



## Isolation and Characterization of Novel Denitrifying Bacterium *Geobacillus* sp. SG-01 Strain from Wood Chips Composted with Swine Manure

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**ABSTRACT:** Nitrate contamination in ground and surface water is an increasingly serious environmental problem and only a few bacterial strains have been identified that have the ability to remove nitrogen pollutants from wastewater under thermophilic conditions. We therefore isolated thermophilic facultative bacterial strains from wood chips that had been composted with swine manure under aerated high temperature conditions so as to identify strains with denitrifying ability. Nine different colonies were screened and 3 long rod-shaped bacterial strains designated as SG-01, SG-02, and SG-03 were selected. The strain SG-01 could be differentiated from SG-02 and SG-03 on the basis of the method that it used for sugar utilization. The 16S rRNA genes of this strain also had high sequence similarity with *Geobacillus thermodenitrificans* 465<sup>T</sup> (99.6%). The optimal growth temperatures (55°C), pH values (pH 7.0), and NaCl concentrations (1%) required for the growth of strain SG-01 were established. This strain reduced 1.18 mM nitrate and 1.45 mM nitrite in LB broth after 48 h of incubation. These results suggest that the *G. thermodenitrificans* SG-01 strain may be useful in the removal of nitrates and nitrites from wastewater generated as a result of livestock farming. (**Key Words:** Compost, Denitrification, *Geobacillus thermodenitrificans*, Nitrates, Nitrites, Swine Manure, Wood Chips)

### INTRODUCTION

Nitrogen compounds released by industrial and domestic activities have been increasingly accumulating in lakes, wetlands, and enclosed areas of the sea (Zhou et al., 2007). In particular, full-scale swine waste treatment systems generate wastewater containing nitrogen compounds that cause environmental pollution. Wood chips are commonly used in swine wastewater treatments in order to alleviate noxious odors and decrease water pollution (de Guardia et al., 2008). Previous studies have examined wood

compost production from bark, sawdust, and wood shavings (McGowin et al., 2001; Zoes et al., 2001; Horiuchi et al., 2003) however, only a few reports have evaluated composting methods using wood chips (Tiquia and Tam, 2000).

The composting process results in biodegradation-induced metabolic heat production that can increase temperatures above 70°C. Only a few specialized microbes can live under these extreme environmental conditions, and the activity and diversity of composting bacterial strains are greatly suppressed at compost temperatures greater than 60°C. Strains related to *Bacillus stearothermophilus* usually dominate under these conditions (Suler and Finstein, 1977; Strom, 1985). Recently, 34 thermophilic bacterial species (grown at 60°C) were isolated during aerobic composting of poultry and cattle manure (Wang et al., 2007), and *Geobacillus thermodenitrificans* was one of those species.

It has been reported that the chemical oxygen demand, sewage sludge, total nitrogen content, and total phosphorous content of swine manure were reduced by passing through filtering system using wood chips (Choi et al., 2008; Gilbert et al., 2008).

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However, the mechanisms involved in reducing the nitrogen compounds have not been identified. Some microorganisms have been associated with the denitrification process, which eliminates fixed nitrogen from the environment and removes excess nitrates during wastewater treatment. Certain bacteria, such as *Pseudomonas stutzeri*, participate in the denitrification process by using nitrate or nitrite as the ultimate electron acceptor for anaerobic respiration (Cheneby et al., 2004; Philippot, 2005b). Biological denitrification is considered one of the most effective processes for removing nitrogen pollutants from wastewater. It is therefore worthwhile to isolate and characterize microorganisms that utilize nitrogen compounds from swine manure composted in wood chips.

In the present study, a thermophilic bacterial strain, SG-01, was isolated from wood chips composted with swine manure and identified. Studies were then performed to determine the optimal temperature, pH and NaCl concentrations for growth of the strain. Finally nitrogen reduction assays were also performed in order to measure nitrogen utilization by the SG-01 strain.

## MATERIALS AND METHODS

### Swine manure composting system

Swine manure was obtained from a swine farm in Kimpo, Korea. Sludge was removed from the swine manure, and liquid waste was sprayed into the filtering system made up of thinning-out tree wood chips piled in a container (10 m width×10 m length×6 m height). The wood chips were used to pack the filter, which was then utilized in the microbial-degradation phase of the composting. The filtering system was spontaneously self-heated in aerobic condition and kept at approximately 60°C for several days. Wood chip temperatures reached a peak of 66°C during the second week of composting and were maintained at that level until d 21 for the thermophilic stage of composting. The average temperature of the filtering system during this process was 60.7°C. Temperatures in the composting piles decreased gradually after the thermophilic stage until they reached ambient temperatures of approximately 27 to 30°C. The pH of the final wood chip compost was 8.3.

