The effect of nisin from *Lactococcus lactis subsp. lactis* on refrigerated patin fillet quality

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**Abstract.** The effect of nisin from *Lactococcus lactis subsp. lactis* with spraying method application on quality of patin fillet during refrigerated storage (4±1°C) was investigated. The quality of patin fillet based on total plate count (TPC), pH, TVB-N, and TBA values during 16 days at 4±1°C. Completely Randomized Design (CDR) was used in one factor (nisin activity) at 0 IU/ml, 500 IU/ml, 1000 IU/ml, and 2000 IU/ml. The observation was done at 0, 4th, 8th, 12th, and 16th days of storage. The result showed that variation of nisin activity significantly affected the quality of fillet according to TPC, pH, and TVB-N values, however no significant difference on the obtained of TBA value. Nisin in 500 IU/ml, 1000 IU/ml, and 2000 IU/ml could extend the shelf-life of fillet until 4th, 8th, and 12th days respectively based on standard in all parameters.

1. **Introduction**

Patin (*Pangasius hypophthalmus*) are freshwater fish that are farmed in Indonesia. Patin fillet has become an export commodity, and it is also in high demand in the global market, especially in the United States and Europe [1]. Fillet has some advantages such as being practical and easy to process; however, fillet can be damaged rapidly because of autolysis, microbial growth, and the oxidation process. Microbial growth is the major factor that leads to spoilage, and fillet then cannot be consumed [2]. Low temperature storage is a common method to decrease the spoilage rate of fillet; however, this method is unable to inhibit growth of psychrophilic bacteria such *Acinetobacter, Pseudomonas spp,* and *Brochothrix thermosphacta* species. In this case, the use of an antimicrobial substance is recommended to retain the quality of fillet during cold storage.

Bacteriocins are ribosomally synthesized peptides from bacteria, especially lactic acid bacteria (LAB) with bactericidal or bacteriostatic effects. Bacteriocins produced by LAB have several properties that make them suitable for food preservation: (i) they are generally recognized as safe (GRAS) substances, (ii) they are inactive and nontoxic on eukaryotic cells, (iii) they become inactivated by digestive proteases, having low effect on the gut microbiota, (iv) they are usually pH and heat-tolerant, (v) they have a relatively broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasm membrane, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation [3]. Nisin is a bacteriocin that has been used as food preservative to inhibit Gram positive bacteria, *Eschericia coli,* and sporing bacteria. Nisin is composed of 34 amino acids with 3354 Da molecular weight produced by *Lactococcus lactis subsp. lactis* [4]. In 1988, nisin
was approved by FDA for specific uses and as a GRAS (Generally recognized as safe) substance [5]. It is also approved by Ministry of Health, Republic Indonesia [6] and in over 40 countries as a food preservative [7].

The application of nisin as food preservative has examined for many products such as cheese, fermented beverage, and canned food to inhibit the growth of Gram positive bacteria and the spores [5,7]. For fishery products, nisin alone or combined with another substance has been used against Listeria monocytogenes, Salmonella sp., Vibrio spp., Clostridium botulinum, and other pathogenic bacteria that usually contaminate the product [4,8]. In this study, the effect of nisin from Lactococcus lactis subsp. lactis with spraying method on the quality of patin fillet were investigated. The patin fillet quality observed based on total plate count (TPC), pH, TVB-N and TBA values during 16 days in refrigerated storage (4±1°C).

2. Material and method

2.1. Sample preparation

Patin siam fish used in the experiments were obtained from Lembah Hijau Multifarm Sukoharjo, Central Java (Indonesia). The fish were immediately filleted and washed with tap water and stored on ice during preparation. The nisin solution was made by dissolving an amount of 1000 mg of nisin powder (activity: 10⁶ IU/g from Sigma-Aldrich Pte Ltd, product number: N5674) in 100 ml HCl (0.02 N) to obtain an initial concentration of 10⁴ IU/ml. About 1.25 ml, 2.5 ml, and 5 ml of this solution were diluted with HCl 0.02 N up to 25 ml to obtain the final concentrations 500 IU/ml, 1000 IU/ml, and 2000 IU/ml respectively [9]. The fillets were randomly assigned into four group consisting of control (0 IU/ml nisin), and three groups sprayed with nisin solution in 500 IU/ml, 1000 IU/ml, and 2000 IU/ml until the whole sample became wet. All samples were packed in plastic plates covered with plastic wrapping, then stored under refrigeration (4±1°C) for 16 days. Total plate count (TPC), pH, total volatile base (TVB), and thiobarbituric acid tests were observed on the the 0, 4th, 8th, 12th, and 16th days of storage days of storage.

2.2. Microbial analysis

Determination of microbial growth in the sample was done using the pour plate method according to SNI 2332.3 [10]. 10 g of fillet were aseptically homogenized with 90 ml sterilized NaCl dilution water (0.85% NaCl w/v) using a mechanical blender (Philips). From this dilution, other decimal dilutions were prepared and plated in Plate Count Agar (PCA) media (Merck) in duplo. Enumeration of the total plate count was determined after two days incubation at 36°C. The data were transferred into logarithms of the number of colony-forming unit per gram of fillet (Log CFU/g).

