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ORIGINAL PAPER

Biodegradation of acetonitrile by adapted biofilm in a membrane-aerated biofilm reactor

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Abstract A membrane-aerated biofilm reactor (MABR) was developed to degrade acetonitrile (ACN) in aqueous solutions. The reactor was seeded with an adapted activated sludge consortium as the inoculum and operated under step increases in ACN loading rate through increasing ACN concentrations in the influent. Initially, the MABR started at a moderate selection pressure, with a hydraulic retention time of 16 h, a recirculation rate of 8 cm/s and a starting ACN concentration of 250 mg/l to boost the growth of the biofilm mass on the membrane and to avoid its loss by hydraulic washout. The step increase in the influent ACN concentration was implemented once ACN concentration in the effluent showed almost complete removal in each stage. The specific ACN degradation rate achieved the highest at the loading rate of 101.1 mg ACN/g-VSS h (VSS, volatile suspended solids) and then declined with the further increases in the influent ACN concentration, attributed to the substrate inhibition effect. The adapted membrane-aerated biofilm was capable of completely removing ACN at the removal capacity of up to 21.1 g ACN/m^2 day, and generated negligible amount of suspended sludge in the effluent. Batch incubation experiments also demonstrated that the ACN-degrading biofilm can degrade other organonitriles, such as acrylonitrile and benzonitrile as well. Denaturing gradient gel electrophoresis studies showed that the ACN-degrading biofilms contained a stable microbial population with a low diversity of sequence of community 16S rRNA gene fragments. Specific oxygen utilization rates were found to increase with the increases in the biofilm thickness, suggesting that the biofilm formation process can enhance the metabolic degradation efficiency towards ACN in the MABR. The study contributes to a better understanding in microbial adaptation in a MABR for biodegradation of ACN. It also highlights the potential benefits in using MABRs for biodegradation of organonitrile contaminants in industrial wastewater.

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Introduction

Organonitriles are of considerable industrial importance as one of the main raw materials in the synthesis of pharmaceuticals, herbicides, pesticides, rubbers, solvents, and extractants. The group of organonitriles includes compounds such as acetonitrile (ACN), acrylonitrile, and benzonitrile (Banerjee et al. 2002; Henahan and Idon 1971; Kao et al. 2006). ACN is of particular importance because of its much quantities used and the potential environmental impacts (Ahmed and Farooqui 1982; Johammsen et al. 1986). The global industrial consumption of ACN was estimated to be over 4×10^4 tonnes in 2001 (Håkansson et al. 2005). ACN is widely used for the manufacture of drug intermediates (e.g. acetaminophen and chiral synthons), pharmaceuticals, pesticides and synthetic rubbers, and as a solvent in laboratory research (Banerjee et al. 2002; Nawaz et al. 1989). As a consequence, ACN is found as a pollutant in industrial wastewater streams associated with its formulation, distribution, and application. ACN can also be released into the subsurface and contaminate groundwater resources as a result of the hydrolysis of pesticides and herbicides. If discharged into the environment, ACN can pose a significant environmental and public health risk, due to its acute toxicity and its carcinogenic and mutagenic potential (Ahmed and Farooqui 1982; Johammsen et al. 1986; Nawaz et al. 1989; Pollak et al. 1991). For these reasons, investigating technologies/processes that can effectively treat ACN-containing wastewater prior to its discharge into the environment is of great practical interest.

Although chemical methods, such as ozone and photocatalytic oxidation are available for removing ACN from wastewater, the harsh reaction conditions, the production of potentially hazardous secondary pollutants and the high operational costs associated with these methods have limited their widespread use (Muñoz et al. 2005). Bioremediation becomes a viable alternative for ACN removal since ACN has been known to be mineralized by microorganisms under aerobic conditions (Håkansson et al. 2005; Nawaz et al. 1991; Li et al. 2007). Many studies in ACN degradation have been reported, particularly on biodegradation using pure bacterial cultures, such as *Candida guilliermondii* CCT 7202 (Dias et al. 2001), *Comamonas testosteroni* and *Acidovorax* sp. (Wang et al. 2004), *Klebsiella oxytoca* (Kao et al. 2006), *Nocardia rhodochrous* (DiGeronimo and Antoine 1976), *Pseudomonas marginalis* (Babu et al. 1995), *Pseudomonas putida* (Nawaz et al. 1989), *Rhodococcus erythropolis* A10 (Acharya and Desai 1997), *Rhodococcus erythropolis* BL1 (Langdanhl et al. 1996), *R. rhodochrous* PA-34 (Bhalla et al. 1992), *Rhodococcus* sp. N 774 (Endo and Watanabe 1989). However, ACN degradation in bioreactor systems for potential engineering applications remains poorly explored (Dhillon and Shivaraman 1999; Manolov et al. 2005; Muñoz et al. 2005; Nawaz and Chapatwala 1991). This is probably due to the fact that microbial activity is hindered by the acute toxicity of ACN. Furthermore, ACN biodegradation is mainly reported in conventional aerobic systems such as completely stirred tank reactors and attached growth reactors. These reactors mainly use mechanical aeration methods that can result in significant loss of ACN via air stripping (Aronstein et al. 1994; Dhillon and Shivaraman 1999; Manolov et al. 2005; Muñoz et al. 2005).

