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TOXICITY TEST OF VANAME SHRIMP (*Litopenaeus vannamei*) SKIN CHITOSAN USING BRINE SHRIMP LETHALITY TEST (BSLT) METHOD

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ABSTRACT

Chitosan is a chitin derivative found in the outer skin of crustaceans such as shrimp and crabs. Chitosan can be used in industry, especially in the pharmaceutical sector, and can also be used to preserve food and fruit. The goal of this research was to employ the BSLT method to determine and measure the quantity of toxicity in chitosan, as well as to analyze the results using probit analysis. This study concerns the extraction of chitin and chitosan: deproteination with NaOH 3.5%, demineralization with 1.5 M HCl, depigmentation with NaOCl 0.315%, and deacetylation with NaOH 60%, specifically, chitin to chitosan transformation, chitosan characterization, FTIR, and toxicity tests. Chitosan was tested using the BSLT method with five test solution concentrations, namely 100, 250, 500, 750, and 1000 g/ml, by calculating the amount of *Artemia salina* L. larval deaths and obtaining lethal concentration (LC_{50}) data. The vannamei shrimp shell chitosan revealed a 58% degree of deacetylation. The toxicity testing demonstrated that chitosan was not harmful to *Artemia salina* L, as shown by an LC_{50} value greater than 1000 µg/ml. Chitosan from Vannamei shrimp has a concentration of 4897.79 µg/ml and is not harmful.

Keywords: Isolation, Vaname Shrimp, Chitosan, BSLT

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INTRODUCTION

Shrimp is widely used in the export industry, especially in frozen form, for local businesses, and for household consumption. High consumption and production of shrimp produce a lot of waste. This waste will have an impact on environmental pollution. Shrimp waste consists of the head, skin, legs, and tail.[1] Shrimp shell waste contains chitin compounds. There are three main components of shrimp shell waste, Protein accounts for 25-44%, calcium carbonate accounts for 45-50%, and chitin accounts for roughly 20%. The abundant amount of chitin allows it to be widely used in various fields.[2]

Chitosan is a polymeric chemical generated from chitin deacetylation that is widely employed in a variety of industrial areas. Chitosan is found in the shells of many marine species, including crabs, shrimp, shellfish, and others.[3] Toxicity tests are performed to determine the presence of toxic effects and to determine the safe use limit of a compound.[4] A toxicity test determines a poison's ability to cause damage when it enters the body and organs that are vulnerable to it.[5,6] A toxicity test utilizing the BSLT method is meant to detect the potential toxicity of a chemical by determining its level of toxicity.[7]

BSLT is a popular approach for toxicity assessment that uses Artemia salina leach larvae as test animals. Because the BSLT approach can be performed fast, cheaply, and readily, it is frequently employed to measure the level of toxicity of a substance.[8] The mortality value is derived utilizing probit analysis to determine the toxicity value using lethal concentration (LC_{50}) in the BSLT approach.[9] The goal of this

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study was to assess the toxicity level of vannamei shrimp (Litopenaeus vannamei) chitosan using the BSLT method against Artemia salina shrimp larvae.

EXPERIMENTAL

Materials and Apparatus

The materials used in this study were Belawan shrimp shell trash, Deli Serdang Regency, A. Salina Leach Eggs, NaOH, HCl, NaOCl, NaCl, and Acetic Acid. The tools used include Beaker glass, measuring cup, funnel, sieve, magnetic stirrer, Hot Plate, Oven, measuring cup, Stirring rod, pH Meter, Furnace, Desiccator, Porcelain Cup, pliers' crucible, crucible cup, Analytical balance, FT-IR Spectrophotometry.

Sample Preparation

In Vannamei, shrimp shell waste is washed with water until it is clean of impurities. They were then sun-dried. After drying, the material was crushed and sieved to obtain a fine powder.[10]

Deproteination Process

The sample, which had been coarsely powdered, was combined 1:10 (w/v) with a 3.5% NaOH solution in a glass beaker. Thereafter, it was stirred at a speed of 50 rpm for four hours at a temperature between 60 and 70 °C. After that, distilled water is used to rinse the residue in the sample until the pH is balanced. It was then weighed after being dried for 24 hours at 80°C in an oven and chilled in a desiccator.[3]

Demineralization Process

After transferring the deproteination results to a new container, mix in 1.5 M HCl solution at a solvent-to-sample ratio of 1:15 (w/v). Heat the mixture at 60-70 °C for 4 hours, stirring with a magnetic stirrer at 50 rpm. The residue is then rinsed with distilled water until the pH is adjusted. It was then weighed after being dried in an oven for 24 hours at 80°C and chilled in a desiccator.[3]

