Synthesis and antibacterial activity of chitosan membrane from shrimp shell waste

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Abstract. Synthesis and antibacterial activity of chitosan membrane was investigated. Chitosan membrane have been successfully by simple method from chitosan extracted from shrimp shell waste. Extraction of chitosan was carried out in four steps: demineralization, deproteinization, decolorization and deacetylation of chitin. The effect of deacetylation temperature on deacetylation process was studied. The results shown that the increase of deacetylation temperature from 30°C to 90°C causes the increase of chitosan deacetylation degree (DD). The increase of deacetylation temperature cause the increment of OH- attack to the amino group thus realizing the effective deacetylation of chitin. The highest chitosan DD was up to 77.99% is achieved under the deacetylation temperature from 90°C and the occurrence of deacetylation structurally demonstrated by the Fourier transform infrared (FTIR) and the XRD characterization. The antimicrobial test results used S. epidermidis and P. acne of chitosan membrane at various deacetylation temperature conditions indicated that no bacterial activity for all variants.

1. Introduction
Shrimp is one of the main fisheries commodities worldwide, especially in Indonesia [1-3]. This commodity is generally managed on an industrial scale with the main product is frozen shrimp meat. During the processing, meat is separated from the head and shells and produces shrimp waste. In spite of the waste are biodegradable but the dumping off large quantities makes the process running slow and causes environmental problems such as odor to source environmental pollutant [3-4].

The recycling of wastes by extraction of commercially materials substances is an alternative solution and provides added value for shrimp shell waste. Chitosan is one of the commercially materials which is product of chitin deacetylation. Many studies have reported that shrimp shell waste is the most important chitin source therefore it is potential waste that have usefulness for chitosan raw materials [3-6]. The unique physicochemical and biological properties of chitosan such as biodegradability, biocompatibility, nontoxicity, and antimicrobial activities make it has been extensively studied for various biofunctionalities [3].

Commonly, antimicrobial activities of chitosan become a great attraction among researchers especially in the current pandemic conditions. The facial masks are still the front line to prevent the transmission of this virus. The increasing needs for masks, the amount of masks raw material, medical waste due to the use of masks, and comfortability are the main focus in mask development technology. The very shortage of masks makes the threat of increasing medical waste due to the use of masks being
the main environmental concern. This is because the antimicrobial properties of masks are still not efficient [7–9]. Antimicrobial properties of masks especially for facial masks is expected to provide facial skin protection from microbial infections such as Propionibacterium acnes, Corynebacterium, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus pyogenes, etc during in use [10]. Chitosan is one of the main candidates for materials that have antimicrobial and biodegradable properties [3]. The most important factor of the antimicrobial properties is surface area [10–13]. The materials which have the large surface area such as in form of membrane, thin film or nanoparticle make the interaction with surface of the microbial cells become more effective and efficient [14–16]. Meanwhile chitosan is also friendly to the body's tissue system so that it can increase comfortability of masks [17–18]. This work presents antimicrobial properties of chitosan membrane against Staphylococcus epidermidis and Propionibacterium acnes. In addition, the effect of chitin deacetylation temperature on the degree of deacetylation of chitosan was also studied.

2. Methods

2.1 Materials

In this work shrimp shells waste from the dried shrimp home industry (Bangka, Indonesia) with a single species of shrimp (Penaeus semisulcatus). The materials used such as hydrochloric acid, sodium hydroxide, polyvinyl alcohol, sodium chloride, glacial acetic acid, sulfuric acid and sodium hypochlorite were purchased from MERCK. The microbial isolate such as S. epidermidis ATCC 12228 and P. acnes ATCC 12223 were provided by IPB culture collection and stored at 4°C.

2.2 Extraction and characterization of chitosan from shrimp shell waste

The extraction procedure of chitosan in this work followed four steps such as demineralization, deproteinization, decolorization and deacetylation of chitin. Before the extraction step is carried out, shrimp shell waste is rinsed to eliminate all other related impurities and dried using sun drying (approximately 27 °C). The powder of shrimp shell waste obtained through ground the dried shrimp shell to pass through a sieve 100 mesh. The powder demineralized using HCl 1.5 M with a solid to solvent ratio of 1:12 at room temperatures while stirred for one hour. The mixture was filtered, washed with distilled water to remove excess of HCl, and dried in an oven. The demineralized shrimp powder used NaOH 2 M with a solid to solvent ratio of 1:6 while stirred for one hour at room temperature. The mixture was filtered, washed with distilled water to remove excess of NaOH, and dried in an oven to obtained chitin. The decolorization of chitin used NaClO 5% with a solid to solvent ratio of 1:10 while stirred for 30 minutes at room temperature. The bleached chitin was filtered, washed with distilled water to remove excess of NaClO, and dried in an oven. In this step to study the effect of deacetylation temperature, the chitin isolated previously was deacetylated at different temperature 30, 60, and 90°C used NaOH 60% with a solid to solvent ratio of 1:10 while stirred for one hour. After each experiment, the mixture was filtered, washed with distilled water to remove excess of NaOH, and dried to obtained chitosan. The chemical element composition and the phase composition of sample spectra characterized used XRF and XRD respectively. The FTIR spectra of chitosan for all variant were recorded in the range 600 - 4000 cm⁻¹. The degree of deacetylation of sample are calculated based on FTIR spectra according to the equation at previous work [3].

