Evaluation of nitrate removal by continuous culturing of an aerobic denitrifying bacterium, *Paracoccus pantotrophus*

K. Hasegawa-Kurisu*, Y. Otani* and K. Hanaki*

*Department of Urban Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan (E-mail: *kiyo@env.t.u-tokyo.ac.jp*; *hanaki@env.t.u-tokyo.ac.jp*)

Abstract Nitrate removal under aerobic conditions was investigated using pure cultures of *Paracoccus pantotrophus*, which is a well-known aerobic-denitrifying (AD) bacterium. When a high concentration of cultures with a high carbon/nitrogen (C/N) ratio was preserved at the beginning of batch experiments, subsequently added nitrate was completely removed. When continuous culturing was perpetuated, a high nitrate removal rate (66.5%) was observed on day 4 post-culture, although gradual decreases in AD ability with time were observed. The attenuation in AD ability was probably caused by carbon limitation, because when carbon concentration of inflow water was doubled, nitrate removal efficiency improved from 18.1% to 59.6%. Bacterial community analysis using the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method showed that *P. pantotrophus* disappeared in the suspended medium on day 8 post-culture, whereas other bacterial communities dominated by *Acidovorax* sp. appeared. Interestingly, this replaced bacterial community also showed AD ability. As *P. pantotrophus* was detected as attached colonies around the membrane and bottom of the reactor, this bacterium can therefore be introduced in a fixed form for treatment of wastewater containing nitrate with a high C/N ratio.

Keywords *Acidovorax* group; aerobic denitrification; C/N ratio; nitrate reduction; *Paracoccus pantotrophus*

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Introduction

Nitrogen loads induced by human activities pose environmental issues such as eutrophication in closed water districts and nitrate contamination in groundwater. As such, efficient nitrate removal from wastewater is essential to prevent such problems from occurring. It is only obvious that highly advanced treatment facilities installed with anoxic tanks are inevitable in the case of wastewater treatments by municipalities in major cities. However, the rate of highly advanced wastewater treatments in Japan remained low at 9.7% in 2001, because of the high investments in building the relevant facilities (survey based on reports of the National Land and Transport Ministry).

As oxygen inhibitions are subjected to the influence of four categories of nitrate-removal-related enzymes (nitrate, nitrite, nitric oxide, and nitrous oxide reductases), accommodating an anoxic condition has hitherto been thought as a prerequisite in nitrate removal by denitrification. However, as findings in 1980 and thereafter have indicated that said categories of enzymes are not subjected to inhibitions even under aerobic conditions, species of aerobic denitrifying bacteria then began to be documented. Briefly, *Paracoccus pantotrophus* (Robertson and Kuenen, 1984), re-named from *Thiosphaera pantotropa* (Ludwig *et al.*, 1993; Rainey *et al.*, 1999); *Alcaligenes faecalis* (Vanniel *et al.*, 1992); *Nitrosomonas eutropha* (Zart and Bock, 1998); *Thauera mechernichensis* (Scholten *et al.*, 1999); *Microvirgula aerodenitrificans* (Patureau *et al.*, 1998); and *Citrobacter diversus* (Huang and Tseng, 2001) are some of the species identified. As frequent repetitions of aerobic-anoxic condition replacements favor these aerobic denitrifying

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(AD) bacteria, the well-known AD bacterium, *P. pantotrophus*, has actually been isolated from the anoxic tank with oxygen-inflow.

As such, said AD bacteria were employed in this study in an attempt to innovate a simple and low-cost nitrate-removal system by merely adding the bacteria in the aerobic tank. To date, AD bacteria have been exploited under various conditions in the evaluation of nitrate removal (Otani *et al.*, 2004). Table 1 shows brief results of the previous study. On comparing the nitrate removal ability of three species of AD bacteria (*P. pantotrophus*, *A. faecalis*, and *M. aerodenitrificans*) under various carbon-added conditions, batch tests were accordingly conducted. From the results, consistently stable nitrate removals were established under aerobic conditions with sodium acetate using *P. pantotrophus* and *A. faecalis.*

Follow-up studies on nitrate removal ability with sodium acetate using *P. pantotrophus* under varying concentrations indicated that bacterial growth exhausted carbon sources in cases where concentrations of organic matter and bacteria were low during the initial stage, thereby depleting an electron donor for denitrification to eventually impair sufficient removal of nitrate. In cases where the concentration of organic matter was low, early-stage bacterial concentration would be low as well; however, if organic matter was continuously added, the bacterial concentration would gradually increase and gain sufficient AD ability. In short, it is possible to realize viable AD as long as a high enough bacterial concentration is maintained, even under conditions where low concentrations of organic matter prevailed.

