Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.abeq.org.br/bjche

Vol. 30, No. 03, pp. 457 - 465, July - September, 2013

# EVALUATION OF THE MICROBIAL DIVERSITY OF DENITRIFYING BACTERIA IN BATCH REACTOR

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*(Submitted: August 30, 2011 ; Revised: July 30, 2012 ; Accepted: August 23, 2012)*

**Abstract** - Microbial communities in an industrial activated sludge plant may contribute to the denitrification process, but the information on the microorganisms present in denitrifying reactors is still scarce. Removal of inorganic nitrogen compounds can be accomplished by the addition of carbon sources to the biological process of denitrification. Ethanol is an economically viable alternative as a carbon source in tropical countries like Brazil, with large-scale production from sugarcane. This paper reports the successful aplication of activated sludge with nitrate and ethanol in a batch anaerobic reactor. The operation lasted 61.5 h with total consumption of nitrate in 42.5 h, nitrite generation (2.0 mg/L) and ethanol consumption (830.0 mg/L) in 23.5 h. Denitrifying cell counts by the most probable number at the start of the operation were lower than at the end, confirming the ability of the inoculum from activated sludge for the denitrification process. The samples from cell counts were identified as *Acidovorax* sp., *Acinetobacter* sp., *Comamonas* sp. and uncultured bacteria. Therefore, these species may be involved in nitrate reduction and ethanol consumption in the batch reactor.
*Keywords*: Activated sludge system; *Acidovorax*; *Acinetobacter*; Nitrate; Ethanol.

## INTRODUCTION

Microorganisms capable of denitrification are widely distributed in nature: soil, sediment, freshwater, sea and wastewater treatment systems (Park & Yoo, 2009).

Many inocula from domestic and industrial sewage treatment plants may contain denitrifying bacteria, mainly in activated sludge systems (Liu *et al.*, 2006; Daniel *et al.*, 2009), where the biological nitrogen removal occurs to promote denitrification, i.e., under heterotrophic anoxic conditions, the organic carbon sources act as electron donors and reduce nitrate to nitrogen gas (Canto *et al*., 2008). The presence of organic carbon and energy sources is necessary for heterotrophic denitrification (Nava *et al*., 2010).

Most of the denitrifying bacteria are encompassed in the Proteobacteria Phylum, including *Acidovorax*, *Comamonas* and *Acinetobacter*, among others. Such bacteria may be present in waste treatment, especially in activated sludge systems, which are able to form molecular nitrogen from nitrate and an exogenous carbon source, such as ethanol. The complete denitrification, i.e., nitrate to nitrogen gas is mediated by bacteria species that normally use oxygen from air as the energy source (aerobic respiration), but they also have the ability to use

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nitrate and nitrite instead of oxygen (anoxic condition). Thus, these bacteria may grow aerobically in the absence of nitrate, or under anoxic conditions in the presence of nitrate. The conversion of nitrate to molecular nitrogen is also known as anoxic respiration (Park & Yoo, 2009).

A wide variety of organic compounds has been used, such as methanol, ethanol, glucose, acetate, aspartate or formic acid and aromatic compounds (Queiroz *et al.*, 2011). However, most of the published research regarding drinking water denitrification involves the use of methanol, ethanol and acetic acid (Park & Yoo, 2009). Ethanol (Daniel *et al.*, 2009), glucose and acetate are some of the external electron donors successfully used for denitrification. Particularly in Brazil, ethanol represents a feasible alternative (Gavazza dos Santos *et al.*, 2004). Ethanol is produced on a large scale in Brazil since 1975, with the National Program for Alcohol (1975–1985). Brazil produces nearly 2.6 x $10^8$ tons of sugarcane, which is processed by 324 sugar mills to produce sugar and ethanol (Borrero *et al.*, 2003). It is abundantly produced from sugarcane and usually costs less than other convenient carbon sources. Nevertheless, the need for additional electron donor sources for exogenous process increases operational costs, possibly representing a drawback for the use of innovative anaerobic process-based technologies (Gavazza dos Santos *et al.*, 2004).

