Functional characterization of heterotrophic denitrifying bacteria in activated sludge

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WASTEWATER TREATMENT FACILITIES are largely accountable for the detrimental enrichment of water bodies with nitrogenous compounds, resulting in eutrophication. Denitrification is of interest as a means of removing nitrates and nitrites from water supplies because they are also hazardous to human health. The integration of biological nutrient removal into conventional wastewater treatment processes has, however, failed to take into sufficient consideration the role of key microorganisms present, specifically denitrifying bacteria. The purpose of the study reported here was to group such heterotrophic denitrifying bacteria using a series of biochemical and molecular analyses, to achieve an improved understanding of their functions. The role of the denitrifying bacteria in reducing nitrate and nitrite was monitored using the colorimetric nitrate reduction test. The genetic diversity of the culture collection was investigated by the use of denaturing gradient gel electrophoresis (DGGE), which enabled the creation of a microbial population profile of eight predominant isolates. Batch experiments were conducted on these isolates, the results of which ultimately confirmed their classification according to their respective functions, namely, incomplete denitrifiers, true denitrifiers, sequential denitrifiers, and exclusive nitrite reducers.

Introduction

Undesirable environmental effects and health risks associated with water pollution by nitrogenous compounds has led to the need to improve wastewater treatment, by integrating biological nutrient removal into conventional activated sludge processes. Denitrification occurs under artificial anaerobic conditions, resulting in the reduction of nitrate to nitrite, nitrogenous oxides (N_2O) and nitrogen gas (N_2) that escape into the atmosphere. Although biological nutrient removal processes succeed in denitrification, the roles of the microbes involved have not been well defined. A deeper understanding of nitrogen removal as well as the biochemistry, physiology and ecology of heterotrophic denitrifying bacteria should therefore contribute to the improved efficiency of biological nutrient removal.[1–3]

Denitrification is considered to remove bioavailable forms of nitrogen from the biosphere. Since the loss of nitrate from agricultural soil reduces crop yields and modifies the natural fauna and flora in the soil, denitrification is not beneficial to agriculture. Furthermore, excess nitrate or nitrite in soil or aquatic ecosystems leads to ecological and animal and human health problems. Excess nitrate can be transformed into its acid forms, which thereby reduces the quality of soil and water.[4–6]

In addition to the ecological problems it creates, excess nitrate stimulates denitrification, resulting in the loss of bioavailable nitrogen used by plants in the form of gaseous N_2O and N_2; the N_2O so emitted adversely affects the ozone layer. Despite these harmful effects, denitrification is valuable in sewage treatment because it converts nitrate to its gaseous reaction products, thereby reducing the amount of available nitrogen in the sewage.[6]

Denitrifying bacteria comprise four different functional groups based on their ability to reduce nitrate and/or nitrite.[1,6] Heterotrophic denitrifiers perform under anaerobic conditions. These functional groups include incomplete denitrifiers, true denitrifiers, sequential denitrifiers, and exclusive nitrite reducers.[1,6] The characterization of these bacteria has previously been conducted using the colorimetric biochemical nitrate reduction test, which indicates their denitrification abilities; however, molecular analysis is also essential to validate the biochemical conclusions.

The objectives of the study reported here were to conduct colorimetric biochemical nitrate reduction tests and molecular methods on denitrifiers to verify their functions. One method was denaturing gradient gel electrophoresis (DGGE) for the rapid analysis of sequence diversity of complex natural microbial populations and that can identify most single base variations when a G–C clamp is added to one primer in the PCR amplification process.[7–11] It is thus capable of providing a practical picture of changes within a microbial community or of the differences between microbial communities, and finds growing application in the investigation of bioreactors used in wastewater treatment.[12]

Materials and methods

Isolation and maintenance of heterotrophic denitrifiers

Mixed liquor samples of activated sludge were obtained from the anoxic zones of the Darvill Wastewater Treatment Works (Pietermaritzburg, KwaZulu-Natal). Heterotrophic denitrifying bacteria were isolated from these samples and were plated onto Casitone Glycerol Yeast Autolysate Agar (CGYA) medium to obtain a pure culture collection.[6] The culture collection was maintained by monthly subculturing and storage at 4°C on fresh CGYA slants.

