The Effect of Coconut Shell Liquid Smoke in Commercial Feed on Total Bacteria of *Pseudomonas Aeruginosa* in the Tilapia’s Kidney (*Oreochromis niloticus*)

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**Abstract:** The cultivation of Tilapia (*Oreochromis niloticus*) has the prospect to be developed with high economic value. One of the obstacles in tilapia cultivation is the attack of *Pseudomonas aeruginosa*. One of the effort to prevent bacteria disease which do not cause a negative impact on fish, the environment, or consumers that is the use of natural materials such as the utilization of coconut shell that has been processed into liquid smoke. Liquid smoke of coconut shell have bioactive compounds such as phenol, carbonyl and organic acids that act as antibacterials. The result of this research shows the effect of giving of liquid smoke in commercial feed for 21 days maintenance can give effect to total *Pseudomonas aeruginosa* on Tilapia fish kidney organ. 1.5% concentration in commercial feed is an effective concentration for the total decrease of *Pseudomonas aeruginosa* in tilapia kidney organ. Total Plate Count (TPC) value at concentration of 1.5% is $1.80 \times 10^4$ CFU/g.

**Keywords:** Coconut Shell Liquid Smoke, *P. aeruginosa*, Tilapia.

1. Introduction

In the era of industrialization, aquaculture is growing fast to meet food consumption of high nutritional value. Tilapia cultivation (*Oreochromis niloticus*) offers to develop the prospect of cultivation into high economic value. The production of tilapia aquaculture in Indonesia has increased significantly year after year up to 914.78 thousand tons in 2013 to 999.69 thousand tons in 2014 and 1.084 million tons in 2015 (Ministry of Maritime Affairs and Fisheries, 2017).

The increasing demand for tilapia has resulted in farmers applying intensive cultivation systems. According to Arini et al. (2013), intensive cultivation with high stocking densities and high amounts of feed will impact on a declining water quality due to an increasing level of waste from the remaining feed and feces. The declining water quality may lead to disease problem in aquaculture fish. The disease that attacks fish may be of bacteria, parasites, fungi, and viruses. Supriyadi (2009) explained that the higher the use of an intensive system of fish farming is therefore the higher the prevalence of infections against bacteria will be.

According to Saad et al. (2014), one of the bacteria that can attack tilapia is *Pseudomonas aeruginosa*. *Pseudomonas* attacks young and adult tilapia and it is usually found in tilapia’s kidney (Lubis et al., 2014). The prevalence of *Pseudomonas aeruginosa* found in tilapia kidney organs is 30% (Elissa et al., 2010). Fish kidney is a main organ in fish excretion system and it also function as lymphomyeloids which plays a role in the formation the blood cells (Susantie and Manurung, 2017). The clinical symptom of fish infested by *Pseudomonas aeruginosa* is septicemia. Septicemia is a systemic disease caused by bacteria in the blood (Elissa et al., 2010).

Problems caused by bacterial on fish, generally can be overcome with antibiotics (Ogawara, 1981).
Krisnaningsih et al. (2005) argued that the main cause of antibiotic resistance is ill-advised or overdose use. Actions taken to prevent bacterial diseases in order not to have a negative impact on fish, the environment, or consumers, namely through the use of natural ingredients. Coconut shell waste processed to liquid smoke can be used as an antibacterial (Yulia and Prayitno, 2016). According to Darmadji (2006), liquid smoke is proven to suppress the growth of pathogenic bacteria such as Escherichia coli, Bacillus subtilis, Pseudomonas sp. and Salmonella sp.

Based on the above background, coconut shell liquid smoke contains phenol and organic acids which can be used as an antibacterial against Pseudomonas aeruginosa. This study was conducted to determine whether the supply of coconut shell liquid smoke in commercial feed influences the amount of Pseudomonas aeruginosa in tilapia kidney (Oreochromis niloticus) and to determine the effective percentage of liquid smoke concentration in commercial feed in reducing the amount of Pseudomonas aeruginosa in tilapia kidney (Oreochromis niloticus).

2. Research methodology

2.1 Location and time of the research

This research was conducted in May - July 2018 inside the Laboratory of Fish Anatomy and Cultivation as well as Microbiology Laboratory and Fish Disease Analyst of Faculty of Fisheries and Marine Airlangga University Surabaya.

