ISOLATION AND ACTIVITY OF NITRIFYING BACTERIA IN INCREASING THE VIABILITY OF SOIL NITROGEN

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Abstract. Nitrogen is essential nutrient for the growth of organisms and become a limiting factor in soils. Nitrifying bacteria consist of ammonifying and nitrifying bacteria. The former oxidize ammonia into nitrite and the later oxidize nitrite into nitrate. A number of nitrifying bacteria has been isolated from a pond sediment using media of *Nitrosomonas* medium and *Nitrobacter* medium by pour plate method. A total of 7 isolates nitrifying bacteria were found from pond sediment. Two isolates were Gram negative and 5 isolates were Gram positive. The population number of the Gram-negative bacteria was 3 x 103 cfu. mL-1, while the Gram-positive bacteria were ranging from 2.0 x 103 to 5.0 104 cfu. mL-1. The nitrifying bacterial isolates were capable of reducing ammonia concentration in the amount of 0.878-10,261 ppm, increasing nitrite concentration ranging from 0.313-1.358 ppm, and increasing nitrate concentration in the amount of 0.215-0.262 ppm.

Keywords : Nitrogen, Nitrobacter, Nitrosomonas, Nitrifying Bacteria

1. Introduction

Soil fertility is the ability of soil to sustain plant growth by providing essential plant nutrients and favourable chemical, physical, and biological parameters. The roles of bacteria in increasing soil fertility are through nutrient recycling such as carbon, nitrogen, sulfur, and phosphorus, decompose dead organic matter and release simple compounds in the soil, which can be taken up by plants, fix atmospheric nitrogen and increase the nitrogen content of the soil, which can be readily absorbed by plants, they also improve soil structure and increase the water-holding capacity of the soil. The availability of nitrogen in soil naturally derives from atmospheric fixation and nitrification process conducted by microbes.

Nitrification process is an oxidation reaction that usually occurs under aerobic conditions, which serves as an intermediate of oxidized and reduced forms of nitrogen in its cycling [1]. The nitrate produced serves as a nutrient for the growth of microbes and plants, and a substrate for denitrification. This has made it important to environmental sustainability and agricultural intensification. Compounds such as ammonium (NH_4), ammonia (NH_3), hydroxylamine (NH_2OH), nitrous oxide (NO), nitrite (NO_2^-), and nitrate (NO_3^-) are the major forms of nitrogen associated with the process. The soil nitrification process is divided into two major phases which are nitritation and nitratation, and the order of microbial oxidation of ammonia via nitrite to nitrate is sequential [2]. Nitritation is the process of oxidizing ammonia to nitrite, while nitratation phase oxidizes nitrite to nitrate. This process is commonly conducted by several group of nitrifying bacteria and archaea in a complex chemical transformation, and they are affected by several factors, such as total soil nitrogen, pH, synthetic fertilizers, chemical nitrification inhibitors, and other agrochemicals [3]. Nitrifying bacteria convert the most

reduced form of soil nitrogen, ammonia, into its most oxidized form, nitrate, which is critical for soil ecosystem function in controlling losses of soil nitrogen through leaching and denitrification. The nitrification process requires the mediation of two distinct genera of bacteria that convert ammonia to nitrites (*Nitrosomonas, Nitrosospira, Nitrosococcus, and Nitrosolobus*) and bacteria that convert nitrites (toxic to plants) to nitrates (*Nitrobacter, Nitrospina*, and *Nitrococcus*).

Nitrifying bacteria such as *Nitrosomonas* play an important role in providing nitrogen to plants and limiting carbon dioxide fixation [4]. *Nitrosomonas* is a genus of ammonia-oxidizing proteobacteria that are important players in wastewater treatment plants, where they get rid of excess ammonia by converting it to nitrite. *Nitrosomonas* are rod-shaped chemolithoautotrophs with an aerobic metabolism. While they do not grow by photosynthesis, their unusual metabolic behaviour involves burning ammonia with oxygen. Long, thin membranes inside the bacteria's cell use electrons from ammonia's nitrogen atom to produce energy. In order to complete cell division, *Nitrosomonas* must consume vast amounts of ammonia, making the division process last for several days. The cells grow either in pairs or short chains. In nitrification *Nitrosomonas* plays the role of oxidizing ammonia to nitrite, which is then converted to nitrate by other bacteria. The members of the genus *Nitrosomonas* for instance *Nitrosomonas aestuarii, N. communis, N. cryotolerans, N. europaea, N. eutropha, N. halophila, N. marina, N. nitrosa, N. oligotropha*, and *N. ureae*. They are found widely distributed in soil or water, where there are large amounts of ammonia, such as lakes or streams into which treated and untreated sewage is pumped. The objectives of the study were to isolate nitrifying bacteria from pond sediment, to know the ability of the bacterial isolates in the nitrification process.

