Effect of nitrogen nanobubble preservation on the quality, microbial population, and storage life of fresh yellowfin tuna (Thunnus albacares)

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Abstract. The objectives of this study were to evaluate the effect of nitrogen nanobubble for storing fresh yellowfin tuna (Thunnus albacares). Yellowfin tuna were stored for 10 days in two types of treatment: nitrogen nanobubble (NNB) and conventional packaging process (CPC). Two methods including qualitative (organoleptic) and quantitative (histamine and mercury content, and total plate count of bacteria) were analyzed to determine the treatments effect on post-harvest quality, microbial population, and shelf-life of the fish. Results show that nitrogen nanobubble is proven to maintain dissolved oxygen levels remain in low conditions (<1 mg/L) during treatment. Although the organoleptic properties for both treatments do not show any significant differences, the lowest histamine and mercury contents are found in treatment NNB at 11.8 mg/kg and 0.14 mg/kg, respectively. The microbes in treatment NNB is achieved levels deemed safe (based on national standards) at 1.8 x 10^4 colonies/g, E. coli content <3 MPN/g, and the presence of Salmonella sp. are not detected. Our results suggest that treatment NNB is preferable for preservation process of fresh yellowfin tuna and might improve to extend the shelf-life.

1. Introduction
Yellowfin tuna is one of superior economic commodity with increment trend in exports reached at 16.57% in periods of 2016-2017. Yellowfin tuna which commonly known as a high nutrition fish; however, it decayed easily due to its great amount of protein containing free amino acids for microorganism metabolism process, ammonia production, amine, organic acid, and sulphuric acid biogenic [1, 2, 3]. The quality reduction might occur right after the fish death because it caused the defence mechanism to be stop. A common method to inhibit those reductions is usage of low
temperature, including both cooling and freezing. This kind of methods helps to delay biochemical processes in the fish body. However, the usage of ice is less effective due to its ability to maintain low temperature merely in short time \[4\]. The needs of technology development to preserve the freshness of fish are absolutes. This technology expected to help preserve freshness of fish in longer time and might leads to prosperity increment of anglers. One of effective technology is a combination of nitrogen gas and nanobubble technology.

Nitrogen gas used in the modified technology to suppress the presence of oxygen and reduced microbial growth, henceforth extend the product shelf life. The inhibitor property of nitrogen gas was used to preserve fruits and vegetables, fresh and processed meat, cottage and quarg cheese, and the concept feasibility was proven to preserve cold fresh milk, particularly as mixture of CO\(_2\)/NO\(_2\) was applied \[5\]. Nanobubble is an emerging technology used to maintain post-harvest quality of foodstuffs. Nanobubble particle have unique properties as large surface area with great negative charge that caused the bubble from coalescence and improve the bubble stability in water. The objectives of this research are to evaluate the effect of nitrogen nanobubble preservation on the quality, microbial population and storage life of fresh yellowfin tuna (Thunnus albacares).

2. Materials and methods

2.1. Materials and tools
The material used is yellowfin tuna of 30 kg obtained from PT. Perikanan Nusantara. Laboratory testing materials used are PCA (Plate Count Agar), BPW (Buffered Pepton Water) 0.1%, BGLBB (Brilliant Green Lactose Bile Broth), LSTB (Lauryl Sulfate Tryptose Broth), ECB (Escherichia Coli Broth), L- EMBA (Levine Eosin Methylene Blue Agar), MR-VP (Methyl Red-Voges Proskauer), KCB (Koser Citrate Broth), SCA (Simmons Citrate Agar), Kovas Voges-Proskauer Reagents (VP), LB (Lactose Broth), SCB (Selenite Cystine Broth), TTB (Tetra Thionate Broth), RV (Rappaport Vassiliadis), XLDA (Xylose Lysine Deoxycholate Agar), HEA (Hektoen Enteric Agar), BSA (Bismuth Sulphate Agar), TSIA (Triple Sugar Iron Agar), LIA (Lysine Iron Agar), LDB (Lysine Decarboxylase Broth), KCNB (Kalium Cyanide Broth), TB (Tryptose Broth), TSB (Trypticase Soy Tryptose Broth), SIM, Kovac Reagents, BHI (Brain Heart Infusion), Urea Broth, Malonate Broth, Phenol Red Lactose Broth, Phenol Red Sucrose Broth, Keratin Crystals Bromcresol Purple Dye Solution 0.2%, Physiological Saline Solution 0.85%, Formalinized Physiological Saline solution, Salmonella Polyvalent Somatic (O) Antiserum A-S, Salmonella Polyvalent Flagellar (H) Antiserum Phase 1 and 2, Salmonella Somatic Group (O) Monovalent Antisera.

