BUKTI REVIEW

JURNAL INTERNASIONAL BEREPUTASI:

Dental Journal

ISSN: 9783728, 24429740

Judul Artikel:

Characteristics of Chitosan from *Penaeus monodon* Shells and Its Effect on The Viscosity of Chitosan-Gelatin Suspension as an *Injectable Bone Substitute Material* on *Socket Preservation*

Dental Journal

Indonesia | Universities and research institutions | Media Ranking

Country
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Dentistry
Dentistry (miscellaneous)

Publisher
Universitas Airlangga, Faculty of Dental Medicine

SCIMAGO INSTITUTIONS RANKINGS

SCImago Media Rankings

SJR 2024
0.258

Q3

H-Index
6

Publication type
Journals

ISSN
19783728, 24429740

Coverage
2019-2025

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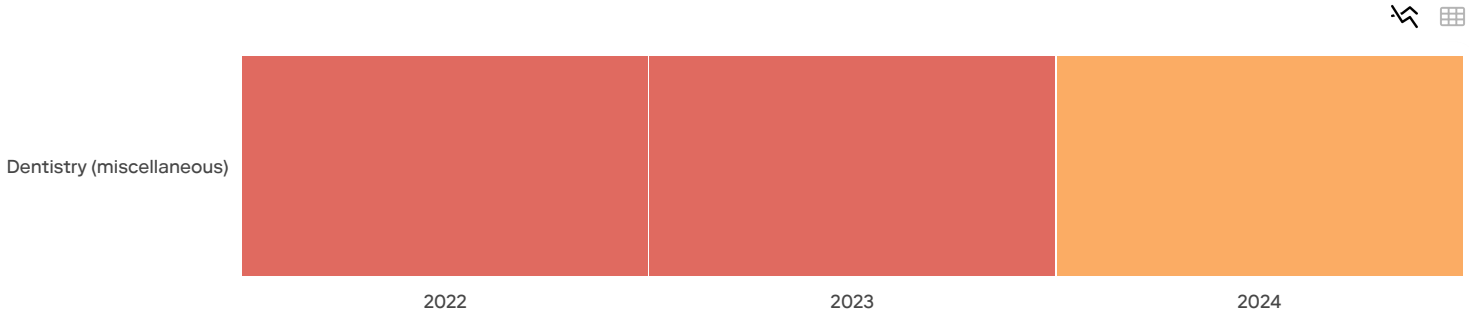

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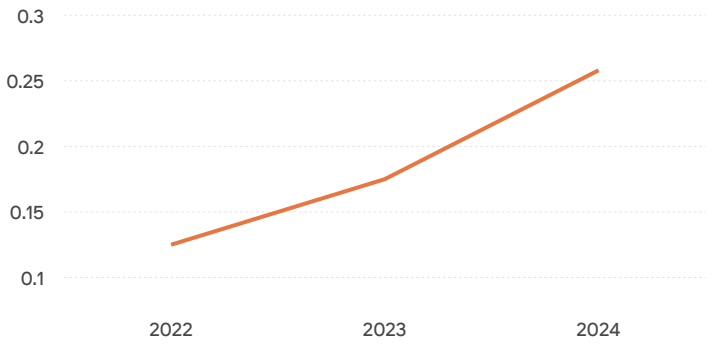

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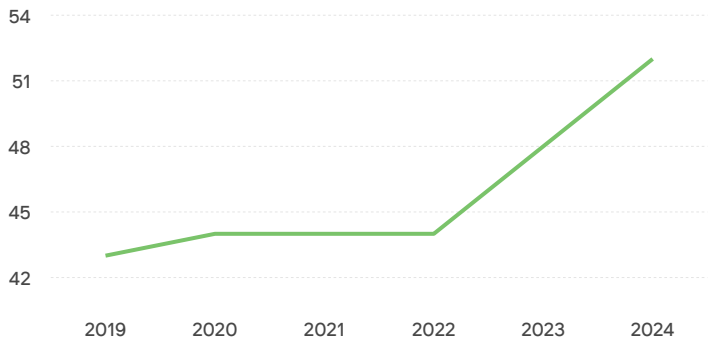

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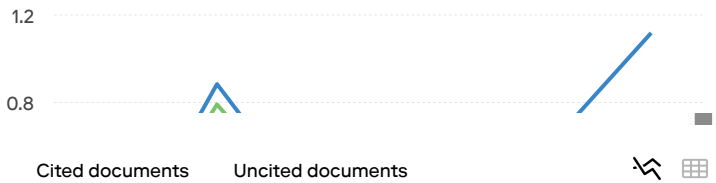

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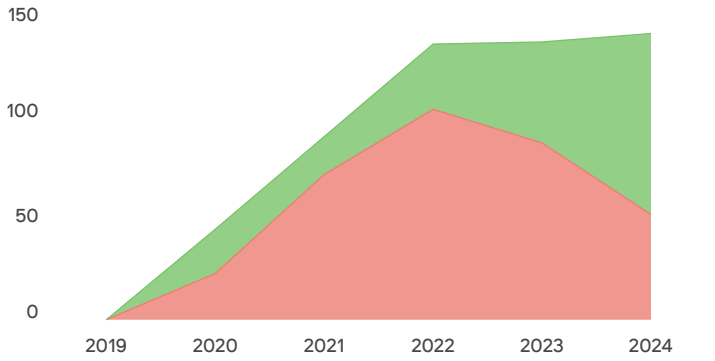

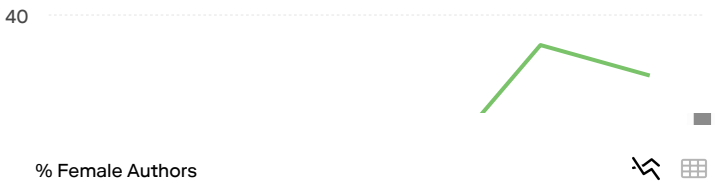

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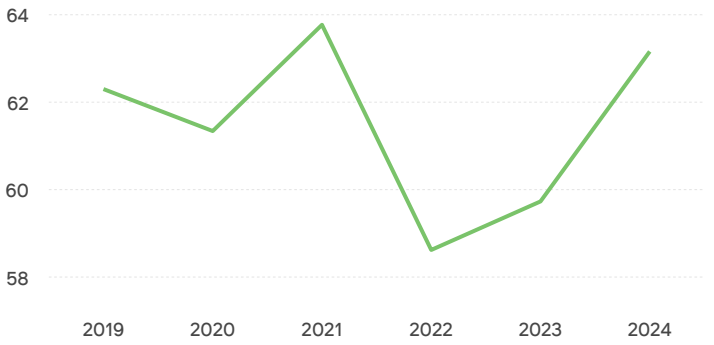