### Isolation and growth of bacteria from the wood chips

Composted wood chips (1 g) were mixed with 5 mL LB media, stirred for 5 min, and incubated at room temperature for 30 min. Sample supernatants (100 µL) were plated on 1.5% LB agar plates and incubated at 55°C for 24 h in a sealed humidified jar to prevent plate drying. Three strains were selected for further investigation based on the colony color, odor, and morphological features.

### Bacterial identification

The bacteria were identified by 16S rRNA sequence homology analysis as follows. Genomic DNA was isolated from these strains and used for the amplification of the 16S rRNA gene fragment as previously described (Sogin, 1990). Briefly, 16S rRNA gene fragment was amplified by the polymerase chain reaction (PCR) using the eubacterial universal primers 27F (5'-AGAGTTTGATCCTGGC TCAG-3') and 1492R (5'-GGTACCTTGTTACGACTT-3'). Polymerase chain reaction (PCR) were performed using a thermal cycler (Takara Bio Inc. Japan) with an initial denaturation step of 94°C for 3 min, followed by 35 amplification cycles consisting of denaturation at 94°C for 60 s, annealing at 60°C for 60 s, and elongation at 72°C for 60 s. The reaction was terminated with a terminal elongation step of 72°C for 3 min followed by cooling at 4°C. The resulting PCR band was used for nucleotide sequence analysis. Nucleotide sequences of approximately 1,450 bp length were analyzed for similarity to the 16S rRNA sequence of the isolated bacteria by using the BLAST program (Altschul et al., 1997). The nucleotide sequences of 16S rRNA were analyzed three times for each strains and aligned using the ClustalW program for multiple sequence alignment (Thompson et al., 1994). The evolution distance matrix was calculated using the Kimura 2-parameter evolution model (Kimura, 1983). The resulting phylogenetic tree was constructed using neighbor-joining analysis (Saitou and Nei, 1987; Felsenstein, 1993). Biochemical identification was also performed using API 50CHB (BioMerieux, Hazelwood, MO) according to the manufacturer's instructions.

### Physiological characterization

The optimum NaCl concentration for bacterial growth was determined using previously described methods (DeFlaun et al., 2007). The effect of temperature on growth was tested in LB broth. An additional study was performed to determine the optimal pH for growth in a buffered system. The LB broth was adjusted to pH values ranging from 5.0 to 9.5 (room temperature) by using the following buffers: 50 mM citrate-Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 5.0 to 6.8), 50 mM Tris-HCl buffer (pH 7.0 to 8.0), and 50 mM glycine-NaOH buffer (pH 8.5 to 9.5). Each LB broth was inoculated with an SG-01 culture that had been grown overnight. Optical density at 600 nm was analyzed by measuring absorbance values every 6 h. The initial optical density of each culture was 0.04. The Specific growth rate is defined as the increase in cell mass per unit time, e.g., grams cells (g) per gram cells (g) per hour: (g·g<sup>-1</sup>·h<sup>-1</sup>).

### Nitrate and nitrite reduction assays

A jar fermenter was used to investigate the SG-01 growth curve and to perform nitrate and nitrite reduction

assays. The pH changes were also monitored during this process. Bacterial cells (1%) were inoculated into 100 mL LB broth containing 2.0% NaCl and supplemented with 100 ppm nitrate (1.18 mM NaNO<sub>3</sub>) or nitrite (1.45 mM NaNO<sub>2</sub>), and then incubated at 55°C for 48 h. The modified brucine-sulfanilic acid method (APHA, 1989) was used to measure nitrate concentrations. Nitrite concentrations were measured using the Griess reaction (Green et al., 1982) Color absorbance was measured at 540 nm by using a spectrophotometer (U-2000, Hitachi, Japan). Un-inoculated samples were used as controls for the nitrate and nitrite reduction assays. Experiments were performed in triplicate, and standard curves were generated for each experiment by using known concentrations of sodium nitrate and sodium nitrite as references.

## RESULTS

### Identification and characterization of the isolated bacteria

Nine different colonies from the samples of the wood chips composted with swine manure were isolated and grouped based on colony morphological characteristics, color, and odor. Biochemical characteristics were analyzed in 3 rod-shaped spore-forming candidate bacteria, SG-01, SG-02, and SG-03.

