Biological nitrate removal from synthetic wastewater using a fungal consortium in one stage bioreactors

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Abstract

A series of lignocellulosic fungi, capable of cellulase and/or xylanase production, were isolated from soil to be used for cellulose degradation and nitrate removal from nitrate-rich wastewater in simple one-stage anaerobic bioreactors containing grass cuttings as source of cellulose. The fungal consortium, consisting of six hyphomycetous isolates, some of which belong to the genera *Fusarium*, *Mucor* and *Penicillium*, was able to remove a significant portion of the nitrate from the treated water. The results were obtained for three bioreactors, i.e. FR, FRp and AFRp, differing in volume and mode of grass addition. Bioreactor AFRp received autoclaved grass, instead of non-autoclaved grass containing natural microbial consortia, as supplied to FR and FRp. Nitrate removal in FR amounted to 89% removal efficiency, while this was 65% and 67% in FRp and AFRp, respectively. The residual chemical oxygen demand (COD) concentration in FR was higher than 600 mg/ℓ, while it was 355 and 379 mg/ℓ in FRp and AFRp, respectively. The similar nitrate removal results for AFRp and FRp indicated that the micro-organisms attached to grass cuttings did not seem to affect the nitrate removal in the reactor. This observation has led to the conclusion that the fungal consortium was, except for being able to degrade cellulose within the grass cuttings, also responsible for nitrate removal from the synthetic nitrate-rich wastewater.

Keywords: bioreactors, acetate, cellulose, COD, fermentation, fungi, nitrate removal

Introduction

Elevated nitrate (NO_3^-) concentrations in groundwater, ranging from 150 mg/ℓ to 850 mg/ℓ (NO_3^--N), are a threat to South African communities relying on groundwater as drinking water (Tredoux, 1993; Meyer et al., 1997; Tredoux et al., 2001). Typical toxic responses to nitrate exposure are methaemoglobinaemia (Ergas and Reuss, 2001), abortion and still-born babies (Bruning-Fann and Kaneene, 1993). Therefore simple- to-operate and cost-effective treatment technologies for nitrate removal from nitrate-contaminated groundwater should be investigated

Robertson and Cherry (1995) as well as Blowes et al. (2000) demonstrated passive *in situ* nitrate removal methods that are mechanically simple and require little maintenance. Waste lignocellulose-containing solids, such as sawdust, grass cuttings and leaf compost, provided the carbon source for heterotrophic denitrification. Filamentous soil fungi of the genus *Fusarium* are known to utilise such waste materials as carbon source, and are also known for their significant denitrification rates (Guest and Smith, 2002; Shoun et al., 1992). Since the first empirical evidence was obtained on fungal denitrification, numerous biochemical and molecular studies were conducted on this phenomenon, using pure cultures of various fungal taxa obtained from culture collections (Shoun and Tanimoto 1991; Shoun et al., 1992; Kobayashi et al., 1996; Shoun et al., 1998; Tsuruta et al., 1998; Zhou et al., 2002; Kumon et al., 2002; Watsuji et al., 2003).

Recent studies have indicated that, utilising organic carbon compounds as electron source, fungal dissimilatory nitrate reduction may occur via two distinct energy-generating pathways (Takaya, 2002). The first respiratory nitrate denitrification occurs under hypoxic conditions and is catalysed by the sequential reactions of nitrate reductase and nitrite reductase, resulting in the formation of nitric oxide – the latter is then reduced via the action of nitric oxide reductase resulting in the formation of the gas nitrous oxide. The second pathway, i.e. ammonia fermentation, occurs under anaerobic conditions and comprises the reduction of nitrate to ammonium, coupled with substrate-level phosphorylation and the catabolic oxidation of electron donors, such as alcohol to acetate (Zhou et al., 2002).

