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Isolation and characteristic of an aerobic denitrifier with high nitrogen removal efficiency

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Paracoccus denitrificans **DL-23, isolated from aerobic domesticated activated sludge, was demonstrated to have high ability of denitrification and heterotrophic nitrification under aerobic condition. After optimization (succinate, COD/N 10, 37°C, 160 rpm), DL-23 removed 420 and 860 mg/l NO_3^- -N within 36 to 60 h of growth, respectively. DL-23 also removed 380 mg/l NH_4^+-N within 24 h with ammonia as nitrogen source. The maximum removal rate was 30.3 mg/l·h. Meanwhile, DL-23 exhibited aerobic nitrite reduction ability with 658 mg/l NO_2^--N within 48 h.**

Key words: Aerobic denitrification, heterotrophic nitrification, nitrogen removal, *Paracoccus denitrificans*.

INTRODUCTION

The conventional nitrogen removal process comprises of nitrification (the conversion of NH_4^+ to NO_2^- and further to NO_3^-) by autotrophs under aerobic conditions and denitrification (the conversion of NO_3^- and NO_2^- to gaseous nitrogen) by heterotrophs under anaerobic conditions. Denitrification is nearly an exclusively facultatively anaerobic or microaerophilic trait (Zumft, 1997). However, the discovery of the aerobic denitrifiers *Paracoccus pantotrophus*, *Pseudomonas* spp. and *Alcaligenes faecalis* (Robertson and Kuenen, 1983*)* broke this theory. Some aerobic denitrifiers are not only capable of performing denitrification under aerobic conditions but also have the phenomenal ability to proceed to heterotrophic nitrification (Joo et al., 2005).

Compared with the conventional nitrogen removal process, the process using aerobic denitrification has several potential advantages (Kim et al., 2008; Khardenavis et al., 2007): (1) the simultaneous nitrification and denitrification in the same reactor largely reduce the construction cost and the operational complexity; (2) less acclimation problems; (3) the alkalinity released during denitrification can partly compensate for the base consumption in the nitrification, while it can reduce the extra alkali costs and avoid the secondary pollution.

To date, many different species of aerobic denitrifier have been reported, but relevant studies are generally focused on the removal of low concentrations of nitrate and ammonia and the nitrogen removal efficiencies, generally are not high, the denitrification efficiency of *Thiosphaera pantotropha* ATCC 35512 in 72 h was only 27.29% (Su et al., 2001). Joo et al. (2005) reported that *A. faecalis* No.4 showed a maximum removal rate of NH_4^+-N under intermediate and high ammonia loads were 23.6 and 26.1 mg-N/l·h, respectively. This study was performed to isolate a high efficiency aerobic denitrifier and investigate the nitrogen removal ability according to various carbon source, C/N ratio, incubation temperature and aeration speed based on a batch experiment. The ability of heterothrophic nitrification of the strain was also discussed.

MATERIALS AND METHODS

Media

Culture medium used in the acclimatization process and for batch test were modified denitrification medium (DM), modified lysogeny broth medium (LB) and nitrification inhibitor medium (NI). The ingredients of the DM were as follows (g/l): KNO_3, 3.0;

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$Na_2HPO_4·7H_2O$, 7.9; KH_2PO_4, 1.5; $MgSO_4·7H_2O$, 0.1; Na-succinate·$6H_2O$, 13; trace element solution, 2 ml; pH 7.0-7.3. The trace element solutions included the following components (g/l): EDTA, 50.0; $ZnSO_4$, 2.2; $MnCl_2·4H_2O$, 5.06; $CaCl_2$, 5.5; $FeSO_4·7H_2O$, 5.0; $(NH_4)_6Mo_7O_{24}·4H_2O$, 1.1; $CuSO_4·5H_2O$, 1.57; $CoCl_2·6H_2O$, 1.61; pH 7.0. LB medium contained (g/l): tryptone 10, yeast extract 5, KNO_3 1 pH 7.2. The NI medium contained (g/l): NH_4Cl 1.5; Na-succinate·$6H_2O$ 11; $MgSO_4·7H_2O$ 0.1; $Na_2HPO_4·12H_2O$ 6.7; KH_2PO_4 1; trace element solution 2 ml, pH 7.0-7.3.

Screening of the aerobic denitrifiers

The aerobic denitrifying organisms were enriched by acclimatizing the sludge taken from a secondary sedimentation tank at the Wenchang Waste Water Treatment Plant (Harbin, China). Fresh sludge was inoculated into a 5 l sequencing batch reactor (SBR). Headspace of 2 l was provided to prevent any solid losses generally caused by foaming. The dissolved oxygen (DO) was controlled at 2.5-5.5 mg/l by air pump and recorded by DO analyzer. The initial pH and temperature in the reactor were maintained at 7 and 28°C, respectively.