2.3. Chemical analyses

The pH value was measured using digital pH meter (Hanna Instrument) at room temperature [11]. The TVB-N value was determined using the Conway micro diffusion method according to SNI 2354.8 [12]. The data of TVB-N value was shown in mg N per 100 gram fillet. To measure the TBA value, 10 g of fillet was homogenized with 50 ml distilled water and transferred into a distillation flask. Then, 47.5 ml distilled water and 2.5 ml HCl (4M) were added to the flask and the mixture was distilled in high temperature. 5 ml of distillate was pipetted in a reaction tub and 5 ml of TBA reagent was added. The mixture was heated in boiling water for 35 minutes then cooled. The absorbance (A) was measured using a spektrofotometer (528 nm) [13].

2.4. Statistical analysis

Completely Randomized Design (CRD) was used in one variable was nisin activity in 0 IU/ml, 500 IU/ml, 1000 IU/ml, and 2000 IU/ml in two replication for each sample. Data were analyzed with an ANOVA test (One Way ANOVA) followed with DMRT (Duncan Multiple Range Test) (α=5%) if there were the variances between samples.
3. Result and discussion

3.1. Microbial analysis
The deterioration process of fish results from three mechanisms: enzymatic autolysis, lipid oxidation, and microbial growth. Microbial growth is the major factor that leads to spoilage, and the product cannot be consumed because of the production of new compounds that are responsible for changes in odor and flavor [2]. Some species of bacteria such as Pseudomonas spp, Shewanella, Micrococcus, Alcaligenes, Photobacterium phosphoreum, and Brochothrix thermosphacta are spoilage microorganisms that are usually present in fishery products, producing amine compounds such as trimethylamine (TMA), dimethylamine (DMA), and other biogenic amines [14]. During storage, the microorganisms have different abilities to tolerate the preservation conditions. In this study, changes of microbial count in patin fillets throughout storage are shown in Fig. 1.

The initial microbial count of control and samples treated ranged from 4.76-5.38 log CFU/g, which showed all the samples were in good condition according to the SNI standard of fresh fish [15]. The TPC value of all the samples increased gradually with storage; however, the sample with nisin had lower counts than the control throughout storage. The control sample exceeded the microbiological acceptability limit of 7 log CFU/g [16] in the 4th day of storage and increased faster than the treated samples. The sample with 500 IU/ml and 1000 IU/ml nisin exceeded the limit after the 8th and 12th days respectively, while the sample with 2000 IU/ml nisin did not reach 7 log CFU/g until the end of storage. The TPC values in the control sample and samples treated with 500 IU/ml, 1000 IU/ml, and 2000 IU/ml nisin at the end of storage were 9.58 log CFU/g, 9.25 log CFU/g, 7.37 log CFU/g, and 6.50 log CFU/g respectively. These results indicated that nisin had an inhibitory effect on microbial growth in refrigerated patin fillet and extended its shelf life, in approach with previous reports that informed the self-life of refrigerated vacuum packaged rainbow trout fillet that was sprayed with nisin (100 ppm) could reach 16 days of storage [17]. These result also accordance with other report that informed the used of 5% nisin solution with dipping method could inhibit microbial growth in pompano fillet about 1.22 log CFU/g lower than control in 12 days [18].

The effect of nisin on the quality of patin fillet is related to its ability to be an antimicrobial substance. Nisin is active against Gram positive bacteria and its spores. The inhibition effect of nisin against Gram positive bacteria is based on pore formation in the cytoplasmic membrane of the target bacteria. It leads to loss of these substances and depletes the proton motive force, which interferes with cellular biosynthesis and causes cellular death [5]. It is also been reported that nisin interferes with cell wall biosynthesis by binding with a peptidoglycan precursor (lipid II) [8]. Whereas the inhibition effect of spores is nisin interacts with membrane sulfhydryl that destroys spor germination [4].

3.2. Chemical analyses

3.2.1. pH
pH had correlations with microbial counts and total volatile base nitrogen (TVB-N) values of the sample. Fish tissue generally has neutral pH and decreases into slight acidity in early storage; however, the pH gradually increased because of microbial activity that produced alkaline compounds such trimethylamine, dimethylamine, and biogenic amines [2,19]. In this study, changes of pH in patin fillets throughout storage are shown in Fig. 2.

The initial pH of patin fillets ranged from 6.35-6.44 and gradually increased for all samples until the end of storage. There were no significant differences (α=0.05) between the control and sample treated with 500 IU/ml nisin; however, the sample treated with nisin 1000 IU/ml and 2000 IU/ml showed significant differences (α=0.05) with the control. The sample with 2000 IU/ml nisin showed the lowest increase of pH among other treatments without any significant differences (α=0.05) until the 12th day of storage. At the end of storage, the pH of each sample reached 8.39; 8.49; 7.48; and 7.25 for the control and samples treated with 500 IU/ml nisin, 1000 IU/ml, and 2000 IU/ml.
respectively. The results indicated that 1000 IU/ml nisin and 2000 IU/ml activities could keep the pH of the fillets under control during storage.