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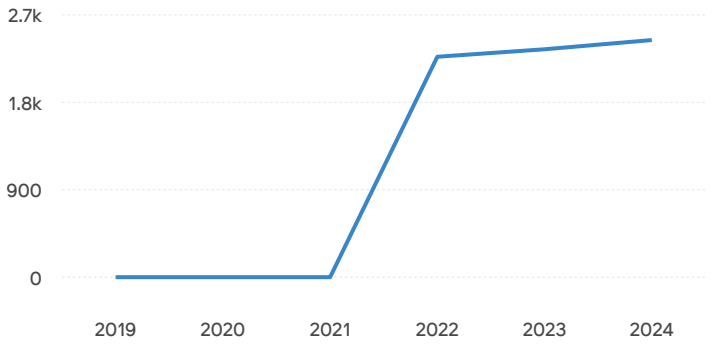

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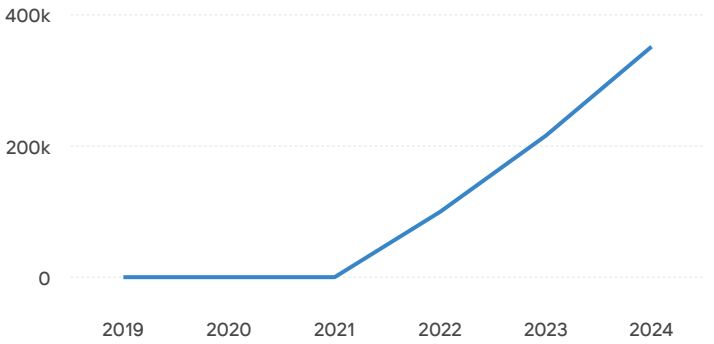

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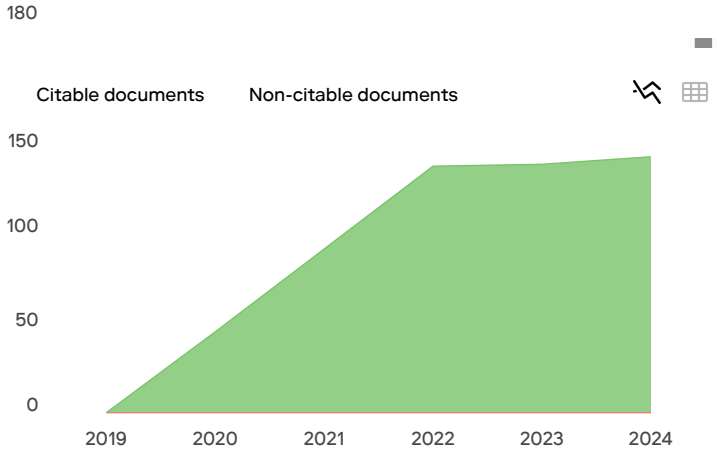

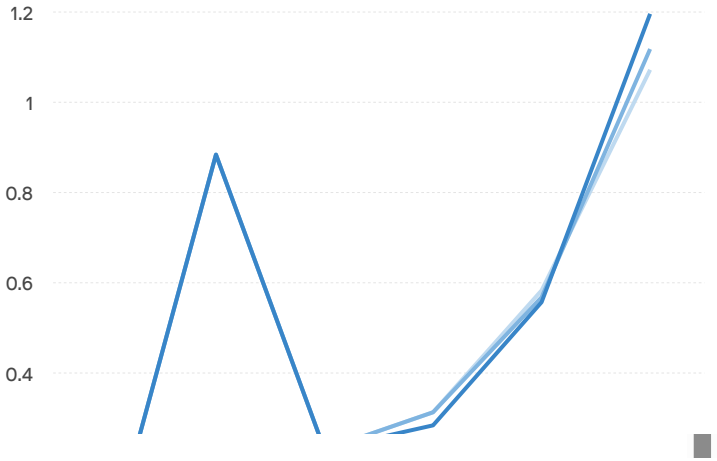

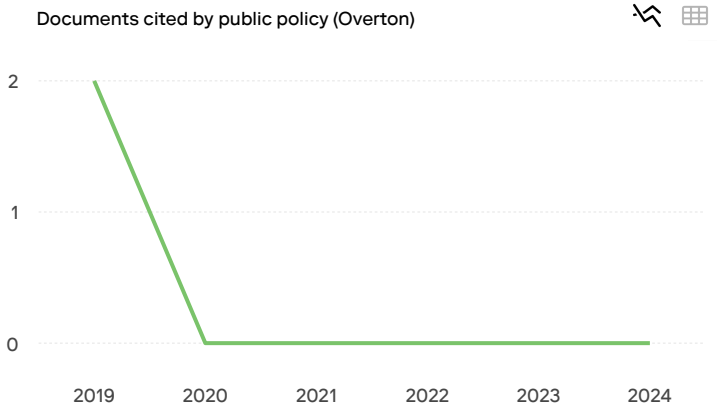

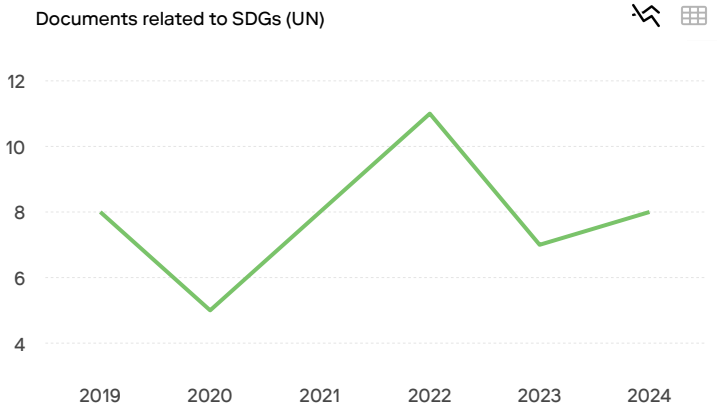

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[DJMKG] Article Review Request

2 messages

Alexander Patera Nugraha <dental.journal.unair@gmail.com> Thu, Dec 28, 2023 at 12:10 PM
To: Rosalina Tjandrawinata <rosalina@trisakti.ac.id>

Dear Rosalina Tjandrawinata,

I believe that you would serve as an excellent reviewer of the manuscript, "Characteristics of Chitosan from Penaeus monodon Shells and Its Effect on The Viscosity of Chitosan-Gelatin Suspension as an Injectable Bone Substitute Material on Socket Preservation ," which has been submitted to Dental Journal. The submission's abstract is inserted below, and I hope that you will consider undertaking this important task for us.