2.3 Synthesis of chitosan membrane

The chitosan membrane was synthesized by reacting chitosan powder with acetid acid 1% with a solid to solvent ratio of 1:50 while stirring for one hour at room temperature to form chitosan gel. The chitosan gel was mixed with 5 ml PVA 10% solution then put into a glass plate and dried at room temperature to obtained chitosan membrane. The chitosan membrane reacted with NaOH 1% by immersion for 5 minutes at room temperature and followed by washing with distilled water to pH 7 and dried at room temperature. After that, the chitosan membrane reacted with 50 mL H₂SO₄ 0.5 N solution by immersion for 5 minutes then washed with distilled water to pH 7 and dried at room temperature.
2.4 Antibacterial test of chitosan membrane

The antibacterial activity was evaluated against *Staphylococcus epidermidis* and *Propionibacterium acne*. A freshly prepared overnight culture containing approximately $2 \times 10^8$ CFU/mL (colony forming units in milliliter) of *S. epidermidis* and *P. acne* was inoculated into petri dish containing chitosan membrane for all varians with diameter 2 cm and incubated at 37°C for 24 h respectively. At the end of 24 hours incubation, the samples were observed for antibacterial activity by observing the zone around the chitosan membrane.

3. Results and Discussion

The chemical element composition of shrimp shells, chitin and chitosan at deacetylation temperature 90°C are presented in Table 1, Table 2, and Table 3 respectively. The dominant element of shrimp shells waste is Ca which reaches 86.56%. The presence of P and S indicated that its not only contain minerals which is represented by Ca, Mg, Si, Fe and Al but also a little amount of protein relatively. In the Figure 1, its clearly that the element Ca in the waste is confirmed as the single phase of calcium carbonate (CaCO$_3$) in forms of calcite. Chitin shrimp shells waste was obtained through demineralization and deproteinization steps used HCl and NaOH respectively.

| Table 1. The chemical element composition of shrimp shells. |
|-----------------------------------------------------------|
| Element | Content (% Wt) | Element | Content (% Wt) |
| Ca      | 86.56          | Si      | 1.35          |
| P       | 3.92           | Fe      | 0.52          |
| S       | 3.22           | Al      | 0.41          |
| Mg      | 1.77           | Others  | 2.25          |

| Table 2. The chemical element composition of chitin shrimp shells waste. |
|-------------------------------------------------------------------------|
| Element | Content (% Wt) | Element | Content (% Wt) |
| Ca      | 35.41          | Mg      | 8.36          |
| Si      | 23.62          | S       | 4.72          |
| Fe      | 10.22          | P       | 4.21          |
| Al      | 9.87           | Others  | 3.59          |

| Table 3. The chemical element composition of chitosan at deacetylation temperature 90°C. |
|----------------------------------------------------------------------------------------|
| Element | Content (% Wt) | Element | Content (% Wt) |
| Ca      | 28.76          | Fe      | 9.12          |
| S       | 24.92          | Mg      | 4.71          |
| P       | 15.22          | Al      | 3.38          |
| Si      | 11.43          | Others  | 2.46          |
For refinement the color of chitin shrimp shells waste used NaClO as bleaching agent. The chemical element composition of chitin shrimp shell waste consists of Ca as the dominant element was 35.41% and another element such as Si, Fe, Al, and Mg (Table 2). The decrease contain of Ca in chitin indicates that the demineralization process in shrimp shell waste has been carried out. This is confirmed by the disappearance of calcite peaks in the diffraction data pattern on chitin. The contain of Ca in chitin is still quite high. The appearance of peaks of CaO, Ca(OH)$_2$ and CaCO$_3$ confirmed the presence of Ca derivative compounds in chitin. Chitin peaks in this work were observed at 20$^\circ$; 23.24$^\circ$ and 26.35$^\circ$ (Figure 2) [19-20].

Based on the analysis of the diffraction data pattern shown in Figure 3, it shows that overlapping peaks of chitosan and chitin at 20$^\circ$. Meanwhile, the other chitin peaks have disappeared and the diffraction data pattern has a tendency to widen peaks. This indicates that there is a decrease in chitin contain and in the crystallinity of the chitosan during the deacetylation process. In addition, there are also several peaks of Ca derivative compounds and this is confirmed from the data chemical element composition of chitosan which was successfully synthesized at a deacetylation temperature of 90$^\circ$C where the Ca content was 28.76% (Table 3).
Figure 3. The XRD pattern of chitosan at deacetylation temperature 90°C.