2.2 Research tools and materials

This research used 20 aquariums of 50 x 30 x 30 cm³, aerators, 20 pieces of aeration stone, aeration hose, vacuum hose, bucket, reservoir, DO meter, ammonia test kit, pH paper, and thermometer. Other equipment included plastic, syringe, measuring cup, beaker glass, erlenmeyer, analytic balance (Ohaus Scout-Pro), spatula, heater and magnetic stirrer (AM4 VUP Scientifica), autoclave (Hirayama, Japan), oven, volume pipettes, cotton, petri dishes, mortar and pestle, vortex (VM-1000), test tubes, test tube racks, laminary flow, incubators, driveways, microtubes, micropipets, turbidimeters, centrifuges, trays, and sectional sets.

The materials used were tilapia (Oreochromis niloticus) of 1.5 months, with body length ranged from 7 to 9 cm and body weight ranged from 5-9 grams, commercial feed, coconut shell liquid smoke, Pseudomonas aeruginosa ATCC 27853 bacteria, Trypticase Soya Agar (TSA) media, Trypticase Soya Broth (TSB) media, Pseudomonas Cetrimide Agar (OXOID) media, glycerin, aquades, physiological NaCl 0.9%, and alcohol 70%.

2.3 Sterilizing tools and materials

The glass tools were sterilized with autoclave. First, they were washed with fresh water, they dried, and finally wrapped with wrapping paper. Next, they were put into an autoclave, and the autoclave was operated with a temperature of 121° C and underwent a pressure of one atmospheric for 15 minutes. After the process was completed, the tools were removed from the autoclave and stored in a sterile container (Sudarno et al., 2016).

2.4 Mixing commercial feed with liquid smoke

This study used pellet-shaped commercial as the test feed and the pellet size was adjusted according to the size of fish mouth opening. The liquid smoke used in this study was grade 1 coconut shell liquid smoke (product of Madaniah from Yogyakarta). Before the feed was given to the tilapia, it was mixed with liquid smoke. The feed was first crushed into flour form and then mixed with liquid smoke with concentrations of 1%, 1.5% and 2% (Sasongko et al., 2014). We used cup to measure the liquid smoke based on the concentration used i.e. 10 ml/kg of feed, 15 ml/kg of feed, and 20 ml/kg of feed. After mixing the feed and the liquid smoke, it was then impressed until it become pellets of 2-3 mm.

2.5 The preparation of tilapia cultivation

The research preparation included preparation of aquariums and media water for maintenance. The
The aquarium used in the study was first sterilized with 400 ppm chlorine and then rinsed with clean water and finally dried (BBL, 2003). The maintenance media used in this study was fresh water. Fresh water was put into 20 aquariums. Each aquarium was filled with 25 liters of water.

The tilapia used for this study came from the Center for Development of Freshwater Fish Cultivation (BPBAT) Umbulan, Pasuruan, East Java. They were placed in an aquarium, undergone five treatments and each treatment used four replications. Each aquarium was filled with 10 fish. Before the research was conducted, the fish was put in an adaption for five days so they were to adapt the new environment and to the test feed. After the adaptation period was over, the fish underwent fasting for 24 hours with the goal of eliminating the effect of the remaining food in the fish's body.

2.6 The preparation of pseudomonas aeruginosa bacteria culture

The bacteria used in this study was *Pseudomonas aeruginosa* ATCC 27853 taken from the Testing Service Unit of the Faculty of Pharmacy, Airlangga University, Surabaya. The first step in preparing the bacterial culture of *Pseudomonas aeruginosa* was to make media *Trypticase Soya Broth* (TSB) by weighing the TSB reagent according to the dosage indicated on the product packaging and dissolving it with distilled water. Next we sterilized it using autoclave at 121°C for 15 minutes. The bacterial culture media prepared was then planted by bacteria from pure culture to the TSB media. Afterwards, it was incubated for 18-24 hours at room temperature (28-30°C).

