Denitrification in municipal waste landfill by *Pseudomonas stutzeri* P-1-5

Yangqing Wang[1,a], Liyan Song[1,b,*], Yu Lei[1,c]

[1]Environmental Microbiology and Ecology Research Center, Chongqing Institute of Green and Intelligent Technology, Chinese Academy of Science, Chongqing, 401122, China

[a]Email: wangyangqing@cigit.ac.cn, [b]Email:songliyan@cigit.ac.cn , [c]Email: leiyu@cigit.ac.cn

*Correspondence to Dr. LiYan Song

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Abstract. Although *in situ* denitrification has been well documented in landfills, few studies have paid attention on the denitrifier in landfills who dominates this process. In this study, a strain P-1-5 was isolated from landfill by *Pseudomonas* selecting medium and identified as *Pseudomonas stutzeri* by 16S rRNA gene sequencing. P-1-5 could use many carbon sources as well as sodium salt of caboxy methyl cellulose. When the initial concentration of nitrate was 415.8 mg/L, the strain completely removed nitrate within 16 h under aerobic condition and 93.1 % nitrate within 48 h under anaerobic condition. The strain is a denitrifer and the optimal culture conditions for nitrate remove was sodium succinate as carbon source, C/N 8，pH 7, and 30 ℃. Results of this study provides the direct evidence that *Pseudomonas* plays important role in nitrogen cycle and insights for the increased understanding of nitrogen management in landfill.

Introduction

China produced 100 billion tons municipal solid waste (MSW) in 2010 and landfilled 70% of them. During the processes of refuse decomposition, landfill leachate and landfill gas are produced. Landfill leachate, a highly contaminated wastewater, might threaten human health and environment such as surface and ground water. Landfills are the important sources of anthropogenic greenhouse gases such as methane [1] and nitrous oxide [2] as it contains large amount organic matter (e.g. cellulose and hemicellulose) and protein [3]. Most previous researches focus on the carbon cycle in landfill by studying functional microorganism isolation (e.g. cellulolytic microorganism) and microbial community structure along refuse decomposition [4]. However, nitrogen cycle in landfill has not been well characterized.

Because landfills are primary anaerobic/anoxic zones, denitrification occurs. Both heterotrophic denitrification and autotrophic denitrification have been confirmed in landfill [5]. The advantage of denitrification includes the simultaneous removal carbon and nitrate without requiring oxygen input and recovering half of the alkalinity consumed during nitrification. Denitrification might inhibit acetogenic, sulfate reduction, and methanogenic processes because nitrate, served as a terminal electron acceptor, are energetically favor over the related terminal electron acceptors in those process [5]. Therefore, understanding the denitrification in landfill is critical for carbon and nitrogen management in landfill.

Previous researches have assessed *in situ*, or partially *in situ*, denitrification at both laboratory and field scale. Burton and Watson-Craik [6] used a landfill cell to denitrify externally nitrified leachate and found that nitrate was efficiently consumed under anaerobic/anoxic landfill conditions. Price et al. [7] evaluated the denitrification activity of active decomposing refuse and well decomposed refuse. Result showed that active decomposing refuse had much higher nitrate removal ability than well decomposed refuse. Although *in situ* denitrification has been well documented, few studies have paid attention on the denitrifier in landfill who dominates this process. Therefore, the study of denitrifier in landfill would reveal insight into the *in situ* denitrification and nitrogen cycle within this niches.

In our recent study we showed that genus *Pseudomonas* was dominant at landfill leachate by 454 pyrosequencing (accounting for 40.9% to 92.4% of total abundance of bacteria) [4] as well as at landfill refuse (data not shown) by Illumina pyrosequencing. Xie et al. [8] also found that *Pseudomonas* is important in aged refuse for leachate treatment by 454 pyrosequencing. *Pseudomonas* is a genus of Gram-negative, δ-*proteobacteria*, belonging to the family *Pseudomonadaceae* containing 223 validly described species (see http://www.bacterio.net/pseudomonas.html). The members of *Pseudomonas* demonstrate a great deal of metabolic diversity and are able to colonize a wide range of niches [9]. *Pseudomonas* also has denitrification ability [10]. However, the reports on the isolation of *Pseudomonas* and its denitrification in landfill is limited.