Elimination of identical isolates

Gram stains were used to analyse the colonial and cellular morphology of the isolates.[6,13] Cultures were inoculated onto CGYA plates and incubated at room temperature. Stains were observed within the first 24 hours of growth, because staining bacterial cultures older than one day may produce variable results. A further four-day incubation period of the inoculated CGYA plates produced well-defined colonies for morphological analysis. Only cultures that exhibited uniform morphology with regard to size, pigmentation, form, as well as those that displayed a single Gram reaction were regarded as 'pure'.

Denitrification screening and subsequent classification

All isolates were screened in triplicate for both nitrate and nitrite reduction over a range of concentrations. These concentrations were established by the addition of 1.0, 0.5 and 0.2 g/l of potassium nitrate (KNO_3) and potassium nitrite (KNO_2), respectively. These isolates were subsequently classified according to the following functional groups: incomplete denitrifiers, true denitrifiers, sequential denitrifiers, and exclusive nitrite reducers. This was achieved by inoculating the bacteria onto nitrate and nitrite media followed by incubation at 20°C for four days. The colorimetric biochemical nitrate reduction test was used to screen the denitrification properties of the isolates.[13] Bacteriological agar at a concentration of 0.1%, instead of 1.5%, was incorporated into the media that semi-solidified it, thus preventing oxygen from diffusing into the media. This achieved anoxic conditions that are essential for most denitrification processes.[13]

Extraction of DNA from isolates

DNA was extracted from whole cells using an adaptation of the boiling method.[7] About 3–4 loopfuls of pure culture was dispensed into sample vials containing 1 ml of sterile distilled water, and vortexed. Sample vials were placed in a water bath and suspensions were boiled at 48°C for 15 min and stored at –4°C until further use.

PCR amplification conditions

The 16S rRNA genes were selectively amplified using PCR by applying oligonucleotide universal primers to target a wide range of bacterial species. PCR was performed in 100 μl reaction mixtures, which included: 10 μl 10 × PCR buffer excluding $MgCl_2$, 1.5 mM $MgCl_2$, 10 μl bacterial DNA, 2 mM deoxynucleoside triphosphate mix (dNTP), forward and reverse

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primers at a concentration of 50 μM and 5 U DNA *Taq* polymerase, respectively.

The universal primers used had the following nucleotide sequences:

P 341 forward (with 40 bp GC clamp): 5′ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3′,[14] corresponding to positions 341–358, and

P 1492 reverse (1492–1512): 5′ TAC GGC TAC CTT GTT ACG ACT T 3′.[15]

The reaction mixture was subject to PCR under the following conditions: an initial step was performed with a hot start at 94°C for 4 min, before polymerase was added, followed by 35 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 4 min in a thermal cycler, and immediately cooled to 4°C (PCR Sprint Temperature Cycling System, Hybaid, U.K.).

The presence of PCR-amplified products was confirmed by analysis on 2.5% agarose gels stained with ethidium bromide.

DGGE of amplified fragments

DGGE of PCR-amplified DNA fragments was conducted using the *Bio-Rad* D Gene™ Denaturing Gel Electrophoresis System (Bio-Rad) and Power Pac 300 (Bio-Rad) and performed in accordance with the manufacturer's instructions and applications guide (catalogue numbers 170-9000 to 170-9070). A 7.5%, 16 × 16 cm gel was used. Optimum concentrations of the denaturing solutions were experimentally determined by varying the concentrations of urea and formamide. Good separation and resolution of DNA into clear bands on the gel demonstrated optimum concentrations of the denaturing solutions. A 30% low density denaturing solution and a 50% high density denaturing solution were used. All DGGE gels were run for 80 min at a constant 200 V at 60°C. Gels were stained with ethidium bromide and photographed under a UV transilluminator. All reagents were of molecular biology grade.

