Comparison of Formulation Methods to Produce Nano-Chitosan as Inhibitor Agent for Bacterial Growth

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Abstract. Chitosan is known as an antibacterial agent. The effective surface area ratio of chitosan can be increased by modification into nanoparticles. Nano-chitosan can be prepared with several simple methods, i.e. precipitation, ionic gelation, or the polyelectrolyte complex method. This study compared these three methods in terms of the targeted product characteristics, i.e. stability of the average nanoparticle size as well as the colloidal dispersion, and the antibacterial characteristics. All three methods resulted in nanoparticle formation, but in the precipitation method significant zeta potential reduction was observed due to the presence of negative ions from the alkali that neutralized the chitosan amine group. The ionic gelation method yielded higher zeta potential and higher inhibition of bacterial growth than those yielded by the polyelectrolyte complex method. Ionic gelation and the polyelectrolyte complex method resulted in much better colloidal dispersion stability than the precipitation method, where a significant particle size increase was observed after one week of storage. This result indicates that both ionic gelation and the polyelectrolyte complex method can be used for forming nano-chitosan for the purpose of food preservation. However, for fishery products it is advisable to use the polyelectrolyte complex method because the TPP usually used in ionic gelation is not allowed to be applied to fish.

Keywords: *anti-bacteria; ionic gelation; nano-chitosan; particle size; polyelectrolyte complex; precipitation; zeta potential.*

1 Introduction

Attention to formulation and application of nanoparticles has developed greatly over the last decade. Nano-chitosan is a nanoparticle material that has high potential for use as food preservative. Nano-chitosan is a transformation of chitosan into nanoparticle size. Chitosan itself is a safe, non-toxic and environmentally friendly biopolymer [1]. The change in particle size of chitosan

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does not change the properties of chitosan mentioned previously in [2,3], but does change its antibacterial properties [4]. Nano-chitosan has been shown to have better antibacterial activity than chitosan solution [5]. This encourages the application of nano-chitosan as food preservative. Several studies on nano-chitosan application to fishery products have been carried out, including application on fillets of silver carp [6], white-leg shrimp [7] and fish fingers [8], in which a positive impact of using nano-chitosan on extending the shelf life of the products was observed.

Using a bottom-up process is a common approach of building nano-chitosan since dissolved chitosan molecules are able to self-assemble in the presence of crosslinkers [9]. In dilute acetic acid to chitosan, chitosan becomes soluble and protonated. Protonation leads to enormous particle sizes in chitosan due to the presence of electrostatic repulsion [10]. Reduction of the particle size is a strategy to optimize the activity of chitosan due to the increased power of the concentrated positive charge and enlargement of the contact surface area. A bottom-up process such as size modification can be carried out using precipitation [11], ionic gelation [12,13] or the polyelectrolyte complex method [14]. All three methods are are widely used because they are simple and inexpensive. The difference between the three methods lies in the chemicals used to transform dissolved chitosan into nanosize particles. The precipitation method utilizes a specific physicochemical property of chitosan, i.e. its insolubility in alkaline solution. In the application of this method, chitosan solution is mixed with NaOH or other alkaline compounds to make it precipitated [11]. On the other hand, ionic gelation and the polyelectrolyte complex method convert chitosan to nano-chitosan by creating crosslinks between the amine groups from the chitosan polymers. The crosslinker can be micro anionic molecules (such as tripolyphosphate) or macro anionic molecules (an oligosaccharide such as gum Arabic), respectively [15].

The formation of nano-chitosan particles is influenced by the solution characteristics (pH, temperature, ratio of chitosan and crosslinker, and the initial concentration of chitosan) and by the presence of other molecules capable of donating their molecular charges [12,16,17]. The utilization of NaOH affects the pH value, while TPP and gum Arabic are dissociated when dissolved in water at neutral pH [14,18] so that each of these influences contributes to nano-chitosan with different characteristics. Replacement of TPP as crosslinker in nano-chitosan synthesis is needed because TPP is known to manipulate the weight of fish due to water retention [19]. So far, the characteristics of nano-chitosans made using the aforementioned methods have not been well established. This study aimed to reveal the advantages and disadvantages of each method in order to find the most appropriate method to produce nano-

chitosan before being applied for food preservation, especially fishery products, which very easily decay due to their high nutritional content [20].

