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# Synthesis of Sulfated Chitosan Through Sulfation Reaction of Chitosan with Chlorosulfonic Acid in N, N-Dimethylformamide, and Antibacterial Activity Test

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**Abstract.** Sulfated chitosan has been successfully synthesized through sulfation reaction between chitosan and chlorosulfonic acid using catalyst and N, N-Dimethylformamide (DMF) solvent. The first step was sulfation of chitosan with 4; 4.5; 5 mL of HClSO3. The formation of sulfated chitosan was supported by an increased solubility in water and the appearance of peaks at 1111 $cm^{-1}$, which indicated the C-O-S group. Sulfated chitosan that has been produced, has a degree of sulfation of 5.6041%; 6.0045%; 6.8051%. Sulfated chitosan shows moderate antibacterial activity and a wide spectrum of antibacterial properties against *Escherichia coli* and *Staphylococcus aureus* bacterias. The increase in the degree of sulfation was proportional to the increase in antimicrobial activity of both bacteria. The highest antibacterial activity based on inhibitory zone diameter was 10 mm for sulfated chitosan that has been produced from 1 g of chitosan with 5 mL of $HClSO_3$ with a degree of sulfation was 6.8051%.

**Keywords:** Antimicrobial Index, Chitosan, Sulfation, Sulfated Chitosan

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## 1 Introduction

Chitin is a cellulose-derived polysaccharide that has an N-acetyl group at the position of the C-2 atom. This compound is mostly found in the outer shell of invertebrate animals such as arthropods, mollusks, and annelids. Chitin is also found in the cell walls of low-grade plants, especially in fungal cells. *crustacea* skin such as shrimp skin contains 20 to 30% of chitin and crab skin contains 15 to 20% chitin while squid cartilage contains 100% chitin (Alimuniar, 1992).

Chitosan is a type of natural polymer that has the general formula $(C_6H_{11}NO_4)n$ or is referred to as (1,4)-2-amino-2 deoksi-β-D-glucose. Chitosan is the main derivative of chitin, where obtaining chitosan is carried out by deacetylation of chitin (Mat, 1995).

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Due to chitosan being insoluble in water and neutral or high pH areas, the use of chitosan is quite limited. Therefore, chemical modification of chitosan is needed to provide water-soluble ingredients that are required to produce new biomaterial (Seedevi et al., 2017). Chitosan is biodegradable, non-toxic, and friendly environment material. Antibacterial activity is one of the most important bioactivities of chitosan. Chitosan has good antibacterial activity against several types of bacteria such *as Agrobacterium tumefaciens*, Corynebacterium michiganense, Escherichia coli, Micrococcus luteus, and Staphylococcus aureus (Mohamed et al, 2013).

Chemical modification of chitosan and chitin has often been done by preparing the derivatives with beneficial properties, such as carboxy methylation, chitosan/chitin oxidized, and sulfated. These derivatives have a widely applications i.e additives in food and cosmetic products, drugs and gene delivery systems, or as stationary phase chiral for HPLC. Sulfated chitosan has the most interesting, not only potential in biological activities, such as anticoagulants, antivirals, antimicrobials, and antioxidants but also because of its cost low as a biomaterial (Vino et al, 2012; Han et al, 2015).

Researchers have previously reported that the anticoagulant activity of polysulfated chitosan was synthesized from sea crabs under semi-heterogeneous conditions (Vongchan et al, 2002). Synthesis of sulfated chitosan was performed through the sulfation reaction of chitosan with chlorosulfonic acid in DMF solvent (Ginting, 2004). In addition, the synthesis of sulfated chitosan was also conducted through a sulfation reaction (Sun et al, 2016). In this study, researchers are interested in the synthesis of the sulfated chitosan through sulfation reaction using chlorosulfonic acid and followed by an antibacterial activity test.

