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Biodegradation of nitrilotriacetic acid (NTA) and ferric–NTA complex by aerobic microbial granules

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ABSTRACT

Development of mixed-culture microbial granules under aerobic conditions in a sequencing batch reactor (SBR), capable of completely degrading a recalcitrant metal chelating agent is reported. In laboratory-scale reactor studies, the microbial granules degraded 2 mM of free nitrilotriacetic acid (NTA) and Fe(III)–NTA completely in 14 and 40 h, respectively. Free NTA was degraded at a specific rate of 0.7 mM $(g\,MLSS)^{-1}\,h^{-1}$, while Fe(III)–NTA was degraded at a specific rate of 0.37 mM $(g\,MLSS)^{-1}\,h^{-1}$. Achievement of significant degradation rates of NTA and ferric–NTA in double-distilled water suggests that the microbial metabolism is not constrained by lack of essential elements. Efficient degradation of recalcitrant synthetic chelating agents by aerobic microbial granules suggests their potential application in a variety of situations where heavy metals or radionuclides are to be co-disposed with metal chelating agents.

1. Introduction

Synthetic chelating agents are used in many industrial applications because of their capability to bind and mask metal ions. Amongst these, nitrilotriacetic acid (NTA) is a synthetic organic metal chelating agent whose metal binding properties are exploited in a wide range of applications. These include detergent, food, pharmaceutical, cosmetic, metal finishing, photographic, textile and paper industries. It is also used as a component in decontamination formulations of nuclear reactors and in nuclear waste processing (Bolton et al., 1996; Witschel and Egli, 2001; White and Knowles, 2003). Chemical decontamination processes use one or a mixture of chelating agents such as ethylenediaminetetraacetic acid (EDTA), NTA, picolinic acid and citric acid. Chelating agents can form very strong complexes with metal ions and radionuclides. NTA has found widespread use in conditioning reactor cooling water, as an anticorrosive and scale inhibitor and in cleanup operations (White and Knowles, 2003). Co-disposal of heavy metals or radionuclides along with synthetic organic chelating agents creates environmental problems because the latter may promote undesirable displacement of toxic heavy metals/radionuclides away from the primary disposal site (Bolton et al., 1996; Thomas et al., 1998). Therefore, it is desirable to remove the chelating agents from the wastes prior to disposal. However, synthetic chelating agents and their metal complexes are recalcitrant and resist biodegradation; only a few bacterial strains are able to degrade them, albeit at relatively lower rates (White and Knowles, 2003). Studies on microbial degradation of NTA (White and Knowles, 2000, 2003) and other chelating agents (Witschel and Egli, 2001; Reinecke et al., 2000; Joshi-Tope and Francis, 1995) revealed that metal complexation has a significant negative effect on biodegradability of the chelant.

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As a result, there is significant research interest in developing effective biological treatment process for degrading synthetic chelating agents.

Immobilized cell systems have been successfully employed in biodegradation of harmful wastes for a number of years (Junter and Jouenne, 2004). Since close packing of cells at high cell densities occurs in immobilized cell systems, high volumetric reaction rates can be realized, where such systems are employed for substrate conversion (Nicolella et al., 2000). Immobilization of microorganisms into biofilms or granules is a process that has been effectively put to use for biological treatment of industrial and municipal wastewaters. Aerobic microbial granules are self-immobilized mixed-culture microbial aggregates that form without any substratum or supporting carrier material (suspended biofilms) and are cultivated under defined operating conditions in biological reactors. Cultivation of microbial granules under aerobic conditions has been reported quite recently and is currently under active investigation as the granules permit the design of compact high-rate treatment processes and thus have the potential to improve the process and cost-efficiency of aerobic treatment plants (Bathe et al., 2005). Such aerobic microbial granules have several advantages as compared to conventional activated sludge flocs: they have compact and dense structure, good settling ability, high biomass retention, large specific surface area and the ability to withstand fluctuating organic loading rates (Morgenroth et al., 1997; Etterer and Wilderer, 2001; Liu and Tay, 2004; DeKreuk and van Loosdrecht, 2004). Recently, the potential of these aerobic microbial granules to degrade toxic pollutants such as phenol has been demonstrated in laboratory-scale reactors (Jiang et al., 2002; Tay et al., 2005). However, the ability of aerobic microbial granules to degrade other recalcitrant xenobiotic substances is not known. Thus, the major objective of the present work was to cultivate aerobic microbial granules and to evaluate their degradative ability for treating recalcitrant synthetic chelating agents such as nitrilotriacetate and its metal complexes.

