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Biodegradation of aniline by *Candida tropicalis* AN1 isolated from aerobic granular sludge

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Abstract

Aniline-degrading microbes were cultivated and acclimated with the initial activated sludge collected from a chemical wastewater treatment plant. During the acclimation processes, aerobic granular sludge being able to effectively degrade aniline was successfully formed, from which a preponderant bacterial strain was isolated and named as AN1. Effects of factors including pH, temperature, and second carbon/nitrogen source on the biodegradation of aniline were investigated. Results showed that the optimal conditions for the biodegradation of aniline by the strain AN1 were at pH 7.0 and 28–35°C. At the optimal pH and temperature, the biodegradation rate of aniline could reach as high as 17.8 mg/(L·hr) when the initial aniline concentration was 400 mg/L. Further studies revealed that the addition of 1 g/L glucose or ammonium chloride as a second carbon or nitrogen source could slightly enhance the biodegradation efficiency from 93.0% to 95.1%–98.5%. However, even more addition of glucose or ammonium could not further enhance the biodegradation process but delayed the biodegradation of aniline by the strain AN1. Based on morphological and physiological characteristics as well as the phylogenetic analysis of 26S rDNA sequences, the strain AN1 was identified as *Candida tropicalis*.

Key words: aniline; aerobic granular sludge; biodegradation; *Candida tropicalis* AN1

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Introduction

Aniline is a widely distributed pollutant in some industrial wastewaters, and has been listed as one of the priority pollutants by US EPA and the Ministry of Environmental Protection of China due to its threat to both environment and human health (Lyons et al., 1985; Khan et al., 2003; Kim et al., 2004; Wang et al., 2007). Thus, the biodegradation of aniline is of great concern and has attracted many researchers' attention. To date, it is well recognized that aniline can be efficiently removed by aerobic biological treatment (Takeo et al., 1998; Zhang et al., 2008; Xiao et al., 2009), and many aniline-biodegrading bacteria such as *Pseudomonas* sp. (Anson and Mackinnon, 1984) have been isolated and identified, from which several aniline-degrading genes or gene clusters were cloned (Meyers, 1992; Fujii et al., 1997; Fukumori and Saint, 1997; Takeo et al., 1998).

Aerobic granular sludge is a novel environmental biotechnology and has been extensively to be used in municipal wastewater treatment (Wang et al., 2004; Sunil et al., 2008). It is a microbial community with high integrity, mainly consisted of aerobic, anaerobic and anoxic microorganisms (Arrojo et al., 2004; Liu and Liu, 2006; Zhou et al., 2007). In previous studies, aniline biodegrading bacteria were usually firstly isolated and purified with low aniline concentrations, and then enriched with higher aniline concentrations to enhance their degrading abilities (Wei et al., 1998; Ren et al., 1998). In this study, however, another approach for the enrichment of aniline biodegrading bacteria was employed, in which certain sludge samples were directly enriched with gradually increased aniline concentrations to form stable aerobic granular sludge. It is speculated that, during the enrichment process, the abundance and population of microorganisms which could survive in the sludge would decrease with increasing aniline concentrations due to the toxicity of aniline. However, because of the synergistic interaction among different microorganisms, the strains which can survive at high aniline concentrations would synergize well with each other and thus possess high potential for aniline removal (Wang and Zhou, 2010). Therefore, it is presumed that aerobic granular sludge or degrading strains isolated from it may possess a higher degradation capacity for aniline than that isolated from wastewater or activated sludge.

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The objectives of the present study were to: (1) study the feasibility of forming aerobic granular sludge with high aniline removal efficiency through acclimation processes with initial activated sludge from wastewater treatment plant; (2) isolate bacteria capable of using aniline as sole carbon and nitrogen sources from the aerobic granular sludge formed; (3) investigate factors including pH, temperature, and second carbon/nitrogen source on the biodegradation of aniline by the isolated strain. These results are helpful for understanding the formation mechanism of the aerobic granular sludge during wastewater treatment processes and the synergistic effects of microbes included in the aerobic granular sludge.

