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Chitinase Activity by Chitin Degrading Strain (*Bacillus Salmalaya*) in Shrimp Waste

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ABSTRACT

Introduction: Chitin degradation by chitinase enzyme can be used on a large scale forbioremediation of seafood waste and is environmentally friendly.

Objective: The main aim of this study was to screening the potential of the strain as chitin degrading agent.

Methods: In this present study, the cell-free supernatant of *Bacillus salmalaya* was determined for its protein concentration. *Bacillus salmalaya139SI* was isolated from agricultural soil and it was identified by staining technique and colony morphology. The production of chitinase by *Bacillus salmalaya139SI* was optimized under different concentration of substrate, pH and temperature.

Results: Strain *139SI* exhibited strong hemolytic activity and the crude protein concentration of *Bacillus salmalaya* was 84.09mg/mL with OD value 0.462. Strain *139SI* were also screened on colloidal chitin agar medium supplemented with mineral salt. Chitinase production was determined by clear zones of hydrolysis produced after 7 days of incubation at 37°C. The maximum chitinase production was observed in Brain Heart Infusion broth supplemented with 1.0% colloidal chitin at pH 7 and temperature 35°C after four days of incubation. Chitinase activity was observed when high concentrations of crude extract of *139SI* able to degrade shrimp shell by showing degradation zone at day 4.

Conclusion: From the results, we concluded that the *Bacillus salmalaya*has potential to be a biofunctional chitinase that could degrade complex polysaccharides present in the organic wastes and applicable in cleaning the environment..

Key Words: Shrimp, Chitinase, Hemolytic activity, *Bacillus salmalaya*, Chitin, Degradation

INTRODUCTION

Shrimp has constituted a primary phase of crustacean consumption in present years. An increase in shrimp waste is unavoidable owing to the increased amount of consumption. Shrimp waste is considered an imperative source of chitin. A critical sum of shrimp waste is delivered in Asia that essentially in Thailand and India.[1] Solid wastes, consisting of the head, shelland tail portions, accumulate owing to shrimp processing. The waste composed of the cephalothoraxes and exoskeleton [2] accounts for 50-70% of the raw materials weight and is usually discarded. The recycling of chitinous waste is extremely important to keep the carbon-nitrogen balance in ecosystem.[2]

Chitin is a sugar-like polymer and is available at a low cost. It appears to be safe for use in humans in the long term and

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has very low toxicity. Chitin plays a protective role in many lower eukaryotes similar to that of cellulose in plants. Metabolism of chitin in synthesis and degradation is essential for different morphogenesis.[2] Chitin occurs as crystalline microfibrils in nature. It can be found as the main component in construction processing of fungal cell walls, mollusca radula, nematode eggshells, worm and arthropod exoskeletons, cephalopod beaks, and fish scales.[3] The absence of chitin in vertebrates and plants makes the chitin metabolism potentially useful parasite-specific target for chemotherapeutic attack.[1]

Marine bacteria for survival of aquatic ecosystem rapidly catabolize chitin. Some bacteria are producing chitinases probably to hydrolyze the diversity of chitins found in nature. As stated by Okonkoet al. (2006) several strains of microorganisms have been selected or genetically modified to increase the efficiency with which they produce enzymes.[4] Chitinases are a large and diverse group of enzymes with different molecular structures, substrate specificity and catalytic mechanisms. Many bacteria, including *Serratia* and *Bacillus*, produce four different chitinases, whereas filamentous fungi produce up to 20 different chitinases. As a result, there has been an increase in demand for chitin derivatives produced by the action of chitinases on chitin polymer for a variety of industrial, clinical, and pharmaceutical applications.[4]

Bacteria are thought to be the primary mediators of chitin degradation in nature. Their importance can be seen in both soil and water systems. The rate of chitin hydrolysis in soil systems is related to the bacterial population and abundance.[5] In addition, the degradation process also depends on factors such as temperature and pH. Thus, degradation of chitin by chitinase enzyme can be used as bioremediation of seafood waste at large scale and eco-friendly to the environment.[6]

The main aim of the current study was to analyze and screening the potential strain as chitin degrading agent. Therefore, the specific objectives were to screen potential of *Bacillus salmalaya* in hydrolysis activity, to optimize cultural conditions for the production of chitinase like concentration, pH and temperature and to screen the potential of the strain as chitin degrading agent on shrimp shell.