A membrane-aerated biofilm reactor has been an innovative biomass immobilization method in biological wastewater treatment (Brindle and Stephenson 1996; Casey and Syron 2008). Membrane-aerated biofilm reactors (MABRs) use hydrophobic gas-permeable membranes to provide bubbleless aeration, achieving higher oxygen transfer efficiency, as compared to conventional bubbled diffuser systems, and hence lower the treatment operation cost (LaPara et al. 2006). Due to bubbleless aeration, MABRs also have the added advantage of minimizing the potential stripping loss of volatile organic compounds (VOCs) encountered in conventional aerated biological systems (Casey et al. 1999; Rishell et al. 2004). In addition, the membranes in MABRs serve as the support for biofilm growth as well.

The aggregation of microorganisms onto the biofilm confers additional benefits such as protection against predation and resistance to chemical toxicity.

Moreover, the horizontal gene transfer (HGT) process allows microbial communities to spread existing catabolic pathways and adapt to the presence of xenobiotics in their environment—has been observed in microbial populations immobilized in biofilms (Molin and Tolker-Nielsen 2003; Springael et al. 2002). Since MABRs can provide aerobic conditions for biodegradation and prevent the loss of ACN through stripping, this reactor design is therefore particularly suitable for effective biodegradation of ACN.

In this study, ACN was used as the only substrate to promote growth of aerobic biofilm with step increase in ACN loading rate achieved through increasing ACN concentrations in the influent without the addition of other easily degrading medium to demonstrate the feasibility that aerobic biofilm can be developed in a MABR for effective ACN removal. The work will provide useful understanding on how membrane-aerated biofilm can be deployed for ex situ treatment at sites where industrial wastewater is required to halt the migration of ACN-contaminated plumes. The process can also broaden the benefits of using MABRs to target remediation of recalcitrant organonitriles.

Materials and methods

Reactor operation

A dead-end MABR with a 650 mm height and 55 mm diameter was set up. The MABR had a working volume of 1.54 l and was constructed with Pyrex glass. The reactor contained a membrane module consisting of hydrophobic microporous polypropylene hollow fibers and had a specific membrane surface area of 84.5 m^2/m^3. Bubbleless aeration was carried out at a gas flowrate of 13.1–16.8 ml O_2/min and a trans-membrane oxygen partial pressure of 20.7–27.6 kPa to maintain a dissolved oxygen (DO) concentration at above 1.5 mg/l in the bulk liquid. Centrifugal pumps were used to recirculate the liquid in the reactor at a rate of 2.3 l/min to achieve a completely mixed condition (Re = 4,073) with a mean fluid velocity of 8 cm/s in the reactor. The reactor was operated at 25 ± 1°C in a temperature-controlled room.

The MABR was inoculated with adapted activated sludge reported to degrade ACN (Li et al. 2007). The initial suspended biomass was at a concentration of 2,000 mg VSS/l (VSS, volatile suspended solids). The reactor was operated in a continuous mode from day 1 to day 5 with an initial ACN volumetric loading rate at 0.4 g ACN/l day (0.27 g ACN/l of influent). After 5 days, a thin biofilm was visible on the membrane surface. The reactor was stopped and all the suspended biomass was removed. The reactor was then fed with a synthetic solution containing ACN as the sole carbon and nitrogen source. The synthetic medium had the following composition (per liter): 500 mg KH_2PO_4, 1,100 mg $Na_2HPO_4 \cdot 2H_2O$, 18 mg $CaCl_2 \cdot 2H_2O$, 23 mg $MgCl_2 \cdot 6H_2O$, 26 mg $MnCl_2 \cdot 2H_2O$, 75 mg $FeCl_2 \cdot 4H_2O$, 2.1 mg $NaVO_3$, 1.7 mg $Na_2WO_3 \cdot 2H_2O$, 4.9 mg H_3BO_3, 3.8 mg $NiSO_4 \cdot 6H_2O$, 2.1 mg $ZnCl_2$, 1.8 mg $CoCl_2$, 2.1 mg $Na_2MoO_4 \cdot 2H_2O$ and 2.9 mg $CuSO_4 \cdot 2H_2O$ (Li et al. 2007). Different influent ACN concentrations were used during the study. Effluent was discharged at a 0.096 l/h, providing a hydraulic retention time (HRT) of 16 h throughout the MABR system. Washing was carried out at a flow rate of 18 cm/s and performed for 1 min on day 60 and day 90 in order to clean out excess biofilm growth during the MABR operation.

