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A Strain of *Pseudomonas* sp. Isolated from Piggery Wastewater Treatment Systems with Heterotrophic Nitrification Capability in Taiwan

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Abstract. A high concentration of NH_4^+ in piggery wastewater is major problem in Taiwan. Therefore, in our study, we isolated native heterotrophic nitrifiers for piggery wastewater treatment. Heterotrophic nitrifier AS-1 was isolated and characterized from the activated sludge of a piggery wastewater system. Sets of triplicate crimp-sealed serum bottles were used to demonstrate the heterotrophic nitrifying capability of strain AS-1 in an incubator at 30°C. All serum bottles contained 80 mL medium, and the remainder of the bottle headspace was filled with pure oxygen. The experimental results showed that 2.5 ± 0.2 mmol L^{-1} NH_4^+ was removed by 58 hours, and, eventually, 1.5 ± 0.5 mmol L^{-1} N_2 and 0.2 ± 0.0 mmol L^{-1} N_2O were produced. The removal rate of NH_4^+ by the strain AS-1 was 1.75 mmol NH_4^+ g $cell^{-1}$ h^{-1}. This strain was then identified as *Pseudomonas alcaligenes* (97% identity) by sequencing its 16S rDNA and comparing it with other microorganisms. Thus, strain AS-1 displays high promise for future application for in situ NH_4^+ removal from piggery wastewater.

The pig industry plays a crucial role in the agricultural sector in Taiwan. However, nitrogen and phosphorus in pig farm effluent must be controlled and decreased to avoid eutrophication of surface waters [7]. Average concentrations of both ammonia and nitrogen and total Kjeldahl nitrogen in raw piggery wastewater after solid–liquid separation are 229 ± 33.5 and 2736 ± 3.9 mg L^{-1}, respectively [17]. Wastewater treatment facilities in Taiwan have attained approximately 40% nitrogen removal from piggery wastewater [17]. Thus, there is a need to introduce heterotrophic microorganisms into piggery wastewater treatment facilities to improve nitrogen removal efficiency.

Simultaneous biologic nutrient removal was thoroughly investigated by assessing autotrophic denitrification, heterotrophic nitrification, and phosphorus removal in full-scale wastewater treatment systems [11]. Parts of simultaneous nitrification and denitrification (SND) rely somewhat on concurrent aerobic ammonia oxidation and anaerobic denitrification by heterotrophic bacteria, which could use poly-β-hydroxybutyrate as an electron donor [22]. Autotrophic *Nitrosomonas* spp. was shown to be capable of aerobic deammonification ($NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$) [2, 15], whereas heterotrophic bacteria were reported to be capable of heterotrophic nitrification coupled with aerobic denitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$) [13, 25]. Heterotrophic nitrification is suitable only for organic wastewaters with high ratios of cyclooctadiene (COD) to nitrogen [24]. Most heterotrophic-nitrifying bacteria are capable of aerobic denitrification, including *Arthrobacter* sp. [3, 26], *Thiosphaera pantotropha* (known as *Paracoccus denitrificans*) [8, 13, 16], *P. denitrificans* [14], and *Alcaligenes faecalis* [12, 25].

Peptone [7, 23] and ammonia sulphate [9] have been used as decreased nitrogen sources to investigate heterotrophic nitrification caused by certain microorganisms [3]. Doxtader and Alexander [6] identified a possible role for β-alanin in the heterotrophic nitrification pathway of *Aspergillus flavus*. Thus, β-alanin has been used as the

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sole source of carbon and nitrogen for isolating heterotrophic nitrifiers [3]. Moreover, pyruvic oxime (7 mM), combined with 0.05% (w/v) yeast extract, has also used to identify the nitrification capability of six denitrifying *Pseudomonas* strains [4]. This study used ammonium acetate as the sole source of carbon and nitrogen to isolate heterotrophic nitrifiers and verified their ammonia oxidation and nitrite decrease capability for further application in piggery wastewater treatment systems in Taiwan.

