The effect of reduced acetic acid concentration on nano-chitosan formulation as fish preservative

I J Winayu¹, N Ekantari¹, I D Puspita¹, Ustadi¹, WBudhijanto² and P S Nugraheni*¹

¹Department of Fisheries, Faculty of Agriculture, Gadjah Mada University, Bulaksumur Yogyakarta 55281, Indonesia
²Department of Chemical Engineering, Gadjah Mada University, Bulaksumur Yogyakarta 55281, Indonesia

E-mail: nugrahenips@ugm.ac.id

Abstract. Nano-chitosan has antibacterial activity and can be used as fish preservative. Acetic acid concentration of 1% is commonly used to produce nano-chitosan but it affected consumer acceptance because of its acidity. This study aimed to reduce the concentration of acetic acid and observed its effects on particle size, pH, and bacterial growth inhibition activity. Besides, it was also observed the effect of nano-chitosan with acetic acid concentration reduction on total bacteria and sensory value when applied on tilapia fillets. The results showed that the higher concentration of acetic acid resulted smaller particle size of chitosan and higher bacterial growth inhibition. The similar trend was also shown by acetic acid solution in same concentration. However, the bacterial inhibition zone of nano-chitosan solution was higher than acetic acid solution for each variation of acetic acid concentrations. This was evidenced by the observation of the clear zone formation of Gram positive and negative bacteria culture grown on nutrient agar. The optimum acetic acid concentration in nano-chitosan production was 0.43% because it was able to inhibit the growth of bacteria on the tilapia fillet and resulted in good consumer acceptance even when tilapia fillet was soaked up to 25 minutes in the nano-chitosan solution.

IIntroduction

Chitosan is a natural polysaccharide obtained from chitin through deacetylation process [1]. Size reduction of chitosan particle into nano-chitosan increases its antibacterial activity [2]. Nano-chitosan is more effective in inhibiting bacterial growth than chitosan itself and it is capable of damaging the bacterial cell membrane such as in Salmonella choleraesuis[3], Staphylococcus aureus, Escherichia coli, Bacillus licheniformis and Vibrio parahaemolyticus[4].

The most widely used method for nano-chitosan production is ionic gelation method because the process is easy and inexpensive. The ionic gelation method is prepared by dissolving chitosan into organic acid and then added with tripolyphosphate (TPP) as crosslinker[5]. The chitosan molecules in organic acid solution become soluble and swollen due to the protonation of chitosan’s amine group with H⁺ groups of acids. The swelling process of chitosan’s positive charge causes a large size of chitosan; thus, reduce the reactivity of the molecules to bind the bacterial cell membrane. Tripolyphosphate addition plays an important role in the formation of chitosan particle size into nano-
chitosan due to the bond constructed between chitosan’s positive groups that have been protonated by acid with TPP negative groups[6]. It makes the positive charge of nano-chitosan being more concentrated and increasing the effectiveness in inhibiting bacterial growth. The common organic acid used to dissolve chitosan is acetic acid [7]. The concentration of acetic acid commonly used for nano-chitosan manufacture is ranged from 1 to 2% (v/v)[7,8,9,10]. The use of 1% acetic acid (v/v) already influenced the taste and smell of the preserved products with nano-chitosan[8], so it is necessary to optimize the amount of acetic acid in nano-chitosan in order to reduce bacterial growth but still acceptable for consumers when it is applied to a fishery product.

2 Experimental Method

2.1 Preparation of Nano-chitosan by Ionic Gelation Method

Chitosan powder (Bio Chitosan Indonesia) were weighed for 0.6 g and then dissolved in acetic acid with varied concentration of 0.2%; 0.4%; 0.6%; 0.8%; and 1% (v/v). The dissolution of chitosan powder into acetic acid solution was sequentially in a small amount to prevent a clot. It was homogenized using a magnetic stirrer for 2 hours. Then sodium tripolyphosphate solution (0.84 g/L) was added in ratio 5:2 of chitosan: TPP (v/v) then stirred again for an hour to produce nano-chitosan with final acetic acid concentrations of 0.14%; 0.29%; 0.43%; 0.57%; and 0.71% (v/v), respectively. This reduction of acetic acid was done gradually until obtained the minimum concentration with high performance (antibacterial and sensory effect) of nano-chitosan.

2.2 Characterization of Nano-chitosan

Nano-chitosan was analyzed for its particle size, pH, and bacterial growth inhibition activity. Particle size analysis was conducted using HORIBA SZ 100 PSA with Dynamic Light Scattering (DLS) technique. The pH measurement was performed to determine the acidity level of the nano-chitosan solution.

