Heterotrophic denitrification by Gram-positive bacteria: *Bacillus cereus* and *Bacillus tequilensis*

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Abstract- Two bacteria were isolated from anoxic denitrifying reactor for treatment of domestic wastewater. The analysis of the 16S rDNA gene sequences showed that the isolated strains were affiliated with *Bacillus cereus* and *Bacillus tequilensis*. Denitrification was compared between *Bacillus cereus* and *Bacillus tequilensis* in this study. Two bacilli were able to denitrify and *Bacillus cereus* was more efficient than *Bacillus tequilensis*. *Bacillus cereus* reduced 80% of high amount of nitrate; however, *Bacillus tequilensis* could reduce 37.4% of nitrate. These heterotrophic bacteria are able to eliminate organic matter with the same trend reducing 74.5% for *Bacillus tequilensis* and 70.2% for *Bacillus cereus*.

Index Terms- Denitrification, *Bacillus cereus*, *Bacillus tequilensis*, 16S rRNA gene.

I. INTRODUCTION

Denitrification is a respiratory process that couples electron transport phosphorylation with sequential reduction of nitrate to nitrogen through the intermediates nitrite, nitric oxide, and nitrous oxide (Delong et al. 2013). Biological denitrification is normally conducted by facultative anaerobes which are in essential need for some food and energy sources which are organic or inorganic (Cast and Flora, 1998; Rijn et al., 2006). This fact classifies denitrifiers into two main groups of heterotrophs and autotrophs. Heterotrophic denitrifiers use various organic carbons, such as methanol, acetate, or glucose that can serve as electron donors and carbon sources for growth. The beneficial aspects of denitrification include control or bioremediation of NO_3^- contaminated waters, which can cause eutrophication. However, nitrate has also been linked to stomach, cancer (Yang et al., 2007; Bouchard et al., 1992), and blue baby syndrome (Comly 1987). Denitrifiers may play an important part in the breakdown of various hydrocarbon compounds. Thus, biological denitrification constitutes an important way to reduce nitrogen oxides and organic matter in environment.

Mostly *Bacteria*, and a few *Archaea*, constitute the vast majority of organisms capable of denitrification. A number of fungal isolates carry out reduction of nitrate to N_2O, but the contribution of this reduction to cell growth is variable (Kim et al., 2009; Nakanishi et al., 2010). Some multicellular eukaryotes carry out denitrification (Risgaard-Petersen et al. 2006). Inspite of the fact that several Gram-positive bacteria are denitrifiers (Zumft, 1997; Manachini et al., 2000; Suharti & de Vries, 2005), the basics on denitrification within this group are understudied and they are notoriously overlooked in community analysis of denitrifiers in the environment because they are not targeted by the available PCR primers designed for denitrification genes (Throbäck et al., 2004). Verbaendert et al. (2011) have studied the denitrification of a large collection of *Bacillus* strains and suggested that denitrification occurred in nearly half of the analysed strains. More recently, a variety of bacilli were tested for gas production under denitrifying conditions and found to be complete denitrifiers (Jones et al., 2011). Genome sequencing has revealed the potential for partial denitrification in some *Bacillus* species. For example, qNor is present in *Bacillus tusciae* strain DSM2912 and some *Bacillus licheniformis* strains, but these are their only denitrification enzymes (Delong et al. 2013).

In order to check the denitrification potential in anoxic denitrifying reactor, we isolated two cultures and compared their denitrification patterns to eliminate organic matter, reduction of nitrates and nitrites. The isolates were *Bacillus cereus* and *Bacillus tequilensis*, both isolated from biofilm of anoxic denitrifying reactor. *Bacillus cereus* and *Bacillus tequilensis* are known to be denitrifiers (Gaston et al., 2006; Verbaendert et al., 2011).

