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Research Article

Production of Water-Soluble Chitosan from Crab Shells (*Portunus* sp.) by Pressurized Hydrolysis Method as an Active Material for Hand Sanitizer

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Abstract

There has been no research on the application of water-soluble chitosan (WSC) derived from crab shells as a hand sanitizer. using a pressurized hydrolysis method. The limited solubility of chitosan at neutral pH restricts its usability. The aim of this study was to produce WSC from crab shells using pressurized hydrolysis methods as an active ingredient for hand sanitizer. Chitosan was depolymerized into WSC by utilizing hydrochloric acid (2, 3, and 4%) and was hydrolyzed using a pressure cooker at a temperature of approximately 110˚C for 1 hour. Isopropyl alcohol was then added to the filtrate at a ratio of 2:1. The selected WSC was treated with 3% HCl and made into 3 different concentrations of 140, 150, and 160 mg/ml, then tested for its antibacterial activity. The WSC hand sanitizer antibacterial test has concentrations of 180, 190 and 200 mg/ml, and for positive control using commercial hand sanitizer, and negative control in the form of basic gel without chitosan. By depolymerizing chitosan using 3% HCl, a high solubility (93.57±0.33) of WSC was achieved, with a degree of deacetylation (DD) value of 78.4%. The results indicated that the concentration of WSC is160 mg/ml and exhibited the strongest inhibition against *S. aureus* and *E. coli*, with clear area values of 7.47 mm and 6.70 mm, respectively. The best hand sanitizer formulation is HS3 (in addition of WSC 200 mg/ml) and the ability to inhibit *S. aureus* bacteria with a clear area value of 5.35 ± 0.57 mm and *E. coli* is 4.70 ± 0.07 mm. This study shows the potential of WSC from crab shells as a sustainable and effective antibacterial active ingredient in hand sanitizers, which requires further research on scalability and wider applications.

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1. Introduction

Chitosan has been extracted from various crustacean shells using different methods, including acid hydrolysis, alkaline hydrolysis, and enzymatic hydrolysis (Mohan *et al.*, 2022). Recent research has explored methods to increase the solubility of chitosan in water, such as chemical modification with acetic anhydride or physical treatments such as ultrasonic irradiation (Aranaz *et al.*, 2021). The antimicrobial properties of chitosan are due to its ability to interact with microbial cell walls and disrupt their integrity. Several studies have demonstrated the effectiveness of chitosan-based hand sanitizers in reducing the survival of pathogenic bacteria and viruses (Ke *et al.*, 2021).

Chitosan is proven to have bioactive, biocompatible, anti-bacterial, and biodegradable properties (Huang, 2019), so that chitosan is more widely applied in the industrial, medical, pharmaceutical, and food processing fields (Wang and Zhuang *et al.*, 2022). Chitosan is not soluble in water, but easily soluble in organic acids, this is supported by the research of Pellis *et al.* (2022) which stated that there were limitations in the application of chitosan due to the low solubility of chitosan in neutral pH, so it was necessary to improve the solubility of chitosan. One way to do this is to make chitosan derivatives, namely water-soluble chitosan so that its application is wider and easier. Water-soluble chitosan can be obtained by simplifying the chitosan chain through the depolymerization process. Chitosan depolymerization assisted by the addition of hydrochloric acid (HCl) will accelerate the deamination process and the cutting of polymer chains in the amine group so that the chitosan chain becomes shorter than the previous chitosan chain (Faustine *et al.*, 2020). The process of deacetylation of crab shells using a 50% NaOH solution produces a very high degree of deacetylation of 96.58% (Saputra *et al.*, 2022).

Das *et al.* (2023) reported that low acid levels resulted in imperfect hydrolysis that assisted by pressure caused the chitosan polymer bonds to be cut into smaller units, and hydrolysis to be complete. The pressurized hydrolysis method under low acid concentration is safer, more efficient and simpler, than the chemical hydrolysis method by direct heating using concentrated acid or by autoclaving. The chemical hydrolysis method directly gives poor quality to the water-soluble chitosan produced, namely low yield value, brownish color, below the standard melting point, incomplete solubility, and noise results on the FTIR spectrum graph (Feng, 2021). The pressurized hydrolysis method was chosen to be easy to apply and maximize the hydrolysis of chitosan. Completely hydrolyzed chitosan has a high degree of deacetylation, and a high degree of deacetylation will increase the antibacterial strength of Ardean *et al.* (2021a). Factors influencing the antibacterial activity of chitosan and water-soluble chitosan modified by functionalization. One of them can be applied as an antibacterial bioactive substance. Chitosan contains lysozyme enzymes and amino polysaccharide groups that can inhibit microbial growth because chitosan consists of positively charged polycations that can damage bacterial cells (Azmana *et al.*, 2021).

Chamidah *et al.* (2019) states that water-soluble chitosan is better when used as an antibacterial than natural chitosan, therefore water-soluble chitosan is very useful when used as a hand sanitizer product or hand sanitizer that is used without using water and serves to kill bacteria or germs (Kusrini *et al.*, 2023). Commercial hand sanitizers usually use alcohol as an active antibacterial substance. The alcohol content is less safe when used repeatedly, so natural ingredients that have antibacterial abilities such as water-soluble chitosan is needed to replace alcohol as the active substance in hand sanitizers to make them safer and not have side effects. However, water-soluble chitosan is usually made from chitosan raw materials with a high degree of deacetylation, because at this stage of the manufacturing process it uses high temperatures. So, in this study, we tried to use chitosan made without heating as the raw material for making water-soluble chitosan. There is quite a lot of research on hand sanitizers made from water-soluble chitosan from shrimp, but there is no hand sanitizer made from water-soluble chitosan from crab shells yet. There is quite a lot of research on hand sanitizers made from water-soluble chitosan from shrimp, but there is no hand sanitizer made from water-soluble chitosan from crab shells. Fitriyana *et al.* (2021) found that the commercial hand sanitizer had the best bacterial inhibitory ability, meanwhile, the chitosan-based hand sanitizer produced the inhibitory ability of bacteria which was close to the commercial hand sanitizer. Water soluble chitosan made from chitosan from crab shells is still not in production, most of it is still at the chitosan stage, for hand sanitizer needs water soluble chitosan is needed.

The purpose of this study was to make water-soluble chitosan from crab shells chitosan raw materials with a DD value below 70% with hydrochloric acid concentration treatment of 2,3 and 4%, and determine the best concentration of HCl in making water-soluble chitosan by applying the pressurized hy drolysis method using a pressure cooker, followed by with chemical analysis and the best results from the chemical analysis is in antibacterial tests, then the best water-soluble chitosan concentration will be increased and included in the hand sanitizer formulation as an active ingredient for hand sanitizers tested for their inhibitory power against *E. coli* and *S. aureus* bacteria.

However, all previous research focused on hand sanitizers made from water-soluble chitosan from shrimp's shells. To the best of our knowledge, very little research has been conducted on hand sanitizers made from water-soluble marine chitosan from crab's shells. The novelty of using a pressurized hydrolysis method to produce water-soluble chitosan lies in its ability to overcome the limitations of traditional chitosan production methods. This innovative approach enhances the solubility, antimicrobial properties, and environmental sustainability of chitosan from a crab's shell, making it a valuable active material for hand sanitizers and potentially other applications (Peter *et al.,* 2021).

2. Materials and Methods

2.1 Materials

2.1.1 The equipments

The equipments used are pressure cooker 0.8-1 bar, gas stove, digital thermometer (Airmen TP. 101), pH meter (Ionix, PH5S), Rion Viscoster VT – 04F (Rion, Ltd. China), digital scale (Vibra AJ-1200E, Japan), universal pH paper (Supelco, 1.095350001), 500 mesh planktonic, measuring cup, beaker, funnel, Fourier Transform Infrared Spectroscopy (FTIR) (Rayleigh - UV1800 V/VIS, China), filter paper, plastic filter, plastic tray, oasis needle, Petri disk, test tube, bunsen, tweezers, vortex.

2.1.2 The materials

Making water-soluble chitosan using the following materials; unheated chitosan with deacetylated degrees of 57.64%, 32% technical HCl (Rofa, RLC2.0068.0500), technical isopropyl alcohol (Rofa RLC2.0033.0500), technical NaOH (Rofa CAS 1310-73-2), distilled water, Mueller Hilton Agar (MHA, Oxoid), Nutrient Broth (NB, Oxoid), Nutrient Agar (Oxoid), disc paper (Oxoid), Chloramphenicol disk (Oxoid), commercial hand sanitizer, *S. aureus* bacteria (ATCC 25923, T1) and bacteria *E. Coli* (ATCC 25922, T1), alcohol.

