Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2015.1119750

57 (2016) 21455–21462
September

Taylor & Francis
Taylor & Francis Group

Isolation of a thermophilic aerobic denitrifier and characterization for its denitrification performance

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Received 13 May 2015; Accepted 25 October 2015

ABSTRACT

At present, no *Chelatococcus daeguensis* strain has been reported to be an aerobic denitrifier. In this study, an aerobic denitrifier was isolated from the biofilm of a field biotrickling filter and its aerobic denitrification activity was evaluated under various conditions in batch reactor experiments at 50°C. Based on biochemical studies and 16S rRNA sequencing analysis, the isolate was identified as *C. daeguensis* strain TAD1. The strain TAD1 was examined to determine the effects of different carbon sources, C/N ratios, and dissolved oxygen concentration on aerobic denitrification activity at 50°C. *C. daeguensis* TAD1 efficiently removed nitrate using disodium succinate as the sole carbon source. Nitrate was hardly reduced when glucose was used at 50°C. The optimal C/N ratio was 9, giving a denitrification efficiency of 96.1% and higher carbon concentrations did not inhibit cell growth and denitrification activity. *C. daeguensis* TAD1 tolerated oxygen levels about 5.1 mg/L. The denitrification efficiency of *C. daeguensis* TAD1 was higher than that of mesophilic bacteria.

Keywords: Aerobic denitrification; Thermophilic; Characterization

1. Introduction

Denitrification is a biological nitrogen reduction process which can transform nitrate or nitrite to environment-friendly nitrogen gas, and many microorganisms have been reported to accomplish the denitrification process in different systems [1]. Traditionally, denitrification process takes place under anoxic environment [2,3]. In last decades, aerobic denitrifiers have been discovered, which can utilize nitrate as electron acceptors at the environment of oxygen present. Robertson et al. [4] isolated three bacteria which could denitrify at the presence of oxygen from a wastewater treatment system. Lukow and Diekmann [5] isolated a strain which can remove nitrogen under an oxygen partial pressure of 30% saturation from a leachate treatment plant. Pai et al. [6] have successfully used *Alcaligenes denitrificans* DCB T25 in fully aerobic wastewater treatment process.

As far as is known, the reported aerobic denitrifiers are mainly mesophilic denitrifiers and only effective in ambient temperature [7–9]. However, the temperature of many industrial outflow wastewaters containing nitrogen pollutants is relatively high (above 45°C), such as fertilizer wastewater, steel production wastewater, and absorption solution of flue gas from power plants [10]. Conventional mesophilic denitrifiers are not suitable to directly treat these

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wastewaters. Therefore, pre-cooling should be carried out as a prerequisite, which will not only increase daily operation costs but also decrease the biological pollutant assimilation rate [11].

From this point of view, it is very essential to develop a thermophilic denitrification process which can directly be conducted on the nitrogen polluted wastewater at a relatively high temperature. Therefore, the author's group has tried to isolate the thermophilic aerobic denitrifier from a practical industrial wastewater treatment plant, in which the influent is high in temperature and contains high concentration nitrate. It has been proved that it is very effective to isolate micro-organism strains acclimating special environment in corresponding environmental condition [12–15].

The purpose of this study was to isolate an aerobic denitrifier which could acclimate thermophilic condition from the biofilm of a field biotrickling filter. The morphology, physiology, biochemistry, and genetic properties were characterized. To optimize the operational condition, the effect of carbon sources, C/N ratios, dissolved oxygen (DO) concentrations, and carbon source concentrations on aerobic denitrification activity of the isolated strain were investigated at a relatively high temperature of 50˚C. In addition, the aerobic denitrification activity of the isolated strain under the optimal conditions was verified and compared with other mesophilic aerobic denitrification bacteria in literatures.