In this study, 19 strains were isolated by *Pseudomonas* selection medium from landfill refuse. The strain with highest denitrification activity was characterized and identified as *Pseudomonas stutzeri* P-1-5 by 16S rRNA gene sequences. P-1-5 had denitrification ability under both aerobic and anaerobic condition, suggesting its main role in nitrogen cycle in landfill. The main factors (carbon source, ration of C/N, temperature, and pH) influencing denitrification of the strain was then investigated. This study will help to better characterize the nitrogen cycle of landfills and provide insights for the increased understanding of nitrogen management at these locations.

Materials and Methods

Sampling site description and sampling. Refuse sample for *Pseudomonas* isolation was obtained from Jiangchungou (JCG) landfill, Xian, China in 15 September 2013. Early study indicated that the relative abundance of *Pseudomonas* was 8.0 % of the total bacteria (total 18062 well treated sequences) in JCG landfill leachate [4]. Refuse were cored using the same protocol as leachate sampling [4]. The samples in drill sleeve were taken by pushing an extensible core pusher. Samples were cored at a depth of 0.8-1.0 m. After retrieval, the refuse cores were sealed in steel sleeve (inner diameter: 150mm, height: 300mm) and maintained at 4 °C. The outer layer of the cores (1-2 cm) was removed in the laboratory under clean condition to eliminate possible contamination. The remaining refuse was homogenized aseptically by manual mixing.

Isolation and identification . Five gram well mixed refuse was dispersed in 50 ml of sterile PBS buffer (pH 7.4). These 10^{-1}-diluted aliquots were serially diluted to 10^{-2} and 10^{-3}. Two hundreds uL of the dilutions was used to inoculate on *pseudomonas* selection agar medium [11]. One liter *pseudomonas* agar medium contained 16 g gelatin peptone, 10 g enzymatic digest of casein, 10 g potassium sulfate, 1.4 g magnesium chloride, and 11 g agar (pH 7.1). One mililiter sterilized CFC and 1ml sterilized glycerol were added into 1 L 45°C sterilized medium to form the *pseudomonas* selection medium. One liter CFC was composed of 10 mg cetrimide, 10 mg fucidin and 50 mg cephaloridine. All *pseudomonas* medium and supplements were purchased from Rishui biotechnology company, Qingdao, China.

DNA extraction from the purified strain was performed using the Ultraclean soil DNA Kit according to the manufacturer's instructions and then quantified by NanoVue plus spectrophotometer (GE, USA). The small-subunit ribosomal DNA was amplified using 16S rRNA general primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The conditions for PCR were using initial denaturation at 95°C for 4 min, followed by 30 cycles of 1 min at 94°C 1 min at 55°C and 1 min at 72°C; and a final extension at 72°C for 10 min. PCR products were visualized by 0.8% agarose gel (w/v) electrophoresis and purified by TIANgel Midi Purification Kit (TianGen, China). The purified DNA fragments were ligated into pMD19-T vector (TaKaRa, China) according to vendor's instruction. Approximately 200 clones were grown in Luria-Bertani (LB) plate supplemented with 50 μg/ml ampicillin. Three clones were picked and cultured overnight at LB broth with same concentration ampicillin. The plasmid was then extracted by TIANprep Mini Plasmid Kit (TianGen, China), purified by TIANpure Mini Plasmid Kit (TianGen, China), and sequenced at BGI Corp (Beijing, China) by ABI 3730 using the 27F primer. The obtained 16S rRNA gene sequences were assembled

with Seqman II 5.0 (DNASTAR) [12] and analyzed using BLASTn program. Phylogenetic trees were constructed by the neighbour-joining [13] method of MEGA version 6.0. Evolutionary distances were calculated using Kimura's two-parameter model and bootstrap values were based on 1000 replications [14]. Sequences were deposited in the GenBank public database under the accession numbers KM201252.