2.1.3 Ethical approval

This study does not require ethical approval because it does not use experimental animals.

2.2 Methods

2.2.1 Production of water-soluble chitosan

The manufacture of water-soluble chitosan begins with making chitosan from crab shells referring to Natalia *et al.* (2021). Furthermore, making chitosan water-soluble refers to Sudianto *et al.* (2020) by modifying the concentration of hydrochloric acid (HCl) and hydrolysis time. Hydrolysis is carried out with a pressure cooker at a pressure of 0.8-1 bar and a temperature of ±110°C for 1 hour, using HCl concentrations of 2%, 3%, and 4%.

Chitosan that has been hydrolyzed under pressure and the addition of HCl is then added with technical isopropyl alcohol 2 times to the chitosan filtrate until a milky white precipitate is formed, then filtered with 500 mesh filter cloth and neutralized using 10% technical NaOH solution until the chitosan filtrate is neutral as evidenced by universal pH paper, after neutral the residue is filtered again and separated from the filtrate. The residue was washed with 100 ml of isopropyl alcohol to remove impurities that were still attached to the water-soluble chitosan residue. The residue was air-dried at room temperature (30°C) for 24 hours to ensure all water had evaporated.

Water-soluble chitosan will be analyzed chemically by looking at the yield, solubility, acidity, viscosity, and deacetylation degree. The best results based on chemical analysis and the value of the degree of deacetylation will be followed by an antibacterial test against *E. coli* and *S. aureus* bacteria. The best inhibition of the selected water-soluble chitosan concentration will be used as the basis for the formulation of a water-soluble chitosan hand sanitizer.

2.2.2 Making of hand sanitizer formulation

Hand sanitizer is made by dissolving water-soluble chitosan first in distilled water (aqua dest), then dissolving CMC-Na with distilled water. The dissolved water-soluble chitosan was then combined with CMC-Na which had dissolved and formed a gel, then 0.2 ml of glycerin and 0.1 ml of propylene glycol were added to provide moisture to the water-soluble chitosan hand sanitizer formulation, and the last step was adding fragrance. 1 drop for each water-soluble chitosan hand sanitizer formulation. The water-soluble chitosan hand sanitizer formulation was vortexed until evenly distributed. Furthermore, the formulation of the water-soluble chitosan hand sanitizer was tested for hedonic physical properties against organoleptic (appearance, smell, and texture) by 30 untrained panelists, as well as dispersion tests, homogeneity, and pH tests on water-soluble chitosan hand sanitizers, and finally continued with an antibacterial test to see the zone of inhibition against *E. coli* and *S. aureus* bacteria, so that the best water-soluble chitosan hand sanitizer can be determined. The formulation of a water-soluble chitosan hand sanitizer was made using the ingredients in Table 1.

Table 1. Formulation of water-soluble chitosan hand sanitizer.

Components	Basis Gel	Concentration	Concentration	Concentration
	0 mg/ml	180 mg/ml	190 mg/ml	200 mg/ml
Water-Soluble Chitosan	-	1.8 g	1.9 g	2 g
CMC-Na	0.05 g	0.05 g	0.05 g	0.05 g
Glycerin	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Propylene glycol	0.1 ml	0.1 ml	0.1 ml	0.1 ml
Distilled water ad	10 ml	10 ml	10 ml	10 ml
Orange Scents	1 drop	1 drop	1 drop	1 drop

Source: Manus, *et al.* (2016)

2.2.3 Chemical analysis procedure

2.2.3.1 Yield (AOAC, 2005)

The yield value of water-soluble chitosan (WSC) was calculated based on the comparison between the weight of WSC obtained and the weight of the initial chitosan. The yield calculation is seen in the formula below

Yield = (Weight of WSC produced) / (Weight of Initial Chitosan) x 100%..(i)

2.2.3.2 Viscosity

The viscosity test of water-soluble chitosan using Rion Viscotester VT-04F, using 2 grams of WSC dissolved in 200 ml of distilled water, then the WSC solution was put into a stainless-steel cup until the mark of the viscometer pendulum was closed. Turn on the viscometer and leave the needle in a constant position, record the constant value.

2.2.3.3 Solubility

The solubility of WSC was tested by adding 1 g of WSC and dissolved in 100 ml (1:100 w/v) (Li *et al.*, 2019) Then filtered with filter paper and dried. The dried filter paper was weighed to constant weight.

Isolation (%) = (Final Weight) / (Initial Weight) x 100 %..(ii)

Solubility (%) = 100% - Insolubility.....................(iii)

2.2.3.4 Degree of acidity

The pH test was carried out according to SNI 06-6989.11:2004, namely by weighing 1 g of WSC and dissolved in a 40 ml beaker containing distilled water, then the pH meter was immersed.

2.2.3.5 Degree of deacetylation

The results of the WSC FTIR test are the basis for calculating the value of the WSC deacetylation degree using Li *et al.* (2019). as follows.

Degree of Deacetylation = 100-[A1655] / [A3450] x 100/1.33..(iv)

Where:

A1655 : Absorbance of the amide group is about 1655 cm-1

A3450 : The absorbance of the hydroxyl group is about 3450 cm-1

1.33 : Comparison of A1655/A3450 against completely deacetylated WSC (100%)

2.2.4 Physical properties test of water-soluble chitosan hand sanitizer formulation

2.2.4.1 Organoleptic water-soluble chitosan hand sanitizer

Organoleptic test (appearance, smell, and texture) which was carried out by hedonic assessment using 30 untrained panelists with a questionnaire referring to SNI 2346-2006 with a value of 1 (very very dislike) – 9 (very very like) to find out the community's acceptance of water-soluble chitosan hand sanitizer products made by comparing them to commercial hand sanitizers. Panelists perform hedonic tests separately at different times.

2.2.4.2 Dispersion of water-soluble chitosan hand sanitizer

The dispersion test was carried out by placing 0.5 grams of water-soluble chitosan hand sanitizer gel as much as 0.5 grams on a flat glass and place another glass on top of the hand sanitizer gel leaving it for one minute. Then measure the diameter formed from the hand sanitizer gel preparation on the glass plate from 3 different sides. Next, add a load on the glass plate of 150 g, let stand for one minute, and recalculate the diameter formed.

2.2.4.3 Homogeneity of water-soluble chitosan hand sanitizer

The homogeneity test refers to Putri (2019), namely by observing the preparation of water-soluble chitosan hand sanitizer gel placed on a transparent glass plate and then observing the presence or absence of granules in the water-soluble chitosan hand sanitizer gel preparation and commercial hand sanitizer. A good hand sanitizer is homogeneous and there are no granules in the gel preparation.

2.2.4.4 Degree of acidity of water-soluble chitosan and water-soluble chitosan hand sanitizer

Testing the degree of acidity of the WSC hand sanitizer was carried out the same as the test of the degree of acidity on water-soluble chitosan.

2.2.5 Microbiological analysis of water-soluble chitosan and water-soluble chitosan hand sanitizer

2.2.5.1 Rejuvenation of test bacteria

Bacteria test of *E. coli* and *S. aureus* by taking aseptically and separately 20 L of bacteria were inoculated on Nutrient Broth (NB) media, and each bacterium in NB media was incubated for 24 hours at 37˚C. Furthermore, each bacterium was taken as much as 1 ose and inoculated on Nutrient Agar (NA) aseptically and separately, and continued with incubation for 24 hours at 37˚C.

2.2.5.2 Water soluble chitosan antibacterial test paper disk method

The disc paper was first soaked in WSC solution with concentrations based on Chamidah *et al.* (2019) used in hand sanitizer from shrimp shells, while we used this concentration from crab shells of 140, 150, and 160 mg/ml for 24 hours and 3 replications. The negative control was also immersed for 24 hours in distilled water which is WSC solvent, while for the positive control, chloramphenicol disc paper was used. The soaked disc paper was then placed on the surface of the Muller Hilton Agar media which had been inoculated by aseptic bacterial cultures, then incubated at 37˚C for 24 hours. The clear zone formed around the paper disc indicates the presence of antibacterial activity and is measured using a caliper (Kadak and Salem, 2020).

2.2.5.3 Antibacterial test of water-soluble chitosan hand sanitizer disc paper method

The WSC hand sanitizer antibacterial test has the same stages as the WSC antibacterial test, only the test material is different in the form of hand sanitizer with concentrations based on Chamidah *et al.* (2019) used in hand sanitizer from shrimp shells, while we used this concentration from crab shells 180 mg/ml (HS1), 190 mg/ml (HS2), and 200 (HS3) for hand sanitation and for positive control using commercial hand sanitizer, and negative control in the form of basic gel without chitosan.

