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ORIGINAL PAPER

Identification of novel denitrifying bacteria *Stenotrophomonas* sp. ZZ15 and *Oceanimonas* sp. YC13 and application for removal of nitrate from industrial wastewater

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Abstract Two novel denitrifying bacteria were successfully isolated from industrial wastewater and soil samples. Using morphological, biochemical/biophysical and 16S rRNA gene analyses, these two bacteria were identified as *Stenotrophomonas* sp. ZZ15 and *Oceanimonas* sp. YC13, respectively. Both of these two bacteria showed efficient NO_3^--N removing abilities under a semi-anaerobic condition without obvious accumulation of NO_2^--N, N_2O-N and NH_4^+-N. NO_3^--N removal from paper mill wastewater was also successful by treatments with either a denitrifier or an immobilization method. Therefore, this study provides valuable denitrifying bacteria in biotreatment of industrial wastewater and other environmental pollution caused by NO_3^-/NO_2^-.

Keywords Biological nitrate removal · *Stenotrophomonas* · *Oceanimonas* · Denitrifying bacteria · Industrial wastewater · Immobilization

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Introduction

Nitrogen pollution has become the most serious environmental problem since it causes algae blooms. Algae decreases water dissolved oxygen so severely that final result is the death of fish and other aquatic organisms. The major sources of nitrogen pollution are the nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_4^+) that mainly originated from the emission of industrial wastewater and the use of excess fertilizers in agricultural fields. Biodenitrification is considered as the most effective process in nitrogen polluted wastewater treatments (Schmidt et al. 2003; Choi et al. 2004; Zhu et al. 2008). For example, nitrate contamination in groundwater could be reduced by natural denitrification using indigenous microorganisms (Mohamed et al. 2003). Denitrifying microorganisms (denitrifiers) capable of reducing NO_3^-, without accumulation of NO_2^- (nitrate reduction, $NO_3^- \rightarrow NO_2^-$) and NH_4^+ (nitrate assimilation, $NO_3^- \rightarrow NH_4^+$) provide potential applications in industrial wastewater cleaning systems. The final products of biodenitrification are the gaseous nitric oxide (NO), nitrous oxide (N_2O) or nitrogen (N_2) (Zumft 1997). Reduction of the emissions of gaseous nitrogen compounds (NO*x*) to the atmosphere is also important, since NO or N_2O may cause greenhouse effect, ozone depletion and photochemical air pollution (Park et al. 2000).

Denitrifiers are widespread in soil, sediment and aquatic ecosystem and include bacteria (Betlach 1982; Knowles 1982; Robertson and Kuenen 1984; Shooner

et al. 1996), archaea (Braker et al. 2001; Philippot 2002; Cabello et al. 2004; Raghoebarsing et al. 2006; Thauer and Shima 2008), and fungi (Kobayashi et al. 1996; Zhou et al. 2002; Watsuji et al. 2003). So far, denitrifying capabilities have been detected in about 150 species within 50 genera (Chen et al. 2002; Shieh et al. 2004; Horn et al. 2005; Jean et al. 2006; Lee and Koo 2007; Liu et al. 2007; Sorokin et al. 2007). Among them, *Pseudomonas*, *Neisseria* and *Bacillus* are the most common denitrifying bacteria (Zumft 1997), and their denitrification processes are carried out under anaerobic/semi-anaerobic conditions. However, *Alcaligenes faecalis* and *Paracoccus denitrificans* have shown the abilities to perform denitrification by simultaneous utilization of oxygen and nitrate as electron acceptors (Rehfuss and Urban 2005).

A number of studies have used active sludge containing mixed microorganisms to treat high nitrogen wastewater (Gabald'on et al. 2007). Isolation and identification of denitrifying bacteria from wastewater revealed that genera *Achromobacter*, *Alcaligenes*, *Aquaspirillum*, *Azoarcus*, *Bacillus*, *Brachymonas*, *Paracoccus*, *Pseudomonas*, *Rhodocyclus*, *Thauera*, were usually present in the mixed cultures that denitrify successfully (Kniemeyer et al. 1999; Sakano et al. 2002; Leta et al. 2004; Yoshie et al. 2004; Rehfuss and Urban 2005; Thomsen et al. 2007; Wang and Lee 2007). However, researches on denitrification abilities of individually cultivated strain in industrial wastewater environments have been limited.