2 Material and Methods

2.1 Materials

The materials used for nano-chitosan preparation were chitosan powder (Bio Chitosan Indonesia), acetic acid (glacial, Ajax Finechem Pty. Ltd.), sodium hydroxide (Merck), tripolyphosphate/TPP (Sigma Aldrich), gum Arabic (local market) and MiliQ water (obtained using equipment made by Millipore Corporation). Inhibition on bacterial growth was tested using 4 strains of bacteria, namely *Bacillus subtilis* (from the culture collection of Quality and Fisheries Product Lab, UGM), *Staphylococcus aureus* (FNCC 0047), *Escherichia coli* (FNCC 0091), and *Vibrio parahaemolyticus* (JCM 2147). Selective media, i.e. *MRS Agar* (de Man, Rogosa and Sharpe agar from Merck), MSA (Mannitol Salt Agar from Oxoid), EMB agar (Eosin Methylene Blue agar from Oxoid) and TCBS agar (Thiosulfate Citrate Bile Salt Sucrose from Merck), were used to grow the bacteria for testing the antimicrobial activity of the nano-chitosan.

2.2 Methods

2.2.1 Preparation of Nano-chitosan Using Precipitation, Ionic Gelation and Polyelectrolyte Complex Method

Chitosan (0.08%, m/v) was dissolved into 100 ml of dilute aqueous acetic acid solution (1%, v/v) and then mixed using a magnetic stirrer for 2 hours to obtain chitosan solution.

The next step was modifying the particle size using three methods. The first method was the precipitation method, using aqueous NaOH solution (with concentrations of 0.1 N and 1 N) added dropwise into chitosan solution using a pipette while the solution was continuously stirred. The alkaline addition was stopped when the solution reached a pH value of 6.3 [21]. The second method was ionic gelation using TPP solution (0.84 g/L) as crosslinker. The TPP was added to chitosan solution with a volume ratio of 5:2 (chitosan:TPP) and homogenized for 30 minutes [22]. The last method was the polyelectrolyte complex method, using the same steps and ratio as with the ionic gelation method. The only difference was in the crosslinker used, i.e. the TPP was replaced by 0.3% (m/v) of gum Arabic solution [14]. This concentration was obtained from our previous study on the optimization of the chitosan-gum Arabic ratio.

2.2.2 Characterization of Particle Size, Zeta Potential, and pH of Nano-chitosan

The nano-chitosans produced by the three respective methods in this study were characterized for particle size and zeta potential using a particle size analyzer (Zetasizer Nano ZSP, Malvern). Both parameters were also measured in the chitosan solution for comparison. To evaluate the contribution of acetic acid on the antibacterial activity of nano-chitosan, the zeta potential of the acetic acid was observed as well. The pH value was analyzed using a Mettler Toledo pH-meter.

2.2.3 Nano-chitosan Activity in Inhibiting Bacterial Growth

All bacteria were planted in selective agars, which were adjusted to suit the type of bacteria. Onto the selective agar that had been solidified in a petri dish, 100 µl of particular bacterial isolate was poured and then flattened using a Drigalsky spatula. After drying, a 5-mm diameter paper disk was placed on the selective agar, continued by wetting the paper dish with 20 µl of the solution tested. The samples were then incubated at 37 °C for 24 hours. The inhibition zone is expressed by a clear zone that appears surrounding the paper disk. The calculation of the inhibition zone was conducted by calculating the difference between the diameter of the clear zone and the diameter of the paper disk.

3 Results and Discussion

3.1 Differences between Nano-chitosan Formulations in the Particle Size Result

Chitosan has multi-properties activity due to its amine group, which is very reactive with other molecules. Chitosan has plenty of hydrogen bonds in the backbone, which makes it hydrophobic. Acid protonates the amine group of chitosan and reduces the hydrogen bonds so that the chitosan readily dissolves in water [23]. Protonation resulted in large molecules due to the repulsive force of the positive charge of chitosan, as is evidenced by the data in Figure 1. Neutralization of the protonated amine group causes chitosan to become insoluble and this is used for nanoparticle formation. However, in pH solution far above the pKa value, gels are formed [21].

The addition of crosslinkers succeeded in reducing the particle size of the chitosan. There were no significant differences between the three nano-chitosan preparation methods in terms of particle size and all methods resulted in a seemingly clear solution, which indicates the absence of precipitation (Figures 2(A), 2(C), and 2(D)). The ionic gelation and polyelectrolyte complex methods

have the same mechanism of formation, by which complexation occurs due to the ionic interaction between the protonated amine group of chitosan and the dissociated crosslinker.