The FTIR spectra of chitosan samples obtained under various deacetylation temperature conditions are illustrated in Figure 4. The broad band at about 3432 cm\(^{-1}\) corresponded to the vibrational OH stretching. The successful deacetylation demonstrated by disappeared the bands nearly at 3255 and 3098 cm\(^{-1}\) originating from stretching of N-H after deacetylation and reduction of band at 1655 cm\(^{-1}\) and 1310 cm\(^{-1}\) assigned to the stretching of C=O in amide bond and CO-NH bending vibration respectively [3]. The stretching vibrations of the glycosidic bond of chitosan polysaccharide structure at 1016 cm\(^{-1}\) also indicated the deacetylation of chitin.

Figure 4. FTIR spectra of chitosan from shrimp shells at various deacetylation temperature conditions.

Deacetylation temperature is one of the important factors that affect the chitosan DD. In this work, the chitin shrimp shell waste deacetylazed at different temperatures 30°C, 60°C and 90°C respectively. As shown in Table 4, with the increase of deacetylation temperature, the chitosan DD increased gradually 74.76% to 77.99%. The highest of chitosan DD is reached at the deacetylation temperature was 90°C. The steric hindrance formed by compact structure of natural chitin mostly influenced the
deacetylation reaction of chitin because its obstructs the attack of OH\(^{-}\) to the amino group. Furthermore, the diffusion rate of OH\(^{-}\) to the surface and the inside of chitin particle would be closely depends on deacetylation temperature. Therefore, the increase of deacetylation temperature facilitated OH\(^{-}\) to resolves the steric hindrance more effective and achieve the chitin deacetylation.

**Table 4.** Degree of deacetylation of chitosan at various deacetylation temperature conditions.

| Deacetylation temperature (°C) | Degree of deacetylation (%) |
|-------------------------------|-----------------------------|
| 30                            | 74.76                       |
| 60                            | 75.17                       |
| 90                            | 77.99                       |

The antimicrobial test results of chitosan membrane at various deacetylation temperature conditions as shown in Table 5. The antimicrobial test results used *S. epidermidis* and *P. acne* of chitosan membrane at various deacetylation temperature conditions indicated that no bacterial activity for all variants (Figure 5).

**Table 5.** The antimicrobial test results of chitosan membrane at various deacetylation temperature conditions

| Types of bacterial               | Deacetylation temperature (°C) |
|----------------------------------|-------------------------------|
| *Propionibacterium acne*         | 30                            |
| *Staphylococcus epidermidis*     | 30, 60, 90                     |

(-) No bacterial activity

**Figure 5.** Photograph of antimicrobial test results of chitosan membrane at various deacetylation temperature conditions against *S. epidermidis*: (a) 30°C, (b) 60°C, and (c) 90°C.

Mechanisms for the antimicrobial action of chitosan was studied such as chelation and kationic-anionic interaction [21-23]. Chelation mechanism occurs when chitosan could chelated with trace elements or essential nutrients to inhibit the growth of bacteria. Meanwhile kationic-anionic interaction mechanism occurs when chitosan could interact with anionic groups on the cell surface and form
polyelectrolyte complexes with bacterial surface compounds, thereby forming an impermeable layer around the cell, which prevents the transport of essential solutes into the cell.

Figure 6. Photograph of antimicrobial test results of chitosan membrane at various deacetylation temperature conditions against P. acne: (a) 30°C, (b) 60°C, and (c) 90°C.

The most important factor of the antimicrobial action of chitosan is surface area and degree of deacetylation. Its provide high affinity with bacteria cells because of the larger surface area of the chitosan could be tightly adsorbed onto the surface of the bacteria cells so as to disrupt the membrane, which would lead to the leakage of intracellular components, thus killing the bacteria cells. The negatively charged surface of the bacterial cell is the target site of the polycation. Its depend on the degree of deacetylation of chitosan. Therefore, the polycationic chitosan with higher surface charge density such as in form of membrane, thin film or nanoparticle interact with the bacteria will be greater than chitosan without in form it.

4. Conclusion
Chitosan has been successfully produced from shrimp shells waste and its antibacterial activity was investigated. In this work, significantly indicated that increasing of deacetylation temperature causes the increase of chitosan DD. The highest chitosan DD was 77.99% is achieved at deacetylation temperature of 90°C. The antimicrobial test results used S. epidermidis and P. acne of chitosan membrane at various deacetylation temperature conditions indicated that no bacterial activity for all variants. However, further studies are needed to maximize the degree of deacetylation of chitosan and the inhibition of bacteria growth factors such as the minimum inhibitory and bactericidal concentrations due to its contribution to the effectiveness of antibacterial activity of the chitosan membrane.

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