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Title:
Characteristics of Chitosan from Penaeus monodon Shells and Its Effect on The Viscosity of Chitosan-Gelatin Suspension as an Injectable Bone Substitute Material on Socket Preservation

Abstract:

Background: *Injectable bone substitute materials are often used to assist the healing process of bone defects. The chitosan synthesized from Penaeus monodon shells was developed into a chitosan-gelatin suspension as an injectable bone substitute for socket preservation.* ***Purpose:*** *To describe the characteristics of chitosan powder and their influence to the viscosity of chitosan-gelatin suspensions. Characterization of chitosan was carried out through morphology, moisture content, ash content, molecular weight, deacetylation degree, and viscosity.* ***Methods:*** *Penaeus monodon shells from Tarakan Waters, Borneo, were prepared for chitosan using three synthesis methods (Group 1-3) and made into a chitosan-gelatin suspension with a chitosan and gelatin ratio of 45:55 (w/w%). Differences in viscosity using the Kruskal-Wallis and Mann-Whitney tests. Effect of molecular weight and deacetylation degree of chitosan powder on the viscosity of the chitosan-gelatin suspension using the Pearson test.* ***Results:*** *Group 2 had moisture content (10.63%), molecular weight (159.68 kDa), viscosity of chitosan powder (5.53 dPa.s), and viscosity of chitosan-gelatin suspension (40.20 c.Ps). Meanwhile, Group 1 had the highest values for ash content (27.83%) and deacetylation degree (93.72%). There was a significant difference in viscosity between chitosan-gelatin suspension groups. The Spearman correlation test showed that the correlation coefficient between the viscosity of the chitosan-gelatin suspension and molecular weight was 0.389 and deacetylation degree was -0.195, respectively.* ***Conclusion:*** *The characteristics of chitosan synthesized from Penaeus monodon have the potential as an injectable bone substitute in socket preservation. The viscosity of the chitosan-gelatin suspension is influenced by gelatin, molecular weight, and deacetylation degree of chitosan powder.*

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Rosalina Tjandrawinata <rosalina@trisakti.ac.id> Fri, Jan 5, 2024 at 9:14 AM

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1. Has this text ever been published in other media? Comment: I did not see this text ever been published in other media		V
2. Is the title appropriate, concise, clear, and describes the contribution of scientific development? (maximum 10 words, covering the variables studied) Comment: The title is clear, and describes the contribution of scientific development but too long, more than 10 word. It is 25 words		V
3. Research report:		
a) Introduction includes the background clearly? Comment: The background is clear enough	V	
b) The purpose clearly? Comment: The purpose is clear	V	
REVIEW	**YES***	**NO***
c) Methods and research design in accordance with the purpose of the study? Comment: Methods and research design in accordance with the purpose of the study	V	
d) The research procedure is described precisely and in detail, thus ensuring internal/external validity? Comment: The research procedure is described precisely and in detail, thus ensuring internal/external validity.	V	

e) The results answer the research question? Comment: The results answer the research question	V	
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Characteristics of Chitosan from *Penaeus monodon* Shells and Its Effect on The Viscosity of Chitosan-Gelatin Suspension as an *Injectable Bone Substitute Material* on *Socket Preservation*

Commented [RT1]: Make the title shorter

ABSTRACT

Background: *Injectable bone substitute materials are often used to assist the healing process of bone defects. The chitosan synthesized from Penaeus monodon shells was developed into a chitosan-gelatin suspension as an injectable bone substitute for socket preservation.* ***Purpose:*** *To describe the characteristics of chitosan powder and their influence to the viscosity of chitosan-gelatin suspensions. Characterization of chitosan was carried out through morphology, moisture content, ash content, molecular weight, deacetylation degree, and viscosity.* ***Methods:*** *Penaeus monodon shells from Tarakan Waters, Borneo, were prepared for chitosan using three synthesis methods (Group 1-3) and made into a chitosan-gelatin suspension with a chitosan and gelatin ratio of 45:55 (w/w%). Differences in viscosity using the Kruskal-Wallis and Mann-Whitney tests. Effect of molecular weight and deacetylation degree of chitosan powder on the viscosity of the chitosan-gelatin suspension using the Pearson test.* ***Results:*** *Group 2 had moisture content (10.63%), molecular weight (159.68 kDa), viscosity of chitosan powder (5.53 dPa.s), and viscosity of chitosan-gelatin suspension (40.20 c.Ps). Meanwhile, Group 1 had the highest values for ash content (27.83%) and deacetylation degree (93.72%). There was a significant difference in viscosity between chitosan-gelatin suspension groups. The Spearman correlation test showed that the correlation coefficient between the viscosity of the chitosan-gelatin suspension and molecular weight was 0.389 and deacetylation degree was -0.195, respectively.* ***Conclusion:*** *The characteristics of chitosan synthesized from Penaeus monodon have the potential as an injectable bone substitute in socket preservation. The viscosity of the chitosan-gelatin suspension is influenced by gelatin, molecular weight, and deacetylation degree of chitosan powder.*

Keywords: *Characteristics, Chitosan, Chitosan-Gelatin Suspension, Penaeus monodon, Viscosity.*

