Biological nitrogen and phosphorus removal by filamentous bacteria in pure culture

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Abstract

The availability of excess nutrients (phosphorus (P) and nitrogen (N)) in wastewater systems causes many water quality problems. These problems include eutrophication whereby algae grow excessively and lead to depletion of oxygen, death of the aquatic life and bad odours. Biological phosphorus removal has gained attention because the condition of wastewater is manipulated in order to facilitate nutrient removal by the microbial communities in the wastewater. It has been reported that filamentous bacteria are capable of removing P at a similar or higher rate to that of heterotrophic bacteria. It has also been reported that conditions that facilitate biological nitrogen removal promote bulking in a biological nutrient removal system. The aim of the project was therefore to evaluate the role of filamentous bacteria in biological nutrient removal (BNR) processes. For denitrification this was achieved by performing the nitrate reduction preliminary screening test followed by batch tests. Neisser staining was used to locate polyphosphate granules in cells. All Neisser positive isolates were evaluated for P accumulation employing batch tests. The findings of this study demonstrated that 29% of the isolates were true denitrifiers, 3% were sequential denitrifiers, 11% were nitrate respirers, 13% were non-denitrifiers and 45% were nitrate respirers at high concentrations (1 g/ℓ and 0.5 g/ℓ) and true denitrifiers at low concentrations (0.2 g/ℓ). The results of the nitrate reduction batch test demonstrated that up to 18.46 mg/ℓ nitrate was reduced to nitrogen gas. 53% of the isolates reduced nitrite, 33% resulted in nitrite accumulation and 9% did not react to nitrite. Of the 38 isolates 16% were positive for the Neisser stain, 34% were positive for the glycogen stain and 79% were positive for the PHB stain. Batch test results showed phosphate accumulation of up to 17.12 mgP/ℓ. It was demonstrated by this study therefore, that filamentous bacteria have the potential to biologically remove nutrients. These research findings will serve as a basis for further investigations.

Keywords: activated sludge, denitrification, glycogen accumulating organisms, filamentous bacteria, phosphorus removal

Introduction

Biological nutrient removal (BNR) has gained attention over chemical nutrient removal because of the high cost of the chemical process and the large sludge volumes produced. (Machnika et al., 2005 and Sarioglu, 2005). BNR has three stages, i.e. the anaerobic compartment, anoxic compartment and aerobic compartment. In the anaerobic compartment phosphate accumulating bacteria (PAOs) are selected for and phosphate is released from the bacteria. Under anoxic conditions denitrification and phosphate uptake take place while nitrification and phosphate uptake take place under aerobic conditions (Van Loosdrecht, 2004).

The process of phosphate uptake involves exposing the PAOs to alternating anaerobic-aerobic conditions. Under anaerobic conditions PAOs take up the carbon source and store it in the form of polyhydroxybutyrate (PHB). This is accompanied by the degradation of internally stored polyphosphate in order to provide energy for carbon source uptake. Phosphate is then released in the form of orthophosphate. Under aerobic conditions PAOs take up orthophosphate to replenish their polyphosphate pools using stored PHB as a carbon and energy source (Barak and Van Rijn, 2000; Mino et al., 1998).

Denitrification is the biological conversion of nitrate to more reduced forms such as dinitrogen gas (N_2), nitrous oxide (N_2O) and nitric oxide (NO). Facultative aerobes that can utilise nitrate instead of oxygen as a final electron acceptor are responsible for denitrification. The breakdown of carbonaceous organics in the denitrification process is similar to that in the aerobic process, the only difference being in the final stages of the electron transfer (Sedlak, 1999). This would indicate the need for strict anoxic conditions in the denitrifying system (Lilley et al., 1997). It has been demonstrated that under acidic conditions denitrification can take place in the presence of oxygen (Martin, 1991). Nitrate will replace oxygen in the endogenous respiration reaction. The rate of denitrification depends on the nature and concentration of the carbonaceous matter undergoing degradation. Most investigations agree that denitrification is a zero-order reaction with respect to nitrate being reduced to very low nitrate concentration levels (Martin, 1991).