The acclimation period was divided into two phases, the first was intermittent aeration mode, and each running period lasted for 24 h, including periods of aeration, settling, discharging and loading. Half of the reactor volume (1.5 l) was replaced by fresh DM medium every 24 h. The initial aeration time lasted for 6 h per day, increased as the total nitrogen (TN) removal rate increased. The second phase was continuous aeration mode, the aeration time kept at 20 h. A strategy of stepwise acclimatization of sludge from low to high concentration of nitrate and chemical oxygen demand (COD) was adopted. At the initial acclimation period, LB was chosen as the main medium and the percent of DM increased gradually. This can prevent the damage on the bacterial cells caused by the sharp change in conditions.

10 ml of the sludge sampling at 30th and 60th were inoculated into 90 ml of the DM in 500 ml Erlenmeyer flask, respectively. Several sterilized glass beads were added to destroy the sludge floc aggregate in order to prevent the anaerobic microenvironment. The flask was inoculated at 28 °C on a rotary shaker at 160 rpm. The resultant bacterial suspension was streaked on DM medium agar plates and incubated at 28°C for 2-3 days. The isolation process was repeated several times until the isolates were found to be pure. After tha the obtained strains were transferred to 50 ml of DM and NI medium in 500 ml flasks, cultured at 28°C and 160 rpm for 48 h. The concentrations of NO_3^--N, NO_2^--N and NH_4^+-N were measured at the end of the culture. The bacterium with the highest NO_3^--N, NO_2^--N and NH_4^+-N removal efficiency was obtained and suspended in 30% glycerol solution at -40 °C for long-term storage.

Bacterial identification

The isolated strain that showed the best nitrogen reduction was grown at 28°C on LB agar. Standard physiological and biochemical characteristics were examined using the Micro-biochemical Tubes (Tianhe Co. Ltd., China). Genomic DNA of DL-23 was prepared using a Bacterial DNA Kit (Omega Bio-Tek, Inc.). Amplification was done by PCR with primers named BSF8/20 (5'-AGAGTTTGATCCTGGCTCAG-3') and BSRI541/20 (5'-AAGGAGGTGATCCAGCCGCA-3'). Amplification products were cloned using a commercially available pMD18-T vector cloning kit and transformed into competent *Escherichia coli* JM109. 16S rDNA sequence (1385 bp) was done by Invitrogen Company (Shanghai, China). The 16S rDNA sequences used for the phylogenetic analysis were derived and compared with those of other bacterial

16S rDNA sequences available in the GenBank database. Kimura's two parameter model was applied for the calculation of evolutionary distance. A phylogenetic tree was constructed by the neighbor-joining method. Bootstrap analyses of 1000 replicates were carried out using MEGA 4.0.

Aerobic denitrification of strain DL-23

To examine the aerobic denitrification capacity of DL-23, batch test was set up in triplicate in sterile 500 ml Erlenmeyer flasks containing 50 ml of DM medium, nine layers of absorbent gauze and glass beads were added to enhance the aerobic condition. Five carbon compounds, methanol, sodium acetate (abbreviated as acetate medium), glycerol, sodium succinate and glucose served as carbon substrates in DM medium. KNO_3 was fixed and provided 420 mg/l NO_3^--N. The amount of each carbon source was adjusted to give a C/N ratio of 15. The DL-23 inoculated in a 50 ml Erlenmeyer flask containing 5 ml of LB medium, cultured at 28°C in a shaking incubator (120 rpm). When the culture reached log phase, the cells were centrifuged at 4000 rpm (4°C, 5 min). The cell pellets were resuspended in sterile water to OD_{600} around 1.5. A 2% (V/V) preculture was inoculated into the mediums and cultured at 28°C and 160 rpm for 60 h. Aliquots of 2 ml were removed periodically for chemical analysis and measurement of cell density. Four C/N radios of medium (5, 10, 15 and 20) were tested by adjusting the carbon concentration. To observe the effects of incubation temperature and dissolved oxygen (DO) concentration on aerobic denitrification, the temperature was adjusted to 20, 28 and 37°C, the shaking speed was set at 80, 120, 160 and 200 rpm representing low to high DO concentrations (Taylor et al., 2009).

The effect of high nitrate load on aerobic denitrification was conducted by adjusting initial nitrate concentration at 800 mg/l. The influence of nitrite concentration on aerobic denitrification capacity of DL-23 was assessed by adjusting NO_2^--N concentrations from 200 to 650 mg/l using $NaNO_2$.

Heterotrophic nitrification of strain DL-23

Heterotrophic nitrification capability of DL-23 was assessed in the medium with NH_4Cl as the sole nitrogen source and sodium succinate as the carbon source. The initial NH_4^+-N was set at 400 mg/l and the C/N ratio of 10, cultured at 37°C, 160 rpm for 60 h. NH_4^+-N, NO_3^--N, NO_2^--N, TN, COD and OD_{600} was measured at 6 h intervals.