Materials and Methods

Isolation. The medium (A) (Table 1) was used to isolate heterotrophic nitrifiers [18, 19, 20]. The sole carbon source in the medium was 0.1% (w/v) ammonium acetate [3]. Inocula for isolation were obtained from raw piggery wastewater, soils, and anaerobic and activated sludge from piggery wastewater treatment facilities. Diluted piggery wastewater, soil, and sludge samples of 0.1 mL 1% (w/v or v/v) were transferred individually into Erlenmeyer flasks containing 150 mL autoclaved sterile medium (A). After 48 hours of incubation at 30°C, some of the culture suspensions in the inoculated flasks were tested for the presence of nitrite [5]. Once nitrite was detected, the remaining 0.1 mL of the culture suspension was streaked on tryptic soy agar plates (Difco) to obtain isolated single colonies.

Qualitative assessment of aerobic ammonia oxidation and nitrate decrease. *Aerobic ammonia oxidation:* The medium (A) agar plates were employed to verify their heterotrophic ammonia oxidation capability. The isolates were inoculated on agar plates containing 18.6 mmol L^{-1} NH_4^+ and incubated at 30°C under aerobic conditions. Nitrite production from the inoculated plates was confirmed after 48 hours of incubation by adding sulfanilamide and N-(1-naphthyl)ethylenediamine reagents and observing color formation [5]. In each case, medium plates without inoculation were used as sterility controls. *Nitrate decrease*. The medium (C) agar plates containing KNO_3 (Table 1) were inoculated with the isolates and incubated at 30°C under aerobic and anaerobic conditions to verify their nitrate decrease capability. Nitrite production was confirmed by observing color formation on the inoculated plates after 72 hours of incubation [5].

Identification of the isolate. The isolates were identified by sequencing their 16S rDNA. Universal primers 16f27 (5'-AGAGTTT GATCMTGGCTCAG-3') and 16r1488 (5'-CGGTTACCTTGTTAG GACTTCACC-3') [1] were used to amplify their 16S rDNA by polymerase chain reaction (PCR) protocol. Finally, the PCR product was verified using an agarose gel based on the size (1.5 kb). The PCR product was cut out from the agarose gel and purified by a QIAquick Gel Extraction Kit (Qiagen). Protech Technology sequenced the resulting 16S rDNA product. Finally, the 16S rDNA sequence of the isolate was compared with that of other microorganisms by way of BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/Blast. cgi).

Biomass measurement. The AS-1 isolate was enriched in medium (A) until optical density ($OD)_{600}$ = 0.55, and the cell suspension of AS-1 was then series-diluted by mixing half-and-half with sterile phosphate buffer (pH 7.5) to a final volume of 20 mL. The OD of each dilution of a cell suspension then was measured immediately at 600-nm wavelength (i.e., OD_{600}). The dry weight of the AS-1 strain was determined using the method of Koch [10]. The linear regression of the OD_{600} versus dry weight of cell mass was calculated based on the corresponding OD_{600} values and dry weights of the strain AS-1 cell mass.

Table 1. List of media used in this study

Function	Component	Medium A[a]	B[b]	C[c]	D[d]
Phosphate buffer	KH_2PO_4 (gL^{-1})	1.5	1.5	1.5	1.5
	$Na_2HPO_4{\cdot}7H_2O$ (gL^{-1})	7.9	7.9	7.9	7.9
N source	NH_4Cl (gL^{-1})	0.3	–	–	0.15
	CH_3COONH_4 (gL^{-1})	1	–	–	–
C source	CH_3COOK (gL^{-1})	–	–	–	2
	$Na_2C_4H_4O_4{\cdot}6H_2O$ (gL^{-1})	–	2.7	2.7	–
NO_2^- source	$NaNO_2$ (gL^{-1})	–	0.035	–	–
NO_3^- source	KNO_3 (gL^{-1})	–	–	2	–
Minerals and trace elements	PS-1 (mL L^{-1})	5	5	5	5
	VS-salt (mL L^{-1})	5	5	5	5
Multivitamins	V8 (mL L^{-1})	1	1	1	1
H^+ strength	pH	7.5	7.5	7.5	7.5

[a]Medium (A) was used to isolate heterotrophic nitrifiers.
[b]Medium (B) was used to test microbial nitrite reduction.
[c]Medium (C) was used to test microbial nitrate reduction.
[d]Medium (D) was used to test microbial ammonia oxidation.

Quantitative assessment of nitrite decrease. Microorganisms were isolated and purified by employing streaking plate techniques from the cell suspension grown in the medium (A) in which nitrite was produced. Nitrite concentrations in the purified isolates then were assessed. Next, 4.5 mL cell suspension was inoculated into 250-mL Erlenmeyer flasks with 150 mL sterile medium (B) and incubated aerobically at 30°C in triplicate. The flasks containing uninoculated sterile medium served as the sterility controls. Samples were examined at 27- and 15-hour intervals to determine nitrite decrease using nitrite color reagents as previously described [5].