The objectives of this research were to: (1) isolate novel denitrifying bacteria and study their biological denitrification activities. Identification of the denitrifiers was performed using morphological, biochemical/biophysical and 16S rRNA gene analyses; (2) evaluate nitrogen removing efficiencies of the denitrifiers in industrial wastewater environments. The novel denitrifying bacteria may have superior potentials in NO_3^-/NO_2^- bioremediation in various environments.

Materials and methods

Sample collection and isolation of denitrifying bacteria

For isolation of denitrifying bacteria, an industrial wastewater sample and a soil sample were used. The wastewater sample originated from a paper mill effluent in Wuhan city, Hubei province, central China. The soil sample was collected from the surface soil horizon (0–15 cm) near the Yellow Sea beach of Yancheng city, Jiangsu province, eastern China. Fresh samples were immediately used for bacterial isolation.

The denitrifying bacteria isolation were carried out by adding 10 g soil or 10 ml wastewater (triplicates) to 90 ml of 0.85% NaCl solution and incubated at room temperature for 30 min with 150 rpm shaking. The extraction solution was serially diluted and plated onto nitrate medium plates [KNO_3 2 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.2 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, $C_4H_4O_6KNa{\cdot}4H_2O$ 20 g l^{-1}, and 1.6% agar; pH 7.2]. The plates were incubated at 37°C for 1 week. Single colonies were restreaked several times to obtain pure isolates.

Qualitative biochemical analysis was at first performed to screen bacteria for denitrification capabilities. Fifty microlitre of each bacterial culture ($OD_{600} = 1.0$) was inoculated into 10 ml nitrate liquid medium in a culture tube with a screw-on cap to create an airtight condition and kept at 37°C for up to 9 days without shaking. Each day, 0.5 ml of each culture was transferred into a microcentrifuge tube and analyzed for their denitrification potential using the alpha-naphthylamine method as described by Wang and Skipper (2004). The strains that showed visible gas and the abilities of nitrate reduction with no obvious nitrite and ammonia accumulation were determined as denitrifying bacteria.

Morphological and biochemical/biophysical analyses of the denitrifying bacteria

Colony morphologies of the isolates were monitored on nitrate medium plates after incubating at 37°C for 7 days. Cell morphologies were examined under a JSM-6390/LV scanning electron microscope (JSM-6390, JEOL, Japan) with 20,000 V accelerating voltage and 20,000 times enlargement. Gram staining was performed using colonies on nitrate plates as described by Bailey and Scott (1966).

Biochemical and biophysical analyses were performed according to Bergey's manual (Holt et al. 1994). Characteristics of the denitrifying isolates, such as optimum grow temperature and pH, catalase and oxidase activities, hydrolysis of starch, gelatin

liquefaction, Voges–Proskauer (V. P.) test, Methyl Red (M. R.) test, indole production, the utilization of sole carbon/nitrogen sources, were tested.

16S rRNA gene identification, DNA sequencing and phylogenetic analysis

DNA of each strain was extracted using standard methods (Sambrook and Russell 2001). The nearly full-length 16S rRNA gene of each denitrifier was amplified by PCR using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Wilson et al. 1990). PCR amplification was performed in a 50 µl volume containing 10 ng DNA, 50 ng of each primer, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 5 µl of 10× PCR buffer [100 mM Tris–HCl (pH 8.3), 100 mM KCl] and 2.5 units of *Taq* DNA polymerase (Fermentas, Hanover, MD, USA). The PCR program consisted of an initial 5 min denaturation step at 94°C; 30 cycles of 45 s at 94°C, 45 s at 49°C, 1.5 min at 72°C; and a final 5 min extension step at 72°C.

The PCR products (~1,460 bp) were separated on a 1% agarose gel and purified using the UltraPure™ PCR Kit (SBS Genetech, Beijing, China). DNA sequencing analysis was performed using ABI 3730XL DNA analyzer by SUNBIO Company (Beijing, China). Sequences were analyzed by BlastN searching tools (http://www.ncbi.nlm.nih.gov/blast). The sequences were edited for the same lengths and compared using ClustalX 1.83 software (Thompson et al. 1997). The phylogenetic tree was constructed using the neighbor-joining distance method with the MEGA 3.1 software (http://www.megasoftware.net; Kumar et al. 2004) and the reliability of the inferred tree was tested by 1,000 bootstrap. Some reference sequences from the GenBank were used in generating the phylogenetic tree for clarification.