METHODOLOGY

Bacillus salmalaya 139SI

Bacillus salmalaya139SI was originally isolated from soil obtained from the private farm in Selangor, Malaysia (2.99917°N 101.70778°E).[7]

Chitin from shrimp shells

Practical grade, chitin powder (Sigma, USA) was used and modified to form colloidal chitin as it is more homogenous distribution in agar media and act as a primary carbon source to bacteria in a medium for analysis of chitinase activity.

Growth condition and Chitinase production.

Single colony of *139SI*, from BHI agar plate was cultured in 1 L of Difco ™, USA.Brain–Heart Infusion (BHI) medium containing 5 g/L KCl, 3 g/L dextrose, 2.5 g/L Na_2HPO_4, 14.5 g/L gelatin, 6 g/L BHI, and 6 g/L peptic digest of animal tissue with additional 1% (w/v) chitin powder from shrimp shell as inducer in a shaking incubator at 150 rpm for 72 h at 35°C.The incubation continued until the OD_{600} equaled 1. The 7 days culture was centrifuged (8000 × g for 20 min) by using (SORVALL ST 16R) centrifuge and filtered through a sterile syringe filter with a pore size of 0.2µm (Minisart syringe filter) to remove all particles and dead microorganisms without any influence on their ingredients. The supernatant formed was a crude extract of chitinase enzyme. The cell-free broth or supernatant was concentrated by freeze-drying and was stored at −20 °C.

Estimation of protein content

Purified and estimation enzymes was estimated by the following method of Lowry et al. (1951)[8] using Bovine serum albumin as the standard.[9]

Preparation of colloidal chitin

Colloidal chitin was prepared from chitin powder (sigma-Chemicals Company, USA) by the modified method of Hsu and Lockwood.[10]

Preparation of chitin agar medium

The chitin medium was prepared using moist form colloidal chitin based on the modified protocols combining elements from Hsu et al. and Ramırez et al.[10,11]

Plate screening of Chitinase activity

Bacillus salmalaya139SI coloniesfrom the plates were subcultured on Brain heart infusion broth (BHI; Difco) and incubated at 30°C for 24 hours. Later, a few drops of culture was placed onto the surface of BHI agar and was spread using sterile spreader or sterile cotton swab. This was incubated at 30°C for overnight. After incubation, the confluent growth of culture was inoculated onto chitin agar using spot inoculation. Plates were incubated at 30°C for about a week (7 days). Following incubation, the bacterial cultures were observed for production of chitinase. On the chitin agar, the clear zone of inhibition were noted around the colonies and was calculated using a Vernier caliper in (cm) and after 3 days of incubation, the chitinolytic index was calculated. The chitinolytic index was calculated using the below equation:

$$\text{Chitinolytic index} = \frac{\text{Diameter of the clear zone-diameter of the colony}}{\text{Diameter of the colony}}$$

Optimization of culture condition

Effect of substrate concentrations on chitinase production

Bacillus salmalaya strain *139SI* was cultivated in different concentrations (0.3, 0.5, 0.8 and 1.0 %) of colloidal chitin enhance with minimal medium to determine the optimum concentration of colloidal chitin for chitinase production.

Effect of pH value on the chitinase production

Bacillus salmalaya strain *139SI* was grown at different pH range of the culture medium from 4 to 10. HCL was used for pH 4; phosphate buffer was used for pH 7 and NaOH for pH 10 in minimal medium containing 1.0% colloidal chitin to determine the optimum pH for chitinase production.

Effect of temperature on the chitinase production

To determine the optimum temperature for chitinase production, *Bacillus salmalaya* strain *139SI* was grown in the culture medium containing 1.0% colloidal chitin and incubated at different temperature of 25°C, 30°C, 35°C, 40°C and 45°C up to 4 days.

Reaction of crude chitinase on shrimp shell

A number of prawn shell were prepared and treated with different concentration of chitinase of strain*139SI*. Then, shell was observed in a month to identify the ability of chitinase in degradation action under microscope (OLYMPUS SZ40).

RESULTS

Hemolytic activity

Bacillus salmalaya strain *139SI*showed positive result as appears clear zone around the bacterial colony accompanied by lightened yellowish discoloration of medium as in figure 1a. The complete lysis exhibits that strain *139SI* have strong Beta (β) haemolysis because it could produce haemolysin substance, which is bacterial protein breakdown of the hemoglobin of the red blood cells and disrupting the structure of the membrane or punching a hole through the membrane.