In this study, we therefore first investigated the time-related changes in nitrate concentration in batch tests to subsequently evaluate the AD ability of *P. pantotrophus* under high early-stage bacterial concentrations. Thereafter, an efficient approach to sustain bacterial cultures using a membrane-separation continuous bio-reactor was adopted, and the AD ability of said bacterium and its competitive relationship with other species were examined under conditions where contamination with external bacteria on a long-term basis was possible.

Methods

Preparation of bacterium

A lyophilized strain of *P. pantotrophus* (ATCC 35512) from the American Type Culture Collection with most optimal growth in ATCC medium-1396 was proliferated under aerobic conditions at 28 °C. Bacteria were then treated with 200 mgN/L (KNO_3) and 4 gC/L (CH_3COONa) and continuously proliferated under said culture conditions. Colonies that indicated an exponential growth phase were rapidly frozen in liquid nitrogen before storage at −80 °C until use. All procedures were performed under sterilized conditions.

Test of initial bacterial density

Batch tests were conducted under various culture conditions to evaluate the AD ability of *P. pantotrophus* in cases where early-stage high bacterial concentrations were designated (Table 2). Conical flasks of 500-mL volume were used. The ATCC medium-1396 was employed as the culture medium, and sterilized CH_3COONa, KNO_3 and NH_4Cl were added to establish the designated concentrations.

Meanwhile, lyophilized bacterium *P. pantotrophus* was thawed and treated with 200 mgN/L (KNO_3) and 4 gC/L (CH_3COONa) under aerobic culture conditions. Cultured *P. pantotrophus* was added to the various appropriate designated early-stage bacterial concentrations. The internal liquid phase of conical flasks was fixed at 250 mL, and bacterial culture was sustained in an aerobic condition by closure of the flask mouth with

Table 1 Results from the previous study and scope of this study

	Evaluation points	Bacteria used	Carbon source	Results	Ref.
1	Carbon source effects	*P. pantotrophus, A. faecalis, M. aerodenitrificans*	Sodium acetate, ethanol, glucose, leucine, peptone	*P. pantotrophus* and *A. faecalis* showed higher ability of nitrate removal when sodium acetate was added.	
2	Conc. effects	*P. pantotrophus*	Sodium acetate	Even if optimum carbon was used, *P. pantotrophus* showed low ability of aerobic denitrification when the concentration was below 500 mgC/L.	Otani *et al.* (2004)
3	Carbon repetitive addition			When carbon was added repetitively and bacterial density reached 1.0, nitrate began to be reduced even under 100 mgC/L.	
4	Initial bacterial density				This paper
5	Continuous operation				

Table 2 Conditions for the batch test of initial bacterial density

	Initial OD_{580}	Carbon [mgC/L]	Nitrate [mgN/L]	Ammonium [mgN/L]
A	2.66	100	20	20
B	2.07	100	20	0
C	1.92	50	20	0
D	1.07	50	20	0

a silicon stopper while revolving at 90 rpm at an internal temperature of 28 °C. The early-stage bacterial concentration registered an optical density of 1.07–2.66 at 580 nm (OD_{580}), because nitrate removal was observed in the previous study (Otani *et al.*, 2004) only when OD_{580} reached above 1.0. Sampling employed sterilized Pasteur glass pipettes with all procedures conducted under strictly sterilized conditions.

