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Oxidation of Hydrogen sulfide, Ammonia nitrogen and Nitrite nitrogen by *Bacillus* sp. Isolated from West Coast of Korea

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서해안에서 분리한 *Bacillus* sp.의 황화수소, 암모니아 질소, 아질산 질소에 대한 산화능

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Abstract

In the aquaculture industry, the biological treatment using probiotics for eliminating hydrogen sulfide, ammonia nitrogen and nitrite nitrogen is receiving attention. In this study, *Bacillus* sp. TO-3, *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12 were isolated from the west coast of Korea and oxidation ability of hydrogen sulfide, ammonia nitrogen and nitrite nitrogen were analyzed. *Bacillus* sp. TO-10 had high oxidation toward hydrogen sulfide by oxidizing 100% and *Bacillus* sp. TO-3 and TO-12 each had hydrogen sulfide oxidation of 49.53% and 69.03%. *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12 had ammonia nitrogen oxidation approximately of 32.7 mg/L, while *Bacillus* sp. TO-3 had 11.4 mg/L of ammonia nitrogen oxidation. *Bacillus* sp. TO-3, *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12 all had high oxidation toward nitrite nitrogen by oxidizing 100% of 137.46 mg/L nitrite nitrogen. Therefore, *Bacillus* sp. TO-3, *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12 are possible probiotics used as water quality improvement of eliminating hydrogen sulfide, ammonia nitrogen and nitrite nitrogen.

Key words : *Bacillus*, Oxidation, Hydrogen sulfide, Ammonia nitrogen, Nitrite nitrogen

Ⅰ. Introduction

The modern aquaculture high rearing density for mass produce and low intensity flow tank system structure causes high concentration of nutrients such as nitrogen (N), phosphorus (P) and specific organic, resulting poor water quality in aquaculture environment (Kang and Yoon, 2004; Piedrahita, 2003). The sediments of metabolic wastes from fish and uneaten feed in the tank cause consumption of dissolved oxygen (DO) and the nutrient anaerobic decomposition by the bacteria occur, hydrogen sulfide (H_2S), ammonia nitrogen (NH_3-N) and nitrite nitrogen (NO_2-N) (Handy and Poxton, 1993; Kioussis et al., 2000).

Hydrogen sulfide occurs when various bacteria decompose nutrient sediments in anaerobic environment using electron acceptor such as sulfate (SO_2-4) (Kim et al., 2011). Hydrogen sulfide is toxic to cultured fish by decreasing hatching rates and killing fry causing an impact on productivity

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(Smith et al., 1976; Beauchamp et al., 1984).

Ammonia nitrogen (NH_3-N) exists as dissolved ammonia (NH_3) and ammoniumion (NH_4^+) in water (Zhou and Boyd, 2016). Ammonia (NH_3) and ammoniumion (NH_4^+) are toxic to cultured fish, especially high concentration of un-ionized ammonia damage the fish gill, decreasing oxygen transportability in blood and decreasing osmoregulation by damaging the erythrocyte and tissue (Tomasso, 1994; Romano and Zeng, 2013).

Nitrite nitrogen (NO_2-N) occurs as an intermediate product of ammonia and nitric acid when ammonia is oxidized by bacteria. Nitrite nitrogen is toxic to cultured fish by combining with the fish erythrocyte, reducing the oxygen transport ability. Therefore, death by asphyxia happen even when air supply is enough in the aquaculture tank. Nitrite nitrogen poisoning doesn't have external symptoms while progressing into chronic. (Russoet al., 1974; Brown and McLeay, 1975).

Aquaculture farms use chemical and physical treatment to eliminate hydrogen sulfide, ammonia nitrogen and nitrite nitrogen. However the chemical treatment occurs secondary containment and the physical treatment is expensive (Gadre, 1989; Mook et al., 2012). Therefore, biological treatment using probiotics is receiving attention in aquaculture for the purpose of water quality improvement. Probiotics are beneficial microorganism to organism. Especially a gram positive strain *Bacillus* sp. is an effective probiotics for water quality improvement (Verschuere et al., 2000; Sahu et al., 2008).

