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A Trial on the Cultivation to Increase the Growth Activity and the Nitrification Activity of *Nitrosomonas europaea*

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The growth and nitrification activities of *Nitrosomonas europaea* are generally very low, and this step has been rate-limiting the microbiological nitrification and denitrification processes of ammonium ions. In the present study, a cultivation method to increase the nitrification activity accompanying the growth of *N. europaea* was attempted as follows. After the oxidation of nitrite to nitrate by *Nitrobacter winogradskyi* was carried out, the culture liquid was recovered by centrifugation, and acetic acid was added to it as a carbon source. *Paracoccus denitrificans* was inoculated into the culture liquid and cultivated so as to consume the nitrate and acetic acid completely. Subsequently, the culture liquid was recovered by centrifugation and after ammonium sulfate was added to the culture liquid, *N. europaea* was grown on the culture liquid. On the basis of this procedure, high nitrification activity accompanying the growth of *N. europaea* was attained.

1. Introduction

It is very important for the nitrogen cycle in nature to increase the activity of the nitrification and denitrification processes. In particular, the nitrification of inorganic nitrogen is carried out by nitrite and nitrate bacteria, and it is well known that since the growth activity of these bacteria is very low, the activity is the rate-limiting step of the nitrification and denitrification processes[1~3].

The treatment of sewage by activated sludge is better than that by the physicochemical method from the viewpoint of energy consumption and secondary environmental pollution. Therefore, it is very important that the nitrification process by microorganisms is stable and highly active to remove inorganic nitrogen efectively. To increase the growth activity of the bacteria related to the nitrification, it is necessary to develop a method so as to obtain a highly concentrated culture liquid of active cells. The following procedure was performed as a cultivation method to increase the nitrification activity. First, after the oxidation of nitrite ions to nitrate ions by *Nitrobacter winogradskyi* was carried out, the culture liquid was recovered by centrifugation, and acetic acid was added to it as a carbon source. *Paracoccus denitrificans* was inoculated into the culture liquid, and cultivated until the nitrate ions and acetic acid were completely consumed. Subsequently, the culture liquid was recovered by centrifugation, and after ammonium sulfate was added to the culture

liquid, *N. europaea* was grown on the culture liquid.

2. Experimental

In the preliminary test, when only *Nitrosomonas europaea* was cultivated, it was very difficult to increase its growth activity, and the oxidation of ammonium ions did not occur sufficiently. Therefore, the following procedure was performed. First, *Nitrobacter winogradskyi* IFO 14297 was cultivated. The culture liquid obtained after the cultivation was used to cultivate *Paracoccus denitrificans* IFO 14907. Subsequently, this culture liquid was used to cultivate *Nitrosomonas europaea* IFO 14298. The cultivation characteristics and the increase in the growth activity were examined.

2-1 Cultivation of *N. winogradskyi*

The culture medium shown in **Table 1** was used to increase the growth activity of *N. winogradskyi.* The pre-cultivation was carried out at 28 ℃ with shaking (100 strokes/min) and repeated several times by inoculating the growth cells into the same fresh culture medium. The cells obtained consumed the nitrite ions completely within the cultivation period. Using the cells with such high activity, the main cultivation was carried out with the same culture medium as follows.

Table 1. Components of the culture medium for cultivating *Nitrobacter winogradskyi* IFO 14297

A)		B)	
$NaNO_2$	0.85 g/l	$CaCl(2H_2O)$	0.02 g/l
$NaHCO_3$	0.53	$MnSO_4(4H_2O)$	0.02
K_2HPO_4	0.50	$MgSO_4(7H_2O)$	0.10
KH_2PO_4	0.50	$CuSO_4(5H_2O)$	0.10 mg/l
NaCl	0.20	Fe(III)EDTA	7.80 mg

A) and B) were prepared separately. Each component in B) was also autoclaved separately.The initial pH of culture liquid was adjusted to 7.8.