Continuous operation using MBR

A membrane-separation continuous bio-reactor (Figure 1) was installed to maintain high bacterial concentrations and afford conservation of the bacteria at various growth stages. The reactor with a 5-L volume was installed in a bath equipped with a heater to maintain a constant designated temperature. The heated bath was consistently filled with circulating warm water (28 °C) to facilitate maintenance of a thermally constant environment. Wastewater removal was executed via a hollow fiber membrane (pore size: 0.1 μm; Mitsubishi Reyon). Frozen-stored *P. pantotrophus* was thawed, and again treated with 200 mgN/L (KNO_3) and 4 gC/L (CH_3COONa) under aerobic culture conditions to adjust the early-stage bacterial concentration at $OD_{580} = 2.4$ before initiating operation by adding said bacterium in the reactor under sterilized conditions. The substrate concentrate was sterilized at 121 °C under high pressure for 20 min and mixed with reverse-osmosis (RO) water before being subjected to inflow to yield 100 mgC/L (CH_3COONa) and 200 mgN/L (KNO_3). The hydrological retention time (HRT) was 7 h, and bacterial removal was not performed during the experimental period. Room air was delivered into the reactor at 13 L/min via a diffuser to persistently maintain aerobic conditions. Sampling was performed with sterilized Pasteur glass pipettes, and all procedures were conducted under sterilized conditions.

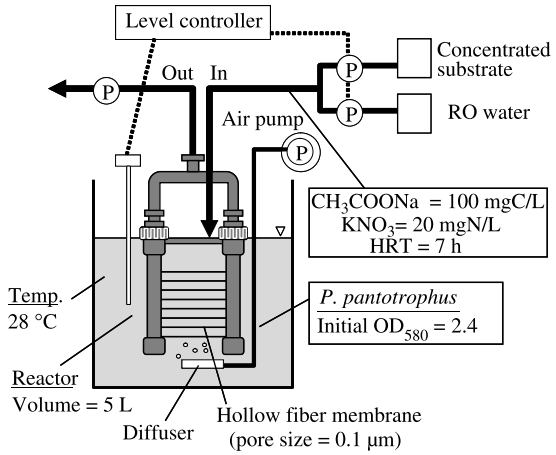

Figure 1 Experimental unit of membrane nitrogen removal process

Parameter analysis of water quality

Parameters including nitrate-N, nitrite-N and acetate ions were analyzed by an ion-chromatograph (Compact IC, Metrohm). On microbial community analysis, the optical concentration at 580-nm wavelength (OD_{580}) was measured with a spectrophotometer (U-2200, Hitachi).

Microbial community analysis

All bacteria were subjected to microbial community analysis with 16SrDNA as the target. DNA extracted from the samples with the Fast DNA spin kit for soil (Qbiogene, USA) was subjected to the analysis using the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method. Region V3 was subjected to PCR, which was performed with primer sets of 357f (*Escherichia coli* positions: 341–357, sequence: 5′-GC clamp-CCTACGGGAGGCAGCAG-3′; target: Bacteria) and 518r (*E. coli* positions: 517–534, sequence: 5′-ATTACCGCGGCTG CTGG-3′; target: Bacteria, Archaea and Eucarya). With PCR products designated for DGGE, the 5′ terminal of forward primer was attached to GC-clamp with a 40-bp GC-rich sequence of 5′-CGCCCGCCGCGCGCGGCGGGCG -GGGCGGGGGCACGGGGGG-3′. Temperature conditions were established according to those of Muyzer *et al.* (1993), and PCR was conducted using a thermal cycler (GeneAmp 9600; Applied Biosystems, USA) with 94 °C: 9 min→(94 °C: 30 s → 53 °C: 30s → 72 °C: 30 s) × 30 cycles → 72 °C: 5 min of temperature conditions. In addition, nitrite-reducing enzyme genes (nirS) subjected to PCR-DGGE were performed according to Braker *et al.* (1998) using primer sets nirS2F (TACCACCC(C/G)GA (A/G)CCGCGCGT) and nirS3(GCCGCCGTC(A/G)TG(A/C/G)AGGAA) where the 5′ terminus of forward primers was attached with GC-clamp with a 40-bp GC-rich sequence (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG). After the PCR products were subjected to electrophoresis with the Dcode System (Biorad, USA) using 130 V under conditions of 60 °C for 5 h and staining with Vistra Green (Amersham Pharmacia, USA), images were then captured using the Fluor-Imager 595 (Molecular Dynamics, USA). The bands separated by PCR-DGGE on gels were first isolated and then subjected thrice to thawing for DNA dissolution to facilitate interpretation of the base sequences. The DNA templates were subjected again to PCR-DGGE with the primer sets (357fGC–518r). As a one-time procedure is unable to refine a DGGE band under normal conditions, 5–6 repeats were performed in order to secure a single target band on DGGE images. After band purification, PCR was performed with the same primer sets, and the products thus obtained were used for sequencing reactions. Sequencing was conducted with the Vistra Sequencing kit using a sequencer (SQ-5500, Hitachi, Japan). Interpreted base sequences were then compared with previously known sequences using the Similarity Search Program BLAST (http://www.ddbj.nig.ac.jp/search/blast-j.html).