In this study, *Bacillus* sp. TO-3, *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12 strains were isolated from the west coast of Korea and oxidation ability of hydrogen sulfide, ammonia nitrogen and nitrite nitrogen were analyzed for development purpose of water quality improvement probiotics.

Ⅱ. Material and methods

1. Strains isolation and identification

TO-3, TO-10 and TO-12 strains were isolated from the west coast of Korea. The water samples were spread in a mineral salt medium appeared in Table 1 and cultured at 35℃ for 48 hours. Strains which have high oxidation of hydrogen sulfide, ammonia nitrogen and nitrite nitrogen were selected and isolated. The selected strains were identified by 16s rRNA gene from Solgent Co., Ltd (Daejeon, Korea).

2. Hydrogen sulfide oxidation analysis

The component of the mineral salts medium used in the hydrogen sulfide oxidation analysis is in <Table 1>. The mineral salts medium was made into anaerobic condition by injecting nitrogen (N) gas for 30 minutes and was autoclaved at 121℃ for 20 minutes. Sodium thiosulfatepentahydrate ($Na_2S_2O_3 \cdot 5H_2O$) 8 g/L was separately made and after making into anaerobic condition and autoclaved, it was combined with the mineral salts medium. The isolated strains TO-3, TO-10 and TO-12 were anaerobic cultured in the mineral salts medium at 35℃ for 24 hours. The cultured medium was collected at 0 hour and 24 hour and was

<Table 1> Mineral salt medium component

Component	Concentration (g/L)
$(NH_4)_2SO_4$	3.0
KH_2PO_4	0.1
$MgSO_4 \cdot 7H_2O$	0.5
$CaCl_2 \cdot 2H_2O$	0.3
$FeSO_4 \cdot 7H_2O$	0.01
NaCl	19.45

centrifuged at 10000 rpm for 5 minutes. The supernatant was used as sample. The hydrogen sulfide concentration was analyzed by the methylene blue method from marine environment official test methods (Ministry of Oceans and Fisheries, 2013). Sample 500 μL was mixed with diamine solution 10 μL and left at room temperature for 20 minutes. Absorbance was measured at 670 nm using microplate spectrophotometer (BioTek Instruments, USA). Sodium sulfide nonahydrate ($NaS{\cdot}9H_2O$) was used as a standard and the sample concentration of hydrogen sulfide (mg S/L) was measured using a standard curve.

3. Ammonia nitrogen oxidation analysis

LB broth containing ammonia sulfate ($(NH_4)_2SO_4$) 0.1 g/L was used as medium for ammonia nitrogen oxidation analysis. Isolated strains TO-3, TO-10 and TO-12 were cultured in the medium at 35℃ for 24 hours. The cultured medium was collected at 0, 6, 12, 24 hour and was centrifuged at 10000 rpm for 5 minutes. The supernatant was separated and used as sample. The ammonia nitrogen concentration was analyzed by indophenol method from marine environment official test methods (Ministry of Oceans and Fisheries, 2013). Sample 2.5 mL was mixed with phenol solution 100 μL and nitroprussidesodium solution 100 μL. Oxidation solution 250 μL was added and was developed at 35℃ water bath for 30 minutes. Absorbance was measured at 640 nm using microplate spectrophotometer (BioTek Instruments, USA). Ammonia sulfate ($(NH_4)_2SO_4$) was used as a standard and the sample concentration of ammonia nitrogen (mg/L) was measured using a standard curve.

