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Original Research Article

Detection of Denitrifying population from upflow anaerobic packed bed column using PCR

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A B S T R A C T

Keywords

Denitrification, Aquaculture, Nitrous oxide reductase, Denitrifying genes, PCR, Direct sequencing.

Molecular identification of denitrifying micro-organisms colonizing an upflow anaerobic packed bed bioreactors was carried out. PCR amplification of *nirK*, *nir*S (nitrite reductase genes) and *nos*Z (nitrous oxide reductase genes) in DNA extracted from the direct environmental samples collected from various regions of the column bioreactors showed positive results. Subsequent sequencing of the PCR amplified products and comparison with the published sequences showed similarity with several important denitrifiers namely *Alcaligenes xylosoxidans*, *Paracoccus sp*., *Nitrospira sp*., *Ochrobactrum sp*., *Halomonas denitrificans* strain DSM 18045, *Cupriavidus sp.* R-31544, *Ralstonia eutropha*, *Comamonas denitrificans*, thereby confirming the presence of denitrifiers in the enriched bioreactors. The study affirms the suitability of simple PCR detection of process specific genes directly from environmental samples in lab scale bioreactors.

Introduction

Nitrate accumulation is an important problem in intensive aquaculture practices such as Recirculating Aquaculture Systems (RAS). An effective means to biologically remove such nitrate is Denitrification; understood as the dissimilatory transformation of nitrate to nitrogen gas. The bacteria involved (Denitrifiers) are primarily aerobic heterotrophic bacteria, having the ability to switch to anaerobic respiration under anoxic conditions, reducing NO3- and NO_2^- to nitric oxide (NO), nitrous oxide (N_2O) and N_2.

The ability to denitrify is principally due to the activity of four enzymes of denitrification namely, (1) nitrate reductase (NAR), (2) nitrite reductase (NIR), (3) nitric oxide reductase (NOR) and (4) nitrous oxide reductase (NOS). NAR enzymes are either membrane bound (Nar) or periplasmic (Nap), catalyzing the reduction of NO3 to NO2 (Carter et al.,1995; Roussef-Delif et al., 2005). Denitrifiers can be distinguished from nitrate reducers by targeting genes encoding the nitrate reductases (Throback 2004). Several studies have been carried

out previously to identify the presence of denitrifiers from various environments. Rosch *et al.,* (2002) studied the biodiversity of denitrifying and dinitrogen fixing bacteria in an acid forest soil. Sachiko *et al.,* (2004) studied with denitrification specific PCR primers, the effect of salinity on nitrite reductase gene diversity in denitrifying bacteria of wastewater treatment systems. Kandeler *et al .,*(2006) studied the abundance of narG, nirS, nirK and nosZ genes of denitrifying bacteria during successions of a glacier foreland using specific primers.

It is possible to confirm through PCR, the presence of denitrification specific gene sequences using specific primers in a nitrate removal system. Positive detection would then confirm the hypothesis that nutrient removal of nitrate nitrogen is taking place through the process of denitrification. The present study used PCR to detect denitrification specific gene sequences from genomic DNA extracted from environmental samples. The samples were drawn from different sampling ports of two upflow anaerobic packed bed columns having different bacterial support media namely coconut coir fibre and a commercially available reticulated plastic medium termed as Fujino spirals respectively.

The nutrient removal performance of the anaerobic columns with respect to their respective media have been previously published (Manoj, 2012). The objective of this study is to ascertain suitability of PCR of DNA extracted directly from environmental samples; in order to identify a particular nutrient cyclic process using specific primer sequences. The reason behind this approach was that in order to study a phylogenetically widespread process such as denitrification, it is better to target specific genes (Scala and Kerkhof, 1998). Braker *et al .,*(1998) has also stated that since denitrifiers are not defined by close phylogenetic relationship, an approach involving 16S rRNA molecules is not suitable for general detection of this physiological group in the environment.

Materials and Methods

Sample Collection

Samples were collected in separate sterile sampling bottles from each of the sample ports of the bioreactors (upflow anaerobic packed bed reactors each with a volume of 3.9 Litres) loaded with the different bacterial support media viz., Coconut coir fibre and Fujino spirals (commercially available reticulated plastic media) (Figure 1). The sample ports have been designated separately for the top, middle, bottom port for Coconut coir packed column (CCTP,CCMP,CCBP) and Fujino spirals packed column (FJTP,FJMP,FJBP) respectively. Samples were also collected from the effluent ports of bioreactor packed with coconut coir media and Fujino spirals; and designated as MC (main column ie., Coconut coir filled column) and FC (Fujino column) respectively.