Carbon utilization. The carbon utilization characters of the isolated strain were tested using the API 50CH systems with the vendor's instruction. Cellulose is major carbon source in landfill [3]. Thus it was tested by the strain. The strain was incubated in flask containing denitrification medium with sodium salt of caboxy methyl cellulose (CMC) as sole carbon at 30°C and150rpm for 24 h.

The basic denitrification medium contains (per liter) 3 g KNO_3, 16.4 g CH_3COONa, 0.2 g $MgSO_4 \cdot 7H_2O$, 3 g K_2HPO_4 and 1 g KH_2PO_4 (pH: 7.0). Before use, 1 L denitrification medium was supplemented with 2 ml trace element solution [15]. The trace element solution contained (per liter) 57.1 g EDTA·2Na, 3.9 g ZnSO4·$7H_2O$, 7.0 g $CaCl_2 \cdot 2H_2O$, 5.1 g $MnCl_2 \cdot 4H_2O$, 5.0 g $FeSO_4 \cdot 7H_2O$, 1.1 g $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, 1.6 g $CuSO_4 \cdot 5H_2O$, and 1.6 g $CoCl_2 \cdot 6H_2O$ (pH: 6.0).

Assessment of denitrification property. Strain *P-1-5* was inoculated in a 250 ml flask (for aerobic culture) or sterve anaerobic bottles (for anaerobic culture) containing 100 ml denitrification medium and incubated on a shaker (150 rpm) for 24 hours at 30 °C under aerobic and anaerobic conditions.

Evaluating denitrification activity of P-1-5. To evaluate how carbon sources affect the denitrification ability, strain P1-5 was cultured in denitrification medium with the addition of the following carbon sources: glucose, sucrose, citrate, sodium acetate, ethanol, methanol, sodium succinate. Three grams of KNO_3 was provided as nitrogen source and the C/N ratio was adjusted to 12. The effects of C/N ratios were measured using sodium succinate as the sole carbon source, and a C/N ratio of 2, 4, 8, 12, and 16, respectively. The removal efficiency in different pH (4, 5, 6, 7, and 8) and temperature (30°C, 40°C, 50°C) were also measured.

Analysis method. The OD value of the culture broth was measured at 600 nm using a spectrophotometer (UV 8500, Japan). Culture samples were centrifuged at 12,000 rpm for 10 minutes and the supernate was used for chemical analysis. TN, nitrate, and nitrite were determined according to standard methods [16]. TN was determined by alkaline potassium persulfate photometry. Nitrite was determined by N-(1-naphthalene)-diaminnoethane photometry method at a wavelength of 540 nm. Nitrate was measured by phenol disulfonic acid photometry method.

Statistics method. All tests were performed in triplicates. All data were expressed as the mean ± standard error.

Results and discussion

Isolated and identification. Nineteen strains of microorganisms were isolated from *pseudomonas* selection agar medium. Among these strains, strain P1-5 showed the highest denitrification activity (completely removed 300 mg/L nitrate in 24h) and was thus selected for the further study.

The strain P1-5 formed small (1-2 mm in diameter), hard, dry, and tenaciously coherent and the freshly isolated colonies were wrinkled appearance on the agar culture media after 36 h of growth at 30°C under aerobic conditions. The strain is gram negative.

The length of 16S rRNA gene sequence of strain P-1-5 is 1057 bp. The comparison of the 16S rRNA gene sequence of P-1-5 and the corresponding sequences of other bacterial strains in GenBank database through BLAST indicated that strain P-1-5 was most similar to two 4-*n*-butylphenol utilizing *Pseudomonas* sp. species isolated from the rhizosphere sediment (99% sequence similarity) [17], an aerobic denitrifier *Pseudomonas stutzeri* specie from activated sludge (99%) [18], an aerobic denitrifier *Pseudomonas stutzeri* (99%) [19], and a model denitrifier *Pseudomonas stutzeri* ATCC 17588 (99%) [10], suggesting that strain P-1-5 might play important role in denitrification in landfill. A phylogenetic tree was constructed according to the neighbor-joining algorithm (Figure 1). The tree topology, supported by high bootstrap values, clearly showed that strain P-1-5 was within the genus *Pseudomonas*.