4. Nitrite nitrogen oxidation analysis

LB broth containing sodium nitrite ($NaNO_2$) 0.1 g/L was used as medium for nitrite nitrogen oxidation analysis. Isolated strains TO-3, TO-10 and TO-12 were cultured in the medium at 35℃ for 24 hours. The cultured medium was collected at 0, 6, 9, 12 hour and was centrifuge at 10000 rpm for 5 minutes. The supernatant was separated and used as sample. The nitrite nitrogen concentration was analyzed by diazotization method from marine environment official test methods (Ministry of Oceans and Fisheries, 2013). Sample 5 mL was mixed with sulfanilamide solution 100 μL and left at room temperature for 5 minutes. Napeutiretillendiamin solution 100 μL was mixed and left at room temperature for 20 minutes. Absorbance was measured at 540 nm using microplate spectrophotometer (BioTek Instruments, USA). Sodium nitrite ($NaNO_2$) was used as standard and the sample concentration of nitrite nitrogen (mg/L) was measured using a standard curve.

5. Heat resistant analysis

Isolated strains TO-3, TO-10 and TO-12 were each cultured in LB broth at 35℃ for 24 hours. Each cultured mediums were incubated in 50℃, 60℃, 70℃ water bath for 15 minutes and were spread at LB agar and incubate at 35℃ for 18 hours. The strains survival rate was analyzed by calculating the CFU/mL.

Ⅲ. Result and Discussion

1. Isolated strains identification

The identification result of isolated strains from the west coast of Korea is shown in <Table 2>. Nineteen strains were isolated from the water samples

<Table 2> Identification of *Bacillus* sp. strains using 16s rRNA sequencing

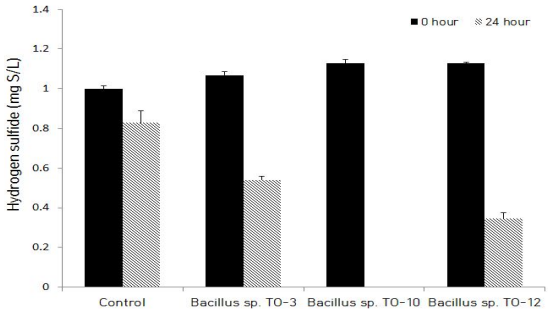
Microorganism	Identified strains	Identity		
		Match	Total	Pct(%)
TO-3	*B. amyloliquefaciens*	1476	1480	99
TO-10	*B. licheniformis*	1476	1480	99
TO-12	*B. subtilis*	1452	1456	99

and three strains which have high oxidation of hydrogen sulfide, ammonia nitrogen and nitrite nitrogen were selected. The selected three strains TO-3, TO-10 and TO-12 were each identified as *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus subtilis*. In this study, the three isolated strains would be named as *Bacillus* sp. TO-3, *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12.

2. Hydrogen sulfide oxidation ability

The result of *Bacillus* sp. strains analyzed of hydrogen sulfide oxidation ability is shown in figure 1. *Bacillus* sp. TO-3 oxidized 49.53% of the hydrogen sulfide by reducing 1.07±0.02 mg S/L hydrogen sulfide to 0.54±0.03 mg S/L for 24 hours. *Bacillus* sp. TO-12 oxidized 69.03% of hydrogen sulfide by reducing 1.13±0.04 mg S/L hydrogen sulfide to 0.35±0.03 mg S/L for 24 hours. *Bacillus* sp. TO-10 oxidized 100% of the 1.13±0.02 mg S/L hydrogen sulfide for 24 hours, which stands out to have the most effective hydrogen sulfide oxidation ability between the three *Bacillus* sp. strains.

Hydrogen sulfide have high toxicity toward cultured fish even in small concentration. Exposure of 0.5 mg/L hydrogen sulfide resulted hyperpnea, apnea and finally respiratory arrest (Torrans and Clemens, 1982). Therefore eliminating hydrogen sulfide is important in aquaculture industry. The result of *Bacillus* sp. oxidation ability of hydrogen sulfide also appeared in Nakada and Ohta(1999); Choi et al.(2018). The *Bacillus* sp. TO-3, *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12 appeared to have similar oxidation ability of hydrogen sulfide comparing with Choi et al.(2018).



