cell viability. As the 139SI has highest concentration of purified chitinase enzyme (87.86 mg/ml), hence their performance was increased especially against various type of fungus, bacteria and degrading chitin-composed material. However, a larger scale trial is needed in the future for degradation of marine waste as chitinase enzyme have potential to be usedas biocontrol agent in controlling plant diseases.

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Authors' Contribution: All authors contributed equally

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Table 1: Preparation of Blank, Tube 1 until Tube 5.

Tube	V. of BSA (ml)	V. of distilled water (ml)	Lowry reagent(ml)	Follin reagent(ml)
Blank	-	1	5	0.5
1	0.2	0.8	5	0.5
2	0.4	0.6	5	0.5
3	0.6	0.4	5	0.5
4	0.8	0.2	5	0.5
5	1.0	-	5	0.5

Table 2: Showing the mean and standard error of inhibition of strain 139SI, 140SI and 141SI against three types of fungus; *R. solani*, *Phytophthora* sp., *Fusarium* sp.

Sample/Fungi	*R. solani*	*Pyhtophthora* sp.	*Fusarium* sp.
139SI	15±9.90	19±10.61	15±11.31
140SI	11.5±4.95	16.5±10.61	11.5±7.78
141SI	13.5±6.36	16.5±7.78	13.5±9.19
Control	16±8.49	15.5±6.36	16±12.73

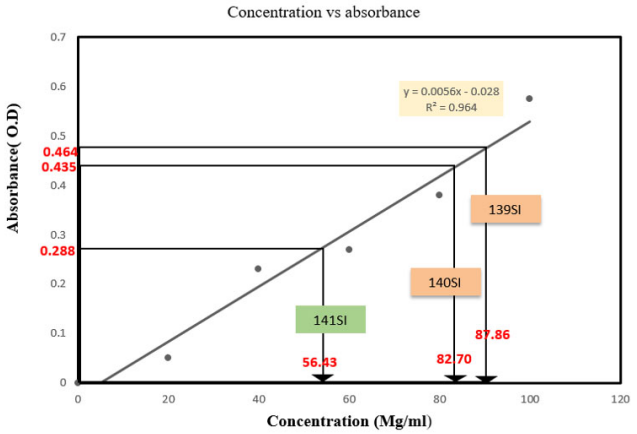

Figure 1: Concentration and absorbance of purified proteins.

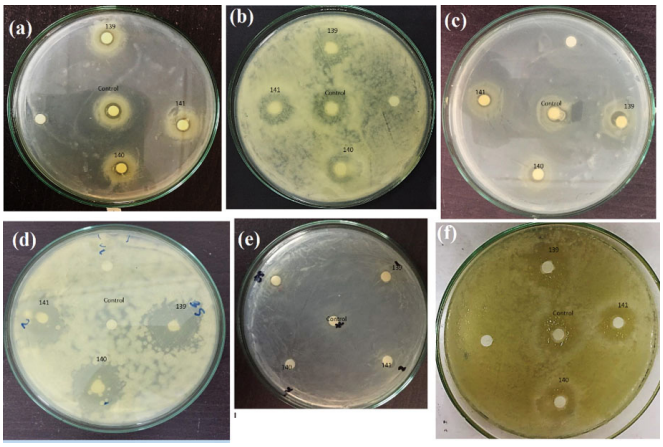

Figure 2: (a) Effect of purified chitinase 139SI on mycelial growth of *R. solani* after 24h incubated. (b) Effect purified chitinase on mycelial growth of *R. solani* after 48h incubated. (c) Purified chitinase against *Phytophthora* sp. After incubated 24 h. (d) Purified chitinase against *Phytophthora* sp. 48h. (e) Effect of purified chitinase on mycelial growth of *Fusarium sp.* after 24h incubated. (f) Effect of purified chitinase on mycelial growth of *Fusarium sp.* after 48h incubated.

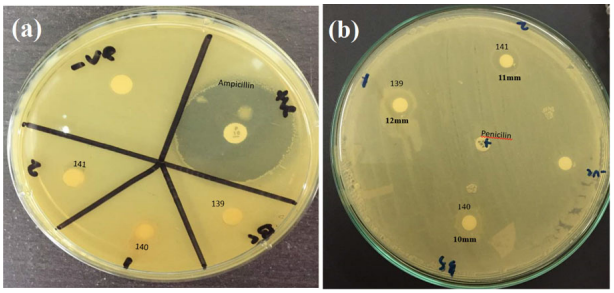

Figure 3: (a) Agar plate shown strain139SI,140SI and 141SI not sensitive to the tested gram positive bacteria, *S. aureus* after incubated 24 h. (b) Strain 139SI has larger antibacterial zone inhibition (12mm) against gram negative bacteria, *E.coli,* compared to both strains of 140SI(10mm) and 141SI (11mm) strain.

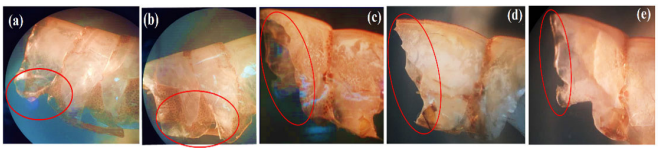

Figure 4: (a)Prawn shell 1 before treatment at 0 day. Red circle showed origin shape and structure before treated and degrade by chitinase enzyme. (b) Red circle showed the degradation area of prawn shell 1 after 7 days; reduction in shell prawn chitin material by 200ug/ml chitinase enzyme from the strain 139SI (c) Prawn shell 2, before treatment at day 0. Red circle showed origin shape and structure before treated and degrade by chitinase enzyme strain139SI. (d) Red circle showed prawn shell 2 part was degraded by 400ug/ml purified chitinase strain139SI as early as 3 days after treatment. (e) Red circle showed prawn shell 2 part was obviously shrunken at day 7.