Assessment of heterotrophic nitrification and aerobic denitrification. Crimp-sealed 118-mL serum bottles containing 80 mL medium were prepared in triplicate (Table 1). These bottles were evacuated and the headspace pressurized with 69 kPa pure oxygen (approximately 98% oxygen analyzed by gas chromatography (GC) coupled with a thermal conductivity detector (TCD) (PerkinElmer) three times before autoclaving. After autoclaving, VS-salt, PS-1, and V_8 solutions were added to the bottles at room temperature. For each isolate, 2.4 mL culture suspension (OD_{600} = 0.18) was inoculated into the triplicate bottles and incubated at 30°C. Samples from the bottles were measured periodically to determine OD_{600}, NH_4^+, NO_2^-, N_2O, and N_2 gas concentrations.

Analysis. OD_{600} was determined by spectrophotometry (Spectronic 20+, Milton Roy) at a wavelength of 600 nm. Moreover, bacterial suspensions were centrifuged at 3600 × g for 20 minutes to allow for measurement of ammonia and nitrite levels in the supernatant before assay was performed. Ammonia concentration was then determined by Nessler assay at a wavelength of 425 nm with a sensitivity for ammonia detection of 400 μg N L^{-1} [5]. Nitrite concentration was determined by colorimetry at a wavelength of 543 nm [5]. To determine O_2 and N_2, 20-μL samples (injection volume) were removed from the headspace of the serum bottles using a pressure-lock syringe (Supelco) and analyzed by GC–TCD [19]. To measure N_2O, 500-μL gas samples were removed from the headspace of the serum bottles, once again using a pressure-lock syringe (Supelco) and analyzed by GC with an electron-capture detector (PerkinElmer) [21]. O_2, N_2, and N_2O were quantified by the methods proposed by Su et al. [19, 21].

Results and Discussion

Microorganisms. Nitrite was found only in the culture suspension in the flasks of medium (A) inoculated with diluted activated sludge. Only two pure cultures, AS-1 and AS-2, were obtained by way of streaking plate techniques from this culture suspension.

Qualitative assessment of aerobic ammonia oxidation and nitrate decrease. The purpose of the qualitative assessments was to test the capabilities of aerobic NH_4^+ oxidation and NO_3^- decrease for the isolates. Observation results revealed that AS-1 grew faster than AS-2 on plates containing NH_4Cl. Almost no nitrite was detected on plates inoculated with AS-1, but nitrite was detected on plates inoculated with AS-2. The result may imply that AS-1 is capable of ammonia oxidation. AS-1 still grew faster than AS-2 on plates containing nitrate under aerobic conditions, and more nitrite was detected on AS-1 inoculation plates after 48 hours than on AS-2 inoculation plates. The observation results may imply that AS-1 can use nitrate as the electron acceptor under aerobic conditions. Neither strain grew aerobically on plates containing NH_4Cl, but lacking additional organic carbons such as acetate. Consequently, both strains were heterotrophic bacteria.

Ammonia oxidation by strains AS-1 and AS-2 under aerobic conditions. A bacterial suspension (7.5 mL; AS-1 $OD_{600} = 0.09$ or AS-2 $OD_{600} = 0.05$) of the strains was inoculated into 500-mL Erlenmeyer flasks containing 250 mL sterile medium (D) and incubated aerobically at 30°C in triplicate. The samples were periodically examined for NH_4^+, NO_2^-, and OD_{600}. Experimental results demonstrated that the OD_{600} of strains AS-1 and AS-2 reached a stationary phase after 57 and 45 hours, respectively. AS-1 completely oxidized 2.7 ± 0.1 mmol L^{-1} NH_4^+ by 33 hours (Fig. 1a), and the removal rate of NH_4^+ was 0.064 mmol L^{-1} NH_4^+-N h^{-1} under aerobic conditions. However, strain AS-2 oxidized 2.6 ± 0.1 mmol L^{-1} NH_4^+ by 153 hours (Fig. 1b), and the removal rate of NH_4^+ was approximately 0.013 mmol L^{-1} NH_4^+-N h^{-1} under aerobic conditions. AS-1 consistently maintained superior ammonia oxidation capability to that of AS-2. Nitrite was not detected after 45 hours in the AS-1 culture suspension. Consequently, AS-1 was selected for advanced study of heterotrophic nitrification.