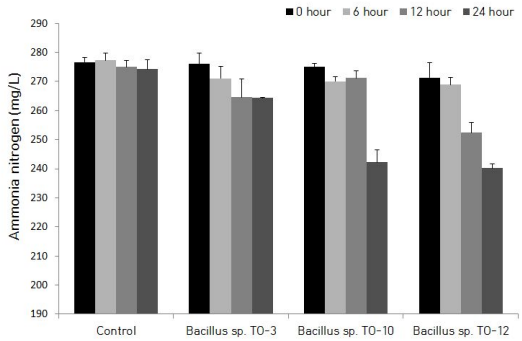
[Fig. 1] Hydrogen sulfide oxidation of *Bacillus* sp. strains.

3. Ammonia nitrogen oxidation ability

The result of *Bacillus* sp. strains analyzed of ammonia nitrogen oxidation ability is shown in figure 2. *Bacillus* sp. TO-3 oxidized 11.4 mg/L ammonia nitrogen by reducing 276±3.67 mg/L ammonia nitrogen to 264±6.33 mg S/L for 12 hours. However after 12 hours ammonia nitrogen concentration didn't appeared reducing. *Bacillus* sp. TO-10 appeared to have oxidation ability after 12 hour. *Bacillus* sp. TO-10 oxidized 32.7 mg/L ammonia nitrogen by reducing 275±1.22 mg/L ammonia nitrogen to 242±4.11 mg/L. *Bacillus* sp. TO-12 oxidized 31 mg/L ammonia nitrogen by reducing 271±5.11 mg/L ammonia nitrogen to

240±1.44 mg/L for 24 hour, which appeared to have similar ammonia nitrogen oxidation ability with *Bacillus* sp. TO-10.

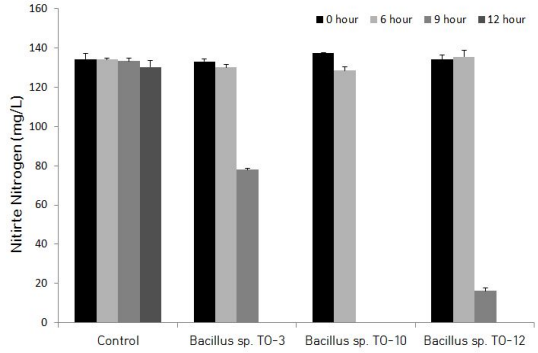
Ammonia nitrogen damage the fish gill, decrease oxygen transportability in blood. Exposure to 40 mg/L ammonia nitrogen caused mortality in cultured fish (Chen and Lei, 1990). Therefore eliminating ammonia nitrogen is important in aquaculture industry. The result of *Bacillus* sp. having oxidation ability of ammonia nitrogen also appeared in Lee et al.(2003). *Bacillus* sp. TO-3, *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12 had a similar ammonia nitrogen oxidation for 24 hours and it is considered the three *Bacillus* sp. strains would continue to oxidize ammonia nitrogen for 48 hours as in Lee et al.(2003).



[Fig. 2] Ammonia nitrogen oxidation of *Bacillus* sp. strains.

4. Nitrite nitrogen oxidation ability

The result of *Bacillus* sp. strains analyzed of nitrite nitrogen oxidation ability is shown in figure 3. *Bacillus* sp. TO-3 oxidized 133.22±1.36 mg/L nitrite nitrogen to 78.05±0.82 mg/L at 9 hour and oxidized 100% of nitrite nitrogen at 12 hour. *Bacillus* sp. TO-10 oxidized 100% of 137.46±0.27 mg/L nitrite nitrogen at 9 hours, resulting the fastest nitrite nitrogen oxidation ability between the three *Bacillus* sp. strains. *Bacillus* sp. TO-12 oxidized 100% of the 134.18±2.18 mg/L nitrite nitrogen at 12 hours.