Analytical methods

All tests were conducted in triplicate, with the exception of the acclimation experiment. OD_{600} was determined by spectro-photometry at 600 nm (U-2800; Hitachi, Japan). Moreover, bacteria suspensions were centifuged at 3000 rpm for 15 min for measurement of nitrogen. NO_3^--N was measured by ultraviolet spectrophotometry and NO_2^--N was measured by the N-(1-naphthalene)-diaminoethane photometry method. Ammonium was determined by photometric determination with Nessler's reagent (APHA et al., 1998). TN was determined by UV spectrophotometry. Intracellular nitrogen content was calculated by subtracting the TN of inoculated medium following centrifugation (4°C, 15 min, 3000 rpm) from the TN of non-centrifuged medium. COD was determined using a COD analyzer (ET99730; Lovibond, Germany). The pH and DO were measured using a pH meter and a DO meter (9010; Jenco, America), respectively.

Table 1. The nitrogen removal ability of 10 bacterial isolates.

Culture condition	Sampling batch(d)	Isolated number	NO_3^--N (420 mg/L)			NH_4^+-N (285 mg/L)	
			OD_{600}	NO_2^--N	NO_3^--N	OD_{600}	NH_4^+-N
Carbon Source: Succinate C/N ratio: 15 Shaking Speed: 160 rpm Temperature: 28 °C	30th	DL-2	2.2	45	27	2.8	12
		DL-4	1.1	167	3.2	3.4	0
		DL-7	1.3	122	18	3.1	8
		DL-8	1.9	71	0	2.7	0
		DL-12	1.6	89	12	3.5	13
		DL-16	1.8	55	23	3	0
	60th	DL-17	2.2	62	0	2.8	0
		DL-20	2.3	31	0	3.1	0
		DL-23	2.5	17	0	2.9	0
		DL-25	2.3	42	0	3.3	0

RESULTS

Isolation and identification of strain DL-23

After 60 days' acclimation, the COD removal rate was above 79%, NO_3^--N removal rate was above 99%, TN was above 60% and the highest TN removal rate reached 67%. The active sludge in the SBR changed from deep dark and diffused to brown yellow and floccose, the settling velocity stayed at 25-30%.

Twenty five bacterial colonies grew on the DM agar-medium, the aerobic denitrification and heterotrophic nitrification abilities of 10 isolates are tabulated in Table 1. Strain DL-23 exhibited the highest NO_3^--N and NH_4^+-N removal efficiency and was selected for further studies.

The results of morphological, physiological and biochemical characteristics of DL-23 are summarized in Table 2. A BLAST search of available data in the GenBank database showed a high similarity (100%) with *P. denitrificans* DSM413 (Figure1). Thus, we designated DL-23 as *P. denitrificans*.

The effect of carbon source on aerobic nitrate removal

Different carbon sources which acted as electron donors affected the denitrification activity of DL-23 (no.1-5; Table 3). DL-23 almost completely removed 420 mg/l NO_3^--N within 36 h in the medium of glycerol, succinate and glucose. However, DL-23 could not effectively remove NO_3^--N in the medium of acetate and methanol. In the glycerol medium, 60.8% of the removed nitrate (260±11.2 mg/l) was converted to cell mass, and thus, 32.7% (139.6±4.7 mg/l) was calculated to be removed by aerobic denitrification. In the succinate medium, 34.6% was removed by aerobic denitrification and 61.2% was

assimilated by DL-23. In the glucose medium, 29% was removed by aerobic denitrification and 65.5% was assimilated by DL-23. The accumulation of nitrite appeared during the culture period. The nitrite concentration in succinate medium increased to 130 mg/l and then rapidly decreases to zero at 30 h (data not shown). The amount of nitrite was still at a low level (<49.3 mg/l) when glycerol was a carbon source. This divergence may be due to the less removal rate of nitrate compared to the succinate culture. In term of important parameters such as nitrate removal efficiency and TN reduce rate, succinate was chose as the best carbon source for further study.

The effect of C/N ratio on aerobic nitrate removal

Table 3 (no.6-9) reveals that the mean nitrate reduction rate at C/N ratio of 5 reached 14.9 mg/l·h within the first 12 h, much higher than other C/N radios. 420 mg/l NO_3^--N was almost reduced within 36 h at the C/N ratios of 5, 10 and 15. At C/N 20, there was a lag phase during the process of nitrate reduction, which was probably caused by the worse adaption of DL-23 to the high carbon concentration. During the test, the nitrite presented different levels of build-up. At C/N 5, the nitrite accumulated was 206.2±0.5 mg/l, which might be due to the delayed synthesis of nitrite reductase along with the exhausted carbon source. The higher concentration of organic carbon did not enhance the denitrification effect, on the contrary, low C/N radio seems more favorable for denitrification, although, it can not support the whole energy and electron need of denitrify 420 mg/l nitrate. The TN removal efficiencies did not vary distinctly between the other C/N ratios except for C/N 5, which was much smaller because of the accumulation of nitrite. In consideration of the TN removal rate and economy of

Table 2. Taxonomical characteristics of *P. denitrificans* DL-23.

