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Modification Of The Method For Identifying Histamine-Forming Bacteria Using Niven Agar With The Double Filtration Preparation Method

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Abstract - The decline in the quality of tuna produces histamine compounds. Histamine is formed at temperatures >4°C. One method for identifying histamine-producing bacteria is carried out using Niven method. The purpose of this study is to create a modification of the Niven method by adding the double filtration method. The modified Niven agar method was applied to tuna samples (cube and ground meat) taken randomly from processing stages located in Jakarta. The determination of the number of histamine-producing bacteria was carried out using the pour plate method on modified Niven agar medium. Observations of bacterial colony growth were conducted at 10, 18, and 24 hours. The research results show that the ALT calculation of sterilized tuna samples indicated that no bacteria grew in those samples, meaning that the colonies grown on Niven media using the modified method were purely BPH colonies intentionally added to the product. Then, the growth results of the two bacteria contaminated in the sterilized tuna (*M. morganii* and *E. aerogenes*) showed that bacterial growth was observed at the 10-hour observation. Up to the 18-hour observation, the bacteria were still noticeably increasing in number, and by the 24th hour, a decrease in bacterial numbers began to appear, signaling that the bacteria had passed the stationary phase. The modified Niven agar method using the double filtration method at the preparation stage can be used as a step for screening and identifying BPH, both from single colonies grown in liquid media and in tuna products, with more accurate identification results.

Keywords: Tuna, Histamine-forming Bacteria, Double Filtration, Niven

I. INTRODUCTION

Tuna fish is a commodity that deteriorates quickly if not stored at low temperatures and can also produce histamine compounds that are harmful to humans who consume it [1]. Tuna belongs to the family Scombridae, with one of its characteristics being a high content of the free amino acid histidine [2]. This amino acid is one of the ten essential amino acids needed by children and infants [3]. The amino acid histidine is especially necessary for infants because it functions as a precursor to histamine, a chemical released

by the immune system during allergic reactions and that stimulates the production of hydrochloric acid in the stomach, which is important during the digestive process [4]. However, the amino acid histidine can also be converted by certain bacteria into histamine, which can be harmful to some individuals.

Histamine is a biogenic amine compound obtained from the breakdown of free histidine amino acid found in fish meat, which is produced biologically through the decarboxylase process of free amino acids and is present in various foodstuffs such as fish, red meat, cheese, and fermented foods [5].

Histamine-producing bacteria are naturally found in fish meat. While the fish is alive, these bacteria do not break down the amino acid histidine present in the fish meat. The breakdown activity only begins when the fish dies. Some histamine-producing bacteria can survive at low temperatures, even at 4°C. It has been detected that some bacteria are able to continue producing histamine up to levels of 300 mg/kg during storage at 4°C for 5 days [6]. Therefore, it is important to ensure that no histamine-producing bacteria are present in the raw tuna entering the export tuna production unit.

Most of Indonesia's tuna is exported to other countries and has long been a top export commodity after shrimp. Therefore, ensuring that the raw materials entering the tuna processing units for export are in prime condition is essential. Effective field monitoring of tuna quality necessitates a rapid and reliable analytical approach. One commonly used technique for detecting histamine-producing bacteria is the Niven method. This study aims to develop a modified version of the Niven method through the incorporation of a double-filtration procedure.

II. MATERIALS AND METHODS

2.1 Methods and tools

The materials used are 6 histamine-producing bacteria: *Enterobacter aerogenes, Hafnia paralvei, Klebsiella pneumonia, Morganella morganii, Klebsiella oxytoca*, and *Proteus mirabilis*. The media used are commercial TSA media (Tryptone Soya Agar, Oxoid brand no. CM0131), formulated Tao agar media, and formulated Niven agar media. Additionally, 2 layers of membrane filters were used (KG-25, Ǿ 25 mm, Advantec, Tokyo, Japan), consisting of one membrane with a pore size of 10 µm (Y100A025A, Ǿ 25 mm, Advantec) as the top filter and a pore size of 0.2 µm (K020A025A, Ǿ 25 mm, Advantec) as the second filter.