PCR Amplification

Samples (150 ml) collected from each port were centrifuged in a cold centrifuge (Hitachi,Japan) at 10,000 rpm for 10 min. The supernatants were discarded and 500 µl of concentrated sediments dispensed aseptically in individual 1.5 ml eppendorf tubes. DNA was extracted using a commercial DNA extraction Kit (QIAGEN QI Amp DNA stool mini kit) following the manufacturer’s protocol.

Table.1 Amplification conditions and primers with amplification protocols used to detect denitrifying population in upflow anaerobic packed bed column bioreactors

<table>
<tr><td>PCR amplification Contents
Total volume (25µl)</td><td colspan="4">THERMAL CYCLER CONDITIONS</td></tr>
<tr><td rowspan="7">(a) 2.5 µl : 10xPCR buffer (50mM Kcl,15mM MgCl2,100mM Tris-Hcl,pH 9.0 at room temperature)
(b) 200 µM of each deoxynucleotide triphosphate
(c) 1.25 U of Taq polymerase
(d) 1.0 mM of each primer
(e) 10-100 ng DNA</td><td colspan="4">1. Initial denaturation of DNA – 94°C for 2 minutes
2. 35 cycles of 30 seconds at 94°C – 1 minute
3. 51°C – 1 minute
4. 72°C – 1 minute
Reaction is completed after 10 minutes at 72°C</td></tr>
<tr><td>SELECTED PRIMERS</td><td>nirK</td><td>nirS</td><td>nosZ</td></tr>
<tr><td></td><td>FlaCu + R3Cu NirKlF + nirK5R</td><td>Cd3Af + r3CD nirSlF + nirS6R</td><td>nosZ – F + nosZ1622R</td></tr>
<tr><td>Other conditions</td><td></td><td></td><td></td></tr>
<tr><td></td><td>Additional 1.0 mM $MgCl_2$, 400 ng/µl BSA, 0.1% Triton-x added for nirK1F nirK5R</td><td>Additional 1.0 mM MgCl2, 400 ng/µl BSA, 0.1% Triton-x added for nirS6R</td><td></td></tr>
<tr><td>For Environmental samples, BSA added in PCR for all primer combinations</td><td>nirK primer combinations : 400 ng/pil</td><td>nirS primer combinations : 1,000 ng/pil</td><td>nosZ primer combinations : 600ng/pil</td></tr>
<tr><td>Annealing temperature altered/optimized</td><td>FlaCu + R3Cu : 57°C</td><td>Cd3Af + r3CD : 57°C</td><td>53°C</td></tr>
<tr><td></td><td>Expected Product size</td><td>< 500 bp</td><td>< 500 bp</td><td>< 500 bp</td></tr>
</table>

Table.2 List of denitrification specific primers referred in this study

Gene	Primer Name and Sequence Primer Sequence	Literature Source
nirS	>KA3-F-nirS from *Paracoccus denitrificans* CACGGYGTBCTGGCGAAGGGCGC	Mergel and Bothe (2002)
nirS	>KA25-R - nirS - *Paracoccus denitrificans* CGCCACGCGCGGYTCSGGGTGGTA	
nirK	>K15-F - nirK –*Alcaligenes faecalis* GGCATGGTACCTTGGCACGTAACCTCGGGC	
nirK	>K16-R - nirK – *Alcaligenes faecalis* CATTAGATCGTCGTTCCAATCACCGGT	
nirS	>nirSCd3Af AACGYSAAGGARACSGG	Kandeler *et al .,*(2006)
nirS	>nirSR3cd GASTTCGGRTGSGTCTTSAYGAA	
nirK	>nirK1F GG(A/C)ATGGT(G/T)CC(C/G)TGGCA	Braker *et al .,*(1998)
nirK	>nirK2F GC(C/G)(C/A)T(C/G)ATGGT(C/G)CTGCC	
nirK	>nirK3R GAACTTGCCGGT(A/C/G)G(C/T)CCAGAC	
nirK	>nirK4R GG(A/G)AT(A/G)A(A/G)CCAGGTTTCC	
nirK	>nirK5R GCCTCGATCAG(A/G)TT(A/G)TGG	
nosZ	>Nos661F CGGCTGGGGGCTGACCAA	Scala and Kerkhof (1998).
nosZ	>Nos2230R TTCCATGTGCAGCGCATGG	
nosZ	>Nos1527F CGCTGTTCHTCGACAGYCA	
nosZ	>Nos1527R CTGRCTGTCGADGAACAG	
nosZ	>Nos1773R ATRTCGATCARCTGBTCGTT	
nosZ	>nosZ-F - nosZ - *Paracoccus denitrificans* CGYTGTTCMTCGACAGCCAG	Mergel and Bothe (2002)
nosZ	>nosZ-R - nosZ - *Paracoccus denitrificans* CATGTGCAGNGCRTGGCAGAA	

