POTENTIAL APPLICATION OF AN AEROBIC DENITRIFYING BACTERIUM AS BIOAGENTS FOR WASTEWATER TREATMENT

LI Ping, LIU De-Li, ZHENG Yong-Liang, JIANG Yue, ZHANG Shan and CHEN Shu-Li

(*College of Life Sciences, Central China Normal University, Wuhan, Hubei* 430079, *P. R. China*)

Abstract: A bacterium strain HS-03 was isolated from the activated sludge plant in a suburb of Wuhan city, China which has been found to be capable of aerobic denitrification. The effect of different inoculated amounts of pre-cultured media was analyzed. The denitrifying rates of this strain under aerobic and micro-anaerobic conditions were compared. The result indicated that the rate was almost not affected by the presence of oxygen. 10m M nitrate was removed more than 90% in 36 hours by this bacterium in the specific medium. During the denitrifying process, nitrite was always kept at very low level. The denitrification potential of this bacterium was evaluated to treat artificial wastewater under fully aerobic conditions. Main biochemical and physiological features of this strain were characterized. The 16S rDNA sequence was compared with the published data in GenBank by using BLAST. The BLAST level of similarity to *Pseudomonas stutzeri* was 99. 1%. These results of phenotype and genotype proved that the strain HS-03 was a new strain belonging to the species of *P. stutzeri*.

Key words: Aerobic denitrification; Wastewater treatment; Identification

CLC number: X172 **Document code**: A **Article ID**: 1000-3207(2006)06-0667-04

Denitrification is the ability of bacteria to use nitrogen oxides (NO_3^- and NO_2^-) as electron acceptors to produce gaseous nitrogen, mainly N_2. The oxidation of organic material coupled to reduction of oxygen leads to a higher energy yield than reduction of nitrate. Oxygen is commonly accepted to be the first choice as electron acceptor[1]. Therefore, denitrifying is generally thought to only occur under almost anaerobic conditions. An efficient wastewater treatment to remove nitrogen components relies on successively exposing water to aerobic and anaerobic conditions. These properties represent a shortcoming of current systems because denitrifying would be inhibited by high oxygen if it only occurring effectively under anaerobic conditions[2], although there are a number of aerobic bacteria which are also facultative anaerobic. To resolve this problem, some new ways are required.

In the last decade, some bacteria that are capable of aerobic denitrification have been reported[3—5]. Most of these bacteria can denitrify in the presence of low levels of oxygen concentrations (down to 2mg/L) but some are able to operate at high oxygen[6]. The biochemical mechanisms are somewhat different between aerobic and anaerobic denitrifying. Aerobic denitrification is a newly discovered way to remove nitrogen from wastewater. Denitrifying and nitrifying bacteria do always occur simultaneously in one biological reactor; however, the level of activity mainly is controlled by the level of oxygen. There are plenty of aerobic bacteria in nature capable of denitrification[7]. Novel denitrifying bacteria are expected to be found. This paper presents data demonstrating that a bacterium strain HS-03 isolated from an actived sludge plant in suburban Wuhan (Hubei Province) was capable of denitrification under fully aerobic conditions and could remove nitrogen effectively from wastewater.

Received date: 2004-11-4; **Accepted date**: 2006-3-9

Foundation item: National Natural Science Foundation of China No. 3017001

Corresponding author: Liu De-Li, E-mail: deliliu2002@yahoo.com.cn

1 Materials and Methods

1.1 Microorganisms The bacterium strain HS-03 was isolated from the actived sludge plant in suburban Wuhan, China. The fungus *Aspergillus oryzae* AF 92012 was maintained in our laboratory.