*Pseudomonas aeruginosa* culture on *Trypticase Soya Broth* media that has grown was then centrifuged at 3000 rpm for 20 minutes (Hossain et al., 2006). The supernatant was then removed and added with a physiological Nacl solution of 0.9% until the volume was the same as the initial volume, then it was *divortexed* until it became homogeneous and later *d reincentrifuged*. This was done twice. After washing with 0.9% physiological Nacl, the bacterial density was calculated using turbidimeters (Kelley et al., 2014). The density of *Pseudomonas aeruginosa* used in this study was 2 x 10^7 CFU/ml (Hossain et al., 2006). If the density of bacteria obtained was too dense, we would do a dilution using the following formula (Petrucci et al., 2007).

\[
V_1 \cdot N_1 = V_2 \cdot N_2
\]

Description:

- \(V_1\) = volume of bacterial suspense in the required media
- \(V_2\) = desired volume
- \(N_1\) = Density of bacterial population in media (cell/ml)
- \(N_2\) = The density of the desired bacterial population (cell/ml)

2.7 Injecting pseudomonas aeruginosa bacteria into tilapia

Tilapia that has undergone fasting, were then injected with *Pseudomonas aeruginosa* with a density of 2 x 10^7 CFU/ml (Hossain et al., 2006). 0.2 ml of *Pseudomonas aeruginosa* suspension was injected intraperitoneally with a 1 ml syringe into the tilapia. The injection was carried out slowly in the intraperitoneal section and closed the eyes of the tilapia with a clean cloth wet condition to avoid the tilapia becoming stressful after the injection (Hardi et al., 2014). The injected tilapia were then put into the aquarium.

2.8 Tilapia cultivation

The breeding of tilapia was carried out for 21 days (Saad et al., 2014). Tilapia was placed in an aquarium with the size of 50 x 30 x 30 cm3 with a volume of 25 liters/aquarium. Each aquarium filled with 10 tilapia injected with *Pseudomonas aeruginosa*. Throughout the breeding, the water quality was well controlled.

Control of water quality included thinning of the remaining food waste and metabolism in the aquarium which was done daily and we changed the water every three days. Measurement and recording of water quality was carried out daily in the morning and evening, including checking on water pH and
temperature. Solubility of oxygen and ammonia was measured every seven days.

Weighing the test fish was conducted every seven days to determine the amount of feed to be given and the growth of test fish. The food was given twice a day as much as 5% of the total body weight of the test fish. Feeding was given twice per day at 09.00 and 16.00 WIB.

2.9 Sampling

We took the sample of tilapias (*Oreochromis niloticus*) from the aquarium and dissected them aseptically for their kidney. Next, the tilapia’s kidney were mashed using mortar and pestle. Afterwards, we weighed them until it reached 0.1 gram and then dissolved them in 0.9 ml physiological NaCl 0.9%.

2.10 Calculation the total plate count (TPC)

Calculation of Total Plate Count (TPC) was carried out aseptically in the laminar flow. The samples which have been dissolved in 0.9% physiological NaCl was taken 100 pl and diluted in multilevel (dilution 10 "1 to 10" 4). Next we took 100 pl from the dilution 10 "2, 10” 3, 10 "4 using the duplo spread plate method on the media of Pseudomonas Cetrimide Agar (OXOID). The composition of the media Pseudomonas Cetrimide Agar (OXOID) was 20% peptone gelatin, magnesium chloride 1.4%, 10% potassium sulphate, cetrimide 0.3%, and agar 13.6%. Next, they were incubated for 18-24 hours at 30° C. Interpretations and calculations of Pseudomonas aeruginosa colonies on petri dishes was conducted based on SNI 01-2332.3-2006.

2.11 Data analysis

Data from the total calculation of Pseudomonas aeruginosa on tilapia kidney using the Total Plate Counter (TPC) method was analyzed using a statistical calculation method of Variance Analysis ANOVA. If the result was significantly different, we should further test them using Duncan's advanced test (Duncan's multiple range test) in SPSS program (Statistical Program Software System) (Kusriningrum, 2010).

| Treatment | TPC (x 10^4 cfu/g) ± SD |
|-----------|-------------------------|
| K1        | 0,00 ± 0,00000          |
| K2        | 5,28 ± 0,02258          |
| P1        | 3,69 ± 0,03138          |
| P2        | 1,80 ± 0,05534          |
| P3        | 1,79 ± 0,08249          |

3. Result and discussion

3.1 Total Plate Count (TPC)

Tabel 1. Total Plate Count (TPC) the bacterium Pseudomonas aeruginosa in tilapias’ kidney (*Oreochromis niloticus*) (CFU/g).