Test	Result
Colony morphology	Round
Margin	Regular
Elevation	Raised
Surface	Smooth
Density	Opaque
Pigment	Light yellow
Gram's	-
Shape	Rod
Size	Short
Arrangement	single
Growth temperature (°C)	15-45
Growth pH	6.0-11.0
H_2S production	-
M-R reaction	+
V-P reaction	+
Gelatin liquefaction	-
Casein hydrolysate	+
Oxidase	+
Catalase	+
Amylase	-
Dextrose	+
Methanol	+
Maltose	+
Mannose	+
Ethanol	+
Phaseomannite	+
Mannite	+
Sorbierite	+
Citrate	-

(+),positive reaction; (−), negative reaction.

carbon source, C/N 10 was selected for the later research.

The effect of temperature on aerobic nitrate removal

The nitrate removal efficiency at 37°C was much higher than at 28°C, the maximum nitrate removal rate reached 24.7 mg/l·h. At 20°C, after a long transient time, the nitrate reduced to the same level as other temperatures. The accumulated nitrite reduced slowly and completely nitrite removal was not observed even after 60 h. The TN removal rate increased from 41 to 52.3% at 37°C compared to 28°C (no.10-12, Table 3). The higher removal rate of nitrate and the TN indicated that temperature played a much more critical role than carbon source and C/N ratio under aerobic denitrification.

The effect of shaking speed on aerobic nitrate removal

The effect of shaking speed on nitrate removal from 420 mg/l was investigated (no.13-16, Table 3). With the increase of shaking speed, there was a corresponding increase in DO concentration (Taylor et al., 2009). 420 mg/l NO_3^--N was almost completely removed within 30 h at 160 and 200 rpm. However, at 80 and 120 rpm, nitrate removal rate was significantly slow (42 h). TN removal efficiency increased with the decrease of culture speed. At 80 rpm, TN reduced was 58.5% while at 200 rpm was 44.6%.

Nitrate removal under high-strength nitrate conditions

Changes in nitrogen compounds and nitrogen balance in nitrate removal under intermediate and high nitrate loads at C/N 10 are shown in Table 3 (no.17-18) and Figure 2. 424.5 and 860.8 mg/l NO_3^--N were removed between 97.7 and 94.8% within 36 and 60 h, respectively. The maximum NO_3^--N removal rates were 23.1 and 40.6 mg/l·h, while 53.2 and 45.4% of the TN was removed, respectively. The growth patterns of DL-23 under two nitrate loads were similar. Quick nitrate reduction and growth of the bio-mass corresponded with the large consumption of COD and accumulation of nitrite. The COD removal rate reached 90 and 81.9% under intermediate and high nitrate loads, respectively. Under the intermediate nitrate load, the concentration of nitrite increased at the beginning of the reaction and peak at 181±13 mg/l at 24 h, reduced to zero rapidly afterwards. However, under high nitrate load, after small down from the peak of 273±9 mg/l, nitrite leveled off at 190±12 mg/l, after which no decrease in the concentrations was observed. This phenomenon may be caused by the cell aging. From the grow curve, we can see that under both nitrate loads, the cell exhibits different degree of autolysis which may be caused by the high pH produced by the denitrification and the toxicity of the high concentration of nitrite.

Utilization of nitrite under aerobic conditions

Nitrite is one of the main intermediate of denitrification and toxic to the bacterium. The results of aerobic denitrification with nitrite as nitrogen source are shown in Table 4.