2. Materials and methods

2.1. Cultivation of mixed-culture aerobic microbial granules

A laboratory-scale column-type sequencing batch reactor (SBR) with a working volume of 3.0 l was used for the cultivation of microbial granules. The dimensions of the SBR were: height 1200 mm and diameter 62 mm, height-to-diameter ratio being 19.4. SBR was operated with a minimum settling velocity ((V_s)min) of 13.2 m h^{-1}. Air was introduced at the bottom of the SBR at a rate of 3.0 l min^{-1} (superficial upflow air velocity 5.1 cm s^{-1}) using porous air stones to promote the formation of small air bubbles. The SBR was operated at ambient temperature ($32 \pm 1\,^{\circ}C$) at a volumetric exchange ratio of 66% and 6 h cycle duration with 60 min anaerobic static fill, 282 min aeration, 3 min settling, 10 min effluent decantation and 5 min idle. Synthetic wastewater (SWW) used in the SBR consisted of sodium acetate (63 mM), nitrilotriacetate (0.26 mM), $MgSO_4 \cdot 7H_20$ (3.6 mM), KCl (4.7 mM), NH_4Cl (35.4 mM), K_2HPO_4 (4.2 mM) and KH_2PO_4 (2.1 mM) dissolved in tap water. NTA was prepared as a stock solution (1.0 g l^{-1}) and its pH adjusted to 7.5 by adding NaOH. It was added to the SWW during the cultivation of granules for promoting enrichment of NTA-degrading strains. Addition of the influent (SWW) at the bottom of the sludge bed and the withdrawal of the effluent were performed using peristaltic pumps, which were operated with the help of electronic timers. The dissolved oxygen concentration in the reactor during the aeration phase was maintained at ~7.5 mg l^{-1}. The pH was not controlled but was observed to vary from 7.6 to 8.2. The SBR was inoculated with 800 ml of activated sludge collected from a municipal wastewater treatment plant at Kalpakkam, South India. The specific oxygen uptake rate (SOUR), specific gravity and biomass concentration (mixed liquor suspended solids (MLSS)) were measured according to standard methods (APHA, 1995). The process of sludge granulation was monitored using the sludge volume index (SVI) at different settling times as described previously (Schwarzenbeck et al., 2004). Acetate in the bulk liquid was occasionally measured using gas chromatography as described in Beun et al. (2002).

2.2. Microscopy

Morphology of the developing granules was studied with the help of a stereozoom microscope (Nikon SMZ-1000 equipped with a digital camera) and confocal laser scanning microscope (CLSM, Leica TCS-SP2 AOBS attached to an inverted microscope Leica DM-IRE2). Development of granules was periodically monitored using stereozoom microscope (Nikon SMZ1000) and images were acquired with an attached digital camera (Olympus DP70). The acquired images were processed using the freeware *ImageJ 1.33x* (downloadable from the site http://rsb.info.nih.gov/ij). Particles having size of over 60 μm were used for calculating mean granule size and circularity using *ImageJ*. A minimum of 200 granules were analysed. For confocal imaging, the granules were stained with 0.01% acridine orange for 1 h and thoroughly rinsed with phosphate buffer saline for 15 min. A stained granule was then directly placed on top of a cover slip. A 60 × 1.2 NA water immersion objective was used for acquiring images. The 488-nm line from an Argon laser was used for excitation, the emission was collected by setting the detection bandwidth between 510 and 550 nm.

2.3. Biodegradation of NTA

Biodegradation experiments using NTA and ferric–NTA complexes were performed in graduated glass cylinders (diameter: 7.6 mm; height: 460 mm) having 1.0 l working volume. Fresh granules collected from SBR were used in experiments dealing with repeated cycles of free NTA degradation. Granules incubated for 4 h with free NTA or iron complexed NTA were used in the experiments carried out with 2 mM of free or complexed NTA. The degradation studies were carried out using double-distilled water, with NTA or Fe–NTA added as the sole source of carbon, nitrogen and energy. To test biodegradation of metal–chelant complex, ferric chloride and NTA were mixed in equimolar (1:1) concentration to obtain

the Fe(III)–NTA complex. The final concentration of the free NTA and Fe(III)–NTA complex in the bioreactor at zero hour was 2 mM. Forty millilitres of pre-cultivated (see above) microbial granules were introduced into 1.0 l for the degradation studies. Reactors containing free NTA and ferric–NTA without granules were used as control. The experiments were repeated twice and all experiments were carried out at room temperature ($32 \pm 1\,^{\circ}C$). Oxygen was introduced into the experimental glass cylinders at the bottom at a flow rate of $2.0\,l\,min^{-1}$ by using an air dispenser. Aliquots were withdrawn periodically, filtered through 0.22 μm filters (Millex GS, Millipore) to remove suspended solids and analysed for NTA. Control reactors containing no biomass were operated in parallel in order to exclude NTA-removal mechanisms other than biodegradation.