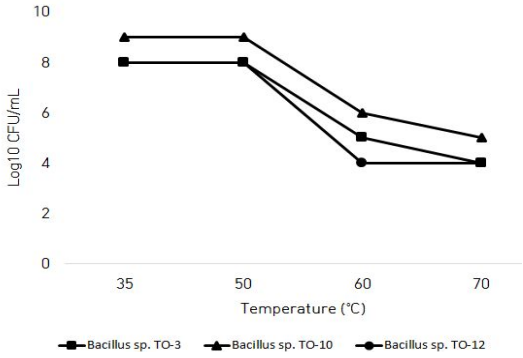
[Fig. 3] Nitrite nitrogen oxidation of *Bacillus* sp. strains.

Nitrite nitrogen is toxic to cultured fish by reducing oxygen transport ability and causing asphyxia. Exposure to 20 mg/L nitrite nitrogen caused mortality in cultured fish (Chen and Lei, 1990). Therefore eliminating nitrite nitrogen is important in aquaculture industry. The result of *Bacillus* sp. having oxidation ability of nitrite nitrogen also appeared in Lee et al.(2003). In this research *Bacillus* sp. oxidized nitrite nitrogen for 48 hours, however nitrite nitrogen still remained. Unlike Lee et al.(2003), *Bacillus* sp. TO-3, *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12 strains had completely oxidized nitrite nitrogen before 12 hours, resulting higher oxidation ability of nitrite nitrogen.

5. Heat resistance

The result of *Bacillus* sp. strains analyzed of heat resistance is shown in figure 4. At 50℃, the three *Bacillus* sp. strains appeared to maintain the same CFU/mL compare with the result at 35℃ which indicates the three *Bacillus* sp. strains have strong heat resistance at 50℃. *Bacillus* sp. TO-3 appeared to have 5 $\log_{10}$ CFU/mL of cell

survivability at 70℃. *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12 appeared to have 4 $\log_{10}$ CFU/mL of cell survivability at 70℃. All three *Bacillus* sp. strains appeared to have high heat resistance at 50℃ and also survive at 70℃. Therefore, the three *Bacillus* sp. strains are able to survive the occurred heat during the process of water quality improvement product.



[Fig. 4] Heat resistance of *Bacillus* sp. strains.

Ⅳ. Conclusion

Three *Bacillus* sp. strains were isolated from the west coast of Korea and oxidation ability of hydrogen sulfide, ammonia nitrogen and nitrite nitrogen were analyzed for development purpose of water quality improvement probiotics. *Bacillus* sp. TO-3, *Bacillus* sp. TO-10 and *Bacillus* sp. TO-12 were each identified as *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus subtilis*. *Bacillus* strains are appropriate and safe probiotics used in aquaculture, known to have effect of increasing fry body weight, digestion ability and reducing the severity of cellular stress (Acella et al., 2010).

Hydrogen sulfide was oxidized each by *Bacillus* sp. TO-3 49.53%, *Bacillus* sp. TO-10 100% and *Bacillus* sp. TO-12 69.03% for 24 hours. Ammonia nitrogen was oxidized each by *Bacillus* sp. TO-3 11.4 mg/L, *Bacillus* sp. TO-10 32.7 mg/L and *Bacillus* sp. TO-12 31 mg/L for 24 hours. All three *Bacillus* sp. strains have oxidized 100% nitrite nitrogen for 12 hours and *Bacillus* sp. TO-10 had the fastest nitrite nitrogen oxidation ability. Due to these results, all three *Bacillus* sp. strains had the oxidation ability toward hydrogen sulfide, ammonia nitrogen and nitrite nitrogen. Above all, *Bacillus* sp. TO-10 had the most effective oxidation ability between the three isolated *Bacillus* strains. All three *Bacillus* sp. strains had strong heat resistance at 50℃ and survived at 70℃. Therefore the three *Bacillus* sp. strains are possible to be used as probiotics for water quality improvement of hydrogen sulfide, ammonia nitrogen and nitrite nitrogen.

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