Estimation of protein content

Bacillus salmalaya strain *139SI* produced 0.32g of chitinase crust. The amount might slightly not exact as we used an old freeze-drying machine. Thus, there was some errors or mistakes happened during freeze drying for instance incomplete drying since the process of removing moisture not working well.Based on the results from the BSA standard graph (figure 1b), *Bacillus salmalaya* strain *139SI* have high amount of crude protein concentration which is 84.09 mg/mL with OD value 0.462. Therefore, it proved chitinase crust of strain *139SI* was pure. That might be due to the chitinase crust was influenced by other substance.

Plate screening of chitinase activity

In the primary screening of chitinase activity as shown in figure 2, *Bacillus salmalaya139SI* produced prominent zone of hydrolysis on the colloidal chitin agar. Clear zone around the spot inoculation was increasing in diameter until day 7 with chitinolytic index 7. This indicated strain *139SI* has chitinolytic activity to breakdown chitin compound in medium.

Effect of substrate concentrations on chitinase production.

Among the four different concentrations tested, the results showed *Bacillus salmalaya* strain *139SI* produce chitinase maximally at the concentration of 1% of colloidal chitin with absorbance value (1.845±0.092), followed by colloidal chitin at 0.8% (1.541±0.103), 0.5% (1.418±0.068). Beyond 0.5%, the substrate concentration decreased the enzyme activity (Table 1). But above 0.5% of colloidal chitin concentration, chitinase production was significantly increased.

Effect of pH value and temperature on the chitinase production.

In order to evaluate the effect of pH of the media on the chitinase production, *Bacillus salmalaya139SI* were grown at different pH (4, 7, and 10). The optimal pH for chitinase production was examined when kept at 37°C. Among the tested pH, neutral condition pH 7 (1.318±0.029) supported the maximum chitinase production. In pH 4, chitinase production was 0.235±0.040 followed by pH 10 was 0.297±0.135 (Table 2). Chitinase production was relatively stable at pH 7. However, for alkaline and acidic condition, it rapidly lost its chitinase production. In addition, from the observation of the broth culture medium, the culture broth for acidic and alkaline are slightly clear compared to pH 7 medium that are cloudier which indicate the cell growth. From this test, it showed an excessively high or low pH led to poor cell growth and chitinase production.

Data presented in table 3 clearly indicated incubation temperature affects many biological processes, including the growth rate and enzyme production. The organism exhibited a good growth as well as chitinase production at 35°C with average 1.732±0.072. The incubation temperature 30°C (1.460±0.036) also found to influence the chitinase production. It has been observed that in both lower and higher temperature the chitinase production decreased.

Reaction of crude chitinase on shrimp shells

Five different concentrations of the crude chitinase was tested on the shrimp shell to check the chitin degradation ability. Among the five concentration, 200 mg/ml until 300 mg/ml of crude chitinase was more effective in degrading ability compared shrimp shell treated with lower concentration. This is because the degradation activity can be seen as early as day 4 and more clearly seen in the following week. At the

end of the month, all concentrations showed potential of the degradation activity (Figure 3). In this study, high concentration of crude chitinase was used to get the result within a month and also high concentration will produce more effective enzyme action mechanism in hydrolyze the chitin. Thus, this study showed the degradation chitin-composed material and action mechanisms of the chitinase enzyme were associated with volume of the treatment given on the shrimp shell.

DISCUSSION

Hemolytic or also referring to hemolysis is the breakdown of the membrane of red blood cells by a substance known as hemolysin. Hemolysin is a group of bacterial protein, which causes the lysis of the red blood cell (RBC) membrane in the growth substrate [12]. Many types of bacterial possess hemolytic proteins. These proteins act by desegregate into the membrane of RBC and disrupting the structure of the membrane.[12] Bacteria are differentiated based their haemolytic properties. In the study by Dadrasnia and Salmah (2015), *Bacillus salmalaya* was found to be a potential degrader of crude oil waste. In the study, haemolytic activity for *Bacillus salmalaya139SI* was detected as the presence of a definite clear zone around the colony.[7] In the other finding by the same authors, *Bacillus salmalaya139SI* also identified as biosurfactant bacteria. As is known, biosurfactant bacteria were all positive in hemolytic activity as it is initial test that always been used to identify biosurfactant producing bacteria. Therefore, hemolytic activity appears to be a good criteria in screening in the search for surfactant-producing strain. As reported by Abu et al., (2018), the bacterial colonies that was streaked on blood agar medium exhibit g β-hemolytic activities and was found to have a potential to produce bioflocculant to remove organic matter.[13] Thus, isolated *Bacillus salmalaya139SI* were represents as members of a novel species of the genus *Paenibacillus* based on the hemolytic activities[7] and also the ability to bind efficiently and degrade animal or human hemoglobin that could provide an effective heme source to ensure its successful growth and proliferation *in vivo*.[13,14]