Tripolyphosphate (TPP) dissociates partially when dissolved at neutral pH [24]. Phosphate groups that bind the amine group with ionic bounds reduce the electrostatic repulsion so that swollen molecules become smaller. The same also occurs in the chitosan-gum Arabic complex. Gum Arabic is a natural anionic polysaccharide with highly branched polysaccharide consisting of a β-(1-3) galactose backbone with linked branches of arabinose, rhamnose, and glucuronic acid. The carboxyl groups (of glucoronic acid) are responsible for the negative charge of the gum Arabic above pH 2.2 [25,26]. The glucoronic acid binds the protonated amine group, leading to crosslinking and molecular shrinkage.

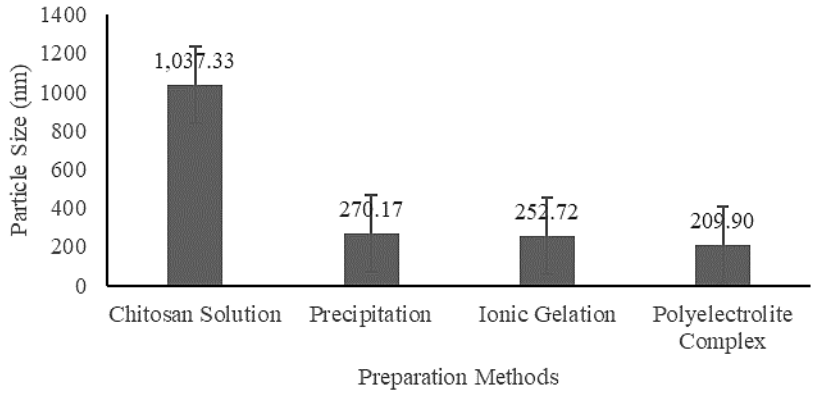

Figure 1 The particle size of chitosan and nano-chitosan solution formulated using various methods.

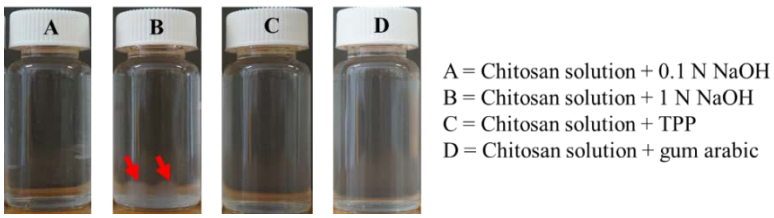

Figure 2 The appearance of nano-chitosan solution prepared with different methods.

The concentration of NaOH used to precipitate chitosan affects the formation of nanoparticles. No agglomeration was observed when mixing the chitosan with

0.1 N of NaOH (Figure 2(A)), whereas application of 1 N of NaOH did show agglomeration, visible as white shadows on the bottom of the bottle (Figure 2(B)). No obvious precipitates were observed in the TPP and gum Arabic cases (Figure 2(C) and 2(D), respectively). The visual change (agglomeration) was also seen in the chitosan-NaOH (0.1 N of NaOH) after storage for one week at room temperature. Nano-chitosan prepared by ionic gelation and the polyelectrolyte complex method showed good stability during one week of storage because they both still exhibited a clear solution. The stability of chitosan-TPP could be classified as highly stable because it still showed a particle size range that was the same as fresh solution after storage of 3 months at room temperature [22]. Moreover, a previous study has shown that storing chitosan-TPP nanoparticles at 25 °C showed high stability for 12 months, whereas storage at 40 °C was stable for 6 months [27].