With the above as background, the aim of this study was to isolate a series of lignocellulosic soil fungi, screen them for cellulase and xylanase production, as well as for anaerobic growth. Subsequently, a consortium of these isolates was evaluated for the ability to degrade cellulose, to produce acetate and remove nitrate from a nitrate-containing feed water in a simple one-stage reactor system containing grass cuttings as source of cellulose.

Materials and methods

Fungal isolation

Moist chambers (1 ℓ conical flasks) for fungal isolation were prepared by wetting 5 g autoclaved (121°C; 15 min) wheat-straw with 15 mℓ filter-sterilised (Sartorius, Minisart; pore size 0.2 µm) nutrient solution consisting of 6.7 g/ℓ yeast nitrogen base (YNB, Difco, ref. no. 239210) supplemented with 200 mg/ℓ filter-sterilised chloramphenicol (Sigma, Cat. No. C0378-25G). The wetted straw was inoculated with 1 g compost-rich soil. Five such aluminium-capped moist chambers were each incubated at 25ºC within a 20 ℓ plastic bag, along with a 1 ℓ conical flask containing 100 mℓ distilled water. After 4 to 14 d of incubation isolation of fungi was conducted by aseptically transferring

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TABLE 1
Identity, characteristics and inoculum size of each fungal isolate in the fungal consortium evaluated for removal of nitrate from the fermentation reactors

Isolate no.	Taxon	+Genbank accession number	* CMC	* RBB-xylan	Anaerobic growth	Dry weight of inoculum (g)
V8	*Penicillium corylophilum*	EF451579	16	15	-	0.19
V20	*Penicillium citrinum*	EF451580	8	0	-	0.19
V25	*Hyphomycetous* sp.	-	8	0	-	0.06
V30	*Mucor circinelloides*	EF451581	2	7	+	0.13
V33	*Hyphomycetous* sp.	-	2	7	-	0.07
V45	*Fusarium oxysporum*	EF451582	7	0	+	0.30
						Total inoculum: 0.96

+ Accession numbers at Genbank (www.ncbi.nlm.nih.gov/Genbank) of the PCR products obtained for isolates representing the bulk of the inoculum.
** Diameter (indicated in mm) of clearing zones observed on the CMC and the RBB-xylan plates, respectively indicating the relative activity of cellulases and xylanases.*

developing fungal mycelia to malt extract agar (MEA, Biolab, Merck Chemicals) plates and incubating them at 30ºC for 7 d. Single-spore cultures of the fungal isolates were then prepared and maintained on MEA slants at 22ºC.

Screening for fungal cellulase and xylanase activity

Agar plates containing CMC (carboxy methyl cellulose, Sigma Cat. No. C-5678) (De Koker et al., 2000) and RBB-Xylan [remazol brilliant blue (RBB, Sigma Cat. No. R-8001) covalently linked to Beechwood 4-O-methyl–D-glucurono-D-xylan (Sigma Cat. No. X-0502)] (Farkas et al., 1985), respectively, as indicator media were employed to screen potential fungal isolates for activity of cellulases and xylanases – the two major hydrolytic enzyme groups associated with wood degradation. The centre of RBB-Xylan and CMC plates was inoculated with plugs (5mm diameter) of one-week-old fungal growth on MEA, and incubated at 30ºC for 1 to 4 d until fungal growth covered a third of the plate's surface. Xylanase activity was indicated by pale clearing zones surrounding fungal colonies on the RBB Xylan plates. Clearing zones indicating cellulase activity were visible only after flooding (for 15 min) with 0.1% aqueous azo dye congo red (New India Chemical Enterprises, Cochin-24), followed by flooding (for 2 to 3 h) with 1N NaCl at 22ºC. Six fungal isolates selected as potentially the best producers of cellulases and xylanases, as indicated by the size of the clearing zones, were thereafter screened for the ability to grow under anaerobic conditions.