Six step increases in ACN loading were studied for the MABR over a period of 90 days. When the suspended sludge was discharged on day 5 in phase 1, ACN in the solution was not completely degraded, probably due to the small quantity of biofilm mass attached to the membrane. The amount of ACN in the influent in the following operation was again maintained at 250 mg/l. Until the effluent showed a complete removal of ACN, the MABR was subjected to a higher ACN loading by increasing ACN concentration in the influent to 320, 460, 800, 950 and 1,100 mg/l on days 20 (phase 2), 36 (phase 3), 48 (phase 4), 55 (phase 5), 73 (phase 6), respectively. These increases consequently provided the MABR with an average ACN loading rate of 5.12, 6.05, 9.08, 15.27, 18.11 and 21.44 g/m^2 day in phases 1, 2, 3, 4, 5 and 6, respectively. Each step increase in ACN loading was carried out only after complete ACN removal was obtained in the previous step. The complete ACN removal period, referred to as "steady-state" was monitored for at least 5 days.

Analytical methods

Mixed liquor suspended solid (MLSS), VSS, pH, specific oxygen utilization rate (SOUR), and DO were measured according to the standard methods (APHA 1998). To measure ACN concentrations, samples were first centrifuged at 13,200×*g* at 4°C for 20 min and the supernatants were analyzed immediately by gas chromatography (6890N, Agilent, USA) equipped with a Agilent HP-5 5% phenyl siloxane capillary column (30 m × 320 μm internal diameter × 0.25 μm film thickness) and a flame ionization detector as previously described by Li et al. (2007). Total organic carbon (TOC) and total nitrogen (TN) were measured by a TOC-VCSH plus nitrogen analyzers (Shimadhu, Japan). Ammonia, nitrite and nitrate were determined using Hach test kits and a UV–vis spectrophotometer (DR 5000, Hach, USA). Biofilm thickness was determined through a non-invasive method (Freitas dos Santos and Livingston 1995) and the images were captured through a VH-Z75 microscope (Keyence, Japan) via a charge-coupled device that was connected to a computer. Biofilm thicknesses were measured at different locations in the biofilms and averaged from three separate measurements at each location.

Acetonitrile biodegradation rates were examined using biofilm mass samples harvested from the MABR at the steady-state at phase 6. A number of 100 ml serum bottles were each filled with 50 ml of the mineral salt medium solution containing acetonitrile concentrations ranging from 30 to 2,500 mg/l, and inoculated with 40 mg VSS of the biofilm mass with acetonitrile-degrading microorganisms (with a VSS of 400 mg/l). Initially, the serum bottles were purged with nitrogen gas to remove any oxygen from the serum bottles. pH in the serum bottles was adjusted to 7.0 using 1 M H_2SO_4 or NaOH. The serum bottles were then sealed with aluminum caps with PTFE/silicone septum and incubated at 25°C and 180 rpm on a rotary shaker. Sterile control tests (without biomass) to record the abiotic loss of acetonitrile were also incubated under the same conditions. Fifteen milliliter (1 atm) of oxygen were regularly added to each serum bottle with a glass syringe. Liquid samples of 1 ml were periodically taken from each batch serum bottle to monitor the concentrations of acetonitrile. All experiments were performed in triplicate. An analysis in the kinetic degradation data was performed with the Haldane's equation for an inhibition substrate,

$$V = \frac{V_{\max}[S]}{[S] + K_s + [S]^2 / K_i} \quad (1)$$

where V and $V_{\max}$ are the specific and the theoretical maximum specific substrate biodegradation rates (mg ACN/g VSS h), respectively, and S, K_s, and K_i are the substrate concentration, half-saturation constant, and inhibition constant (mg ACN/l), respectively. Furthermore, the ability of the biofilm mass to degrade acrylonitrile, and benzonitrile compounds was also evaluated on day 90 according to the method described above.

Scanning electron microscopy

Biofilm samples were obtained from the MABR by cutting off the hollow fiber membrane with biofilm grown on it. The samples were soaked in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer saline (PBS) (130 mM NaCl and 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) for 4 h. The samples were then washed three times for 20 min each in a 0.1 M sodium cacodylate buffer. The fixed samples were subsequently dehydrated in a series of 50, 70, 80, 90, 95, and 100% ethanol solutions for 10 min each, and stored in 100% ethanol. Samples were then dried in a critical-point drier using liquid CO_2 and coated with gold particles for 40 s before the scanning electron microscopy (SEM) analysis. The coated samples were examined with a SEM (JSM-5310LV, JEOL, Tokyo, Japan) at 20 kV.

DNA extraction

Genomic DNA of the ACN-degrading biofilm mass was extracted according to the method described by Kowalchuk et al. (1997). Approximately 200–300 mg (wet weight) of biofilm mass was harvested in duplicate during the steady state operation of the MABR and used immediately for DNA extraction. This involved bead beating followed by extraction with saturated phenol (pH 8.0), phenol/chloroform (1:1), and chloroform. The amount of extracted DNA was quantified using a UV–vis spectrophotometer (Jasco V-550, Japan) at the wavelength of 260 nm. The extracted DNA was precipitated overnight with a sodium acetate–ethanol mix at −20°C and dissolved

in sterile deionized water. Extracted DNA samples were stored in a −20°C freezer before analysis.