II. MATERIALS AND METHODS

II.1. Identification of denitrifying strains

Isolates ADR1 and ADR2 were isolated from an anoxic denitrifying reactor treatment of domestic wastewater on nitrate–sucrose-agar (NSA) plates. Total DNA was extracted as described by Pitcher et al. (1989). The isolates were identified by molecular methods which consist to amplify the 16S rRNA gene of the strains by PCR and to determine 16S rDNA sequence by direct sequencing. The PCR amplification was performed with the primers pA (AGAGTTTGATCCTGGCTCAG) and pH (AAGGAGGTGATCCAGCCGCA) designed by Edwards et al. (1989) and used by other (McLaughlin et al., 2002). PCR amplification conditions were 95°C for 5 min followed by 30 cycles of 95°C for 40 s, 55°C for 1min, and 72°C for 2 min and a final 10-min extension step at 72°C. Sequencing of the PCR amplicon was done in ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems) using the POP-7 polymer and ABI PRISM Genetic Analyzer Data Collection and ABI PRISM Genetic Analyzer Sequencing Analysis software.16S rRNA gene was compared with those available in EMBL database (https://www.ebi.ac.uk/Tools/sss/ncbiblast/nucleotide.html) using BlASTN. Reference and isolates sequences were aligned

with SINA (Pruesse et al., 2012) and then imported in MEGA 5.1 (Tamura et al., 2011). Phylogenetic tree was constructed by maximum likelihood.

II.2. Denitrification studies of the isolates

Denitrification experiments were performed in the medium, modified from Vossoughi et al. (1982), with the following composition (per liter of distilled water): $FeSO_4 \cdot 7H_2O$ 0.002g, $CuSO_4 \cdot 5H20$ 0.02 g, $ZnSO_4 \cdot 7H_2O$ 0.02 g, $MnSO_4 \cdot H_2O$ 0.12 g, $MgSO_4$ 0.8 g, $CaCl_2 \cdot 2H_2O$ 0.04 g, K_2HPO_4 3.2 g, yeast extract 0.3g, KNO3 4.7g, sucrose 3g, at pH 7.5. The isolates were grown in nutrient broth for 24 h and centrifuged at 10.000 rpm for 7 min. The cell pellet was washed twice with Nacl 0.9% and resuspended in Nacl 0.9% with absorbance of 1.0 at 620 nm for isolates and 25 ml of this was inoculated in 250 ml Erlenmeyer flasks containing 250 ml of the medium. The flasks were incubated at 30°C under static conditions up to 6 days by sampling at an interval of every 24 h for estimating growth, nitrate, nitrite and organic matter.

II.3. Analytical methods

Chemical oxygen demand (COD), nitrate and nitrite were estimated according to the standard methods for the examination of water and wastewater (APHA, 1995). Bacterial growth was estimated by dry cell weight (DCW) measured by drying the cells at 105 °C to constant weight for 12 hrs.

III. RESULTS

III.1. Characterization of the isolates

The two cultures under study, ADR1 and ADR2, were isolated from the anoxic denitrifying reactor for domestic wastewater treatment in nitrate-sucrose-agar. The isolates were subjected to 16S rRNA gene sequence analysis. A species level match is based on a similarity greater than or equal to 99% (Drancourt et al., 2000).

Table I: Determination of denitrifying isolates by partial 16S rRNA gene sequence similarity

	Isolate ADR1	Isolate ADR2
Similar species	*Bacillus cereus*	*Bacillus tequilensis*
Accession No.	KF484678	JX315319
% Similarity	99	99

Isolate ADR1 was observed to be gram-positive bacilli, and BLAST results of partial 16S rRNA gene showed 99% identity with *Bacillus cereus*; Isolate ADR2 Gram-positive bacilli, showed 99% similarity with *Bacillus tequilensis* with partial 16S rRNA gene sequence (Table I). Phylogenetic positions of isolates are shown in (Figure 1), where the isolates ADR1 and ADR2 clustered with *Bacillus cereus* and *Bacillus tequilensis*, respectively.