2. Materials and methods

2.1. Experimental mediums

In order to screen the aerobic denitrifiers, modified screening medium (MSM) and bromothymol blue (BTB) medium were used. The contents of MSM include: KNO_3, 1.5 g/L; $Na_2HPO_4{\cdot}7H_2O$, 7.9 g/L; KH_2PO_4, 1.5 g/L; NH_4Cl, 0.3 g/L; $MgSO_4{\cdot}7H_2O$, 0.1 g/L; disodium succinate, 10.0 g/L; trace element solution, 2 mL/L; pH 7–7.5. The contents of BTB medium include: KNO_3, 5.0 g/L; $Na_2HPO_4{\cdot}7H_2O$, 7.9 g/L; KH_2PO_4, 1.5 g/L; $MgSO_4{\cdot}7H_2O$, 0.1 g/L; disodium succinate, 15 g/L; BTB (1% in ethanol), 1 mL/L; agar, 10.0 g/L; trace element solution, 2 mL/L; pH 7.0.

Denitrification medium (DM) was employed to determine denitrification activity of the aerobic denitrifiers. The contents of DM include: KNO_3, 0.6 g/L; $Na_2HPO_4{\cdot}7H_2O$, 7.9 g/L; KH_2PO_4, 1.5 g/L; $MgSO_4{\cdot}7H_2O$, 0.1 g/L; disodium succinate, 4.0 g/L; trace element solution, 2 mL/L; pH 7–7.5.

The trace element solution for micro-organism growth consists of the following components: EDTA, 50.0 g/L; $ZnSO_4$, 2.2 g/L; $CaCl_2$, 5.5 g/L; $MnCl_2{\cdot}4H_2O$, 5.06 g/L; $FeSO_4{\cdot}7H_2O$, 5.0 g/L; $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 1.1 g/L; $CuSO_4{\cdot}5H_2O$, 1.57 g/L; $CoCl_2{\cdot}6H_2O$, 1.61 g/L; pH 7.0.

2.2. Screening procedures for aerobic denitrifiers

Aerobic denitrifying micro-organisms were enriched from the biofilm sampled from a field biotrickling filter at Ruiming coal-fired power plant (Guangzhou, China). Firstly, the biofilm samples were transferred into flasks prefilled with 100 mL of MSM broth, then enclosed with six layers of gauze. The flasks were cultured in a shaking incubator (140 rpm) at 30˚C for 2 d. Secondly, 15% (V/V) of bacteria suspension liquid was inoculated into freshly autoclaved MSM broth and cultured at 34˚C. In order to isolate the aerobic denitrifiers acclimating relatively high temperature, the second procedure was repeated every other day, but the culture temperature was increased by 2˚C until the temperature reached 50˚C. The nitrate consumption, pH variation, and the quantity of bubble generation in culture solutions were monitored daily, which can indicate the denitrification activity of micro-organisms.

After the culture procedure at increasing temperatures, the re-screening procedures were carried out to acquire the isolates. The resulting bacterial suspension was streaked on BTB agar plate medium at 50˚C for a continuous 3 d. Then blue colonies were selected from the BTB agar plates, transferred to DM broth and incubated at 50˚C for 2 d. The selection of blue colonies is because the denitrification strains may utilize the nitrate which will cause an increase in pH value of the medium and lead to the final blue color indicator [16]. This rescreening was repeated for three times. Finally, the acquired isolates were identified and coincidently assured that there was only one strain in present. It was named as TAD1 in this study and used in the following experiment.

2.3. Identification of the isolated strain

The 16S rRNA sequence of TAD1 was determined by sequencing polymerase chain reaction (PCR) amplified rRNA. The total bacterial DNA was extracted and purified according to the method described by Ozeki et al. [17]. PCR was conducted using genomic DNA (0.1 μg) as the template. The primers used for 16S rRNA PCR amplification were F27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and R1522 (5′-AAGGAGGTGATCCAGCCGCA-3′) [18]. Typical amplification procedures include: pre-denaturation at 94˚C for

4 min, followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 54℃ for 30 s, and extension at 72℃ for 2 min, with a final extension at 72℃ for 10 min. The PCR products were sequenced by Invitrogen Company (Shanghai, China). The 16S rRNA sequences (1,385 bp) were examined in blastn (NCBI, USA) for similarities. The sequence data of the isolated strain had been submitted to the DDBJ/EMBL/GenBank databases with accession no. HM000004.