The tools used in the research include Erlenmeyer flasks, scales, incubators, water baths, measuring glasses, test tubes, Petri dishes, inoculating needles, and membrane filters of 10 mm and 0.2 mm. The materials used are peptone, yeast extract, glucose, L-histidine, sodium agar, bacto agar, bromothymol blue, NaCl, MgSO4.7H2O, MgCl2.6H2O, CaCl2.2H2O, KCl, NaBr, SrCl2.6H2O, H3BO3, NaF, and KI. Six standard cultures were used, including Hafnia paralvei, *Morganella morganii* ATCC 258330, Klebsiella pneumoniae ATCC 13883, Klebsiella oxytoca ATCC 700324, and Proteus mirabilis ATCC 7002.

2.2 Sampling

A total of 4 tuna fish samples were randomly taken from the frozen storage room of a tuna export company in Jakarta. The tuna taken was in the form of processed cubes with quality suitable for export.

2.3 BPH Growth on TSA Medium, Tao Agar Medium, and Niven Agar

A total of 6 BPH isolates (*Enterobacter aerogenes, Hafnia paralvei, Morganella morganii*) ATCC (American Type Culture Collection, a non-profit organization that collects, stores, and distributes standard reference microorganisms, cell lines for research and development) 25830, *Klebsiella pneumoniae* ATCC 13883, *Klebsiella oxytoca* ATCC 700324, and *Proteus mirabilis* ATCC 7002) from slant agar were refreshed by streaking a single loop of BPH culture onto Tryptone Soya Agar (TSA) media, and incubated at 35°C for 24 hours. A single colony obtained from TSA was subsequently grown on Tryptone Soya Broth (TSB) media and incubated at 35°C for 24 hours. After incubation, serial dilutions were performed to achieve the desired cell density (10^3 – 10^4 colonies/ml). Serial dilution was carried out by taking 1 ml of the sample from the TSB media and adding it to the first 9 ml of sterile TSB media, then taking 1 ml from the TSB in the first tube to add to the TSB in the second tube, and so on.

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The six isolates with certain densities were grown on three different types of agar media, namely Niven Agar, Tao Agar, and TSA, using the pour plate method and incubated at 35°C for 24 hours. The growth of BPH was observed at 18 and 24 hours.

2.4 BPH Growth in Modified Niven Agar Method

The Niven agar method was modified by adding a double filtration stage during the sample analysis process. This step refers to the method developed by [7]. A total of 1 ml of BPH culture with a density of 10^3 colonies/ml was filtered using 2 prepared filters of different sizes. A filter membrane with a pore size of 10 µm was placed on top of a 0.2 µm filter membrane, followed by rinsing with 10 ml of sterile saline solution. After that, the 0.2 µm filter was placed on the surface of the solidified Niven agar medium. The Petri dish containing the agar and filter membrane was incubated at 35°C for 24 hours.

Observation of BPH colony growth was carried out at 10, 18, and 24 hours. As a control, BPH cultures were also grown on Tao medium [7] and TSA medium. Two BPH isolates with a density of 10^9 – 10^10 colonies/ml were then contaminated onto sterilized tuna fish and analyzed using a modified Niven method.

2.5 Validation of BPH identification method using Niven Agar with the modified method

To obtain tuna free from bacterial contamination, the tuna is sterilized using irradiation (20 kGy) carried out at the National Nuclear Energy Agency (BATAN) facility. Two packages of tuna cubes measuring 1x1x1 cm are packed in plastic, weighing 460 g per package or 1 lb.

Irradiated tuna samples were first taken in 25 g portions to count the total bacteria after irradiation and before the experiment was conducted. Then, 25 g of these samples were contaminated with two BPH isolates (*E. aerogenes and M. morganii*) by immersing the tuna sample in the isolate culture for 1 minute, and then drained. The treatment was carried out with two repetitions. The isolates used were pure isolates that had been grown for 24 hours at 35°C.

After the treatment, the tuna samples were placed in a diluent medium (*phosphate buffer*) and subjected to serial dilution. Next, 1 ml of the sample was incubated on the test medium using the pour plate method. The test media used were Niven Agar with a modified method and Tao Agar. Incubation on both media was carried out at 35°C for 48 hours. Growth observation of the isolates was conducted at 10, 18, 24, and 48 hours.

