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Simultaneous nitrification and denitrification by diverse *Diaphorobacter* sp.

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Abstract Eight bacterial isolates closely related to *Diaphorobacter* sp. were isolated from activated biomass surviving on wastewater laden with dyes and nitro-substituted chemicals and were identified by 16S rDNA sequence analysis. The isolates showed sequence similarity of 99–100% to other *Diaphorobacter* strains such as ZY 2006b, F2, NA5, PCA039, *D. nitroreducens* KSP4, and KSP3 and 98–99% sequence homology to *D. nitroreducens* NA10B (type strain JCM 11421). Neighbor-joining tree revealed that all the eight strains formed tight cluster together and also showed close clustering with other *Diaphorobacter* strains. Isolates demonstrated the ability to perform simultaneous nitrification and denitrification under aerobic conditions. Strains HPC 805, 815, 821, and 856 gave highest chemical oxygen demand removal (85–93%) and ammonia removal (92–96%), which correlated well with higher growth rates of the cultures. Simultaneously, complete removal of nitrate supplied in the medium in presence of ammonium and acetate (electron donor) was observed in addition to aerobic nitrite release from ammonium. Thus, the above strains showed ability to perform partial nitrification followed by further aerobic removal of common intermediate nitrite, which indicated their potential application in treatment systems for treatment of high-nitrogen-containing wastewaters.

Keywords *Diaphorobacter nitroreducens* · Simultaneous nitrification denitrification · Ammonia-oxidizing bacteria

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Introduction

Removal of nitrogen by biological means is a widely adopted method for treatment of high-nitrogen-containing wastewaters due to its effectiveness and cost benefits. This process is normally carried out by different groups of bacteria by aerobic removal of ammonium (nitrification by autotrophic bacteria) and anaerobic conversion of the nitrate and nitrite to N_2 gas (denitrification by heterotrophic bacteria) under different aeration rates (Knowles 1982; Metcalf and Eddy 1991; Sharma and Ahlert 1977).

Various processes have been developed based on biological nitrification and denitrification which include short-cut nitrification and denitrification, anaerobic ammonium oxidation (Annamox), completely autotrophic nitrogen removal over nitrite (CANON) process, and oxygen-limited autotrophic nitrification–denitrification process which find widespread applications throughout the world (Peng and Zhu 2006). However, nitrification and denitrification via common intermediate nitrite (partial nitrification) has been found to be technically most feasible and economical method which has been employed successfully on a full scale in single reactor system for high ammonia removal over nitrite process (Mulder et al. 2001).

The latest advancement in biological nitrogen removal has been the development of a highly energy-efficient combination of process of partial nitrification with Annamox in a single reactor (CANON process; Third et al. 2005). This combined process consisted of addition of nitrifying culture to enriched Annamox culture in a reactor to initiate the CANON process in continuous mode which on stabilization was able to remove nitrogen for extended period at rate of 0.08 kg N m^{-3} day^{-1}.

Concurrent nitrification and denitrification (called simultaneous nitrification denitrification—SND) by aerobic

conversion of ammonium (NH_4^+) to N_2 gas has been known to occur naturally in biomass films or activated sludge flocs by virtue of the difference in dissolved oxygen (DO) levels at different points (von Munch et al. 1996). Certain groups of heterotrophic bacteria such as *Alcaligenes faecalis*, *Thiosphaera pantotropha*, *Comamonas*, etc. have also been known to possess activity of SND (Ferguson 1994; Joo et al. 2005; Otte et al. 1996; Patureau et al. 1997; Robertson and Kuenen 1988; van Niel et al. 1992). A novel bacterium belonging to family *Comamonadaceae* capable of performing SND was isolated from activated sludge and was named as *Diaphorobacter nitroreducens* (Khan and Hiraishi 2002).

Joo et al. (2005) demonstrated the effect of C/N ratios in removal of ammonia by an *A. faecalis* strain in presence of acetate and found C/N ratio of 10 to be optimum for ammonia removal, but at lower C/N ratio of 5, 40% of ammonium remained unconsumed. From the nitrogen balance, the authors found that, at C/N ratio of 10, up to half of the ammonium nitrogen was converted to intracellular nitrogen. Similar experiments on effect of C/N ratios on removal of ammonium in a sequencing batch reactor inoculated with sludge by Chiu et al. (2007) revealed that ammonium removal was better at high C/N ratio of 19.7 and reduction in C/N ratio to 6.3 resulted in decrease in ammonium removal efficiency.