2.4. NTA analysis

NTA was analysed by spectrophotometry and by high-performance liquid chromatography (HPLC). The spectrophotometric assay was developed to estimate the concentration of NTA in SWW because the HPLC analysis was time consuming for routine analysis. The photometric assay was used for analysis of NTA in the absence of iron, while HPLC was used for analysing NTA in the presence of iron. Prior to sample collection, aeration was stopped and the granules were allowed to settle. Clear liquid sample was collected and stored in a freezer ($-10\,^{\circ}C$) until analysis. Samples containing NTA were incubated in the presence of 10 mM (final concentration) $CuSO_4$, resulting in the formation of copper–NTA complex. After the addition of the reagent, the samples were incubated for 5 min at room temperature; the precipitated complexes of Cu^{2+} with phosphates (present in the growth medium) were removed by filtration through a 0.22 μm filter. Subsequently, the absorption of the filtrate was measured at 305 nm by using a Shimadzu UV–Visible spectrophotometer.

NTA was also analysed through HPLC by the method of Parkes et al. (1981), after some modification. Copper sulphate, instead of cupric nitrate, was used for generating copper–NTA complex, because it does not interfere with the estimation of NTA (cupric nitrate was found to give a small peak very close to NTA peak). An HPLC (Waters 501) equipped with reverse phase column (Bondapak™ C18) and UV–visible detector (Waters 486) was used. The mobile phase consisted of 0.4% tetrabutyl ammonium hydroxide, 10% methanol in double-distilled water; vacuum filtered (0.22 μm) after adjusting the pH to 7.5. Samples for HPLC were prepared as mentioned above for spectrophotometry. Twenty microlitres of the sample were injected into the HPLC column and flow rate was maintained at $1.5\,ml\,min^{-1}$. Copper–NTA complex was detected at 254 nm as a single sharp peak with a retention time of 2.6 min.

3. Results

3.1. Development of aerobic microbial granules

Seed sludge had a morphology consisting of fluffy, irregular and loose flocs. The average size of the seed sludge flocs was approximately 60 μm. The average $SVI_{10\,min}$ and $SVI_{30\,min}$ of the seed sludge were 333 and $219\,ml\,g^{-1}$, respectively. MLSS was $840\,mg\,l^{-1}$. Microscopic examination of the seed sludge showed dominance of filamentous bacteria typical of bulking sludge (Fig. 1A). Following inoculation, the colour of the activated sludge in the SBR gradually changed from black to yellow during the granulation process. The biomass concentration decreased to $480\,mg\,MLSS\,l^{-1}$ and then stabilized at $1210\,mg\,MLSS\,l^{-1}$ on the 13th day of SBR operation. The filamentous growth observed in the reactor during the initial periods disappeared after about 14 days and both $SVI_{10\,min}$ and $SVI_{30\,min}$ decreased to $77\,ml\,g^{-1}$ on the 12th day of reactor operation, indicating transformation of seed flocs into granules. Dense, round granules became prevalent within 22 d after reactor startup (Fig. 1B). Examination of the granules using CLSM showed that they were mostly dominated (surface as well as interior regions) by rod and cocci-shaped bacteria; filamentous bacteria were absent (Fig. 1C). Several cell clusters of nearly spherical shape, consisting of tightly packed bacterial cells were frequently observed within the granule (Fig. 1C). General characteristics of the microbial granules are shown in Table 1.

3.2. Analysis of free and complexed NTA

The samples containing NTA were incubated with the $CuSO_4$ reagent for forming copper–NTA complex before spectrophotometric analysis. $CuSO_4$ reagent alone did not show any absorption at 305 nm. The absorption of copper–NTA complex at 305 nm showed a linear relationship between 0.1 and 3.0 mM of NTA (Fig. 2). The minimum detection limit was about 0.1 mM. The extinction coefficient was determined to be $\varepsilon_{305} = 0.386\,mM^{-1}\,cm^{-1}$.

In the case of HPLC, an already existing method (Parkes et al., 1981) was modified and used for NTA analysis. Use of copper sulphate instead of cupric nitrate improved the sensitivity of photometric and HPLC analysis of NTA. The two methods showed good correlation ($R = 0.98$). Therefore, photometric method was used for routine estimation of NTA in distilled water as well as in microbiological culture media, while HPLC was used for estimating NTA in the presence of iron in the distilled water and in the culture media.

3.3. Reactor performance

Fig. 3 shows the removal of acetate as well as NTA during SBR cycle period. Most of the acetate was consumed in the anaerobic fill period and within the first 30 min of the aeration period. Most of the NTA was removed during the aeration phase, particularly after acetate removal. Complete removal of influent NTA during SBR cycle period was observed during the reactor operation.