Nitrite decrease by strains AS-1 and AS-2. A cell suspension (4.5 mL; AS-1 $OD_{600} = 0.42$ or AS-2 $OD_{600} = 0.25$) of the isolates was individually inoculated into 250-mL Erlenmeyer flasks containing

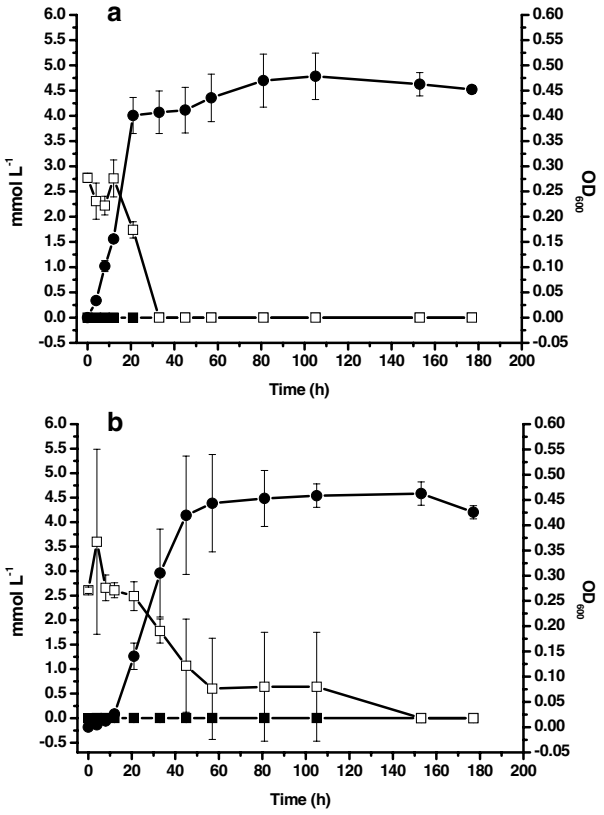

Fig. 1. Changes in NH_4^+ (open square), NO_2^- (solid square), and OD_{600} (solid circle) by oxidizing 2.8 mmol L^{-1} NH_4^+ with (a) AS-1 and (b) AS-2 in triplicate under aerobic conditions.

150 mL medium (B) in triplicates. The flasks then were incubated aerobically at 30°C. The OD_{600} at 42 hours was 0.10 ± 0.01 (initial $OD_{600} = 0.02 \pm 0.00$) and 0.07 ± 0.01 (initial $OD_{600} = 0.01 \pm 0.00$) for AS-1 and AS-2, respectively. The initial nitrite reading in the culture suspension was 0.33 ± 0.02 and 0.35 ± 0.01 mmol $L^{-1}$for AS-1 and AS-2, respectively. Nitrite was significantly decreased in all the AS-1 culture suspension samples by 27 hours ($NO_2^- = 0.08 \pm 0.04$ mmol L^{-1}) and was undetectable by 42 hours. However, nitrite decrease was slower in all AS-2 culture suspension samples than in the AS-1 samples.

Identification of AS-1. AS-1 (here termed "SU3") was Gram-negative, rod-shaped, and identified as *P. alcaligenes* (97% identity) based on comparison of its 16S rDNA sequence with that of *P. alcaligenes* isolate LB19 using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/).

Herotrophic nitrification and aerobic denitrification by AS-1. The results of these time course experiments indicated that 2.5 ± 0.2 mmol L^{-1} NH_4^+ was totally

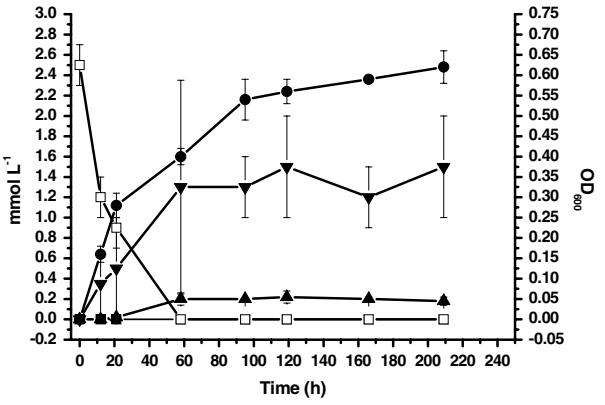

Fig. 2. Changes in NH_4^+ (open square), NO_2^- (closed square), N_2O (closed triangle), N_2 (closed inverted triangle), and OD_{600} by oxidizing 2.8 mmol L^{-1} NH_4^+ with the strain AS-1 in aluminum crimp-sealed serum bottles.