2.4. Aerobic denitrification activity assays

Although the isolated strain could stimulate color change of the BTB medium, it is important to measure its nitrogen removal ability to confirm the denitrification capability of TAD1. The bench-scale apparatus used for aerobic denitrification activity assays is shown in Fig. 1.

The denitrification reactor was completely closed except for the gas inlet and outlet. In order to avoid the hetero-infection through the inlet and outlet, two pre-sterilized bacteria filters (0.25 μm) were equipped at the inlet and outlet, respectively. The experiment methods are as follows. The isolated bacterium was transferred to modified DM of 500 mL and cultured in thermostatic water bath at 50℃. Argon was firstly bubbled in reactor to strip off the air and to avoid the interference of N_2 in air for the experimental results. Then, mixed oxygen and argon were supplied by sprayer at the bottom of the reactor to maintain specific DO concentration in the reactor. Except for test of the DO concentration, DO was maintained at about 4.0 mg/L.

Different carbon sources, C/N ratios, and DO concentrations tests were all carried out in DM broth. DM only contains nitrate as the sole nitrogen source. In carbon sources test, glucose, sodium citrate, sodium acetate, and disodium succinate were added to DM as the carbon source, respectively. For each of the carbon sources added, C/N ratio was kept constant and in accord with C/N ratio in DM described in Section 2.1. In C/N ratios test, a series of C/N ratios, adjusting to 1, 3, 5, 6, 7, 9, 12, 15, 18, and 20, were tested with disodium succinate used as the sole carbon source. In DO concentration test, gas flow rate was kept constant by flowmeter and different DO concentrations were controlled by the altering oxygen partial pressure in mixture gas. To ensure the accuracy, all the experiments were performed in triplicate. All reported results are average value of three independent experiments.

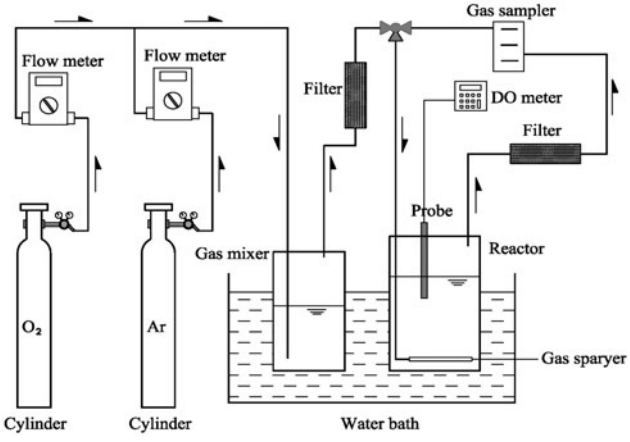

Fig. 1. Aerobic denitrification activity assay system.

2.5. Analytical methods

DO and pH were measured with a multi-function water quality monitor (Multi 340i, Germany WTW). Nitrate and nitrite concentrations were analyzed by ion chromatography (DX-500, Dionex Corporation). Nitrogen (N_2) was analyzed by a gas chromatograph (GC-2014, Shimadzu, Japan). The morphology of the bacteria was examined with a transmission electron microscopy (Hitachi H800, Japan).

3. Results and discussion

3.1. Identification of the isolated aerobic denitrifying bacterium TAD1

The isolated strain in this study was identified as a pure strain TAD1. It was short and rod shaped, motile