2.3 Analysis Data

One way ANOVA and Tukey's Advanced Test (SPSS IBM version 23 software) were used to analyse the effect of hydrochloric acid (HCl) concentration on the characteristics of WSC.

3. Results and Discussion

3.1 Results

3.1.1 Characteristics of water-soluble chitosan

The water-soluble chitosan in this study was made from processed chitosan with a deacetylation degree of 57, 64%. The method chosen in the manufacture of water-soluble chitosan is the pressurized hydrolysis method with hydrochloric acid as the solvent which refers to the modified research of Sudianto *et al.* (2020). The characteristic value of water-soluble chitosan can be seen in Table 2.

Table 2. Characteristics of water-soluble chitosan.

Characteristics	HCl Concentration		
	2%	3%	4%
Color	Yellowish white	Yellowish white	Brownish yellow
Odor	Odor less	Odor less	Odor less
Form	Powder	Powder	Powder
Yield (%)	85.5±0.91[a]	83.37±0.73[b]	80.43±0.5[b]
pH	4.90±0.24[a]	5.85±0.34[a]	5.79±0.37[a]
Viscosity (cPs)	67±2.16[a]	69±0.82[a]	69.3±0.94[a]
Solubility (%)	93.10±0.086[a]	93.57±0.33[a, b]	92.77±0.25[b]
Degree of Deacetylation (%)	64.80	78.40	51.71

Note: The difference in letters in superscript indicates a significant difference

3.1.2 Color, odor, and form of water-soluble chitosan

Water-soluble chitosan in this study produced a yellowish-white color, powder, and odorless for the treatment of 2% and 3% HCl concentrations, while for water-soluble chitosan with 3% HCl concentration it produced a brownish white color, powder form, and odorless. Chitosan test results are shown in Figure 1. The color of water-soluble chitosan at 2% and 3% HCl concentration treatment is in accordance by commercial chitosan, namely yellowish white and by GRAS standards (Permadi *et al.*, 2022). This is because the chitosan produced is odorless and in powder form. Chitosan in 4% HCl treatment produces a brownish yellow color. The brownish yellow color is thought to be caused by an imperfect neutralization process, causing the browning process to occur. The brownish yellow color is also caused by HCl and the interaction of amino sugars which form the brown fur fural component due to the amino glycosyl enolization reaction (Oktarlina *et al.*, 2022). The color, odor and form of water-soluble chitosan are shown in Table 3.

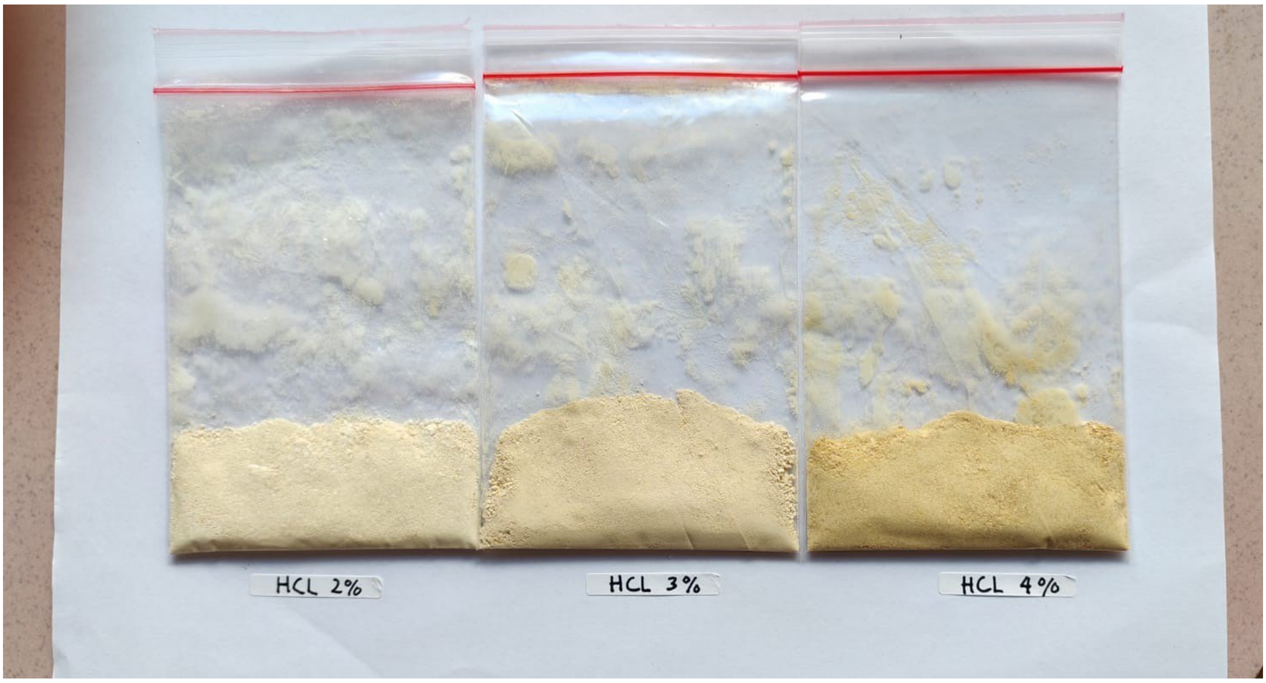


Figure 1. Colour of water-soluble chitosan test result.

Table 3. Color, odor, and form of water-soluble chitosan test results.

WSC	Results		
	Color	Odor	Form
HCl 2%	Yellowish - white	No Smell	Powder
HCl 3 %	Yellowish - white	No Smell	Powder
HCl 4 %	Brownish - Yellow	No Smell	Powder
Commercial*	White	No Smell	Powder

3.1.3 Water soluble chitosan yield

The yield of water-soluble chitosan in this study was quite high, ranging from 80.43 - 85.50%, based on the results of statistical tests with analysis of variance and Tukey's follow-up test at 5% level $p<0.05$ which indicated a significant difference between the concentration treatments. HCl on water-soluble chitosan yield value. The yield of the research results is higher when compared to the results of glu cosamine from Cahyono (2018), 65.33%; Meata *et al.* (2019), which is 66.53 – 70.13%, but still, by the results of water-soluble chitosan from Sudianto *et al.* (2020) which ranges from 80 - 90%. The results of the water-soluble chitosan yield is presented in Figure 2.

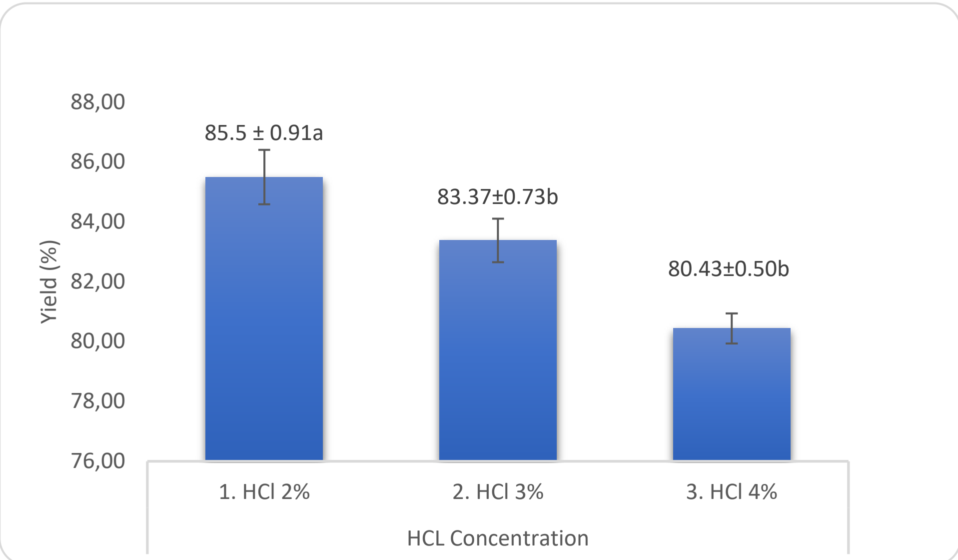


Figure 2. Graph of water-soluble chitosan yield.