Results and discussion

There were no Gram-positive cocci present. Gram-negative heterotrophic bacteria are predominant in activated sludge plants and were so in the culture collection. Gram-positive heterotrophic denitrifying bacteria are believed to play minor roles in the activated sludge process, so little research has been conducted on them.[13]

Denitrification screening and subsequent classification

The denitrification properties of the isolates were assessed using the colorimetric nitrate reduction test. The bacteria that reduced nitrate to nitrite were classified as incomplete denitrifiers.[6,13]

If no nitrate was left in the medium, because it had already been reduced by the bacteria to nitrite and then further reduced to N_2, isolates that displayed this type of reaction were classified as true denitrifiers.[6,13]

Table 1. Comparison of the classification of the denitrifying isolates after biochemical and molecular analyses.

Classification	Number of isolates	
	Biochemical analysis (NO_3^- reduction test)	Molecular analysis (DGGE)
Incomplete denitrifier	73	3
True denitrifier	47	2
Sequential denitrifier	54	2
Exclusive nitrite reducer	5	1

Isolates that displayed incomplete denitrification on the nitrate medium and nitrite reduction on the nitrite medium were classified as sequential denitrifiers. Additionally, cultures that displayed incomplete denitrification at high nitrate concentrations but displayed true denitrification at low nitrate concentrations were also classified as sequential denitrifiers.[6]

Cultures that displayed no nitrate reduction on nitrate media, but effected nitrite reduction when grown on nitrite media, were classified as exclusive nitrite reducers.[6,13] The intensity of the colour reactions indicated the extent to which nitrate and nitrite were reduced.

Table 1 displays the results of comparing the isolates based on biochemical tests and molecular techniques. Molecular analysis clearly indicated that those isolates that were regarded as different based on the Gram reaction, as well as colonial and cellular morphology, belonged to the same species but were of different strains. Thus, 73 morphologically different incomplete denitrifiers were reduced to just 3 predominant isolates. The classification of the denitrifying isolates by means of biochemical analysis verified the results of previous research.[6] Of the 179 isolates, biochemical analysis revealed that there were 47 true denitrifiers, 54 sequential denitrifiers and 5 exclusive nitrite reducers. However, molecular analysis indicated 3 incomplete denitrifiers, 2 predominant true denitrifiers, 2 sequential denitrifiers and only 1 exclusive nitrite reducer.

Molecular analysis of denitrifying isolates

Agarose gel electrophoresis was carried out on all PCR-amplified 16S rDNA fragments. Fluorescent bands represented a positive result for the presence of DNA in the samples. For DGGE gels, bands that had the same horizontal alignment in each lane of the gel indicated organisms with DNA fragments of the same length but different nucleotide sequences.

Nitrate and/or nitrite reduction by predominant isolates

Batch experiments were conducted under anoxic conditions on the remaining 8 predominant isolates, which could not be characterized on the basis of the nitrate reduction test alone. The capacities of these isolates to reduce nitrate and/or nitrite were determined by measuring the amount of nitrate consumed and that of nitrite remaining or converted to N_2 after the batch experiment. This was achieved using a Technicon Autoanalyser II.

Incomplete denitrifiers were represented by isolate 56, true denitrifiers were represented by isolate 88, the sequential denitrifiers were represented by isolate 147, and the exclusive nitrite reducers were exemplified by isolate 176.

Incomplete denitrifiers

Figure 1 depicts the ability of isolate 56 to reduce nitrates and/or nitrites. This is an example of an incomplete denitrifier, which was capable of reducing nitrate to nitrite, but incapable of further reduction

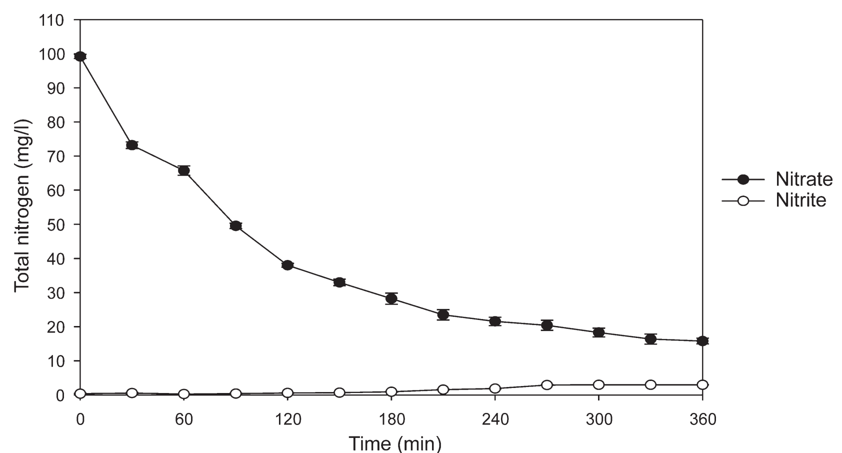

Fig. 1. Graph showing the amount of nitrate and/or nitrite reduced by isolate 56.

of the nitrite. There was a gradual increase in nitrite concentration with time. For this organism, the amount of nitrate declined in proportion to the increase in nitrite.