3.2 Zeta Potential and pH value of Nano-chitosan Prepared with Different Methods

Although the nano-chitosans resulted from three methods previously discussed did not show differences in particle size, they were not the same with respect to zeta potential values. The zeta potential represents the electrostatic potential of the electrical double layer surrounding a nanoparticle in solution [28]. Zeta potentials above a value of +30 mV indicate a stable colloid, which prevents agglomeration due to the presence of repulsion of charged particles, which has benefit for storage [29]. Evidence of neutralization of chitosan charges, and hence reduction in zeta potential value, was observed in the precipitation method (Figure 3). The reduction of zeta potential also occurred in the cases of the ionic gelation and polyelectrolyte complex methods because several amine groups of chitosan were used to crosslink with the TPP and the gum Arabic. The ionic gelation method resulted in lower zeta potential than the polyelectrolyte complex method. Gum Arabic, when dissolved at neutral pH, undergoes dissociation and contributes to the negative charge in the solution. This negative charge is reduced when the gum Arabic is mixed with chitosan solution, which has a low pH (2.7). The negative charge of the gum Arabic decreases along with the decrease in pH value of the solution [30] due to the protonation of the carboxylic groups of the gum Arabic [31]. Goncalves *et al.* [32] have proved that at acidic conditions, the negative zeta potential of gum Arabic decreases from around -20 at pH 6 to around -10 at pH 3, and this continues along with a decreasing pH value. Therefore, reduction of the positive charge of chitosan due to be used in the crosslinking process, does not influence the zeta potential of the nano-chitosan because of the contribution of the protonated gum Arabic.

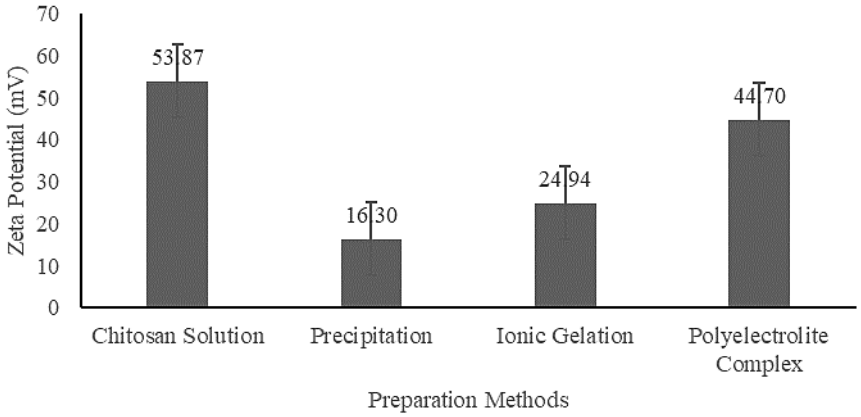

Figure 3 Effect of different formulation methods on the zeta potential of the nano-chitosan.

Figure 4 shows that the initial pH value of the chitosan solution was 2.7 and only had a slight increase with the addition of crosslinker. The TPP, when dissolved in water, had a pH value 9.04 while the gum Arabic exhibited a pH value of 5.19. As both crosslinkers had a higher pH value than the chitosan, mixing them with chitosan might only slightly have increased the pH value because the volume ratio was only 5:2 (chitosan:crosslinker). The precipitation method resulted in the highest pH value of the nano-chitosan since this method utilizes the mechanism of transforming the soluble state to an insoluble state when chitosan approaches the pKa value [21].

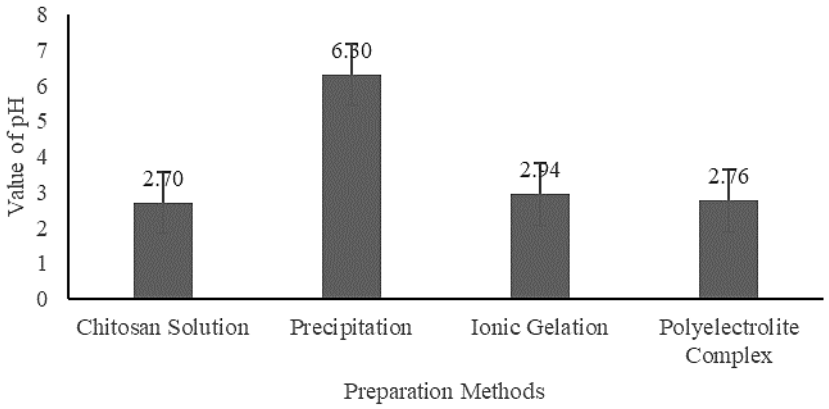

Figure 4 The pH value of nano-chitosan produced by various methods.