In the present study, we attempted to isolate bacteria from activated biomass treating chemical and dye industries wastewater and capable of carrying our SND. A total of eight strains were characterized which showed potential for SND under aerobic conditions and belonged to the β-proteobacteria of *Comamonadaceae* family.

Materials and methods

Isolation and identification

The eight bacterial cultures were isolated from activated biomass from a common effluent treatment plant (CETP) treating wastes from chemical and dye industries and maintained in 30% glycerol stocks. The genomic DNA of the isolates was extracted according to Sambrook et al. (1989), and molecular taxonomic position of the isolates was determined by partial 5' 16S rDNA sequence comparison. The 16S rDNA was amplified, and the sequences were aligned on the CLUSTAL_X program (Thompson et al. 1997), and a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) and further evaluated by bootstrap sampling (Felsenstein 1985). The scale bar represents 0.1 fixed mutations per site, and bootstrap values were derived from 1,000 analyses. The phylogenetic tree was displayed using the TREEVIEW program (Page 1996), and the 16S rDNA sequences were deposited into the NCBI GenBank.

The genotypic variance in between the different isolates was determined by random amplification of polymorphic DNA (RAPD) analysis using standard primer (Amersham) with the following sequence 5′ d[AAGAGCCCGT] 3′. The RAPD reaction mixture consisted of the following components in a total volume of 50 μl: autoclaved distilled water—31.0 μl; 10X polymerase chain reaction (PCR) Buffer (with 15 mM of $MgCl_2$)—5.0 μl; dXTPs (200 μmol)—2.5 μl; primer (25 pmol)—5.0 μl; *Taq* polymerase (3 U μl^{-1})—1.5 μl; template DNA—5.0 μl; mineral oil—two drops. RAPD PCR program was carried out in Thermocycler (Perkin Elmer) for 40 cycles with each cycle consisting of three major steps, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min. The different band patterns were observed by agarose gel electrophoresis (2% gel).

Growth medium and culture conditions

A complex mineral medium as described by Blaszczyk (1993) was used for cultivation and studies on simultaneous nitrification and denitrification by the isolates. The composition of the medium (C/N ratio=8) was as follows (g l^{-1}): K_2HPO_4—7.0; KH_2PO_4—3.0; sodium citrate·$2H_2O$—0.5; $MgSO_4 \cdot 7H_2O$—0.1; $(NH_4)_2SO_4$—1.0 (7.5 mM NH_4^+–N); $FeSO_4 \cdot 7H_2O$—0.05; KNO_3—1.8 (20.9 mM NO_3^-–N); CH_3COONa—10.0. Initial pH was set at 7.0±0.2, and 250-ml capacity conical flasks containing 100-ml medium were used in the experiments, which were incubated at 30°C in an orbital shaker at 150 rpm. The experimental conditions remained the same in all the subsequent experiments unless specifically stated.

Studies on growth and SND by the isolates

One hundred microliters of the different *Diaphorobacter* strains viz HPC 805, HPC 815, HPC 820, HPC 821, HPC 834, HPC 847, HPC 848, and HPC 856 was inoculated from glycerol freezer stocks in 5-ml Luria–Bertani broth tubes. After 24 h of incubation, the cultures were centrifuged at 7,000 rpm for 10 min, washed twice with sterile distilled water, and resuspended in minimum amount of distilled water. The optical density was measured at 600 nm against distilled water blank and 1-O.D. cells were inoculated into experimental flasks. Samples were withdrawn at 24-h intervals for estimating the changes in pH, biomass, chemical oxygen demand (COD), nitrate, nitrite, and ammonium concentration. All physico-chemical analyses were performed as per standard methods (APHA, AWWA, WPCF 2005). Specific growth rates (μ) were calculated by the following formula:

$$\mu = \frac{d[X]}{[X] \times d[T]}$$

where [*X*] is the actual cell density expressed as O.D. at time [*T*], [*dX*] is change in cell density over a fixed interval of time *d*[*T*].