Under different nitrite loads from 202.3±5.7 to 658±11.5 mg/l, nitrite was removed completely within 18 to 48 h. Furthermore, with the increase of nitrite, TN degradation rate increased from 30.9 to 62.6%. During the aerobic phase, an increase of NO_3^--N from 7.2±1.9 to 54.2±6.8 mg/l
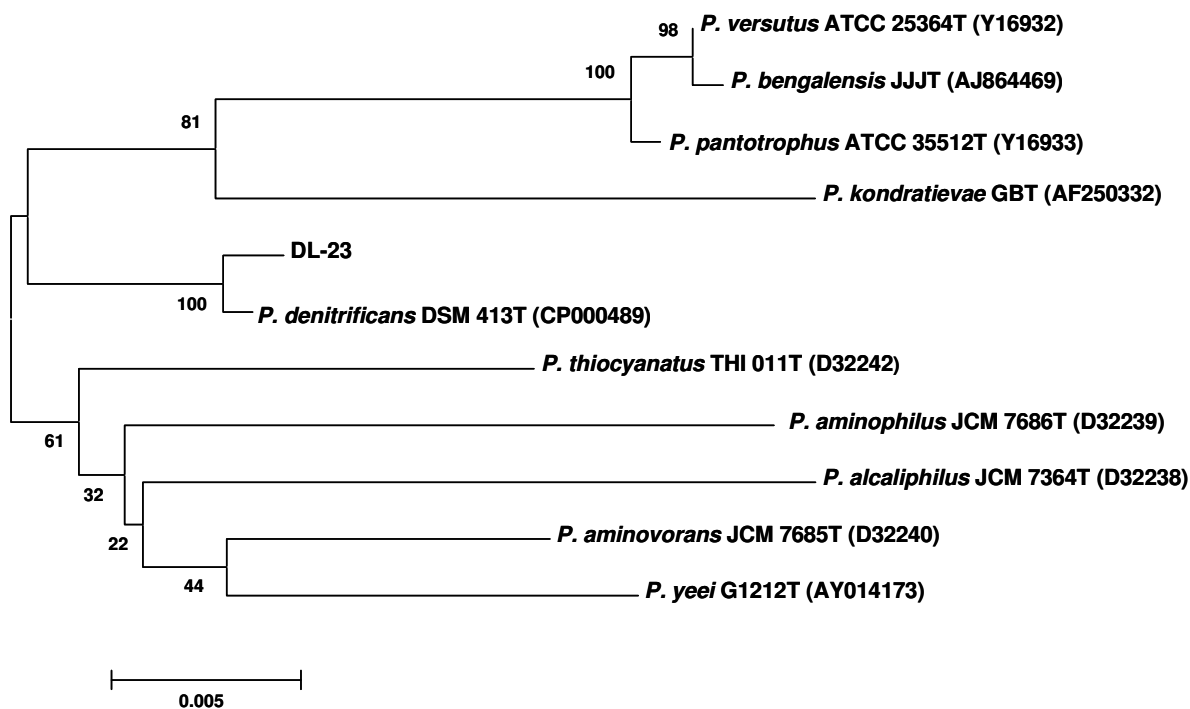
Figure 1. Phylogenetic tree based on a comparison of the 16S rDNA gene sequence.

was obtained, the large accumulation of nitrate maybe caused by the lack of carbon source. The NO_2^--N oxidation informed heterotrophic nitrify ability of DL-23. So far, the ability of bacteria to use nitrite aerobically for growth and denitrify is seldom assessed, DL-23 exhibited a highly nitrite tolerant and nitrite reduction capability, indicate that DL-23 has mechanisms that produce tolerance to reactive nitrogen species (Doi et al., 2009).

Heterotrophic nitrification by *P. denitrificans* DL-23

Most aerobic denitrifying bacteria are capable of heterotrophic nitrification, including *P. pantotrophus* (Gupta, 1997), *A. faecalis* (van Niel et al., 1992) and *Arthrobacter* sp. (Brierley and Wood, 2001). Figure 3 shows the variations of biological nitrogen transformation and cell growth of DL-23 at the condition of 37°C, C/N 10 and 160 rpm. After a 12 h lag phase, DL-23 began to grow and the OD_{600} increased quickly. Maximum growth was observed between 12 and 30 h and reached the maximum growth rate of 0.17/ h. Meanwhile, NH_4^+-N and COD decreased significantly with the growth of the strain. The maximum removal rate of NH_4^+-N was 30.3 mg/l·h and completely removed in 36 h. NO_3^--N began to accumulate after 12 h. No nitrite was observed during the whole process. From the nitrogen balance, 79% of the ammonia originally provided was detected in cells of the

bacterium. The percentage of nitrogen lost in the flask culture was estimated to be 18.8% which was presumed to be converted to N_2 gas through denitrification.

DISCUSSION

After the domesticating of activated sludge by adopting the method of intermittent and continuous aeration conditions, 25 strains were isolated. The isolates of 60th exhibited better denitrification abilities than 30th which implied that the continuous aeration mode performed an effective selection pressure on aerobic denitrifier. Compared with the aerobic denitrification, the heterotrophic nitrification of the isolates exhibited no distinct differences because the nitrification process was not influenced by oxygen concentration. The optimum carbon source clearly showed that the characteristics of the carbon source had significant influence on aerobic denitrification process. The nitrogen removal rate by using succinate and glycerol was significantly higher than other organic substances (no.1-5, Table 3). The effect of carbon sources on the growth of DL-23 was determined by the decomposition and transformation of carbon source. Best denitrification occurred when succinate was carbon source. It is not accorded with the conclusion that the activity of periplasmic nitrate reductase increases with the extent of reduction of the carbon substrate   (Richardson

Table 3. Nitrogen balance of nitrate removal in shaking culture experiment by DL-23.