Screening for anaerobic fungal growth

Cultures of each of the six selected fungal isolates were prepared by inoculating a semi-synthetic medium contained in sterile Petri dishes with a plug (0.5 cm diameter) of one-week-old growth on MEA. The semi-synthetic medium consisted of yeast extract (Biolab from Merck (Pty) Ltd, South Africa) (1.0 g), $MgSO_4.7H_2O$ (0.4 g), glucose (Saarchem from Merck (Pty) Ltd, South Africa) (10.0 g), casamino acids (2.0 g; Bacto, Ref. No. 223050), neopeptone (2.0 g; Difco, ref. no. 223050), agar (18.0 g; Biolab from Merck (Pty) Ltd, South Africa), Cat. No. HG000BX1.500), $NaNO_3^-$ (6.0 g), KCl (0.5 g), KH_2PO_4 (1.5 g), ZnSO4.7H_2O (44.0 g), H_3BO_3 (22.0 g), $MnCl_2.4H_2O$ (10.0 g), $FeSO_4.7H_2O$ (10.0 g), $CoCl_2.6H_2O$ (3.4 mg), $CuSO_4.5H_2O$ (3.2 mg), $Na_2MoO_4.2H_2O$ (3.0 mg), EDTA (100.0 mg; Cat. No. 223 60 20 EM), and was dissolved in 1 000 mℓ H_2O. All mineral salts used were analytical reagent grade and obtained from major retailers. The inoculated plates were incubated at 22°C in anaerobic jars (Oxoid, Hants in UK), in combination with gas generating kits (anaerobic system BR0038B, Oxoid). Aerobic control plates for each isolate were also prepared. After one week of incubation the plates were screened for the presence of developing fungal colonies.

Identification of fungal isolates

Preliminary identification of the six selected isolates was achieved by using the keys and descriptions of Domsch et al. (1980). Subsequent identification with molecular techniques was achieved by extracting genomic DNA from those isolates representing the bulk of the inoculum to be used for nitrate removal from the synthetic wastewater (Table 1), followed by analyses of the internal transcribed spacer (ITS) region of the ribosomal genes.

Biomass for DNA extraction was obtained by harvesting fungal growth in malt extract broth, incubated at 22°C for 5 d, using miracloth (Calbiochem). The biomass was subsequently subjected to quick-freezing in liquid nitrogen, and stored overnight at -80°C before genomic DNA was isolated using the method of Raeder and Broda (1985). The ITS region was amplified by the polymerase chain reaction (PCR) using universal ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') oligonucleotide primers as described by White et al. (1990). The PCR reactions were performed by using Taq polymerase (Fermentas) in a Perkin-Elmer 2400 thermal cycler. The conditions under which the PCR reactions were performed are as follows: denaturation for 2 min at 95°C and 30 s at 94°C, followed by 30 cycles of annealing for 30 s at 55°C, elongation for 1 min at 72°C, denaturation for 1 min at 94°C followed by a final elongation step of 5 min at 72°C. The PCR products were purified by column chromatography (Nucleospin^R Extract II, Separations) and sequenced using a Perkin Elmer ABI PRISM Model 3100 genetic sequencer. The data from the forward and reverse sequences were compared and aligned by using DNAMAN for WINDOWS Version 4.13 (Lynnon Biosoft). The fungal isolates were identified by comparing known sequences using the BLAST program of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast). Information on the sequences representing the fungal isolates was subsequently submitted to Genbank (www.ncbi.nlm.nih.gov/Genbank).

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Evaluation of the fungal consortium to remove nitrate from nitrate-rich feed water

Inoculum preparation

One-week-old fungal growth of each of the six selected fungal isolates, suspended in autoclaved (121°C; 15 min) 100 mℓ malt extract broth (Biolab, Merck Chemicals) that was contained in a sterile 1 ℓ conical flask and incubated on a rotary shaker (160 r/min; throw 50 mm) at 25°C, was used to inoculate the three fermentation reactors (FR, FRp and AFRp). The dry weight of the inoculum representing each strain, determined according to the analytical procedures as described for VSS in *Standard Methods* (1985) is listed in Table 1.