A hypothesis for the cause of bulking by the low food-to-microorganism (low F/M) filamentous bacterial group in a BNR system was proposed by Casey et al. (1999). In this hypothesis it is stated that the floc-forming organisms are able to reduce nitrate to di-nitrogen gas through the denitrification intermediates. Casey et al. (1999) state that in a BNR system, competition between floc-forming and filamentous bacteria for mutually growth-limiting substrate is influenced by the inhibition of substrate utilisation of floc-formers under aerobic conditions. If denitrification is incomplete at the onset of the aerobic phase, the intracellular denitrification intermediates inhibit the aerobic cytochrome *o* of floc formers and their substrate utilisation ability is therefore inhibited. At this stage filamentous bacteria utilise the substrate and proliferate in the system, leading to bulking (Casey et al., 1999).

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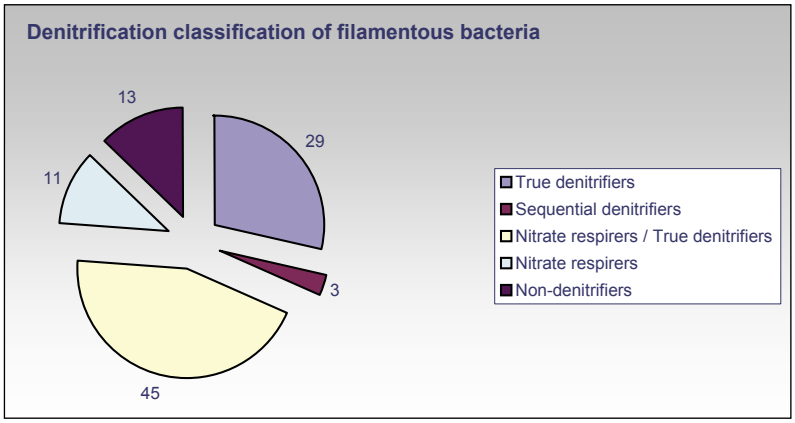

Figure 1
Denitrification classification of isolates in percentage

It was recently discovered by Machnika et al. (2005) that filamentous bacteria can remove nutrients biologically. They state that filamentous bacteria are also capable of removing phosphorus at a rate similar to or higher than that of heterotrophic bacteria in the activated sludge. The aim of this study was therefore to evaluate the role of filamentous bacteria in BNR.

Experimental

Isolation of pure cultures of filamentous bacteria

The cultures of filamentous bacteria used to conduct this research were isolated, cultivated and purified according to Ramothokang et al. (2003). A Gram stain was conducted to verify purity of isolates and also as a characterisation tool.

Denitrification

Preliminary screening

Pure cultures of filamentous bacteria were each screened for their denitrification ability at 1 g/ℓ, 0.5 g/ℓ and 0.2 g/ℓ nitrate and nitrite concentrations as potassium salts respectively, employing the nitrate reduction method devised by Drysdale et al. (1999). The colorimetric biochemical reduction test was used to screen for nitrate and nitrite reduction (Cappucino and Sherman, 1992).

Batch test

Isolates exhibiting a positive result for denitrification were then studied further in duplicate in the form of a batch test by inoculating 5 mℓ of two-day-old starter cultures into 145 mℓ of CGYA broth (Atlas, 1993) supplemented with 0.1 g/ℓ potassium nitrate that had been sparged with nitrogen gas for 1min to create anoxic conditions. A volume of 5mℓ of sample was drawn every hour. Nitrogen concentration (as nitrate and nitrite) was therefore monitored hourly for 6h and concentrations were analysed using an auto-analyzer (Technicon Auto Analyzer AAII, Dermotech South Africa). Slow continuous stirring was employed to keep filamentous bacteria in suspension. The cultures were grown at room temperature (20 to 25°C). The control was an uninoculated broth.

Phosphorus removal

Evaluation of storage polymers

The Neisser, PHB (Jenkins et al., 1993), and Glycogen stain (Periodic Acid Schiff's method by McManus, 1946 as cited online at http://www.hoslink.com.hist/6.HTM, accessed September 19, 2003) were employed to determine polymer storage (regions) within the filamentous bacteria.