In 2004, Stoykovet al.[15] mention that expression of inducer can generate of a signal and increase in the production of the chitinase. It also has been reported by Sato and Araki, (2008) that showed the significance of medium composition for the production of chitinase from *Bacillus cereus* by supplementing the medium directly with chitin.[16] The culture medium was incubated until reach OD value equaled 1 because it was the highest value culture density (cell number) of the strain with a constant growth rate, thereby exhibiting a maximum chitinase production. In the study by Salmah and Dadrasnia (2015), the maximum production of biosurfactant also measured until the OD value equaled to 1 and was found to be a potential degrader of crude oil waste.[7]

In the study by Budi et al., (2000), *Paenibacillus* species have been reported to have high activity of cell wall degrading enzymes and chitinase, making this species commonly applied as biocontrol agents.[3] To give comprehensive proof, Kumar et al., (2012) mentioned that different species of *Bacillus* has been reported to produce chitinase and the result from their findings, *Bacillus amyloliquefaciens SM3* produced highest chitinolytic activity compared to other isolated chitinolytic bacteria.[17] However, in the study by [18] he reported different strain will give different abilities to secrete extracellular degradative enzymes.

Results of this research found that *Bacillus salmalaya* strain *139SI* have a potential to be mediators of chitin degradation and may be useful for biotechnological applications and production of transgenic microorganisms with superior biocontrol capabilities. Chitin degradation could therefore be explored as a general model for understanding microbial degradation of biopolymers in the biosphere.

In this study, high concentration of crude chitinase was used to get the result within a month and also high concentration will produce more effective enzyme action mechanism in hydrolyze the chitin. Thus, this study showed the degradation chitin-composed material and action mechanisms of the chitinase enzyme were associated with volume of the treatment given on the shrimp shell.

In the study by Sorokulova et al., (2009), they reported that the *B. cereus* strain performed better in shell waste decomposition and was used for large-scale fermentation in 12 L of 10% shrimp shell waste broth.[19] The similar report was supported by Abirami et al., 2016 observed that *Bacillus licheniformis*SSCL10 rapidly degrade the shrimp shell completely within 12 days while other isolate Bacillus subtilis took more days for degradation activity.[20] From the result obtained in this study, chitinase from *Bacillus salmalaya139SI* has efficiency in hydrolytic activity and it can also be used for the degradation of other chitin materials.Therefore, chitin degradation needs to be explored as a general model for understanding microbial degradation of biopolymers in the biosphere.

CONCLUSION

Chitinase are ubiquitous proteins that are widely distributed among all kingdom of life. Chitinase as the name indicates is involved in the breakdown of chitin. The result concluded that *Bacillus salmalaya* are novel mesophilic bacterial strains that has strong hemolytic activity shown the best and ability to produce a huge amount of chitinase in short time. This enzyme may also be useful in the management of sea-food waste industries. Colloidal chitin as the sole source of carbon can prove to be economical in terms of the fermentation expenditure. Neutral pH along with a temperature around 35°C

facilitates the highest yield. This work revealed that strain *139SI* also effective in hydrolyze chitin medium and degrading shrimp shell at concentration 200, 250 and 300mg/ml of crude chitinase. *Bacillus salmalaya139SI* makes it a potential candidate for the bioremediation of seafood waste at large scale. Strain *139SI* performance was increased since it has highest concentration of protein chitinase enzyme. However, much attention and research needed for multiple potential applications in the future such as nanobiotechnology application involves drug and gene delivery or in agriculture, food and environmental protection.

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Authors' Contribution: All authors contributed equally.

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Table 1: Table showing production of chitinase by *Bacillus salmalaya* in different colloidal chitin concentration after 24h, 72h and 120h and results are presented as mean ± SD.The experiments are carried out in triplicates (n=3).