Results and discussion

Initial bacterial density effects

The results of batch tests conducted where early-stage high bacterial concentrations were designated are shown in Figure 2. While no nitrate removal was observed at the early stage under low initial bacterial density in the previous study (Otani *et al.*, 2004), nitrate removal was initiated just after the start-up under any experimental conditions. It indicated that high initial bacterial concentration makes it possible to reduce nitrate even under lower C/N conditions.

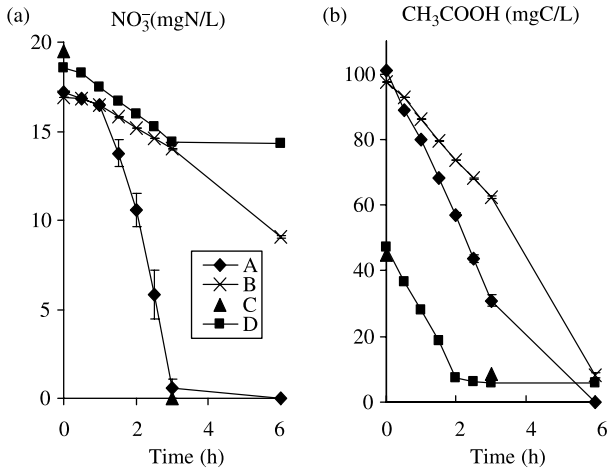

Figure 2 Temporal changes of (a) nitrate and (b) carbon under high bacterial density

Progress of denitrification during continuous operation

Results of nitrate removal during the continuous culture operation are shown in Figure 3. Although the ratio of nitrate removal increased to 66.5% on day 4, gradual time-related decreases in the denitrification rate were thereafter observed, indicating eventually a reduced ratio of 18.1% on day 20 after the start-up. Throughout the experiment, increase of bacterial density was observed and acetate was not detected in the effluent, implying that the carbon element was totally exhausted due to bacterial growth.

When the inflow carbon concentration was doubled from 100 mg to 200 mg C/L on day 23, increases in the denitrification ability were noted followed by further denitrification increases to 59.6% on day 24 after the start-up. This also implied that carbon shortage caused the decrease of nitrate removal ratio. While the MBR system is suitable for keeping bacterial density, it also brings carbon shortage due to bacterial growth.

Microbial community changes during continuous operation

Many studies were conducted about continuous nutrient removal using pure cultures; however, rare attention has been paid to contamination from outside. As the pump, inflow

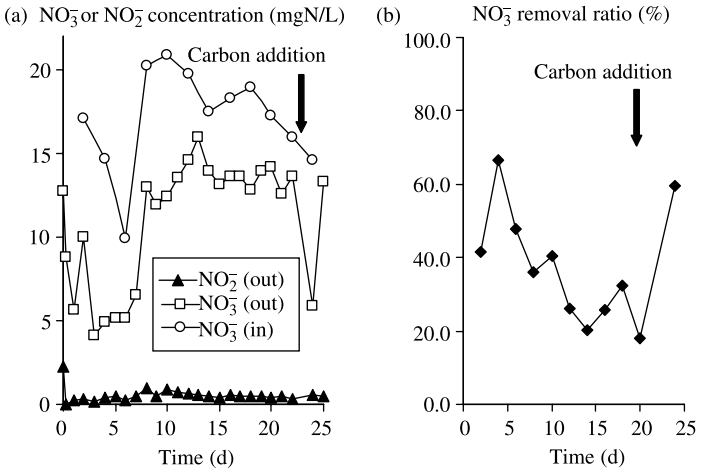

Figure 3 Nitrogen removal during continuous operation (a) temporal changes in concentrations (b) temporal changes in nitrate removal ratio