1 Materials and methods

1.1 Sludge sample and liquid medium

The activated sludge was collected from an aeration tank of Nanjing Chemistry Factory Wastewater Treatment Plant (China) which was used to treat chemical wastewater containing aniline and nitrobenzene.

The liquid medium used for cultivating microorganisms was mineral salt medium containing 1 g K_2HPO_4, 2.5 g NaH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$ and 0.2 g KCl in 1 L nonionic water, adjusted to pH 7.4 with 2 mol/L NaOH, and sterilized at 121°C for 20 min.

1.2 Enrichment of aerobic granular sludge and isolation of aniline-degrading bacteria

Sludge sample of 10 mL was firstly inoculated into 90 mL liquid medium described above containing 10 mg/L aniline as the sole carbon and nitrogen sources, and cultivated on a rotary shaker at 180 r/min and 28°C for 7 days. Then 10 mL sub-samples were collected and re-inoculated into the same liquid medium but with higher concentration of aniline. After the consecutive cultivation process, the concentrations of aniline in the medium steadily increased from 10 to 1000 mg/L, and aerobic granular sludge were successfully obtained (Wang and Zhou, 2010). The aerobic granular sludge was stirred and diluted with sterile water, and then spread onto agar plates in which 600 mg/L aniline and 1.5% (*W*/*W*) agar were added into the mineral salt medium. After 72 hr incubation at 28°C three strains with the potential of degrading aniline were obtained and named as AN1, AN2 and AN3, respectively. The three strains were cultivated at 28°C for 72 hr in flasks containing 100 mL mineral salt medium supplied with 100 mg/L aniline as carbon source. After that, 10 mL strain cultures were inoculated into 250 mL flasks containing 90 mL mineral salt medium amended with 600 mg/L aniline to test their biodegradation abilities. These flasks were then shaken on a rotary shaker at 180 r/min and 28°C. Samples were collected from each flask at 6 hr intervals and centrifuged at 12,000 r/min for 10 min before being subjected to determine the remaining concentration of aniline.

1.3 HPLC measurement

Determination of aniline was performed on HPLC (Waters 1525, USA) equipped with both UV (2487) and fluorescence detector (2475) and a reverse phase C18 column (4.6 mm × 150 mm, 5 μm particle size). The mobile phase was a mixture of water/acetonitrile 30/70 (*V*/*V*), delivered by a pump at a flow rate of 1 mL/min (Zhao et al., 1997). The UV wavelengths were set at 254 nm and the column was held at 30°C. Under these conditions the retention time of aniline was about 2.5 min. The calibration curve was established between the peak areas and the concentration of aniline, which was used to calculate the concentration of aniline in the experiment. The data presented were the average values derived from three measurements and their relative standard deviations were less than 10%.

1.4 Effect of initial aniline concentration on the biodegradation of aniline by the strain AN1

The effect of initial aniline concentration on the biodegradation of aniline by AN1 was studied using five different aniline concentrations: 100, 200, 400, 600 and 800 mg/L. AN1 culture of 10 mL was inoculated into 250 mL conical flasks containing 90 mL mineral salt medium with the presence of certain aniline concentration (100, 200, 400, 600 or 800 mg/L) to give the final AN1 density of 10^6 cells/mL, then all these flasks were shaken on a rotary shaker at 180 r/min and 28°C. All experiments were performed in triplicate. The 5 mL samples were collected from each flask at 6 hr intervals and then centrifuged at 12,000 r/min for 10 min. The supernatant was diluted and used to determine the aniline concentration.