Colloidal chitin concentration %	Absorbance (O.D) at 600nm			Average (Mean ± SD)
	24h	72h	120h	
0.3	1.203	1.276	1.314	1.264±0.056
0.5	1.342	1.434	1.477	1.418±0.068
0.8	1.462	1.503	1.657	1.541±0.103
1.0	1.741	1.883	1.912	1.845±0.092

Table 2: Table showing production of chitinase by *Bacillus salmalaya* in broth amended with 1.0% of colloidal chitin in different pH value after 24h, 72h and 120h and results are presented as mean ± SD.The experiments are carried out in triplicates (n=3).

pH value	Absorbance (O.D) at 600nm			Average (Mean ± SD)
	24h	72h	120h	
4	0.281	0.205	0.219	0.235±0.040
7	1.285	1.341	1.329	1.318±0.029
10	0.283	0.298	0.310	0.297±0.135

Table 3: Table showing production of chitinase by *Bacillus salmalaya* in broth amended with 1.0% of colloidal chitin at pH 7 with different temperature of incubation and results are presented as mean ± SD. The experiments are carried out in triplicates (n=3).

Temperature °C	Absorbance (O.D) at 600nm				Average (Mean ± SD)
	24h	48h	72h	96h	
25	1.163	1.178	1.213	1.192	1.187±0.021
30	1.467	1.464	1.411	1.498	1.460±0.036
35	1.656	1.686	1.786	1.801	1.732±0.072
40	1.362	1.393	1.407	1.495	1.414±0.057
45	1.252	1.212	1.173	1.196	1.208±0.033

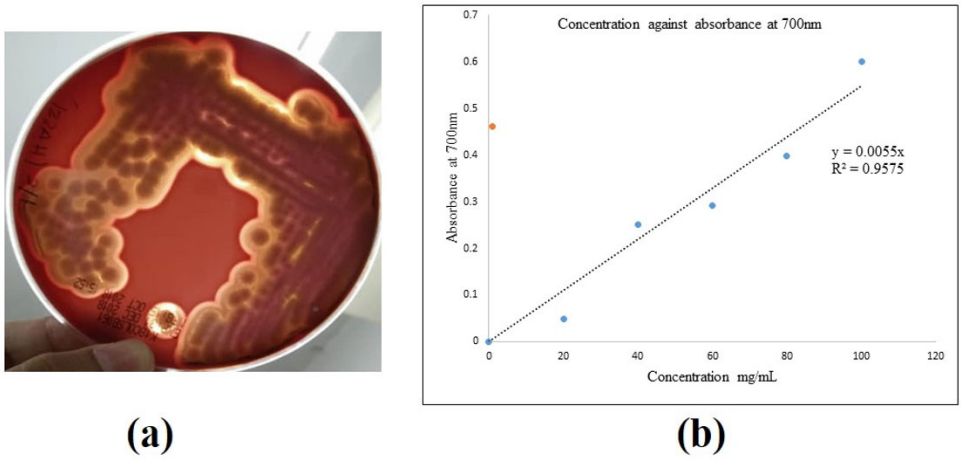

Figure 1: (a) Red arrow showed clear zone produced by pure colonies of *139SI* after 24h incubated at 37°C under aerobic conditions. *Bacillus salmalaya* strain *139SI* have strong haemolytic activity on the blood agar medium (b) figureshowing concentration of chitinase of strain *139SI* plotted against absorbance at 700nm on BSA standard graph. The crude protein concentration of *Bacillus salmalaya139SI* was 84.09 mg/mL and its absorbance was 0.462 O.D.