The stoichiometric relationships describing the bacterial energy reaction (Park & Yoo, 2009) is written as follows, when ethanol is used as a carbon source:

$$0.69\ C_2H_5OH + NO_3^- + H^+ \rightarrow\ 0.14\ C_5H_7NO_2 + 0.43\ N_2 + 0.67\ CO_2 + 2.07\ H_2O$$

Although this equation reveals the stoichiometric quantity of ethanol required for nitrate dissimilation, additional ethanol is required for de-oxygenation and cell synthesis. In practice, 25% to 30% of the ethanol required is used for bacterial cell synthesis. When dissolved oxygen is present, the ethanol requirement is correspondingly higher. Therefore, a common working value of the weight ratio of substrate to nitrate ($C:N0_3$) is nearly 3 (Park & Yoo, 2009).

There are only a few references about batch reactors and the denitrification process. These configurations can be used to investigate nutritional requirements (Maintinguer *et al.*, 2008). The denitrification process with complex carbohydrates is being evaluated in batch reactors because the particulate organic matter that is present in these systems can difficult the operation in other configurations, such as with starch (Iamamoto, 2006). Moreover, the biomass remains retained in the batch reactor in all of the operational periods when compared with continuous flow reactors. These facts can contribute to the total nitrate consumption verified in batch reactor (Etchebehere *et al.*, 2001; Gavazza dos Santos *et al.*, 2004).

The nitrate removal with activated sludge inoculum has been studied particularly in countries of temperate climate. There are few reports of studies on nitrate removal and molecular biology with activated sludge inoculum from tropical countries like Brazil. In this sense, the first aim of our study was to evaluate the denitrification process with activated sludge as inoculum from tropical climates. The second goal of our study was to carry out a microbial characterization of the inoculum aiming to identify the potential organisms specifically involved in the denitrification process.

This work studied the microbial diversity of denitrification in a batch reactor fed with ethanol and nitrate using techniques of molecular biology and traditional methodology of microbiology.

## MATERIAL AND METHODS

### Batch Reactor

The experiment was carried out with sludge from the Sewage Treatment Plant activated sludge system at Volkswagen São Carlos (São Carlos – SP - Brazil).

The batch reactors were prepared in triplicate in Duran® flasks of 2 L, where 1 L was reaction medium, 10% (v/v) inoculum (100 mL/L).

The nutritional medium (Zinder, 1984) was prepared with the following composition (g/L): $NH_4Cl$, 0.5, $K_2HPO_4$, 0.4; $MgCl_2.6H_2O$, 0.1, $CaCl_2.2H_2O$, 0.05. The trace metals solution used was (10 ml/L): NTA (4.5 g/L); $FeSO_4.7H_2O$ (0.556 g/L); $MnSO_4.H_2O$ (0.086 g/L); $CoCl_2.6H_2O$ (0.17 g/L) $ZnSO_4.7H_2O$ (0.21 g/L), $H_3BO_3.NiCl_2$ (0.19 g/L); $Na_2MoO_4.2H_2O$ (0.02 g/L); the vitamins solution used was (10 mL/L): Biotin (0.002 g/L), Folic Acid (0.002 g/L), Thiamine (0.005 g/L), Riboflavin (0.005 g/L), Nicotinic Acid (0.005 g/L), Calcium Pantothenate (0.005 g/L), Pyridoxine (0.01 g/L), Vitamin B12 (0.0001 g/L), Lipoic Acid (0.005 g/L) and p-aminobenzoic acid (0.005 g/L). The sodium bicarbonate solution (0.1 g/L) was used as a buffer to maintain pH 7.0. Finally, 0.68 g $NaNO_3$/L was added to obtain 0.12 g $N\text{-}NO_3$/L and ethanol (1.65 g/L) for a $C:NO_3$ ratio equal to 3.

The reactors were submitted to a $N_2$ atmosphere

(99.99%) for 20 min after the distribution of the solutions. After that, they were capped with butyl rubber stoppers, wrapped and kept at 25 ºC ± 1 ºC, with agitation at 120 rpm operated during 61.5 h.