Bacteria used in this study were Bacillus licheniformis (collection culture of Quality and Fisheries Product Lab, UGM), Staphylococcus aureus (FNCC 0047) (Gram-positive), Escherichiacoli(FNCC 0091), and Vibrio parahaemolyticus (JCM 2147) (Gram-negative). These four types of bacteria are often found in fishery products because of the possibility of contamination during the process. So, by using this bacterial strain the antibacterial test will give early information the probability of nano-chitosan for preserving fish. Each bacterium was derived from a pure culture that was inoculated on TSB medium (Tryptone Soya Broth) and incubated at 37°C for 24 hours. The activity of bacterial growth inhibition was performed using paper disc method[11]. The bacterial isolates were planted on nutrient agar(NA) in a petri dish, then flattened using drigalsky. Paper disc with diameter of 5 mm was moistened with 20 μl of nano-chitosan solution, placed on the agar media, then incubated at 37°C for 24 hours. Inhibition activity on bacterial growth was proven by clear zone formation around the paper disk. Inhibition zone was calculated by subtracting clear zone diameter with diameter of paper disk.

2.3 Nano-chitosan Application on Tilapia Fillet

Tilapia fillet was soaked in nano-chitosan solution with acetic acid concentrations of 0.14%; 0.29%; 0.43%; 0.57%; 0.71% (v/v). The soaking procedure was carried out by dipping the tilapia fillet into solution with the ratio of tilapia filet: solution = 1:1 for 3 minutes. Total plate count of bacteria was measured on those samples. As comparison, the sample treated with only acetic acid solution (similar concentration used in nano-chitosan treatment) was also analyzed.

The sensory analysis was performed by the trained panelists that were selected by a series of triangle test. The samples of tilapia fillet were prepared previously by soaking in nano-chitosan solutions for 3 minutes or 25 minutes then cooked in the microwave at 40°C for 8 minutes. Panelists were asked to describe whether the presence of acid in the product was detectable. The threshold test was also performed to reinforce the explanation in the description test[12]. The test was carried out...
using an acetic acid solution in varied concentration to get the smallest concentration of acetic acid recognized by the panelists.

3 Results and Discussion

3.1 Characterization of Nano-chitosan

Table 1. Particle size and pH of nano-chitosan with variation of acetic acid concentration

| Acetic acid concentration in nano-chitosan | pH   | Particle size (nm) |
|-------------------------------------------|------|--------------------|
| 0.14%                                     | 3.9  | 162.5±0.00         |
| 0.29%                                     | 3.7  | 157.7±0.00         |
| 0.43%                                     | 3.5  | 153.4±42.63        |
| 0.57%                                     | 3.4  | 149.2±0.70         |
| 0.71%                                     | 3.3  | 134.3±32.38        |

Reduction of acetic acid concentration affects the particle size of nano-chitosan (Table 1). Acetic acid is used to protonate amine groups of chitosan. The sufficient amount of acetic acid causes protonation to take place so that many positive charges is available to crosslink with the negative charge of TPP and form a smaller particle size. Reduction of acetic acid concentration up to 0.14% only slightly changes the size of the nano-chitosan particles. The smaller pH value of nano-chitosan compare to the pKa value of chitosan (6.5) is advantageous for the stability aspect of the solution since the high pH value triggers agglomeration.

![Fig 1. Effect of reduced acetic acid concentration in nano-chitosan on its antibacterial activity measured by inhibition zone on Gram-positive bacteria (Bacillus licheniformis and S. aureus) and Gram-negative bacteria (E. coli and V. parahaemolyticus) culture grown on NA.](image)

The effectivity of nano-chitosan in inhibiting the bacterial growth is shown in Fig 1. Nano-chitosan and acetic acid solution have a similar trend for bacterial inhibition but the activity of nano-chitosan
was higher than the acetic acid solution in every concentration for all types of bacteria. The presence of nano-chitosan disrupts the permeability of membrane cell of bacteria so that cells experience nutrient deficiencies to grow and cause cells death [13]. The bacterial cell wall of the Gram-positive and Gram-negative has a different structure that affects the reaction process between bacteria and nano-chitosan. Gram-negative bacteria are more sensitive to the presence of chitosan because the outer part of the cell membrane consists of lipopolysaccharide, lipoprotein, and phospholipids, which easily interact with the protonated amino group of chitosan due to the presence of anionic carboxyl groups and phosphate groups on the membrane cell layer. Whereas in Gram-positive bacteria, the outer part of the cell membrane consists mainly of peptidoglycan that leads to the lower ability of chitosan to adhere to the cell membrane [14,15]. However, in Gram-positive bacteria, amine groups from protonated chitosan are still possible to interact with negatively charged from teichoic acid located in the outer part of the cell membrane[16].