Enzyme assay

Cell-free extracts were prepared by subjecting pre-grown cultures to lysis by ultrasonication in presence of 20-mM phosphate buffer (disodium hydrogen phosphate+potassium dihydrogen phosphate, pH 7.0). The ultrasonicated samples were placed in a sonication bath for 30 min to detach the membrane-bound enzymes which were separated from the cell debris by centrifugation. Formation of nitrite from nitrate in presence of centrifuged cell-free extract in the reaction mixture was taken as a measure of nitrate reductase activity (Sanderson and Cocking 1964). Similarly, the disappearance of nitrite from reaction mixture in presence of cell-free extract was considered as a qualitative measure for nitrite reductase activity (Wray and Filner 1970). Protein concentration in the cell-free extract was determined by Folin-phenol reagent (Lowry et al. 1951) to determine the specific activity of the enzymes. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the transformation of 1 μmol of substrate per minute. The amount of enzyme activity per unit of protein was defined as the specific activity of a given preparation of material.

Results

The eight cultures were isolated from activated biomass of a CETP treating dye and chemical industry wastewater and tentatively numbered as HPC 805, HPC 815, HPC 820, HPC 821, HPC 834, HPC 847, HPC 848, and HPC 856. The genotypic characterization of the eight isolates was carried out by 16S-rDNA-sequence-based phylogenetic analysis. Partial sequencing of the 16S rDNA of the isolates (674–675 bases) was carried out and deposited in the NCBI GenBank with the accession numbers AY996989, AY996993, AY996996, AY996997, DQ131848, AY997013, DQ131850, and DQ131852, respectively (Kapley et al. 2007). Homology search revealed that all the isolated cultures shared sequence similarity of 99–100% to other *Diaphorobacter* strains such as ZY 2006b, F2, NA5, PCA039, *D. nitroreducens* KSP4, KSP3, and *Acidovorax* sp. 3DHB1. In addition, the isolates showed 98–99% sequence homology to *D. nitroreducens* NA10B (type strain JCM 11421) and *Acidovorax* sp. LW1 (type strain DSM 13225). With respect to other members of the family *Comamonadaceae*, the isolates showed sequence similarities of 95–98% to *A. aerodenitrificans*, *A. avinae* C1, *Acidovorax* sp. 1916, *Alicycliphilus* sp. R24604, R24611, *A. denitrificans* K601, *Alicycliphilus* sp. A8, *Aquaspirillum* sp. EMB 325, and *Simplispira* sp. R28033.

Figure 1 shows a neighbor-joining phylogenetic tree as prepared from the partial 16S rDNA sequences. Because complete sequences of closely related strains were available in the NCBI GenBank, while partial sequences of our isolates were available, neighbor-joining tree was prepared by taking partial lengths (800 bases) of the related sequences from NCBI GenBank from 5′ end. Partial sequences of type strains from the same group viz β-proteobacteria and other groups (one each) were included for demonstration of genetic relatedness. All our isolates were clustered together with HPC 820, HPC 821, HPC 856, HPC 815, and HPC 848 forming a tight cluster while HPC 805, HPC 847, and HPC 834 showing a slight divergence from this cluster. The eight strains also formed a close cluster with other *Diaphorobacter* strains and *Acidovorax* sp. LW1, in addition to strains belonging to *Alicycliphilus*, *Aquaspirillum*, and *Simplispira.* The tree showed a clear evolutionary divergence of this group of β-proteobacteria from type strains belonging to other groups such as *Acinetobacter*, *Citrobacter*, *Rhizobium*, *Campylobacter*, *Geobacter*, *Bacillus*, and *Methanobacterium*. Genetic variance between the eight different strains was confirmed by RAPD, which showed different band patterns in the different strains (Fig. 2).

The ability of the different *Diaphorobacter* strains to grow under conditions of SND was studied, and the growth profiles were evaluated in a time series analysis and expressed graphically in the form of specific growth rate (μ). It is seen from Fig. 3, that the growth rates were higher for isolates HPC 821 (1.016 O.D. ml^{-1} h^{-1}) and HPC 815 (0.762 O.D. ml^{-1} h^{-1}) with a decrease observed in the order HPC 820 > HPC 847 > HPC 848 > HPC 805 > HPC 856 > HPC 834.

In addition to growth, the removal of COD and ammonia by the different strains was monitored and is shown in Fig. 4. Strains HPC 805, HPC 815, HPC 821, and HPC 856 gave highest COD removal (85–93%) and ammonia removal (92–96%), while lower values were observed for remaining strains (70–80% COD removal, 80–85% ammonia removal). This correlated well with the higher growth rates seen in case of the above strains.