1.5 Effect of initial pH on the biodegradation of aniline by the strain AN1

The effect of initial pH on the biodegradation of aniline by the strain AN1 was also studied in 250 mL conical flasks. The same as before, 10 mL of strain AN1 culture was inoculated into flasks containing 90 mL mineral salt medium with the presence of 400 mg/L aniline to give the final AN1 density of 10^6 cells/mL. The pH values of medium were then adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0, respectively, using 2 mol/L H_2SO_4 or 2 mol/L NaOH. These flasks were then shaken on a rotary shaker at 180 r/min and 28°C. Samples were also collected from each flask at 6 hr intervals and centrifuged at 12,000 r/min for 10 min before being subjected to determine the aniline concentration.

1.6 Effect of temperature on the biodegradation of aniline by the strain AN1

Strain AN1 culture of 10 mL was inoculated into 250 mL conical flasks containing 90 mL mineral salt medium with the presence of 400 mg/L aniline to give the final AN1 density of 10^6 cells/mL. The pH values of medium were adjusted to 7.0 using 2 mol/L H_2SO_4 or 2 mol/L NaOH. Then these flasks were shaken on rotary shakers at 180 r/min and five different temperatures, i.e., 15, 20, 25, 28 and 35°C, respectively. Samples collection and treatment procedures were the same as before.

1.7 Effect of second carbon or nitrogen sources on the biodegradation of aniline by the strain AN1

To study the effect of second carbon or nitrogen sources on the degradation of aniline, different dosages (1, 3, and 5 g/L) of glucose as a second carbon source as well as different dosages (1, 3, and 5 g/L) of ammonium chloride as a nitrogen source were added into the flasks containing 90 mL mineral salt medium, 10 mL strain AN1 culture and 400 mg/L of aniline. Only aniline added as sole carbon and nitrogen source was set as control. The pH values of medium were adjusted to pH 7.0 using 2 mol/L H_2SO_4 or 2 mol/L NaOH and incubated on a rotary shaker at 180 r/min and 28°C.

1.8 DNA extraction and PCR amplification

The strain AN1 was grown on the agar medium (20 g/L D-glucose, 10 g/L peptone, 5 g/L yeast extract and 20 g/L agar) for 48 hr at 25°C. Two or three loops of this culture were suspended in 500 μL sterile nonionic water. Biomass collected on a glass fiber membrane (GF/C, Whatman) was washed off with a PBS (130 mmol/L NaCl, 10 mmol/L sodium phosphate buffer pH 7.2) and then centrifuged at 8000 r/min for 10 min in a 2-mL centrifuge tube at 4°C . DNA was obtained by simply disrupting the cells through freezing in liquid nitrogen for 5-min, followed by a 5-min heat shock at 100°C (Xiao et al., 2009).

PCR reactions were performed with the NL1 gene (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACG-3′), denaturation was firstly performed at 94°C for 6 min, and then 40 cycles were completed with an initial denaturation at 94°C for 3 min, followed by 36 cycles with a temperature profile of 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min. An extension period of 5 min at 72°C was carried out at the end of the 36 cycles (Baleiras Couto et al., 2005).

The recombinant plasmid containing a DNA fragment about 615 bp from the strain AN1 was sequenced with shotgun by Shanghai Biological Engineering Company (China) and then assembled using the Big Dye package (USA). The PCR products were analyzed using ABI 377, and the 26S rD1/D2 sequence of the strain AN1 was made against the sequences in the GenBank using the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov). Phylogenetic tree was constructed using the Meg Align program (Saitou and Nei, 1987).

2 Results and discussion

2.1 Formation of aerobic granular sludge and isolation of aniline degrading strains from aerobic granular sludge

During the processes of acclimation, a large amount of floccus was firstly formed at the end of acclimation period with 100 mg/L aniline, and granular matters began to form in the following acclimation processes with much higher aniline concentrations. The quantity and size of these granular matters increased gradually with time prolonged. Yellowish granular sludge was formed after about three months of acclimation, which was consistent with our previous results (Wang and Zhou, 2010). Microscopic examination revealed that the aerobic granular sludge was mainly composed of yeasts, filamentous bacteria or fungi, and relatively low abundance of bacteria.