**Physical-Chemical and Chromatographic Analysis**

The total volatile solids (TVS) and the nitrate consumption were determined according to APHA, 2005, spectrophotometrically. The nitrite analysis was performed by flow injection (FIA – APHA, 2005). The volatile fatty acids and alcohols were determined by gas chromatography in a Shimadzu GC-2010, equipped with a flame ionization detector, autosampler for headspace COMBI-PAL - AOC model 5000 and HP-INNOWAX column (30 m x 0.25 mm x 0.25 mm film thickness), according Maintinguer *et al.*, 2008).

**Quantification of Denitrifying Bacteria**

The Most probable Number (MPN) of denitrifying bacteria was performed at five times the dilution at the beginning and at the end of the operation of the batch reactor, according to Tiedje (1982), adapted for liquid samples. The cell counts by the MPN method were made after 15 days of incubation, according to APHA, 2005. The culture medium composition and, nitrate and ethanol concentrations used in the MPN assays were similar to those of the batch reactor operation, as mentioned earlier.

**Molecular Biology**

The samples for analysis of the 16S rRNA were obtained from the highest positive dilution counts (MPN) of denitrifying bacteria at the end of the test from the batch reactors.

Total genomic DNA of the samples was acquired after cell lysis with glass beads (Sigma) and phenol-chloroform extraction as previously described by Griffiths *et al.* (2000) modified.

The amplification by the polymerase chain reaction (PCR) was performed with a bacterial domain primer set for the 16S rRNA gene, 27 forward (5'-AGAGTT TGATCCTGGCTCAG-3') and 1100 reverse (5'-AGGGTTGCGCTCGTTG-3') (Lane, 1991). The PCR (Thermo cycler Eppendorf AG - Hamburg 22,331) amplification was carried out with initial denaturation at 94 ºC for 5 min followed by 30 cycles of denaturation at 94 ºC for 45 s, annealing at 55 ºC for 45 s, extension at 72 ºC for 1.45 min and final extension at 72 ºC for 7 min and cooling at 4 ºC.

Samples of PCR (Polymerase Chain Reaction) products (16S rRNA) were cloned into the plasmid vector pGEM (Promega Easy Vector System I) according to the manufacturer's specifications. The clones were randomly selected and amplified by PCR. Nucleotide sequencing was performed on an automated ABI 310 PRISM sequencer (Dye terminator Cycle Sequencing Kit, Applied Biosystems, USA) in accordance with the manufacturer's instructions using an M13 forward primer (50-GTAAAA CGA CGG CCA G-30) (Messing, 1983). The PCR (Thermo cycler Eppendorf AG – Hamburg 22, 331) amplification was carried out with initial denaturation at 94 ºC for 2 min followed by 25 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min, extension at 72 ºC for 1 min; and final extension at 72 ºC for 7 min and cooling at 4 ºC.

The nucleotide sequences were processed and they were aligned with the Seqman program (Lasergene DNAstar package) to remove the signals from the vector and low quality bases. The aligned sequences were determined by the BLAST search program on the NCBI web site compared with the 16S rRNA gene organism sequences represented in the database Genbank (http://www.ncbi.nlm.nih.gov) and Ribosomal Data Base Project (http://rdp.cme.smu.edu). The phylogenetic tree was constructed by the Neighbor-Joining method (Saitou & Nei, 1987) using the program MEGA version 4.1 (Kumar *et al.*, 2008). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies. The known sequences of *Aspergillus niger* (FJ828924.1) were added and it was used as out-group.