The eluted DNA were immediately stored at -20°C and used as template for PCR. Specific PCR protocols for amplification were carried out for each denitrification gene primer set as detailed in Table 1. PCR amplifications were carried out in a total volume of 25 µl. The reaction mixture consisted of 22 µl of red dye PCR mix (Bangalore Genei, India), 1µl each of forward and reverse primers at a concentration of 40 pico moles and 1 µl of DNA. PCR amplification was carried out in the cycling conditions with an initial denaturation at 94°C for 2 minutes, 30 cycles with denaturation at 94°C for 1 minute, annealing at 51°C for 1 minute, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes.

The PCR amplified products were separated by gel electrophoresis. About 8 µl of the PCR products were mixed with 4 µl of gel loading solution (40% sucrose, 0.1 M EDTA (pH 8.0), 0.5 % sodium dodecyl sulphate, and 0.05 % bromophenol blue) and resolved on a 1.5 % agarose gel in Tris-acetate-EDTA buffer (0.04 M Tris-acetate, 0.001 M EDTA with 0.8 mg of Ethidium Bromide/ml) for 1.5 h at 60 V. The products were visualized and documented in a gel documentation unit (Vilber Lourmet, France) and photographed under UV illumination.

PCR reactions were carried out to detect the presence of denitrifying bacteria from the extracted whole DNA population. The primers used to amplify the specific genes for denitrification namely *nir*K, *nir*S (Nitrate reductase) and *nos*Z (nitrous oxide reductase) are listed in Table.2 Direct sequencing of positive PCR products (amplicons) was carried out in an automated sequencer (Applied Biosystems) utilizing the commercial sequencing service (Ocimum Biosolutions, India). The sequences were identified on the basis of sequence similarity, by comparison with the sequences available in the databases of National Centre for Biotechnology Information using BLAST program. Unique sequences were submitted to GenBank.

Results and Discussion

PCR amplification of nirK1, nirK2, nirS2 and nosZ showed positive results in all the samples. NirS1 amplification also gave positive results in all the samples except for the middle port of the column packed with coconut coir as media. The results of the positive bands for the various primers by PCR are illustrated in Figures 2 (a,b,c). In the figures,"M" represents the molecular marker and the numbers are sequentially as arranged in Table 3.

The PCR products were gel purified using a commercial gel purification kit (Bangalore Genei, India). Direct sequencing was carried out using commercial automated sequencing services (MWG, Bangalore, India). Sequence analysis using BLAST N program (NCBI BLAST) showed homology with prominent denitrifying species namely *Alcaligenes xylosoxidans*, *Paracoccus sp*., *Nitrospira sp*., *Ochrobactrum sp*., *Halomonas denitrificans* strain DSM 18045, *Cupriavidus sp*. R-31544, *Ralstonia eutropha*, *Comamonas denitrificans*. The major organisms identified have been tabulated in Table 4. The sequences were aligned in CLUSTAL and a phylogenetic relationship between the bacteria (reported 1: Uemoto and Saiki (2000) studied the removal of nitrogen by denitrification using *Nitrosomonas europaea* and *Paracoccus denitrificans*, in a bioreactor, packed as gel envelopes.