Cellulose degradation by the fungal consortium in absence of nitrate in cellulose degradation reactor (CDR)

To test the cellulose degradation ability of the fungal consortium, 600 mℓ fungal consortium was mixed with 500 g grass cuttings in an anaerobic reactor (Vol. 2 ℓ) The contents of the grass reactor were mixed on a daily basis after which samples were taken. The samples were filtered and analysed for the VFA concentration and pH. Tap water (200 mℓ/d) was added to replace the volume in the reactor due to sample taking.

Fermentation reactor (FR) with two (non-autoclaved) grass additions

The fermentation reactor (FR) with a volume of 20 ℓ (Fig.1) was fed with nitrate-rich (NO_3^--N: 583 mg/ℓ) water through an inlet at the bottom of the vessel. The effluent was discharged at the top of the reactor. A 2 ℓ perforated container filled with grass cuttings was mounted inside the FR. A recycle stream was pumped from the top of the FR through the perforated container, so that the recycle water contacted the partly degraded grass for optimal COD, e.g. acetate, generation (Fig. 1).

The experiment was initiated by mixing inocula from the six fungal isolates with the grass inside the basket of the reactor resulting in a total of 600 mℓ of fungal inoculum, representing *ca.* 1.0 g (dw) fungal mycelium (Table 1). The reactor was initially operated under batch-mode conditions until nitrate removal was observed. Thereafter FR was fed continuously with nitrate-rich feed water. Fresh grass cuttings (250 g) were added on Day 2 and Day 25. The total experimental period was 45 d.

Fungal fermentation reactor (FRp) receiving non-autoclaved grass

In a repeat study FR was operated for 110 d similar to the methodology explained above, but now grass cuttings were added on a more regular basis. Initially, 700 g grass cuttings (35 g grass/ℓ) were added to FR, whereafter 25 g grass was added on Days 14, 23, 30, 37, 43, 46, 51, 55 and 65. From Day 67 to Day 75 the grass addition was 50 g/d, and 100 g/d from Day 75 to Day 110. In this (repeat) study FR was referred to as FRp

Fungal fermentation reactor (AFRp) receiving autoclaved grass

A 2 ℓ bioreactor (AFRp) was operated similarly to FRp, but with the grass cuttings autoclaved prior to addition to bioreactor

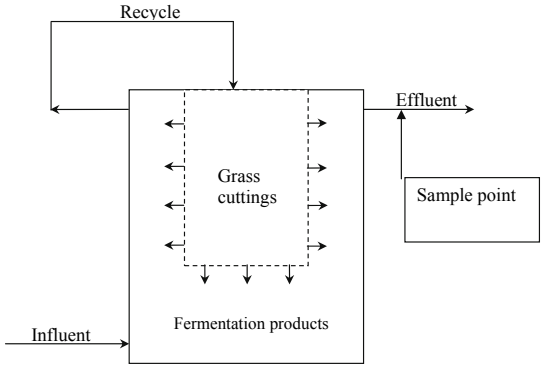

Figure 1
The configuration of the fungal fermentation reactor (FR) used in this study

AFRp. Such elimination of all microbial activity from the grass would indicate the extent to which the fungal consortium was the sole contributor to the denitrification process occurring in FRp. The experimental period of AFRp was 129 d. The AFRp reactor initially received 100 g grass/2 ℓ (50 g grass/ℓ). Fresh grass (40 g) was added on Days 9, 28, 39, 44, 52, 56, 59, 65, 71 and 80. Daily grass addition was 10 g/d from Days 87 to 94, and 20 g/d from Days 95 to 129.

Periodic analyses

Manual determinations of nitrate, COD and pH were carried out according to the analytical procedures described in *Standard Methods* (1985). With the exception of the pH determination, all analyses were carried out on filtered samples (Whatman No.1).