2. Methods

2.1 Isolation of nitrifying bacteria

A total of 1 g pound sediment was diluted with 9 mL aquades in a tube reaction, and subsequently made a serial dilution until 10-4. Amount of 1 mL suspension each of the 10-3 and 10-4 dilutions was inoculated onto both a selective medium of *Nitrosomonas* (g/L: $(NH_4)_2SO_4$ 2.0, K_2HPO_4 1.0; NaCl 2.0, $MgSO_4$ 0.5, $FeSO_4$ 0.4, $CaCO_3$ 0.01, Phenol red 0.025, Agar 15.0, pH 7) and a selective medium of Nitrobacter (g/L: $NaNO_2$ 0.2, K_2HPO_4 0.5, NaCl 0.5, $MgSO_4$ 0.5, $FeSO_4$ 0.5, Na_2CO_3 1.0, Agar 15.0, pH 7) in duplo by a pour plate method. The plate cultures were incubated at room temperature for 7 days. The number of bacterial colonies growth was counted by using a plate count method. The pure culture was made by taking a loopful of colony and inoculate onto the selective medium by a quadrant streak-plate method. The plates were incubated at room temperature for 48 hours. Each of the growing single colony was then transferred onto slanted *Nitrosomonas* medium for nitritation bacteria and *Nitrobacter* medium for nitratation bacteria as stock cultures. Characterization of bacterial colonies included form, size, color, margin, elevation, and optic.

2.2 Assay of Nitrification Activity of Bacterial Isolates

Each of the 6 mL cultures of isolates SA12, SA14, SA25, SA26, and SA37 (logarithmic phase) was inoculated into 150 mL Nitrosomonas broth medium, and a similar amount of the cultural isolates BA11, BA12, BA26, and BA38 (logarithmic phase) were also inoculated into 150 mL Nitrobacter broth medium. All of the cultures were incubated in a shaker incubator at 120 rpm and 30°C for 12 days. Measurements of ammonia, nitrite, and nitrate concentrations were conducted at incubation times of 0, 2, 4, 6, 8, 10, and 12 days.

A measurement of ammonia concentration (SNI 06-2479-1991) was done by 2 mL of the culture added with 1-2 drops of K-Na Tartrate reagent, and homogenized. An amount of 0.4 mL Nessler reagent was added, and then let sit for 10 min until the solution's color changed to yellow. The absorbance of the solution was then measured using a spectrophotometer at wavelength 420 nm. The concentration of ammonia was calculated based on a standard curve of ammonia with a regression equation of y = 0,1763x + 0,0164.

The measurement of nitrite concentration is referred to SNI 06-6989.9-2004. An amount of 2 mL culture sample was added with 0.04 Sulfanilamide 1% and 0.04mL N-(1-Naphthyl) ethylenediamine (NED) 0.1%, the solution was then homogenized and let stand for 10 min. The solution's color changed to pink until violet, and further measured its absorbance using a spectrophotometer at a wavelength of 432 nm. The absorbance values were converted based on a standard curve of nitrite with a regression equation of y = 0,859x + 0,0443.

The measurement of nitrate concentration referred to SNI 06-2480-1991, by using a Brusin method. An amount of 2 mL culture sample was added with 0.2 mL NaCl, 1 mL H_2SO_4, and let sit until cooled. A total of 0.05 mL Brusin sulfate was added into the solution, homogenized, and let stand for 60 min until the solution's color changed to yellow. The concentration of nitrate was measured using a spectrophotometer at a wavelength of 432 nm. The absorbance values found were converted based on a standard curve of nitrate with a regression equation of y = 0,4498x + 0,0161.