S/N	Carbon source	Culture condition			Initial NO$_3^-$-N (mg/l) [(Mean±S.D)	Final concentration (mg/l)			TN reduced (denitrification ratio) [(Mean±S.D)(%)]
		C/N ratio	Shaking speed (rpm)	Temperature (℃)		NO$_3^-$-N [(Mean±S.D)(h)]	NO$_2^-$-N	Intra-Cellular N [(Mean±S.D)	
1	Glycerol	15	160	28	427.5±4.7	27.9±5 (36)	0	260±11.2	139.6±4.7 (32.7)
2	Succinate	15	160	28	417.4±6	17.6±6.3 (36)	0	255.5±9.6	144.3±5.8(34.6)
3	Glucose	15	160	28	420.4±4.5	23.1±4.5 (36)	0	275.5±7.5	121.9±6.3(29)
4	Methanol	15	160	28	424.3±5.3	102.2±4.5 (48)	147.2±5.4	105.1±14.4	69.8±3.3(16.5)
5	Acetate	15	160	28	419.5±6	342.4±5.6 (48)	12.1±5.9	56.5±7.8	8.5±3.2(2.0)
6	Succinate	5	160	28	417.2±5.4	29.0±3.3 (48)	206.2±0.5	167.7±5.4	14.3±3(3.4)
7	Succinate	10	160	28	420.2±4.6	14.4±2.6 (36)	0	246.7±10.8	159.1±4.4(37.9)
8	Succinate	15	160	28	422.8±5.8	21.1±5.3 (36)	0	250.2±10.3	151.5±4.8(35.8)
9	Succinate	20	160	28	414.4±6.6	21.9±3.7 (48)	0	245.9±13.5	146.6±3.9(35.4)
10	Succinate	10	160	20	421.3±3.2	19.2±2.3 (60)	122.1±13.2	161.8±12.6	118.2±5.2(28.1)
11	Succinate	10	160	28	425.8±5.9	15.5±2.5 (36)	0	235.6±7.6	174.7±6.1(41)
12	Succinate	10	160	37	419.7±4.7	13.0±3.5 (36)	0	187.2±5.8	219.5±4.8(52.3)
13	Succinate	10	80	37	418.2±6.3	3.2±8(42)	0	170.5±11.2	244.5±8(58.5)
14	Succinate	10	120	37	412.8±3.2	4.3±6.6(42)	0	185.3±8.6	223.2±7.6(54.1)
15	Succinate	10	160	37	420.3±4.5	7.0±3.4(36)	0	205.6±14	207.7±4.4(49.4)
16	Succinate	10	200	37	417.2±5.7	9.7±5.6(36)	0	221.5±13.3	186±6.6(44.6)
17	Succinate	10	160	37	424.5±3.1	9.7±4.1(36)	0	188.9±9.1	225.9±7.4(53.2)
18	Succinate	10	160	37	860.8±6.1	45±8.3(60)	190.2±12	235.2±8.3	390.4±15.1(45.4)

Error bars: mean ± S.D. of two replicates.

and Ferguson, 1992). There maybe two reasons explaining this phenomenon. Firstly, succinates were chosen as the main carbon source during the whole acclimation period. Secondly, as the intermittent of citric acid cycle, succinate could involve in synthetic biology directly. Although, some carbon sources could enhance the denitrification efficiency, the C/N ratio must be properly controlled. In sufficient carbon source conditions, the aerobic denitrification efficiency of DL-23 was not raised with the increase of C/N ratio. Within C/N ratio of 10 to 15, DL-23 exhibited similar denitrify ability. Format carbon source

density is the key to denitrification. Denitrify rate will be reduced under too low and too high concentration of the carbon source. At an insufficient carbon concentration, the electron flow is too low to provide enough energy for cell growth, and caused accumulation of intermediate such as nitrite (no.6, Table 3). When excess carbon substrates were added, it inhibits the growth of the bacteria and prolong the denitrification period (no.9, Table 3). Therefore, the optimization of carbon source concentration was very critical. Compared to the relatively strict request of the C/N ratio of *citrobacter diversus* (Huang and Tseng,