The PCR products for 16S rRNA (approximately 1,450 bp) were isolated and sequenced. Sequence homology analysis revealed that SG-01 had 98 to 99% homology with the *Geobacillus* genus. However, the sequences of the 3 strains were not-identical and the strain SG-01 was phylogenetically related to *Geobacillus thermodenitrificans* species (99% similarity with 16S rRNA of the strain 465<sup>T</sup>). The phylogenetic position of the studied strains among

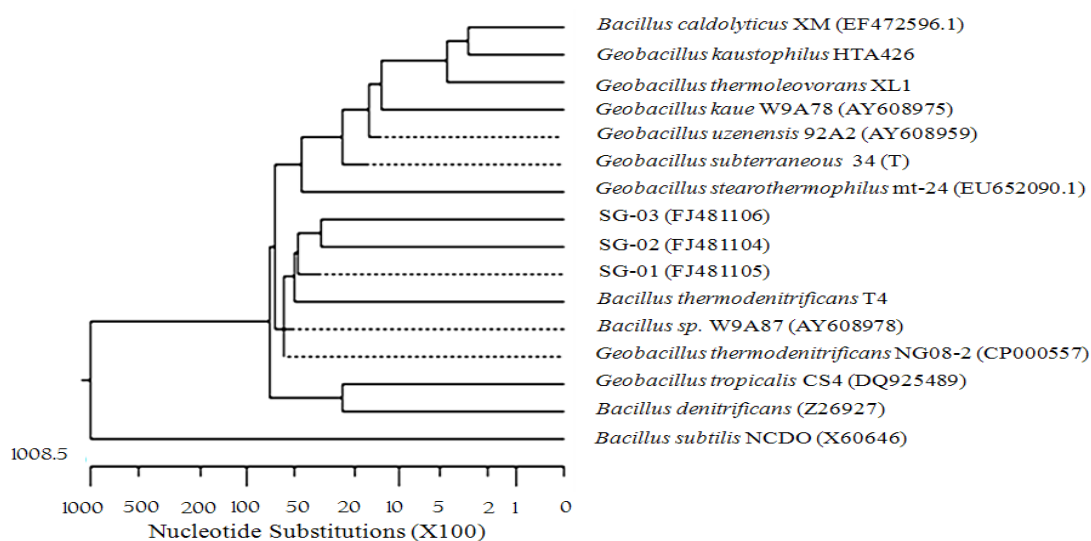
other *Geobacillus* species is shown in Figure 1.

The results of biochemical analyses using the API 50CHB kit revealed that the SG-01, SG-02, and SG-03 strains were biochemically unique based on the utilization of L-arabinose, fructose, inositol, melibiose, sucrose, raffinose, and glycogen (Table 1). The SG-02 and SG-03 strains used L-arabinose, melibiose, and sucrose, whereas the SG-01 strain used glycogen. It was also found that the SG-02 strain used inositol as a carbon and energy source.

Manachini et al. (2000) reported that *G. thermodenitrificans* type strain DSM 465T grows well at 50 to 65°C, with optimum pH values of 6 to 8 and in the presence of 3% NaCl. It is capable of reducing nitrates and nitrites to gas and of anaerobic gas production from nitrate. A common feature between SG-01 strain and DSM 465T strain was observed but specific growth rate of SG-01 strain was not as high as those for other *Geobacillus* sp. (>0.1). More than 70% of the isolates from the wood chip compost piles were classified as SG-01 strains based on the colony morphological characteristics, growth patterns, and 16S rDNA sequence data. This suggests that *Geobacillus thermodenitrificans* is a common inhabitant of composts at 60°C under aerobic conditions, as earlier reported (Hatsu et al., 2002).

### Physiological characteristics of strain SG-01

Optimal conditions for the growth of strain SG-01, including temperature, pH, and NaCl concentration, were investigated. The specific SG-01 strain growth rates were 0.024 at 50°C, 0.027 at 55°C, 0.026 at 60°C, and 0.025 at 65°C. Poor growth was observed at 70°C and 37°C. The optimal temperature for the growth of SG-01 was 55°C. The optimum pH for SG-01 growth was 7.0. No growth was detected at pH values greater than 9.5. The strain grew in



**Figure 1.** Neighbor-joining phylogenetic dendrogram, based on 16S rRNA gene sequences, indicating the positions of the strains studied among those of the other *Geobacillus* species. Bootstrap percentages (based on 1,000 samplings) are given at nodes.