The corresponding nitrite released due to simultaneous nitrification of ammonia and denitrification of nitrate supplied in the medium increased with time, and a maximum NO_2^-–N concentration of 19.85 mM was detected after 96 h in case of strain HPC 815. The NO_2^-–N concentration observed in case of strains HPC 805 was slightly lower (17.85 mM), which decreased to 14.3 mM for HPC 821, HPC 848, and HPC 856, while lower values were observed for the remaining strains (Fig. 5). Further denitrification of nitrite was indicated by the decrease in levels of NO_2^-–N after 96 h; however, no NO_3^- was detected in the medium. Final pH increased to 9.0–9.5 at the end of incubation for all the isolates due to the utilization of acetate from the medium.

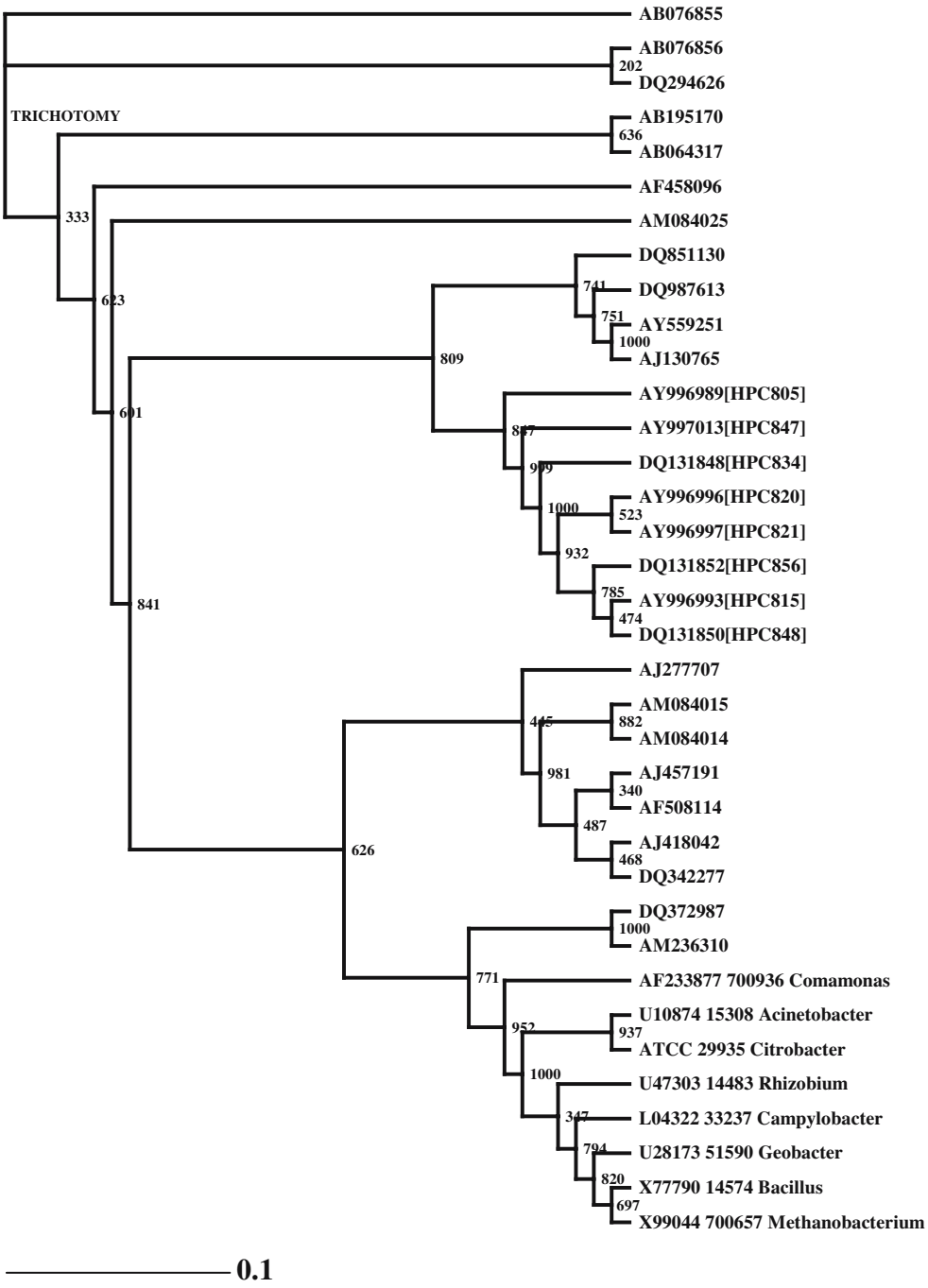

Fig. 1 Neighbor-joining matrix tree based on partial 16S rDNA sequences showing phylogenetic relationships between our laboratory isolates, other members of *Comamonadaceae*, and type members from other groups. Names of the different cultures along with the accession numbers for the 16S rDNA sequences are given in the *parentheses*. Bootstrap values are mentioned at branching points

Maximum activity of nitrate reductase was observed in HPC 856 (0.39 U ml^{-1}) with a specific activity of 0.075 U mg^{-1} protein followed by 0.17, 0.09, and 0.01 U activity in case of HPC 805, HPC 815, and HPC 