2001), the suitable C/N ratio for DL-23 varied broadly, C/N ratio varied from 10 to 15 could both satisfy the need for reducing 420 mg/l NO$_3^-$-N. Temperature also played an obvious effect on the denitrify activity of DL-23. Low temperature (20℃) caused the nitrite to accumulate seriously which might be due to the low activity of nitrite reductase at 20℃. There were no significant distinction on the removal of nitrate between 28 and 37℃, but the removal rate of TN increased obviously at 37℃, the intracelluar nitrogen at 28℃ is 235.6±7.6 mg/l, higher than 187.2±5.8 mg/l at 37℃. High temperature enhanced the activity of
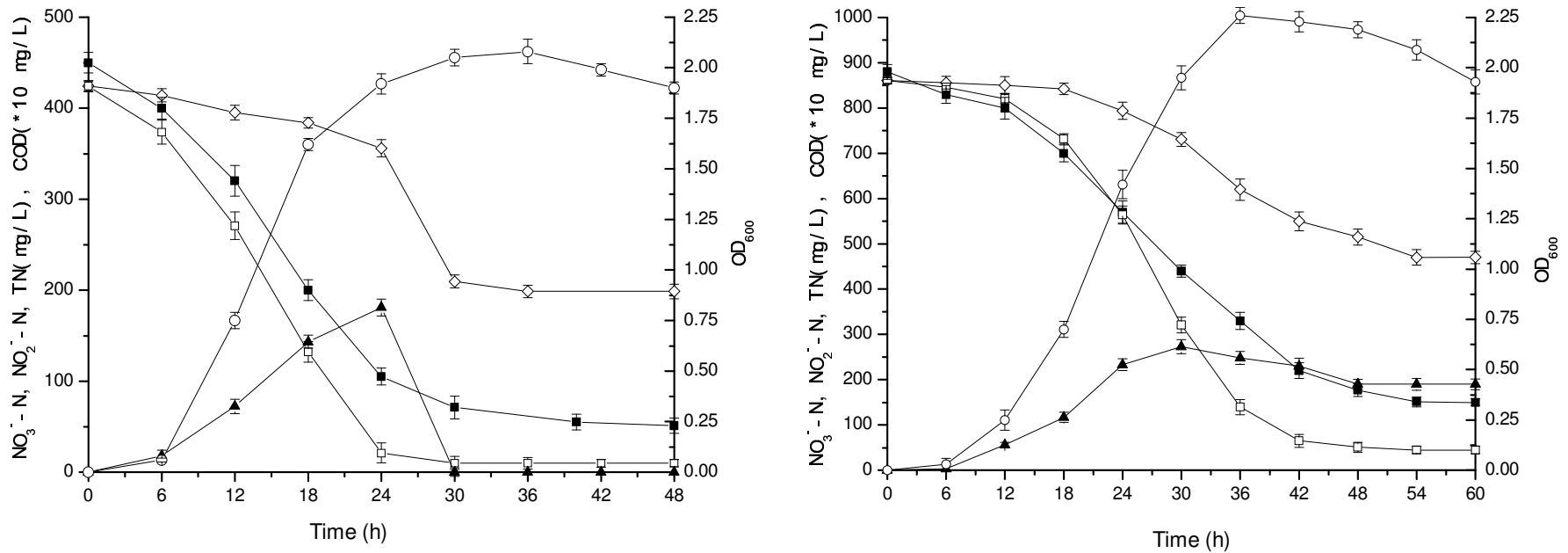
Figure 2. Characteristics of nitrate removal under intermediate (A) and high (B) NO_3^--N loads. Symbols: closed squares, COD; open squares, NO_3^--N; Open circles, OD_{600}; closed triangles, NO_2^--N; open diamonds, TN. Error bars: mean ± S.D. of two replicates.

Table 4. Nitrogen balance of nitrite removal in aerated batch experiment by DL-23.

Culture condition	Initial NO_2^--N(mg/l)	Final concentration (mg/l)			TN reduced (denitrification ratio) [(Mean ± S.D)(%)]
		NO_2^--N [(0)(h)]	NO_3^--N (Mean ± S.D)	Intracelluar N (Mean ± S.D)	
	202.3±5.7	(18)	7.2±1.9	132.5±4.4	62.6±8.1 (30.9)
Carbon Source: Succinate	250.2±6.5	(18)	13.7±3.3	121.5±3.7	115±7.7 (46)
C/N ratio: 10	357.3±4.9	(24)	17.6±4.2	130.3±5.3	209.4±12.7 (58.6)
Shaking Speed: 160 rpm	456.4±8.2	(36)	23.2±3.9	162.1±3.4	271.1±15.6 (59.4)
Temperature: 37 ℃	545.5±7.6	(48)	35.5±7.5	173.8±6.6	336.2±16.2 (61.6)
	658.0±11.5	(48)	54.2±6.8	192.2±9.3	411.6±18.3 (62.6)