Depigmentation Process

Each demineralization process's output is transferred to a different beaker, and for an hour, a 0.315% NaOCl solution is heated to 40 °C with a sample-to-solvent ratio of 1:10 (w/v). After cleaning with distilled water until the pH was neutral, the residue was weighed, dried in an oven with milk at 80 °C, and cooled in a desiccator.[11]

Deacetylation Process

The manufacture of chitosan is obtained through the chitin deacetylation process. Each depigmentation result was placed in a separate beaker, then a 60% NaOH solution with a solvent-to-sample ratio of 1:20 (w/v) was added, and the beaker was heated at 80-100°C for 1 hour. The residue was neutralized with distilled water, dried for 24 hours in an oven at 80oC, and then chilled in a desiccator before being weighed.[11]

Chitosan Characterization

Water content

The two chitosan samples were weighed up to 0.5 g and then placed in a different porcelain dish, the weight of which was recorded. For two hours, the sample was cooked in an oven at 100-105°C. It was then refrigerated for 30 minutes in a desiccator before being weighed until a consistent weight was achieved.[12]

Ash Level

The sample is weighed up to 0.5 g and then placed in a different crucible, the weight of which is known. The sample is then ashed in a furnace at 600°C till white ash is recovered. After that, the cup is cooled in a desiccator and weighed.

Chitosan Solubility

Chitosan solubility is a parameter that can be used as a reference for chitosan quality standards. Chitosan's quality increases with its solubility. Chitosan was dissolved in acetic acid in a 1:100 (g/ml) ratio at a concentration of 2%.[13]

Brine Shrimp Lethality Test (BSLT) Toxicity Test

Method Artificial Seawater Making

To make artificial seawater, dissolve 38 g of salt in 1 L of water. Then filtered with Whatman paper.[14]

Artemia Egg Hatchery Copy Leach

Shrimp eggs hatch in both dark and light vessels. The dark zone contains eggs and aerators, while the other zone contains lights to provide lighting for hatching shrimp eggs.[9] The vessel is given a perforated screen that becomes a way for the hatched larvae to move naturally toward the light. One liter of synthetic seawater is placed into the container. Next, place one spoon—a teaspoon—into the black section of the egg after giving it a one-hour soak in water to clean it beforehand. Aluminum foil is placed over the portion of the container that is dark. 40-watt fluorescent lights are used to light the brightest area of the container, maintaining a hatching temperature of 25 to 30 °C. The eggs of shrimp are soaked for 48 hours for them to hatch. Within 18 to 48 hours, the eggs hatch and move on their own to a bright area, releasing the shrimp larvae from the egg or eggshell. Now that they are actively moving, the larvae are prepared to serve as test subjects for study.[15, 16]

Preparation of Test Solution

The test was performed with a test solution made into five concentrations of 0 µg/ml (negative control), 1000, 750, 500, 250, and 100 µg/ml in artificial seawater, each with three repetitions.

Toxicity Test

In the toxicity test using the BSLT method, 48-hour-old Artemia salina Leach lava was used. prepared vials for each concentration and replicated 3 times with a size of 10 ml. After filling each vial with 10 ml of sample solution, 10 A. salina Leach larvae were put into each vial containing the test chemical. Without the inclusion of a sample, the negative control (blank) was treated the same as the test solution. The number of dead larvae in each vial after 24 hours was manually calculated. For 24 hours, observations were made. The toxicity level was determined by counting the number of dead larvae. That is if the shrimp larvae did not move for a few seconds after being observed.[15]

Data Analysis

The sample toxicity test is based on the LC50 value, which is determined by probit analysis (probability unit) and can kill Artemia salina Leach up to 50% of the time. The percentage death rate can be used to analyze the impact of toxicity.[18]

RESULTS AND DISCUSSION

Chitosan Isolation from Vaname Shrimp Skin

The goal of isolating chitin from shrimp waste is to keep it apart from calcium carbonate and protein. Protein that is covalently attached to the chitin functional group will be separated during the deproteination stage, which is when the protein in the waste from shrimp shells dissolves in the base. The deproteination step was carried out by reacting chitin with a strong base of NaOH in a beaker heated at 70°C for 4 hours. During the deproteination reaction, a few bubbles emerge on the solution's surface, and the solution thickens somewhat and turns crimson.[11, 19] The next process is the demineralization stage. At this point, the deproteination products are mixed with a 1.5 N HCl solution. A substantial reaction happens, resulting in a considerable amount of foam and bubbles, which lasts around 5-10 minutes. This is induced by the surface production of CO_2 and H_2O gases. The formation of CO_2 in production is an indicator of the ongoing reaction of hydrochloric acid with mineral salts contained in shrimp shell waste. The demineralization process aims to remove organic salts or mineral content present in shrimp shells. The main mineral content is $CaCO_3$. The next stage is followed by the depigmentation process. The sample was treated with a 0.315% solution of sodium hypochlorite. The purpose of this process is to remove the color of the chitin.21 Pigment removal aims to give an attractive appearance to the chitosan produced. Applying the deacetylation reaction method to chitin yields chitosan molecules. To achieve deacetylation, add 60% NaOH and heat to between 80 - 100° C. This approach uses NaOH, a strong base, to change the acetyl group (CH_3CO) in chitin into an amine group (NH_2). By increasing the amount of hydroxyl that is accessible for the hydrolysis process, the addition of NaOH increases the likelihood that the carbonyl group will be eliminated as a result of the hydroxyl's addition, leading to the production of a hydrogen amine group.[20]