1.2 Medium The medium used to pre-culture the bacterium strain HS-03 was the LB medium. The medium for pre-culturing the fungus *A. oryzae* was CZ' ($g \cdot L^{-1}$): Glycerol 10.0, $NaNO_3$ 0.85, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5, $FeSO_4 \cdot 4H_2O$ 0.01, K_2HPO_4 1.0. The pH was adjusted to 7.5. The specific medium (SC) was used to evaluate the denitrifying capability of the bacterium strain HS-03. It was composed by ($g \cdot L^{-1}$) $NaNO_3$ 0.85; sodium succinate 4.72; casamino acid 5.0; Na_2HPO_4 7.9; KH_2PO_4 1.5; $MgSO_4 \cdot 7H_2O$ 0.10; Trace solution 2.0ml, pH 7.2. Trace solution ($g \cdot L^{-1}$): $CuSO_4 \cdot 5H_2O$ 4.0, $FeSO_4 \cdot 7H_2O$ 0.7, $FeCl_3 \cdot 6H_2O$ 7.0, $CoCl_3 \cdot 6H_2O$ 0.2, $NaMO_4 \cdot 2H_2O$ 3.4, $CaCl_2 \cdot 2H_2O$ 2.0, pH 7.5. The 'artificial' wastewater (AWW) (Nanki et al., 2003) was used to evaluate the potential of wastewater treatment with this bacterium. It contained the following ingredients ($g \cdot L^{-1}$): $NaNO_3$ 0.85, polypeptone 0.6, bruillon extract 0.41, urea 0.1, sodium succinate 0.03, KH_2PO_4 0.1, KCl 0.014, $MgSO_4 \cdot 7H_2O$ 0.02, $CaCl_2 \cdot H_2O$ 0.018, tap water 1000ml. The pH value was adjusted to 7.2.

1.3 Analytical methods The concentrations of NO_3^- were determined by Brucine sulfanilic acid method at 410nm[8]. NO_2^--N was estimated by fading of methyl orange decoloration photometrically[9].

1.4 Determination of mol% G+C content and 16S rDNA gene sequence The mol% G+C content was determined after cell lysis with lysozyme and isolation of DNA by chloroform/isoamyl alcohol. The gene was amplified by PCR with 1μl (8ng) of total DNA as the template. The primers were universal bacterial 16S rDNA primers: forward primer 5' AGAGT TTGATCCTGGCTCAG 3' and reverse primer 5' GGTTACCTTGTTACGACTT3'[10]. After pre-denature at 94℃ for 3min, PCR was performed by 30 cycles at 94℃ for 30s, 46℃ for 30s, 72℃ for 1min and followed by a final extension at 72℃ for 10min. The 16S rDNA sequence was compared with the published data in GenBank by using BLAST.

1.5 Estimation of aerobic denitrification capability HS-03 was pre-cultivated overnight in LB medium and fungi *A. oryzae* was pre-cultured in CZ' medium for one week. 2ml pre-culture was inoculated into 500-ml Erlenmeyer flask containing 100ml SC or AWW. Each culture was stirred at 200 rpm, 30℃ on a rotary shaker. The bottles were closed with cotton plugs under aerobic conditions and rubber stoppers under micro-anaerobic conditions. Samples were taken from the bottles periodically for measurements. All experiments were run in triplicate.

2 Results

2.1 Measurement of denitrifying rates The relationship between denitrification and the amount of strain HS-03 pre-culture inoculated in the treatment medium is shown in Fig. 1. The denitrifying rates went up quickly before inoculated volume reached 2ml per 100ml medium, while it only raised 12.3% by adding another 2.5ml of the pre-culture. Therefore, the inoculated amount of 2ml per 100ml medium was thought to be the most economical and effective inoculated volume per unit treatment medium.

The denitrifying rates of the strain HS-03 under aerobic and micro-anaerobic conditions were compared. In the specific medium SC, 10mM nitrate was removed at over 90% in 30 hours under microanaerobic condition, while 36 hours were needed under aerobic condition (Fig. 2). This indicated that the denitrifying rate was almost not affected by the presence of oxygen. From Fig. 2, it can be seen that the concentration of NO_2^- was always kept at very low levels, indicating that nitrite was not accumulating during the denitrification process.