Polymerase chain reaction and denaturing gradient gel electrophoresis

Polymerase chain reaction primers P2 (5′-ATTAC CGCGGCTGCTGG-3′) and P3 (5′-CGCCCGCCGC GCGCGGCGGGCGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG-3′) were used to amplify the variable V3 region of the bacterial 16S rRNA gene (corresponding to positions 341-534 in the *Escherichia coli* sequence) (Muyzer et al. 1993). A touchdown PCR thermal profile technique was performed (Watanabe et al. 1998) with a GeneAmp 2700 PCR System (Applied Biosystems, USA) using a 50 μl (total reaction volume) mixture containing 25 μl of 2× GoTaq Green Master Mix (Promega Co., USA), 1 μl of 10 μM each primer, 22 μl of nuclease-free water, and 1 μl of DNA extract (concentration of 100 ng/μl). Successful PCR was confirmed through a 2.0% agarose gel in 1× TAE buffer solution stained with ethidium bromide.

The PCR-amplified fragments were separated by DGGE using a DCode universal mutation detection system (Bio-Rad Laboratories, USA) as described previously (Muyzer et al. 1993). A 30 ml 40–60% urea–formamide denaturant gradient gel [8% (w/v) acrylamide solution (40% acrylamide and bisacrylamide, 37.5:1 stock solution)] in 50× TAE buffer was used. A 45 μl of PCR amplicons from DNA of biofilm mass samples were loaded in each gel well. Electrophoresis was conducted in 1× TAE buffer solution at 85 V and 60°C for 15 h. After electrophoresis, the gel was stained with ethidium bromide for 15 min and then destained for 1 h with 1× TAE buffer solution, and visualized by a SynGene Bio Imaging system (GeneGenius, UK). SynGene GeneTools software was used for DGGE band pattern analysis. Binary Dice coefficient was applied to calculate the similarity of lane patterns (each lane refers to biofilm mass sample from different sampling day).

Results

Formation of ACN-degrading biofilm and reactor performance

Activated sludge from a pharmaceutical reclamation plant was first acclimated over a 30-day period to allow the biomass to adapt to ACN at the concentration of 200 mg/l. No SOUR activity was detected during the initial incubation, indicating that the unacclimated sludge was unable to oxidize ACN. Then, the adapted sludge gradually showed evident improvements in the ACN degradation activity, and SOUR activity was detected at up to 10.6 mg O_2/g VSS h.

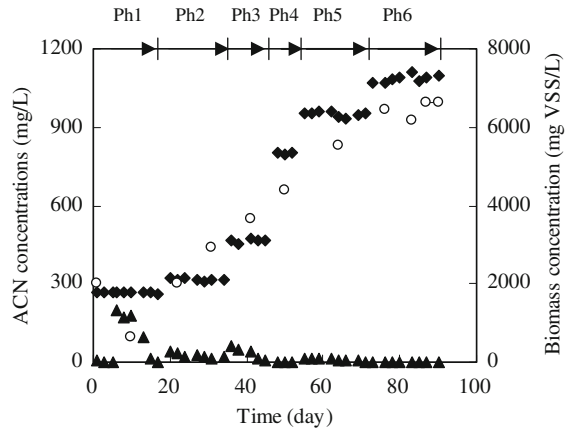

Fig. 1 Profiles of ACN concentrations in influent (◆) and effluent (▲), and biomass concentration in the MABR (○)

Adapted activated sludge with a suspended biomass concentration of 2,000 mg VSS/L was used to seed the MABR for the development of ACN-degrading biofilm. This initial seeding provided the reactor with ACN degradation activity during the first 5 days' biofilm cultivation phase (Fig. 1). In this period of time, the effluent ACN concentration was reduced to below 0.5 mg/l. After 5 days of operation, a thin biofilm was visible on the surface of the membranes. Then, the suspended solids within the MABR were removed and the reactor was restarted. The biofilm eventually grew to become the only form of biomass in the reactor, as evidenced by the gradual increase in the mean biofilm thickness and decrease in the SS of the effluent (Fig. 2).