FTIR Analysis of Isolated Chitosan Purity

The purity test of chitosan separated using Infrared Spectrophotometry revealed absorbance at 3400-2400 cm^{-1} (range O-H), which is 3116.97 cm^{-1}. There is an absorption at the number 1640–1550 cm^{-1} (range N–H), namely at the wave number 1641.42 cm^{-1}. Moreover, 1315.97 and 12.42.16 cm^{-1} of absorption occur at wave numbers 1350–1000 cm^{-1} (range C–N). Next, absorption occurs at wave number 804.97 cm^{-1}, which is within the range of 1000-650 cm^{-1} (C-H).

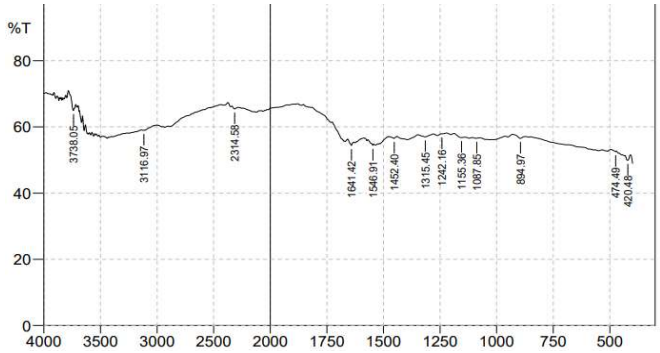

Fig.-1: FTIR Chitosan Vaname Analysis (*Litopenaeus vannamei*)

The proportion of deacetylation can also be used to determine the quality of chitosan. The proportion of deacetylation degree in vaname shrimp shell chitosan was 58% in this investigation. Several aspects contribute to the low DD chitosan research findings, including the stirring factor, temperature, and the type of habitat or shrimp rearing adopted.

Chitosan Characterization

The chitosan obtained was analyzed to assess its quality. Among the tests carried out included organoleptic examination (texture, color, and odor), water content test, ash content test, and solubility in 2% acetic acid. The study's characterization of chitosan results was compared to the quality of SNI No. 7949, Year 2013.

Table-1: Chitosan Characterization

Parameters Organoleptic Examination	SNI (No. 7949, Year 2013)	Vaname Shrimp Skin Chitosan
Texture, Color, Smell	Light brown to white	Powder, yellowish-white, odorless
Water content	≤ 12 %	5,945%
Ash Level	≤ 5%	1,9%
Chitosan is 2% soluble in glacial acetic acid	-	Late
Degree of Deacetylation	≥ 75 %	58%

Chitosan Toxicity Test Results with Test Method BSLT

This toxicity test was carried out using the BSLT method using Artemia salina L test animals because the method is quite easy, inexpensive, and does not require a long time to get the results. Artemia salina, which can be used in toxicity tests when in the naupli stage or larval stage, The larvae used were 48-hour-old larvae because the larvae were in a sensitive state when they were 48 hours old. This is because the organs in Artemia salina L. were fully formed at the age of 48 hours. Artemia salina can drink artificial seawater that has been given test samples with various concentrations so that death is caused by shrimp shell chitosan in various concentrations. Chitosan, which has been dissolved into standard mother liquor, is made into 5 concentrations, namely concentrations of 100, 250, 500, 750, and 1000 µg/ml, with a negative control containing only salt water and shrimp larvae. The addition of negative control aims to determine the effect of salt water on larval mortality so that we can see the effect of larval death from the added money extract. In this BSLT test, each concentration was repeated 3 times to minimize data errors in the research process. Observations on larvae were carried out 24 hours after the concentration treatment. Calculation of larval mortality is done by observing the larvae for a few seconds. Mortality in larvae is calculated when there is no more larval movement within a few seconds.[20]

Based on the data above, the number of larvae used in each treatment was 10 larvae with 3 treatments, so a total of 30 larvae were used for one concentration.