Table.3 Results of PCR amplification against specific primers

No. In Gel	**Code (Sampling Port Location) and Support Medium**	**Primer Set and Results**				
		NirK1 FlaCu+R3Cu	**NirK2 NirK1F+nirK5R**	**NirS1 Cd3Af+r3CD**	**NirS2 nirS1F+nirS6R**	**nosZ nosZF+nosZ1622R**
1	CCTP : Top Port (Coconut coir media filled column)	+	+	+	+	+
2	CCMP : Middle Port (Coconut coir media filled column)	+	+	+	+	+
3	CCBP: Bottom Port (Coconut coir media filled column)	+	+	+	+	+
4	MC: Effluent Port (Coconut coir media filled column)	+	+	-	+	+
5	FJTP: Top Port (Fujino spirals media filled column)	+	+	+	+	+
6	FJMP: Top Port(Coconut coir media filled column)	+	+	+	+	+
7	FJBP: Bottom Port(Coconut coir media filled column)	+	+	+	+	+
8	FC: Effluent Port(Coconut coir media filled column)	+	+	-	+	+

Table.4 List of major denitrifying bacteria identified by direct sequencing of positive PCR amplicons

Support Medium used in Upflow anaerobic packed bed column	Gene	Denitrifiers that showed similarity (%)	GenBank Accession number	Related Denitrification work
Coconut coir	nirK	(a) Alcaligenes xylosoxidans (99%)	AB013078.1	
		(b) Paracoccus sp. (100 %)	AM230885	Uemoto and Saiki (2000)[1] Barak and Rijn (2000)[2]
		(c) Nitrospira sp. (95 %)	EF016121.1	
	nirS	(a) Halomonas denitrificans strain DSM 18045 (97%)	FJ686166.1	Domenecha *et al* .,(2010)[3]
		(b) Cupriavidus sp. R-31544 (91%)	AM403575.1	Domenecha *et al* .,(2010)[3]
		(c) Ralstonia eutropha (83 %)	AM260480.1	Wang and Lee (2007)[4]
	nosZ	(a) Halomonas denitrificans DSM 7281 (97 %)	FJ686162.1	Domenecha *et al* .,(2010)[3]
		(b) Halomonas nitroreducens CECT 7281 (94 %)	FJ686162.1	Domenecha *et al* .,(2010)[3]
		(c) Pseudomonas denitrificans (99 %)	AF016059.1	Cattaneo *et al* .,(2003)[5]
Fujino spirals	nirK	(a) Ochrabactrum sp. (100 %)	AM230869.1	Doi *et al* .,(2009)[6]
		(b) Nitrospira sp. (95 %)	DQ846876.1	
		(c) Blastobacter denitrificans (95 %)	AJ224906.1	
	nirS	(a) *A.eutrophus (83 %)*	X91394.1	
		(b) *Halomonas Koreensis (83 %)*	FJ686156.1	Domenecha *et al* .,(2010)[3]
		(c) *Cuprivadus sp.* R-31543 (91 %)	AM403574.1	
	nosZ	(a) *A.eutrophus (83 %)*	X91394.1	
		(b) *Comamonas denitrificans (77 %)*	DQ865931.1	
		(c) Halomonas denitrificans strain DSM 18045 (97%)	FJ686166.1	Domenecha *et al* .,(2010)[3]

They have strongly indicated in their study that the higher nitrogen removal rates achieved were as a consequence of cooperation between the *Nitrosomonas europaea* and *Paracoccus denitrificans* present in the gels.

2: Barak and Rijn (2000) have reported that *Paracoccus denitrificans* can effectively reduce combined phosphate and nitrate.

3: Domenecha *et al* .,(2010) reported that the denitrifying capability should be considered as an important phenotypic and phylogenetic discriminatory marker within *Halomonas* genus during a comprehensive study of the denitrifying species namely *Halomonas ventosae*, *Halomonas denitrificans* and *Halomonas koreensis*.

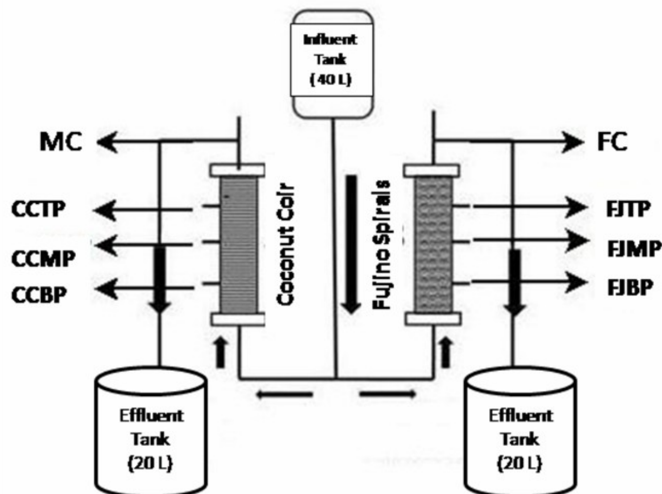
4: Wang and Lee (2007) reported the isolation of *Ralstonia eutropha* from wastewater treatment system manufactured with Polyacrylonitrile fibre (PAN). Their study revealed that *Ralstonia eutropha* in conjunction with other PAN mixed strains could consume up to 1,446 mg/L acrylamide by denitrification.