III. RESULTS AND DISCUSSION

3.1 BPH growth on TSA medium, Tao Agar Medium, and Niven Agar

The growth of BPH isolates on TSA, Niven, and Tao media is shown in Figure 2. In general, all six bacteria were able to grow on the three media with densities ranging from 10^8 to 10^10 colonies/ml. The growth of the six bacteria on Tao agar and Niven media was not significantly different compared to growth on TSA as a control. Tao agar and Niven media can be used to count BPH growth in the form of pure and single cultures. According to [8], TSA media is representative for growing bacteria. Niven media can detect histamine-producing bacteria [9], [10], [11], and [12].

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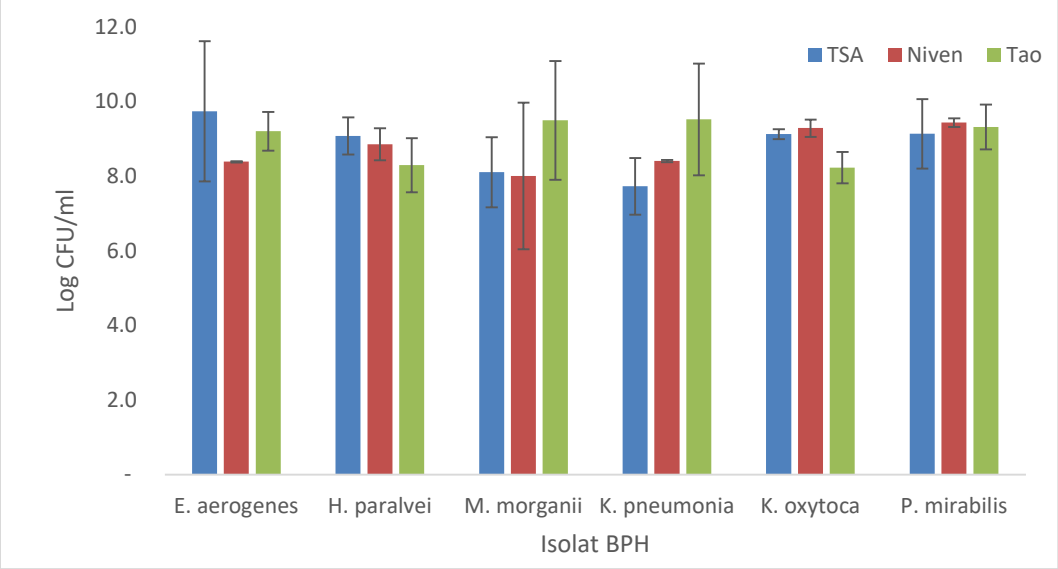

Figure 2. Growth of BPH isolate (log CFU/ml) in the test medium after incubation at 35°C for 24 hours

In general, colony morphology on TSA medium is cream-colored, purple on Niven medium, and blue on Tao medium. There is a color change in colonies on Niven and Tao media after 24 hours of incubation. This is likely because Tao and Niven Agar media contain L-histidine and pH indicator dyes, namely bromothymol blue and bromocresol purple. L-histidine is converted by BPH into histamine, causing a pH change so that the media and resulting colonies will have a specific color that facilitates identification [13]. Specifically, a blue halo appears around colonies growing on Tao agar, and colonies turn dark purple on Niven agar after 24 hours of incubation.

The growth of BPH colonies on three different media and at different incubation times is presented in Table 3. The growth of the six BPH isolates on the three media was satisfactory at 18 hours of incubation, with an average density of 10 log colonies/ml. This indicates that at that observation time, all the bacteria had reached the stationary phase. Bacterial growth at 24 hours was higher compared to 18 hours. The growth of *M. morganii* and *E. aerogenes* on the three media was higher than that of the other bacteria.

Table 3. Number of BPH colonies (log colonies/ml) on the test media after incubation at 35°C for 18 and 24 hours

Isolate Type	Incubation 18 hours			Incubation 24 hours		
	TSA	Niven	Tao	TSA	Niven	Tao
E. aerogenes	10.88	10.86	10.62	TBUD	TBUD	TBUD
H. paralvei	10.78	10.72	10.04	TBUD	TBUD	TBUD
M. morganii	10.75	10.82	10.51	10.99	10.88	10.51
K. pneumonia	9.70	10.00	10.11	10.79	10.46	11.09
K. oxytoca	10.36	10.26	10.51	10.54	10.89	10.93
P. mirabilis	10.56	10.61	10.40	10.57	10.75	TBUD

Note: TBUD: Too Many to Count

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3.2 BPH Growth Using the Modified Niven Method

On Niven medium, the growth of one of the BPH (*M. morganii*) was already visible at the 10-hour observation, but for other types of BPH it was only seen at the 18-hour observation. This difference occurs because the lag phase of the two bacterial species often differs. The lag phase in bacteria occurs when a culture enters a new/different medium from before, during which the bacteria undergo metabolic adaptation of the cells in order to survive in the new environment [14].