During phase 1 (day 1–day 19), the MABR was fed with ACN at 250 mg/l. From day 6 to day 13, ACN concentrations in the effluent persisted at levels above 90 mg/l. This was due to the discharge of the suspended solids on day 5, resulting in a sharp decrease in the biomass concentration in the reactor, thus low biological activity (Figs. 1, 2). However, improvements in biofilm mass characteristics and reactor performance became noticeable after this

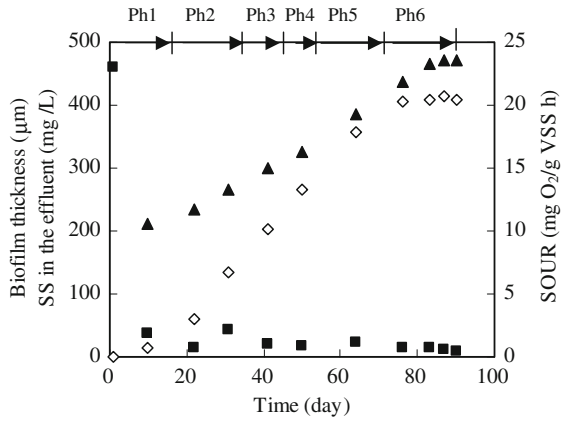

Fig. 2 Profiles of mean biofilm thickness (◇), SS in the effluent (■), and SOUR (▲)

initial biomass attachment period. ACN concentrations in the effluent reduced steadily from day 15 and were below the detection limit (0.4 mg/l) by day 80. Biofilm mass concentrations reached 640 mg VSS/L during phase 1 and increased in the following phases. Similar patterns were also observed in the mean biofilm thickness, with decrease of SS in the effluent during this period. The mean biofilm thickness was 0 on day 0 but increased to 15 μm on day 10. SS in the effluent was 460 mg MLSS/L on day 1 but decreased to below 37 mg MLSS/L from day 10.

The ACN loading of the reactor was increased while the HRT was kept to remain constant at 16 h. Effluent ACN concentrations fluctuated within the first 3–5 days after each step increase in the influent ACN concentration but fell rapidly thereafter in phases 2 and 3. The increases in ACN loading were accompanied with the increases in biofilm mass concentration and biofilm thickness in the reactor, improvements in SOUR and the reduction in SS in the effluent in phases 4, 5 and 6. Nevertheless, the reactor recovered from this initial disturbance, and the increase in biofilm mass concentration and biofilm thickness and the reduction in SS in the effluent improved and reached 6,620 mg VSS/L, 0.41 mm, and 10 mg/l, respectively, at the end of phase 6 (Figs. 1, 2). Increases in ammonia concentration were stoichiometrically proportional to the amounts of ACN degraded. No nitrite or nitrate was detected before phase 4. However, the increases in nitrite and nitrate concentrations were concomitant to the decrease in amounts of ammonia in the reactor effluent after phase 5 with the existence of nitrifying bacteria in the biofilms (data not shown).

Morphological and physiological characteristics of biofilms

Scanning electron microscopy images in Fig. 3 confirm the biofilm mass growth on the surface of the membrane. It can be seen that the biofilm mass had well-distributed channels and pores that would favor the transfer of substrates and oxygen in the biofilm in two opposite directions. The outer surface of the biofilms consisted of rod-shaped bacteria embedded in a large amount of extracellular polymeric substances matrix that enhanced the formation of the biofilm and protected microorganisms against detrimental environment (Borucki et al. 2003; Tolker-Nielsen and Molin 2000). Although ACN is known to be a toxic organic solvent that induces deterioration of microbial cells, the adapted acetonitrile-degrading microorganisms attached to the membrane surface and developed into a biofilm. This

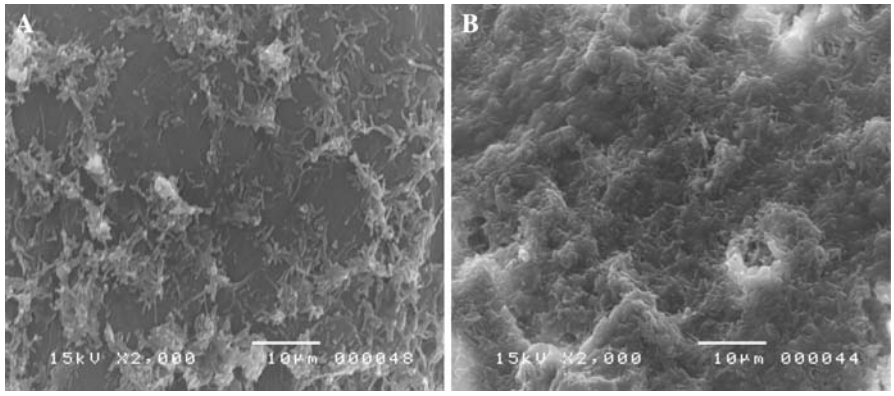

Fig. 3 SEM images of the biofilm mass from the MABR on day 6 (**a**) and on day 90 (**b**). Scale bar: 10 μm

result is in contrast with other studies in MABR where biofilm attachment and formation was found difficult (Casey et al. 1999; Terada et al. 2005).

Specific oxygen utilization rates activity was measured for the reactor biofilm mass in batch incubations with an ACN concentration of 250 mg/l to monitor the change in metabolic activity (Fig. 2). The acclimated sludge on day 0 showed an initial metabolic activity toward ACN, as indicated by a SOUR of 10.6 mg O_2/g VSS h. After day 6, the SOUR values showed an upward trend from phase 1 to 6 and finally stabilized at 23.6 mg O_2/g VSS h from day 76 to the end of the operation study. The steady-state SOUR values achieved at the end of the experiment were approximately twofold higher than the initial values.