Batch test

Neisser-positive pure filamentous bacterial cultures were grown anaerobically for 24 h in CGYA broth with glucose (Glycogen stain positive isolates) or acetate (PHB stain positive isolates) to deplete phosphorus in polyphosphate granules. A volume of 35 mℓ of the respective medium was added into a 40 mℓ anaerobic vial. This is a pre-requisite in order to identify a PAO (Lacko et al., 2003). Cultures were grown at room temperature. Cells from anaerobic incubation were suspended into a respective 115 mℓ CGYA broth containing 10 mg/ℓ K_2HPO_4. Phosphate concentration was monitored every hour for 6 h using the auto-analyzer. A control comprised 150mℓ of an uninoculated broth (Jørgensen and Paulii, 1995).

Results and discussion

Denitrification (preliminary screening)

Results of this study show that filamentous bacteria can be grouped into five groups of denitrifiers as per Drysdale et al. (1999) namely true denitrifiers, sequential denitrifiers, nitrate respirers, non-denitrifiers and those that can be nitrate respirers at high concentrations and true denitrifiers at low concentrations.

True denitrifiers

According to Fig. 1, 29% of the total isolates were made up of true denitrifiers. True denitrifiers have nitrate and nitrite reductase enzymes in their cytoplasm. The presence of these enzymes allows these bacteria to reduce both nitrate and nitrite simultaneously. Denitrification occurs under anoxic conditions where these enzymes are induced (Cappucino and Sherman, 1992; Drysdale et al., 2001; Ramdhani, 2005; Robertson and Kuenen, 1992).

Sequential denitrifiers

Current findings showed that only 3% were sequential denitrifiers (Fig. 1). Sequential denitrifiers can reduce nitrate to nitrite when grown in nitrate media, and when grown in nitrite media, nitrite is reduced to nitrogen gas (Drysdale et al., 1999). Nitrite reductase enzyme is inhibited in the presence of nitrate and this results in the build-up of nitrite in the nitrite media as shown in Table 1 (Ramdhani, 2005).

Nitrate respirers

Bacteria that can only reduce nitrate were observed in this

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study and they are called nitrate respirers. These bacteria lack the enzyme that facilitates nitrite reduction (nitrite reductase enzyme). Only 11% of the total isolates were nitrate respirers (Fig. 1). Findings by Drysdale et al. (1999) state that nitrate respirers prefer aerobic environment as compared to anoxic.

Nitrate respirers-true denitrifiers

At least 45% of the isolates in this study comprised of nitrate respirers at high nitrate concentrations of 1 g/ℓ and 0.5g/ℓand at low nitrate concentration of 0.2 g/ℓ these isolates were true denitrifiers (Fig. 1). This suggests that these isolates have both the nitrate and nitrite reductase enzymes but the nitrite reductase enzyme is inhibited at high nitrate concentration (Drysdale et al., 1999). These isolates may be responsible for bulking due to their affinity for low nitrate concentration and their inability to accumulate nitric oxide intracellularly. Floc-forming bacteria under anoxic conditions accumulate nitric oxide and it prevents oxygen utilisation under aerobic conditions. If these (nitrate respirers – true denitrifiers) organisms are present at the point conditions switch from anoxic to aerobic and denitrification is incomplete, they will proliferate due to their ability to grow under aerobic conditions and their affinity for low nitrate concentration (Casey et al., 1999). The findings of this study agree with the hypothesis proposed by Casey et al. (1999), which states that if denitrification is incomplete at the onset of the aerobic phase, the intracellular denitrification intermediates inhibit the aerobic cytochrome *o* of the floc formers and their substrate utilisation is therefore inhibited. This is when the filamentous bacteria utilise the substrate and proliferate in the system, leading to bulking sludge (Casey et al., 1999).