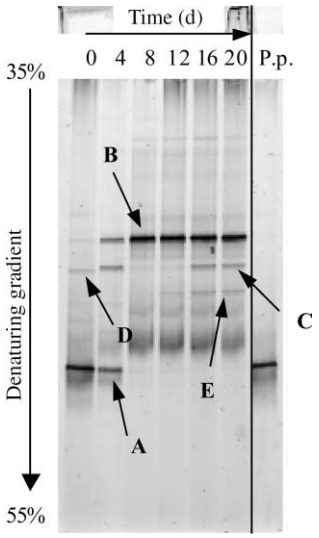

Figure 4 Temporal changes of bacterial community in the continuous reactor

tubing, and other possible bacteria-contaminated routes were present, time-related microbial community changes in the culture tank should be confirmed. The analysis results of extracted suspending samples in the culture tank with the PCR-DGGE (Figure 4) revealed interestingly that the well-demarcated band A of *P. pantotrophus* on initiation of experiment appeared less defined on day 4 and diminished by day 8 after start-up of the experiment. A different band B appeared on day 4, followed by other predominating species in the tank on day 8 after start-up.

According to sequencing results of the respectively detected bands (Table 3), the most closely related species to the predominant bacterium (B) was *Acidovorax* sp. 'samrlab 133385' (Accession No. AY093698). This bacterium is generally found in activated sludge. Microbes were not only suspended in the culture tank, but were also found adhered to the membrane and level-sensor. The adhered microbe colonies were sampled on termination of experiment (day 25 after start-up) to confirm if they had been replaced completely from strain *P. pantotrophus*. From suspending samples in the culture tank on day 20 after start-up (Figure 5), although band B (s20) appeared to be dominant, strain *P. pantotrophus* of the bacterial colonies adhered to the membrane (m1, m2) and the bottom of reactor (b1, b2) posted in a more predominating fashion. Despite the fact that bands A and B prevailed in bacterial samples adhering to the level-sensor (L1, L2), more intensely defined bands C–E appeared as well, suggesting that species other than those of bands A and B were propagating well near termination of experiments. From samples (1, 2) taken at the same site, no particularly marked differences in the microbial community analysis were encountered.

Table 3 Sequence similarities of excised DGGE bands

Band	Closest relative in database (accession No.)	Similarity
A	*Paracoccus pantotrophus* strain LMG (Y17511)	98%
B	*Acidovorax* sp. 'smarlab 133815' (AY093698)	100%
C	*Flavobacterium* sp. 'smarlab biomol-2300973' (AY230767)	100%
	Chryseobacterium sp. WCI (AY054744)	100%
D	Uncultured proteobacterium clone MT11 (AF058383)	100%
E	Uncultured bacterium clone Sta 1-41 (AY289470)	100%

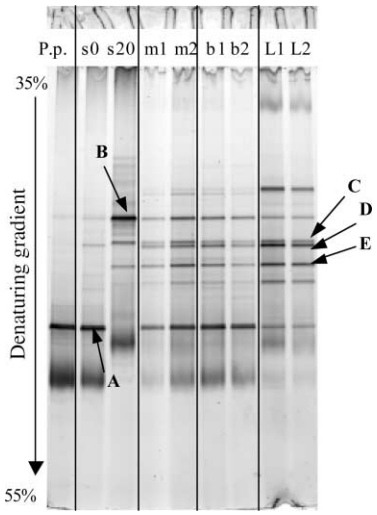

Figure 5 Differences of bacterial community structure in each place of the continuous reactor
P.p.: *P. pantotrophus*, pure culture; s0: suspended sample at day 0; s20: suspended sample at day 20; m: membrane attached; b: bottom attached; L: level sensor attached

Nitrate removal ability of predominating microbial communities

In the previous section, predominating species other than strain *P. pantotrophus* were observed in the suspending microbial communities. Therefore, the batch experiment was carried out in order to clarify whether the replaced bacteria were involved in AD or not. Using the suspending samples respectively taken on days 9 and 19 after start-up, the changes of nitrate content were monitored with KNO_3 treatment under aerobic conditions. After extraction, the respective samples were added to a cryotube under sterilized conditions before the frozen-stored bacteria at $-80\,^{\circ}C$ were thawed, and incubated in media previously treated with 200 mgN/L (KNO_3) and 4 gC/L (CH_3COONa) under aerobic conditions at 28 °C. According to changes in nitrate and nitrite concentrations (Figure 6), nitrate was exhausted on day 2 without signs of nitrite accumulation, implying that certain species among the microbial communities displayed AD ability.