Removal capacities of ACN by the adapted biofilm in the MABR were examined to determine the corresponding changes with the increase in loadings (phases 1-6). Specific ACN removal capacities were insignificant during the first 17 days due to the small amount of the adapted biofilm mass on the membrane surface. From day 17 onwards, ACN removal rates increased and reached 5.7 g ACN/m^2 day on day 31 (phase 2), 8.3 g ACN/m^2 day on day 41 (phase 3), 15.3 g ACN/m^2 day on day 50 (phase 4), 17.9 g ACN/m^2 day on day 64 (phase 5) and 21.4 g ACN/m^2 day on day 83 at the last phase 6 (Fig. 4).

The ability of the stabilized biofilm mass to degrade ACN was evaluated by using reactor biofilm

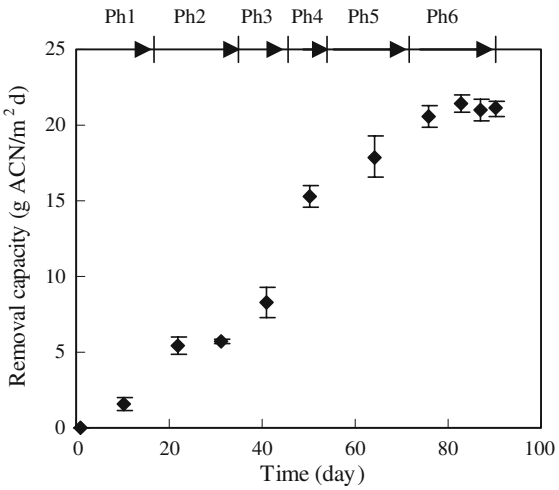

Fig. 4 Profile of acetonitrile removal capacity of membrane-aerated biofilm (*vertical bars* represent the standard deviations calculated from duplicate measurements)

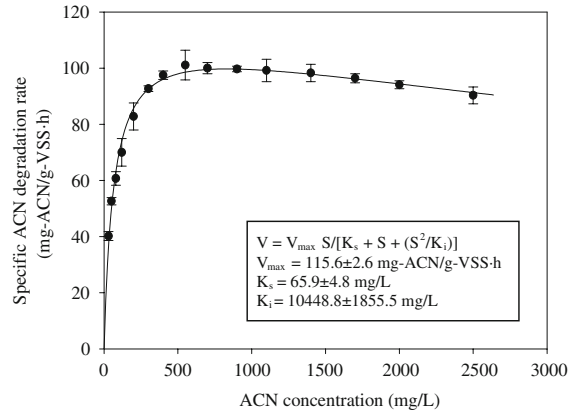

Fig. 5 Specific ACN degradation rates of membrane-aerated biofilms at different ACN concentrations ($R^2 = 0.9879$) (•, experimental results; —, Haldane fit)

mass sampled during days 73–90 (phase 6) to monitor ACN concentration disappearance over time in batch cultures incubated at different ACN concentrations. The specific ACN biodegradation rate increased with the increase of ACN concentrations from 30 to 550 mg/l to achieve the peak of 101.1 mg ACN/g-VSS h. Above 550 mg/l in influent ACN, biodegradation rates showed a mild decline with further increases in the ACN concentration. There was a good fit between the biodegradation data and the Haldane equation, with the following calculated kinetic parameters: $V_{max} = 115.6 \pm 2.6$ mg ACN/g-VSS h, $K_s = 65.9 \pm 4.8$ mg/l, and $K_i = 10{,}448 \pm 1{,}855.5$ mg/l (Fig. 5).

The ACN-degrading biofilms were also able to degrade acrylonitrile and benzonitrile as the sole carbon and nitrogen source (Table 1). The results show that specific degradation rate of 89.3 mg acrylonitrile/g VSS h or 43.6 mg benzonitrile/g VSS h were achieved in the batch bioreactor. Although the rates of acrylonitrile and benzonitrile were lower than those of acetonitrile degradation by adapted biofilms in the current study, these values were compatible to those of Li et al. (2007) who reported the specific degradation rate of acrylonitrile or benzonitrile up to 74 mg acrylonitrile/g VSS h or 29 mg benzonitrile/g VSS h, respectively by suspended activity sludge. Biodegradation pathways of organonitrile observed with the adapted biofilms were similar to those reported by Li et al. (2007) using activated sludge consortia with acetonitrile-degrading microorganisms. Biodegradation of acetonitrile and

Table 1 Degradative capacity and specific oxygen utilization rate by acetonitrile-degrading biofilms consortium on day 90 on various typical organonitriles

Parameters	Substrates		
	Acetonitrile	Acrylonitrile	Benzonitrile
SSDR (mg/g-VSS h)[a]	101.1	89.3	43.6
SOUR (mg O_2/g-VSS h)	23.6	19.4	9.5

[a] Specific substrate degradation rate

acrylonitrile showed a two-step pathway, with the generation of acetamide followed by acetic acid and ammonia for acetonitrile and acrylamide followed by acrylic acid and ammonia for acrylonitrile. However, biodegradation of benzonitrile appeared to be a one-step process, with the direct production of benzoic acid and ammonia, without formation of benzamide. These results indicate that the adapted biofilms possessed a broad substrate utilization capability and exhibited great potential and flexibility for actual applications in bioremediation of various organonitrile compounds.