VFA analyses were conducted using a gas chromatograph (Hewlett Packard HP 5890 Series II) equipped with a flame ionisation detector (FID), while the data were analysed using the Agilent ChemStation™ software. The column used was an HP-FFAP, 15 m x 0.530 nm, 1 µm. The GC/FID programme can be summarised as follows: initial oven temperature 30°C, for 2 min, temperature programmed to increase thereafter from 80°C to 200°C at 25°C/min, with temperature hold for 1 min at 200°C, FID temperature 240°C. The carrier gas (N_2) flow rate was set at 1 mℓ/min.

Results and discussion

Identity and characteristics of fungal isolates

The six fungal isolates showing superior cellulase and xylanase activity were representatives of *Mucor* and the *Hyphomycetes.* Some of the latter belonged to the genera *Penicillium* and *Fusarium* (Table 1). Screening for anaerobic growth on semi-synthetic medium revealed that the isolates representing *Mucor* and *Fusarium* were capable of both aerobic and anaerobic growth, while the isolates representing *Penicilium* and an as yet unidentified hyphomycetous species was capable of aerobic growth only.

Cellulose degradation of the fungal consortium in absence of nitrate

Cellulose degradation using the fungal consortium showed that acetate (160 mg/ℓ after 30 d) was produced from grass cuttings to which the fungal consortia had been added (Fig. 2). A number of fungal species are well known for their ability to degrade grass cellulose, producing acetate (Lynd et al., 2002). This acid can be used as electron donor for the biological nitrate removal (Eq. (1)) (Metcalf and Eddy, 1991; Akunna et al., 1993)

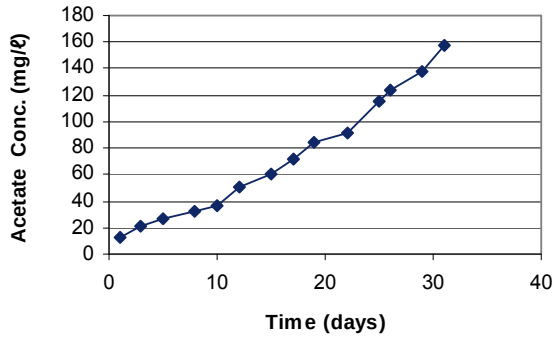

Figure 2
Acetate production from grass cuttings and fungal consortium

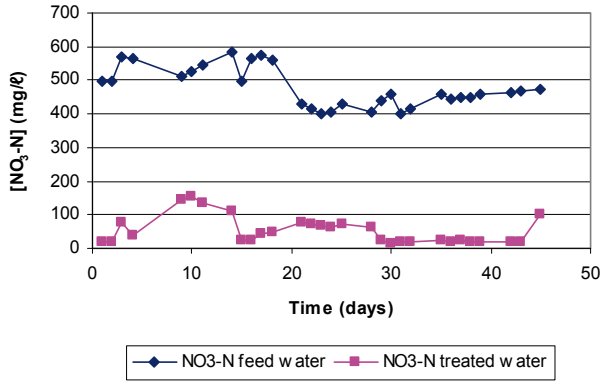

Figure 3
Nitrate concentration in feed and treated water in FR over 45 d

$$8\ NO_3^- + 5\ CH_3COOH \rightarrow 4N_2 + 10\ CO_2 + 6\ H_2O + 8OH^- \quad [1]$$