**Table 1.** Comparative characteristics of *G. thermodenitrificans* strains

Characteristics	Strains					
	SG-01	SG-02	SG-03	NG80-2 <sup>1</sup>	DSM 465 <sup>1,2</sup>	HRO10 <sup>3</sup>
Isolated origins	Wood chip compost	Wood chip compost	Wood chip compost	Deep subsurface oil	Sugar beet juice	Soil
Optimum temperature (°C)	55			65	50-65	50
Optimum pH	7.0			ND	6-8	6.5-7.5
NaCl range (% w/v)	0-2			ND	0-3	ND
Nitrate reduction	Yes			Yes	Yes	ND
Utilization of						
L-Arabinose	-	+	+	+		
Galactose	-	-	-	+		
Fructose	+	+	-	+		
Inositol	-	+	-	+		
α Methyl-D-Glucoside	-	-	-	+		
N-Acetyl-Glucoseamine	-	-	-	+		
Salicin	+	+	+	-		
Melibiose	-	+	+	+		
Sucrose	-	+	+	+		
Raffinose	+	+	-	-		
Glycogen	+	-	-	-		
D-Turanose	-	-	-	+		

ND = No data. <sup>1</sup> Source: Feng et al. (2007). <sup>2</sup> Source: Manachini et al. (2000). <sup>3</sup> Source: Ezeji et al. (2005).

liquid media at NaCl concentrations of 0 to 3%. The optimal NaCl concentration was 2.0%, showing a specific growth rate of 0.025. The specific growth rate of strain SG-01 decreased at NaCl concentrations greater than 3.2%.

#### Nitrate and nitrite reduction

The SG-01 growth curve was investigated using a jar fermenter at 55°C and 2.0% NaCl for 48 h. The maximum cell density of  $2.87 \times 10^8$  CFU/mL of the strain SG-01 was reached in the LB broth at 12 h (Figure 2A). The pH was not changed during the fermentation period (pH 7.0). Nitrogen reduction by SG-01 in nitrate-and-nitrite-containing LB broth was investigated. Nitrate was rapidly reduced within 12 h, and approximately 91.8% of the nitrate was removed within 48 h (Figure 3A, 3B). The nitrate values in uninoculated controls did not decrease. These results suggest that strain SG-01 may reduce nitrate to nitrogen under aerobic conditions. Approximately 99% of the nitrite was reduced in 18 h, and nitrites were no longer detected at 48 h. Nitrite values of the inoculated controls did not decrease. The denitrifying ability of strain SG-01 was stronger than those of other microorganisms (Kariminiaae-Hamedaani et al., 2004). These results suggest that SG-01 could effectively remove nitrates and nitrites from wastewater.