## RESULTS AND DISCUSSION

The nitrate was completely consumed after 42.5 h of operation (Figure 1). The nitrite generation was reduced (2.0 mg/L at 18.5 h) and occurred within 23.5 hours of operation. 1.06 g ethanol/L (36% consumption) was observed after 13.5 h of operation for initial concentration of 1,650 mg/L. The ethanol consumption at the end of the experiment was 54% (0.77 g/L in 61.5 h). These results were nearly those of Gusmão *et al.* (2006). The authors (op.cit.) observed 98.9% of nitrate consumption in 14 h of operation, with purified cells of granular sludge from an up-flow anaerobic sludge blanket (UASB) treating wastewater from a poultry slaughterhouse (DAKAR-Tietê – SP – Brazil), with nitrate (350 N-$NO_3$ mg/L), ethanol (377 mg/L) and benzene (10 mg/L) as carbon sources.

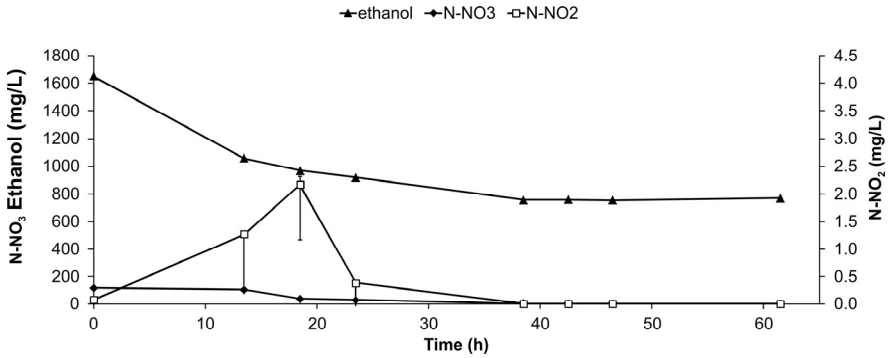


**Figure 1:** Temporal variation of ethanol (▲), nitrate (♦) and nitrite (□) in the anoxic reactor

Methanol (197.78 mg/L) and n-butanol (23.50 mg/L) were detected at the start of the experiment. These alcohols (metanol and n-butanol) were possibly present in the inoculum. The concentration of the alcohols showed little variation until the end of the test, indicating that they were not consumed under this denitrifying condition (Figure 2).

The maximum acetic acid generation was 16.7 mg/L at 61.5 h of operation. The values of STV at the beginning and at the end of the operation of the batch reactor were 5.14 g/L and 11.40 g/L, respectively, indicating an increase of 122% in biomass. These results showed that the operational conditions imposed on the batch reactor benefited the development and permanency of the bacterial consortium under denitrifying conditions.

In this study the process of denitrification was observed, as was also described by other authors using different configurations for the reactors.

Callado & Foresti (2001) operated anaerobic reactors fed with synthetic substrate simulating domestic sewage to remove the largest fraction of carbonic matter and to promote the substrate nitrification, denitrification and biological phosphate removal in the same batch cycle, in a sequential anaerobic/aerobic/anaerobic batch reactor. The system was operated for 41 days with 84 cycles of 12 hours, at the temperature of 28±1 °C. The authors (op.cit,) observed that the denitrification occurred under alternate aerobic and anoxic conditions in the reaction phase. Both of the processes occurred only when sodium acetate (500 mg/L) was added at the beginning of the anoxic phase.

Etchebehere *et al*. (2001), tested acetate (40 mmol/L) and glucose (13 mmol/L) as carbon sources for denitrification in an anaerobic batch reactor with potassium nitrate (20mmol/L) for three different inocula: sludge from an anoxic reactor (laboratory scale) to remove carbon and nitrogen from the leachate of a sanitary landfill; sludge from an UASB methanogenic reactor treating malting and sludge from an anoxic reactor fed with acetate and nitrate. Nitrate was completely consumed from the three samples of sludge tested, as observed in the present study. The authors (op. cit.) concluded that acetate is a better carbon source than glucose.