Quantitative analysis of nitrate removing efficiencies of the denitrifying bacteria

The nitrate broth (nitrate plate medium without agar which contains 2 g l^{-1} KNO_3 = 277 mg l^{-1} NO_3^--N) was used to determine the denitrification efficiencies of these two strains. One milliliter of each denitrifying bacterial culture (OD_{600} = 1.0) was inoculated into each 100 ml nitrate medium in a flask with a special rubber stop, to which was linked a gas collection set and the inoculums were incubated at 37°C for up to 9 days without shaking (a semi-anaerobic condition). Controls were prepared using the same nitrate broth without inoculating a denitrifying bacterium. At regular intervals, 10 ml of each culture was centrifuged at 12,000 revmin^{-1} and the supernatant was filtered using CAD-40 a Macroporous Resin column (Sanxing Resin, Anhui, China).

The concentrations of NO_3^--N, NO_2^--N, NH_4^+-N, and the growth of denitrifying bacteria were determined using a UV spectrophotometer (DU800, Beckman, CA, USA). The growth of each denitrifier was monitored by measuring the absorbance value at 600 nm. The NO_3^--N concentration was calculated by the absorbance value at 220 nm subtracted with the background absorbance value at 275 nm (National Environmental Protection Administration of China 2006). The absorbance of the NO_2^--N was measured at 540 nm after mixing 1 ml of each culture with 1 ml sulfanilamide and 1 ml *N*-1-naphthylethylenediamine × 2HCl (diazo-coupling reaction, Greenberg et al. 1992). The absorbance of the NH_4^+-N was measured at 420 nm according to the ammonium–Nessler's reagent colorimetric method (Greenberg et al. 1992). The nitrate removal efficiency was calculated as [(NO_3^--N initial−NO_3^--N final−NO_2^--N final−NH_4^+-N final−N_2O-N final)/NO_3^--N initial] × %N_2O concentrations in the headspace (about 175 ml) were measured with a gas chromatograph containing an electron capture detector (ECD) and a packed Poropak Q column (CP-3800, Varian, CA, USA). Temperature of the detector and oven were 330°C and 55°C, respectively. N_2 containing CO_2 (5%) was supplied as a carrier gas and the flow rate was 1 ml min^{-1}. The dissolved oxygen (DO) concentration was monitored by use of a DO meter (Model DO200, YSI, OH, USA).

Removal of nitrate from industrial wastewater

The industrial wastewater was collected from the same paper mill for bacterial isolation in October 2007. The wastewater had a pH of 7.16 and an initial concentration of NO_3^--N of 18.94 mg l^{-1}, which was about 4 times higher than the discharge standard of China (5 mg l^{-1}). The amount of NO_2^--N and NH_4^+-N of the wastewater were very low (lower than the discharge standard of China, data not shown).

The NO_3^- contamination of the wastewater was mainly come from the stalks of plants. One milliliter of each denitrifying bacterial culture ($OD_{600} = 1.0$) was added into each flask containing 100 ml wastewater then sealed and incubated at 37°C for up to 9 days without shaking. The concentrations of NO_3^--N, NO_2^--N, NH_4^+-N, NO_2 emission and DO value were determined as previously described.

Removal of nitrate from industrial wastewater by immobilizing the denitrifiers

In order to recycle the microbial cells, the cells of each denitrifier were immobilized in a hybrid matrix of particles made by calcium alginate (CA) and polyvinyl alcohol (PVA). The spherical particles was prepared as follows: (1) 1% sodium alginate and 8% PVA were dissolved in the water at 90°C and gently mixed for 1 h; (2) After complete dissolution and when the temperature was 37°C, 10 ml of this solution was mixed with about 1 g cells (fresh weight). The sodium alginate–PVA-cell mixture was dropped into a saturated boric acid solution containing 2% $CaCl_2$ to form spherical particles. The formed spherical particles were soaked 12 h for gel hardening and then washed three times with water.