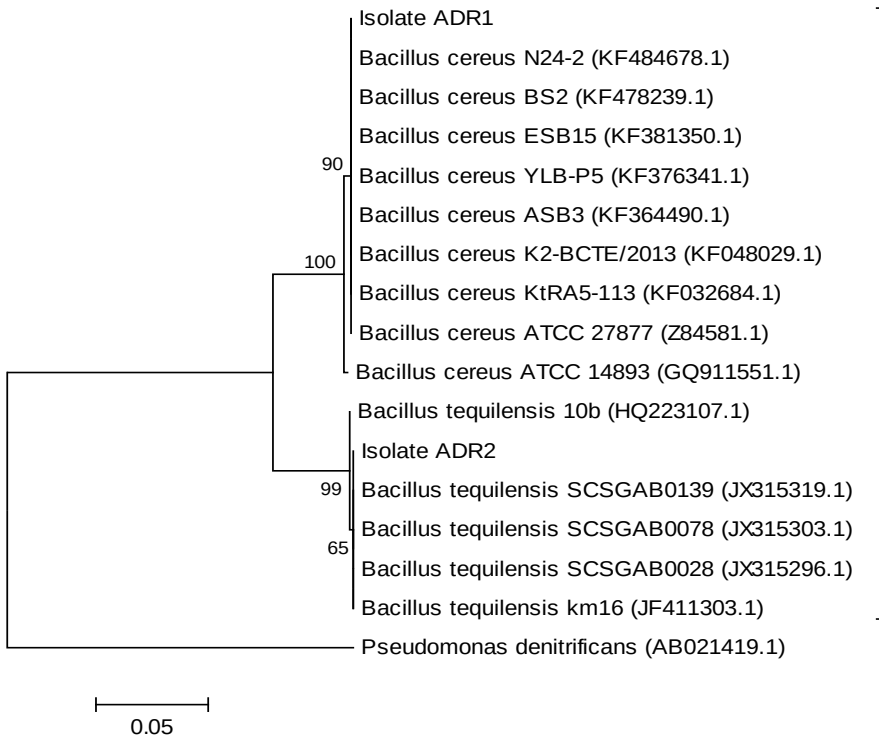

Figure 1: Phylogenetic tree constructed by neighbor-joining method showing position of the isolates with other related cultures. Bootstrap analysis of 10000 resampling by maximum-likelihood method was used to reconstruct tree. Parenthesis contains the accession number of the cultures. *Pseudomonas denitrificans* (AB021419) was used as an outgroup.

III.2. Denitrification Pattern of the isolates

The nitrate reduction and nitrite accumulation of both isolates were monitored in batch mode up to 6 days in 250-mL Erlenmeyer flasks under static condition. Nitrate was reduced to 7.5 mM from 36.96 mM in 6 days by *Bacillus cereus* (80%). *Bacillus tequilensis* could reduce nitrate from 36.96 mM to 23.14 mM (37.4%) (Figure 1a). The nitrate is reduced to nitrogenous oxides by isolates that use nitrate instead of oxygen as electron acceptors and organic matter as carbon and energy source. This fact is confirmed in our results when the COD removal by *Bacillus cereus* and *Bacillus tequilensis* followed the same trend and organic matter decreased from 6040 mg/l to 1800 mg/l (70.2%) by *Bacillus cereus* and from 6040 mg/l to 1540 mg/l (74.5%) by *Bacillus tequilensis* (Figure 2d). The nitrites resulted of the reduction of nitrate reached 294 mM for isolate ADR1 and then it decreased until 28.82 mM, however, the nitrites produced by isolate ADR2 concentration reached 161.14 mM at the beginning of the study and decreased until 2.76 mM (Figure 2b). These results are in accordance with those of nitrate reduction. We observed that *Bacillus tequilensis* is less efficient in nitrate reduction and thus produced a little amount of nitrites. Despite the facts that isolate ADR1 could denitrify more than isolate ADR2. The growth of the second species is more important, this result is showed in (Figure 2c) where the biomass expressed as dry cell weight per liter in (DCW/l).