Characterization of microbial community

Denaturing gradient gel electrophoresis was used to generate genetic fingerprints to provide information on the composition and diversity of the microbial communities in the stabilized ACN-degrading microbial consortium of the biofilm mass in the reactor. DNA was extracted from reactor biofilm mass that was sampled once every few days, from day 73 to day 90 in phase 6. PCR amplification and DGGE analysis of community 16S rRNA genes were performed in replicate on pooled aliquots of extracted nucleic acids. Identical fingerprints were obtained for replicate samples. Figure 6 shows representative DGGE fingerprints of amplified 16S rRNA gene fragments from the reactor biofilm mass. Each band on the DGGE profile corresponded to a gene fragment of unique 16S rRNA sequences and accordingly represented a specific species in the microbial community. The intensity of a band represents the relative abundance of the corresponding microbial species (Fang and Liu 2002; Zhang and Fang 2001). A gradual succession in the microbial community was observed, and the fingerprint patterns were similar for

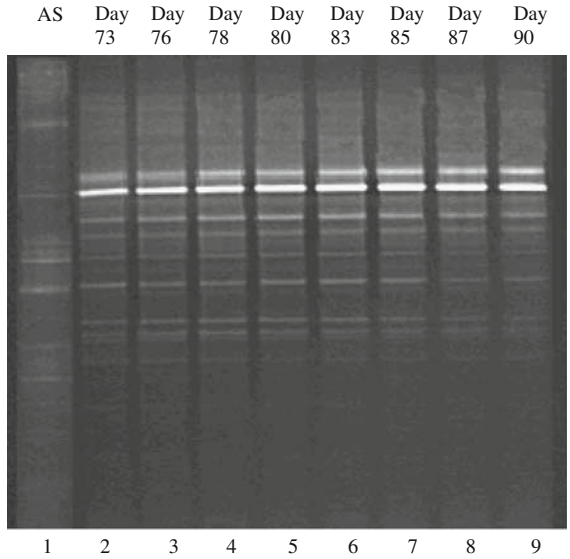

Fig. 6 DGGE fingerprints of PCR-amplified 16S rRNA gene fragments from ACN-degrading biofilm mass. *AS* refers to inoculum of activated sludge. The *number* above each lane stands for the day sampled and the *number* below each lane stands for lane number

different biofilm mass samples, indicating a highly stable microbial community. The biofilm mass characteristics and reactor performance were also consistent with little variation during this period. The matching Dice coefficient analysis clustered the 9 lanes into two groups based on fingerprint similarity. Group 1 contained DGGE fingerprints from seed sludge (AS) (similarity of 0.167–0.288 comparing lane 1 with lanes 2-9) and was associated with reactor start-up; Group II contained DGGE fingerprints from days 73 to 90 and was associated with the development and steady state of biofilm mass (similarity $\geq$0.8 between lane 2 and lane 9) (data not shown).

Discussion

This study shows that it was possible to grow biofilms in a MABR for biodegradation of ACN as the sole carbon and nitrogen source, even though biodegradation of ACN in other reactors often have the problem of stripping loss to the atmosphere, when air bubbling is used as the aeration method (Håkansson et al. 2005; Manolov et al. 2005; Muñoz et al. 2005). ACN loss via stripping can be prevented by using MABRs that achieve bubbleless aeration. In this study, no loss of

ACN was detected since the MABR was dead-ended; therefore all the ACN was biodegraded.

The basis for biofilm formation in the reactor is through a repetitive selection for activated sludge consortium cultivated in the MABR such that adapted components are retained in the system while unadapted and dispersed biomass are washed out. The selection pressures that drive the formation of biofilm can be classified as either microbial or hydraulic in nature. Any reactor system that can exert the appropriate combination of microbial and hydraulic selection pressure should conceivably be capable of achieving biofilm formation. However, it should be pointed out that the successful formation of biofilm is not always to occur (Casey et al. 1999; Rothemund et al. 1996). The successful biofilm formation may depend, for instance, on the acclimation procedure. In this study, a moderate selection pressure was first applied during the start-up stage by operating the MABR with a HRT of 16 h and a low starting ACN concentration of 250 mg/l. The procedure favored the retention of sufficiently active biomass in the MABR by avoiding the combined effect of toxicity and the hydraulic biomass washout. This process is of great significance because microorganisms that are well suited for the treatment of recalcitrant and toxic compounds are usually considered slow growers (Fayolle et al. 2001; Millette et al. 1998).