The working volume was 500 ml and the initial cell turbidity was 0.5 from the absorbance at a wavelength of 540 nm. Air from an air compressor was humidified by a water reservoir after passing through an air filter. The humidified air was supplied to the bioreactor. The cultivation temperature was 28℃. The air in the bioreactor was released through a gas outlet. The sample liquid was removed by passing through a microtube pump via a cell trapping filter attached to one end of the tube and recovered to the collector attached to the other end of the tube. The sample liquid (5 ml) was taken at the beginning of the cultivation and then at 24 h intervals. The concentration of nitrite ions and nitrate ions was measured with an ion chromatograph (DIONEX 2000i/sp), and the pH of the culture liquid was also measured at the same time. Once the nitrite ions in the culture liquid were consumed completely, the culture liquid was centrifuged aseptically at 1000 x *g* for 30 min, and the cells were recovered. The dry weight of the cells at the beginning and the end of cultivation was measured using some of the recovered cells, the remaining cells were washed with saline solution (0.85%NaCl) and stored at –80℃ after glycerin was added to the cell suspension to a concentration of 20%. After centrifugation, the supernatant, which contained nitrate ions, was stored in the refrigerator, and later used to cultivate *P. denitrificans*.

2-2 Cultivation of *P. dentrificans*

To increase the growth activity of *P. dentrificans*, the culture medium shown in **Table 2** was used. The pre-cultivation was carried out at 28℃ for 3 days with shaking (100 strokes/min). At the end of cultivation, the cells, which were recovered by centrifugation (10000 x *g*, 20 min), were washed aseptically with the saline solution. The main cultivation was performed in the bioreactor described above. The culture

Table 2. Components of the culture medium for cultivating *Paracoccus denitrificans* IFO 14907

Polypeptone	5	g/l
Yeast extract	1	
$MgSO_4(7H_2O)$	0.5	

The initial pH of culture liquid was adjusted to 7.0.

medium (500 ml) shown in Table 2 was added to the bioreactor. The cell turbidity was 0.5. The procedure after the beginning of cultivation was the same as that described in section 2-1.

The denitrification was performed as follows. After the supernatant containing nitrate ions described in section 2-1 was filtered with a glass fiber filter (GS-25), 5 ml of 1 M acetic acid was added to the supernatant (500 ml). The supernatant was adjusted to pH 7.0, loaded in a 1000 ml Erlenmeyer flask (working volume: 500 ml), and the high growth-active *P. denitrificans* mentioned above was inoculated into the supernatant. The anaerobic cultivation to denitrificate nitrate ions was performed at 28℃ with mild agitation. The sample liquid (5 ml) was taken at the beginning of cultivation and then at 24 h intervals. The concentration of nitrate ions and the pH of the sample liquid were measured. The cultivation was completed when the nitrate ions were consumed completely. The cells were recovered aseptically by centrifugation (10000 x *g*, 20 min). The complete consumption of acetic acid in the supernatant was confirmed by ion chromatography. The denitrificated supernatant, which was tentatively named the denitrificated liquid, was stored in the refrigerator and later used to cultivate *N. europaea*.

2-3 Cultivation of *N. europaeae*

To increase the growth activity of *N.*

Table 3. Components of the culture medium prepared by modifying the culture medium indicated by IFO.

A)		B)	
$(NH_4)_2SO_4$	0.6 g/l	$MgSO_4(7H_2O)$	0.36 g/l
NaCl	0.36	$FeSO_4(7H_2O)$	0.036
KH_2PO_4	1.2	C)	
		$CaCO_3$	9.0 g/l

A), B) and C) were prepared separately. Each component in B) was also autoclaved separately.
The initial pH of culture liquid was adjusted to 8.0.