INTRODUCTION

The incidence of tooth extraction in general will have a repercussion on alveolar bone resorption. Alveolar bone resorption may occur horizontally or vertically. The large shrinkage nonetheless will occur in the horizontal dimension, especially on the buccal side of the alveolar ridge. The changes in the dimensions of the alveolar bone in post-extraction human tooth sockets showed a decrease in the height of the alveolar crest by 1.53 mm, a decrease in the width of the alveolar bone by 3.87 mm and 1.67 mm decreased alveolar height on the buccal side.[1] The process of socket wounds healing after tooth extraction results in a decrease in volume and changes in the shape of the alveolar bone that can interfere with the tooth extraction. Thus, socket prevention treatment must be carried out immediately to be able to reconstruct the alveolar bone in preparation for implant treatment, so that results are obtained that meet the criteria for successful implant treatment both aesthetically and functionally.[2]

The use of bone substitute material in the post-extraction socket can prevent alveolar ridge shrinkage and help the bone healing process. Scaffold is one of the substitute materials that is commonly used but has several disadvantages, namely being easily brittle and porous, poor mechanical strength by cause of weak bonds between particles, easy to biodegrade, limited and rigid shape.[3,4,5] Disadvantages of bone substitute materials in scaffold have prompted researchers to seek a form of bone substitute material that is effective and keen to cover these disadvantages and gain more leverage advantages. One of the forms of bone substitute material considered to gain improvement in covering the lack of scaffold form is injectable gel suspension. The advantages of administering bone substitute material in the form of a suspension are evident to set in the bone results from its high mechanical properties, execution to fulfill all parts of the bone, apparent to adapt to the anatomical shape of the bone, sterile and attainable.[4,5,6] The suspension is in the form of a gel so it is easy to apply by injection, in suspension preparations injectable bone substitute material can enter into the pores of the bone so that it can fill the bone and adjust the anatomical shape of the bone, the suspension can also be a drug delivery agent good quality, the suspension has high mechanical properties, and the suspension is sterile.[7]

Injectable Bone Substitute (IBS) is often used to treat osteoporosis, a disorder of bone fragility that can increase the risk of fractures. There are two types of IBS, namely IBS which contains ionic hydraulic cement which can harden in vivo after being injected, and IBS which contains a suspension which can harden when injected. The role of injectable bone substitutes can also be alleviated as drug agents that facilitate the healing process of bone defects. The synthesizing IBS material based on HA-Gelatin with the addition of 10% alendronate with a

suspension ratio of 45:55 which has the best viscosity value, is not toxic and can harden through the setting time test.[8] The manufacture of injectable bone substitute suspension based on hydroxyapatite composites with gelatin has an applicable viscosity value, especially at 25°C, which is 30.4 dPa.s to 39.4 dPa.s and has injectibility percentage value of 98.22% to 98.64%, respectively.[6] The suspension of injectable bone substitute material can be said to be applicable if the viscosity value is ≤ 40 dPa.s because the suspension will be able to flow over a wider area and be able to penetrate the pores of the damaged bone.[9] The viscosity is a material property that mitigates the thickness of liquid and is related to the resistance of analyzed fluid in flowing.[8] Viscosity is the internal frictional force of a fluid, the molecules that compose a fluid friction against each other as the fluid flows. Viscosity is affected by pressure, temperature, additives in suspension, size, and molecular weight. The presence of additional materials such as suspension materials will increase the viscosity because the binder and a bonding reaction will occur between the substances. Viscosity will be directly proportional to the size and molecular weight so that the larger the size and molecular weight of a substance, the more viscosity increases. The viscosity will increase if there are more double bonds.[10,11]

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Materials that can be used as bone substitute materials can be natural polymers, namely chitosan.[4,12] Chitosan is produced through the process of deacetylation of chitin compounds. Chitin is the main component in the shells of hard crustacean animals made of chitin, or what is known as the exoskeleton, such as crabs, shrimp, clam shells, and crabs. Chitosan has a quality standard, namely the degree of deacetylation. The degree of deacetylation (DD) is the number of acetyl groups from chitin that are converted into the active group NH_2 in chitosan so that it can affect the work of chitosan in its application. The use in the biomedical field of chitosan must have a value degree of deacetylation ≥ 80%.[13,14] The ideal requirement for chitosan to be used in the biomedical field is to have a degree of deacetylation ≥ 70. The optimum deacetylation rate will be obtained if the concentration of NaOH used is 50%. A concentration of 50% produces better deacetylation if the deacetylation is carried out at a low temperature.[15] The higher the degree of deacetylation, the more chitosan will have cationic properties, a property that can stimulate cell adhesion, as a cell modulator, differentiation, cell movement, synthesis, and cell function.[16,17]

This research proposed to develop the chitosan synthesized from shells of tiger shrimp (Penaeus monodon) from Tarakan waters, East Kalimantan, as a source of chitosan. These shrimps are traditionally cultivated; hence they have good quality and are much thicker and bigger than other types of shrimp, affected by higher chitosan content.[18] Based on the above, the researcher will construe the characteristics of chitosan synthesized from *Penaeus monodon*

with various methods and evaluate its effect on viscosity and cytotoxicity of chitosan-gelatin suspension as an ideal requirement for a candidate to be injectable bone substitute material in socket preservation.

MATERIALS AND METHODS

This research was carried out by making chitosan powder from *Penaeus monodon* shell, Tarakan Waters, Borneo, Indonesia. The Penaeus monodon's shell was cleaned with water and then dried up under the sun. Afterward, the shells were blended delicately and homogenized by 200 mesh to gain finer powder.

This research was divided into three synthesized method groups that were treated by 75°C deacetylation temperature for all groups, such as Group 1: Deproteinization, Depigmentation, and Deacetylation stages; Group 2: Demineralization, Depigmentation, and Deacetylation stages and Group 3: Deproteinization, Demineralization, Depigmentation, and Deacetylation stages. **Deproteinization Stage:** The stage was initiated by putting Penaeus monodon's shell powder (200 g) into 2000 ml NaOH 3,5% (Merck, USA) 1:10 (b/v), accordingly stirring this solution with 75°C for 2 h on a magnetic stirrer and filtered with filter paper. Afterward, the solution was cleaned with 1000 ml aquadest until it became neutral pH powder. Lastly, it was roasted inside a 75°C oven for 24 h until it became powder, called chitin. **Demineralization Stage:** The stage was undertaken by adding 3000 ml HCI 1 M (Merck, USA) 1:15 (b/v) into chitin powder (200 g) accordingly stirring this solution for 1 h inside room temperature on a magnetic stirrer and filtering by filter paper. Afterwards, the solution was filtered and cleansed by 1000 ml aquadest until it gained neutral pH powder. Finally, the powder was roasted inside a 75°C oven for 1 h until dried, which produced chitin powder. **Depigmentation Stage:** The stage was undertaken by putting chitin powder (200 g) gained from the deproteinization or demineralization stage into 2000 ml acetone (Merck, USA) 1:10 (b/v) for 20 h. Afterwards, the solution was filtered and cleaned by aquadest until it gained neutral pH powder. The powder then was roasted in a 75°C oven for 1 h until dried. **Deacetylation Stage:** The stage was undertaken by putting powder (100 g) gained from the depigmentation stage into 1000 ml NaOH 50% (Merck, USA) 1:10 (b/v). Afterward, the solution was soaked at 75°C temperature. The solution was filtered and cleansed by 1000 ml aquadest until it gained neutral pH powder. Finally, the solution was roasted inside a 75°C oven for 1 h until dried, that called chitosan.

