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Assessment of denitrifying bacterial composition in activated sludge

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ABSTRACT

The abundance and structure of denitrifying bacterial community in different activated sludge samples were assessed, where the abundance of denitrifying functional genes showed *nir*S in the range of 10^4–10^5, *nos*Z with 10^4–10^6 and 16S rRNA gene in the range 10^9–10^{10} copy number per ml of sludge. The culturable approach revealed *Pseudomonas* sp. and *Alcaligenes* sp. to be numerically high, whereas culture independent method showed betaproteobacteria to dominate the sludge samples. *Comamonas* sp. and *Pseudomonas fluorescens* isolates showed efficient denitrification, while *Pseudomonas mendocina*, *Pseudomonas stutzeri* and *Brevundimonas diminuta* accumulated nitrite during denitrification. Numerically dominant RFLP OTUs of the *nos*Z gene from the fertilizer factory sludge samples clustered with the known isolates of betaproteobacteria. The data also suggests the presence of different truncated denitrifiers with high numbers in sludge habitat.

1. Introduction

In natural environments nitrate is present in minimal amounts and a high competition exists for its utilization among organisms including plants (Kaye and Hart, 1997). In contrast, effluent of some industries contain very high amounts of nitrate (Zala et al., 1999; Fernandez-Nava et al., 2008) which, when released without treatment percolate into ground water, rivers and lakes causing eutrophication or contaminating the drinking water. The WHO guideline value for nitrate and nitrite in drinking water is 50 mg l^{-1} and 3 mg l^{-1}, respectively, as consumption of high nitrate concentration causes methemoglobinemia in infants and other health hazards. Biological denitrification is widely used to remove the high levels of nitrate by activated sludge processes.

Denitrification takes place in bacteria by a four step reaction of reducing nitrate to nitrite, nitric oxide, nitrous oxide and to dinitrogen with its respective reductases encoded by *nar*, *nir*, *nor* and *nos* genes respectively (Shapleigh, 2006). However, truncation of the denitrification ability is found in some bacteria (Wood et al., 2001; Casella et al., 2006). The denitrifying apparatus is distributed diversely among the bacteria (Shapleigh, 2006), due to which the functional genes are targeted to assess the denitrifier populations. Though polyphyletic distribution is seen, the denitrification studies are carried out in limited number of organisms (Bergaust et al., 2011).

The nitrate removal processes are being viewed mainly from the engineering perspective for the design of reactors, and the micro-scale biology is grossly neglected in most cases (Wilderer et al., 2002). In order to remove the high concentration of nitrogenous oxides efficiently, it is necessary to understand the abundance, structure and activity of the denitrifying bacteria present in the sludge. Also, the insights gained from the microbiology of wastewater treatment processes will help comprehend the effect it has on atmospheric nitrogen cycle. However, very few studies are reported on composition of denitrifiers based on the use of functional genes in sludge habitats. Most of the culture independent studies on abundance and diversity of denitrifying bacteria in activated sludge is reported using only FISH probe-designed populations (Ginige et al., 2005; Morgan-Sagastume et al., 2008).

Thus, the objective of this study was to assess the denitrifying bacterial composition in different activated sludge samples including denitrifying reactor sludge, by both culture dependent and independent means. The functional genes of denitrification are targeted in this study to analyze the abundance and diversity of denitrifying bacteria by culture independent method. Cultivation of denitrifiers including truncated ones in nitrate reduction, and denitrification patterns of representative complete denitrifiers is also investigated.

2. Methods

2.1. Activated sludge samples

Four different activated sludge samples used in this study, their sources and the characteristics are listed in Table 1. Total organic carbon (TOC) in the sludge was measured in TOC analyzer (Shimadzu corp., Japan). Suspended and dissolved solids were measured by standard methods, and different cations were

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Table 1
Characteristics of the activated sludge samples.

Sample name	DaS	GS	WL	NL
Source	Denitrifying reactor of a Fertilizer factory	Aeration tank of fertilizer factory	Aeration tank of municipal Effluent treatment plant	Aeration tank of Common effluent treatment plant in an industrial area
TOC (mg L^{-1})	1020.4	1055.6	687.6	910.8
Suspended solids (mg L^{-1})	64	10	151	248
Total dissolved solids (mg L^{-1})	1330	2350	2500	8570
Calcium (ppm)	2810	59.086	285.63	98.904
Magnesium (ppm)	32.266	14.036	71.509	45.292
Iron (ppm)	16.572	5.084	10.729	26.625
Manganese (ppm)	8.381	0.308	0.729	0.686
Copper (ppm)	BDL	BDL	0.536	0.333
Nitrogenous compounds[a] (ppm)	400–600 (NO_3–N)	–	–	30–45 (NH_3–N)

BDL = below detection limit.
[a] As provided by the plant operators.

measured by inductively coupled plasma spectrometer (ICP) (Optima 3300 RL, Perkin Elmer). All these characteristics of sludge samples were analyzed at Sophisticated Instrumentation Centre for Applied Research and Testing (SICART, Gujarat).