3. Results And Discussion

3.1. Isolation of nitrifying bacteria

Nitrifying bacteria were isolated from pond sediments using *Nitrosomonas* and *Nitrobacter* selective media. The isolation results from *Nitrosomonas* medium obtained 5 isolates which were coded as isolates SA12, SA14, SA25, SA26, and SA37, each of which has a density of consecutive colonies of 1,3 x 104; 3,0 x 103; 4 x 104; 1,0 x 104; and 3,0 x 103 CFU. mL-1. The isolation results from *Nitrobacter* medium obtained 4 isolates which were coded as isolates BA11, BA12, BA26, and BA38, and each had a consecutive colony density of 2 x 104; 1,9 x 104; 3,0 x 103; dan 2,0 x 103 CFU.mL-1. The results of the phenetic characterization of the nine bacterial isolates showed that 2 isolates (SA14 and BA26) were Gram negative, had white colonies, and were small in size, and 7 isolates (SA12, SA25, SA26, SA37, BA11, BA12, BA38) were Gram positive, with colony forms that were round (Table 1).

Table 1. Characteristics of cell and colony of nitrifying bacterial isolates

Isolate code	Gram reaction	Characteristics of bacterial colony					
		Form	Size	Color	Edge	Elevation	Optic
SA12	+	round	small	white	flat	raised	opaque
SA14	-	round	small	clear transparent	flat	raised	transparant
SA25	+	round	small	white	flat	raised	translucent
SA26	+	round	small	white	flat	flat	opaque
SA37	+	round	small	white	flat	raised	translucent
BA11	+	round	medium	clear transparent	flat	raised	transparent
BA12	+	round	small	white	flat	raised	opaque
BA26	-	round	small	white	flat	raised	translucent
BA38	+	round	small	cream	flat	raised	translucent

3.2. Nitrification Activity of the Bacterial Isolates

The nitrification activity of bacterial isolates based on data on changes in levels of ammonia, nitrite and nitrate in the media. The results of measuring ammonia levels in the activity assay of isolates SA12, SA14, SA25, SA26, and SA37 during 12 days of incubation showed that the ammonia levels of all isolates decreased from the first day to the 6th day, then on the 12th day the levels rose again (Table 1). The high reduction in ammonia levels was especially shown by isolates SA12 and SA26, namely from initial levels of 11,217 ppm respectively decreasing to 0.956 and 1,875 ppm after 6 days of incubation.

The results of measuring ammonia levels in the medium containing isolates BA11, BA12, BA26, and BA38 tended to fluctuate every 2 days, the first two days decreased then the next 2 days was increased (Table 2). The highest reduction in ammonia levels was shown by isolates BA12 and BA38, which were able to reduce ammonia levels from 1.132 ppm to 0.242 and 0.259 ppm respectively.

Table 2. Ammonia concentration on the treatments of isolates SA12, SA14, SA25, SA26, and SA37

Days of Incubation	Ammonia Concentration (ppm)				
	SA26	SA25	SA14	SA12	SA37
4	3.798	6.311	8.307	2.216	4.161
6	1.875	7.139	2.079	0.956	4.445
8	2.896	9.544	2.584	5.239	2.465
10	9.623	10.542	11.087	9.090	10.542
12	12.045	13.537	12.278	13.157	12.828

Table 3. Ammonia concentration on the treatments of isolates BA11, BA12, BA26, and BA38

Days of Incubation	Ammonia Concentration (pp)			
	BA11	BA12	BA26	BA38
4	4.161	4.161	4.161	4.161
6	4.445	4.445	4.445	4.445
8	2.465	2.465	2.465	2.465
10	10.542	10.542	10.542	10.542
12	12.828	12.828	12.828	12.828

The results of measuring nitrite concentrations in the treatment isolates SA12, SA14, SA25, SA26, and SA37 showed the opposite pattern to the ammonia measurements (Table 3). These results indicated that these isolates can be grouped as nitrite bacteria, namely a group of bacteria that are able to oxidize ammonia to nitrite. The highest production of nitrite was shown by isolate SA26 with nitrite concentration of 0.313 ppm (nitrite concentration at the day-0 was 0.056 ppm) at incubation time of 6 days. In contrast, isolates BA11, BA12, BA26, and BA38 produced nitrite concentrations that were similar to the pattern of ammonia concentrations (Table 4). As the ammonia concentration increased, the concentration of nitrite was also increased. These results indicated that isolates BA11, BA12, BA26, and BA38 tended to belong to the group of nitrate bacteria. The highest production of nitrite was shown by isolate BA12 in the amount of 1.358 ppm (nitrite concentration at the day-0 was 0.086 ppm) following incubation of 6 days.