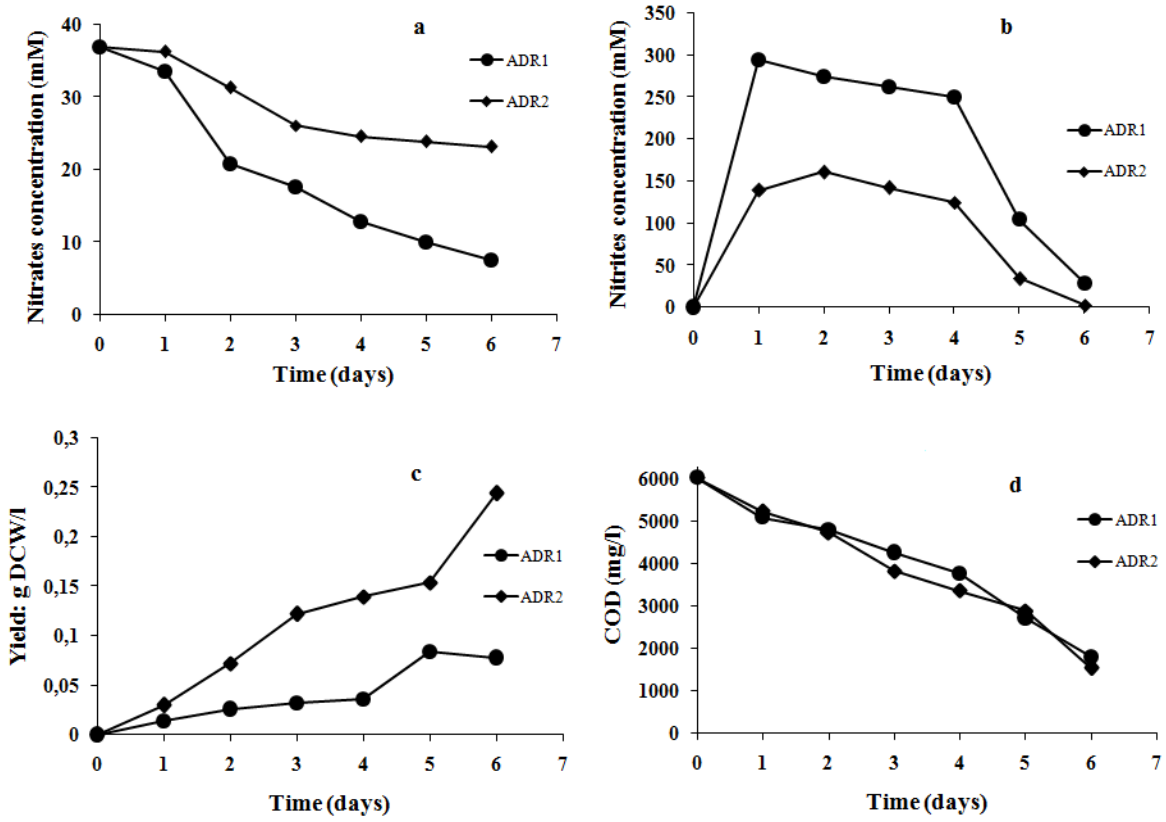

Figure 2: Denitrification pattern of the isolates ADR1 and ADR2. (a) nitrate consumption, (b) nitrite accumulation, (c) Growth, (d) Organic matter (COD).

IV. Discussion

Based on 16S rRNA gene sequencing isolates were affiliated with *Firmicutes*. The ability to denitrify has been identified in taxonomically diverse bacteria, including members of the *Aquificae*, *Deinococcus-Thermus*, *Firmicutes*, *Actinobacteria*, *Bacteroides*, and *Proteobacteria* phyla (Zumft 1997). Isolate ADR1 was related to *Bacillus cereus*. *Bacillus cereus* is a heterotrophic bacterium able to degrade organic matter under nitrate reducing conditions. Dou et al. (2010) reported that *Bacillus cereus* could transform benzene to phenol and benzoate, and then used phenol and benzoate as carbon and energy source. Also, Zhao et al (2009) used the denitrifying *Bacillus cereus* to remove nitrogen and organic matter from reclaimed wastewater used as landscape water. *Bacillus cereus* is most likely involved in biogeochemical nutrient cycling, as it produces a wide range of extracellular enzymes and can grow on decaying organic matter (Borsodi *et al.* 2005). *Bacillus tequilensis* could reduce nitrate to nitrogen, thus this species is a true denitrifier (Gatson et al. 2006). As reported by Das et al. (2014), *Bacillus tequilensis*

was chemoorganotrophic and could use hydrocarbons as sole carbon source.