Table-2: Test Results of Chitosan Vaname Shrimp Shell Toxicity Test (*Litopenaeus vannamei*)

No	Concentration (µg/ml)	Treatment 1		Treatment 2		Treatment 3		Number of Larva which dead	Average % Mortality
		Larvae Dead	% Mortality	Larvae Dead	% Mortality	Larva Mati	% Mortality		
1	Blanko	0	0	0	0	0	0	0	0
2	100	0	0	1	10	0	0	1	3,3
3	250	0	0	1	10	1	10	2	6,7
4	500	2	20	1	10	0	20	3	10,0
5	750	1	10	2	20	2	20	5	16,7
6	1000	2	20	2	20	4	30	8	26,7

From the data above, it can be seen that from the low concentration of 100 µg/ml to the highest concentration of 1000 µg/ml, there is a mortality percentage of 3.3–26.7%. While the blank did not give mortality to the larvae. Artemia salina concentrations that vary in each test vial have a different number of deaths of Artemia salina. This shows that each concentration has a different effect on the mortality of A. salina larvae. The greater the concentration, the greater the mortality of larvae.[20] The data obtained from Table-2 was then analyzed using a probit analysis table to obtain the lethal concentration 50 (LC_{50}) value. The LC_{50} concentration can kill up to 50% of the people. of the experimental animals. A straight-line regression equation can be used to obtain the LC_{50} value by entering the value (50% likelihood of test animal fatality) as y and producing x as the log concentration value.

Table-3: Activity Index Value Calculation

C (µg/ml)	N (Number of Larva)	R (Dead Larva)	P % Mortality	X (log C)	Y (Probit Value)	XY	X^2
100	30	1	3,3%	2,0000	3,1616	6,3232	4,0000
250	30	2	6,7%	2,3979	3,5015	8,3964	5,7501
500	30	3	10,0%	2,6990	3,7184	10,0359	7,2844
750	30	5	16,7%	2,8751	4,0339	11,5977	8,2660
1000	30	8	26,7%	3,0000	4,3781	13,1343	9,0000
				ΣX = 12,9720	ΣY = 18,7935	ΣXY = 49,4874	ΣX^2 = 34,3005

The antilog value of x, which eventually becomes the LC_{50} value parameter, is shown to identify the presence of biological activity in a chemical against test animals by counting the number of larvae that perished as a result of the action of a predefined concentration of the drug.19 Probit analysis of vaname shrimp shell chitosan obtained a graph of a straight-line equation y = 1.1293 x + 0.8287. The graph below depicts the log concentration vs the probit value calculated from the percentage of larval death. The y value was then supplied, which was the probit value of 50% of the test animals, and the x = 3.69362 value was entered; the antilog LC_{50} value was 4897.79 µg/ml. A substance is stated to be hazardous if its LC_{50} value is 1000 g/ml, and if its LC_{50} value is greater than 1000 g/ml, the compound is not potentially dangerous. The high and non-toxic LC_{50} result was owing to the low mortality of A. salina larvae at each concentration, i.e., mortality did not surpass 50% of the number of larvae tested at this concentration.

CONCLUSION

The results of Vaname Shrimp Chitosan (Litopenaeus vannamei) obtained a %Deacetylation Degree of 58%, which stated that the results did not meet the requirements of the Indonesian National Standard,

namely %DD chitosan 75%. The LC_{50} value obtained from chitosan of vannamei shrimp (Litopenaeus vannamei) against *Artemia salina* Leach was 4994.16 µg/ml, demonstrating to *Artemia salina* Leach that chitosan was not poisonous.

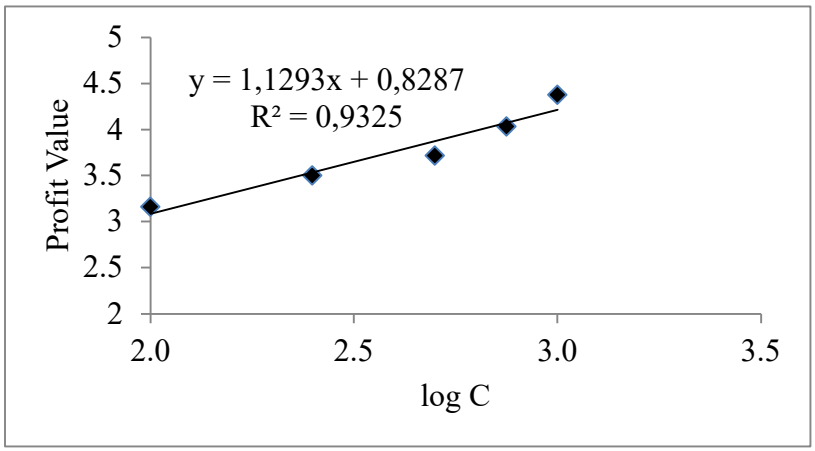

Fig.-2: Linear Regression Graph of Vaname Chitosan Concentration (Litopenaeus vannamei) Against Probit Value

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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