europaeae, the culture medium shown in **Table 3** was used. The pre-cultivation was carried out at 28℃ for 5 days in the shaking flask (500 ml) with shaking (100 strokes/min). The sample liquid (5 ml) was taken at the beginning of cultivation and at 24 h intervals. The concentration of nitrite ions was measured by ion chromatography. At the completion of cultivation, the cells were recovered by centrifugation (10000 x *g*, 30 min) and washed aseptically with saline solution (0.85%NaCl). The recovered cells were inoculated into a 2000 ml flask containing the culture medium (1000 ml) shown in Table 3, or the denitrificated liquid to which was added a directed concentration of ammonium sulfate. The cell turbidity was 0.5. The main cultivation was then begun. The sample liquid (5 ml) was taken at the beginning of cultivation and at 24 h intervals, and the concentration of nitrite ions was measured by ion chromatography. At the same time, the pH and the cell turbidity of culture liquid were also measured. The dry weight of the cells at the beginning and the end of cultivation was measured using some of the recovered cells, the remaining cells were washed with saline solution (0.85% NaCl) and stored at –80℃ after glycerin was added to the cell suspension to a concentration of 20%. Using the

recovered cells, the consumption rate of ammonium sulfate was examined. The cultivation was carried out as follows. The culture medium (1000 ml) shown in Table 3, or the denitrificated liquid to which was added a directed concentration of ammonium sulfate, was added to the three flasks (2000 ml) separately. After the beginning of cultivation with shaking (100 strokes/min), the pH of culture liquid was intermittently adjusted to 8.0. The sample liquid (5 ml) was taken at the beginning of cultivation and at 24 h intervals, and the concentration of nitrite ions was measured by ion chromatography. The cultivation was carried out 5 times by repeating the addition of the recovered cells to the fresh medium. The consumption rate of ammonium sulfate was examined. Using the cells that had their activity increased with the above procedure, the effect of the initial concentration of ammonium sulfate on the consumption rate of ammonium sulfate was examined. In this case, the pH of culture liquid was intermittently adjusted to 8.0.

3. Results and discussion

3-1 Cultivation of *N. winogradskyi* and *P. denitrificans*

Figure 1 shows the results of the cultivation of *N. winogradskyi*. In 3 days of cultivation, nitrite ions in the culture liquid were consumed perfectly, but the concentration of nitrate ions reached the maximum. The cell dry weight reached the maximum (ca. 110 mg dry weight/l-culture liquid) in 2 days of cultivation. This value was ca. 2.2 times higher than that at the beginning of cultivation. On the other hand, in the case of cultivation of *P. denitrificans*, the cell turbidity reached the maximum in 2 days of cultivation, and decreased slightly after 3 days of cultivation. However, the cell dry

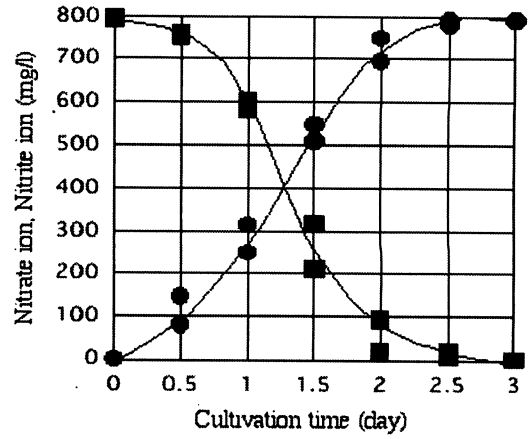

Fig. 1. Time course of the concentration of nitrite ions and nitrate ions in the cultivation of *Nitrobacter winogradskyi*. ■; Nitrite ions, ●; Nitrate ions.