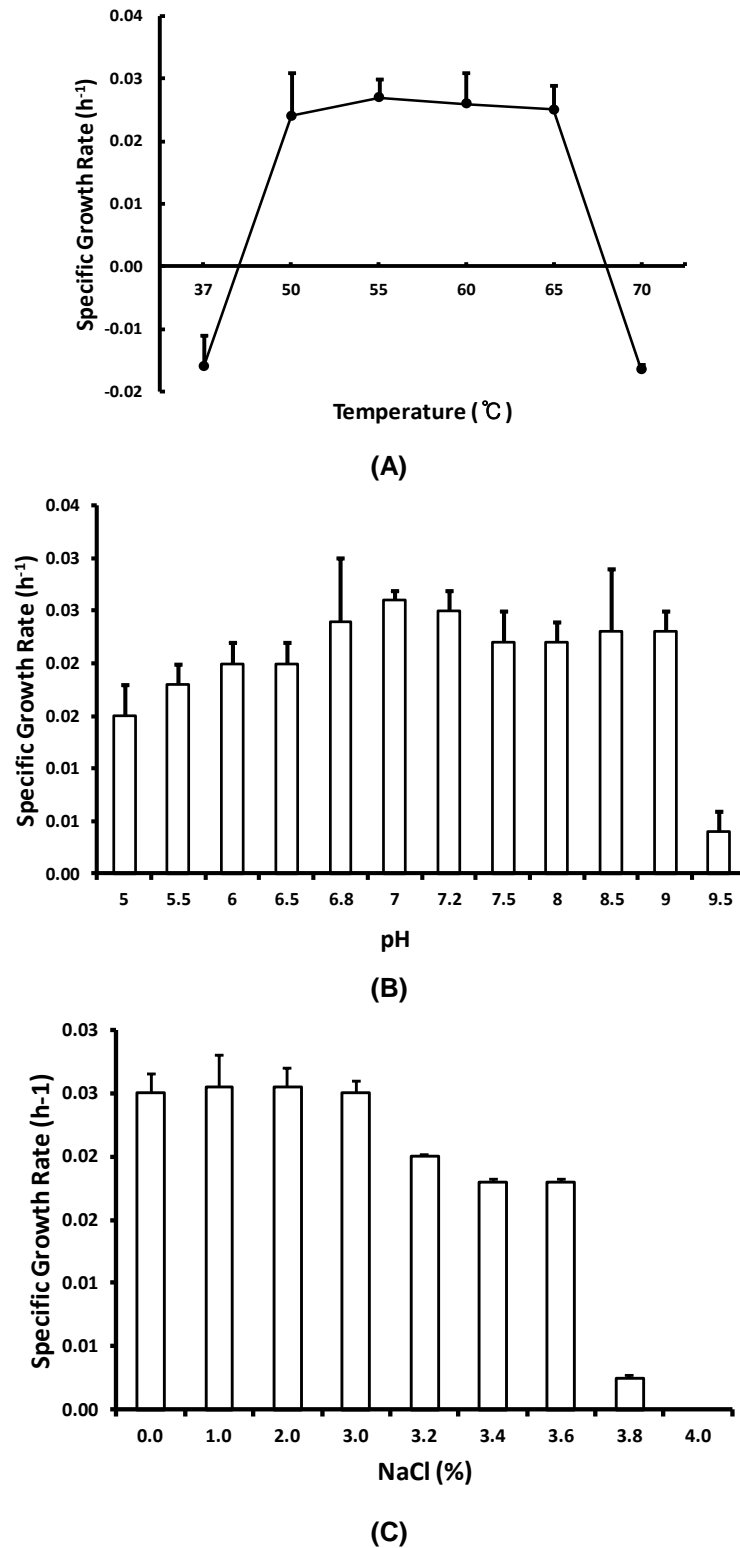
#### DISCUSSION

We isolated microorganisms from composted wood

chips with swine manure. One of the isolates, *G. thermodenitrificans* SG-01 was further characterized. The physiological characteristics of *G. thermodenitrificans* are summarized in Table 1. The SG-01 strain differs biochemically from NG80-2 and HRO10 with respect to optimal temperatures and pH values for growth.

