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Nitrate Removal by *Pseudomonas fluorescens* K4 Isolated from a Municipal Sewage Treatment Plant

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The removal of nitrogen compounds from a wastewater is essential and it is often accomplished by biological process. An aerobic nitrate-removing bacterium was isolated from a municipal sewage treatment plant and soil. On the basis of its morphological, cultural and physiological characteristics and 16S rRNA sequencing data, this strain was identified as *Pseudomonas fluorescens,* and named as *P. fluorescens* K4. The optimal conditions of the initial pH and temperature of media for its growth were 7.0~8.0 and 30℃, respectively. *P. fluorescens* K4 was able to remove 99.9% of nitrate after 24 h in a culture. The strain could grow with a nitrate concentration up to 800 mg/l and was able to remove 99.9% of nitrate after 104 h of incubation. The optimal electron donor was sodium citrate for a nitrate removal. The strain K4 showed a capability of a complete nitrate removal when the initial C/N ratio was 1.0. An effect of the initial seed concentration was observed for a cell of 10% (v/v) for a nitrate removal. Especially *P. fluorescens* K4 could completely remove 200 mg/l ammonium for 3 days.

Key Words : Nitrate, Denitrification, Isolation, Identification, *Pseudomonas fluorescens*

1. Introduction

The traditional biological nitrogen removal mechanism involves nitrification and denitrification steps. Nitrification process makes use of three groups of chemo-litho autotrophic bacteria: well known as 'aerobic' ammonia and nitrite oxidizers and anaerobic ammonia oxidizers[1)]. They obtain energy for a growth from the oxidation of inorganic nitrogen compounds. Denitrification process use chemotrophs, which can use organic and inorganic electron donors. Those that utilize organic electron donors are heterotrophs. A more limited group of autotrophs can utilize H_2 and reduced sulfur. Denitrification proceeds in a stepwise manner in which a nitrate is sequentially reduced to a nitrite and N_2 gas[2)].

In recent years, research on a biological nitrogen removal has been focused towards either improving the efficiencies and energy saving mechanisms of a process or towards identifying new microorganisms and processes that perform better. These issues are especially relevant when high ammonium concentrations need to be removed, e.g., landfill leachates, supernatants from a sludge digestion, effluents from anaerobic treatment plants and agro-industrial wastewater, etc[3~5)].

Nitrate is the end product of a biological nitrification and is accumulate during wastewater treatment processes, unless a denitrification occurs[6)]. A high strength nitrate wastewater discharge may cause an alarming increase in the nitrate level of a ground water and water sources[7)]. Bacterial nitrate removal is a dissimilatory nitrate reduction, which involves four metabolic steps where a nitrate is sequentially reduced to nitrogen via a nitrite, nitric oxide and nitrous oxide. The dissimilatory nitrate reduction is influenced by the concentration of electron donor and acceptor since the ratio of carbon to nitrogen (C/N ratio) has to be more than about 3[8,9)]. This is a reason why or-

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ganic compounds such as methanol or acetate have been added to the wastewater treatment bioreactor for a denitrification[10,11].

Various biological denitrification technologies have been established in order to solve the problem of a nitrate in drinking water and groundwater. The existing technologies differ in the nature of the bacterium used for this purpose and in the technical setup of the equipment.

In this work, we describe a newly isolated nitrate-removing bacterial strain *Pseudomonas fluorescens* K4 from the municipal sewage treatment plant, which was carried out for the effect of a variation of the concentration of nitrate, electro donor, C/N ratio and initial seed concentration on a nitrate removal.

2. Materials and Methods

The aerobic nitrate-removing bacteria were isolated from the sludge of a municipal sewage treatment plant in Daejeon, Korea. An amount of 1 g of a sample was suspended in sterile saline and shaken for 2 h in a shaking incubator. Serial dilutions of the sample were then individually inoculated on to Giltay medium[12]. The Giltay medium used in this study comprised of the following: 0.1%(w/v) KNO_3, 0.1%(w/v) asparagine, 0.85%(w/v) sodium citrate, 0.05%(w/v) $MgSO_4 \cdot 7H_2O$, 0.005%(w/v) $FeCl_2 \cdot 6H_2O$, 0.1%(w/v) KH_2PO_4, 0.02% (w/v) $CaCl_2 \cdot 2H_2O$ and 1%(w/v) BTB solution (Bromothymol blue 1 g in 100 ml ethanol) in distilled water(pH 7.0~7.2).