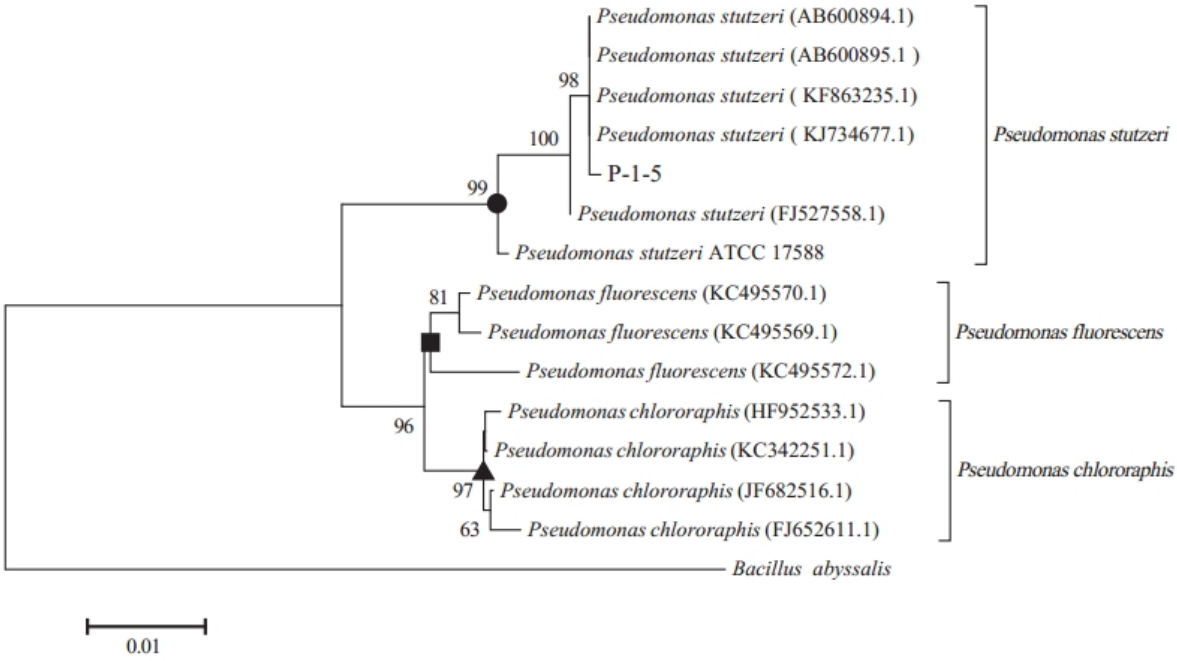

Fig.1. Phylogenetic relationship of the strain P-1-5 with partial 16S rDNA sequences (1100-1500bp) of know bacteria based on the Neighbour-Joining analysis and bootstrap values (1000 replications). The scale bar represents 1% substitutions per nucleotide sequence position. Numbers at nodes represent bootstrap values. Only bootstrap values above 50% are displayed. Strain P-1-5 (1057bp) is depicted in bold. Strain Pseudomonas stutzeri is indicated with a filled circle (●). Strain Pseudomonas fluorescens is indicated with a filled square (■). Strain Pseudomonas chlororaphis is indicated with a filled triangle(▲). Strain Bacillus abyssalis was used as outgroup.

Assessment of the carbon utilization by *P. stutzeri* P-1-5 strain. Strain P-1-5 could use 41 (total 54) kinds of carbon source, including mannitol，L-Arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-Mannose, L-Sorbose, L-rhamnose monohydrate, sorbitol, Methyl alpha-D-Mannopyranoside, Methyl-a-D-glucopyranoside, N-Acetyl glucosamine, nitrilosides, arbulin, esculin, salicin, D-(+)-Cellobiose, D-maltose, D-lactose, melibiose, D-sucrose, inulin, D-(+)-Trehalose dehydrate, D-(+)-Melezitose Hydrate, D-Raffinose, starch, glycogen, xylosic alcohol, gentiobiose, D-Turanose, D-Lyxose, D-Tagatose, D-Fucose, L-Fucose, D-Arabitol, L-Arabitol, potassium gluconate, 2-keto-Potassium gluconat, and 5-keto-Potassium gluconate. It also could use CMC as sole carbon source under aerobic condition.