3.1.4 Degree of acidity (pH) water soluble chitosan

The degree of acidity in this study was carried out by dissolving water-soluble chitosan of different concentrations of as much as 1 gram and putting into a 40 ml beaker containing distilled water, then a pH meter was inserted into the solution to read the acidity degree of the water-soluble chitosan test results. The acidity (pH) values of water-soluble chitosan were 4.9±0.24 - 5.83 ± 0.34 respectively. The treatment of different HCl concentrations did not have a significant effect on the value of the degree of acidity, because $p>0.05$ based on the results of the analysis of variance and the Tukey 5% further test. The highest value was in water-soluble chitosan with 3% HCl treatment and the lowest value was in water-soluble chitosan with 2% HCl treatment. The extraction of H+ ions during chemical hydrolysis using hydrochloric acid occurs excessively, causing different pH values (Gumilar *et al.*, 2023). The graph of the degree of acidity can be seen in Figure 3.

3.1.5. Water soluble chitosan viscosity

Water-soluble chitosan was also tested for viscosity to determine the quality of the water-soluble chitosan produced. Viscosity tests are carried out to determine the viscosity value of a solution at a certain concentration and temperature (Pambudi *et al.*, 2018). The results of statistical tests showed that there was no significant difference between $p>0.05$ for each treatment with different HCl concentrations on the viscosity value of water-soluble chitosan. The viscosity value of water-soluble chitosan in this study ranged from 67 to 69.33 cPs and was included in the low category because the viscosity value was < 200 cPs (Cahyono, 2018). The graph of the viscosity value of water-soluble chitosan can be seen in Figure 4.

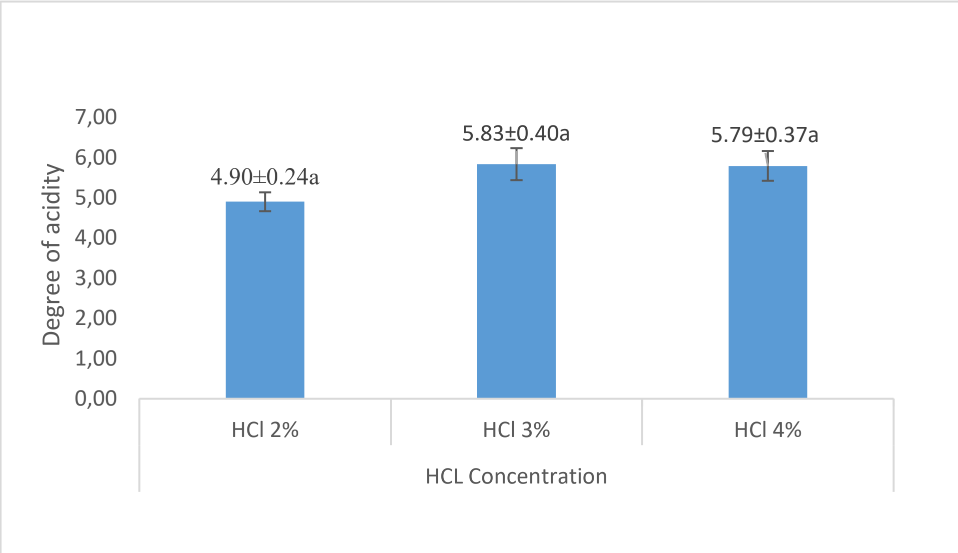


Figure 3. Graph of the degree of acidity of water-soluble chitosan.

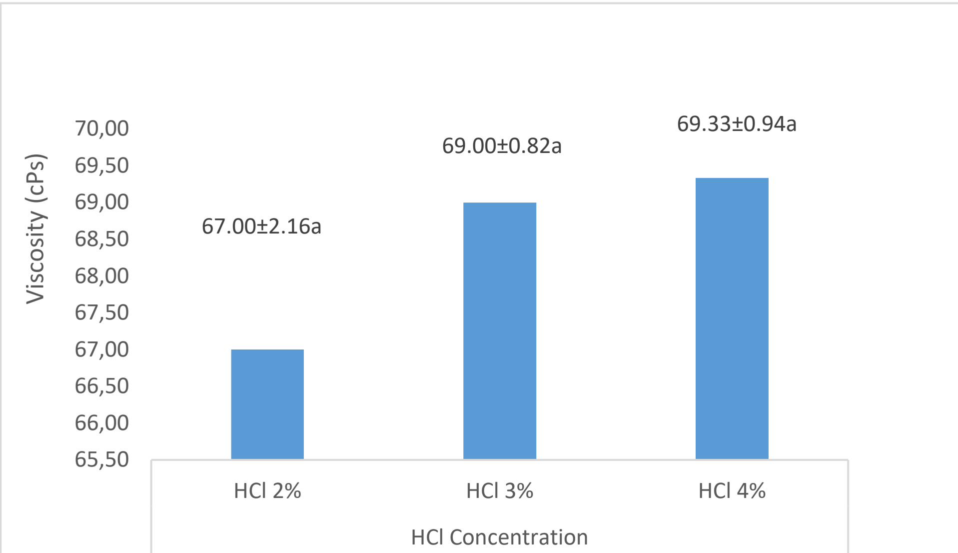


Figure 4. Water Soluble Chitosan Viscosity Graph.

3.1.6 Solubility of water-soluble chitosan

A solubility test is carried out to see the homogeneity of the dissolved chemical that will be dissolved in the solvent. The results of statistical tests showed that there was a significant difference of $p < 0.05$ between different HCl concentration treatments on the solubility value of water-soluble chitosan. The solubility of water-soluble chitosan from this study was very high, the result of the solubility ranges from 92.77±0.25 – 93.57±0.33%. The graph of the solubility of chitosan can be seen in Figure 5.

3.1.7 Degree of deacetylation of water-soluble chitosan

The degree of deacetylation was determined based on the results of the FTIR test. The value of the highest degree of deacetylation of water-soluble chitosan was found in the treatment of 3% HCl concentration of 78.4% which was calculated based on the Hahn *et al*. (2020) formula by comparing the absorbance value of the amide group at a wavenumber of

1655 cm-1 and the hydroxyl group at a wavenumber of 3450 cm-1. The FTIR graph can be seen in Figure 6.

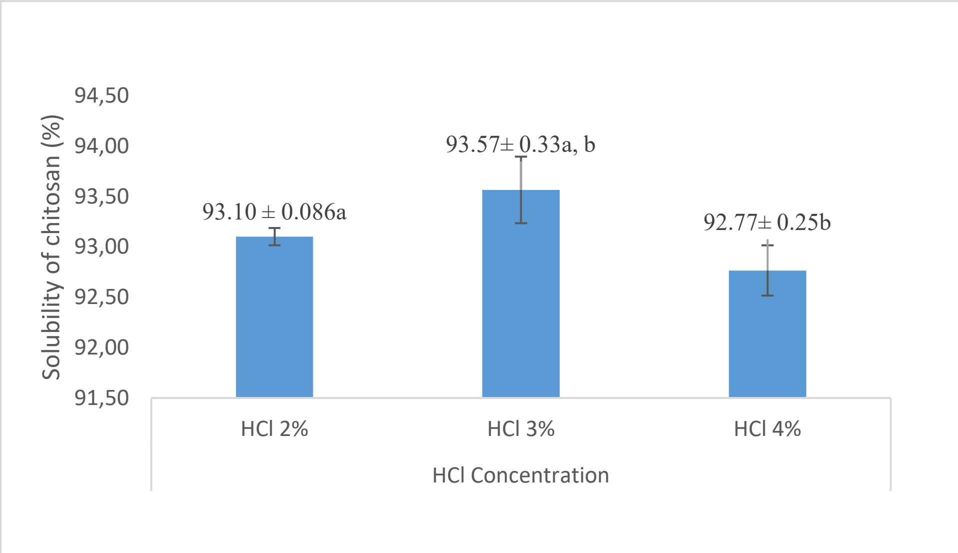
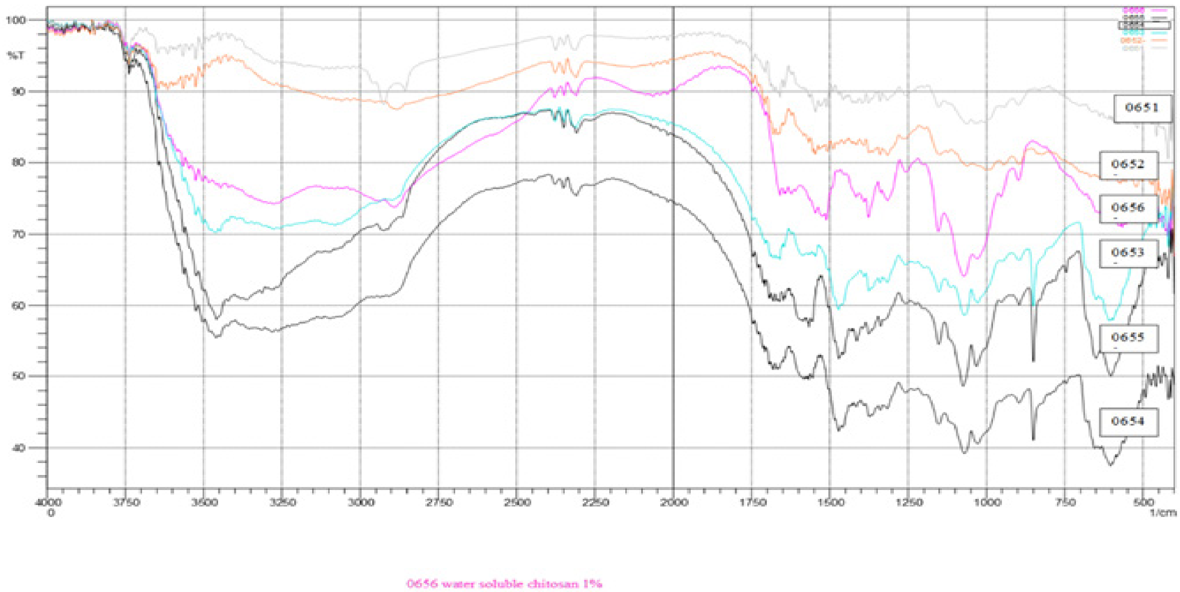


Figure 5. Solubility graph of water-soluble chitosan.