5: Cattaneo *et al* .,(2003) used *Pseudomonas denitrificans* in a study on denitrification of simulated wastewater containing nitrates and methanol as carbon source in two systems namely fluidized bed biofilm reactor (FBBR) and a stirred tank reactor (STR).

6: Doi *et al* .,(2009) studied the role of *Ochrobactrum anthropi* as a novel denitrifier that has evolved reactive nitrogen oxide tolerance mechanisms. They reported a superior performance of the *Ochrobactrum* strain when compared to other denitrifiers in their study namely *Pseudomonas aeruginosa*, *Ralstonia eutropha* and nitrate-respiring *Escherichia coli*.

Figure.1 Depiction of sampling ports used to study representative regions of column bioreactors

(a)



(b)

Figure.2 (a)Amplified products of nirK (Nitrite reductase) gene **(b)** Amplified products of nirS (Nitrite reductase) gene **(c)** Amplified products of nosZ (nitrous oxide reductase) gene

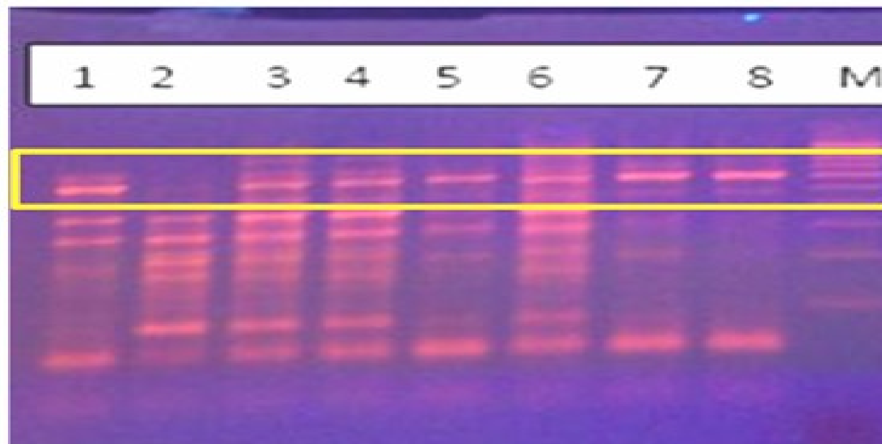


Fig.2 (a)

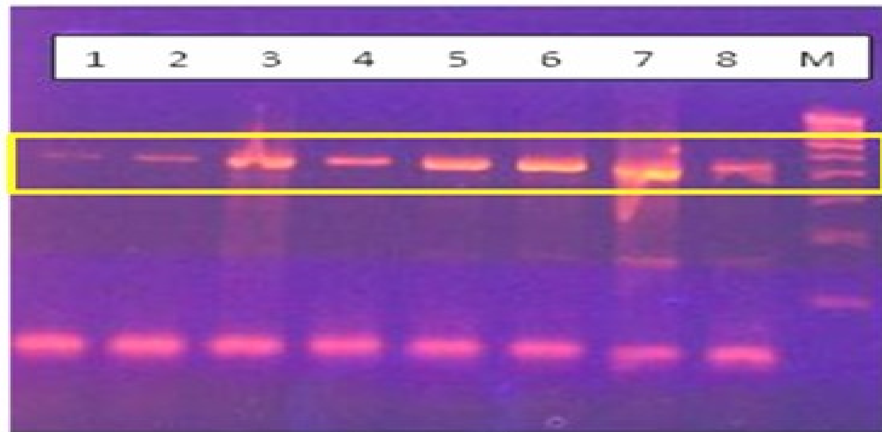


Fig.2 (b)