TABLE 1
Nitrite reduction batch test results in mg/ℓ

Org. no.	At time 0 h (mg/ℓ)	After 6 h (mg/ℓ)	N reduced (mg/ℓ)	N accumulated (mg/ℓ)
17	1.58	0.15	1.43	
15	0.68	0.00	0.68	
7	0.45	0.13	0.32	
14	0.29	0.05	0.24	
12	2.50	2.38	0.12	
36	0.38	0.28	0.10	
21	0.09	0.00	0.09	
28	0.23	0.16	0.07	
32	0.07	0.00	0.07	
8	0.06	0.00	0.06	
2	0.07	0.01	0.06	
29	0.17	0.11	0.06	
35	0.09	0.03	0.06	
30	0.28	0.23	0.05	
11	0.05	0.00	0.05	
1	0.11	0.08	0.03	
38	0.02	0.00	0.02	
22	1.42	1.41	0.01	
34	0.06	0.05	0.01	
19	0.77	2.04		1.27
27	0.18	1.38		1.20
16	0.10	0.94		0.84
25	0.17	0.86		0.69
26	0.80	1.34		0.54
37	0.00	0.34		0.34
23	0.39	0.50		0.11
31	0.18	0.24		0.06
20	0.17	0.22		0.05
9	0.09	0.13		0.04
18	0.04	0.05		0.01
13	0.00	0.00	0.00	
24	0.36	0.36	0.00	
33	0.00	0.00	0.00	

Non-denitrifiers

Findings showed that 13% of the isolates were non-denitrifiers (Fig. 1). These isolates lack both nitrate and nitrite reductase enzymes, therefore they have no role in the denitrification process (Drysdale et al., 1999).

Denitrification (batch test)

57% of the isolates had the ability to reduce nitrite. This could be due to the presence of sequential denitrifiers as shown in a study by Ramdhani (2005). Sequential denitrifiers reduce nitrite only when grown in nitrite media (Drysdale et al., 1999). 33% of the isolates accumulated nitrite. Nitrite accumulation is dominant among nitrate respirers due to their inability to reduce nitrite because they lack the nitrite reductase enzyme (Table 1).

It is therefore indicated by this investigation that filamentous bacteria can reduce nitrate at a rate similar or even higher than that of heterotrophic bacteria. Reduction of up to 18.46 mg/ℓ nitrate was observed which is higher than that of 7.46 mg/ℓobserved by Lacko et al. (2003) using *Alcaligenes* spp. which had been incubated for 6h with an initial concentration of 11.29 mgNO_3-N/ℓ. Results showed that 24% of the isolates proved to reduce nitrate in the range of 10 to 20 mg/ℓ nitrate. Manipulation of filamentous bacteria and enhancement of their nutrient removal capabilities may therefore have a substantial contribution in the BNR system since the presence of nitrogen is implicated with eutrophication and poor water quality. In addition, 30% of the isolates reduced nitrate in the range of 5 to 10 mg/ℓ, which is in the range observed by Lacko et al. (2003). At least 45% of the isolates showed weak nitrate reduction of 0 to 5 mg/ℓ which was also observed in a study by Lacko et al. (2003) (Table 2).

Phosphorus removal

With regard to phosphorus, 16% (Fig. 2) of the isolates had the ability to accumulate phosphate which was indicated by the presence of polyphosphate granules within the cells.

If subjected to alternating anaerobic and aerobic conditions bacteria can accumulate phosphate in the aerobic conditions if their internal phosphate had been depleted under anaerobic conditions, provided the correct carbon source is provided (Mino et al., 1997). Most of the isolates preferred acetate as compared to glucose as a carbon source. This was determined by means of

the glycogen stain (glucose) and PHB stain (acetate). These findings agree with a study by Mino et al. (1997) which states that acetate (Ac) is a preferred carbon source for PAOs under anaerobic conditions (Fig. 2). For the purpose of the study Ac and Gl presented on the graphs (Figs. 3 and 4) depict acetate (Ac) and glucose (Gl) respectively, which were used as carbon sources for phosphate release and accumulation determination. The number represents the isolate number. Batch test results of this study showed that filamentous bacteria could accumulate phosphate at a rate similar or higher than that of heterotrophic bacteria. This was shown by an accumulation of up to 17.12 mgP/ℓ by isolate Ac-27 (Fig. 3) that is close to that of 17.5 mgP/ℓ by *Acinetobacter lwoffie* observed by Sarioglu (2005), but it was lower than the 28.5 mgP/ℓ and 74.4 mgP/ℓ removal observed by Machnika et al. (2005) using a mixture of filamentous bacteria found in foam. The reason for high phosphate accumulation by foam filamentous bacteria may be due to the presence of diverse and mixed filamentous PAOs populations therein, as opposed to pure cultures used in the current investigation.