The colony color of BPH isolates on modified Niven media and Tao Media is presented in Table 4. In general, colonies on modified Niven media are purple, while on Tao media they range from green to blue. This color difference occurs because histamine-producing bacteria form purple colonies against a yellow medium background. The histamine produced will increase the medium's pH, causing the color to change from yellow to purple [15] The difference in colony color on the two media may be due to the use of different color indicators in each medium. The color variations that appear on Tao or Niven agar media when bacteria are cultured indicate a shift in the medium's pH as histamine is formed by BPH utilizing histidine in the medium. The more blue/purple it is, the more basic it becomes. On Niven medium, most of the colonies that grow are blue to purple, whereas on Tao medium at the same hour, purple color is not yet visible. If the observation of purple color is compared between the purple color on Niven medium and the modified method with regular Niven medium, it can be concluded that the intensity is the same for the 18th hour observation, the only difference being that in modified Niven, the colonies are more separated compared to colonies growing on regular Niven medium. From these observations, it is known that modified Niven medium has the same ability as regular Niven agar medium to grow BPH. However, on the modified Niven medium, only bacterial colonies that can pass through the second membrane filter can grow, so it can be concluded that this method is selective.

Table 4. Colony color of BPH isolates on modified Niven and Tao media

Isolat	Modified Niven	Tao
E. aerogenes	Transparent colony, purple-colored	Bluish-green colony
H. paralvei	Purple hallo with a diameter of up to 6.2 cm	Greenish brown
M. morganii	Purple cologne, purple ink	Bluish-green colony
Klebsiella pneumonia	Transparent colony	Transparent colony
K. oxytoca	Purple colony	Green Colony
P. mirabilis	Bluish-purple colony	Greenish-brown cologne

The growth of HAP in tuna on Modified Niven medium is presented in Table 5. The density of M. morganii and E. aerogenes isolates used were 2.05 x 10^10 colonies/ml each. The ALT calculation results of sterilized tuna samples showed that no other bacteria grew in the samples, so the colonies that grew on the modified Niven medium were the HAP colonies intentionally added to the product. The growth of both bacteria followed the same pattern, with an increase in bacterial count at hour 18 and a decrease at hour 24. There was a difference between the number of bacteria contaminated on sterile tuna and the number of bacteria that grew on the tuna meat. This was likely caused by suboptimal absorption of the bacteria into the meat (soaking time, isolate solution, and its amount).

Table 5. BPH growth in tuna analyzed using modified Niven medium

Treatment	Number of BPH (log CFU/g) after incubation for		
	10 hours	18 hours	24 hours
Tuna + *E. aerogenes*	5.88 ± 0.71	8.00 ± 1.78	6.97 ± 0.37
Tuna + *M. morganii*	6.17 ± 0.59	7.38 ± 1.37	6.76 ± 0.29

Table 5 shows that with Niven media, this modified method can already detect the presence of BPH in tuna during a shorter incubation period, so the use of Niven media with this modified method can be recommended to companies because it can show results in a short amount of time. In tuna with E. aerogenes at a 10-hour incubation period, the estimated histamine produced is 89.628 ppm.

IV. CONCLUSION

Niven and Tao media are equally effective in supporting BPH growth. In Niven medium using the non-filtration method, new bacterial colonies were visible after 24 hours of incubation. In Tao medium, colonies started to appear after 5 hours of incubation, but growth was slow. Meanwhile, in Niven medium with the modified method (with the addition of Tao filtration), bacterial colonies became visible after 10 hours of incubation, and growth was rapid. It can be concluded that Niven medium with added filtration, as in the Tao method, results in selected BPH growth that can be observed after 10 hours of incubation (faster than in regular Niven medium). Modified Niven medium can be used in the company because it supports quality control in monitoring the number of BPH in tuna.

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