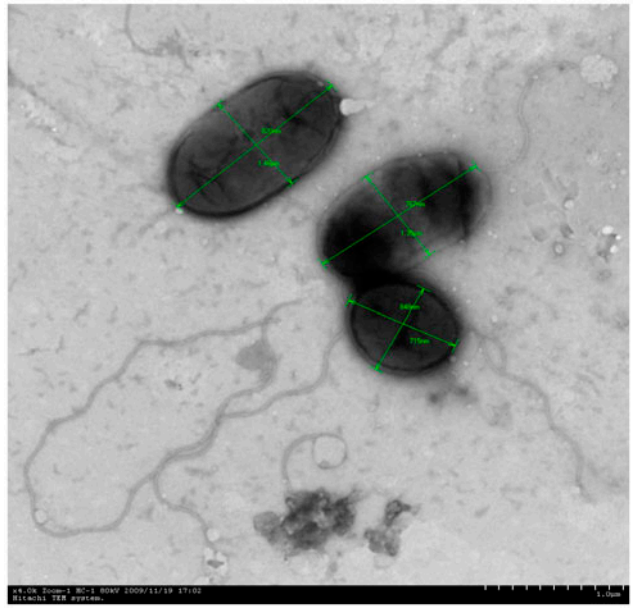

Fig. 2. Transmission electron microscope picture of TAD1 strain.

with single polar flagellum, 0.78 μm in diameter and 1.27 μm in length (average value of multi-bacteria). The morphology of the TAD1 strain can be shown as Fig. 2. It stained Gram negative and showed catalase and oxidase activity. Obvious heterotrophic growth was observed at pH values between 6.0 and 9.5 and at temperature of 20–52℃; optimal growth occurred at pH 7.0–8.0 and 35–40℃. The detailed taxonomical characteristics of the aerobic denitrifying strain TAD1 are shown in Table 1. A BLAST search of 16S rRNA with available data in the DDBJ/EMBL/GenBank databases showed a high similarity (99.0%) with *Chelatococcus daeguensis* strain K106, as is shown in Fig. 3 [19]. Thus, the isolated strain TAD1 may be classified into species of *C. daeguensis*.

Table 1
Taxonomical characteristics of the aerobic denitrifying strain TAD1

Test	Result
Morphological test	
Colony morphology	Round
Margin	Regular
Elevation	Raised
Surface	Smooth
Density	Opaque
Pigment	Light yellow
Gram's reaction	
Shape	Rod
Sized	Short
Physiological test	
Growth temperature (℃)	20–52
Growth pH	6.0–9.5
Biochemical test	
Oxidase test	+
Catalase test	+
Oxidation/fermentation (O/F)	O
Phenylalanine ammonialyase	–
H_2S production	–
Voges Proskauer test	–
Gelatin liquefaction	–
Nitrate reduction	+
Lysine decarboxylase	–
o-Nitrophenyl-β-D-Galactopyranoside	–
Methyl red test	–
Arginine dihydrolase	+
Lecithase test	–
Citrate utilization	+
Urease test	–
Indole production	–

Notes: +: positive reaction; –: negative reaction.

3.2. Aerobic denitrification characteristics of TAD1

3.2.1. Effect of carbon sources on denitrification activity

For aerobic denitrifiers, carbon source is a critical growth rate limitation factor [20,21]. Organic compounds are required as both carbon source for cell growth and energy source for the denitrification process [22]. Thus, carbon source is utilized in three pathways in nitrate reduction process: cell growth, respiration, and denitrification [7]. Glucose, sodium citrate, sodium acetate, and disodium succinate were tested as carbon sources for *C. daeguensis* strain TAD1. The effects of different carbon sources on the denitrification activity of TAD1 at 50℃ were shown in Fig. 4.

It can be seen from the figure that TAD1 can effectively remove nitrate when sodium acetate and disodium succinate were used as carbon source, especially for disodium succinate, the aerobic denitrification efficiency can reach up to 96.1% after 24 h at 50℃. This may be attributed to that disodium succinate is one of the intermediates in the tricarboxylic acid cycle (TCA cycle). Thus, succinate may support maximum growth rate of micro-organisms [23]. Sodium citrate used as carbon source lead to lower nitrate removal efficiency than sodium acetate and disodium succinate. It is noted that when glucose was used as carbon source, nitrate can hardly be removed in the experimental process. It may be because that glucose used as carbon source may repress nitrate reductase or electrons transferring from glucose to the nitrate reductase [24]. Therefore, disodium succinate is optimal carbon source for denitrification reaction of TAD1.