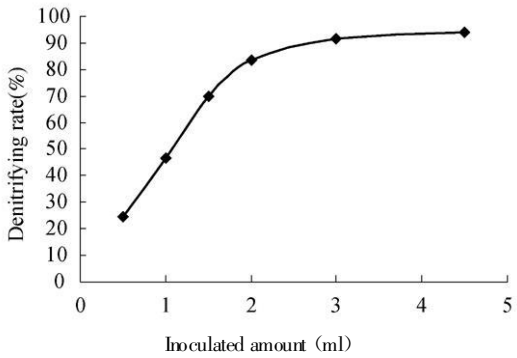

Fig. 1 The relationship between denitrifying rates and different inoculated amounts of the bacterial strain HS-03 pre-culture in every 100ml medium

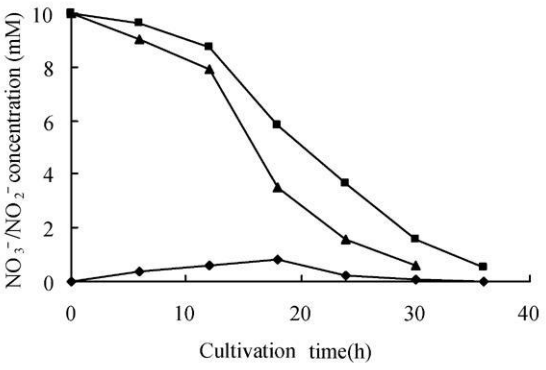

Fig. 2 The denitrifying capability of strain HS-03. ■NO_3^- under aerobic condition, ▲NO_3^- under micro-anaerobic condition, ◆NO_2^- under aerobic condition

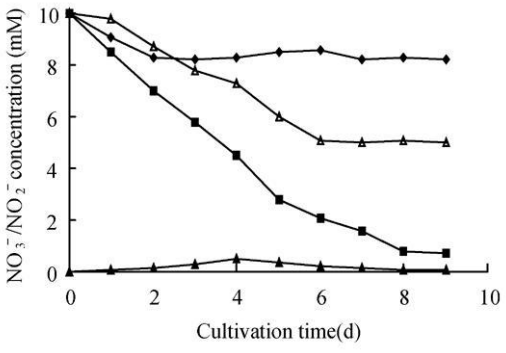

Fig. 3 The denitrifying capability in AWW under aerobic condition. ◆NO_3^- strain HS-03, △NO_3^- fungi *A. oryzae*, ■ NO_3^- strain HS-03 mixed with fungi *A. oryzae*, ▲NO_2^- strain HS-03 mixed with fungi *A. oryzae*

The capability of strain HS-03 to remove nitrogen from AWW was evaluated under aerobic conditions. The denitrifying rate was only reach to 17% after cultivation for two days even if the carbon source was added. However, when the strain was mixed with fungus *A. oryzae* precultured in CZ' medium for one week, the final nitrogen removal rate was over 90%, which was much higher than that of the HS-03 strain and the fungus *A. oryzae* in AWW respectively (Fig. 3). Moreover, the concentration of NO_2^- was also kept at very low level. During the denitrifying process, sodium succinate and glycerol were added as carbon source.

2.2 Identification of this bacterium strain

HS-03 was identified as a Gram negative strain with a slightly curved rod and rounded ends. The mol% G+C content of DNA was 53.55%. It could produce acid from glucose and hydrolyze ornithine, lysine and arginine. Most of the physiological and chemical characteristics accord with those of *Pseudomonas stutzeri* in Manual of Determinative and Systematic Bacteriology[11]. The 16S rDNA sequence was compared with the published data in GenBank by using BLAST. The BLAST level of similarity to *P. stutzeri* was 99.1%. The nucleotide sequence data have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB193828. These results proved that the strain HS-03 was a new isolate belonged to the species of *P. stutzeri*.