The statistical analysis results of variance analysis (ANAVA) showed that the administration of coconut shell liquid smoke in commercial feed with different concentrations suggested significantly different effect (p <0.05). The treatments of K1 and K2 were negative control and positive control and they were used as a comparison between treatments P1, P2, and P3. The results from Duncan's multiple distance test showed that, when compared with the control treatment, treatment P3 was the best treatment. However, treatment P3 was not significantly different (p > 0.05) to that of treatment P2. The result of P1 treatment showed that P1 treatment was the worst treatment. Result of P1 treatment was significantly different (p <0.05) to those of treatment P2 and P3.
The total decline in bacteria occurred when the feed with coconut shell liquid smoke interacted with the tissues/organ in the fish body; the compound in coconut shell liquid smoke the fish needed has been infected by Pseudomonas aeruginosa even in a small amount, the important substances to fish such as phenols and acidic compounds will be absorbed by their tissues/organs. Fish has an immune response system that works as a defense mechanism against attacks from bacteria pathogens. Fish kidney functions as lymphomyeloid system capable of inducing its immune response (Alifuddin, 2002). According to Pasaribu and Wina (2017), the mechanism of liquid smoke shall inhibit the growth of bacteria by breaking the cytoplasm, DNA or cell nucleus from bacteria. Phenol with its -OH group can dissolve the lipids on its cell wall, consequently disrupting the performance of cytoplasmic membrane and inhibiting ATP-ase bonds that cause lysis. As the result, the bacterial growth is inhibited.

3.2 Tilapia specific growth rate

| Treatment                  | SGR± SD         |
|----------------------------|-----------------|
| K1                         | 2.0325± 0.11396 |
| Tilapia Specific Growth     |                 |
| P2                         | 1.8940± 0.18354 |
| P3                         | 1.7077± 0.18354 |

Note: K1 = negative control treatment; K2 = positive control; P1 = ACTK 1%; P2 = ACTK 1.5%; P3 = ACTK 2%; Letter notation superscript which is different in one column shows there are significant differences (p<0.05).

The results of statistical analysis of specific growth rate data indicate that the administration of liquid smoke in commercial feed with different concentrations showed a significant difference (p < 0.05). The results from Duncan's distance indicate that a good specific growth rate is P2 treatment. The value of a low specific growth rate is treatment P1. P2 treatment when compared with treatments P1 and P3, the results of the statistical analysis showed that there were significant differences between treatments (p < 0.05). The high value of specific specific growth rate in P2 treatment (compared to P1 and P3) shows that commercial feed added with liquid smoke with a concentration of 1.5% can stimulate fish growth and the specific growth rate of weight is not significantly different from treatment K1 (negative control), whereas Statistical analysis showed that P1 treatment was significantly different from P3 treatment. P1 and P3 treatments were significantly different from K2 treatment (positive control).

The increase in the specific growth rate of tilapia proves that the addition of coconut shell liquid smoke in commercial feed with different concentrations can influence the rate of growth of fish. According to research by Wang et al. (2012) showed that the use of liquid smoke can be used as additives in livestock, besides that according to Yamauchi et al. (2010) reported that liquid smoke given to livestock can function as a natural antibiotic. Such conditions are basically inseparable from the role of chemical compounds contained in coconut shell liquid smoke so that the performance of livestock production is increasing. Liquid smoke contains acidic compounds such as lactic acid and butyrate, both types of acids are very necessary to optimize the metabolic process of nutrients in the digestive tract (Sari et al., 2014).

3.3 Water quality

Water quality is the most important factor in fish farming because it is one of the supporting factors for fish growth. Inadequate or poor water quality may cause the fish become stressful and they may catch a disease. Control of water quality needs to be done by way of conducting Shift Pond, changing the water, and measuring water quality parameter which include its temperature, pH, dissolved oxygen
(DO), and ammonia levels.

The study used a thermometer to measure the temperature. The water temperature ranged from 27°C to 29.4°C, which was good temperature for aquaculture water based on SNI 7550 (2009) which states that the optimum water temperature for tilapia maintenance ranges from 25°C to 30°C.