2.2. Isolation, screening and Amplified Ribosomal DNA Restriction analysis (ARDRA) of denitrifying bacteria

Isolation of denitrifiers from sludge samples was done using three different media, peptone nitrate medium (PNB), G2M11 and G4M3 (Heylen et al., 2006a). For each sludge sample, serial dilutions were made up to 10^{-4}–10^{-6} and from each dilution 0.1 ml was plated on respective medium. The plates were incubated at 30 °C for 7 days. Isolated colonies with different colony morphologies were picked and inoculated in PNB, which was further screened by nitrate reduction test.

16S rRNA gene was amplified using the primers 27F and 1541R (Table 2) from the genomic DNA of the isolated denitrifying bacteria and ARDRA was performed using the restriction enzymes *Alu*I and *Rsa*I separately. The digested amplicons were run on 8% polyacrylamide gels and silver stained for further analysis. ARDRA profiles were manually analyzed and binary data for presence or absence of bands were computed and the dendrogram was plotted using NTSys software (Exeter software, NY) with UPGMA method.

2.3. Denitrification studies of the isolates

Denitrification experiments were performed in MM2 medium (Srinandan et al., 2010) consisting of sodium succinate 7.9 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, K_2HPO_4 0.2 g, $FeSO_4{\cdot}7H_2O$ 0.05 g, $CaCl_2{\cdot}2H_2O$ 0.02 g, $MnCl_2{\cdot}4H_2O$ 0.002 g, $NaMoO_4{\cdot}2H_2O$ 0.001 g, KNO_3 1.0 g, yeast extract 1.0 g, pH 7.0, distilled water 1000 ml. The isolates were grown in PNB for 18 h and centrifuged at 10,000 rpm for 7 min. The cell pellet was washed twice with phosphate buffered saline (PBS) and resuspended in PBS with absorbance of 0.5 set at 600 nm for all the isolates and 2 ml of this was inoculated in 250 ml Erlenmeyer flasks containing 100 ml MM2 medium for denitrification studies. The flasks were incubated at 30 °C under static conditions up to 17 h by sampling at an interval of every 3 h for estimating nitrate and nitrite. The experiment was performed in triplicates.

2.4. Analytical methods

The brucine sulfate method was used to determine nitrate according to Jenkins and Medsker (1964). Nitrite was estimated according to APHA (1995).

2.5. DNA extraction from sludge samples

Extraction of genomic DNA from sludge was done according to Zhou et al. (1996). Extraction buffer consisted of 100 mM Tris–HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, 1% CTAB and 1% polyvinylpyrrolidone (PVP). 15 ml of activated sludge sample was mixed with extraction buffer and incubated at 30 °C for 30 min in shaking condition. Sodium dodecyl sulfate (SDS) was added to this mixture at 2% final volume and incubated for two hours at 65 °C with intermittent shaking. Centrifugation was done for 10 min and phenol–chloroform extraction of the DNA was carried out. The aqueous phase was precipitated with 0.6 volume of isopropanol at room temperature and the crude nucleic acids pellet obtained by centrifugation

Table 2
PCR Primers used in the study.

Primer name	Target gene	Sequence 5′–3′	Reference
*nos*ZF1 (1184–1203)	Nitrous oxide reductase	WCSYTGTTCMTCGACAGCCAG	Henry et al. (2006)
*nos*ZF2 (1617–1640)	Nitrous oxide reductase	CGCRACGGCAASAAGGTSMSSGT	Henry et al. (2006)
*nos*ZR2 (1864–1884)	Nitrous oxide reductase	CAKRTGCAKSGCRTGGCAGAA	Henry et al. (2006)
*nir*Scd3aF (916–935)	Nitrite reductase	GTS AAC GTS AAG GAR ACS GG	Throback et al. (2004)
*nir*SR3 cd (1322–1341)	Nitrite reductase	GAS TTC GGR TGS GTC TTG A	Throback et al. (2004)
16S 27F (27–48)	16S rRNA	GAGAGTTTGATCCTGGCTCAG	Pillai and Archana (2008)
16S 340F (340–357)	16S rRNA	CCTACGGGAGGCAGCAGA	Pillai and Archana (2008)
16S 534R (518–534)	16S rRNA	ATTACCGCGGCTGCTGG	Pillai and Archana (2008)
16S 1541R (1522–1541)	16S rRNA	AAGGAGGTGATCCAGCCGC	Pillai and Archana (2008)

Parenthesis = position in the respective genes.