3 Discussion

Fully aerobic denitrification is a rather newly discovered metabolic activity that obviously can be carried out by many different bacteria[4,6]. In the past we have learned that some nitrifyers are also facultative denitrifiers to be able to switch when oxygen levels gradually decline to critical values[5]. This study has shown that fully aerobic denitrification can be performed by a bacterium HS-03 selected from an actived sludge plant. The data demonstrated that denitrification can certainly occur simultaneously under aerobic conditions. The denitrifying rate of the bacterium HS-03 was much higher in the specific medium. Furthermore, HS-03 can treat an artificial wastewater effectively when it was applied in a mixed culture with the fungus *A. oryzae*. The denitrifying rate was much higher than that of these two microorganisms separately. The success of this aerobic denitrification process applying to artificial wastewater treatment may serve as an alternative to costly many-stage anaerobic denitrification treatment procedures.

Recently, the presence of nitrogenous substances in wastewater discharges has attracted attention because of the role of nitrogen in eutrophication of receiving waters[12]. Nitrogenous substances exhibit toxicity towards aquatic life, present a public health hazard, and affect the suitability of wastewater for reuse. To prevent eutrophication, biological denitrification, as a new effective way, has been used in removing nitrate from wastewater. However, most bacteria perform denitrification under anaerobic conditions. Here, we reported that the denitrification by bacterium HS-03 and fungus *A. oryzae* will be very useful to remove nitrogen from the wastewater. The mechanism of co-denitrification between these two microorganisms will have to be further investigated before it

can be applied at a commercial scale.

References:

[1] Frette, L., Gejlsbjerg B. & Westermann P., 1997. Aerobic denitrifiers isolated from an alternating actived sludge system. FEMS Microbiology Ecology 24: 363—370

[2] Ferguson, S. J., 1994. Denitrification and its control. Antonie Leeuwenhoek 66: 89—110

[3] Roberson, L. A., E. V. Niel & R. Torreman, 1988. Simultaneous nitrification and denitrification in aerobic chemostat culture of *Thiosphaera pantotroph*. Applied and Environmental Microbiology 54: 2812—2818

[4] Chen, F., Q. Xia & L. K. Ju, 2003. Aerobic denitrification of *Pseudomonas aeruginosa* monitored by online NAD (P) H fluorescence. Applied and Environmental Microbiology 69: 6715—6722

[5] Takaya, N., M. A. Sakairi, Y. Sakaguchi, I. Kato, Z. Zhou & H. Shoun, 2003. Aerobic denitrification bacteria that produced low levels of nitrous oxide. Applied and Environmental Microbiology 69: 3152—3157

[6] Su, J. J., B. Y. Liu & C. Y. Liu, 2001. Comparison of aerobic denitrification under high oxygen atmosphere by *Thiosphaera pantotropha* ATCC 35512 and *Pseudomonas stutzeri* SU2 newly isolated from the actived sludge of a piggery wastewater treatment system. Journal of Applied Microbiology 90: 457—462

[7] Paturead, D., E. Zumstein & J. P. Delgenes, 2000. Aerobic denitrifiers isolated from diverse natural and managed ecosystems. Microbial Ecology 39: 145—152

[8] American Public Health Association, 1992. Standard methods for the examination of water and wastewater. 18th Edition, Part 4000, American Public Health Association, Washington, DC

[9] Kuang, Y. F., P. H. Deng & Y. J. Feng, 2004. Decoloration photometic determination of nitrite by fading of methyl orange. Chemical World 4: 181—182

[10] Weisburg, W. G., S. M. Barns & D. A. Pelletier, 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173: 697—703

[11] Dong, X. Z. &M. Y. Cai, 2001. Manual of determinative and systematic bacteriology. Beijing. China. Science Press. pp 166—168

[12] Mensinga, T. T., G. J. Speijers & J. Meulenbelt, 2003. Health implications of exposure to environmental nitrogenous compounds. Toxicol Rev, 22: 41—51