821, respectively. Nitrate reductase activities were very low in case of the remaining isolates. Reduction in the concentration of nitrite from reaction mixture in presence of cell-free extract of all the isolates indicated the existence of nitrite reductase activity in all the *Diaphorobacter* strains.

Discussion

The efficiency of wastewater treatment plants for nitrogen removal by SND can be judged from the amount of ammonia oxidized to nitrite and further to nitrate followed by reduction of nitrate under anoxic conditions. The process of SND can be considered economical provided nitrification stops at nitrite formation level (partial nitrification), which instead of nitrate serves as substrate for denitrification and is reduced to N_2,

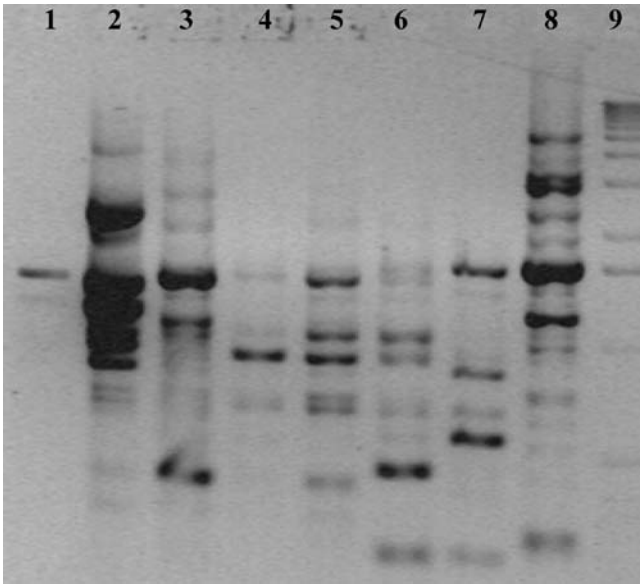

Fig. 2 RAPD profile of different *Diaphorobacter* strains (*lane 1* HPC 805, *lane 2* HPC 815, *lane 3* HPC 820, *lane 4* HPC 821, *lane 5* HPC 834, *lane 6* HPC 847, *lane 7* HPC 848, *lane 8* HPC 856, *lane 9* 1Kb ladder)

thus resulting in reduction in usage of oxygen and reducing power. Conventionally, this process has been carried out in presence of organic matter or added substrates such as acetate, methanol, ethanol, citrate, etc. as electron donors, in addition to intracellular polymers such as polyhydroxybutyrate (Joo et al. 2005; Khan and Hiraishi 2001; Yoo et al. 1999; Zhao et al. 1999).