Fermentation reactor (FR) with two (non-autoclaved) grass additions

Recently, fungi have been recognised to perform denitrification at even greater rates than bacteria (Guest and Smith, 2002). Shoun et al. (1992) found that 9 of the 39 fungi tested for the ability to denitrify, showed complete denitrification to nitrogen gas. In most cases nitrous oxide (N_2O) was the product of the denitrification of nitrate or nitrite. In our study nitrate removal was observed in FR (Fig. 3), with the percentage nitrate removal approaching 100% between Days 29 and 45, and averaging 89% during the entire experimental period. The graphs in Fig. 3 show that the nitrate concentration in the treated water of FR was generally lower than 100 mg/ℓ, and notably between Days 29 and 45, when the nitrate concentration in the treated water was < 25 mg/ℓ. The reactor COD concentrations exceeded 500 mg/ℓ during the entire experimental period, mostly being > 800 mg/ℓ (Fig. 4), which enabled continuous nitrate removal in FR. These results indicated a strong relationship between high reactor COD concentrations resulting in high nitrate removal efficiency. This finding indicated that part of the available COD, e.g. acetate, was readily usable by the nitrate-removing fungi. The relatively high residual COD concentration comprising other cellulose degradation products, were seemingly not utilised by the fungi for nitrate removal. It can be observed from the VFA profile in FR over time that mainly acetic acid was produced (600 mg/ℓ), which decreased rapidly due to being utilised for nitrate removal (Fig. 5). When fresh grass was added on Day 25, only acetic acid was produced and utilised for nitrate removal during the next 10 d.

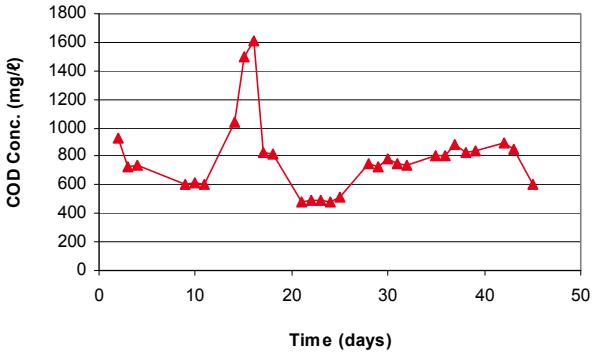

Figure 4
COD concentration in FR

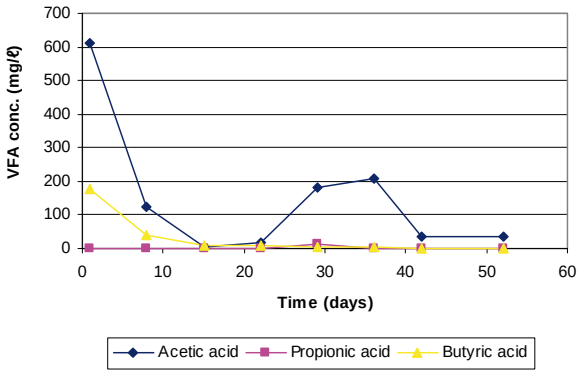

Figure 5
VFA concentration in FR

Fermentation reactor (FRp) with periodic (non-autoclaved) grass additions

The overall percentage nitrate removal efficiency in FRp was 65%, notably less than the 89% nitrate removal efficiency recorded for FR. This result can be ascribed to the lower COD concentration in reactor FRp, which was ca. 50% of the COD concentration in FR (Figs. 4 and 6a). It can be observed from Fig. 6a, that the COD concentration in the reactor fluctuated considerable. It was high on days when fresh grass was added, while it decreased due to nitrate removal. The obtained results indicated that in order to achieve sustained nitrate removal, a high reactor COD concentration is required. No residual acetate, propionate or butyrate was detected in FRp during the experimental period, indicating that all VFA produced was utilised as substrate for nitrate removal. The residual COD concentration, measured in the effluent, comprised other cellulose degradation products, which were not utilised by the fungal consortium as carbon and energy source.

Fermentation reactor (AFRp) receiving autoclaved grass periodically

It can be observed from the graphs in Figs. 7 and 8 that the overall percentage removal efficiency in FR was 65%, while this was 67% in AFRp. These results indicate that nitrate was indeed removed by fungal consortia, since all grass added to AFRp was autoclaved prior to addition to the reactor. The similar results obtained from both reactors FRp (non-autoclaved grass) and AFRp (autoclaved grass) implied that the denitrifying microbes naturally occurring on the grass played a negligible role in removing nitrate from FRp.