After 3 day incubation at 30℃, we selected dilutions exhibiting a color change(green to blue). The inoculation mixture with a serial dilution was then spread on to a plate of the same medium containing 1.5%(w/v) agar and incubated at 30℃ until a color change[12]. The blue colony was selected and then tested in batch nitrate removal experiments.

The isolate was identified using 16S rRNA sequencing of PCR products of an extracted genomic DNA, API20NE and 20E rapid test series(BioMerieux, France)[13,14]. The partial 16S rRNA sequences were compared, based on the NCBI(National Center for Biotechnology Information, www. ncbi.nlm.nih.gov) database using the BLAST search.

In order to determine the optimum culture conditions for a nitrate removal, the flasks were cultivated at various pH values, temperatures, different electron donors and different nitrates. The C/N ratios of the culture medium were adjusted by an addition of NH_4NO_3 and sodium citrate. For a shaken culture in a bottle, stock culture was inoculated into 50 ml of a Giltay medium in a 250 ml bottle and a culture was allowed to proceed for 24 h under a shaken culture. Cultivations were performed at 30℃ and 110 rpm for 2~4 days in a shaking incubator.

Cell growth was estimated photometrically following the optical density of the broth at 650 nm by a spectrophotometer (Lambda 2S, Perkin Elmer). During the fermentation, the pH value was measured periodically (off-line) by pH-meter (Orion. EA 940) with a glass probe. Nitrate and ammonium concentrations were measured using DR-4000 spectrophotometer (Hach Co.).

Culture samples were centrifuged at 10,000 rpm for 10 min and the supernatants were obtained. One ml of the supernatants was put into the test kits(Nitrate Nitrogen Standard Solution and NH_4-N Standard Solution, respectively), mixed with reagents and then the nitrate and ammonium concentration were analyzed.

3. Results and Discussion

The arbitrarily named K4, 5 and 16 were selected among the 13 different aerobic nitrate-removing bacteria isolated from the soil and sludge of the municipal sewage treatment plant, based on the blue colony diameter. We assayed for a nitrate removal of K4, 5 and 16 in a liquid culture that contained 0.3% NH_4NO_3 substituting the KNO_3 of the Giltay medium. The strain K4, 5 and 16 denitrified NO_3^- most efficiently(560 mg NO_3/l was removed) (Fig. 1). Therefore, strain K4 was selected for further nitrate removal tests.

The microorganism was identified as *Pseudomonas fluorescens* on the basis of the morphological, physiological, biochemical results(Table 1) and a nucleotide sequence analysis of 16S rRNA. In the 16S rRNA sequence analysis, 98.2% of similarity was observed between the sequence of the isolate strain K4 and the sequence of *P. fluorescens* in the database(RID: 1158279672-16519-162900856267.BLASTQ1)(data not shown). Thus, the strain was named as *P. fluorescens* K4.

Denitrification is known to occur at between 5~35℃ of the growth temperatures. Particularly, the optimal

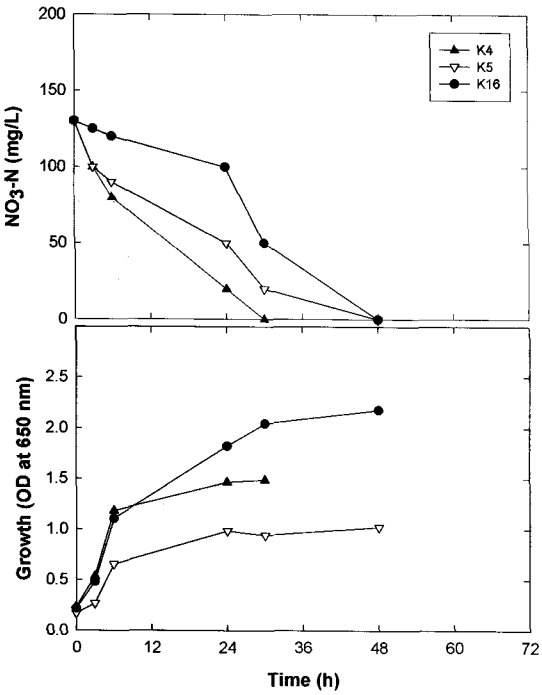

Fig. 1. Nitrate removal by strain K4, K5 and K16 under aerobic condition. Cells were cultivated in 0.1% NH_4NO_3 medium for 2 days at pH 7.0 and 30℃.