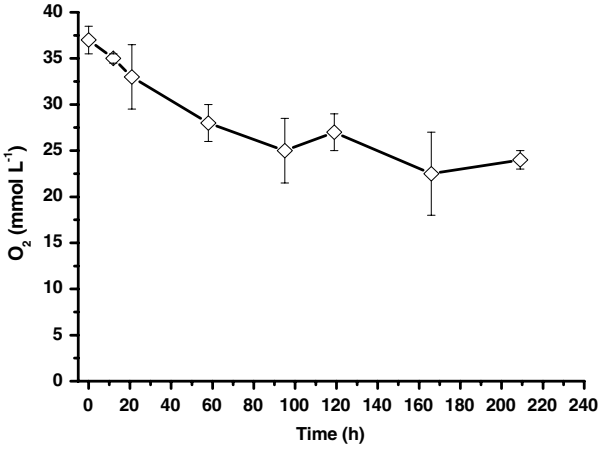

Fig. 3. Change in oxygen in the headspace by oxidizing 2.8 mmol L^{-1} NH_4^+ with the strain AS-1 in aluminum crimp-sealed serum bottles.

removed (Fig. 2), and 24.3% of the oxygen in the headspace was consumed by 58 hours (Fig. 3). The removal rate for AS-1 was 0.0034 mmol L^{-1} NH_4^+ h^{-1}. Moreover, the OD_{600} at 58 hours was 0.40 ± 0.02 and equaled 1.97 ± 0.08 mg cell using the linear regression equation. The OD_{600} values were converted to dry cell weights using a linear regression equation of $Y = 4.9742X - 0.02365$ (where Y represents the cell mass in milligrams, and X represents the OD_{600} value). Thus, a removal rate of 1.75 mmol NH_4^+ $(g\ cell)^{-1}$ h^{-1} was achieved for AS-1. No nitrite was detected during the experimental period; nitrogen gas was produced by 12 hours; and nitrous oxide was detected at 21 hours. Growth of AS-1 entered a stationary phase after 95 hours. All NH_4^+ was removed after 209 hours. Eventually, 1.5 ± 0.5 mmol L^{-1} N_2 and 0.2 ± 0.0 mmol L^{-1} N_2O were produced.

The oxygen levels in the headspace of the crimp-sealed serum bottles remained at 24.0 ± 1.0 mmol L^{-1}. Ammonium ion was completely removed, and oxygen concentration within the closed serum bottles was decreased markedly (37 ± 1.5 mmol L^{-1} to 28 ± 2.0 mmol L^{-1}) by 58 hours (Figs. 2 and 3). This decrease implied that ammonium ion was oxidized aerobically. However, the decrease of nitrite to produce nitrous oxide or nitrogen seemed to consume far less oxygen than ammonium ion oxidation (Figs. 2 and 3). Nitrogen production suggested that AS-1 was capable of heterotrophic nitrification. The removal rate of ammonia was proportional to the growth of the AS-1 (Fig. 2). This study confirmed AS-1 to be a heterotrophic nitrifier.

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Literature Cited

1. Bennasar A, Guasp C, Lalucat J (1998) Molecular methods for the detection and identification of *Pseudomonas stutzeri* in pure culture and environmental samples. Microb Ecol 35:22–33
2. Bock E, Schmidt I, Stüven R, Zart D (1995) Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. Arch Microbiol 163:16–20
3. Brierley EDR, Wood M (2001) Heterotrophic nitrification in an acid soil: Isolation and characterization of a nitrifying bacterium. Soil Biol Biochem 33:1403–1409
4. Castignetti D, Hollocher TC (1984) Heterotrophic nitrification among denitrifiers. Appl Environ Microbiol 47:620–623
5. Daniels L, Hanson RS, Phillips JA (1994) Chemical analysis. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. Washington, DC: American Society for Microbiology, pp 537–539, 541–542
6. Doxtader KG, Alexander M (1966) Nitrification by heterotrophic soil microorganisms. Soil Sci Soc Am Proc 30:351–355
7. Eylar OR, Schmidt EL (1959) A survey of heterotrophic soil microorganisms from soil for ability to form nitrite and nitrate. J Gen Microbiol 20:473–481
8. Gupta AB (1997) Thiosphaera pantotropha: A sulphur bacterium capable of simultaneous heterotrophic nitrification and aerobic denitrification. Enzyme Microb Technol 21:589–595
9. Johnsrud SC (1978) Heterotrophic nitrification in acid forest soils. Holarctic Ecol 1:27–30
10. Koch AL (1994) Growth measurement. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. Washington, DC: American Society for Microbiology, pp 260–261
11. Littleton HX, Daigger GT, Strom PF, Cowan RA (2003) Simultaneous biologic nutrient removal: Evaluation of autotrophic denitrification, heterotrophic nitrification, and biologic