The nitrate removal process carried out by fungi may be ascribed to ammonia fermentation known to occur in anaerobic

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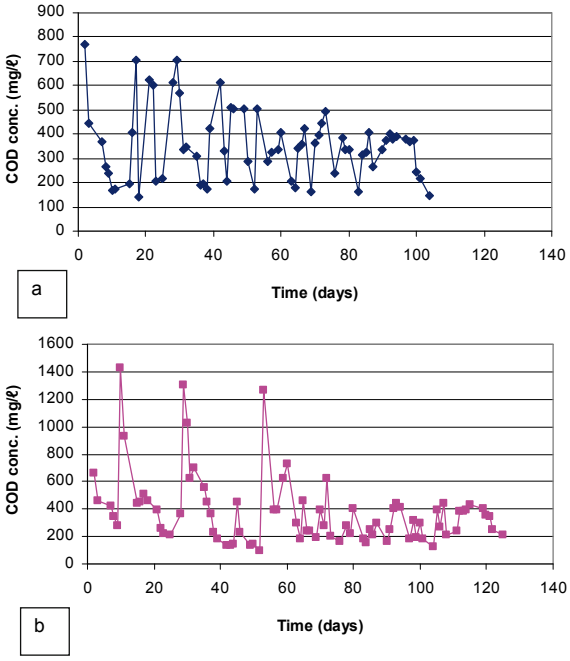

Figure 6
COD concentration in FRp and AFRp during the experimental periods

cultures of *Fusarium oxysporum*, comprising the reduction of nitrate to ammonium coupled with substrate-level phosphorylation and the catabolic oxidation of carbon-containing electron donors (Zhou et al., 2002). With the exception of denitrification of NO_3^- by *Fusarium*, most fungi reduce only NO_2^-, with N_2O being the major denitrification product. However, the unique characteristic of fungal co-denitrification, in which fungi can utilise nitrogen compounds other than nitrate/nitrite, and still produce N_2O and N_2, renders fungi more recalcitrant to inhibition (Kumon et al., 2002; Guest and Smith, 2002). This phenomenon is as yet unidentified in bacteria and empirical data suggest that the mechanism of the process differs among fungal groups.

The VFA concentrations in FRp and AFRp were mostly too low to be detected. After an initial stage when the COD concentration in AFRp periodically exceeded that in FRp, similar COD concentrations (ca. 400 mg/ℓ) were observed in both reactors FRp and AFRp (Figs. 6a and 6b). It can be observed from the graphs in Figs. 6a and 6b that fluctuations in COD concentration correlated to the grass addition. Initially the increase in the COD concentration in AFRp was even more pronounced than in FRp, with a higher level of COD being produced than needed for denitrification. When less grass was added more regularly to the reactors, the COD concentration in both reactors became more stable but presumably too low for increased nitrate removal. Figure 6b shows that after Day 60, the COD concentration in AFRp was on average 400 mg/ℓ, which proved to be too low for sustained nitrate removal. When comparing the COD concentration in FR to that in FRp and AFRp, it is evident that in FR a sustained COD concentration could be maintained, while 250 g grass was added only twice. The difference may be ascribed to the longer experimental periods for FRp and AFRp (110 and 129 d, respectively, compared to 45 d for FR). It can be hypothesised that the fungal consortium lost its cellulose degradation ability with time. It was observed in Figs. 6a and b, that the COD concentrations in FRp and AFRp decreased with time. The results in FR indicated that a residual COD concentration in the treated water is essential for sustained nitrate removal. This corroborates results from other investigations where an additional electron donor was required *in situ* for effective denitrification to proceed (Ergas and Reuss, 2001; Hendriksen and Ahring, 1996; Nollet and Verstraete; 1996; Korom, 1992).