The biodegradation kinetics of aniline by the strains (AN1, AN2, and AN3) is shown in Fig. 1. After 36 hr of incubation, 67.8%, 16% and 7.5% of 600 mg/L aniline added were degraded by the strain AN1, AN2 and AN3, respectively. The biodegradation capability of the strain AN1 for aniline was remarkably higher than that of either AN2 or AN3, thus the strain AN1 (Fig. 2) is regarded as the preponderant strain for the biodegradation of aniline in the aerobic granular sludge acclimated.

The effect of initial aniline concentration on the biodegradation of aniline by the strain AN1 was also investigated. As shown in Fig. 3, when the initial aniline concentration was below 800 mg/L, the strain AN1 could successfully degrade aniline added and the degradation rates depended on the initial concentrations. Linear correlation analysis of the curves in Fig. 3 indicated that the biodegradation rate could reach as high as 17.8 mg/(L·hr) at the initial aniline concentration of 400 mg/L. And the linear equation ($R^2 = 0.91$) was:

$$y = -17.8x + 369.5 \quad (1)$$

where, y (mg/L) is the residual aniline concentration in the medium and x (hr) is the reaction time.

When the initial aniline concentration was 100, 200, 600 or 800 mg/L, the degrading rate was 8.3 mg/(L·hr) ($R^2 = 0.94$), 11.9 mg/(L·hr) ($R^2 = 0.91$), 13.3 mg/(L·hr) ($R^2 = 0.98$) and 8.0 mg/(L·hr) ($R^2 = 0.95$), respectively. Our further study revealed that the biodegrading rate of aerobic granular sludge formed by the strain AN1 could reach 33.6 mg/(L·hr) (Wang and Zhou, 2010). Although previous researches have found that *Delftia tsuruhatensis* strain could consume aniline at 1000 mg/L in 4 days (Sheludchenko et al., 2005) and *Delftia* sp. AN3 isolated was capable to degrade 5000 mg/L aniline in 7 days (Liu et al., 2002), the degradation rates calculated using linear

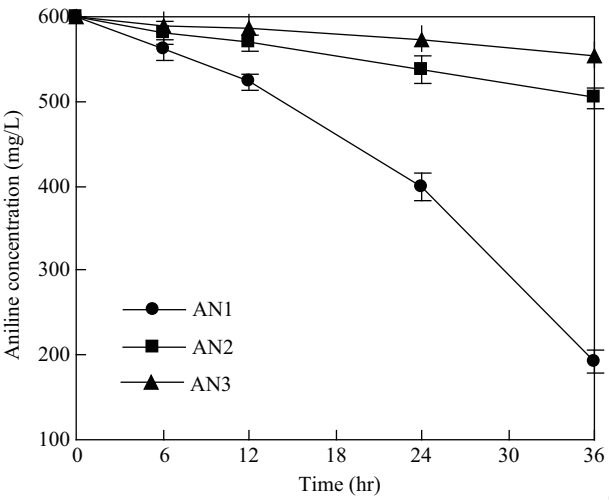

Fig. 1 Biodegradation of aniline by strain of AN1, AN2 or AN3, respectively.

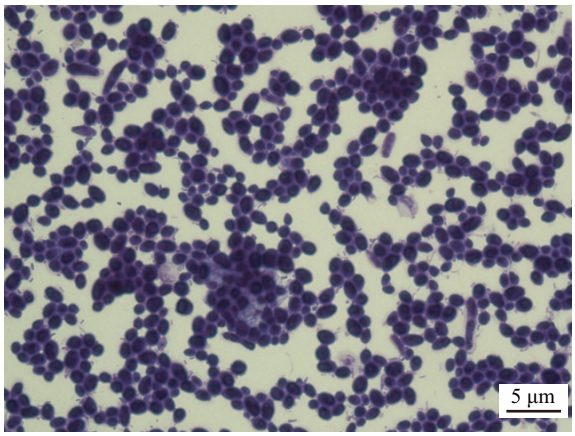

Fig. 2 Photomicrograph of strain AN1 isolated from the aerobic granular sludge (10 × 100).