Table 4. Nitrite concentration on the treatments of isolates SA12, SA14, SA25, SA26, and SA37

Days of Incubation	Nitrite Concentration (ppm)				
	SA26	SA25	SA14	SA12	SA37
4	-0.029	-0.007	-0.027	0.010	-0.033
6	0.369	0.039	0.052	0.047	0.087
8	0.096	0.022	0.002	0.021	0.044
10	0.051	0.079	0.040	0.031	0.050
12	0.044	0.029	0.026	0.037	0.066

Table 5. Nitrite concentration on the treatments of isolates BA11, BA12, BA26, and BA38

Days of Incubation	Nitrite Concentration (pp)			
	BA11	BA12	BA26	BA38
4	0.224	0.231	0.061	0.258
6	0.667	1.444	0.673	0.962
8	0.044	0.979	0.372	0.372
10	0.347	0.968	0.735	1.847
12	0.122	0.474	0.520	0.565

The results of measuring nitrate concentrations in the treatment of isolates SA12, SA14, SA25, SA26, and SA37 showed that nitrate levels tended to increase on incubation until the 6th day, then decreased until the 10th day of incubation, and then increased again on the 12th day of incubation (Table 5). On the other hand, nitrate levels in the BA11, BA12, BA26, and BA38 isolates tended to be the opposite, where nitrate levels rose on the 6th to 8th day, then decreased until the 10th day, and began to rise again at 12 days of incubation (Table 6). The highest increase in nitrate levels in SA isolates was shown by isolates SA37 and SA14 at 0.169 and 0.215 ppm, respectively (nitrate concentration at day-0 was 0.104) at an incubation time of 12 days. The ability of the BA isolate group to increase high nitrate levels was demonstrated by isolates BA12 and BA38, namely 0.182 and 0.262 ppm (nitrate concentration at day-0 was 0.051), on the 8th day of incubation.

Table 6. Nitrate concentration on the treatments of isolates SA12, SA14, SA25, SA26, and SA37

Days of Incubation	Nitrate Concentration (ppm)				
	SA26	SA25	SA14	SA12	SA37
4	0.078	0.113	0.138	0.098	0.115
6	0.129	0.106	0.113	0.151	0.120
8	0.058	0.133	0.106	0.073	0.084
10	0.073	0.091	0.078	0.086	0.102
12	0.171	0.160	0.215	0.191	0.273

The results showed that the nitrification process had taken place as a result of the activity of bacterial isolates which were shown by the decrease ammonia concentration from the initial concentration. The decrease in ammonia concentration was followed by the increased concentration of nitrite and nitrate, especially until the incubation time of

8 days (Table 1 – Table 6). According to Hong [5], nitrification is a process of oxidizing ammonia to nitrite followed by oxidizing nitrite to nitrate. A decrease in ammonia concentration that is not accompanied by the formation of nitrites or nitrates indicates that the nitrification reaction is not occurring [6].

Table 7. Nitrate concentration on the treatments of isolates BA11, BA12, BA26, and BA38

Days of Incubation	Nitrate Concentration (ppm)			
	BA11	BA12	BA26	BA38
4	0.011	0.026	0.033	0.058
6	0.142	0.066	0.122	-0.011
8	0.313	0.131	0.249	0.307
10	0.029	0.051	0.124	0.149
12	0.089	0.082	0.233	0.142

The research results showed that the formation of nitrate decreased after 8 days of incubation (Table 5, Table 6). This decreased can be occurred due to the nitrate can be utilized as an alternative of electron acceptor for heterotrophic bacteria in low oxygen condition [7]. A decrease in nitrate can also occur due to the denitrification process, namely nitrate is reduced and can be converted into free nitrogen [8]. An increase in nitrate concentration accompanied by a decrease in ammonia indicates that the nitrification process is effective. The potential of nitrifying bacteria at a concentration of 250 ppm $(NH_4)_2SO_4$ and obtained an average reduction in ammonia of 3.2 – 2.1 ppm after the 8th day of incubation, and the highest average increase in nitrate was 20.5 ppm [9,10,11].

4. Conclusion

It can be concluded that all bacterial isolates tested belong to nitrifying bacteria that were capable of reducing ammonia concentration in the amount of 0.878-10,261 ppm, increasing nitrite concentration ranging from 0.313-1.358 ppm, increasing nitrate concentration in the amount of 0.215-0.262 ppm.

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