weight reached the maximum (ca. 3.8 g dry weight/l-culture liquid) in 3 days of cultivation. This value was ca. 70 times higher than that at the beginning of cultivation. The growth rate of *N. winogradskyi* was extremely low compared with that of *P. denitrificans*. Since *P. denitrificans* is a kind of heterotroph, the effect of methanol or glucose as a carbon source on the consumption rate of nitrate ions was examined. When the concentration of each substrate was higher than 10 mM as a final concentration, a sufficient consumption rate of nitrate ions was attained (data not shown). However, if the supernatant after cultivation of *P. denitrificans* is used as the culture liquid of *N. europaea* , no organic carbon sources should be present in the culture liquid since *N. europaea* is a kind of autotroph. Therefore, *N. winogradskyi* was first cultivated in the culture medium shown in Table 1, which increased the growth activity. To the recovered supernatant, acetic acid was added to a final concentration of 10 mM. Using this as the culture liquid, *P. denitrificans* was cultivated. Nitrate ions (800 mg/l) added were consumed completely in 2.5~3 days of cultivation, though the pH

of the culture liquid increased from 7.0 to 8.0. This indicates that highly effective denitrification occurred. The supernatant after the cultivation, which was tentatively named the dentrificated liquid, was used to cultivate *N. europaea*.

3-2 Comparison of cultivation of *N. europaea* with different culture liquids

The culture liquid used to cultivate *N. europaea* was either that shown in Table 3 or the denitrificated liquid added ammonium sulfate. **Figure 2** shows the results of cultivation with the denitrificated liquid added ammonium sulfate. The cell turbidity reached ca. 7.6 times that of the initial in 2.5 days of cultivation. In 3 days of cultivation, i.e. the finish of cultivation, the cell dry weight was ca. 355 mg dry weight/l-culture liquid (ca. 6.6 times of the initial cell dry weight). This indicates that *N. europaea* having high growth activity was obtained. The results of cultivation with the culture liquid shown in Table 3 were not shown as the cell turbidity and the cell dry weight could not be measured

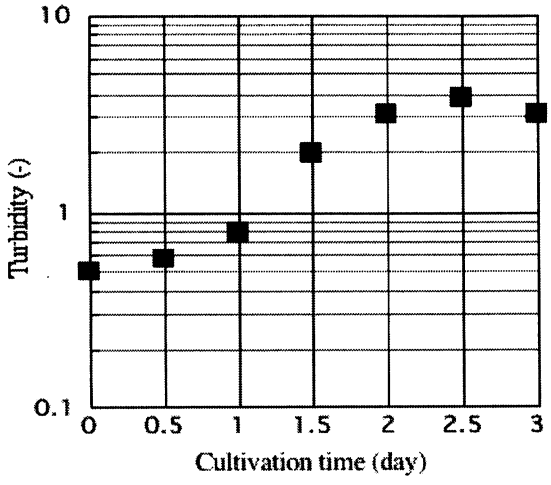

Fig. 2. Time course of the cell turbidity of *Nitrosomonas europaea.*

accurately. Because the culture liquid contained a large quantity of solid $CaCO_3$. **Figure 3** shows the production of nitrite ions from ammonium sulfate by using

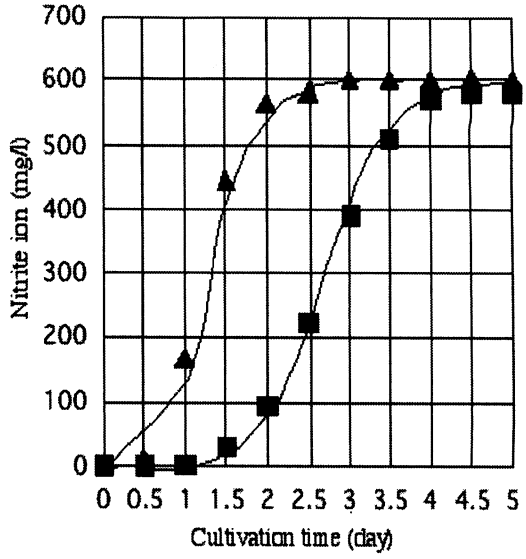

Fig. 3. Effect of different culture liquids on the production of nitrite ions from ammonium sulfate by *Nitrosomonas europaea.* Initial pH of the culture liquid was adjusted to 8.0. ▲; Denitrificated liquid as culture liquid, ■; Medium in Table 3 as culture liquid.