PCR-DGGE was performed on the DNA sample (similar to that supplied for PCR-DGGE analysis for region V3) with nirS as the target to further confirm if denitrification ability was present or not. From results of the nirS analysis (Figure 7), a new nirS band appeared on day 4, followed by gradual then complete disappearance of

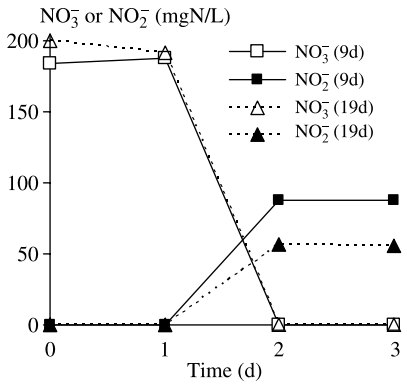

Figure 6 AD ability of replaced cultures

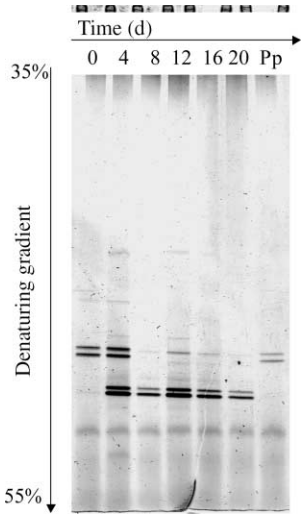

Figure 7 Community analysis using nirS; 0–20: the day when the sample was taken; P.p.: *P. pantotrophus*, pure culture

the *P. pantotrophus*-derived nirS band on day 8. The fact that a different nirS band predominated from day 8 to 20 implies that strains with predominating AD abilities other than those appropriated in the present study were present.

Discussion

P. pantotrophus possesses two types of dissimilative nitrate reductases: the membrane-bound nitrate reductase (NAR) that is expressed under anoxic conditions and conversely the periplasm-type nitrate reductase (NAP) that is expressed under aerobic conditions (Bell and Ferguson, 1991). As a high reduction potency is required in said NAP-induced nitrate reduction (Richardson and Ferguson, 1992), the organic matter which serves as a source of furnishing reduction potency could be taken as the limiting factor in the present aerobic system using said bacterium. According to Ellington *et al.* (2002), the nap operon effect is especially subjected to and determined by the type of organic matter; the higher the reduction potency of the organic matter concerned (e.g. butyrate, etc.), the higher the effect on the nap operon regulation. The organic matter used was indeed the limiting factor even in the continuous operation in this study; viz., nitrate reduction was in fact promoted by addition of the organic matter. MBR is considered to be a good system for keeping high bacterial density, but it also has the weak point as shortage of carbon source due to the high density.

The fact that strain *P. pantotrophus* can be simply replaced by *Acidovorax* group in the suspending phase of the continuous culture operation and remains adhered to the membrane surface and in the bottom of the reactor implies that the bacterium affixed to the carrier to sustain a high bacterial concentration, rendering it as a possible useful candidate in the treatment of wastewater containing high concentrations of organic substances.

Conclusions and perspectives

The following results using *P. pantotrophus* were obtained.

(1) Nitrate removal was confirmed in aerobic conditions where high microbe concentrations were sustained. However, organic matter still served as the limiting factor even in the continuous culture operation, where denitrification was realized under aerobic conditions; the transiently attenuated denitrification rate recovered when the concentration of organic matter was increased, especially in continuous operation.

(2) Although *P. pantotrophus* prevailed as residual adherents within the system in the membrane-separation continuous operation, other microbe communities (with the *Acidovorax* group as the focal species) remained predominant in the suspending phase.
(3) With predominating microbes displaying AD ability in the suspension phase, adopting *P. pantotrophus* would be favorable for wastewater treatment with a high C/N ratio, or useful if said bacterium could be carrier-fixed and added into the treatment system.

Furthermore, exploring and identifying AD manifesting species with higher potencies other than that of strain *P. pantotrophus* is a useful alternative that warrants future pursuit.

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