Previous results substantiate findings that initial nitrite production by actively denitrifying bacterial communities is usually high and may, especially if accompanied by high initial nitrate concentrations, result in nitrite build-up.[15–17] This build-up is a result of the predominant presence of incomplete denitrifiers within denitrifying bacterial communities. It has been shown previously that these incomplete denitrifying bacteria lack key nitrite reductase enzymes that enable the true denitrifiers to reduce nitrites.[16]

True denitrifiers

Figure 2 depicts the ability of isolate 88 to reduce nitrates and/or nitrites. This exemplifies a true denitrifier, which was capable of reducing both nitrate and nitrite simultaneously. Thus for this isolate, the amount of nitrate decreased in proportion to the reduction in nitrite present.

These bacteria all contained nitrate and nitrite reductase enzymes enabling them to reduce nitrate, via nitrite, to gaseous nitrogen.[15,16,18] In our study, two classes of true denitrifiers were analysed.[1,6] The class II type displayed true denitrification in all three test concentrations of KNO_2 and KNO_3. In nitrite media, however, it was either incapable of nitrite reduction or could reduce nitrites at only low KNO_2 concentrations. This inability to reduce nitrite at high concentrations may be due to nitrite toxicity.[19] Nitrite is toxic to many organisms at high concentrations because it removes protein from cell membranes.[19]

Sequential denitrifiers

Figure 3 depicts the ability of isolate 147 to reduce nitrates and/or nitrites. This is an example of a sequential denitrifier, which is capable of reducing both nitrate and nitrite simultaneously. Nitrite reduction is inhibited in the presence of nitrate, however, which results in nitrite accumulation during nitrate reduction.[6]

It has been suggested that the possible cause of this type of phenomenon is that the nitrate ion is the preferred electron acceptor in cellular respiration, since it is capable of accepting five electrons in comparison with nitrite's three.[20]

The reasons for nitrite accumulation are unbalanced reduction reactions for nitrate and nitrite, or delayed induction of nitrite reductase as compared to nitrate reductase.[21]

Exclusive nitrite reducer

Figure 4 displays the ability of organism 176 to reduce nitrites. This is an example of an exclusive nitrite reducer, which exhibits non-denitrification characteristics in nitrate media but is capable of efficient nitrite reduction in nitrite media.[6, 13]

This group of organisms was isolated and characterized from working samples but little is known about their roles in the practical treatment of activated sludge.[16] These bacteria lack nitrate reductase enzymes and are therefore incapable of contributing to nitrate reduction. Although they were not isolated in large numbers, their contribution to denitrification requires a more accurate assessment, considering their efficiency in the reduction of high concentrations of nitrite as observed in this study.

Previous research focused on the grouping of denitrifiers according to their denitrifying potential using the nitrate reduction test.[13] Our findings serve to substantiate previous work by our having used batch tests to characterize the isolates according to their denitrifying abilities and molecular techniques to eliminate the duplicate testing of the initial isolates, which reduced the number from 179 to 8 predominant isolates. Although