The pH measurement in this study was carried out using a pH meter and the result was 7. This result was good for aquaculture water based on SNI 7550 (2009) which states that the optimum pH in tilapia enlargement ponds is between 6.5 and 8.5.

This study used a DO meter for its DO measurement. The result ranged from 3.99 to 5.96 mg/l. The result for both aquaculture water were within the optimum range based on SNI 7550 (2009) i.e. the optimum DO in tilapia enlarges ponds ranges around >3 mg/l.

The measurements of ammonia level measurements in this study were carried out using ammonia test kit. The results of ammonia measurements ranged from 0 to 0.5 mg/l. This range was sufficient for aquaculture waters of the yield according to Centyana et al. (2014) which stated that an optimum ammonia in tilapia water shall not exceed 0.5 mg/l. In contrast, SNI 7550 (2009) states that an optimum ammonia in tilapia enlargement ponds is around <0.02 mg/l. The difference in results was due to the use of ammonia kit tests in ammonia measurements hence the results showed less accurate. Based on the measurement result of water quality for 21 days, the breeding period of tilapia (Oreocromis niloticus) showed that the condition of waters in the breeding media was normal and did not show any differences in the required water quality for tilapia cultivation.

4. Conclusion and suggestion

Based on the results of our research regarding the effect of coconut shell liquid smoke on the all Pseudomonas aeruginosa bacteria in tilapia kidney (Oreochromis niloticus), we concluded that the application of coconut shell liquid smoke in commercial feed had an impact on all Pseudomonas aeruginosa in tilapia’s kidney (Oreochromis niloticus) and the effective concentration of coconut shell liquid smoke in commercial feed against the total reduction of Pseudomonas aeruginosa in tilapia kidney (Oreochromis niloticus) was 1.5% with a total plate count (TPC) of 1.80 x 104 CFU/g.

This study suggests to have further research on the provision of liquid smoke in commercial feed with different concentrations in the breeding of tilapia.

5. References

[1] Alifuddin, M. 2002. Immunostimulan Pada Hewan Akuatik. Jurnal Akuakultur Indonesia, 1(2) : 87-92.

[2] Arini, E., dan R. R. Diasrari. 2013. Pengaruh Kepadatan yang Berbeda terhadap Kelulushidupan dan Pertumbuhan Ikan Nila (Cyprinus carpio) pada Sistem Resirkulasi dengan Filter Zeolit. Journal of Aquaculture Management and Technology, 2(3) : 37-45.

[3] Balai Budidaya Laut (BBL). 2003. Penanganan Penyakit Ikan Budidaya Laut. ISBN : 979-98017-1-0, No 12. Lampung. Hal 24.

[4] Darmadji, P. 2006. Asap Cair Pengawet Aman. Tabloid. Dwi mingguan, Vol 1. No. 22. Agrina. Jakarta. 11 hal.

[5] Elissa, N. M. E., E. N. Abou El-Ghiet, A. A. Shaheen, and A. Abbass. 2010. Characterization of Pseudomonas Species Isolated from Tilapia “Oreochromis niloticus” in Qoroun and Wadi-El-Rayen Lakes, Egypt. Journal Global Veterinaria, 5 (2) : 116-121.

[6] Hardi, E. H., C. A. Pebrianto, G. Saptiani. 2014. Toksisitas Produk Ekstraseluler dan Intraseluler Bakteri Pseudomonas sp. pada Ikan Nila (Oreochromis niloticus). Jurnal Veteriner, 15(3) : 312-322.

[7] Kelley, C. D., A. Krolick, L. Brunner, A. Bruklund, D. Khan, W. P. Ball, and M. W. Shirk. 2014. An Affordable Open-Source Turbidimeter. Journal Sensors, 14 (1) : 7142-7155.

[8] Kementerian Kelautan dan Perikanan. 2017. Tilapia Jadi Komoditas Unggulan Perikanan Budiday.

[9] Kementerian Kelautan dan Perikanan. 2017. Tilapia Jadi Komoditas Unggulan Perikanan Budiday.
http://news.kkp.go.id/index.php/tilapia-iadi - komoditas-unggulan-perikanan-budidaya/. 28 November 2017.