Strain P-1-5 is nutritionally versatile, readily utilizing different kind of monosaccharides, disaccharides, polysaccharides and their derivants, and some carbon compounds typically used by *Pseudomonas stutzeri* (e.g., starch and maltose). The carbon metabolism profile indicated that strain P-1-5 has remarkable biochemical diversity, especially the ability of sugar utilization. CMC utilizing bacteria, including *Eubacterium*, *Clostridium*, *Cellulomonas*, *Microbacterium*, *Bacillus*, and *Brevibacillus*, have been founded at landfill [20]. The isolated *Pseudomonas* P-1-5 from landfill refuse could also use CMC, implying that strain P-1-5 has the ability of cellulose biodegradation. Strains of *P. stutzeri* have been isolated from contaminated soil [10], where degradative and contaminant-resistant strains have to develop relevant ecological activities. For example, *Pseudomonas* has been reported to be capable of degradation trichloroethylene [21]. Landfill is typical contaminated environment with various contaminants (e.g., XOCs and ammonium) [4]. In combination, the isolated P-1-5 might play important role in carbon cycles.

Assessment of the dentrification of *P. stutzeri* P-1-5 strain. The strain P-1-5 could remove nitrate in both aerobic and anaerobic conditions. Under aerobic condition, the strain could completely remove 415.8 mg/L nitrate in 16h (Fig 2a). At the same time, nitrite accumulation occurred during 4-16h and the highest concentration of the accumulated nitrite was 49.1±3.5 mg/L at 12h. Under anaerobic condition, the nitrite accumulated during the whole process (0-48h), the highest concentration of the accumulated nitrite was 104.0 ± 3.0 mg/L at 28h (Figure 2b). After 48h incubation, 93.1% of the total nitrate (415.8 mg/L) was removed. Meanwhile, the concentration of nitrite was dramatically reduced to 17.8±1.7 mg/L.

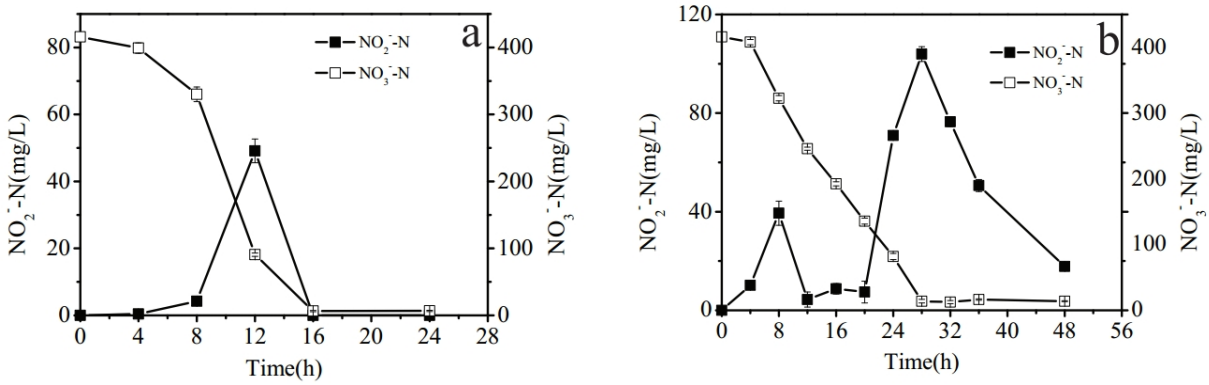

Fig. 2. Nitrate removal performance under aerobic (a) and anaerobic (b) conditions by strain P-1-5. Symbols: nitrite (■); nitrate (▼). Error bars: standard deviation (SD) of three replicates.

Many *P. stutzeri* strains are denitrifers. Among them, one group of the isolated strains reduce nitrate to nitrogen gas without accumulating nitrite, another group of the isolated strains possesses a two phase denitrification process, and the remaining isolates accumulate nitrite at low concentration [22]. Therefore, the strain *P. stutzeri* P-1-5 belongs to the groups of nitrite accumulated type but differs from the *Pseudomonas Stutzeri* D6 [23], which was a two phase denitrifiction bacteria isolated from the activated sludge of a municipal wastewater treatment plant.