3.2 Effect of reduction of acetic acid concentration on tilapia fillet
The effect of the concentration acetic acid reduction on tilapia fillet was observed immediately after the sample was soaked in nano-chitosan or acetic acid solution. The higher number of bacteria were suppressed with the use of higher concentrations of acetic acid. The initial total amount of bacteria in tilapia fillets before being treated with nano-chitosan or acetic acid solution was 2.30 log cfu/mL. Even in the lowest concentration, acetic acid (0.14%) was able to reduce the number of bacteria (Fig 2).

The statistical analysis shows that the number of bacteria logs in tilapia after immersion in nano-chitosan solution was significantly different (P <0.05) with acetic acid solution, indicating a higher antibacterial activity of nano-chitosan compare to that of acetic acid solution

![Graph showing the total amount of bacteria in tilapia fillets after soaking in nano-chitosan prepared in varied concentration of acetic acid and acetic acid solution](image)

The total amount of bacteria in tilapia fillets after soaking in nano-chitosan prepared in varied concentration of acetic acid and acetic acid solution

The acetic acid also has an antibacterial property[7] so it is expected that the optimal results in inhibiting bacterial growth were obtained from the combination of acetic acid and nano-chitosan. The effectiveness of acetic acid in reducing the growth of L. monocytogenes was reported previously. The results showed that the acetic acid with the concentration of 1% [8] provide a higher bacterial inhibitory effect than 0.3% [7]. This result is in line with the data of the total bacteria count and inhibition zones that was conducted in this study. Acids are very influential on the antibacterial activity of nano-chitosan. The H+ group of acetic acid is capable of protonating amine groups from chitosan, which causes the amine group to be more reactive in interacting with bacterial cells that have a negative charge so that it can cause lysis and inhibit growth.

Sensory evaluation of tilapia fillet treated by soaking in nano-chitosan for 3 minutes showed no effect on taste and flavor of the product due to the low absorption of nano-chitosan into tilapia fillet.
Meanwhile, the addition of acetic acid concentration greatly influences consumer acceptance in term of taste and flavor for the sample soaked in nano-chitosan for 25 minutes. The taste and flavor of the acid were not detectable until the concentration of acetic acid reached 0.43%. The remaining acidic taste was detected (Table 2). Threshold values of acetic acid that can be detected by human organs is very low. The threshold value was also evaluated in order to obtain the information of minimum concentration detected by the human senses that will facilitate in formulating the product. Panelists started to detect the difference between solution sample with minerals water starting at 0.004% acetic acid concentration while not yet explaining the taste (absolute threshold). The introduction of flavor types and the different impression of flavor that started to be detected were at a concentration of 0.0075% (recognition threshold and difference threshold). The terminal threshold that can be detected was at 0.04% acetic acid concentration so it is indicating that all panelists were able to sense the difference between sample and mineral water.

Table 2. Sensory evaluation of the taste and flavor of tilapia fillet acid with nano-chitosan treatment

| Concentration of acetic acid in nano-chitosan | Taste of acid on fillet after soaking for | Flavor of acid on fillet after soaking for |
|---------------------------------------------|------------------------------------------|------------------------------------------|
|                                             | 3 minutes | 25 minutes | 3 minutes | 25 minutes |
| 0.14%                                       | undetected | undetected | undetected | undetected |
| 0.29%                                       | undetected | undetected | undetected | undetected |
| 0.43%                                       | undetected | undetected | undetected | undetected |
| 0.57%                                       | undetected | start detected | undetected | Detected |
| 0.71%                                       | undetected | detected | undetected | Strong detected |

Based on the data of total bacterial and sensory evaluation on tilapia fillets, it was found that the concentration of 0.43% acetic acid to produce nano-chitosan showed good bacterial inhibition results without changing the taste and aroma of tilapia fillets so that it gives chance to be utilized further on fish preservation.

4 Conclusion

The acetic acid concentration used for formulating nano-chitosan affects the consumer acceptance and the effectiveness in inhibiting bacterial growth. The antibacterial activity of nano-chitosan with various concentrations of acetic acid was higher than acetic acid itself. Nano-chitosan prepared by using 0.14-0.43% acetic acid to preserve the tilapia fillet were accepted by the consumers, even when it soaked up to 25 minutes. The optimum concentration of acetic acid in terms of its ability to reduce bacterial growth and consumer acceptance was at 0.43% acetic acid concentration. Furthermore, research on the effect of nano-chitosan on fish shelf life can be carried out, with reduced acetic acid concentration.

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