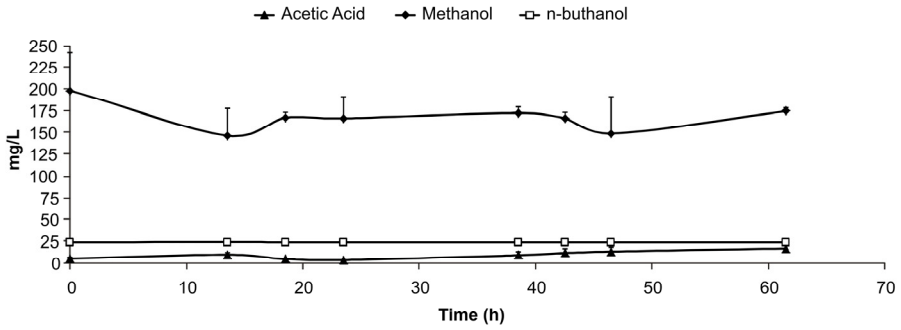


**Figure 2:** Temporal variation of methanol (◆), *n*-butanol (□) and acetic acid (▲) in the anoxic reactor.

Gavazza dos Santos *et al.* (2004) studied the denitrification process carried out in batch reactors fed with synthetic wastewater simulating nitrified effluents from domestic sewage treatment plants using three electron donor sources: methanol (53.3 mg/L), ethanol (38.3 mg/L) and methane (besides the synthetic wastewater). The authors (op.cit.) observed that the most effective electron donor was ethanol, which completely removed nitrite and nitrate, as observed in this work.

Iamamoto (2006) obtained nitrogen removal greater than 84% in a sequential batch reactor fed with starch and ammonium alternating anoxic and aerobic conditions (2h/2h cycles) and 2 mg $O_2$/L for the following concentrations: 125 mg N-$NH_4$/L and 0.95 g starch/L, 250 mg N-$NH_4$/L and 1.9 g starch/L, 500 mg N-$NH_4$/L and 3.8 g starch/L, with inoculum from an activated sludge system of a Sewage Treatment Station (Flores da Cunha Rio Claro – SP – Brazil). Ethanol (1,500 mg/L) was also tested as carbon source together with 500 mg $NH_4$-N/L. The authors (op.cit.) observed that nitrite and nitrate removal was complete (100%), as observed in the present study, demonstrating the feasibility of using ethanol in the denitrification process.

The denitrifying cell counts by MPN at the beginning of the operation of the batch reactor were lower (1.1 x $10^{10}$ MPN/mL) than at the end of the operation (1.2 x $10^{19}$ MPN/mL) (Figure 3). These results are higher than those reported in the literature, described below.

Etchebehere *et al.* (2001) enumerated denitrifying cells by the most probable number (MPN) in a basal medium supplemented with yeast extract (0.5 g/L), potassium acetate (1.84 g/ L) and potassium nitrate (0.72 g/ L) and obtained 9,6 x $10^6$ MPN/mL with sludge from the anoxic reactor of a leachate treating system. Callado and Foresti (2001) used sodium acetate as carbon source in a sequential anaerobic/ aerobic/anaerobic batch reactor and found more MPN denitrifying bacteria at the beginning of the operation (2.5 x $10^6$ MPN/mL) than at the end (3.5 x $10^5$ MPN/mL). The authors (op.cit.) concluded that this decrease did not affect the denitrification process.

Iamamoto (2006) obtained the same order of magnitude in the MPN of denitrifying bacteria at end of the operation of a sequential batch reactor with 250 mg N-$NO_3$/L (3.9 x $10^6$ MPN/mL) and 500 mg N-$NO_3$/L (1.1 x $10^6$ MPN/mL), both with addition of starch (1,900 mg/L) as carbon source and inoculum from activated sludge from a wastewater treatment plant (Rio Claro – SP - Brazil).

The denitrifying bacteria were favored by the nutritional condition imposed in the anoxic reactor. This fact confirmed the ability of the inoculum from activated sludge for denitrification process.