Freshly spherical particles were incubated in to the wastewater and incubated at 37°C for 6 days. The concentrations of NO_3^--N, NH_4^+-N and NO_2^--N were determined as previously described.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of ZZ15 and YC13 are posted in the NCBI GenBank database as EU636000 and EU636001, respectively.

Results

Isolation and identification of denitrifying bacteria

Denitrifying bacteria were isolated by a semi-anaerobic cultivation using nitrate medium, qualitatively and quantitatively analyzed for NO_3^- and NO_2^- degradation and observed gas production in the tubes. Two denitrifying bacterial strains, ZZ15 and YC13, were successfully identified from the wastewater and the soil sample, respectively. They were both Gram-negative, short rod cells when grown in nitrate broth (Fig. 1; Table 1). They could grow aerobically and semi-anaerobically, but their denitrification abilities were only observed under a semi-anaerobic condition (data not shown).

Physiological analyses showed that strain ZZ15 utilized more types of carbon sources (glucose, maltose, sucrose) than YC13 (Table 1). Both strains can use nitrate or nitrite as a sole nitrogen source. They were both negative for oxidase/catalase/indole reactions and lack of gelatin liquefaction abilities. Furthermore, YC13 was positive for starch hydrolysis (Table 1).

The 16S rRNA gene sequence of ZZ15 exhibited a 99% identity with a growth-promoting rhizobacterium *Stenotrophomonas maltophilia* 17A1 (EF580914), and that of YC13 showed a 99% 16S rRNA gene identity with a marine denitrifier *Oceanimonas denitrificans* F13-1 (DQ097665).

Based on the above analyses, the identification results showed that isolated bacteria were them as *Stenotrophomonas* sp. ZZ15 and *Oceanimonas* sp.YC13. The phylogenetic relationships among ZZ15, YC13 and some GenBank 16S rRNA gene sequences are shown in Fig. 2.

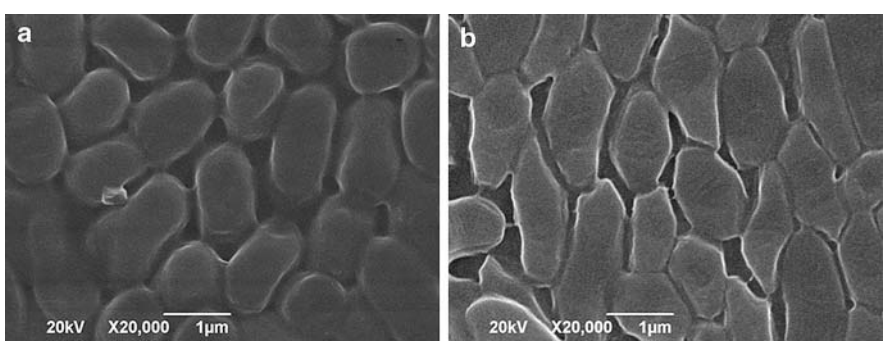

Fig. 1 Scanning electron microscopy (*SEM*) photographs of ZZ15 (**a**), and YC13 (**b**) cells in nitrate broth. *Scale bars* 1 μm

Table 1 Important characteristics of denitrifying bacteria ZZ15 and YC13

Characteristics	ZZ15	YC13
Originated from	Waster water sample	Soil sample
Colony color and sharp	White, irregular	Orange, round and central elevated
Cell morphology	0.5–2.0 μm long, irregular rods	0.5–2.0 μm long, short rods
Gram staining	Negative	Negative
Temperature optimum (°C)	37	37
pH optimum	7	7
Catalase, oxidase reactions	−	−
Starch hydrolysis	−	+
V. P. test	−	No growth
M. R. test	+	No growth
Indole production, gelatin liquefaction	−	−
Acetate, citrate, ethanol, glycerol	+	+
Glucose, maltose, sucrose	+	−
Methanol, L-arginine	−	−
Nitrate, nitrite	+	+
16S rRNA gene identification	*Stenotrophomonas* (99%)	*Oceanimonas* (99%)