In aerobic condition, the nitrate removal efficiency of *P. stutzeri* P-1-5 is higher than that of the reported *Pseudomonas Stutzeri* YG-24 [19] (fully remove 15-200 mg/L nitrate) and *P. stutzeri* strain YZN-001 [24] (fully remove 275.1 mg /L nitriate). In anaerobic condition, the nitrate removal efficiency of *P. stutzeri* P-1-5 is still high (removed 378.4 mg/L nitrate). Wan et al. [25] reported that *Pseudomonas* sp. yy7 could survive and remove up to 40 mg nitrite. The reason that *P. stutzeri* P-1-5 could survive at higher nitrite (104.0 ± 3.0 mg/L) and still had denitrification ability in anaerobic condition might due to the strain was isolated from landfill, a high nitrogen contaminated environment.

Influence of the dentrification ability of *P. stutzeri* P-1-5 . Figure 3a shows the cell growth and nitrate removal efficiency of P-1-5 after 24 h of cultivation in media containing different sources of carbon (sodium succinate, sodium acetate, ethanol, methanol, sodium citrate, glucose and sucrose). Sodium succinate, sodium acetate, and ethanol were more favorable for cell growth (1.54 ± 0.03, 1.54 ± 0.03, and 1.26 ± 0.01, respectively) and nitrate removal (92.4 %, 90.5 %, and 86.3 %, respectively). Within them, sodium succinate was the most preferred source. Sodium acetate has been used as external carbon source to remove nitrate in some microorganism, such as *Vibrio diabolicus* SF16 [26] and *Pseudomonas* sp. ADN-42 [27]. Sodium acetate is also favorable carbon source for strain *P. stutzeri* P-1-5. Additionally, sodium succinate was the optimal carbon source of *P. stutzeri* P-1-5 for nitrate remove, which differed from other isolates. Different carbon sources have obvious effect on the nitrate removal efficiency, might due to their redox potentials . In this study, sodium acetate is the most oxidizing compound while glucose and sucrose are the most

reducing compounds among the five carbon sources. Between sodium citrate and sodium succinate, the sodium citrate is more oxidizing. This implied that carbon source for nitrate removal might be also a physiochemical feature of microorganism.

Figure 3b shows that C/N ratio effects on the cell growth and nitrate removal efficiency. Low C/N ratio (2) was not favorable for the cell growth (1.06 ± 0.00) and nitrate removal efficiency (44.5%). When the C/N ratio went up from 2 to 4, the cell growth (1.56 ± 0.48) and nitrate removal efficiency (97.6%) increased and kept stable even when the C/N ratio went up to 8, 12 and 16, suggesting C/N ratios ≥ 4 is minimal requirement for efficient nitrate removal. It is well known that the C/N ratio is the metabolic characters of microorganism. The optimal C/N (8) for *P. stutzeri* P-1-5 was similar to that of *Pseudomonas Stutzeri* YG-24 (8) [19] and *Pseudomonas* sp. yy7 (8) [25].