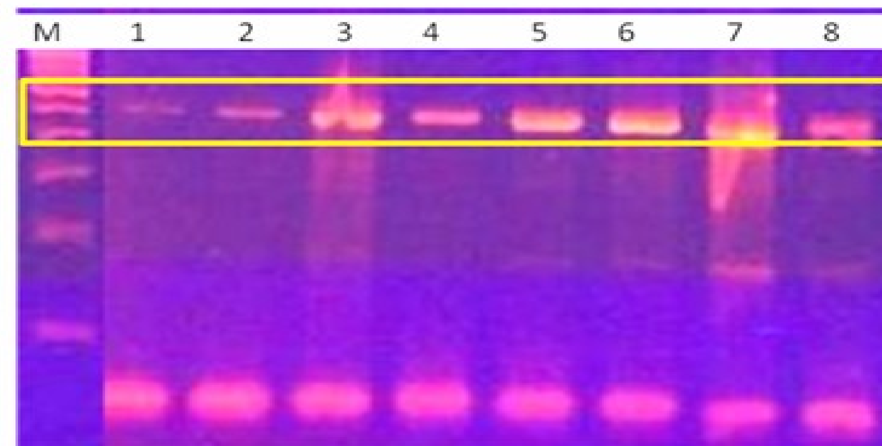
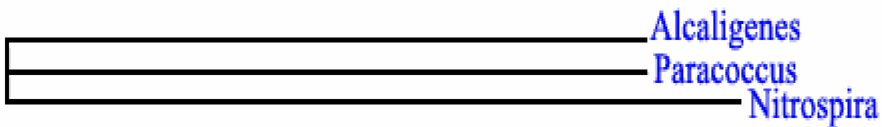


Fig.2 (c)

Figure.3 Phylogenetic relationship of identified denitrifying bacteria

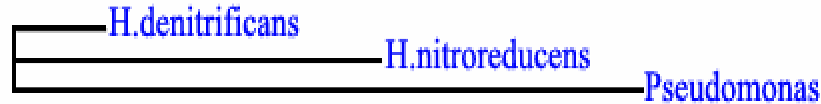
Phylogeny of identified sequences (Coconut coir as support medium)

nirK



nirS

nosZ



Phylogeny of identified sequences (Fujino spirals as support medium)

nirK

nirS

nosZ

from each gene and support medium) were carried out using Clustal W2 Phylogeny tool. The results are presented in Figure 3.

The positive PCR results accompanied by the results of the direct sequencing clearly indicate the presence of denitrifying bacteria within either of the upflow column reactors. The primary reason can be attributed to the enriched nature of the column reactor; specific for denitrifiers since it was enriched with a rich carbon source, Methanol. There remains however, the possibility of detection of additional bacteria that can be detected by other molecular methods, which was not carried out in this study.

Molecular identification of denitrifying microorganisms based on *nir*K, *nir*S and *nos*Z genes and confirmation by direct sequencing showed greater than 90 % similarity to several prominent denitrifying species namely *Alcaligenes xylosoxidans*, *Paracoccus sp*., *Nitrospira sp., Ochrobacterium sp., Halomonas denitrificans* strain DSM 18045, *Cupriavidus sp*. R-31544, *Ralstonia eutropha*, *Comamonas denitrificans*.

It is important to draw the other implications of the study from the positive detection of *nir*K and *nir*S genes.

The presence of ammonia oxidizing microbes can also be hypothesized from this positive detection. Lam *et al .,*(2009) has demonstrated the presence of *nir*S putative nitrite reductase (*nir*S) gene as a molecular marker to study the presence and potential activity of anammox in the environment by reverse transcriptase PCR. NirS is postulated to participate in the oxidation of nitrite to nitric oxide, which forms hydrazine together with ammonium in a process catalyzed by the hydrazine hydrolase (Strous *et al*., 2006).

It is important to note the incidence of these bacteria in other studies conducted to determine their relative importance and significance. Yoshie *et al*., (2004) has reported the incidence of *nir*K sequences during a study on the microbial ecology of nitrite-reducing bacteria in two series of metallurgic wastewater treatment systems (MWTSS) comprising of anaerobic packed bed and fluidized bed with different fluidity conditions. Interestingly, Heylen *et al .,*(2006) used *Curpivadus sp.* as one of the pure denitrifying cultures for genetic sequence analysis of cnorB and qnorB, both encoding nitric oxide reductases was performed on pure cultures of denitrifiers, for which previously nir genes were analyzed. The study affirms the suitability of simple PCR detection of process specific genes directly from environmental samples in lab scale bioreactors.

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References

Barak.Y., and Rijn, J.V. 2000. Atypical polyphosphate accumulation by the denitrifying bacterium *Paracoccus denitrificans*. Appl. Environ. Microbiol. 66(3):1209-1212.