enzyme of aerobic denitrify. The nitrate reduction profiles of DL-23 under various DO concentrations indicated that low concentration of DO reduced removal efficiency of nitrate but increased removal rate of TN. Thus, the electron flowed to the transport chain of denitrification more when DO proportion reduced the size of the electron by oxygen. This phenomenon illustrates that oxygen played an important role in denitrification of DL-23.
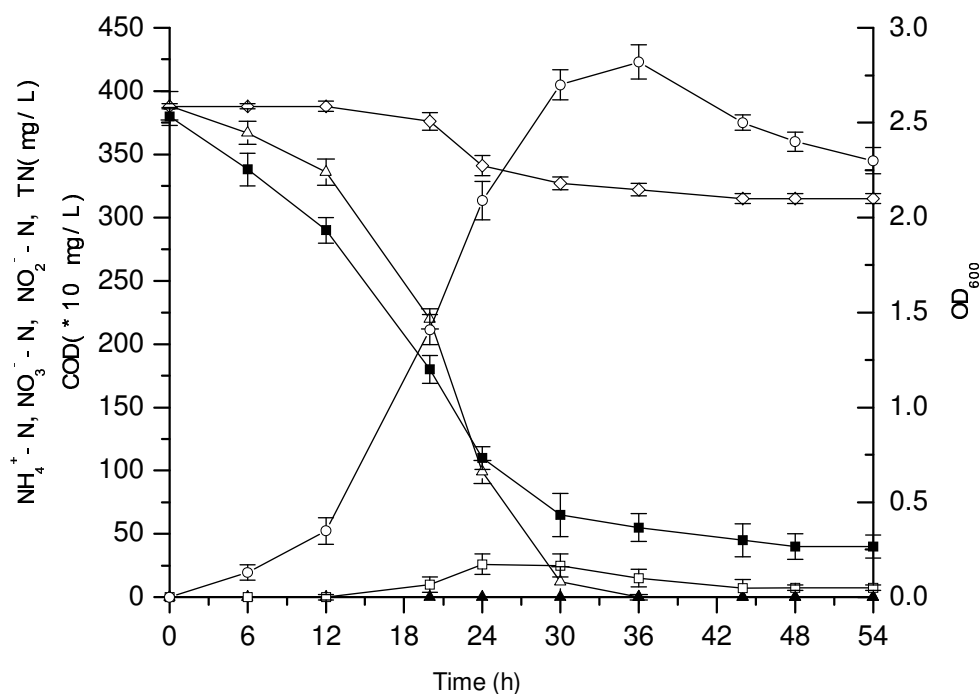
Figure 3. Characteristics of ammonia removal in NI medium in shaking culture. Symbols: open triangle, NH_4^+-N; closed square, COD; open square, NO_3^--N; closed triangle, NO_2^--N; open diamond, TN; Open circle, OD_{600}. Error bars: mean ± S.D. of two replicates.

Although, DL-23 exhibited high efficient denitrify activity under aerobic conditions, it is much as a facultative aerobe, low oxygen concentration was favorable for synthesis and activity of the denitrify enzyme.

The results show that the optimum culture of denitrification was succinate as carbon source, C/N ratio of 10, at 37°C and 160 rpm. Under this conditions, 420 mg/l nitrate could be significantly removed within 36 h; the nitrate removal efficiency was 97.7%, with a maximum removal rate high to 23.1 mg/l·h, TN and COD removal efficiency reached 53.2 and 90%, respectively which means that high efficient and synchronous removal of nitrate and organics under the aerobic conditions was achieved. Between a certain initial nitrate concentrations range from 202.3±5.7 to 658.0±11.5 mg/l, strains DL-23 could precede to nitrogen removal effectively, the removal rate of TN increased as nitrate load was added.

At present, the reported average nitrogen removal rate of aerobic denitrifiers were major at about 4.50-7.1 mg/l·h. Huang and Tseng, (2001) reported that under the C/N ratio of 4-5, NO_3^--N decreased from 180.9 to 2.1 mg/l by *C. diversus* after 40 h culture while the DO concentration remains within the range 3.1-3.8 mg/l. Zhou et al. (2007) showed that *Pseudomonas aeruginosa* NBRC 12689 could removed 330 mg/l of NO_3^--N within 2 d under condition of 30°C and 140 rpm. *Pseudomonas putida* AD-21 studied by Kim et al. (2008) exhibit a nitrate

removal rate of 7.1 mg/l·h and a nitrate removal efficiency of 70% at culture condition of C/N 8 and 30°C. The nitrogen removal rate of DL-23 is 3-9 times of theirs. Furthermore, while the nitrate reducing, the COD also degraded well enough, all these data proved that DL-23 is a well candidate for the applications in industrial or agricultural wastewater treatment systems where nitrate and COD concentrations tend to be high.

P. denitrificans DL-23 has heterotrophic nitrification-aerobic denitrification ability as such ammonia could be converted into gas products under aerobic conditions. Also, it exhibited the ability of simultaneous nitrification and denitrification by single strain. Under the best heterotrophic nitrification conditions, DL-23 demonstrated an high efficiency of ammonium removal, of the mean NH_4^+-N removal rate is faster than 2.50 mg/l·h strain NHR (Zhao et al., 2010) and 3.52 mg/l·h of strain A1 (Yang et al., 2011). Although, the most part of NH_4^+-N is incorporated into microbial cell as a nitrogen source, the fast NH_4^+-N removal rate and low nitrification products by the DL-23 can also decrease TN in wastewater, even though denitrification is not well occurred.

Conclusions

P. denitrificans DL-23 was isolated from the acclimatized

aerobic sludge. It has a high efficiency of aerobic denitrification ability. When the initial nitrate concentration was 420 mg/l, 97.7 and 53.2% of nitrate and total nitrogen were removed under the optimal aerobic conditions, respectively. In addition, it is also capable of heterotrophic nitrification and denitrification, 380 mg/l NH_4^+-N was completely removed within 30 h and 18.8% total nitrogen reduced. Finally, it had an amazing resistant to the high concentration of nitrite. The results suggest that *P. denitrificans* DL-23 may be a good candidate for aerobic wastewater treatment.

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