The tools used are gas regulators, gas hoses, nitrogen nanobubble generator, NanoDoz (Nanox), connector cables, transformers / stabilizers, and water tanks with a capacity of 150 L. The tools used for microbial testing are petri dishes, test tubes, Durham tubes, serology tubes 10 mm x 75 mm, volumetric pipettes, media bottles, colony counters, scissors, tweezers, inoculation needles, stomacher, Bunsen burner, pH meter, weighing scale, magnetic stirrer, vortex shaker, incubator, water bath, autoclave, sterile cupboard (clean bench), refrigerator, freezer.

2.2. Methods
The research conducted in Perikanan Nusantara, Ltd., Bitung, and sample test analysed in Testing and Certification of Fisheries Product Centre/Balai Pengujian dan Sertifikasi Hasil Perikanan (BPSHP) North Sulawesi. Grade-C of yellowfin tuna was stored in cold storage for 5-10 days. The yellowfin tuna was incubated with nitrogen nanobubble which produced by nanobubble machine. The nanobubble machine was soaked in styrofoam basin sized 150 L which has been filled by seawater at volume 100 L. After the machine being installed, the nitrogen gas was discharged into inlet pipe which was controlled by gas regulator at flow of 2 mL/min. Dissolved oxygen (DO) levels was monitored every 15 minutes with calibrated DO meter. During nanobubble generation, ice was added until water temperature reach below 30 C. Two treatments being conducted including: (i) NNB treatment, grade C
tuna incubated in nitrogen nanobubble water and (ii) CPC treatment, grade C tuna being processed in conventional preservation method using ice as a control.

The shelf life of yellowfin tuna was determined using direct method in which the tuna was stored under conditions similar to those that it would actually encounter. The organoleptic test was held at laboratory of Perikanan Nusantara, Ltd after being kept for 10 days. The test followed a national standard SNI number 01-2346-2006. A randomized number code of samples were given to the panelists to be scored. The scoring was given in hedonic scale (score 1-7), considered by 30 panelists. The data was analyzed statistically using one way-ANOVA. The laboratory test was based on national standard SNI 2897:2008 including (a) Total Plate Count, (b) MPN E. coli test with estimation test, affirmation test, and isolation-identification through indole biochemical test, methyl red, voges-proskauer and citrate (IMViC), (c) Salmonella sp. test through Salmonella growth in selective media with pre-enrichment and enrichment then continued by biochemical and serolog test.

3. Results and discussion

3.1. Organoleptic test

The results of organoleptic properties for each treatments including color, texture, and aroma was shown at figure 1 below. The organoleptic test results of NNB treatment showed a red-pale color with spongy and compact texture and fish specific odor. The fish meat as an entirety still considered to be grade C. The cracky and hollow parts of the meat were formed as residual of recurrent grading process. The pale meat with soft texture was shown in treatment CPC. There was a few of reddish meat which can be classified as grade C, but the whole fish in CPC treatment were ranked as reject grade.

The results represented that addition of nitrogen nanobubble is effective to maintain the fish quality, measured from physical, texture, and aroma. Figure 2 showed the capability of nitrogen nanobubble is effective in reducing oxygen gas content at level of 0 mg/L in water media. The requirements to preserve freshness of fish in good condition are to prevent lipid oxidation and aerob bacteria growth in a way of inducing an environment with minimum amount of oxygen through addition of nitrogen in nanobubble form. Oxygen can be reduced by absorbing air (vacuum) or adding an inert gas, namely nitrogen or carbondioxide [6]. Parry [7] and Hawa et al. [8] evaluated that nitrogen can take role as filler and prevent food from damage. Alatossava et al. [9] in his research showed that addition of nitrogen gas gave a great effect in suppressing total amount of bacteria in pasteurized milk.