Braker G., A. Fesefeldt and Witzel, K.P. 1998. Development of PCR Primer Systems for Amplification of Nitrite Reductase Genes (nirK and nirS) To Detect Denitrifying Bacteria in Environmental Samples", Appl. Environ. Microbiol. 64(10): 3769-3775.

Carter J.P., Y.H. Hsiao, S. Spiro and Richardson, D.J. 1995. Soil and

sediment bacteria capable of aerobic nitrate respiration, Appl. Environ. Microbiol. 61:2852-2858.

Cattaneo C. , C. Nicolella and Rovatti,M. 2003. Denitrification Performance of *Pseudomonas denitrificans* in a Fluidized-Bed Biofilm Reactor and in a Stirred Tank Reactor, Eng. Life Sci. 3(4): 87 – 192.

Doi Y. , N. Takaya and. Takizawa, N. 2009. Novel Denitrifying Bacterium *Ochrobactrum anthropi* YD50.2 Tolerates High Levels of Reactive Nitrogen Oxides", Appl. Environ. Microbiol.75: 16.

Domenecha C.M.G. , F.M. Checaa, V. Béjara and Quesada, E. 2010. Denitrification as an important taxonomic marker within the genus Halomonasstar. Syst. Appl. Microbiol. 33(2): 85-93.

Heylen Gevers K., D. , B.V. Lieven, W.JokeGeets, N.Boon and Paul De Vos. 2006.The incidence of nirS and nirK and their genetic heterogeneity in cultivated denitrifiers, Environ Microbiol Rep. 8(11): 2012 – 2021.

Kandeler E. , K.D.D. Tscherko, D. Bru and Philippot. L. 2006 Abundance of narG, nirS, nirK, and nosZ Genes of Denitrifying Bacteria during Primary Successions of a Glacier Foreland, Appl. Environ. Microbiol.72(9): 5957-5962.

Lam P. , G. Lavik, M.M. Jensen, V.D.J. Vossenberg, S.M.D. Woebken, G. Dimitri, R. Amann, M.S.M. Jetten and Kuypers, M.M.M. 2009. Revising the nitrogen cyce in the Peruvian oxygen minimum zone", Proceedings of the National Academy of Sciences USA, 106:4752-4757.

Manoj Valsa Remony and Vasudevan Namasivayam. 2012. Removal of nutrients in denitrification system using coconut coir fibre for the biological treatment of aquaculture wastewater. J Environ Biol. 33(2):271-6.

Roussel-Delif L., S. Tarnawski, J. Hamelin, L. Philippot, M. Aragno and Fromin,N. 2005. Frequency and diversity of nitrate reductase genes among nitrate-dissimilating Pseudomonas in the rhizosphere of perennial grasses grown in field conditions, Microb Ecol. 49:63-72.

Rösch.C, A. Mergel and Bothe, H. 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil", Appl. Environ. Microbiol.68:3818-3829.

Sachiko Yoshie, Y.N. Noda,S.T. Akira, Hirata and Inamori, Y. 2004. Salinity Decreases Nitrite Reductase Gene Diversity in Denitrifying Bacteria of Wastewater Treatment Systems", Appl. Environ. Microbiol, 70(5):3152-3157.

Scala, D.J. and Kerkhof, L.J. 1998. Nitrous oxide reductase (nosZ) gene-specific PCR primers for detection of denitrifiers and three nosZ genes from marine sediments, FEMS Microbiol Lett. 162(1): 61- 68.

Schreier,H.J., N. Mirzoyan and Saito, K. 2010. Microbial diversity of biological filters in recirculating aquaculture systems, Curr. Opin. Biotechnol. 21:318-325.

Throbäck, I.J., 2006. Exploring Denitrifying Communities in the Environment, Doctoral thesis, Swedish University of Agricultural Sciences.

Throbäck, I.N., K. Enwall , A. Jarvis 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(3):401-17.

Uemoto,H., and Saiki, H. 2000. Nitrogen removal reactor using packed gel envelopes containing *Nitrosomonas europaea* and *Paracoccus denitrificans*, Biotechnol. Bioeng. 67(1): 80-86.

Wang, C.C., and Lee, C.M. 2007. Isolation of the Acrylamide Denitrifying Bacteria from a Wastewater Treatment System Manufactured with Polyacrylonitrile Fiber, Curr. Microbiol. 55:4.