Other PHB (Ac-22, Ac-30 and Ac-34) isolates showed weak phosphate accumulation of between 0.567 mg/ℓand 2.139 mg/ℓ (Fig. 3). Phosphate accumulation of between 0.567 mg/ℓ and 2.139 mg/ℓwas also observed for glycogen positive isolates (Gl-10 and Gl-24) (Fig. 4).

TABLE 2
Nitrate reduction batch test results in mg/ℓ

Org. no.	At time 0 h (mg/ℓ)	After 6 h (mg/ℓ)	N reduced (mg/ℓ)
16	23.90	5.44	18.46
31	16.27	3.53	12.74
11	15.20	2.75	12.45
14	16.17	4.09	12.08
34	15.02	2.94	12.08
13	19.27	7.22	12.05
38	14.89	3.26	11.63
8	15.12	4.43	10.69
21	12.54	2.74	9.80
25	14.19	4.69	9.50
27	9.85	0.44	9.41
17	17.27	8.02	9.25
29	16.88	7.81	9.07
22	9.07	0.15	8.92
30	14.57	5.72	8.85
26	17.29	11.96	5.33
18	13.08	7.76	5.32
12	24.82	19.74	5.08
19	7.29	2.39	4.90
36	16.58	12.05	4.53
37	15.50	11.44	4.06
32	20.71	16.88	3.83
35	19.85	16.23	3.62
28	20.90	17.52	3.38
20	18.92	16.00	2.92
1	22.55	20.04	2.51
23	20.35	17.94	2.41
2	17.16	15.19	1.97
9	20.33	18.50	1.83
7	17.48	15.75	1.73
15	11.09	9.84	1.25
24	20.67	19.48	1.19
33	15.62	14.63	0.99

Conclusions

The results of this study demonstrate what was previously unknown, i.e. that filamentous bacteria can remove nutrients (N and P) at a rate that is higher than or similar to that of heterotrophic bacteria, which are known to be the primary nutrient- removing bacteria. Current findings also contribute to improving our understanding of the behaviour and possible contribution of filamentous bacteria to bulking conditions Although current investigations focused on pure culture and determining the capacity of filamentous bacteria to remove nutrients, the findings serve as a good indication of the potential of these organisms and also substantiate the ability of filaments to remove nutrients *in situ*. Findings of this research also validate the current thinking, i.e. enhanced biological phosphorus removal in activated sludge systems is not solely attributed to a single group of micro-organisms but rather a collective function of diverse groups of bacteria including filaments.

Current work

All the isolates are currently being identified with the application of a molecular technique, fluorescent *in situ* hybridisation (F*IS*H) and their identity are being confirmed by genetic sequencing of the DNA for genetic-level identification.