## 2 Materials and Methods

### 2.1 Equipments

In this study, the equipments used were glassware, oven, hot plate, magnetic bar, analytical balance, dialysis membrane, universal indicator, desiccator, autoclave, burette, scalpel, filter paper, and a set of fourier transform infrared (FT-IR) spectrophotometry.

### 2.2 Materials

The main materials used were chitosan, methanol, chlorosulfonic acid, N, N-Dimethylformamide (DMF), hydrochloric acid (HCl), sodium hydroxide (NaOH), phenolphthalein, acetic acid ($CH_3COOH$), nutrient agar, *Staphylococcus aureus* bacteria isolate.

### 2.3 Fabrication of $SO_3$-DMF Sulfated Complex

As much as 4 mL $HClSO_3$ was added drop by drop into 30 mL (DMF) while stirring at 0 to 4ºC. The reaction mixture was then stirred for 15 minutes without cooling until the ($SO_3$-DMF)

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solution reached room temperature. The same procedure was conducted for variations of 4.5 ml and 5 L of $HClSO_3$.

### 2.4 Synthesis of Chitosan Sulfate

Sulfated chitosan was synthesized using the Gamzazade method established in 1997. As much as 1 g chitosan was mixed into the DMF solution. Then, suspended chitosan in the DMF was incorporated into the $SO_3$-DMF sulfated complex. The mixture is then stirred at room temperature for 5 hours. Then The swelled chitosan was neutralized with NaOH 20% and precipitated with methanol. The precipitate was then dissolved with distilled water and filtered, and the filtrate obtained was dialyzed using a dialysis membrane in distilled water for 48 hours. The result obtained was dried in a freezing room, then tested sulfation degree titration method, and then analyzed by FT-IR spectrophotometry and tested antibacterial activity.

### 2.5 Determination of Sulfation Degree

As much as 0.1 g sulfated chitosan was soaked in 1o mL NaOH 1N for 3 days. After 3 days, NaOH was titrated with HCl 0.1N using phenolphthalein as an indicator. At the end of the titration process was when there is a color change from pink to colorless. The degree of sulfation was obtained through equality :

$$\mathrm{DS} = \frac{(\mathrm{V\ initial} - \mathrm{V\ end})\ \mathrm{x\ N\ HCl\ x\ Weq\ SO3}}{\mathrm{weight\ of\ sample}}\ \mathrm{x}\ 100\%$$

Description :

$V_{initial}$ = Volume of HCl blank

$V_{end}$ = volume of sample

### 2.6 Determination of Antibacterial Activity

Determination of the antibacterial activity was performed by agar diffusion method where the paper disc (Ø 6 mm) which has been soaked with sulfated chitosan in distilled water and contacted directly with the inoculated medium by *E.coli and S.aureus*, then it can be observed the inhibition zone formed after incubated for 24 hours. The active areas around the disc show the sensitivity of bacteria against sulfated chitosan used as a test material which was expressed by the diameter of the inhibition zone.

The inhibition zone diameter was measured in millimeters (mm) using a caliper by measuring the total diameter of the clear/cloudy zone around the disc.

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## 3 RESULT AND DISCUSSION

### 3.1 $SO_3$-DMF Sulfated Complex

In a sulfation reaction to organic compounds can be used chlorosulfonic acid reagents in solvent N, N-dimethylformamide (DMF), or pyridine. In addition to chlorosulfonic acid, the reagent used to perform sulfation, such as sulfuric acid, and oleum sulfur trioxide in both pyridine and sulfur dioxide (Muzzarelli et al, 1968). In this study, SO3-DMF sulfated complex was prepared by the addition of HClSO3 in DMF at 0 to 4ºC. $SO_3$-DMF complex formed was a dipolar ion, so it is easily attacked by nucleophiles derived from hydroxyl groups and amine groups of chitosan.

### 3.2 Synthesis of Sulfated Chitosan

In this study, chitosan used was derived from shrimp shells with a minimum deacetylation degree of 73.06%. The sulfated chitosan compound was prepared by reacting chitosan with the $SO_3$-DMF sulfated complex. The formed sulfate chitosan is pale yellow.