The changes in physical appearance of fish meat was suspected to occur because of lipid breakage during storage which caused by amino acid reaction with carbonyl compound resulted from lipid oxidation and leads to brown pigment production [10]. Quality degrading of tuna meat texture occurred during storage because of the bacterial growth which caused the meat to be in spoiled condition [11]. Suwetja [12] informed that the bacterial decomposition started after rigor mortis stage happened or after the fish meat was no longer compact. Junianto [13] revealed that the changes of fish odor was caused by protein decomposition from bacterial activity, so that there was a positive correlation between bacterial population and fish odor. The shifting of odor took place because there was bacterial activity to decompose protein and lipid [14]. Buckle et al. [15] stated that bacterial growth at food product will generate unpleasant odor due to protein and lipid decomposition, and the odor of bacteria itself.
Figure 1. The organoleptic test results of yellowfin tuna after 10 days of incubation (a) NNB treatment; (b) CPC treatment.

Figure 2. Nitrogen nanobubble performance in reducing oxygen gas content.

3.2. Histamine test
Histamine test considered as one of the test parameters because the content of histamine in a certain amount used as one of the parameters to observe the decline in fish quality because histamine is a
product of the decomposition of free amino acids in fish after death. Histamine test results for each treatment showed in table 1. Table 1 showed histamine content in treatment NNB was 11.80 mg/kg, and treatment CPC was 12.27 mg/kg. This value is still within the safe limit set by SNI 2729-2013 and SNI 4110-2014 of maximal 100 mg/kg. The histamine content in each treatment was not much different from the results of other studies (table 2) and was below the threshold of food safety standards of several export destination countries (table 3).

Table 1. Histamine test results of yellowfin tuna after 10 days of incubation.

| Parameters | Unit | Sample | Requirements |
|------------|------|--------|--------------|
|            |      | NNB    | CPC          | (SNI 2729-2013) | (SNI 4110-2014) |
| Histamine  | mg/kg| 11.80  | 12.27        | Max. 100        |                  |

Histamine Histamine is an active primary heterocyclic biological amine compound that is formed in the post mortem phase of fish meat of the scombroid and non scombroid families that contains a lot of free histidine and is formed by decarboxylation of the amino acid histidine by the exogenous decarboxylase enzyme produced by microbes in fish [16, 17]. The process of formation of histamine in fish is strongly influenced by the activity of the enzyme L-Histidine Decarboxylase (HDC) [18]. Histamine caused poisoning known as histamine fish poisoning (HFP), often occurs after consuming marine fish that contain lots of free histidine, which is a precursor of histamine [19].

Removal of dissolved oxygen due to presence of nitrogen nanobubble is critical to inhibit the growth of histamine-forming bacteria. The nitrogen displaced all the oxygen needed for bacterial metabolism, hence slowing growth by proportional amount. Daniels et al. [20] confirmed those findings by replacement of bacterial growth atmosphere with 100% nitrogen. The reduction of metabolism activity was expected to cause the low level of histamine in NNB treatment.

Table 2. Results of histamine levels in scrombidae.

| No. | Researcher          | Research location   | Fish species            | Histamine levels (mg/kg) |
|-----|---------------------|---------------------|-------------------------|--------------------------|
| 1   | Guizani et al. 2005 | Muscat, Sultanate Oman | Yellowfin tuna (Thunnus albacares) | 27.8                     |
| 2   | Patage et al. 2005  | Cochin, India       | Little Tuna (Euthynnus affinis), Indian Mackerel (Rastrelliger kanagurta) | 16.0, 17.5               |
| 3   | Du et al. 2002      | Gainesville, Florida | Yellowfin tuna (Thunnus albacares), Skipjack Tuna | 7.2, 8.9                 |
| 4   | Staruszkiewicz et al. 2004 | Hawai, US | Katsuwonus pelamis, Yellowfin Tuna (Thunnus albacares) | 8.9, 9.7                 |
| 5   | Ko 2006             | Banyuwangi, Indonesia | Albacore Tuna (Thunnus alalunga) | 8.9-21.9                 |
Table 3. Histamine content allowed in several countries.

| No. | Country       | Criteria (mg/kg) | Remarks              |
|-----|---------------|------------------|----------------------|
| 1   | European Union| 100              | Wrong handling       |
|     |               | 200              | Dangerous            |
| 2   | USA           | 50               | Not allowed          |
| 3   | Canada        | 100              | Symptoms of decay    |
| 4   | Germany       | 100              | Symptoms of decay    |
| 5   | Denmark       | 100              | Dangerous for health |
| 6   | Sweden        | 100              | Dangerous for health |