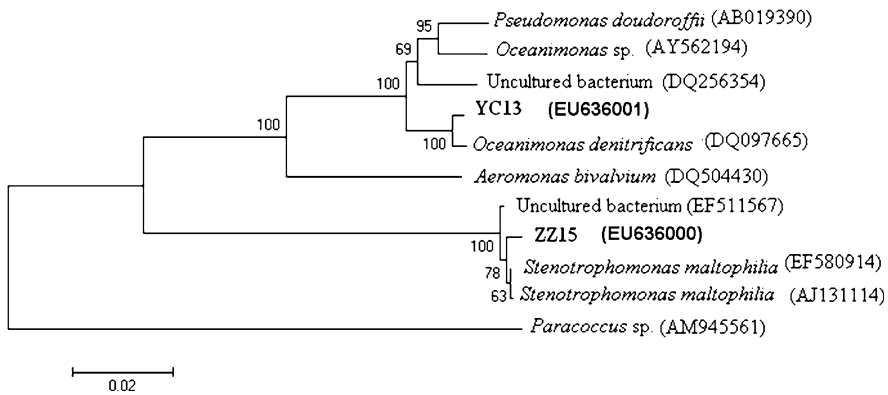

Fig. 2 A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationship among ZZ15, YC13 and other species. The 16S rRNA gene sequence of *Paracoccus* was used as an out-group to root the tree. GenBank accession numbers are given in *parentheses*. The bootstrap numbers indicate the value of 100 replicate trees supporting the branching order. Bootstrap values greater than 60% are shown at branch points. The *scale bar* represents two nucleotide substitutions per 100 nucleotides

The nitrate removing efficiencies of ZZ15 and YC13

The nitrogen reduction process of ZZ15 and YC13 in the nitrate broth are shown in Fig. 3. From the initial NO_3^--N concentration of about 270 mg l^{-1} for both of the bacterial treatments, in 1 day, the NO_3^--N concentrations decreased rapidly and reached at 62.73 mg l^{-1} and 106.05 mg l^{-1}, for ZZ15 and YC13, respectively. A total of 72.5% (for ZZ15, Fig. 3a, □) and 54.8% (for YC13 Fig. 3a, △) of the NO_3^--N was degraded, After 2.5 days, NO_3^--N removal efficiencies for denitrifier treatment samples stayed in higher than 90% (96.0% for ZZ15 and 92.9% for YC13). In the 3rd day, the removal efficiencies increased to a maximum of 96.6% and 95.3%, for ZZ15 and YC13, respectively (Fig. 3a). In the whole 9 days, for the samples without adding