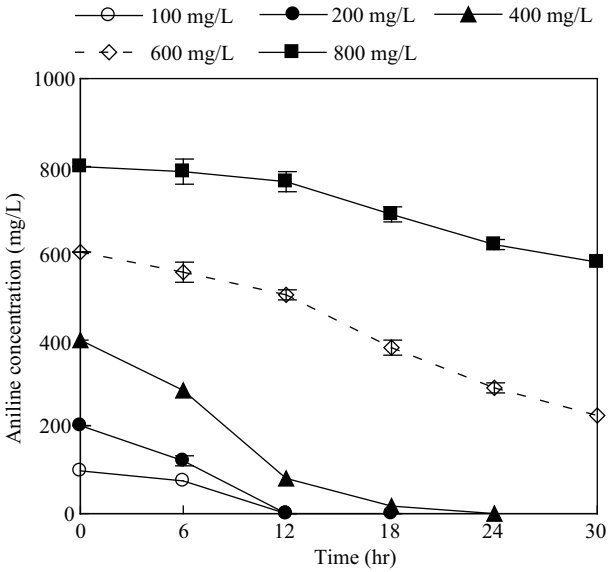

Fig. 3 Effect of initial concentrations on the biodegradation of aniline by strain AN1.

correlation analysis were only 10.42 and 29.76 mg/(L·hr) which were lower than that achieved by aerobic granular sludge formed with the strain AN1. Therefore, the strain AN1 has superiority over other isolated strains for aniline biodegradation due to its ability to form aerobic granular sludge which possessed much higher biodegradation capacity for aniline.

2.2 Effect of initial pH on the biodegradation of aniline

The effect of initial pH on the biodegradation of aniline is shown in Fig. 4. It was found that when the pH was 5.0 or 9.0, aniline biodegradation process was significantly inhibited, implying the restricted growth of the strain AN1 at such pH values. However, when the pH was at the range of 6.0 and 8.0, more than 50% of aniline added could be biodegraded by the strain AN1 in 24 hr. Aniline concentration was dropped from 400 to 17.8 mg/L in only 18 hr especially at pH 7.0, which mean that the biodegradation efficiency could reach as high as 95.6%. Our results are consistent with previous studies which have found that most degrading bacteria, such as *Pseudomous* (Hinteregger et al., 1992) and *Frateuria* (Murakumi et al., 1999), could degrade aniline at a narrow pH range of 6.0 and 8.0.

2.3 Effect of temperature on the biodegradation of aniline

As shown in Fig. 5, the optimal temperature for the growth of the strain AN1 was 28–35°C. Aniline biodegradation efficiency was the fastest at this temperature range as aniline concentration decreased from 400 mg/L to 24.3–16.5 mg/L in only 18 hr and could be completely degraded in 24 hr. However, aniline biodegradation efficiency was dramatically decreased when the temperature dropped to 15°C, indicating that the lower temperature is not beneficial for the biodegradation of aniline by the strain AN1.

2.4 Effect of second carbon and nitrogen sources on the biodegradation of aniline by AN1

The effect of glucose as a second carbon source or ammonium chloride as a second nitrogen source on the biodegradation of aniline by the strain AN1 was also investigated. As shown in Fig. 6, the aniline degradation efficiency could reach as high as 93.0% within 18 hr when only aniline was added as a sole carbon source (CK). After adding 1 g/L glucose as the second carbon source, the aniline degradation efficiency was slightly increased by 5.5% within the same period.