3.2.2. Effect of C/N ratio on denitrification activity

In general, carbon compounds are indispensable electron donor for nitrate reduction in denitrification process [25], so C/N ratio is a critical factor which influences the denitrification efficiency. In order to investigate the effect of C/N on denitrification, carbon source and initial nitrate concentration was, respectively, fixed as disodium succinate and 75.04 mg/L, but the C/N was variable in range of 1–20 by affording different initial disodium succinate concentration. The effect of C/N ratios on nitrate removal by TAD1 is presented in Fig. 5.

It can be seen that denitrification efficiency increases as more carbon was added. The removed nitrate increases from 10.84 to 45.08 mg/L with increasing C/N ratio from 1 to 7. As the C/N ratio was increased to 9, the removed nitrate reached 72.11 mg/L. 96.1% of the nitrates in the medium were removed by TAD1. This high nitrate removal

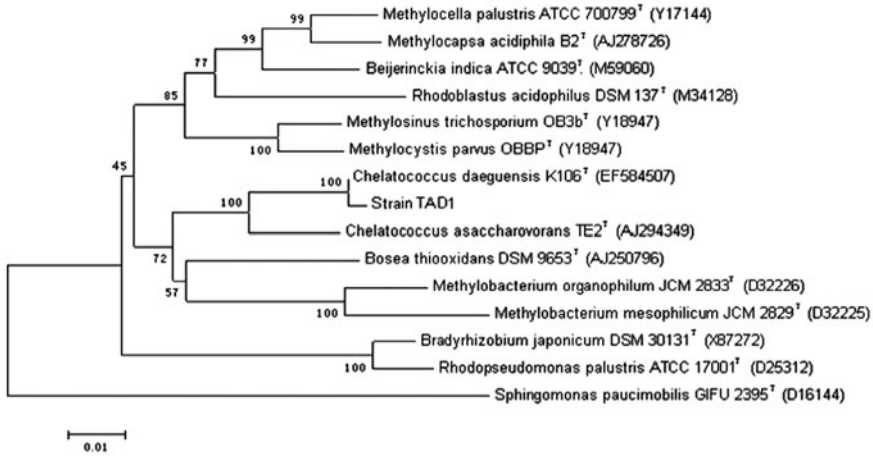

Fig. 3. Phylogenetic tree based on a comparison of the 16S rDNA gene sequence (The phylogenetic tree was generated using the neighbor-joining method. Bootstrap values, expressed as percentages of 1,000 replications, are given at branching points. Bar shows ten nucleotides substitutions per 1,000 nucleotides).

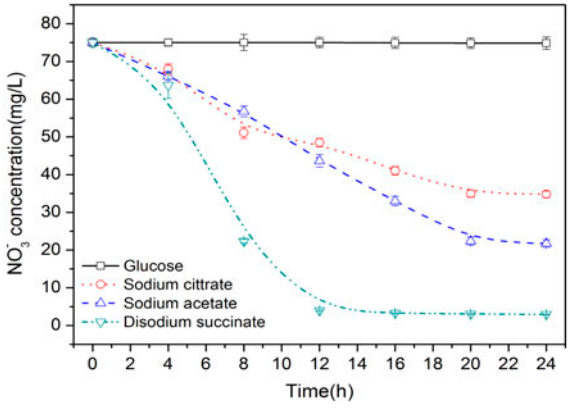

Fig. 4. Effect of different carbon sources on denitrification activity.

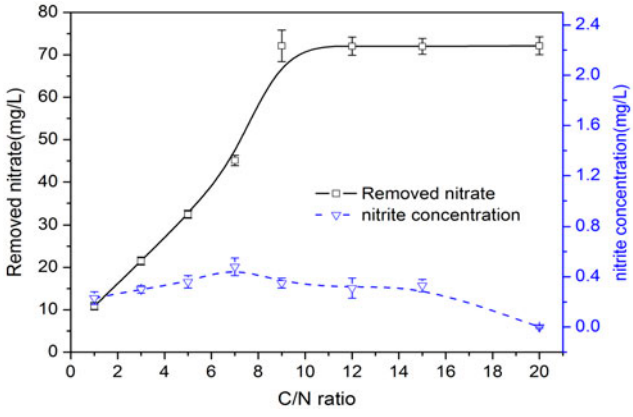

Fig. 5. Effect of the C/N ratio on nitrate removal and nitrite accumulation.