Note:
0651 comercial chitosan,0652 chitosan0653 water soluble chitosan 4%,0654 water soluble chitosan 3%.
0655 water soluble chitosan 2 %, & 0656 water soluble chitosan 1%

Figure 6. The FTIR spectrum results of water-soluble chitosan.

3.1.8 Testing of water-soluble chitosan antibacterial against S. aureus and E. coli

Testing the antibacterial activity of water-soluble chitosan using the paper disc method by looking at the clear zone formed around the paper disc area. The selected water-soluble chitosan was treated with 3% HCl and made into 3 different concentrations of 140, 150, and 160 mg/ml, then tested for its antibacterial activity.

Antibacterial testing against gram-positive bacteria using *S. aureus* bacteria. The results of the analysis of antibacterial variance on *S. aureus* bacteria showed a significant difference between each concentration treatment where $p<0.05$, followed by the Tukey test of 5%. The zone of inhibition of water-soluble chitosan against *S. aureus* bacteria ranged from 4.10 to 7.50 mm. The test results of *S. aureus* bacteria are shown in Table 4.

The highest water-soluble chitosan inhibitory power was at a concentration of 160 mg/ml with an average value of 7.47 mm in the category of medium antibacterial strength and the lowest value was at a concentration of 140 mg/ml with an average value of 4.40 with a weak antibacterial strength category. The positive control (K+) used chloramphenicol disk and was included in the strong category because it was in the range of 11-20 mm, and the negative control (K-) used distilled water as a solvent for chitosan, which was water-soluble and had no inhibitory power. The gram-negative bacteria used in this study was *E. coli*. The test results of *E. coli* bacteria are shown in Table 5.

Table 4. Test results of water-soluble chitosan inhibition against *S. aureus* bacteria.

No	Concentration (mg/ml)	Diameter Zone Clear (mm)			Average (mm)
		1	2	3	
1	140	4.85	4.10	4.25	4.40
2	150	5.90	6.10	6.65	6.22
3	160	7.50	7.40	7.50	7.47
4	Control (+)	12.21	13.73	13.90	13.28
5	Control (-)	0	0	0	0

Note: the value 0 has no resistance

Table 5. Test results of water-soluble chitosan inhibition against *E. coli* bacteria.

No	Concentration (mg/ml)	Diameter Zone Clear (mm)			Average (mm)
		1	2	3	
1	140	3.45	3.00	3.25	3.23
2	150	5.50	5.80	5.50	5.60
3	160	6.65	6.75	6.70	6.70
4	Control (+)	11.23	13.48	15.20	13.30
5	Control (-)	0	0	0	0

Note: the value 0 has no resistance

3.1.9 Test results of water-soluble chitosan hand sanitizer physical properties

The physical properties of water-soluble chitosan hand sanitizers need to be tested to see the acceptance of the community and the quality of the water-soluble chitosan hand sanitizers made. Water soluble chitosan hand sanitizer must be made by SNI 06-2588-1992. The quality of hand sanitizer liquid synthetic detergent according to SNI 06-2588-1992 is shown in Table 6.

The physical properties of the water-soluble chitosan hand sanitizer formulation were tested, with three different water-soluble chitosan hand sanitizer formulations (180, 190, and 200 mg/ml) and one commercial product.

3.1.10 Organoleptic test

The organoleptic of the water-soluble chitosan hand sanitizer was tested by hedonic testing, using 30 untrained panelists who are the general public. Panelists conduct hedonic tests separately and at different times to avoid bias towards each panelist's assessment. Organoleptic was observed with parameters (appearance, smell, and texture). The results of the organoleptic test were statistically processed using SPSS.

The following are the results of the panelists' calculations can be seen in Figure 7.

Table 6. Quality standard of hand sanitizer liquid synthetic detergent (SNI 06-2588-1992).

Test Type	Standard Requirements
Active substance content	Min 5%
pH	4.5 - 8.0
Liquid emulsion	Stable
Additional Substances	According to applicable regulations
Spread (cm)	5 - 7

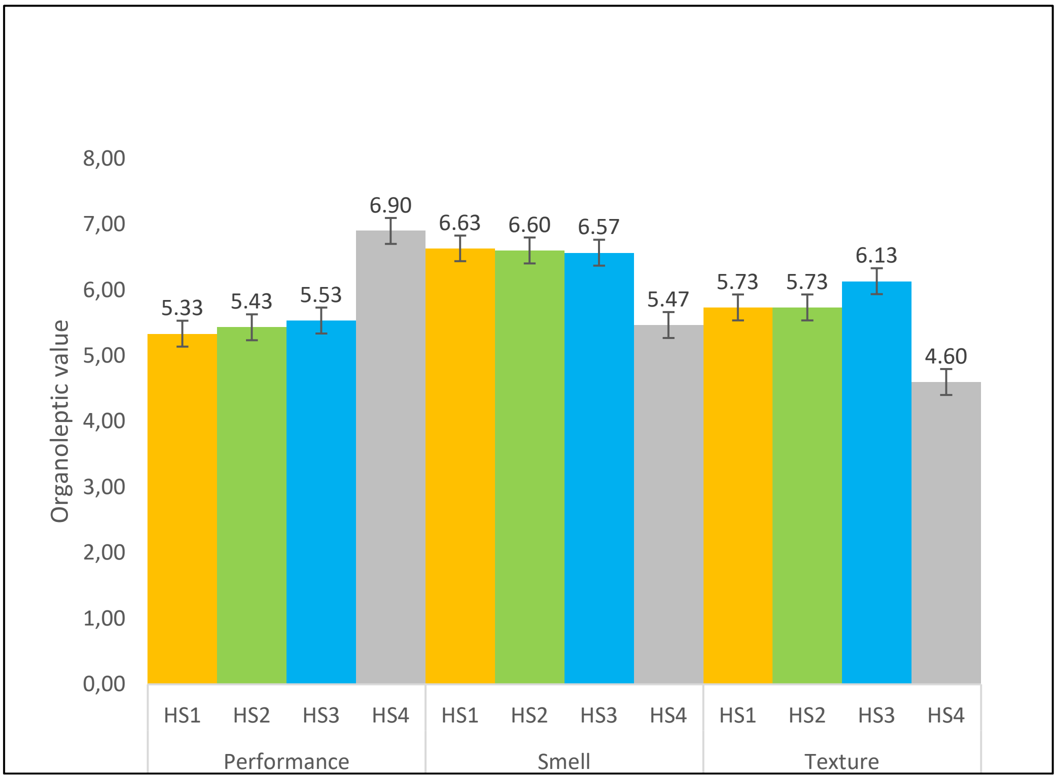


Figure 7. Graph of organoleptic test of water-soluble chitosan hand sanitizer formula.

3.1.11 Smell

Odor value of water-soluble chitosan hand sanitizer for HS1, HS2, and HS3 turned out to be quite liked by the public because they got a value range of 6.06 - 6.12 which was rounded up to 6 (somewhat like) because the water-soluble chitosan hand sanitizer has an orange aroma, compared to HS4 (commercial hand sanitizer) which smells neutral with the value of 5.19 is rounded up to 5. The smell of water-soluble chitosan hand sanitizer is somewhat preferable to that of commercial hand sanitizers that contain alcohol and are slightly pungent. From the odor value, water-soluble chitosan hand sanitizer is superior to commercial hand sanitizers.