Partial nitrification is essential for economical operation of wastewater treatment systems by promoting accumulation of heterotrophic ammonia-oxidizing bacteria (AOB) and the washout of nitrite-oxidizing bacteria (NOB) responsible for oxidation of nitrite to nitrate (Laanbrock and Gerards 1993). The above explanation holds true for mixed bacterial systems in wastewater treatment plants where these two

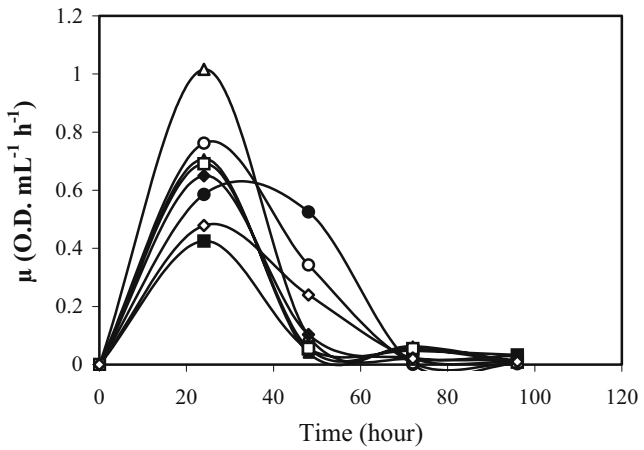

Fig. 3 Growth profiles of *Diaphorobacter* strains in denitrifying medium (*filled circle* HPC 805, *open circle* HPC 815, *filled inverted triangle* HPC 820, *open triangle* HPC 821, *filled square* HPC 834, *open square* HPC 847, *filled diamond* HPC 848, *open diamond* HPC 856)

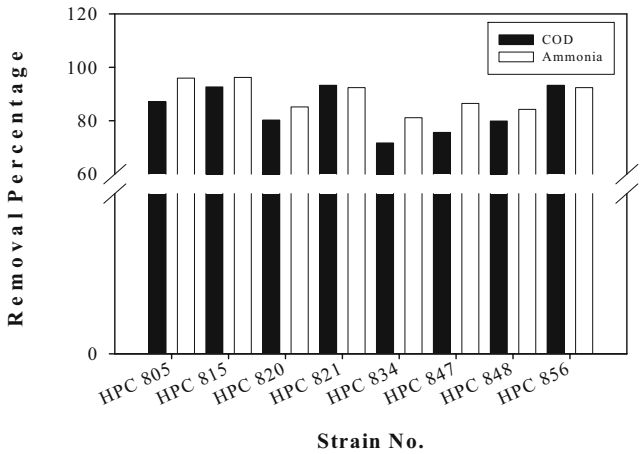

Fig. 4 Percentage COD and ammonia removal by *Diaphorobacter* strains at the end of 96 h of incubation

groups of bacteria coexist and their numbers varied based on the surrounding levels of DO, pH, ammonium concentration, temperature, sludge age, etc., with higher levels of ammonium favoring faster growth of AOB (Peng and Zhu 2006). Peng and Zhu have also reviewed the different growth patterns which were known to occur within the anaerobic AOB belonging to *Planctomycetes* (fast growth pattern at lower substrate and high ammonium concentrations and slow growth pattern in presence of high substrate concentration). However, similar growth patterns were also observed among the aerobic *Diaphorobacter* strains in our study because, according to Wong-Chong and Loehr (1978) and Gibbs et al. (2004), ammonium concentrations higher than 3.5 mg NH_3–N l^{-1} inhibited further nitrification of nitrite to nitrate and allowed the denitrification to proceed under aerobic conditions. The lack of nitrate formation was attributed to the fact that pure cultures of heterotrophic AOB did not possess the ability of forming nitrate from nitrite, a reaction which was carried out by NOB in wastewater treatment systems (Peng and Zhu 2006).

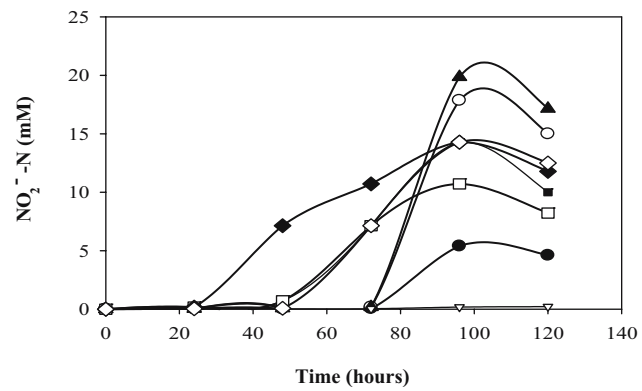

Fig. 5 Nitrite release by *Diaphorobacter* strains in denitrifying medium (*filled circle* HPC 805, *open circle* HPC 815, *filled inverted triangle* HPC 820, *open triangle* HPC 821, *filled square* HPC 834, *open square* HPC 847, *filled diamond* HPC 848, *open diamond* HPC 856)