Commented [RT3]: A chart / diagram at least of this part will be very useful to make it easier to understand

The physicochemical properties of chitosan synthesized from *Penaeus monodon* shell with various synthesized methods were characterized to analyze chitosan morphology, moisture content, ash content, molecular weight, deacetylation degree, and viscosity.

Morphological characteristic: The chitosan morphological characteristic was analyzed by Scanning Electron Microscope (SEM) with 15 kV voltage to evaluate the morphology illustration by 200x resolution.

Moisture content measurement: The moisture content test were carried out using gravimetric analysis. The sample (0.5 g) inside the porcelain cup had been known as the weight. The sample was heated inside a 100-105°C oven for 1-2 h. Then, it was refrigerated inside a desiccator for 30 minutes. Thenceforth, it was weighed and heated inside the oven, then it was refrigerated inside a desiccator and repeated by the constant weight. The replication was undertaken three times for each sample group, then the average calculation was undertaken in the end. This moisture content calculation was undertaken as follows:

$$\textbf{Moisture content}\ (\%) = \frac{[\textbf{Initial weight}\ (\textbf{g}) - \textbf{Dry weight}\ (\textbf{g})]}{\textbf{Initial weight}\ (\textbf{g})}\ \textbf{x 100}$$

Ash content measurement: The ash content test was undertaken by gravimetric analysis. The porcelain cup was weighed, and then chitosan (1 g) was put inside that cup. Afterward, chitosan was soaked by adding 1 mL concentrated H_2SO_4 and slowly heated until it became very burnt. Then, when it was finally cooled, add 1 mL H_2SO_4 inside the cup. It is heated slowly until (600 + 50) °C temperature and retained at a similar temperature until the residual becomes burnt. Finally, the cup is refrigerated inside a desiccator and weighed. The process of heating and cooling process was repeated until gaining constant weight result. Ash content was calculated, as follows:

$$\textbf{Ash content}(\%) = \frac{(\mathbf{W_2} - \mathbf{W_1})}{\mathbf{W}}\ \textbf{x 100}$$

W_1 is initial weight tested (g), W_2 is ignited crucible weight (g) and W_3 is residue ignited with crucible (g). The replication was undertaken three times for each sample, then the average calculation was undertaken at the end.

Molecular weight measurement: The molecular weight test used the Ostwald viscosity method. The initial chitosan powder was weighed. After that, it was placed into a centrifuge tube and dissolved into 50 ml of 1% acetate acid. Accordingly, it was set into viscometer Ostwald. Afterward, it was smoked until the upper line and flowed until the lower

line. The flow time is calculated. Finally, the result was connected to the weight molecule by Mark and Houwink equality, as follows:

$$[\eta]\mathbf{SC} = \frac{[\mathbf{2}(\eta\mathbf{sp} - \mathbf{ln}\eta\mathbf{r})]^{\mathbf{0,5}}}{\boldsymbol{C}}$$

[ɳ]SC is intrinsic viscosity score, ηsp is specific viscosity score (ηsp = ɳr – 1), ɳr is relative viscosity, ln is natural log, and C is solvent

$$[\eta] = \mathbf{k}\,[\mathbf{M_v}]^{\propto}$$

M_v is chitosan molecule average weight viscosity, ∝ and k are constants (∝ = 0,83 and k = 1.4 x 10^{-4} acetate acid solution and [ɳ] is intrinsic viscosity.

Determinate Deacetylation Degree (DD): The infrared spectrophotometry was used to determinate chitosan functional groups and deacetylation degree. The method used in this study was the Fourier Transform Infrared Spectroscopy (FTIR) instrument (Perkin Elmer, UK) method. The deacetylation degree was calculated by using Baxter's formula equation:

$$\mathbf{DD}\ (\%) = \mathbf{100} - \left[\left(\frac{\mathbf{A_{1655}}}{\mathbf{A_{3450}}}\right) \times \left(\frac{\mathbf{100}}{\mathbf{1.33}}\right)\right]$$

A_{1655} is an absorption degree at 1655 cm^{-1} of the Amide-I band which measures group N-acetyl content. A_{3450} is the absorption degree at 3450 cm^{-1} which measures the hydroxyl band. Factor 1.33 is ratio A_{1655} dan A_{3450} for fully N-acetylated chitosan.

The chitosan-gelatin suspensions were prepared. Begin with mixing the two solutions with a gelatin: Na-CMC ratio of 3: 1. The gelatin solution that has been made with a concentration of 5% is put into a 225 ml beaker which is then mixed with 75 ml of 2% Na-CMC solution. Stirred until the solution is homogeneous. Chitosan-gelatin suspension for each group was prepared by mixing chitosan and gelatin with a ratio of 45:55. Making gelatin and chitosan suspension (ratio 50:50) is done by mixing 5% (w/v) gelatin solution which has been mixed previously with 300 ml of 2% (w/v) Na-CMC solution with 1% chitosan solution (w /v) as much as 300 ml into a beaker and stirred continuously for 6 hours at a temperature of 40°C. Preparation of gelatin and chitosan suspension (ratio 45:55) is done by mixing 5% (w/v) gelatin solution which has been mixed previously with 300 ml of 2% (w/v) Na-CMC solution with 1% (w/v) chitosan solution. /v) as much as 270 ml into a beaker and stirred continuously for 6 hours at 40°C.