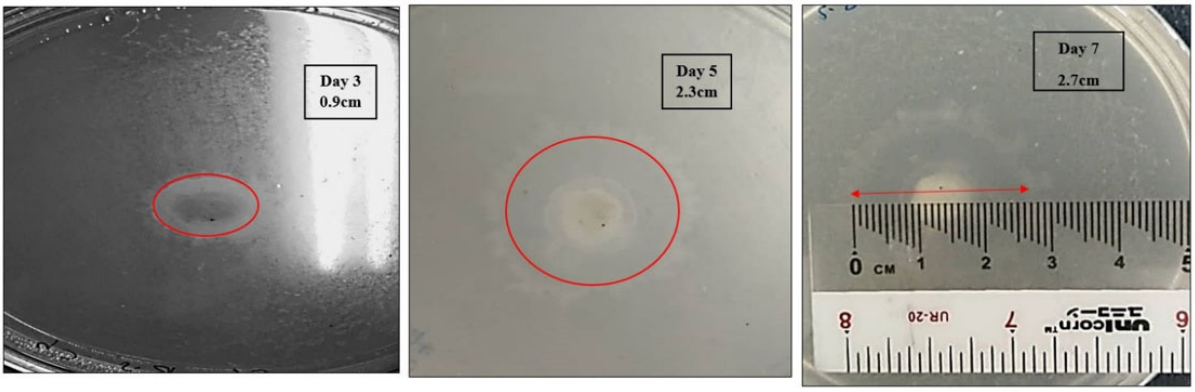

Figure 2: Figures showed days 2, 5 and 7 with different diameter of zone hydrolysis on colloidal chitin agar produced by *Bacillus salmalaya139SI*.

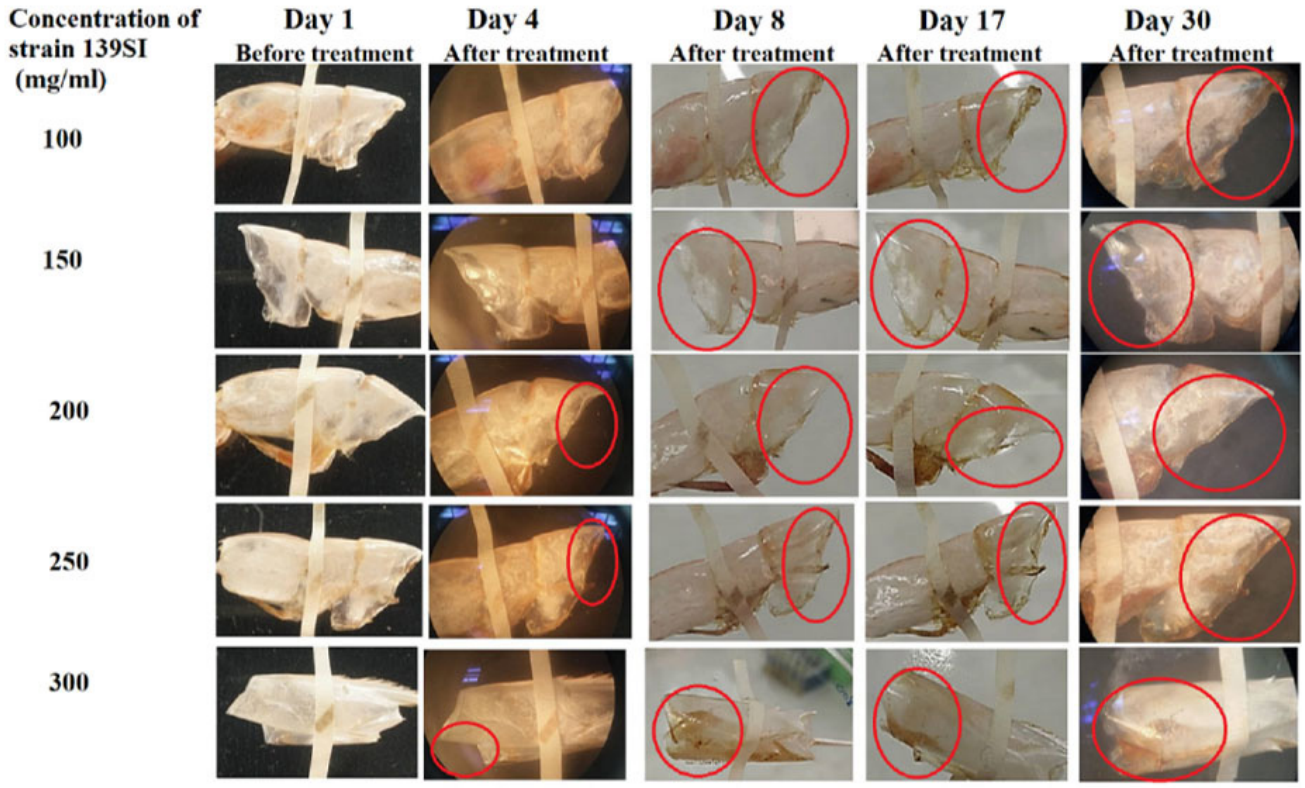

Figure 3: Figure showing day 1,4, 8,17 and 30results and red circle above showed prawn shell was degraded by different concentration of strain*139SI* after treatment.