Our results supported this theory, and simultaneous nitrification of ammonium at high rates by the eight *Diaphorobacter* isolates leading to formation of nitrite was observed, followed by denitrification resulting in a decrease in nitrite concentration on further incubation. In our study, presence of 7.5 mM NH_4^+–N (212 mg l^{-1} NH_4^+–N) allowed the complete reduction of 20.9 mM NO_3^-–N (162 mg l^{-1} NO_3^-–N) under shake flask conditions indicating efficient SND through common intermediate nitrite. The percent conversion of total available nitrogen (N; 7.5 mM NH_4^+–N+ 20.9 mM NO_3^-–N=28.4 mM NO_3^-) to nitrite varied between 17 and 70% for the different strains, and the absence of ammonium and nitrate indicated incorporation of the remaining amount of supplied nitrogen (30–83%) into the newly synthesized bacterial biomass. However, studies are required for evaluating the quality of off-gas with respect to the composition of gaseous products of denitrification especially to ascertain the absence of long-lived greenhouse gas such as N_2O. Thus, from the above studies, it was concluded that this group of bacteria belonged to the heterotrophic AOB capable of performing partial nitrification with characteristic absence of nitrate formation in the medium.

A periplasmic nitrate reductase (NAP) has been suggested to be involved in the aerobic conversion of nitrate to nitrite. NAP activity was not repressed by either ammonium or oxygen (Bedzyk et al. 1999; Bell et al. 1990). Celen and Kilic (2004) isolated 39 cultures with NAP activity in presence of different nitrogen sources (nitrate alone, nitrate+ ammonium, ammonium alone), and it was found that, when ammonium and nitrate were present together, aerobic nitrate reduction did not lead to high nitrite accumulation in contrast to higher nitrite accumulation when nitrate was the sole nitrogen source. From our studies, it was clear that both nitrate and nitrite reductase activities were present in all strains of isolated *Diaphorobacter*; however, further purification and characterization of the enzymes was required.

Very few reports are available on the diversity of this genus with three strains being isolated by Khan and Hiraishi (2002), which were named *D. nitroreducens* NA10B, KSP3, and KSP4, respectively. Similarly, Heylen et al. (2006) studied the diversity of denitrifying bacteria in sludge and of the 68 isolates belonging to the β-proteobacteria of family *Comamonadaceae*; only a single isolate belonging to genus *Diaphorobacter* (*D. nitroreducens*) was found to occur. Considering the rarity of this genus in effluent treatment plants, our study revealed a relatively high number of isolates of genus *Diaphorobacter* with capability of carrying out SND at different rates. Accordingly, further studies have to be carried out for SND by these bacteria in presence of alternative electron donors such as high-organic-containing wastewaters especially effluents from distilleries and dye industries. In addition, the characterization of purified nitrate and nitrite reductases from these organisms needs to be performed along with studies on nitrogen balance during the SND process.

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References

APHA AWWA WPCF (2005) Standard methods for the examination of water and wastewater, 21st ed. Washington D.C.

Bedzyk L, Wang T, Ye RW (1999) The periplasmic nitrate reductase in *Pseudomonas* sp. strain G-179 catalyzes the first step of denitrification. J Bacteriol 181:2802–2806

Bell LC, Richardson DJ, Ferguson SJ (1990) Periplasmic and membrane-bound respiratory nitrate reductase in *Thiosphaera pantotropha*. FEBS Lett 265:85–87

Blaszczyk M (1993) Effect of medium composition on the denitrification of nitrate by *Paracoccus denitrificans*. Appl Environ Microbiol 59(11):3951–3953

Celen E, Kilic MA (2004) Isolation and characterization of aerobic denitrifiers from agricultural soil. Turk J Biol 28:9–14

Chiu Y-C, Lee L-L, Chang C-N, Chao AC (2007) Control of carbon and ammonium ratio for simultaneous nitrification and denitrification in a sequencing batch bioreactor. Int Biodeterior Biodegr 59:1–7

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Ferguson SJ (1994) Denitrification and its control. Antonie van Leeuwenhoek 66:89–110

Gibbs BM, Shephard KA, Third KA, Cord-Ruwisch R (2004) The presence of ammonium facilitates nitrite reduction under PHB driven simultaneous nitrification and denitrification. Water Sci Technol 50(10):181–189