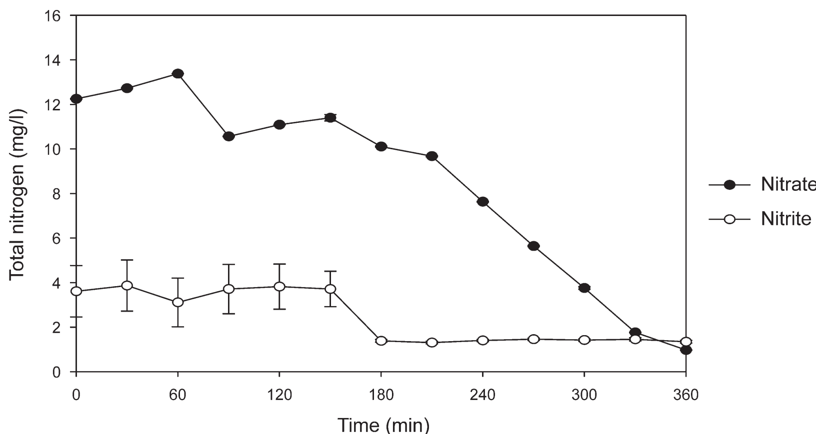

Fig. 2. Graph showing the amount of nitrate and/or nitrite reduced by isolate 88.

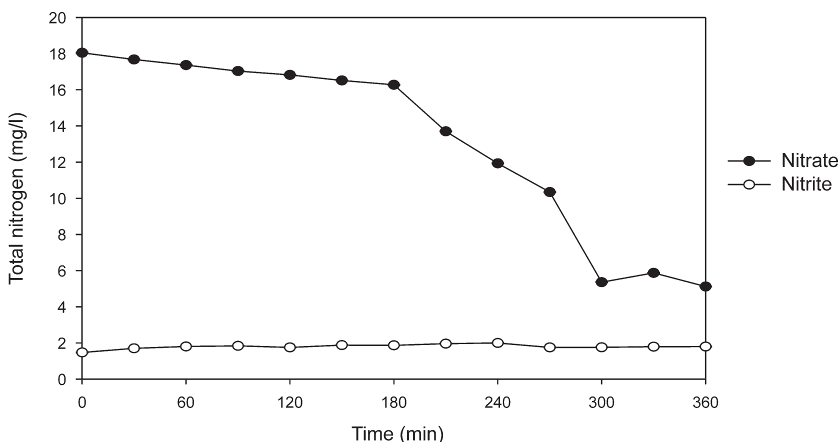

Fig. 3. Graph showing the amount of nitrate and/or nitrite reduced by isolate 147.

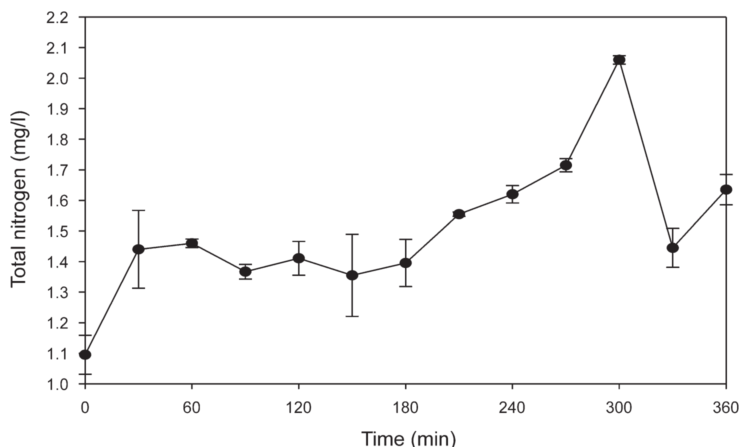

Fig. 4. Graph showing the amount of nitrite reduced by isolate 176.

we used small-scale, culture-dependent techniques, our findings could be representative of the microbial contribution to denitrification in full-scale wastewater treatments.

Conclusions

This study confirmed the existence of a diverse community of heterotrophic bacteria involved in nitrogen removal during wastewater treatment, of which incomplete denitrifying bacteria were significantly involved. We identified a large number of incomplete denitrifiers (73 isolates), as well as sequential denitrifiers (54), true denitrifiers (47), and exclusive nitrite reducers (5).

DGGE was primarily used to establish the diversity of the four functional groups of denitrifiers. Identification of the respective isolates by nucleotide sequencing will be the focus of future research. DGGE profiling revealed 8 predominant isolates, which consisted of 3 incomplete denitrifiers, 2 true denitrifiers, 2 sequential denitrifiers and 1 exclusive nitrite reducer. These isolates were not previously characterized according to their denitrifying abilities.

This research provides a foundation for improving our understanding of the microbial contribution to denitrification and the functional role of denitrifying bacteria in wastewater treatment processes.

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