However, when more than 1 g/L glucose was added, the aniline degradation efficiency within the same period even decreased drastically (Fig. 6a). The same phenomenon could also be observed in the tests of adding ammonium chloride as a second nitrogen source. It was found that addition of 1 g/L ammonium chloride could slightly enhance the biodegradation process (Fig. 6b), but even more addition of ammonium chloride would lag or delay the biodegradation process of aniline by AN1. The enhanced degradation of aniline was due to the fact that extra glucose or ammonium chloride could be co-metabolized as a second carbon or nitrogen source to enhance the growth of AN1. However, when an excessive second carbon or

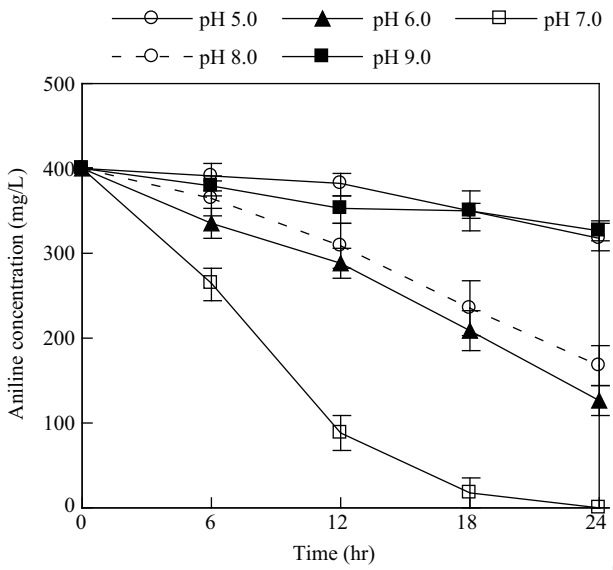

Fig. 4 Effect of initial pH on the biodegradation of aniline by strain AN1.

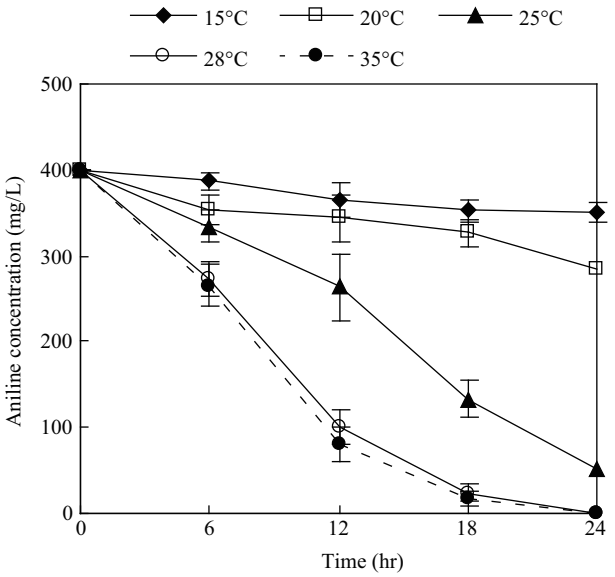

Fig. 5 Effect of temperatures on the biodegradation of aniline by strain AN1.