phosphorus removal in full-scale systems. Water Environ Res 75:138–150
12. Papen H, von Berg R, Hinkel I, Thoene B, Rennenberg H (1989) Heterotrophic nitrification by *Alcaligenes faecalis*: NO_2^-, NO_3^-, N_2O, and NO production in exponentially growing cultures. Appl Environ Microbiol 55:2068–2072
13. Robertson LA, VanNiel EW, Torremans RAM, Kuenen JG (1988) Simultaneous nitrification and denitrification in aerobic chemostat of *Thiosphaera pantotropha*. Appl Environ Microbiol 54:2812–2818
14. Robertson LA, Cornelisse R, De Vos P, Hadioetomo R, Kuenen JG (1989) Aerobic denitrification in various heterotrophic nitrifiers. Antonie Van Leeuwenhoek 56:289–299
15. Schmidt I, Bock E (1997) Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas eutropha*. Arch Microbiol 167:106–111
16. Stouthamer AH, de Boer APN, van der Oost J, van Spanning RJM (1997) Emerging principles of inorganic nitrogen metabolism in *Paracoccus denitrificans* and related bacteria. Antonie Van Leeuwenhoek 71:33–41
17. Su JJ, Liu YL, Shu FJ, Wu JF (1997) Treatment of piggery wastewater treatment by contact aeration treatment in coordination with the anaerobic fermentation of three-step piggery wastewater treatment (TPWT) process in Taiwan. J Environ Sci Health A Tox Hazard Subst Environ Eng 32:55–73
18. Su JJ, Liu BY, Lin J, Yang JB (2001) Isolation of an aerobic denitrifying bacterial strain NS-2 from the activated sludge of piggery wastewater treatment systems in Taiwan possessing denitrification under 92% atmosphere. J Appl Microbiol 91:853–860
19. Su JJ, Liu BY, Liu CY (2001) Comparison of aerobic denitrification under pure oxygen atmosphere by *Thiosphaera pantotropha* ATCC 35512 and *Pseudomonas stutzeri* SU2 newly isolated from the activated sludge of a piggery wastewater treatment system. J Appl Microbiol 90:457–462
20. Su JJ, Liu BY, Chang YC (2001) Identification of an interfering factor on chemical oxygen demand (COD) determination in piggery wastewater and elimination of the factor by an indigenous *Pseudomonas stutzeri* strain. Lett Appl Microbiol 33:440–444
21. Su JJ, Liu BY, Chang YC (2003) Emission of greenhouse gas from livestock waste and wastewater treatment in Taiwan. Agric Ecosyst Environ 95:253–263
22. Third KA, Burnett N, Cord-Ruwisch R (2003) Simultaneous nitrification and denitrification using stored substrate (PHB) as the electron donor in an SBR. Biotechnol Bioeng 83:706–720
23. Van Goole AP, Schmidt EL (1973) Nitrification in relation to growth in *Aspergillus flavus*. Soil Biol Biochem 5:259–265
24. Van Loosdrecht MCM, Jetten MSM (1998) Microbiologic conversions in nitrogen removal. Water Sci Technol 38:1–7
25. VanNiel EW, Braber KJ, Robertson LA, Kuenen JG (1992) Heterotrophic nitrification and aerobic denitrification in *Alcaligenes faecalis* strain TUD. Antonie Van Leeuwenhoek 62:231–237
26. Verstraete W, Alexander M (1972) Heterotrophic nitrification by *Arthrobacter* sp. J Bacteriol 110:955–961