3.3. Mercury test
Mercury test results for each treatments are shown in table 4 below. Table 4 showed mercury content in treatment NNB is 0.141 mg/kg, whereas in treatment CPC is 0.155 mg/kg. Those values were deemed safe based on national standard SNI 2729-2013 and SNI 4110-2014 which is appointed at maximum 0.5 mg/kg. Mercury (Hg) is one of dangerous heavy metals, very toxic, and has bioacumulative property [21, 22]. Mercury that contaminated water could generate serious biological effect which leads to degradation of quality and quantity of fish resources because of contamination of heavy metals which accumulated at the body of aquatic animals through food web [23, 24, 25].

Table 4. Mercury test results for each treatment after 10 days of incubation.

| Parameters | Unit | Sample | Requirements |
|------------|------|--------|--------------|
|            |      | NNB    | CPC          |
| Mercury    | mg/kg| 0.141  | 0.155        |
|            |      | Max. 0.5                      |

Halstead [26] proven that toxic chemical compound could not be degraded inherently in sea, with the result that it would not disturb the living of organism. The sediment mercury altered to be methyl mercury (CHrHg(I)) by sulfate reducing bacteria known as methylation process. Methyl mercury entered into food web and accumulated in human body [27]. If the fish that contain methyl mercury consumed by human continuously, it directs to accumulation of metals that will become hazard and might lead to mortality [28, 29]. Yusuf et al. [30] also proven that toxicities of methyl mercury gave slight effect to gastrointestinal but serious risk in neurology, including: soreness of genital labia, tongue and movement (arm and feet), confusion, hallucination, irritability, sleep disorder, ataxia, amnesia, speaking and thinking disorder, damaged of hearing, unstable emotion, comma, and mortality.

The mercury-forming bacteria was investigated to be dominantly gram-negative bacteria (Escherichia and Enterobacter), a few of gram-positive (Staphylococcus sp. and Streptococcus sp.), and those belonging to genera Bacillus. The absence of aeration was proven to decrease production of methyl mercury [31]. As mentioned before, the presence of nitrogen nanobubble removed the oxygen available so that the bacterial growth was inhibited and the formation of methyl mercury was reduced as well [20].

3.4. Microbial contamination
Microbial contamination test including total microbe analysis (TPC), E. coli, and Salmonella sp. were applied to identify total colony in yellowfin tuna. The results of microbial contamination for each treatment were shown at table 5. Microbiology testing including quantitative to determine quality and shelf life of food, qualitative test to assign the safety level, and bacterial indicator test to specify sanitation levels [32]. Results showed that both treatments were achieved levels deemed safe based on national standard SNI 2729-2013 and SNI 4110-2014. The results were also showed that treatment...
NNB succeeded in depress bacterial growth with total TPC value $1.8 \times 10^4$ colony/g, $E. \ coli <3$ MPN/g, and negative for *Salmonella*. Buckle et al. [15] and Harmain et al. [33] informed that the factors influencing microorganism growth are nutrition supply, pH, aw, temperature, time, and oxygen availability. *Salmonella* sp. is a gram-negative bacterium that could cause gastro entities, enteric fever, septicemia, and diarrhea [34, 35]. The total number of microbial for both treatments was likely due to the absence of dissolved oxygen.

### Table 5. Results of total number of microbial colonies testing in each treatment.

| Parameters | Unit   | Sample  | Requirements |
|------------|--------|---------|--------------|
| TPC/ALT    | Colony/g | 1.8 x 10$^4$ | >3 x 10$^4$ |
| $E. \ coli$ | MPN/g   | <3     | <3           |
| *Salmonella* | g      | (-)    | Negative / Max. 25 |

### 4. Conclusion
The NNB technology application for tuna preservation is effective to maintain the post-harvest quality measured by its physical appearance, texture, aroma, and by its chemical properties based on organoleptic, histamine, and mercury test results. The total microbes in treatment NNB is achieved levels deemed safe (based on SNI) at $1.8 \times 10^4$ colonies/g, $E. \ coli$ content $<3$ MPN/g, and the presence of *Salmonella* sp. are not detected. The quality of fresh yellowfin tuna maintains for 10 days. NNB treatment is successful in preserving the tuna freshness according to the research results.

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