3.3 Effect of Nano-chitosan Prepared with Different Methods in Inhibiting Bacterial Growth

In the case of decaying fish, the quality of the fish is predominantly affected by bacterial activity. Although nano-chitosan also has anti-fungal activity, this is not discussed in this paper because the goal of this work was to prepare a fish preservative agent and hence the work was focused on antibacterial activity. The preparation method affects the ability of the nano-chitosan to inhibit bacterial growth. Figure 5 shows that the particle size and zeta potential of chitosan play mutually important roles in inhibiting bacterial growth. The combination of smaller particle size and higher zeta potential generate optimal bacterial growth inhibition activity, as was shown in the results from the polyelectrolyte complex method and the ionic gelation method.

The precipitation method produced the lowest antibacterial activity because despite the same particle sizes as yielded by both other methods, the zeta potential of the particles resulted from precipitation was the lowest among the three methods tested in this study. With such low zeta potential value, the inhibition induced by the nanoparticles from the precipitation method for the growth of *E. coli*, *Staphylococcus aureus*, and *Vibrio parahaemolyticus* was even lower than the inhibitory effect of 1% of acetic acid solution used to dissolve the chitosan. Holappa *et al*. [33] also showed that increasing the pH value above 6 causes a decrease in nano-chitosan activity in inhibiting bacterial growth due to the neutralization of nano-chitosan's charges as indicated by a zeta potential decrease.

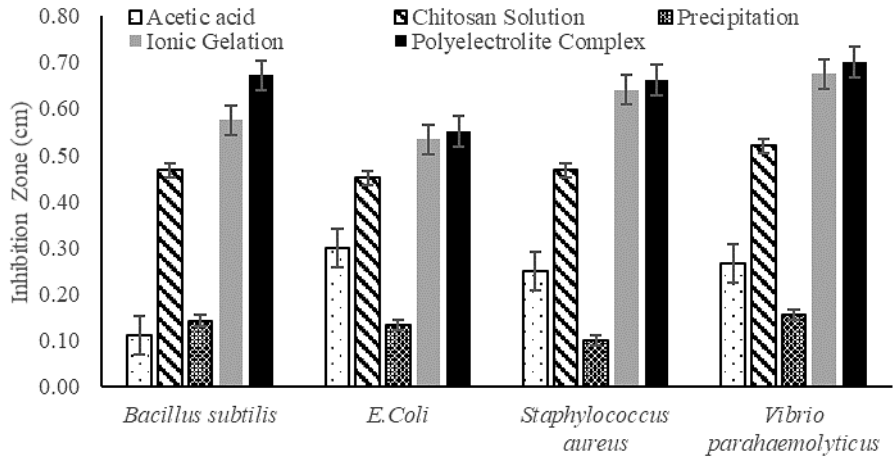

Figure 5 Activity of nano-chitosan in inhibiting bacterial growth.

The ionic gelation method exhibited good stability and a positive effect on food preservation [6-8] but there is a constraint to its application on fishery products

because it leads to water retention, resulting in a tendency to manipulate the product weight [33]. This drawback of ionic gelation nano-chitosan with TPP is considered dishonest to consumers and hence its application on fish products should be avoided, although there are no toxicity issues. Instead, application of complex chitosan-gum Arabic is suggested for application on fishery products. The application of chitosan-gum Arabic complex is so far more directed to the development of tissue engineering, drug delivery [34] and other purposes. Only very limited information for application in food preservation is available. Given the potential indicated by this study, the mechanism of chitosan-gum Arabic complex nanoparticle formation and its application as food preservative agent needs to be further investigated.

4 Conclusions

The preparation method of nano-chitosan affects the size of the resulted nano-chitosan particles and its ability to inhibit bacteria growth. The alkali-precipitation method is considered inappropriate for formulating nano-chitosan as food preservative because although it produces a small particle size, it also decreases the zeta potential due to the neutralization effect. The resulted particles also indicate a higher agglomeration tendency, which makes it unstable during storage. Both the ionic gelation method (using TPP as croslinker) and the polyelectrolyte complex method (using gum Arabic as crosslinker) produced the expected size range of nano-chitosan particles, exhibited good size stability during storage, and showed high bacterial inhibition activity. Between the two methods, the polyelectrolyte complex method led to the highest inhibition activity. This shows the high potential of the polyelectrolyte complex method for application as a natural and affordable food preservative agent. However, this study was merely meant as a preliminary study to explore the potential of the three methods and the consequences of each method. The polyelectrolyte complex method needs to be investigated further in relation to various aspects of food preservation, especially related to fishery products, so that the potential of gum Arabic to be used as an alternative crosslinker to replace TPP, which is banned in the fishery industry, can be evaluated.

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