Viscosity Measurement: The viscosity test of chitosan powder and chitosan-gelatin suspension was carried out using a Brookfield viscometer. Measurements for each group were carried out six times. The sample was immediately put into a 300 ml glass breaker and then heated to room temperature at 25°C. Then, the sample was measured for viscosity with rotor number 1. The results displayed are numbers indicating the viscosity level of the chitosan suspension in cPa.s units as a function of temperature.[6]

Commented [RT4]: Beaker glass?

RESULTS

The morphology of *Penaeus monodon* shell and chitosan isolation method effect on the sample morphology was observed by SEM are shown in **Figure 1**. *P. monodon* shell powder illustrated an irregular and rough morphology form. The morphology form of *P. monodon* shell powder was almost similar with isolated chitosan powder morphology both by Group 1. Furthermore, Group 2 showed a more delicate material surface by forming (a well-defined round shape). Then, Group 3 showed more delicate and fibrous surface morphology.

The percentage yield of chitosan is determined by using the ratio of the obtained chitosan dry wet after various synthesized methods and the starting material of Penaeus monodon. **Table 1** showed the percentage yield of chitosan in Group 1 was 10.5%. Group 3 showed the lowest percentage yield of chitosan, 8.2%. On the other hand, Group 2 showed the highest percentage yield of chitosan, 20%.

The moisture content, ash content, and viscosity of chitosan synthesized from *P. monodon* with various methods are shown in **Table 1**. In this study, the synthesized chitosan from *P. monodon* of Group 1 showed the lowest moisture content of 6.53%, but this group also showed the highest ash content of 27.83%. Group 2 showed the highest moisture content of 10.63% followed by 1.2% of the ash content. The moisture content of Group 3 was 8.73%. Furthermore, Group 3 showed the lowest ash content by 1.06%. The viscosity of Group 3 was 5.07 dPa.s. On the other hand, Group 2 showed the highest viscosity by 5.53% and Group 1 showed the lowest viscosity by 4.6%.

Table 1 also shows that the chitosan synthesized from *P. monodon* with various methods may produce various molecular weights and deacetylation degrees. Group 2 showed the highest molecular weight, 159,68 kDa. The deacetylation degree of Group 2 was 87,87%. The lowest molecular weight was gained by Group 3, which was 37,12 kDa followed by the deacetylation degree, which was 86,22%. Whereas, the highest deacetylation degree was gained, by Group 1, which is 93,72%. the molecular weight of Group 1 was 65,98 kDa.

FTIR analysis was employed to identify the functional groups of chitosan derived from *P. monodon* shell through various synthesis methods. In **Figure 2**, the infrared spectrum of chitosan reveals distinctive features. The determination of DD of chitosan involved a robust band appearing at 3444.7 cm-1 (Group 1), 3365 cm-1 (Group 2), and 3437.7 cm-1 (Group 3), affirming the presence of NH and OH stretching, along with intramolecular hydrogen bonds. Additionally, the existence of acetylated residues of amide I (NHCOCH3) was confirmed in the bands around 1655.8 cm−1 for all three groups. Notably, in proximity to this band, several peaks emerged, attributable to the N-H bending of amide II bands and indicating C–H bending vibrations of -CH2. These findings align with results reported in previous studies.[19,20,21]

Table 2 shows the viscosity of chitosan-gelatin suspension. The lowest viscosity of chitosan (Group 1)-gelatin suspension was 27.30 c.Ps. The highest viscosity was obtained in the chitosan (Group 2)-gelatin suspension, which was 40.20 c.Ps. The viscosity of chitosan (Group 3)-gelatin suspension was 37.25 c.Ps. Statistical analysis showed that there were significant differences between groups (p=0,00). The Spearman correlation test was carried out to determine the effect of the degree of deacetylation and the molecular weight of chitosan on the viscosity of the chitosan-gelatin suspension. The results of the Spearman correlation test showed that the correlation coefficient between molecular weight and viscosity of chitosan-gelatin suspension was 0.389, which has a moderate correlation level and is positive. Meanwhile, the results of the Spearman correlation test between the deacetylation degree and viscosity of chitosan-gelatin suspension showed that the correlation coefficient was -0.195, which has a weak correlation level and is negative.

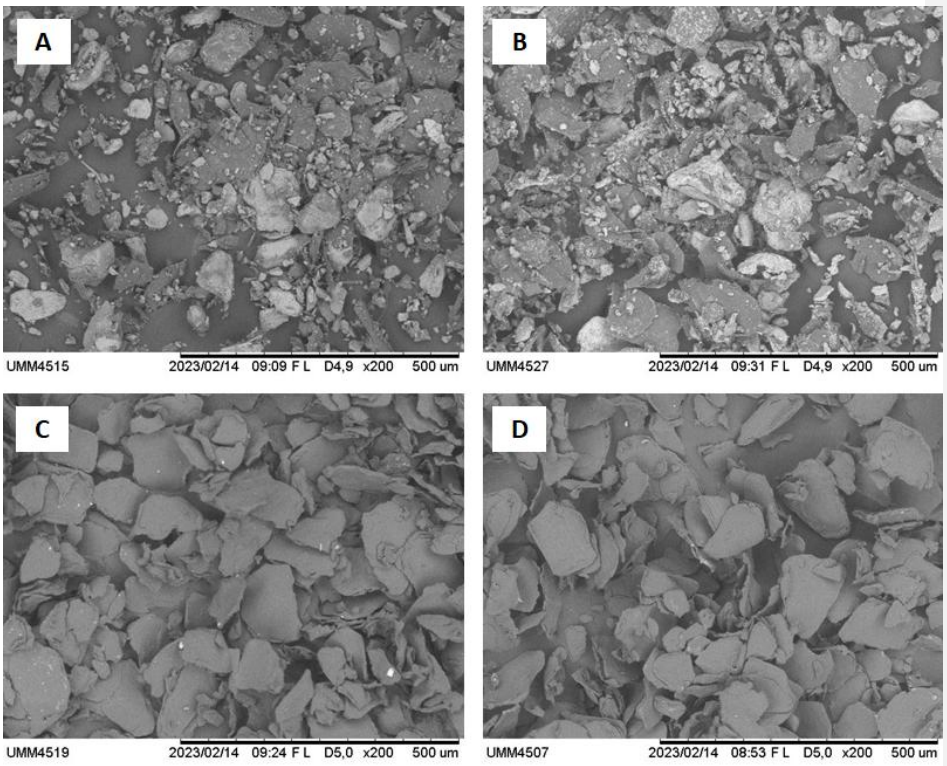
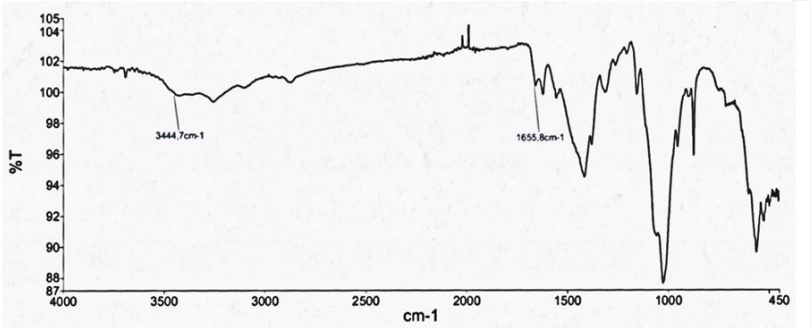
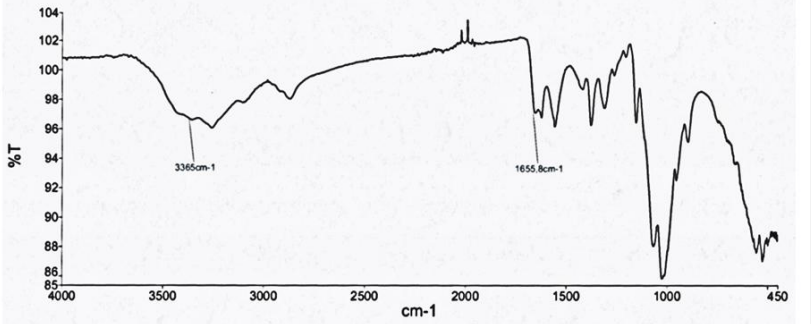