3.1.12 Texture

The texture of the water-soluble chitosan hand sanitizer HS1 and HS2 got a value of 5.35 – 5.38 which was rounded up to 5 (neutral) while HS3 got a value of 5.69 which was rounded up to 6 (somewhat like), the texture of the water-soluble chitosan hand sanitizer gave taste, soft after use, while for HS4 the score was 4.21 rounded up to 4 (rather dislike) because commercial hand sanitizers use alcohol and after use, they give the impression of a mat and dry.

3.1.13 Homogeneity

The homogeneity of the hand sanitizer was tested on a glass plate, and after observing on HS1, HS2 and HS3 there were few fine grains, while HS4 (commercial hand sanitizer) had no fine or homogeneous grains. According to Bahri *et al.* (2021), a good hand sanitizer that meets the requirements of SNI 06-2588-1992 is a hand sanitizer that does not show any lumps or coarse granules in the gel.

3.1.14 Spreadability

The dispersion value of water-soluble chitosan hand sanitizer was calculated based on statistical analysis and $p<0.05$, which means that there was a significant difference between the treatment of water-soluble chitosan concentration added to the hand sanitizer formulation and the spreadability value of the water-soluble chitosan hand sanitizer gel preparation. The dispersion value of water-soluble chitosan hand sanitizer can be seen in Figure 8.

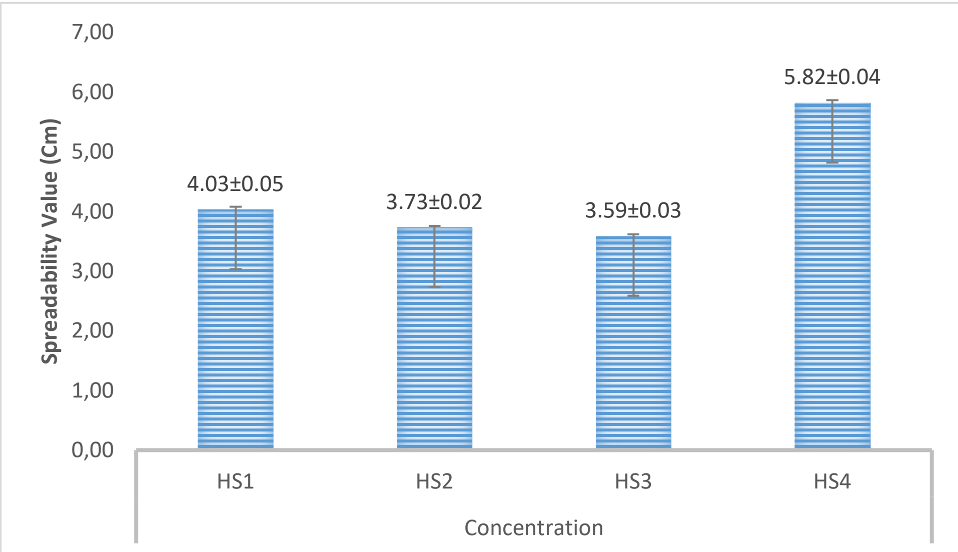


Figure 8. The spread ability of water-soluble chitosan hand sanitizer.

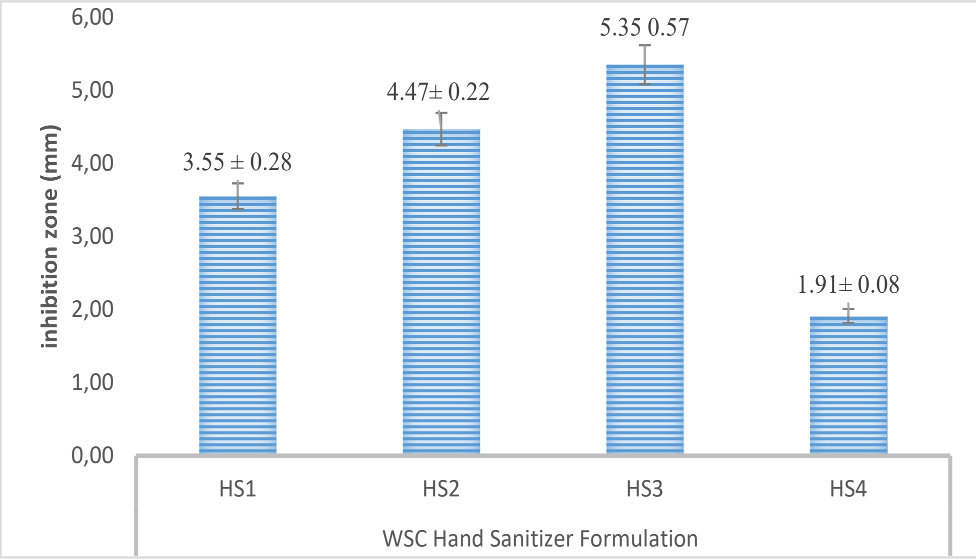
3.1.15 Degree of acidity (pH)

The acidity value of the water-soluble chitosan hand sanitizer formulation ranged from 6.05 to 6.28. There was no significant difference $p<0.05$ between the treatment of water-soluble chitosan concentration and the acidity value of the hand sanitizer produced. SNI 06-2588-1992 states that the required pH value for a good commercial hand sanitizer is in the range of 4.5 - 8.0. The acidity value of each concentration of water-soluble chitosan hand sanitizer has met the quality standard of SNI 06-2588-1992 hand sanitizer because if the pH value is low (acidic) it can cause skin irritation, while the skin will be scaly if the pH value of the hand sanitizer is too high (base) (Ariyanthini *et al.*, 2021).

3.1.16 Testing of water-soluble chitosan hand sanitizer antibacterial against S. aureus and E. coli

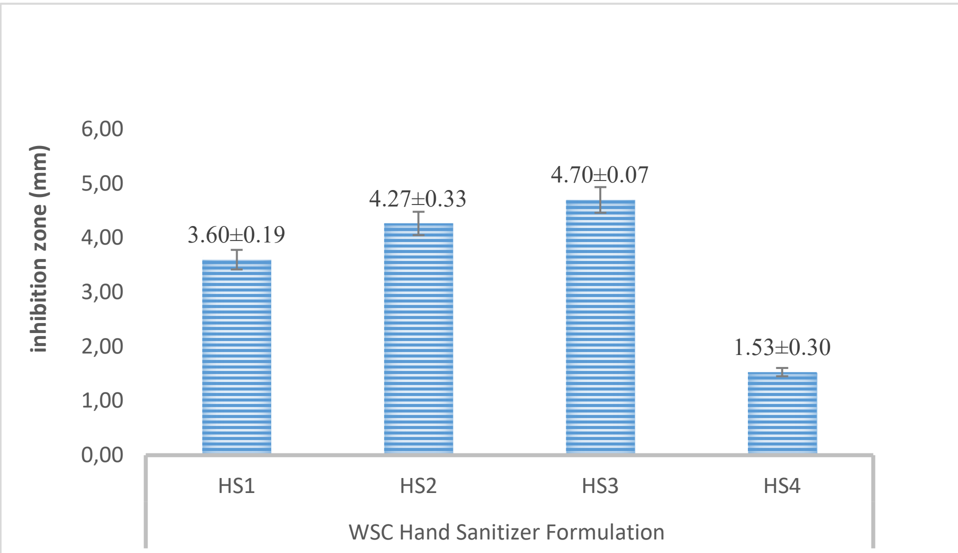
After the physical test on the water-soluble chitosan hand sanitizer was carried out, then continued with the antibacterial hand sanitizer test. Water-soluble chitosan to be included in the hand sanitizer formulation refers to the results of the antibacterial activity of water-soluble chitosan, where the concentration of water-soluble chitosan is increased to 180, 190, and 200 mg/ml due to the dilution of water-soluble chitosan interacting with solvents and other substances as the filler in hand sanitizer formulations. The positive control used as a comparison of water-soluble chitosan hand sanitizers used commercial hand sanitizers, while the negative control used was a gel-based hand sanitizer without the active ingredient of water-soluble chitosan. The results of the water-soluble chitosan hand sanitizer test results against *S. aureus* bacteria can be seen in Figure 9.

The results of the water-soluble chitosan hand sanitizer antibacterial test with analysis of variance showed a significant difference between each water-soluble chitosan hand sanitizer formulation where $p<0.05$, followed by the 5% Tukey test. The inhibitory power of the hand sanitizer formulation against *S. aureus* bacteria was between 3.55 ± 0.28 – 5.35 ± 0.57 mm and was categorized as weak, while the positive control had a smaller value of 1.91 ± 0.08 and fell into the weak category. The inhibition zone in the range of 5 is a weak category (Achmad *et al.*, 2020). The results of the antibacterial hand sanitizer test against *E. coli* bacteria can be seen in Figure 10.