Heylen K, Vanparys B, Wittebolle L, Verstraete N, Boon N, de Vos P (2006) Cultivation of denitrifying bacterium: optimization of isolation conditions and diversity study. Appl Environ Microbiol 72(4):2637–2643

Joo H-S, Hirai M, Shoda M (2005) Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* No. 4. J Biosci Bioeng 100(2):184–191

Kapley A, Baere T-De, Purohit HJ (2007) Eubacterial diversity of activated biomass from a common effluent treatment plant. Res Microbiol 158(6):494–500

Khan ST, Hiraishi A (2001) Isolation and characterization of a new poly(3-hydroxybutyrate) degrading denitrifying bacterium isolated from activated sludge. FEMS Microbiol Lett 205:253–257

Khan ST, Hiraishi A (2002) *Diaphorobacter nitroreducens* gen. nov., sp. nov., a poly(3-hydroxybutyrate) degrading denitrifying bacterium isolated from activated sludge. J Gen Appl Microbiol 48:299–308

Knowles R (1982) Denitrification. Microbiol Rev 46:43–70

Laanbrock HJ, Gerards S (1993) Competition for limiting amounts of oxygen between *Nitrosomonas europaea* and *Nitrobacter winogradsky* grown in mixed continuous cultures. Arch Microbiol 159:453–459

Lowry OH, Rosenbrourgh NJ, Farr NJ, Randall JR (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Metcalf X, Eddy X (1991) Wastewater engineering: treatment, disposal and reuse, 3rd edn. McGraw-Hill, New York, pp 429–433

Mulder JW, van Loosdrecht MCM, Hellinga C, van Kempen R (2001) Full-scale application of the SHARON process for the treatment

of rejection water of digested sludge dewatering. Water Sci Technol 43(11):127–134

Otte S, Grobben NG, Robertson LA, Jetten MSM, Kuenen JG (1996) Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions. Appl Environ Microbiol 62:2421–2426

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Patureau D, Bernet N, Moletta R (1997) Combined nitrification and denitrification in a single aerated reactor using the aerobic denitrifier *Comamonas* sp. strain SGLY2. Water Res 31:1363–1370

Peng Y, Zhu G (2006) Biological nitrogen removal with nitrification and denitrification via nitrite pathway. Appl Microbiol Biotechnol 73:15–26

Robertson LA, Kuenen JG (1988) Heterotrophic nitrification on *Thiosphaera pantotropha* oxygen uptake and enzyme studies. General Microbiol 134:857–863

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanderson GW, Cocking EC (1964) Enzymic assimilation of nitrate in tomato plants I. Reduction of nitrate to nitrite. Plant Physiol 39:416–422

Sharma B, Ahlert RC (1977) Nitrification and nitrogen removal. Water Res 11:897–925

Third KA, Paxman J, Schmid M, Strous M, Jetten MSM, Cord-Ruwisch R (2005) Treatment of nitrogen-rich wastewater using partial nitrification and Anammox in the CANON process. Water Sci Technol 52(4):47–54

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins JD (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

van Niel EWJ, Braber KJ, Robertson LA, Kuenen JG (1992) Heterotrophic nitrification aerobic denitrification in *Alcaligenes faecalis* strain TUD. Antonie van Leeuwenhoek 62:231–237

von Munch E, Lant P, Keller J (1996) Simultaneous nitrification and denitrification in bench-scale sequencing batch reactors. Water Res 30:277–284

Wong-Chong GM, Loehr RC (1978) Kinetics of microbial nitrification: nitrite-nitrogen oxidation. Water Res 12:605–609

Wray JL, Filner P (1970) Structural and functional relationships of enzyme activities induced by Nitrate in Barley. Biochem J 119:715–725

Yoo H, Ahn K-H, Lee H-J, Lee K-H, Kwak Y-J, Song K-G (1999) Nitrogen removal from synthetic wastewater by simultaneous nitrification and denitrification (SND) via nitrite in an intermittently aerated reactor. Water Res 33(1):145–154

Zhao HW, Mavinic DS, Oldham WK, Koch FA (1999) Controlling factors for simultaneous nitrification and denitrification in a two stage intermittent aeration process treating domestic sewage. Water Res 33(4):961–970