Figure 1. Morphology of *Penaeus monodon* shell and chitosan using SEM with 200x resolution. The morphology form of *Penaeus monodon* shell powder (A). The morphology form of chitosan powder of Group 1 (B). The morphology form of chitosan powder of Group 2 (C). The morphology form of chitosan powder of Group 3 (D).

Group 1



Group 2



Group 3

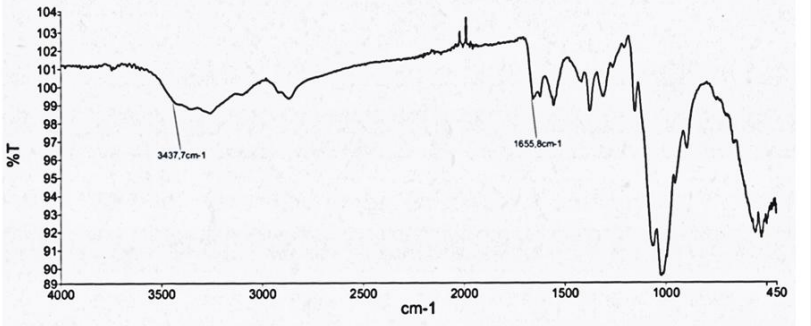


Figure 2. FTIR spectra of *Penaeus monodon* shell and chitosan powders.

Table 1. Yield and Physicochemical characteristics of chitosan from *Penaeus monodon* Shells.

	Group 1	Group 2	Group 3
Yield (%)			
Chitosan powder from *Penaeus monodon* shell (g)	100	100	100
Yield of chitosan (%)	10.5 %	8.2 %	20 %
Physicochemical Properties (%)			
Moisture content (%)	6.53	10.63	8.73
Ash content (%)	27.83	1.2	1.06
Viscosity (dPa.s)	5.07	5.53	4.6
Molecular weight (kDa)	65.98	159.68	37.12
Deacetylation degree (%)	93.72	87.87	86.22

Table 2. Viscosity of Chitosan-Gelatin Suspensions.

	Group 1	Group 2	Group 3
Viscosity of Chitosan-Gelatin suspension (c.Ps)	27.30	40.20	37.25

DISCUSSION

The morphology of the material and the effect of various methods on synthesized chitosan powder from *Penaeus monodon* were observed by SEM. The difference in the surface materials can be seen in Figure 1. This study proved that the demineralization stage of the method affected the form and chitosan material surface. The *P. monodon* shell powder as the starting material and the Group 1 powder that was synthesized without the demineralization stage showed similar morphology, such as the irregular form and rough surface. On the other hand, the Groups 2 and 3 that synthesized with the demineralization stage exhibited similar morphology, such as more delicate surface morphology. The demineralization stage as one of the stages for synthesizing chitosan powder aims to remove mineral content, especially $CaCO_3$ and $Ca_3(PO)_4$ contained in the shrimp shell by the acid treatment.[21] The deproteinization

disrupted of chemical bonds between chitin and proteins; the demineralization removed all the minerals and the deacetylation removed the acetyl groups.[22] The steps have a significant effect on the surface morphology and size of chitosan powder. The appearance of Group 3 which used the 4-step method (deproteinization, demineralization, depigmentation, and deacetylation) presented a smooth surface with a fibrous structure and was smaller than other groups. These results indicated that the synthesized method of chitosan exhibits changes in the shape and surface morphology.

Commercial chitosan during storage may be affected by moisture absorption, due to its characteristic which is a hygroscopic material in nature. This study showed chitosan moisture content is affected by the synthesized process. The deproteinization step affected the moisture content of chitosan powder. It was proven by Group 2 that the 3-step used method (demineralization, depigmentation, and deacetylation) showed the highest moisture content compared to Group 1 and Group 3 which included the deproteinization steps method. A similar study also proved that the moisture content of chitin from shrimp shell showed the moisture content after deproteinization was decreased compared to before deproteinization.[23] The commercial chitosan should contain moisture less than 10%.[24]

The demineralization step affected the ash content of chitosan powder. The ash content percentage of chitosan describes the effectiveness of the demineralization process to eliminate minerals. The explanation because the chitosan ash content is calculated from the residue inorganic weight ratio towards chitosan weight. Group 1 which used the 3-step method (deproteinization, depigmentation, and deacetylation) has the highest ash content because there was no demineralization stage in this method. The ash content is an indicator of the demineralization process which aims to remove minerals and carbonates. The ash content is should not exceed 1% to get good quality chitosan. The ash content of chitosan is an important indicator in determining chitosan purity. The more ash content is removed, the higher the quality of the degree of purity of chitosan.