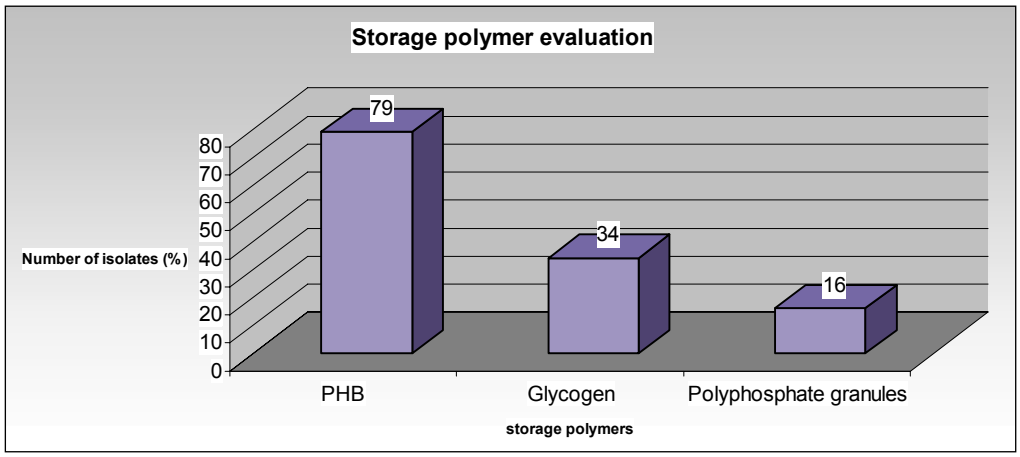

Figure 2
Storage polymer results in percentage

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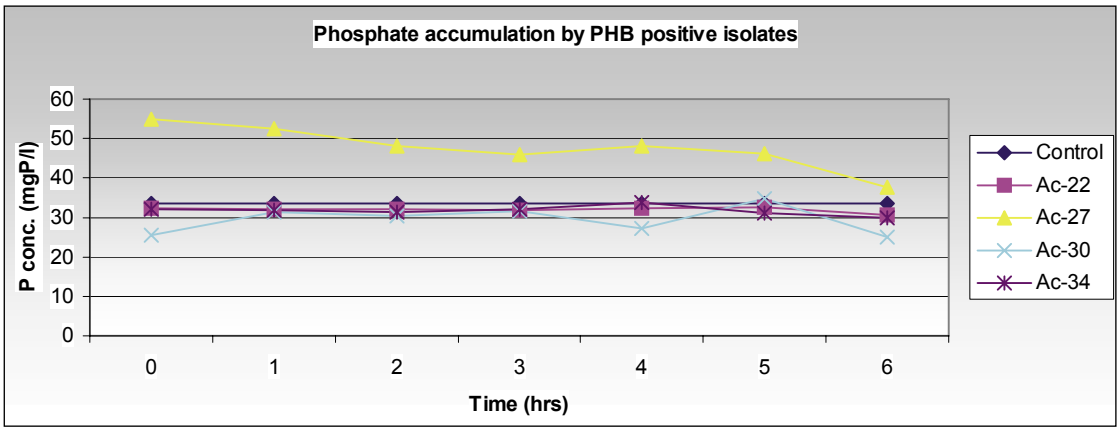

Figure 3
Phosphate accumulation by PHB positive isolates on acetate

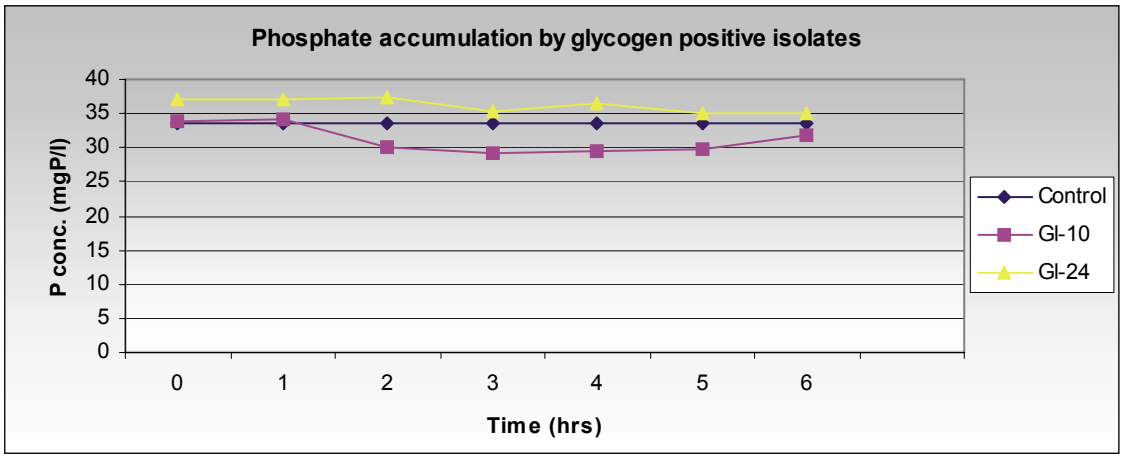

Figure 4
Phosphate accumulation by glycogen-positive isolates on glucose

Recommendations

It is recommended that the BNR system be manipulated in such a manner that the ability of filamentous bacteria to remove nutrients be optimised to achieve better BNR, without compromising the rest of the activated sludge system and without inciting bulking or causing any system perturbations . Future research should focus on determining the role of PAO's and denitrifiers *in situ* collectively, and using novel molecular techniques such as combined Fluorescent *in situ* hybridisation – microautoradiography (F*IS*H-MAR) to elucidate the roles of these microorganisms in biological nutrient removal.

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