[9] Krisnaningsih, M., W. Asmara, dan M. Wibowo. 2005. Uji Sensitivitas Isolat Escherichia coli Patogen pada Ayam terhadap Beberapa Jenis Antibiotik. Jurnal Sain Veteriner, 23(1) : 13-18.

[10] Lubis, D. A., H. Syawal, M. Riauwaty. 2014. Identifikasi Bakteri Patogen Pada Ikan Nila (Oreochromis niloticus) di Kecamatan Marpoyan Damai Kota Pekanbaru. Universitas Riau. 8 hlm.

[11] Ogawara, H. 1981. Antibiotic Resistance in Pathogenic and Producing Bacteria with Special Reference to Beta Lactam Antibiotics. Journal Microbiological Reviews, 45 (4) : 591-619.

[12] Pasaribu, T., Wina E. 2017. Komparasi Aktivitas Tiga Jenis Asap Cair terhadap Pertumbuhan Mikroba secara In Vitro. Prosiding Seminar Nasional Teknologi Peternakan dan Veteriner. Bogor. Hal 679-685.

[13] Petrucci, R. H., W. S. Harwood, F. G. Herring and J. D. Madura. 2007. Kimia Dasar Prinsip-Prinsip dan Aplikasi Modern. Terjemahan : S. S. Achmadi. Penerbit Erlangga. Jakarta. Hal 119-120.

[14] Saad, S. A., Ketkat and F. A. Mohammed. 2014. Changes Assosiated with Pseudomonas Infection in Cultured Oreochromis Spesies and its Relations to Economic Losses of Fish Production Farms. Journal Agriculture veterinary, 1(3): 127-137.

[15] Sasongko, P., W. Mushollaeni dan Hermawan. 2014. Aktivitas Antibakteri Asap Cair dari Limbah Tempurug Kelapa terhadap Daging Kelinci Asap. Jurnal Buana Sains, 14 (2) : 193-197.

[16] Standar Nasional Indonesia (SNI). 2006. Cara Uji Mikrobiologi - Bagian 3 : Penentuan angka lempeng total (ALT) pada produk perikanan. Badan Standarasisi Nasional ICS 67 050. Jakarta. 11 hal.

[17] Standar Nasional Indonesia (SNI). 2009. Produksi Ikan Nila (Oreochromis niloticus, Bleeker). Kelas Pembesaran di Kolam Air Tenang. Badan Standarisasi Nasional ICS 61 250. Jakarta. 8 hal.

[18] Sudarno, R. Kusdarwati, Rozi, D. D. Nindarwati, dan L. A. Sari. 2016. Petunjuk Praktikum Mikrobiologi. Fakultas Perikanan dan Kelautan Universitas Airlangga Surabaya. Surabaya. Hal 17-36.

[19] Supriyadi dan Bastiaawan, D. 2009. Penyebaran Penyakit Streptococciasis pada Pusat Budidaya Ikan Air Tawar. Proseding Seminar Pengendalian Penyakit Udang IV di Purwokerto. Hal 168-172.

[20] Susantie, D., dan U. N. Manurung. 2017. Identifikasi Bakteri Patogen pada Ikan Nila (Oreochromis niloticus) di Lokasi Budidaya Ikan Air Tawar Kabupaten Sangihe. Jurnal Budidaya Perairan, 5(03): 11-17.

[21] Wang, H. F., C. Wang, W. M. Wang, J. X. Zhang, J. X. Liu, & B. Dai. 2012. Effect of Bamboo Vinegar as an antibiotic Alternative on Growth Performance and Fecal Bacterial Communities of Weaned Piglets. Journal Livestock Science, 144:173-180.

[22] Yamauchi, K., J. Ruttanavut, and S. Takenoyama. 2010. Effects of dietary bamboo charcoal powder including vinegar liquid on chicken performance and histological alterations of intestine. Journal of Animal and Feed Science, 19 : 257-268.

[23] Yulia dan Prayitno. 2016. Efektifitas Konsentrasi Asap Cair (LiquidSmoke) Dari Tempurung Kelapa Terhadap Angka Kuman Pada Tahu. Jurnal Vokasi Kesehatan, 2(2):173-177.