Note: HS1 = WSC 180 mg/ml; HS2 = WSC 190 mg/ml; HS3 = WSC 200 mg/ml; HS4 = Hand sanitizer commercial

Figure 9. Graph of hand sanitizer inhibition against *S. aureus.*



Note: HS1 = WSC 180 mg/ml; HS2 = WSC 190 mg/ml; HS3 = WSC 200 mg/ml; HS4 = Hand sanitizer commercial.

Figure 10. Graph of hand sanitizer inhibition against *E. coli.*

uble chitosan is high. The optimal concentration for water-soluble chitosan from crab's shell inhibition falls in the medium category. Physical features of an organoleptic water-soluble chitosan from crab's shell hand sanitizer: neutral appearance, somewhat similar fragrance, neutral texture, similar with a commercial for not homogenous, dispersion and pH. The most effective hand sanitizer formulation from crab's shell water soluble chitosan is HS3 (including 200 mg/ml of water-soluble chitosan), which has a weak inhibition zone for *S. aureus* bacteria and *E. coli.*

3.2 Discussions

3.2.1 Characteristics of water-soluble chitosan

Water soluble chitosan in this study produced a yellowish white color, powder form and no odor is by commercial chitosan in Suptijah (2012) and meets the standards in GRAS (2012). The yield of water-sol-

3.2.2 Color, odor, and form of water-soluble chitosan

The color of water-soluble chitosan in the 2% and 3% HCl concentration treatment is by commercial chitosan in Suptijah (2012), namely yellowish white and in accordance with the standards in GRAS (2012) because the chitosan produced is odorless and in powder form. Chitosan in 4% HCl treatment produces the same brownish yellow color as reported by Cahyono (2018) and Kusumaningsih *et al.* (2004). The brownish yellow color is thought to be due to an imperfect neutralization process, which causes the browning process. The brownish yellow color is also due to HCl and amino sugars interacting which form a brown furfural component due to the amino glycosyl enolization reaction (Afridiana, 2011). The brownish yellow color is thought to be caused by an imperfect neutralization process, causing the browning process to occur. The brownish yellow color is also caused by HCl and the interaction of amino sugars which form the brown furfural component due to the amino glycosyl enolization reaction (Oktarlina *et al.*,2022)

3.2.3 Water-soluble chitosan yield

The high yield indicates the hydrolysis process is going well, so the water-soluble chitosan yield is high. Chitosan hydrolysis is influenced by pressure from the hydrolysis method, temperature, and acid concentration to glucosamine (Cahyono, 2018), a similar mechanism also affects the formation of water-soluble chitosan. The higher the concentration of HCl causes the yield of water-soluble chitosan to decrease. Meata *et al.* (2019) reported that the amount of glucosamine yield was influenced by the acid concentration and the high temperature used during the reaction rate; Cahyono (2018) also reported that the yield value decreased in line with the increasing concentration of HCl and heating time. In a chemical reaction, the concentration of the reagent will decrease in line with the increasing reaction results, with the pressure on the chitosan hydrolysis process, it functions to cut polymer bonds into monomers (smaller units) so that water-soluble chitosan is formed.

3.2.4 Degree of acidity (pH) water-soluble chitosan

Hydrochloric acid used in the hydrolysis process in the manufacture of water-soluble chitosan causes the neutralization step to be quite long and must be repeated. The neutralization step of water-soluble chitosan was carried out using isopropyl alcohol which has a pH ranging from 5-6. Neutralization with isopropyl alcohol is useful for removing impurities that remain in the water-soluble chitosan residue, resulting in a pH that is close to neutral. Extraction of H+ ions during chemical hydrolysis using hydrochloric acid does not cause different pH values (Nurjannah *et al.,* 2016).

3.2.5. Water-soluble chitosan viscosity

Cahyono (2018) states that the pressure on the hydrolysis process affects the cutting of polymer bonds into monomers, while Dwiyitno *et al.* (2017) reports that heating causes depolymerization which causes the chitosan polymer chains to break and form shorter chains (monomers). The large number of polymers that become monomers results in a low molecular weight, where the molecular weight is related to the intrinsic viscosity so that if the molecular weight is low, the viscosity will be below. This is what causes the viscosity of the water-soluble chitosan of the test results to be low.

3.2.6 Solubility of water-soluble chitosan

The solubility of dissolved chemicals is also influenced by the degree of acidity, temperature, and pressure used. Many acetyl groups that substitute H+ ions for hydroxyl and amine groups during the depolymerization process cause the solubility of water-soluble chitosan to be higher (Dwiyitno *et al.*, 2017). Chitosan can be dissolved in water because of the presence of OH and NH_3Cl in the smallest carbon units, which are produced from the pressurized hydrolysis process with hydrochloric acid so that the Cl- ions from HCl bind to the amino groups of chitosan and form NH_3Cl.

3.2.7 Degree of deacetylation of water-soluble chitosan

The FTIR spectra test was carried out to see the characteristics of the chitosan functional groups present in the water-soluble chitosan of the test results, in addition to determining the value of the degree of chitosan deacetylation. The stretching OH and NH stretching functional groups in this study are in the absorption band at a wavenumber of 3396 cm 1. The stretching CH functional group is seen in the absorption band at wave number 2939 cm-1, the C=O amide functional group is seen in the absorption band at wave number 1638 cm-1. The FTIR spectra of water-soluble chitosan in this study has shown the presence of amide absorption bands and hydroxyl groups that characterize chitosan in general. The results of the water-soluble chitosan spectra have little noise at wavelengths 3700-4000 but show a strong, firm, and wide absorption band at wavelengths 400-3700 as reported by Ahmad and Ayub (2022).

The treatment of HCl concentration with the highest DD value was at 3% HCl concentration, while the value of the degree of deacetylation at 2 & 4% HCl concentrations was still below the quality standard of chitosan, namely > 70%. The low value of the degree of deacetylation at concentrations of HCl 2 & 4% is suspected because the hydrolysis process did not run perfectly so that the acetyl group was not cut off maximally which resulted in the DD value not reaching 70%. In addition, the initial DD value of chitosan was only 57.64%, so the value of water-soluble chitosan was not too high. Based on the characteristic values of color, odor, shape, pH, viscosity, solubility, and degree of deacetylation, the 3% HCl treatment was selected as water-soluble chitosan to be tested for its antibacterial activity against *S. aureus* and E. *coli* bacteria.

3.2.8 Testing of water-soluble chitosan antibacterial against S. aureus and E. coli

The results of *E. coli* antibacterial test with analysis of variance showed a significant difference between each concentration treatment where $p<0.05$, followed by the Tukey test of 5%. The inhibition zone of water-soluble chitosan against *E. coli* bacteria ranged from 3.00 – 6.75 mm in the weak and moderate categories, the inhibition zone of positive control (K +) in the range of 11-20 mm was included in the strong category, while the negative control did not have inhibition. Hayati *et al.* (2023) reported that the inhibition zone in the range of 5 was in the weak category, 6-11 mm in the moderate category, 11-20 mm in the strong category, and >20 mm in the very strong category. The antibacterial inhibition of *S. aureus* is greater than the inhibition of *E. coli* as seen from the large clear zone formed, this is presumably because *S. aureus* bacteria is a gram-positive bacterium with a single cell wall structure and is simpler than *E. coli* bacteria which is a gram-negative bacterium that have a more complex cell wall (three layers). Zhou *et al.* (2022) stated that antibacterial compounds are easier to achieve work targets (damaging cell walls) in gram-positive bacteria because they have a simple cell wall structure.

Different inhibitions of antibacterial activity can also be influenced by the solvent used in dissolving the bioactive substances. Wang *et al.* (2020) reported that one type of polar solvent is water, the use of a polar solvent (water) is thought to facilitate water-soluble chitosan bioactive substances in penetrating the cell walls of *S. aureus* compared to *E. coli*. The cell walls of gram-positive bacteria are also composed of teichoic acid which is a water-soluble polymer and has the function of transporting in and out of positive ions, this is what causes bioactive compounds to easily enter the cell walls of polar gram-positive bacteria, causing damage to the peptidoglycan layer which is polar than the nonpolar lipid layer (Riu *et al.*, 2022). The inhibition ability of weak and moderate water-soluble chitosan is also suspected because the initial chitosan raw material used has a low degree of deacetylation so that the antibacterial ability of water-soluble chitosan is also not too strong (Jin *et al.*, 2021). The DD of water-soluble chitosan is only 78.4%. DD values above 90% can only be obtained if the initial sample has a DD value above 75%, this is also confirmed by Kadak *et al.* (2023) which states that the value of the initial deacetylation degree affects the value of the final deacetylation degree. The higher the DD value of chitosan, the more acetyl groups are replaced with amine groups so that the antibacterial ability is getting stronger. The working system of chitosan as an antibacterial is the presence of N atoms (amine groups) which have a positive charge that will bind to a negative charge on the surface of the bacterial cell wall which causes changes in cell permeability (Ardean *et al.*, 2021b) and an imbalance of internal cell pressure that causes lysis (Egorov *et al.*, 2023). Water-soluble chitosan has inhibitory power against *S. aureus* and *E. coli* bacteria, although the inhibitory power is categorized as moderate. The results of the water-soluble chitosan antibacterial test are the basis for adding the water-soluble chitosan active substance to the hand sanitizer formulation.