efficiency of *C. daeguensis* TAD1 at C/N ratio of 9 may benefit from the increasing activity of the nitrate reductase or the increase in cell density in broth. Further increases in the C/N ratio did not affect the nitrate removal, and denitrification efficiency of *C. daeguensis* TAD1 remained at a high level when C/N ratio was increased from 9 to 20. This result was different from that reported by Huang and Tseng [7], who pointed out that denitrification efficiency decreased and the growth of the denitrifiers were inhibited at high carbon concentrations. Therefore, the optimal C/N ratio for the aerobic denitrifier TAD1 in this study was about 9 at 50°C, which is obviously higher than that of *Citrobacter diversus* whose optimal C/N ratio was 4–5 at 25°C [7]. This may be attributed to the fact that in high temperature condition, *C. daeguensis* TAD1 needs more energy for cell growth and aerobic denitrification process [26]. Nitrite was not detected to accumulate obviously when the C/N ratio was increased from 1 to 20. Nitrite produced from nitrate reductase was quickly reduced by high nitrite reductase activity. This may be because the high temperature enhanced the activity of the denitrifier and reduced the accumulation of nitrite.

3.2.3. Effect of dissolve oxygen on denitrification activity

Some studies have demonstrated that there is a critical DO concentration value for effective aerobic denitrification process, and denitrification rate decreases significantly when DO concentration exceeds the critical value [4,24,27]. The effect of DO

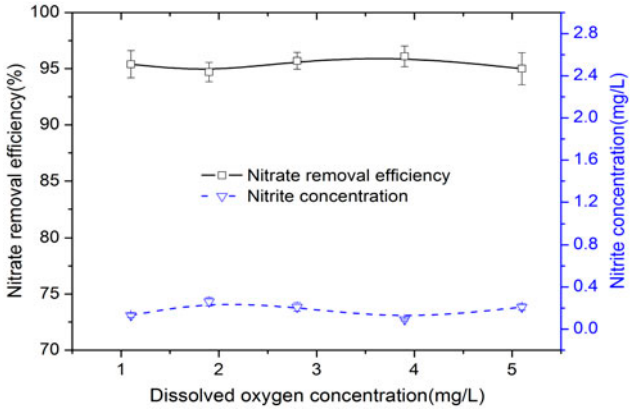

Fig. 6. Denitrification effects of strain TAD1 at various DO concentrations.

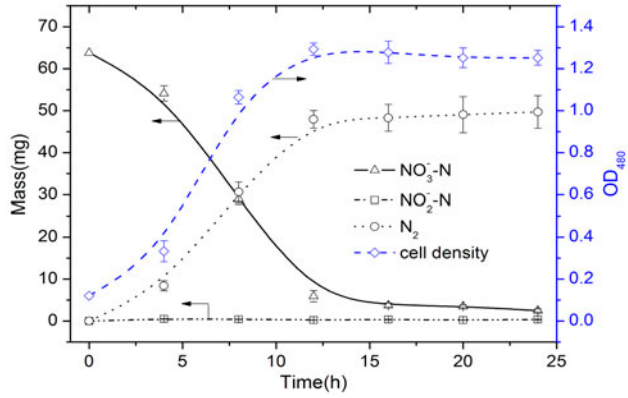

Fig. 7. Variation of NO_3^--N, NO_2^--N, N_2 and cell growth of strain TAD1 at optimal conditions.

concentration on the denitrification of *C. daeguensis* TAD1 was displayed in Fig. 6.

When DO concentration increased from 1.1 to 5.1 mg/L, denitrification efficiency was almost constant and remained at about 96%. It shows that nitrate reductase is not inhibited by oxygen, and nitrate removal efficiency can be remained at a steady level. It is reported that a well-known aerobic denitrifier *Pseudomonas* sp. has a maximum DO tolerance about 4 mg/L [7], so *C. daeguensis* TAD1 has a higher tolerance of DO concentration than *Pseudomonas* sp. Because of limitation of solubility of atmospheric oxygen in water at atmosphere and temperature of 50˚C, more experiments that DO concentration was over 5.1 mg/L were difficult to accomplish. So it is not very sure that it is the exact critical value for TAD1 in our study, but this critical value is beyond 5.1 mg/L definitely. This reveals that TAD1 has higher oxygen tolerance capacity than other aerobic denitrifiers. Nitrite was almost not detected as the DO concentration was increased from 1.1 to 5.1 mg/L. This may be because that DO concentration does not affect nitrite reductase activity.