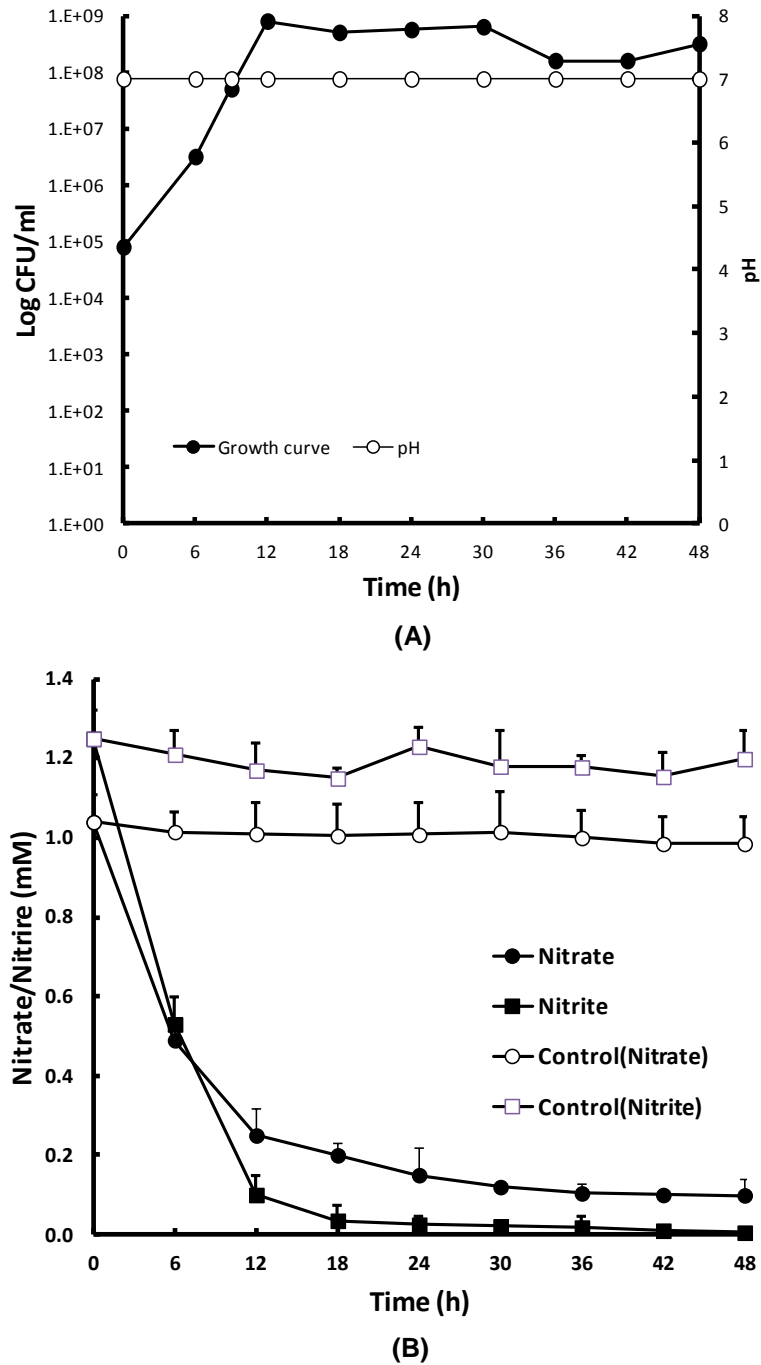
Previous studies have shown that composting temperatures can significantly influence the rate of the composting process (Tiquia et al., 1997). Several researchers have found diverse bacteria in hot composts (Peters et al., 2000; Dees and Ghiorse, 2001) and an increase in respiratory activity at temperatures greater than 65°C (Beffa et al., 1996). The results of those studies provided evidence against the general understanding that bacterial activity and diversity in compost reduce dramatically at temperatures greater than 60°C. A range of *Geobacillus* species can denitrify nitrates to nitrogen gas (Nazina et al., 2005), and reduction of nitrates and nitrites by cultures similar to strain SG-01 was observed in the present study. However, the SG-01 strain effectively removed approximately 90% of the nitrates and 100% of the nitrites in LB broth within 48 h of incubation. These results corroborate the findings of previous studies (Suthersan and Ganczarczyk, 1986; Turk and Mavinic, 1986).

Nitrate contamination in ground and surface water is an increasingly serious environmental problem; thus, biological denitrification is an essential process in the removal of nitrogen pollutants from aqueous ecosystems



**Figure 2.** Optimal conditions for the specific growth rate of *G. thermodenitrificans* SG-01. Growth was determined in LB broth at various (A) temperatures (°C), (B) pH, and (C) salinity (% NaCl) after 24 h. Values are expressed as the mean (n = 3) and the error bars represents standard deviation (SD).

(Van de Pas-Schoonen et al., 2005). Denitrification is a respiratory process in which soluble nitrogen oxides, nitrates, and nitrites are reduced to NO, N<sub>2</sub>O, and N<sub>2</sub> gases, respectively, under oxygen-limited conditions (Philippot, 2005a). Nitrite reductase is the key enzyme involved in denitrification. It catalyzes the reduction of soluble nitrite



**Figure 3.** The growth curve (A) and the nitrate and nitrite reduction values (B) of *G. thermodenitrificans* SG-01 cultured in LB broth for 48 h. The initial nitrate and nitrite concentrations were 1.18 mM and 1.45 mM, respectively. Values are expressed as the mean (n = 3) and the error bars represents standard deviation (SD).

into gas (Henry et al., 2004). Nitrite reductase can be found in other denitrifying or nitrite-reducing bacteria such as *P. stutzeri* and *P. aeruginosa* (Ward, 1995; Rinaldo et al., 2007). Livestock wastewater contains high levels of ammonia nitrogen, which causes nitrite buildup rather than normal nitrification. This inhibition of nitrification (Anthonisen et al., 1976) always depends on nonionic ammonia concentrations (free ammonia,  $\text{NH}_3$ ) and nonionic

nitrogen salt concentrations (free nitrous acid,  $\text{HNO}_2$ ).

It should be noted that the denitrification experiments in the present study were only performed under laboratory conditions, and future research should be more reflective of actual environmental conditions. Nonetheless, our results suggest that *G. thermodenitrificans* SG-01 may be useful for the effective removal of nitrates and nitrites from wastewater, particularly liquid swine manure.

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