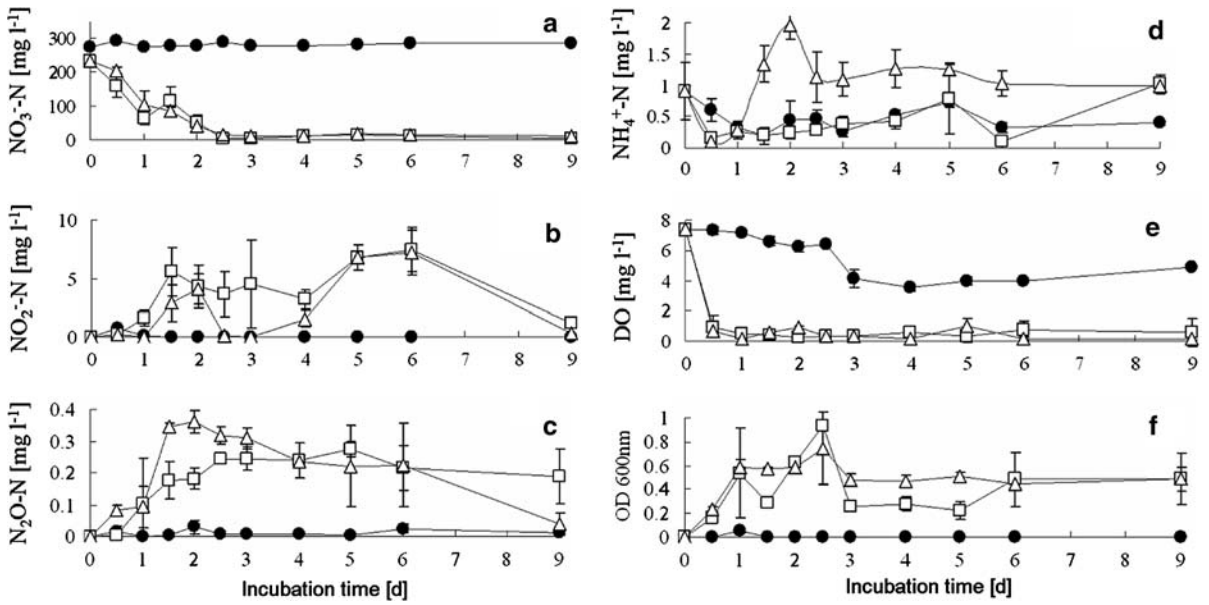

Fig. 3 Nitrate removing efficiency tests of ZZ15 and YC13 in nitrate broth. **a–f** The concentration of NO_3^--N, NO_2^--N, N_2O-N, NH_4^+-N, DO and OD_{600} of each denitrifier and control, respectively. ●, nitrate broth only; □, nitrate broth inoculated with ZZ15; △, nitrate broth inoculated with YC13

a denitrifer, the NO_3^--N concentration remained almost the same (Fig. 3a, ●).

Through the 9 day incubation period of each treatment, the levels of NO_2^--N (Fig. 3b), N_2O-N (Fig. 3c) and NH_4^+-N (Fig. 3d) accumulation were all very low (under 10 mg l^{-1}), indicating the completion of denitrification reactions.

The reduction of NO_3^--N occurred along with the decreasing of dissolved oxygen (Fig. 3e) and the increasing of cells of each bacterium (Fig. 3f). The value of dissolved oxygen fell down quickly in the first 0.5 day for denitrifier treatment sample (0.96 mg l^{-1} for ZZ15, 0.65 mg l^{-1} for YC13, initial DO value 7.40 mg l^{-1}). After the DO values maintained at lower values of less than 1 mg l^{-1}, effective denitrification began, nitrate removal rate move to a high degree (Fig. 3a vs. e). The DO values of the control only declined slightly, from 7.40 to 4.88 mg l^{-1}.

Removal of NO_3^--N from industrial wastewater

The initial NO_3^--N concentration of the industrial wastewater was 18.94 mg l^{-1}. But after adding ZZ15 and incubation for 1.5 days, the NO_3^--N concentration decreased to below the national discharge standard (1.90 mg l^{-1}, 89.34% removed, Fig. 4a). For YC13, it took 2 days to reach the NO_3^--N national discharge standard (3.37 mg l^{-1}, 82.21% removed, Fig. 4b). In the whole NO_3^--N removing process, the NO_2^--N, NH_4^+-N and N_2O-N accumulation were insignificant indicating the completion of denitrification reactions. The change of DO values between inoculated sample and non-inoculated sample was almost the same (Fig. 4a, b).

Both strains showed relatively lower speeds of NO_3^--N degradation in the wastewater samples than in the nitrate broth (Fig. 3). The control wastewater samples also showed certain levels of NO_3^--N degradation, but without adding the denitrifying bacteria, the NO_3^--N could not reach the national discharge standard in 9 days (Fig. 4), or even in 15 days (data not shown).

Removal of NO_3^--N from industrial wastewater by immobilizing the denitrifiers

Bacterial immobilization experiments were performed in the same condition as described in the wastewater treatment. It took 3 days to reach the national discharge standard of NO_3^--N without the accumulation of NO_2^--N and NH_4^+-N (Fig. 5). The immobilized strains showed relatively lower efficiency of NO_3^--N degradation than using denitrifiers in the wastewater treatments (Fig. 4).