different culture liquids. The production rate of nitrite ions appeared to be high in the case of cultivation with the denitrificated liquid added ammonium sulfate. Using the cells obtained from this cultivation, a complete cultivation, that is, ammonium ions were consumed completely in the every cultivation, was carried out 5 times with fresh culture liquid. In the third cultivation, the consumption rate of ammonium sulfate reached the maximum, and was maintained at this level after the third cultivation (data not shown). **Figure 4** shows the consumption rate of ammonium sulfate in the different culture liquids with the cells recovered after the third cultivation. The rate appeared to be high in the case of cultivation with the denitrificated liquid added ammonium sulfate. **Figure 5** shows the effect of the initial concentration of ammonium sulfate on the initial consumption rate of ammonium sulfate. When the ammonium sulfate concentration was higher than 6000 mg/l, the rate reached a

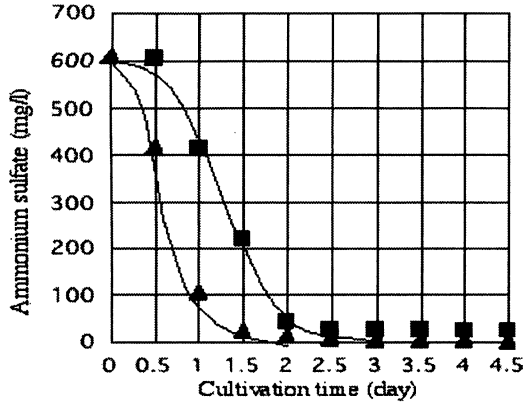

Fig. 4. Time course of the consumption of ammonium sulfate in different culture liquids by *N. europaea*. The symbols in the figure are the same as in Fig. 3.

plateau. The rate appeared to be high in the case of cultivation with the denitrificated liquid added ammonium sulfate. The cause of the increase in the activity remains unclear. On the other hand, Tokuyama T. and K. Asano investigated the effect of additives on ammonia-oxidative systems of *N. europaea*[4], and found that the addition of

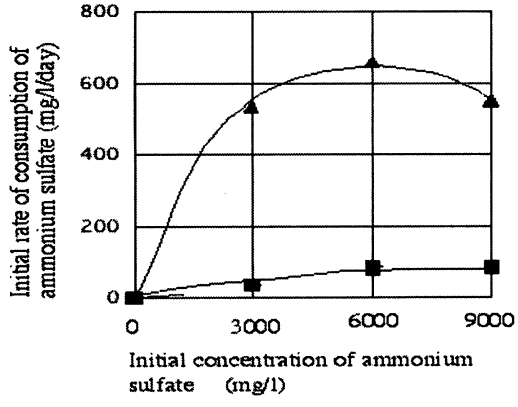

Fig. 5. Effect of the initial concentration of ammonium sulfate on the initial consumption rate of ammonium sulfate by *N. europaea*. The directed concentration of ammonium sulfate was added to the culture liquid. The symbols in the figureare the same as in Fig. 3.

1 mM $MgCl_2$ or 5 x 10^{-5} mM ATP to fragments of the cell bodies activated the oxidation of ammonia and hydroxylamine. The cause of high nitrification and growth activities in the present study may be due to the activation of the metabolic pathway of the oxidation of ammonium ions on the basis of a kind of activator in the denitrificated liquid.

4. Conclusion

It has been reported that the growth activity of *N. europaea* is generally low. In the present study, the supernatant of the culture liquid of *N. winogradskyi* was used as the culture liquid of *P. dentrificans* with the addition of acetic acid. When the supernatant obtained after *P. dentrificans* was cultivated, i.e. the denitrificated liquid, was used to cultivate *N. europaea* with the addition of ammonium sulfate, the growth activity of *N. europaea* markedly increased. However, the cause of the increase in the activity remains unclear.

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