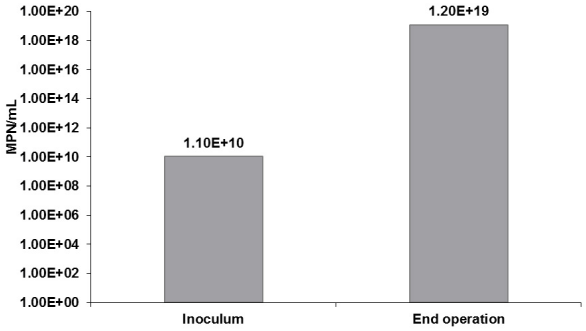


**Figure 3:** MPN of denitrifying bacteria from the inoculum and at the end of operation of the batch reactor

Fifty clones were obtained from the sample analyzed for cloning and sequencing of the 16S rRNA gene fragments of the microbial consortium. However, clones with values less than 180 nucleotides were not incorporated into the phylogenetic analysis because they were not sufficient to be compared with the database described below. The sequences from cloning and sequencing were obtained from the MPN highest dilution positive sample ($10^{-18}$) (Table 1).

*Acinetobacter* sp. was identified with the similarities of: 98% (clones 1, 8, 13, 15, 33, 43, 45, 52, 54, 62, 67, 83 and 2, 4, 18, 20, 23 and 27) and 99% (clones 6, 15, 16, 37 and 38). It is a Gram-negative bacterium, non-motile, oxidase-negative, non-fermentative, in pairs and belongs to the Proteobacteria Phylum, Moraxellaceae Family. It is an important microorganism in soil, where it can contribute to mineralization, for example, of the aromatic compounds (Geng *et al.*, 2006). Wang *et al.* (2007) isolated *Acinetobacter* strains in a sample from an activated sludge system (Jizhuangzi –Tianjin - China). This strain was capable of biodegrading phenol (1.1 g/L) by free and immobilized cells. Geng *et al.* (2006) isolated phenol degrading bacteria in a sample from an Activated Sludge Treatment System (Singapore). Biochemical tests showed that these microorganisms can grow in the presence of ethanol, glucose, sucrose and aromatic compounds such as toluene, phenol and benzoate. It was described as a new species, *Acinetobacter* EDP3. The authors (op.cit.) concluded that this species can be used to remove phenolic compounds or for *in situ* bioremediation of phenol in soils. Cai *et al.* (2009) identified 58 resistant bacteria from soil contaminated by arsenic. The *Acinetobacter, Agrobacterium, Arthrobacter, Comamonas, Rhodococcus, Pseudomonas* and *Stenotrophomonas* strains were identified in high concentrations (20 mM Ar/L). *Acinetobacter* sp. identified in this work had their growth due to the operational conditions imposed and could contribute to denitrification.

**Table 1: Clones identification of the Bacteria Domain**

| Clones | Microorganisms | Acess | Similarity (%) | Base pairs (average) | References |
|---|---|---|---|---|---|
| 1, 8, 13, 33, 43, 45, 52, 54, 62, 67, 83 | *Acinetobacter* sp. | AY673994.1 | 98 | 427 | Wang *et al.* (2007) |
| 2, 4, 18, 20, 23, 27 | *Acinetobacter* sp. | DQ374459.1 | 98 | 391 | Roy *et al*. (not published) |
| 9 | Ancultured Rhodocyclaceae Family | AY945917.1 | 98 | 454 | Liu *et al*. (2006) |
| 6, 15, 16, 37, 38 | *Acinetobacter* sp. | EU073072.1 | 99 | 434 | Cai *et al* (2009) |
| 24 | *Comamonas* sp. | EU312978.1 | 98 | 449 | Lu *et al.* (not published) |
| 25, 28, 34, 36, 42 | *Acidovorax* sp. | AB076844.1 | 99 | 453 | Khan *et al*. (2002) |
| 26 | *Comamonas* sp. | DQ3017871 | 97 | 455 | Hong *et al.* (not published) |
| 65 | *Acidovorax* sp. | EF033514.1 | 97 | 453 | Gentile *et al.* (2007) |
| 25, 28, 34, 36, 42 | *Acidovorax* sp. | AB076844.1 | 99 | 453 | Khan *et al*. (2002) |

Clones 24 and 26 were similar to *Comamonas* sp. with similarities of 98% and 97%, respectively. *Comamonas* are Gram-negative bacteria belonging to Proteobacteria Phylum, Comamonadaceae Family. Etchebehere *et al.* (2001) isolated Gram-negative denitrifying bacteria from an anoxic reactor used for landfill leachate treatment in Montevideo (Uruguay). The isolated species had similarity with *Comamonas terrigena*. However, this microorganism was considered a new species, named *Comamonas nitrativorans*. It was described as a gram-negative bacteria, polar flagellum mobile, aerobic and chemoorganotrophic. This species grew in ethanol, acetate and butyrate, nitrate, nitrite and can reduce nitrate to $N_2$.