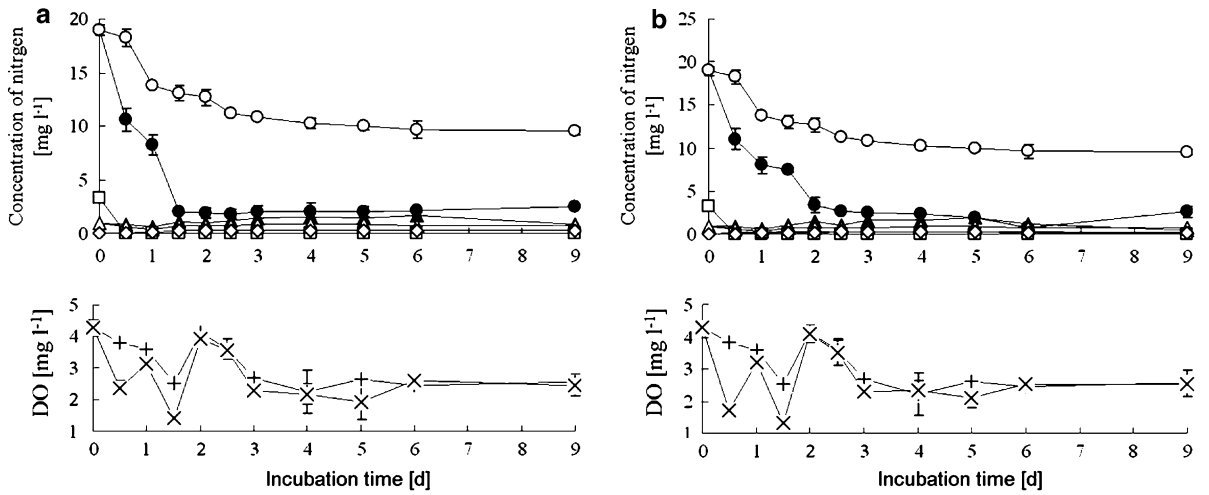

Fig. 4 Time course of nitrogen concentration, cell dry weight and DO in the industrial wastewater treated with ZZ15 (**a**) or YC13 (**b**). ●, concentration of NO_3^--N, wastewater treated with a denitrifier; ○, concentration of NO_3^--N, wastewater only; ■, concentration of NO_2^--N, wastewater treated with a denitrifier; □, concentration of NO_2^--N, wastewater only; ▲, concentration of NH_4^+-N, wastewater treated with a denitrifier; △, concentration of NH_4^+-N, wastewater only; ◆, concentration of N_2O-N, wastewater treated with a denitrifier; ◇, concentration of N_2O-N, wastewater only; ×, DO, wastewater treated with a denitrifier; +, DO, wastewater only

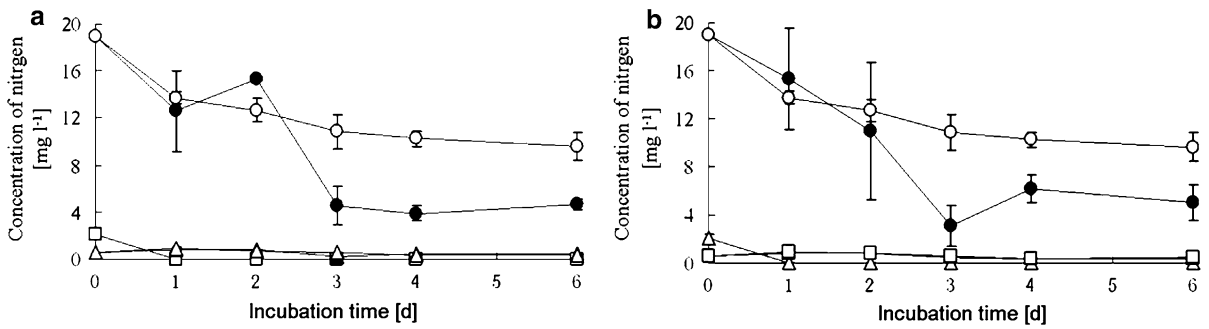

Fig. 5 Time course of nitrogen concentration in the industrial wastewater treated with immobilized spherical particles ZZ15 (**a**) or YC13 (**b**). ●, concentration of NO_3^--N, wastewater treated with a denitrifier; ○, concentration of NO_3^--N, wastewater only; ■, concentration of NO_2^--N, wastewater treated with a denitrifier; □, concentration of NO_2^--N, wastewater only; ▲, concentration of NH_4^+-N, wastewater treated with a denitrifier; △, concentration of NH_4^+-N, wastewater only

Discussion

For the potential application in environmental bioremediation, the denitrifying bacteria were screened not only for abilities to reduce nitrate, but also with no increase of nitrite, ammonia and nitrous oxide during denitrification. That was important due to the fact that excess NO_2^- and NH_4^+ are very harmful to aquatic organisms (Philips et al. 2002) and nitrous oxide is a greenhouse gas. Two novel denitrifying bacteria were isolated from the wastewater and soil samples and identified as *Stenotrophomonas* sp. ZZ15 and *Oceanimonas* sp.YC13, respectively. They were efficiently able to degrade NO_3^--N in a semi-anaerobic condition without obvious accumulation of NO_2^--N, NH_4^+-N and N_2O-N.