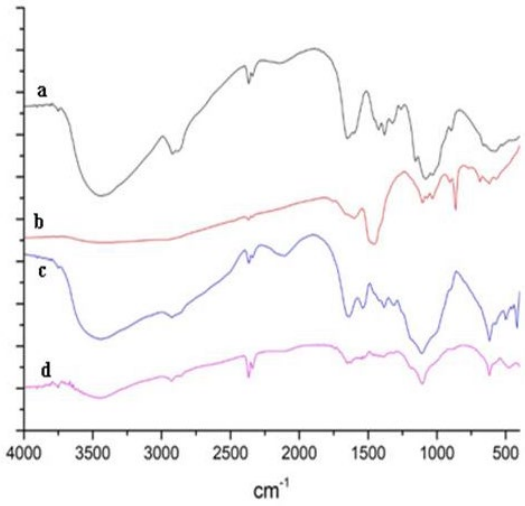


Figure 1. FT-IR spectra of (a) chitosan, (b) KS 1, (c) KS 2, (d) KS 3

Description :

KS 1 = sulfated chitosan from the reaction between 1 g of chitosan with 4 mL of $HClSO_3$

KS 2 = sulfated chitosan from the reaction between 1 g of chitosan with 4.5 mL of $HClSO_3$

KS 3 = sulfated chitosan from the reaction between 1 g of chitosan with 4 mL of $HClSO_3$

The FT-IR test results indicated in figure 1 show that the three typical bands found in the group's KS 1, KS 2, and KS 3 were the bands at 3410; 3448; 3425 $cm^{-1}$ respectively, which indicated the presence of OH groups, the bands 1597; 1643; 1645 $cm^{-1}$, respectively, which assigned N-H bonds, the bands at 1103; 1111; 1111 $cm^{-1}$, which showed C-O-S bonds and the

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S=O group that identifies the formation of –O-sulfate. In addition, figure 1 also shows the presence of C-O-S contributes to the band of 1111 $cm^{-1}$, which indicated the presence of sulfated chitosan. In the formation of sulfated chitosan, the occurrence of peak change at 1080 $cm^{-1}$ becomes 1103, 1111, and 1111 $cm^{-1}$ which indicated the sulfate group has been substituted for the chitosan.

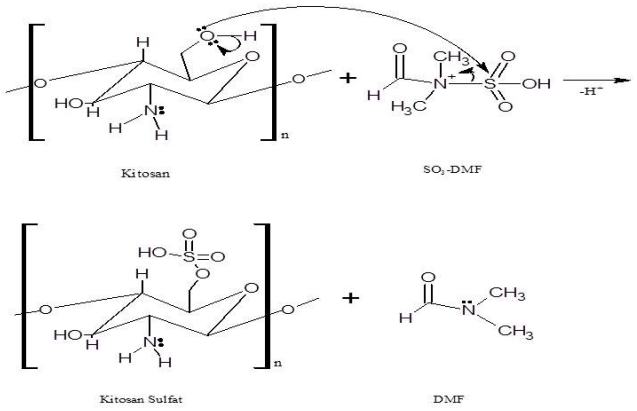


Figure 2. Reaction mechanism of sulfated chitosan formation

Furthermore, sulfation reaction of chitosan with chlorosulfonic acid in N, N Dimethylformamide solvent at room temperature to form sulfated chitosan. The addition of DMF into a chlorosulfonic acid solution to form an $SO_3$-DMF complex. In alkaline solution, sulfated chitosan polyanion and their chain conformations are the same as the arrangement of the chain in the β-1.4 glucopyranose, where the presence of intramolecular hydrogen bonds between the oxygen ring and hydroxyl at the C-3 atom makes this compound more stable. The reaction mechanism of sulfated chitosan formation is presented in figure 2.