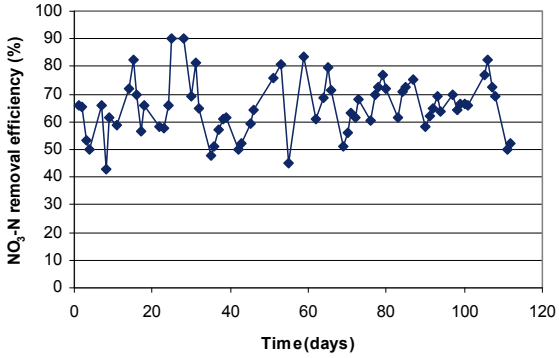

Figure 7
NO_3^--N removal efficiency (%) in FRp over 110 d

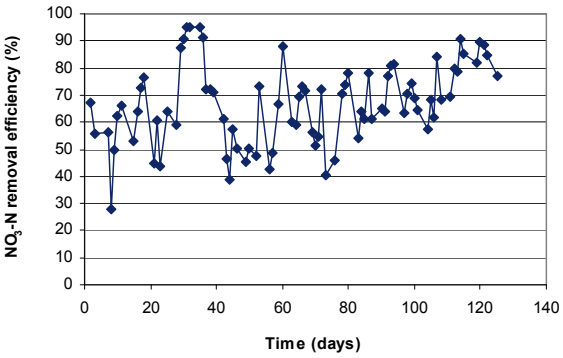

Figure 8
NO_3^--N removal efficiency (%) in AFRp over 125 d

Evaluation of bioreactor performance in terms of nitrate removal during the entire experimental periods

The results, based on the averages of daily sample analyses obtained for the different parameters measured in the three bioreactors, i.e. FR, FRp and AFRp, are listed in Table 2. All three reactors showed satisfactory nitrate removal: 89% of the nitrate was removed in FR, while the percentage nitrate removal efficiency in FRp and AFRp was 65% and 67%, respectively. These percentage removal values showed a positive correlation with the COD concentrations in the reactors, which were 790 mg/ℓ, 355 mg/ℓ and 379 mg/ℓ for FR, AFRp and FRp, respectively. It can thus be observed from the nitrate removal and the COD concentration in the treated water, that at a relative high residual COD concentration, the percentage nitrate removal increased. These findings support the general contention that organic

TABLE 2
The NO_3^--N removal parameters in bioreactors FR, FRp and AFRp

Parameters	FR	FRp	AFRp
pH	8.13	8.01	8.01
NO_3^--N in (mg/ℓ)	478	390	378
NO_3^--N out (mg/ℓ)	52	135	126
COD (mg/ℓ)	790	355	379
NO_3^-removal (mg/ℓ)	426	255	251
% NO_3^- removal	89	65	67

carbon compounds, measured by COD determination, are used as electron source for fungal nitrate reduction.

Both FRp and AFRp received frequent additions of grass cuttings, but the latter received autoclaved grass instead of non-autoclaved grass containing consortia of naturally occurring microbes. However, similar nitrate removal results were obtained for both these reactors (Table 2). This result indicates that the fungal consortium alone (AFRp) was responsible for the nitrate removal to the same extent as the nitrate removal by a mixed consortium of microbes attached to the grass cuttings, supplemented with the six fungal isolates (FRp) and thus that these six isolates were major contributors to nitrate reduction in the two reactors.

Conclusions

The results of this study showed that:

- A consortium of lignocellulosic soil fungi, capable of cellulase and xylanase production, can degrade cellulose in grass to produce acetate
- The acetate produced could function as reduced organic electron donors for nitrate removal from nitrate-rich feed water using simple one-stage bioreactors
- The nitrate removal efficiency was dependent on the residual COD concentration in the treated water
- High nitrate removal efficiency of 89% was achieved in FR
- Comparable nitrate removal efficiencies of 65% and 67% were obtained in FRp and AFRp, respectively, when FRp received normal grass additions and AFRp received autoclaved grass additions
- The fungal consortium was responsible for nitrate removal in AFRp.

While it has to be taken into account that the reactor effluent still needs to be treated to decrease the residual COD concentration and to remove microbial biomass before potable water can be obtained, this study showed that cellulose degradation and nitrate removal was due to the activity of a consortium of lignocellulosic soil fungi, capable of cellulase and xylanase production.

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