In the previous study informed that the used of 100 ppm nisin sprayed on rainbow trout fillet that was kept under vacuum showed no significant difference (p≥0.05) with a control until the end of storage. Another report also reported that the used of 5% nisin with dipping method on pompano fillet showed no significant difference (p≥0.05) with a control during storage [18]. These differences might be because the species and the conditions used in the experiments were different [20].

3.2.2. Total Volatile Base Nitrogen (TVB-N)
TVB-N is widely used as an indicator of fish spoilage. This measurement based on total amount of ammonia (NH₃) produced by the deamination of amino acid of spoilage bacteria, dimethylamine (DMA) produced by autolytic enzymes during storage, and trimethylamine (TMA) produced by the reduction of trimethylamine oxide (TMAO) of spoilage bacteria that are formed in non-fermented food products during storage [21, 22]. In this study, the changes of TVB-N in patin fillets during storage are shown in Fig. 3.

The initial TVB-N of patin fillet was varied at 11.39 mg N/100 g, 15.84 mg N/100 g, 13.36 mg N/100 g, and 16.42 mg N/100 g for the control, sample treated with nisin 500 IU/ml, 1000 IU/ml, and 2000 IU/ml, respectively. The TVB-N of all samples gradually increased until the end of storage. Samples with 1000 IU/ml nisin and 2000 IU/ml had much lower TVB-N values than the control; however, the sample with 500 IU/ml nisin had the highest values of the other treatments. This might be caused by the low effectivity of nisin at 500 IU/ml, meaning it could not inhibit spoilage bacteria in the samples. However, in the higher activities (1000 IU/ml and 2000 IU/ml) nisin could maintain the TVB-N values below those of the control until the end of storage. Previous study showed that the used of nisin at the lowest concentration (0.1%) with the dipping method in grass carp fillet gave a higher TVB-N value than control in the end of storage, whereas at the higher concentration (0.2%) nisin could maintain the TVB-N value below that of the control [23]. In this study, the control and samples treated with 500 IU/ml nisin exceeded the acceptability limit of 35 mg N/100 g fillet [21] on the 4th day of storage, the sample with 1000 IU/ml nisin and 2000 IU/ml exceeded the limit on the 16th day of storage. The TVB-N values in the control sample, and the samples treated with 500 IU/ml nisin, 1000 IU/ml, and 2000 IU/ml at the end of storage were 307.54 mg N/100 g, 424.25 mg N/100 g, 79.47 mg N/100 g, and 51.90 mg N/100 g, respectively.

3.2.3. Thiobarbituric Acid (TBA)
The TBA is one of the indicator related to lipid oxidation occurring in the fishery product during storage, it is also closely related to off-odour and off-flavor attributes [24]. The maximum level of TBA value indicating the good quality of fishery product (frozen, chilled, or stored with ice) is 5 mg malonaldehyde/kg, while the fish may be consumed up to the level of 8 mg malonaldehyde/kg [25]. In this study, the changes of TBA value in patin fillets during storage are shown in Fig. 4. All the samples had very low initial TBA values at 0.05-0.06 mg malonaldehyde/kg and gradually increased during storage. However, no significant differences were found the any samples at the end of storage. The similar results also reported in the previous study that examined the effect of nisin on the TBA values of pompano fillet during refrigerated storage [18]. The results showed that during the entire storage period, TBA values of sample treated with nisin were similar to the control, which indicated that nisin had no effect on reducing the lipid oxidation of fish. The addition of 100 ppm nisin alone in the buffalo meat sausage also did not alter the TBARS (thiobarbituric acid reactive substances) level from that of the control [26].

At the end of storage, the TBA values of all the samples were still below the maximal acceptable limit of 8 mg malonaldehyde/kg. The control sample and sample treated with nisin 500 IU/ml, 1000 IU/ml, and 2000 IU/ml reached 0.37 mg malonaldehyde/kg, 0.31 mg malonaldehyde/kg, 0.33 mg malonaldehyde/kg, and 0.34 mg malonaldehyde/kg, respectively.
Figure 1. Changes of microbial count in patin fillets during storage

Figure 2. Changes of pH in patin fillets during storage

Figure 3. Changes of TVB-N in patin fillets during storage
4. Conclusion
The microbial and chemical analyses of the patin fillet during 16 days cold storage (4±1°C) have examined to explain the effect of nisin as food preservative on the quality of patin fillet. According to TPC, pH, and TVB-N results, patin fillets quality could be maintained by the use of nisin at 1000 IU/ml and 2000 IU/ml. Due to all standards, patin fillets sprayed with nisin activity 1000 IU/ml and 2000 IU/ml could extend the shelf life until 8th and 12th days of storage respectively, while control and sample with nisin activity 500 IU/ml have been unable to consume in the 4th day of storage.

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