### 3.3 Results of Sulfation Degree

The results of determining the sulfation degree were presented in Table 1.

**Table 1**. Results of determining the sulfation degree of sulfated chitosan

| Sample | Sulfation Degree (%) |
|---|---|
| KS 1 | 5.6042 |
| KS 2 | 6.0045 |
| KS 3 | 6.8051 |

A sulfation degree is a degree that shows how much sulfate content was found in chitosan. The determination results of the sulfation degree of sulfated chitosan with variations in the concentration of chlorosulfonic acid show that the increase in the value of sulfation degree was

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caused by the high concentration of a sulfating agent ($HClSO_3$) used. According to Dhuhita and Arti (2010), an increase in the degree of sulfation will increase its hydrophilic properties and absorbs a lot of air.

### 3.4 Antibacterial Activity Test Results of Chitosan and Sulfated Chitosan

Antibacterial activity testing of chitosan and sulfated chitosan sulfate was conducted on *Escherichia coli* and *Staphylococcus aureus* bacterias using the agar diffusion method, the results of the antibacterial activity test can be seen in Table 2.

**Table 2**. Antibacterial activity test of chitosan and sulfated chitosan

| Bacteria | Treatment | Paper Disc Diameter (mm) | Inhibitor Zone Diameter (mm) |
|---|---|---|---|
| ***Staphylococcus aureus*** | Chitosan 6% | 6 | 8.4 |
| | KS 1 | 6 | 8.0 |
| | KS 2 | 6 | 8.3 |
| | KS 3 | 6 | 9.5 |
| ***Escherichia coli*** | Chitosan 3% | 6 | 9.2 |
| | KS 1 | 6 | 6.8 |
| | KS 2 | 6 | 9.0 |
| | KS 3 | 6 | 10.0 |

The chitosan and sulfated chitosan test results indicate the presence kill zone (radical zone) on Escherichia coli and an inhibitor zone (radical zone) on *Staphylococcus aureus* testing. The kill zone indicated by there was a clear area around the disc while the inhibitor zone showed by area which looks infertile or more cloudy compared to the areas that do not affect by substances (Ayu et al, 2014).

The sulfated chitosan solution proved to be able to kill the activity of the bacteria on *Escherichia coli* and inhibit the *Staphylococcus aureus* bacteria. Classification of antibacterial power was classified according to Davis and Stout (1971), namely: inhibition zone diameter of 5 mm categorized as weak, inhibition zone diameter of 5 to 10 mm categorized as moderate, inhibition zone diameter of 10 to 20 mm categorized as strong and inhibition zone diameter of 20 mm categorized very strong or more. Thus, the formed sulfated chitosan has a strength moderate antibacterial.

These results also show that the antibacterial activity of KS3>KS2>KS1 against both bacteria, This is due to the high degree of sulfation in chitosan, the higher its antibacterial activity. The higher sulfation degree of chitosan showed that more sulfate groups substituted the chitosan.

Sulfated chitosan has sulfate ($SO_3H$) and amine ($-NH_2$) groups were positively charged and very reactive, so it can bind the negatively charged bacterial cell wall.

According to Dewi et al. (2006), the bactericidal mechanism of various cationic antibacterial commonly was through interaction and breakdown of the membrane/ cell wall structures. The criteria based on Davis and Stout (1971) showed that sulfated chitosan has moderate activity. However sulfated chitosan has a wide spectrum. It meant that the sulfated chitosan has antibacterial activity against negative Gram bacteria (*Escherichia coli*) and positive Gram. bacteria (*Staphylococcus aureus*).

## 4 Conclusion

Sulfated chitosan is successfully produced from chitosan by employing the sulfation reaction which demonstrates antibacterial activity properties. From three different $HClSO_3$ concentrations, an $HClSO_3$ concentration of 5 mL gives the largest sulfation degree and kill zone diameter on *Escherichia coli* and inhibition zone diameter on *Staphylococcus aureus*.

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