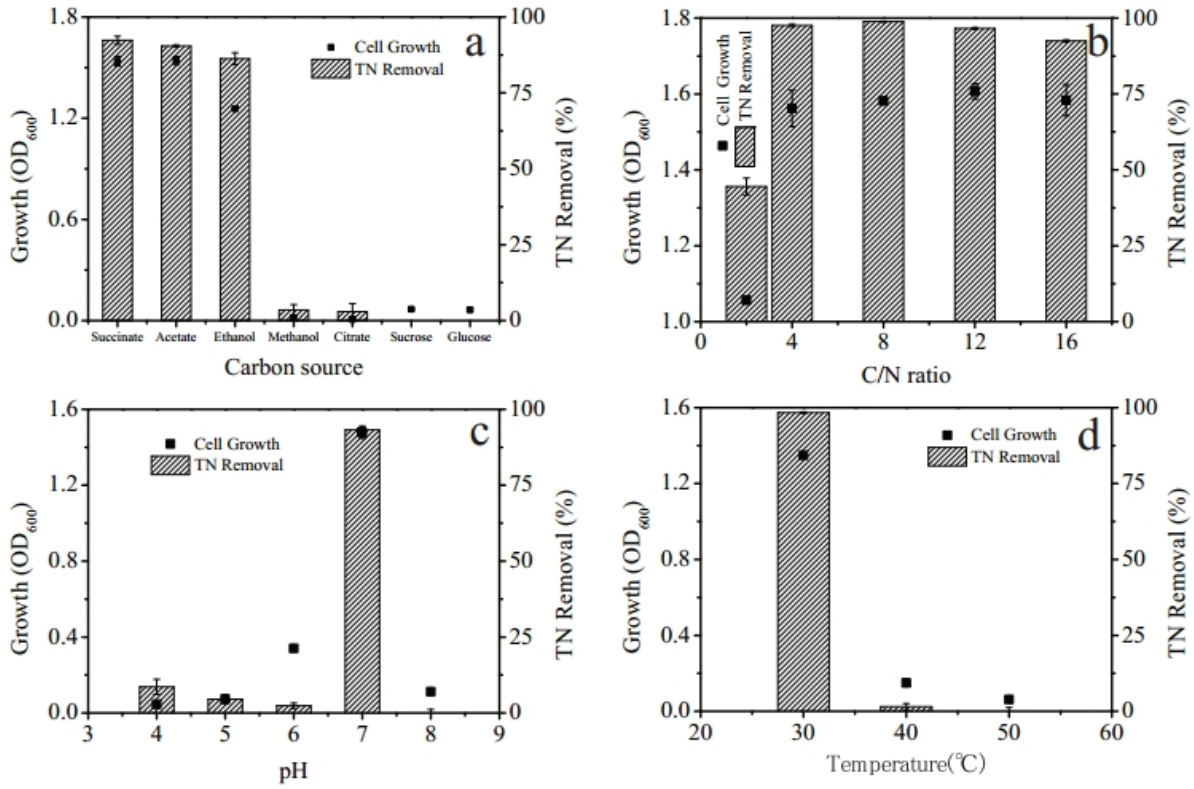

Fig.3. Optimization of carbon source (a), C/N (b), pH and temperature (c) for the cell growth and nitrate removal efficiency of strain P-1-5.

Figure 3c shows the pH effects on the cell growth and nitrate removal efficiency. The pH range (3-9) in this study is typical pH range of landfill during refuse decomposition (initial aerobic phase (IAP), anaerobic phase (ANP), initial methanogenic phase (IMP), and stable methanogenic phase (SMP)) [28, 29]. Only neutral pH was favorable for the cell growth (1.48 ± 0.03) and nitrate removal efficiency (93.2%). The optimal pH range for denitrifers is between 6-8 [30]. Thus, the optimal pH for *P. stutzeri* P-1-5 is within the range.

Figure 3d shows the temperature effects on the cell growth and nitrate removal efficiency. The optimal temperature for *P. stutzeri* P-1-5 was 30 °C, with high value of the cell growth (1.35 ± 0.01) and nitrate removal efficiency (98.4 %). When the temperature increased to 40 °C and 50 °C, there was no or slightly nitrate removal and low cell growth.

Regarding to the varying environmental condition in refuse decomposition (e.g., initial aerobic phase, anaerobic acid phase, initial methanogenic phase, and stable methanogenic phase), how *P. stutzeri* P-1-5 plays denitrification role in refuse decomposition is unknown. Further study of the genus *Pseudomonas* in real landfill condition is necessary.

Conclusion

The bacterium *Pseudomonas stutzeri* P-1-5 was isolated from landfill and found to remove nitrate under both aerobic and anaerobic conditions. The strain completely removed 415.8 mg/L nitrate within 16 h under aerobic condition and removed 387.1 mg/L nitrate within 48 h under anaerobic condition. The strain is a denitrifer and the optimal culture conditions for nitrate remove was sodium succinate as cabochon source, C/N 8，pH 7, 30 ℃. Although further studies are required, the results of this study provides the direct evidence that *Pseudomonas* plays important role in nitrogen cycle in landfill.

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