Despite the fact that diverse denitrifiers have similar denitrification apparatus, each organisms have own activity. In this study, we compared the denitrification of two bacilli, ADR1 and ADR2, isolated from a denitrifying reactor and identified as *Bacillus cereus* and *Bacillus tequilensis*. The nitrate reduction rate is higher in *Bacillus cereus*. However, two isolates have nitrite accumulation. Carlson and Ingraham (1983) revealed different patterns of denitrification between *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*. Betlach and Tiedje (1981), showed that transit nitrite accumulation in *Alcaligenes sp.* and *Pseudomonas fluorescens* was due to the differences in the reduction rates of nitrate and nitrite. The growth estimated by dry cell weight is more important in *Bacillus tequilensis* than *Bacillus cereus*. Otherwise, the increase of cell number leading to enhance the quantity of biomass and the sludge in the system of wastewater. Thus, *Bacillus cereus* is more efficient because this strain reduces more amount of nitrate than *Bacillus tequilensis* and produces less sludge.

V. CONCLUSION

The above results showed that isolates ADR1 and ADR2 isolated from denitrifying reactor were identified as *Bacillus cereus* and *Bacillus tequilensis*. The experimental results showed that *Bacillus cereus* could reduce 29.46 mM of nitrate and degrade 4240 mg/l of organic matter within 6 days. However, *Bacillus tequilensis* is less efficient and could reduce 13.82 mM of nitrate and 4500mg/l of organic matter. In addition, *Bacillus cereus* produces less biomass avoiding clogging of wastewater treatment system. These results concerning *Bacillus cereus* showed that the isolated bacterium could potentially remediate wastewater with high level of nitrate and organic matter.

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REFERENCES

[1] APHA (American Public Health Association) (1995) Standard Methods for the Examination of Water and Wastewater, 19th ed, Washington DC.

[2] Betlach; MR, Tiedje JM, (1981) Kinetic explanation for accumulation of nitrite, nitric oxide, and nitrous oxide during bacterial Denitrification. Appl. Environ. Microbiol. 42: 1074-1084.

[3] Borsodi AK, Micsinai A, Rusznyak A, Vladar P, Kovacs G, Toth EM and Marialigeti K (2005) Diversity of alkaliphilic and alkalitolerant bacteria cultivated from decomposing reed rhizomes in a Hungarian soda lake. Microbial. Ecol. 50: 9-18.

[4] Bouchard DC, Williams MK, and Surampalli RY (1992) Nitrate contamination of groundwater: source and potential health effects. Journal AWWA 84: 85–90.

[5] Carlson CA, Ingraham JL (1983) Comparison of Denitrification by Pseudomonas Pseudomonas stutzeri, Pseudomonas aeruginosa, and Paracoccus denitrificans. Appl. Environ. Microbiol. 45: 1247-1253.

[6] Cast KL, Flora JRV (1998) An evolution of two cathode materials and the impact of copper on bio-electrochemical denitrification. Water. Res. 32: 63–70.

[7] Comly HH (1987) Cyanosis in Infants caused by nitrates in well water. J. Americal Med. Assoc. 257: 2788-2792.

[8] Comly HH (1987)Cyanosis in Infants caused by nitrates in well water. J. Americal. Med. Assoc. 257: 2788-2792.

[9] Das R and Kazy SK (2014) Microbial diversity, community composition and metabolic potential in hydrocarbon contaminated oily sludge: prospects for in situ bioremediation. Environ Sci Pollut Res. DOI 10.1007/s11356-014-2640-2.

[10] DeLong EF, Lory S, Stackebrandt E, Thompson F (2013) The prokaroytes Prokaryotic Physiology and Biochemistry. Springer-Verlag Berlin Heidelberg p682.

[11] Dou J, Ding A, Liu X, DuY, Deng D, Wang J (2010) Anaerobic benzene biodegradation by a pure bacterial culture of *Bacillus cereus* under nitrate reducing conditions. Journal of Environ. Sci. 22: 709–715.