3.3. Aerobic denitrification activity determination

Based on the study of the influential factors for aerobic denitrification activity, the aerobic denitrification activity at the optimal conditions can be determined. The optimal conditions include disodium succinate as carbon source, C/N ratio at 9, culture temperature at 50˚C and DO concentration at arbitrary level. Under these conditions, the results for variation of NO_3^--N, NO_2^--N, N_2 and the growth of strain TAD1 vs. the time were shown in Fig. 7.

It can be seen from the figure that cell growth exhibited no obvious lag phase at begin of the cultivation. When cell growth reached the exponential phase, the cell concentration, which is expressed by optical density at 480 nm (OD_{480}), increased quickly from 0.33(OD_{480}) to 1.29(OD_{480}), and the NO_3^--N dramatically decreased to 5.92 mg within 12 h. The results of nitrogen gas produced by TAD1 indicated that the amount of nitrogen gas increased with the increasing of *C. daeguensis* TAD1 growth rate. These results demonstrated that *C. daeguensis* TAD1 have ability of aerobic denitrifying and produce nitrogen gas from nitrate under thermophilic condition of 50˚C. At about 12 h later, the amount of nitrogen gas produced reached a plateau. During the whole denitrification process, NO_2^--N accumulation was not observed.

Moreover, optimal denitrification efficiency for *C. daeguensis* TAD1 at a relative high temperature is comparable to other reported aerobic denitrifiers at mesophilic temperatures. The results for comparison are listed in Table 2. It can be seen that at 50˚C the maximum aerobic denitrification efficiency of *C.*

Table 2
Comparison of denitrification efficiency for mesophilic bacteria and *C. Daeguensis* TAD1

Bacteria source	Temperature (˚C)	Denitrification efficiency (%)	Refs.
Citrobacter diversus	25	90.8	[7]
Pseudomonas putida	30	57.6	[8]
Delftia tsuruhatensis	30	60.5	[8]
Pseudomonas putida TD-21	30	95.9	[9]
C. daeguensis TAD1	50	96.1	This study

daeguensis TAD1 was 96.1%, which is slightly higher than *Pseudomonas putida* TD-21 and obviously higher than other mesophilic bacteria. Therefore, the proposed *C. daeguensis* TAD1 may act as a good alternative for aerobic wastewater treatment at the condition of relative high temperature where conventional mesophilic aerobic denitrifiers is ineffective.

4. Conclusions

An aerobic denitrifier strain *C. daeguensis* TAD1 has been successfully isolated from the biofilm of a field biotrickling filter. The isolated strain was gram-negative and short rod shape. A BLAST search of 16S rDNA with available data in the DDBJ/EMBL/GenBank database showed that the isolated strain has a high similarity (99.0%) with *C. daeguensis* strain K106. It was demonstrated that the *C. daeguensis* TAD1 could accomplish denitrification process in thermophilic and aerobic environment. The TAD1 strain could efficiently remove nitrate using sodium acetate and disodium succinate as carbon source. Especially when disodium succinate was used as carbon source, aerobic denitrification efficiency reached 96.1% after 24 h at 50˚C. The optimal C/N ratio for this aerobic denitrifier TAD1 was about 9. Tolerated oxygen level for TAD1 was about 5.1 mg/L, which was higher than that of *Pseudomonas* sp. Comparative study revealed that the *C. daeguensis* TAD1 had higher denitrification activity than the known mesophilic bacterial strains. Therefore, TAD1 may be promising in wastewater treatment under thermophilic condition.

Acknowledgments

This research was financially supported by the National Natural Science Foundation of China (Grants No. 51378217 and No. U1360101).

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