temperature for the denitrification was reported to be 30℃[15]. The effect of various temperatures(25, 30 and 40℃) was examined in a 0.1% NH_4NO_3 medium. The optimal temperature for the nitrate removal was observed to be 30℃. After 24 h of incubation, the nitrate removal efficiency of strain K4 was 99.9%. There was no nitrate removal at 40℃. Cell could not grow at 40 ℃(data not shown).

The pH is an important factor for a biological nitrate removal[14]. The effect of the initial pH on a nitrate removal was tested in the range of pH 5.0~9.0. A high level of a nitrate removal was observed over a broad pH range between 6.0 and 8.0, and it was a maximum at pH 7.0. Generally, the optimal pH range accepted for a denitrification by denitrifiers. This is probably because the activity of the nitrate reductase was inhibited[7,14,15].

It is important to select a carbon source that would ensure the most effective process of a nitrate reduction as well as provide a readily available and cost-effective process[7]. The following compounds were applied to demonstrate the effect of a carbon source on the rate of the nitrate removal by *P. fluorescens* K4:

Table 1. Taxonomical characteristics of the isolated strain K4

Contents	Characteristics
Morphological characteristics	
Cell shape	rod
Gram stain	-
Spore formation	-
Motility	+
Cultural characteristics	
Colony shape	circular, undulate, convex
Colony surface	smooth
Colony colony	white
Biochemical characteristics	
Growth in air	-
Growth anaerobically	-
Acid from glucose	-
Oxidase	+
Catalase	+
O/F test	Oxidation
Yellow pigmentation	-
Arginine dihydrolase	+
Nitrate reduction	+
Indole production	-
Urease	-
β-Glucosidase	+
β-Galactosidase	-
Gelatin hydrolysis	-
Assimilation : glucose, arabinose, mannose, mannitol, gluconate, N-acetyl-glucosamine, malate	+
Assimilation : maltose, caprate, adipate, citrate	-

glucose, methanol, ethanol, acetic acid and sodium citrate. As shown in Fig. 2, the optimal electron donor was sodium citrate for the nitrate removal.

The nitrate removal rate was observed with different initial nitrate concentrations. As shown in Fig. 3, the strain was able to remove 99.9% of the nitrates tested after 24~104 h in an incubation.

To investigate the effect of the initial seed concentration, a culture was provided at different initial seed concentrations of 5%(v/v), 10%(v/v) and 15%(v/v). *P. fluorescens* K4 could remove well when the initial seed concentration was 10%(v/v).

At different initial concentrations of the nitrate and sodium citrate as a sole carbon source, strain K4 showed a capability of a complete nitrate removal

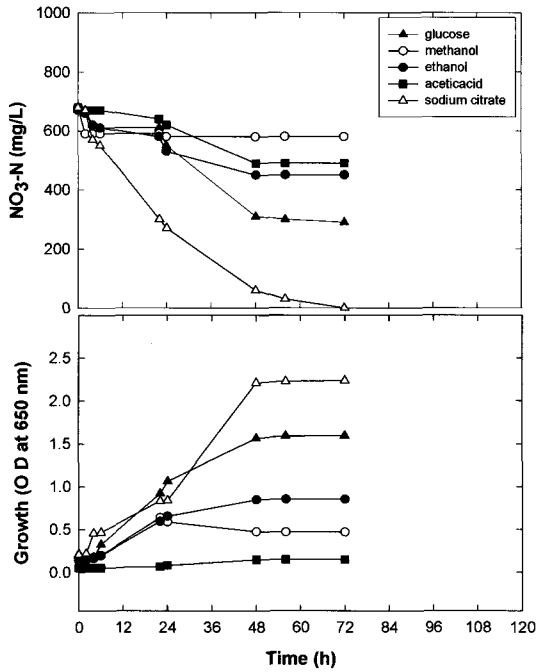

Fig. 2. The effect of different electron donor on the cell growth and nitrate removal by *P. fluorescens* K4. Cells were cultivated in 0.5% NH_4NO_3 medium for 3 days at pH 7.0 and 30℃.