Therefore, *Comamonas* species identified in this study were present in the inoculum of activated sludge and could contribute to the denitrification that occurred in the tests.

The clones 25, 28, 34, 36, 42 and 65 were similar to *Acidovorax* sp. with similarities of 99% and 97%, respectively (Table 1). *Acidovorax* sp. belongs to the Proteobacteria Phylum; it is easily found in activated sludge systems. A lot of *Acidovorax* species act as regulator microorganisms of microbiological treatment processes in activated sludge systems. Several *Acidovorax* species are used in plastic biodegradation and in the removal of other organic contaminants, including nitrophenols, nitrobenzene and polychlorinated biphenyls through denitrification (www.cebl.autokland.ac.nz/ecogenomics/index.html). Khan *et al.* (2002) isolated denitrifying bacteria species degrading PHBV (poly-3-hydroxy-butyrate-co-3-hydroxyvalerate) from three activated sludge systems, used to treat municipal sewage (Nagoya, Osaka, and Toyohashi - Japan). The 37 clones analyzed showed similarity to organisms belonging to the Betaproteobacteria class. Most clones showed similarity with the *Acidovorax* species, confirming that this species was involved in the PHBV degradation under denitrifying conditions. Gentile *et al.* (2007) isolated similar species to *Acidovorax*, *Delftia acidovorans*, *Pseudomonas*, *Chryseobacterium* and *Achromobacter* from a denitrifying reactor operated with ethanol (40.0 g/L) and lactic acid (40.0 g/L) as carbon sources; they tested electron donors separately and nitrate (1.9 g $NaNO_3$/L; 2.3 g $KNO_3$/L) was used as nitrogen source. The authors (op. cit.) concluded that complete nitrate reduction to $N_2$ was done by *Acidovorax* species, whereas *Achromobacter* sp. and *Delftia acidovorans* were responsible for incomplete nitrate denitrification to nitrite. Therefore, *Acidovorax* species were present in the samples analyzed in this study and they could be involved in the denitrification that occurred in the batch reactor.

Uncultivated bacterial strains from the Rhodocyclaceae Family were identified with 98% similarity (clone 9 - AY945917.1 and clones 46, 84 AY945905), as shown in Table 1. The Rhodocyclaceae Family members are Gram-negative bacteria belonging to the Betaproteobacteria class. They are denitrifying aerobic rods with versatile metabolic capacity. Most species live in aquatic habitats and oligotrophic soil conditions. A lot of them occur in wastewater and play an important role in the biological decontamination treatment of such sites (http://en.wikipedia.org/wiki/Rhodocyclaceae). Liu *et al.* (2006), fed a denitrifying reactor with quinoline (40 mg/L) – a toxic amine used in the manufacture

of dyes, pesticides and synthetic fuels, glucose (180 mg/L), nitrate and potassium phosphate (C/N/P of 150:30:1). The inoculum was from the second sedimentation tank of the sewage treatment system of Industry Shanghai Coking & Chemical Factory (Wujing, Shanghai - Japan). The quinoline removal was 90.2% after the steady-state reactor period (6 weeks). Molecular biological analyses of inoculum and from the denitrifying reactor revealed uncultured bacteria, *Thauera* and *Azoarcus* in both tests. According to the authors, the percentages of clones affiliated with bacteria belonging to *Azoarcus* and *Thauera* were 74% in the denitrifying reactor and 4% in the inoculum, respectively. The knowledge of micro-organisms from environmental samples is dependent on laboratory conditions with pure cultures (Pace, 1997). However, less than 1% of the bacteria from different ecosystems are known (Amann, 1995), or roughly 99% have not been studied and identified. Therefore, in this work we expected to obtain similar sequences of uncultivated bacteria.