Substrate concentration can affect the granulation process by selecting and enriching different bacterial species and influencing bioactivity of the granules (Moy et al. 2002). For instance, a higher substrate concentration generally results in a thicker biofilm with a decreased density (García Encina and Hidalgo 2005). A premature increase in substrate concentration before reaching any improvement in settling ability would likely lead to severe biomass loss that could not be adequately compensated by the growth of new biomass (Tay et al. 2005). Therefore, a progressive increase in ACN concentration in the feed was effected only when the reactor had achieved the complete removal of the preceding ACN concentration. By performing timed step increases in ACN loading, the selection pressures that were created allowed the biofilm mass to adapt to the higher ACN concentrations as the sole carbon and nitrogen source without any deterioration in either the biofilm mass or effluent quality. Different strategies have been adopted in previous studies where a cosubstrate was used to boost biofilm growth. For example, the addition of glucose as a cosubstrate along with *p*-nitrophenol has been conducted to promote formation of the *p*-nitrophenol-degrading biofilm or granule for immobilization and also the added glucose was to ensure an adequate acclimation of the attached biofilms for *p*-nitrophenol degradation (Bhatti et al. 2002; Yi et al. 2006).

Biofilm mass harvested from the MABR on day 85 had a maximum specific ACN biodegradation rate of 115.6 mg ACN/g VSS h. This biodegradation rate appears to be higher than those reported in the literature. For example, Li et al. (2007) obtained acetonitrile degradation rates at up to 83 mg ACN/g VSS h in the bioreactor with suspended activated sludge consortium. One explanation was that the repetitive selection of the microorganisms attached to the membrane surface served to adapt the chemical toxicity that might exist in the surrounding milieu.

Acetonitrile biodegradation kinetics in the biofilms was fitted against the Haldane model for substrate inhibition. Although higher ACN concentrations are known to inhibit growth of ACN-degrading microorganisms, the high K_i value of 10,448 mg ACN/l in this study indicates that biofilm microorganisms were more resilient to the inhibitory effects of ACN toxicity. This is consistent with earlier findings that the biofilm structure sheltered microorganisms in the biofilms against the toxicity effects of high ACN concentrations in the surrounding environment (Håkansson et al. 2005; Manolov et al. 2005; Muñoz et al. 2005). Compared to other suspended growth systems or bubbled aeration systems, membrane-aerated biofilms from the MABR are less susceptible to inhibition effects and volatilization loss of substrate and hence can potentially be used to degrade higher concentrations of ACN.

Activated sludge from a pharmaceutical plant was chosen as the inoculum in this study because of its microbial diversity, which increased the likelihood of selection for specific bacterial strains with catabolic capability towards ACN. The reactor biomass successfully acclimated to the presence of increased ACN loads, as evidenced by the corresponding increases in the specific rate of ACN transformation (Fig. 4). Adaptation of microbial communities to the presence of xenobiotics in their environment may arise as a result of a combination of different mechanisms, such as an increase in the population size of the organisms

that degrade the target xenobiotic, mutations of various kinds (e.g., single nucleotide changes or DNA rearrangements) that result in resistance to or degradation of the target xenobiotic, and acquisition of genetic traits by horizontal gene transfer (Spain and Van Veld 1983). Interestingly, there is an evidence to suggest that genes involved in the degradation of ether fuels might be transferred horizontally between bacteria belonging to the *Rhodococcus* group that is known to be capable of degrading ACN (Acharya and Desai 1997; Beguin et al. 2003; Endo and Watanabe 1989; Kobayashi et al. 1990; Langdanhl et al. 1996). Although the extent to which each of the adaptive mechanisms contributed to the development of ACN-degrading biofilm has yet to be determined, it is likely that such adaptation selected individual or groups of microorganisms that best suited to tolerate and degrade ACN, and these microorganisms eventually swept over and dominated the microbial community.

Denaturing gradient gel electrophoresis analysis revealed that the biofilms contained highly stable microbial community. This is probably a result of the selection pressure exerted by the MABR operation, which would favor the enrichment of certain bacterial species to the detriment of others. Furthermore, the toxicity or recalcitrance of ACN is likely to have contributed to the limited diversity observed, as only a handful of isolates have so far been characterized and known to utilize ACN as a sole carbon and energy source (Kao et al. 2006). Hence, many mechanisms could be involved in the enhancement of metabolic efficiency in the adapted biofilms. First, ACN metabolic activity could be enhanced by the retention of specific ACN-degrading microorganisms in the biofilms. Second, this enhancement could be the result of syntrophic interactions between the biofilm populations in the biofilms. Finally, metabolic enhancement could result from an increase of specific degradation activity through the exchange of genetic material among the bacteria in the biofilms.

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