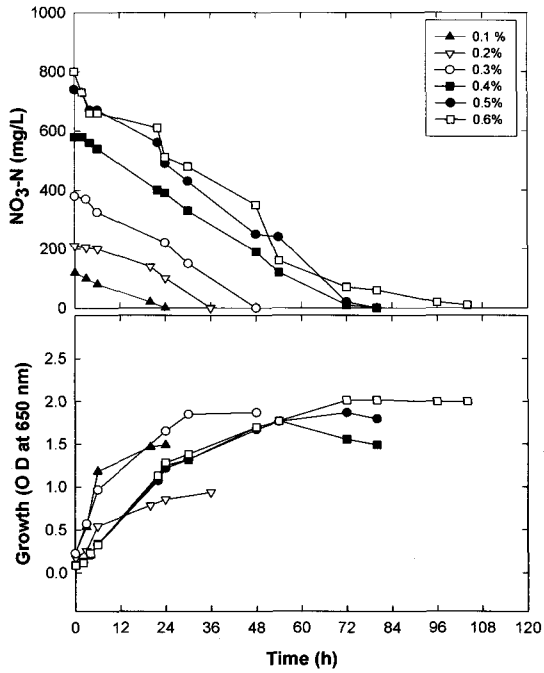

Fig. 3. The effect of nitrate concentration on the cell growth and nitrate removal of *P. fluorescens* K4. Cells were cultivated in 0.1~0.6% NH_4NO_3 medium for 4 days at pH 7.0 and 30℃.

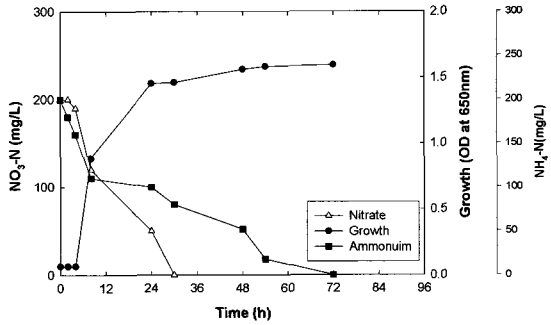

Fig. 4. Cell growth, nitrate removal and ammonium removal by *P. fluorescens* K4. Cells were cultivated in 0.1% NH_4NO_3 medium for 3 days at pH 7.0.

when the initial nitrate concentration was up to 600 mg/l NO_3-N. Among the initial C/N ratios of 0.4, 0.6, 0.8 and 1.0, a complete nitrate removal was observed at the C/N ratio of 1.0. Alves et al.[16] reported that with a increasing C/N ratio, the nitrate removal rate also increased from 15 to 93%. Rouse et al.[7] also employed a fluidized bed for a denitrification process, but in their experiment they used ethanol as the carbon source at a C/N ratio of 1.0. They achieved a 60~70% nitrate reduction, but for a C/N ratio in the range of 1.3~2.3 they recorded a complete removal of the nitrates. Other researchers have used methanol for a denitrification at a C/N ratio of 3:1 and have also achieved a complete reduction of nitrates[16,17].

To investigate the effect of ammonium removal by *P. fluorescens* K4, batch experiments were conducted at 0.1% NH_4NO_3 medium. Fig. 4 shows the nitrate removal rate, cell growth and ammonium concentration after 72 h cultivation. After *P. flurorescens* K4 removed the nitrate completely at 0.1% NH_4NO_3 medium, the ammonium level was steadily declined.

4. Conclusions

In this study, the aerobic nitrate-removing bacteria were isolated from municipal sewage treatment plant. The microorganism was identified as *Pseudomonas fluorescens* K4 on the basis of the API-20NE test series and nucleotide sequence analysis of 16S rRNA. The optimal temperature for the nitrate removal was observed to be 30℃. A high level of a nitrate removal was observed over a broad pH range between 6.0 and 8.0, and it was a maximum at pH 7.0. The optimal electron donor was sodium citrate for the nitrate removal. *P. fluorescens* K4 was able to remove

99.9% of the nitrates tested after 24~104 h in an incubation. A complete nitrate removal was observed at the C/N ratio of 1.0.

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