The phylogenetic tree obtained with consensus Bacteria domain primers in molecular biology analyses of the experiment is illustrated at Figure 4.

The coefficients of similarity between the different groups of microorganisms were 97% to 99% and they indicated the presence of phylogenetically related species, based on 16S rRNA gene partial evaluation sequences. Known sequences of species were from the NCBI database with *Aspergillus niger* (FJ828924.1) as an out-group sequence of.

Therefore, *Acidovorax*, *Comamonas* and *Acinetobacter* species identified in this study were present in the activated sludge system of Volkswagen São Carlos Motors and they could be involved in the nitrate reduction and ethanol consumption.

## CONCLUSIONS

The potential of the inoculum from an activated sludge system and the use of ethanol as carbon source in a denitrification process have been demonstrated in a batch reactor.

The MPN denitrifying bacteria values obtained in this study, combined with results for the removal of nitrate, revealed that there were denitrifying bacteria in the inoculum from an activated sludge system, which were favored by the nutritional conditions imposed.

The identified clones had phylogenetic affiliation in the Proteobacteria phylum, Alphaproteobacteria and Gamaproteobacteria class, with *Acidovorax* sp., *Acinetobacter* sp., *Comamonas* sp. and uncultured bacteria. These bacteria could be involved in the nitrate reduction and consumption of ethanol in anoxic batch reactor.

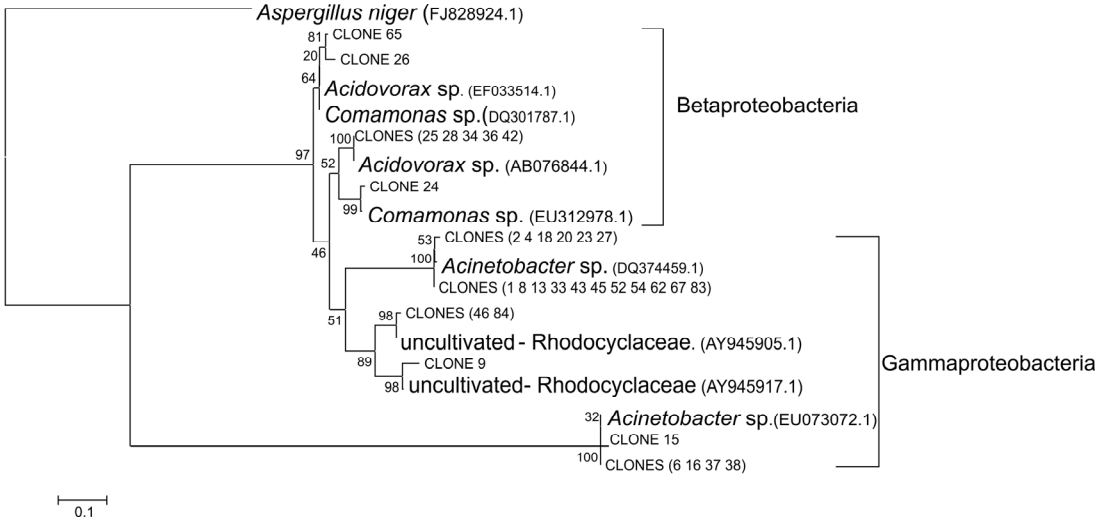


**Figure 4:** Consensus phylogenetic tree based on sequences from clones with primers for Bacteria Domain (27 Forward and 1100 Reverse) obtained from the MPN highest dilution positive ($10^{-18}$) sample. The bootstrap values present on the tree indicate the percentages of 500 replicates. GenBank accession numbers are listed after species names. Out-group sequence of *Aspergillus niger* (FJ828924.1)

## ACKNOWLEDGMENTS

The authors gratefully acknowledge grants received from FAPESP and CNPq.

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