3.2.9 Test results of water-soluble chitosan hand sanitizer physical properties

Physical properties of organoleptic water-soluble chitosan hand sanitizer with parameters of neutral appearance (5), somewhat favorable odor (6), neutral texture (5) and somewhat favorable (6), not homogeneous, spread ability 3.59-4.03 cm, pH value 6.05-6.28. From the results of several physical evaluations, it was shown that the hand sanitizer of water-soluble chitosan met the preparation requirements that could be accepted. Physical evaluation is important to conclude that these preparations can be applied. In addition, the concentration of the extract did not show a significant effect on each of the results of the physical evaluation of the preparations.

3.2.10 Organoleptic test

The value of the appearance of water-soluble chitosan hand sanitizer for HS1, HS2, and HS3 is in the neutral category with a value range of 5.1 – 5.28 which is rounded up to 5, and for HS4 (commercial) it is in the rather like category with a value of 6.23 rounded up to 6. Based on the hedonic value of the appearance, it can be concluded that the appearance of commercial hand sanitizers is preferred by the general public, compared to commercial hand sanitizers, this is presumably because the appearance of water-soluble chitosan hand sanitizers still looks cloudy and not transparent like HS4 (commercial hand sanitizer). The higher the concentration of the chitosan used, the cloudier the color of the hand sanitizer gel (Kaban *et al.,* 2022).

3.2.11 Smell

The smell of water-soluble chitosan hand sanitizer is somewhat preferable to that of commercial hand sanitizers that contain alcohol and are slightly pungent. From the odor value, water-soluble chitosan hand sanitizer is superior to commercial hand sanitizers. The aroma is given to the aroma of oranges because the essence of oranges is added to the preparation.

3.2.12 Texture

Water-soluble chitosan hand sanitizer based on hedonic value on texture is still superior to commercial hand sanitizers, this is presumably due to the use of water-soluble chitosan natural ingredients in hand sanitizer formulations, thus providing a soft taste and coupled with the presence of glycerin in water-soluble chitosan hand sanitizer formulations. which moisturizes the skin.

3.2.13 Homogeneity

The homogeneity of water-soluble chitosan hand sanitizer is still not good, because there are still a few lumps of CMC-Na that have not completely dissolved, while for water-soluble chitosan it has completely dissolved before being combined into the formulation. The stirring process with the vortex is thought to be still not long enough which results in the remaining fine grains. The results of the homogeneity test of commercial hand sanitizers are better than water-soluble chitosan hand sanitizers which are still below the requirements of SNI 06-2588-1992. The homogeneity of chitosan hand sanitizer should be improved to completely dissolve the CMC-Na lumps before being mixed in the formulation and the mixing process with a vortex must be ensured until all the granules smooth can be dissolved completely

3.2.14 Spreadability

The test value for dispersing power of water-soluble chitosan hand sanitizer is 3.59 – 4.03 cm, where this value does not meet the quality standard of SNI 06-2588-1992, namely the standard requirements for good dispersion for commercial hand sanitizers ranging from 5-7 cm, and the value of the HS4 hand sanitizer has met commercial quality standards. The higher the concentration of water-soluble chitosan in the hand sanitizer formula affects the spreadability of the hand sanitizer formula itself. The dispersion value is also influenced by the use of humectants, namely glycerin and propylene glycol, which causes the spreadability of the hand sanitizer gel to increase (Somwanshi *et al*., 2023). From the picture, it can be seen that the smallest dispersion is in HS3, which is 3.59 ± 0.03 cm. In other words, the HS3 water-soluble chitosan hand sanitizer formula is very solid, this is not good because high dispersion affects the antimicrobial ability of the hand sanitizer gel to spread evenly on the surface skin (Arifin, 2021).

3.2.15 Degree of acidity (pH)

SNI 06-2588-1992 states that the required pH value for a good commercial hand sanitizer is in the range of 4.5 - 8.0. The acidity value of each concentration of water-soluble chitosan hand sanitizer has met the quality standard of SNI 06-2588-1992 hand sanitizer because if the pH value is low (acidic) it can cause skin irritation, while the skin will be scaly if the pH value of the hand sanitizer is too high (base) (Ariyanthini *et al*., 2021).

3.2.16 Testing of water-soluble chitosan hand sanitizer antibacterial against S. aureus and E. coli

The results of the water-soluble chitosan hand sanitizer antibacterial test against *E. coli* bacteria with analysis of variance showed a significant difference between each water-soluble chitosan hand sanitizer formulation where $p<0.05$, followed by the 5% Tukey test. The inhibitory power of the hand sanitizer formulation against *E. coli* bacteria was between 3.60 ± 0.19 - 4.70 ± 0.07 mm and was categorized as weak, while the positive control had a smaller value of 1.53 ± 0.30 and fell into the weak category. The inhibition zone in the range of 5 is a weak category (Achmad *et al*., 2020). Commercial hand sanitizers have a lower inhibitory value than water-soluble chitosan hand sanitizers, this indicates that hand sanitizers with natural ingredients have the potential to replace commercial hand sanitizers made from alcohol.

The inhibition of water-soluble chitosan hand sanitizers was seen to be the greatest in *S. aureus* bacteria compared to *E. coli,* in other words, water-soluble chitosan hand sanitizers damaged the cell walls of *S. aureus* bacteria compared to *E. coli,* this was confirmed by Wulandari *et al.* (2022), who reported that *S. aureus* bacteria are more easily penetrated by antibacterials from water-soluble chitosan hand sanitizers compared to *E. coli.* bacteria, due to the low lipid content (1 - 4%) of gram-positive bacteria compared to gram-negative bacteria which contain lipids. high (11-12%) and complex cell wall structure (three layers), so that water-soluble chitosan hand sanitizers more easily diffuse into the cell walls of gram-positive bacteria which have many pores in the peptidoglycan layer, and cause metabolism to be disrupted. Disrupted metabolism results in damage, so that the bacterial cell wall defense becomes weak and abnormal, followed by It only enlarging the pores on the bacterial cell wall, causing lysis and ending in death (Buijs *et al*., 2023). Water-soluble chitosan in hand sanitizers also causes cross-linking between chitosan polycations and anions on the surface of the bacterial cell wall, resulting in a change in membrane permeability (Li *et al*., 2020). Ghimire *et al*. (2024) also stated that there is a lone pair of electrons in chitosan that attracts Ca2+ minerals from the bacterial cell wall resulting in lysis, so the bacteria cannot survive and die.

4. Conclusion

The pressurized hydrolysis method in an acid atmosphere with a pressure cooker can produce water-soluble chitosan from crab shell, and the best treatment is the treatment of 3% HCl concentration. The best water-soluble chitosan inhibition is a concentration of 160 mg/ml. The best hand sanitizer formulation is HS3 (addition of water-soluble chitosan 200 mg/ml) with an inhibition zone of 5.35±o.57 mm for *S. aureus* bacteria and 4.70±0.07 mm for *E. coli* which is in the weak category. Further research may explore the scalability of pressure hydrolysis, and the broad-spectrum antibacterial efficacy of chitosan produced from crab shells.

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Authors' Contributions

All writers contributed to the final script. Each author contributed as follows: Niken and Dessy collected the data, authored the text, and designed the figures. Aef and Fera developed the major conceptual ideas and Khamhou critically revised the text. All authors discussed the findings and contributed to the final text.

Conflict of Interest

We certify that we have no personal or business interest in, or potential for personal gain from, any of the organizations or initiatives, nor do any of our relatives or affiliated businesses.

Declaration of Artificial Intelligence (AI)

We claim that no artificial intelligence (AI) tools, services, or technologies were employed in the creation, editing, or refinement of this manuscript. All content presented is the result of the independent intellectual efforts of us, ensuring originality and integrity.

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