[12] Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, and Raoult D (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clin. Microbiol. 38: 3623-3630.

[13] Edwards U, Rogall T, Blöcker H, Emde M, and Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic. Acids. Res. 17:7843–7853.

[14] Gatson JW, Benz BF, Chandrasekaran C, Satomi M, Venkateswaran K and Hart ME (2006). *Bacillus tequilensis* sp. nov., isolated from a 2000-year-old Mexican shaft-tomb, is closely related to Bacillus subtilis. Int. J. Syst. Evol. Microbiol. 56: 1475–84.

[15] Jones CM, Welsh A, Throback IN, Dorsch P, Bakken LR and Hallin S (2011) Phenotypic and genotypic heterogeneity among closely related soil-borne N2 and N2O-producing Bacillus isolates harboring the nosZ gene. FEMS Microbiol. Ecol. 76:541–552.

[16] Kim SW, Fushinobu S, Zhou S,Wakagi T and Shoun H (2009) Eukaryotic nirK genes encoding copper-containing nitrite reductase: originating from the protomitochondrion? Appl. Environ. Microbiol. 75:2652–2658.

[17] Manachini PL, Mora D, Nicastro G, Parini C, Stackebrandt E, Pukall R and Fortina MG (2000) Bacillus thermodenitrificans sp. nov., nom. rev. Int. J. Syst. Evol. Microbiol. 50: 1331–1337.

[18] McLaughlin RW, Vali H, Lau PCK, Palfree RGE, De Ciccio A, Sirois M, Ahmad D, Villemur R, Desrosiers M and Chan ECS (2002) Are there naturally occurring pleomorphic bacteria in the blood of healthy humans? J. Clin. Microbiol. 12:4771–4775.

[19] Nakanishi Y, Zhou S, Kim SW, Fushinobu S, Maruyama J, Kitamoto K,Wakagi T, Shoun H (2010) A eukaryotic copper-containing nitrite reductase derived from a NirK homolog gene of Aspergillus oryzae. Biosci. Biotechnol. Biochem. 74:984–991.

[20] Pitcher DG, Saunders NA and Owen RJ (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8:151-156.

[21] Pruesse E, Peplies J and Glöckner FO (2012) SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics. 28:1823-1829.

[22] Rijn JV, Tal Y and Schreier HJ (2006) Denitrification in recirculating systems: theory and applications. Aquacul. Eng. 34, 364–376.

[23] Risgaard-Petersen N, Langezaal AM, Ingvardsen S, Schmid MC, Jetten MS, Op den Camp HJ, Derksen JW, Pina-Ochoa E, Eriksson SP, Nielsen LP, Revsbech NP, Cedhagen T, van der Zwaan GJ (2006) Evidence for complete denitrification in a benthic foraminifer. Nature. 443:93–96.

[24] Suharti and de Vries S (2005) Membrane-bound denitrification in the Gram-positive bacterium Bacillus azotoformans. Biochem. Soc. Trans. 33: 130–133.

[25] Tamura K and Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10:512-526.

[26] Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28: 2731-2739.

[27] Throbäck IN, Enwall K, Jarvis A and Hallin S (2004) Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol. Ecol. 49: 401–417.

[28] Verbaendert I, Boon N, De Vos P, Heylen K (2011) Denitrification is a common feature among members of the genus *Bacillus*. Syst. Appl. Microbiol. 34:385–391

[29] Vossoughi M, LAroche M, Navarro JM, Faup G and Leprince A (1982) Denitrification ne continu à l'aide de microorganismes immobilisés sur des supports solides. Water Res. 16 : 995-1002.

[30] Yang CY, Wu DC and Chang CC (2007) Nitrate in drinking water and risk of death from colon cancer in Taiwan. Environ. Int. 33: 649-653.

[31] Zhao S, Hu N, Chen Z, Zhao B and Liang Y (2009) Bioremediation of Reclaimed Wastewater Used as Landscape Water by Using the Denitrifying Bacterium *Bacillus cereus*. Bull. Environ. Contam. Toxicol. 83:337–340.

[32] Zumft W (1997) Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61: 533-616.

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