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The molecular weight of chitosan is also an important parameter because it affects physiochemical characteristics and chitosan biology, such as hydrophilicity, viscosity, moisture absorption, biodegradability, antimicrobial activity, and chitosan mucho adhesion. These results presented that the deproteinization and or demineralization steps influenced the molecular weight of chitosan. The highest molecular weight of chitosan was presented in Group 2 which used the 3-step method without deproteinization. This deproteinization steps should be disrupted the chemical bonds between chitin and proteins. Furthermore, the molecular weight of chitosan depends on how many monometric units are in the biopolymer.

When the biopolymer only contains monomeric forms of 2-amino-2-deoxy-D-glucopyranose, the degree of deacetylation is 100 percent. The deacetylation degree is the relation between units of 2-acetamido-2-deoxy-Dglucopyranose.[25] This study proved that Group 1 which used the 3-step method and Group 3 which used the 4-step method showed lower molecular weight yet higher deacetylation degree. The properties and uses of chitosan are influenced by the deacetylation degree and molecular weight. This showed that repeated use of NaOH in the deproteination and deacetylation processes is more effective in breaking off more acetyl groups so that the deacetylation degree will be lower. Repeated use of NaOH produces longer polymer chains and greater molecular weight. The isolation process of the deproteinization, depigmentation, and deacetylation methods only uses NaOH at the deacetylation stage so it tends to cut the chitin main chain and produces chitosan with a short polymer chain. The use of NaOH alone in the deacetylation stage will result in the release of a few acetyl groups and a low molecular weight so that the deacetylation degree will be higher.[26]

Chitosan is a product of chitin deacetylation which is a long-chain polymer of glucosamine (β-1,4-2 amino-2-deoxy-D-Glucose). FTIR was used to identify the functional group present in the chitosan. The determination of the deacetylation degree was done by two absorption bands, a characteristic band that represents the acetylated residues of amide I that were seen for the three groups. Also confirmed that carboxymethylation takes place only at both amino groups and hydroxyl groups with a value of deacetylation degree from chitosan about >86% for the three groups. However, the difference was seen in intensities due to different reactions of acid treatment (demineralization stage) except in Group 1. Some band was weakened after the deproteination and demineralization process.[20]

The viscosity value of chitosan is directly proportional to its molecular weight. The greater the viscosity value, the greater the chitosan molecular weight. In this research, chitosan powder was produced using the Deproteinization, Demineralization, Depigmentation, and Deacetylation process (Group 3) which had the lowest molecular weight and showed the lowest viscosity value, namely 4.6 c.Pas. Meanwhile, chitosan powder produced using the Demineralization, Depigmentation, and Deacetylation processes only (Group 2) which has the highest molecular weight shows the highest viscosity value, namely 5.53 c.Pas. This means the chitosan polymer molecular chain of Group 2 is longer than Group 3. When chitosan is added to a solvent, the solvent gradually diffuses into the polymer aggregates resulting in the swelling of the polymer, and all chain segments of the polymer molecule in solution are unfolded and fully solvated.[27]

This study also showed the correlation between molecular weight and viscosity of chitosan-gelatin suspension through the Spearman correlation test, which turned out to have a positive correlation value so it can be said to have a positive relationship, which means there is a relationship in the same direction where the greater the molecular weight, the greater the viscosity. The higher molecular weight of chitosan may be higher the viscosity of chitosan-gelatin suspension. However, the Spearman correlation test in this study showed a moderate level of correlation between the molecular weight of chitosan powder and the viscosity of chitosan-gelatin suspension. This is possible due to the intermolecular interactions between chitosan and gelatin. Gelatin molecules bind to chitosan macromolecules through electrostatic interactions and hydrogen bonds, it is thought that a single chitosan macromolecule binds to 3-4 gelatin macromolecules. Chitosan is a lyophilized component and gelatin is a blocking polyelectrolyte. The electrostatic interactions between charged amino groups in chitosan and carboxyl groups of gelatins contribute to the stabilization of chitosan-gelatin complexes.[28,29] A similar study reported that the viscosity of chitosan solution is influenced by its molecular weight, but the presence of electrolytes also influenced the viscosity, in which the viscosity is sharply reduced in the presence of sodium acetate up to a concentration of about 2 g/dl.[27]

The results of the Spearman correlation test on the correlation between deacetylation degree and viscosity of chitosan-gelatin suspension was -0.195, so it has a weak level of correlation and is negative, so it can be said to have an opposite relationship, where the greater the degree of deacetylation, the viscosity decreases and vice versa. This is possible because the three types of chitosan synthesis methods in this research can produce chitosan powder which has a high degree of deacetylation, namely in the range of 86.22 - 93.72%. So even though there seems to be a tendency for a correlation between the degree of deacetylation and the viscosity of chitosan-gelatin suspension, the level of correlation is weak. A higher deacetylation degree of chitosan may lower the viscosity of the chitosan-gelatin suspension. Increasing the value of the degree of deacetylation in the group results in the stiffness of the chitosan chain in solution decreasing and the solubility of chitosan increasing. The high value of the degree of deacetylation causes the cationic charge of chitosan to also increase. Increasing the cationic charge in the solution will produce a repulsive force, which will make the chitosan polymer, which was previously coiled, open into straight chains, and the viscosity of the solution will decrease.[30]

In regards to the results of this study, it can be concluded that the chitosan synthesized from *P. monodon* used four stages of synthesis such as deproteinization, demineralization, depigmentation, and deacetylation showed the most suitable characteristic to be used as

injectable bone substitute materials on socket preservation. The viscosity of the chitosan-gelatin suspension is influenced by gelatin, molecular weight, and DD of chitosan powder. Thus, this chitosan-gelatin suspension can be applied to socket preservation treatment by modifying the chitosan-gelatin suspension for exhibiting an appropriate setting time.

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4. Bukti review diterima oleh pengelola jurnal

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E-ISSN: 2442-9740; P-ISSN: 1978-3728