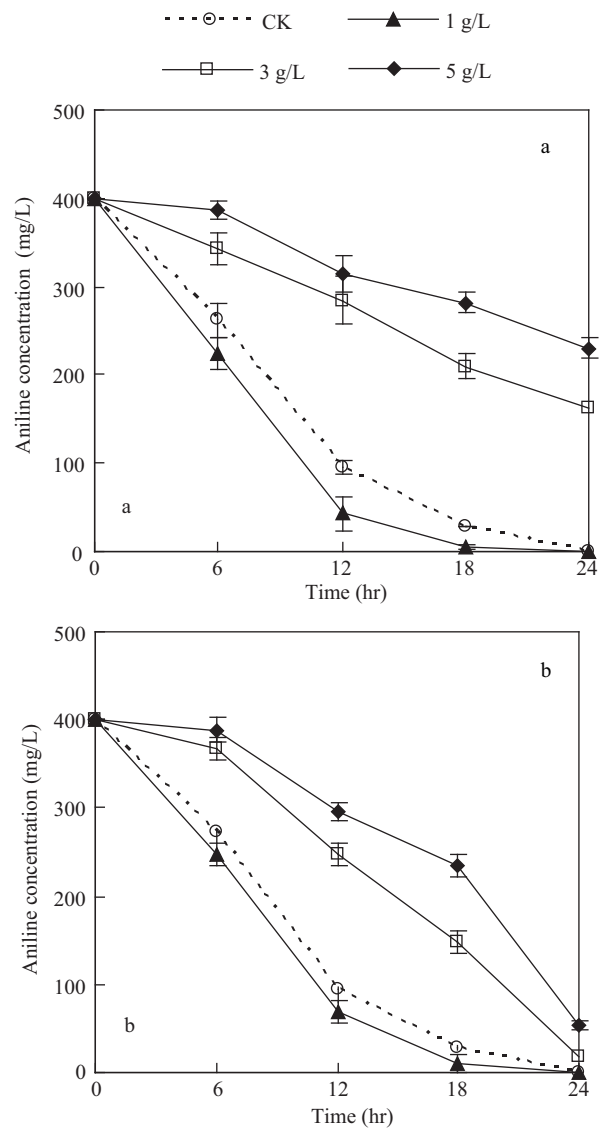

Fig. 6 Effect of different doses of glucose (a) as second carbon and ammonium chloride (b) as second nitrogen on the biodegradation of aniline by strain AN1.

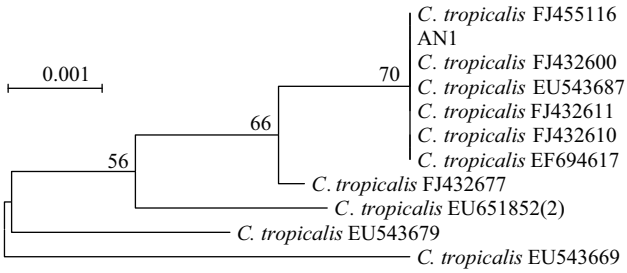

Fig. 7 Phylogenetic tree drawn from neighbor-joining analysis based on the 26S rDNA D1/D2 sequence of strain AN1 and close strains.

nitrogen source was present, the degradation of aniline was suppressed since glucose and ammonium chloride could be more easily assimilated than aniline by microorganisms (Wei et al., 1998; Liu and Xie, 2007).

2.5 PCR reaction sequencing of region 26S rD1/D2

The strain AN1 was found to be closely related to *C. tropicalis* based on the sequence of 26S rDNA D1/D2, and a better comparison between the strain AN1 and 10 high similar strains was performed by drawing a phylogenetic tree as shown in Fig. 7.

The complete sequence of the 615 bp 26S rDNA fragment form the strain AN1 had been deposited in the GenBank database (accession no. HM231275). The similarity of the strain AN1 with other *C. tropicalis* strains was more than 99%. Based on the phylogenetic analysis of 26S rD1/D2 sequences, the strain AN1 isolated from the aerobic granular sludge was identified as *C. tropicalis* species.

3 Conclusions

The strain, AN1, isolated from the aerobic granular sludge can use aniline as sole carbon, nitrogen, and energy source, and the optimal pH and temperature range for the biodegradation of aniline by AN1 are pH 7.0 and 28–35°C, respectively. At an initial aniline concentration of 400 mg/L, the biodegradation efficiency reaches 93.9% in 18 hr, and 100% in 24 hr, with a biodegradation rate of 17.8 mg/(L·hr). In the light of phylogenetic analysis of 26S rDNA sequences, the strain AN1 was identified as *C. tropicalis*. To our knowledge, it is the first report that the aniline-degrading bacteria is successfully isolated from aerobic granular sludge. Further work should be performed to study the role of the isolated strain in the formation mechanisms of aerobic granular sludge.

Acknowledgments

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