Previously, two strains of *Stenotrophomonas* were reported to reduce NO_3^--N, but the final products were NO_2^--N (Heylen et al. 2007). Furthermore, recently, a denitrifier *Stenotrophomonas maltophilia* X0412 (DQ359944) was isolated from a shrimp pond and showed the ability of aerobic denitrification. The 16S

rRNA gene sequence of our strain *Stenotrophomonas* sp. ZZ15 showed only a 97% identity to X0412. The highest homologous sequence to the ZZ15 16S rRNA gene in the GenBank was that of *Stenotrophomonas maltophilia* 17A1 (99%), but no denitrification ability of 17A1 was reported. Thus we consider ZZ15 as a novel denitrifying bacterium that has the ability of denitrification under a semi-anaerobic condition.

Although the 16S rRNA gene sequence of *Oceanimonas denitrificans* F13-1 (DQ097665) was posted in GenBank in 2006, no detailed study about this denitrifier has been reported. Thus, our strain *Oceanimonas* sp. YC13 should also be considered as a novel denitrifying bacterium.

ZZ15 and YC13 showed efficient NO_3^--N removal abilities in both nitrate broth and industrial wastewater. Even though the speed of NO_3^--N degradation in wastewater was relatively slower than in nitrate broth, ZZ15 or YC13 still efficiently removed NO_3^--N efficiently. Wastewater is a more complicated environment and there are different factors may affect the nitrate removal rate. In the wastewater environment, ZZ15 is considered as an indigenous denitrifier, while YC13 is considered as a non-native bacterium. However, the denitrification ability of YC13 was almost as efficient as ZZ15 indicating that some non-native bacteria could also adapt and be applied in industrial wastewater treatment system.

Due to the complexity of the industrial wastewater environments, it was predicted that by the use of mixed cultures, nitrogen was removed more efficiently than by use of a single species. Van de Pas-Schoonen et al. (2005) reported that a complex culture rather than a single strain was responsible for denitrification in a man-made ecosystem. Takaki et al. (2008) reported that by co-cultivation of *Ralstonia pickettii* K50 with an actinomyces *Streptomyces griseus*, a significant enhancement of denitrification activity occurred in an artificial wastewater. In this study, in order to increase the nitrogen removal efficiency, we have also tested a mixture of ZZ15 and YC13 together to perform denitrification in both pure culture and in industrial wastewater. The mixed culture worked almost the same as a single one (data not shown). A possible explanation of observed results is the fact that mixing the ZZ15 and YC13 resulted in competition for carbon source or other nutrients utilization. The cultivation of the wastewater bacteria on nitrate medium during the whole wastewater treatment process (9 days) was also performed, and results indicated that the major bacteria in the plates were the inoculated strains (ZZ15 or YC13-data not shown). This observation confirmed that ZZ15 or YC13 played an important role in the nitrogen removing process.

During the process of denitrification, the reduction of NO_3^--N occurred along with the decreasing of dissolved oxygen and the increasing of cells of each bacterium. This indicates that the denitrifiers need some oxygen to growth. When the concentration of DO lower than 2 mg l^{-1} (in wastewater), or even 1 mg l^{-1} (in pure culture), effective denitrification began, indicating that the denitrification was performed in a semi-anaerobic condition.

The two novel denitrifying bacteria have potentials for further applications in industrial wastewater cleaning systems, since it would be relatively easy to construct fix column system to remove nitrogen. We have immobilized the denitrifiers with filling materials and the results proved that ZZ15 and YC13 could still perform denitrification successfully. This will expands the use of these denitrifiers to construct biological nitrogen treatment column equipments to recycle the denitrifiers from the bioreactor effluents after completion of biological degradation.

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