3.4. Biodegradation of NTA in batch experiments

In degradation studies carried out using 1.0 l glass cylinders, concentration of NTA was monitored at regular intervals using spectrophotometry or HPLC. Degradation of different initial concentrations of free NTA by microbial granules was studied. Degradation of 0.8 mM of free NTA by microbial

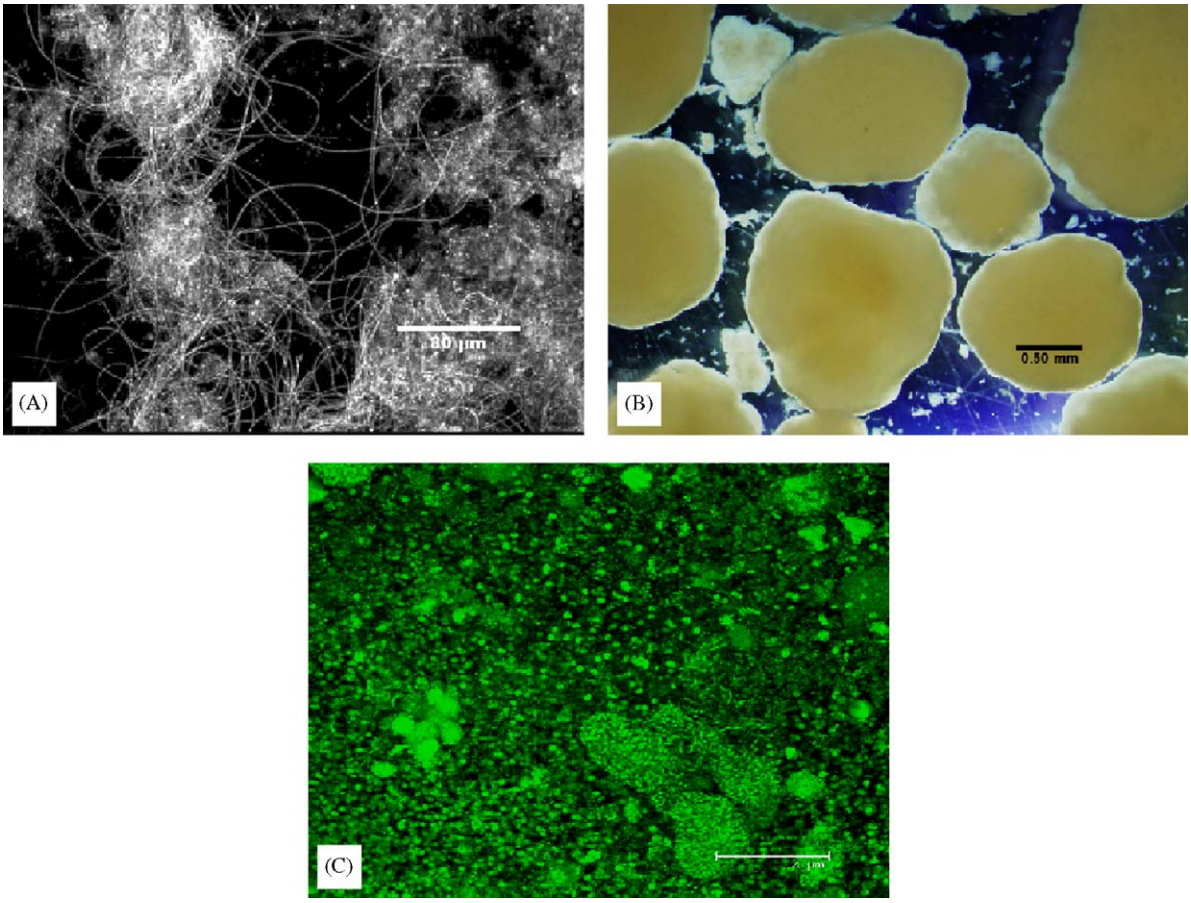

Fig. 1 – Morphological features of seed sludge and mature aerobic microbial granules. (A) Morphology of seed sludge showing predominantly filamentous growth, (B) morphology of microbial granules, (C) microstructure of a microbial granule as revealed by confocal laser scanning microscopy.

Table 1 – Characteristics of aerobic microbial granules cultivated in SBR fed with SWW containing acetate and NTA

Parameter	Value
Sludge Volume Index (ml g^{-1})	76
MLSS[a] (mg l^{-1})	1210
Average size (mm)	1.1
Circularity[b]	0.78
SOUR[a] (mg O_2 g^{-1} MLSS h^{-1})	2.4
Specific gravity	1.5

Granules collected from the SBR after 14 days of SBR operation were used for analysis

[a] MLSS: mixed liquid suspended solids; SOUR: specific oxygen utilization rate.

[b] Circularity is a measure of roundness (ratio of minor axis to major axis).

granules is shown in Fig 4A. NTA consumption was faster during cycles 2, 3 and 4 as compared to cycle 1. However, NTA removal was retarded during cycle 5. For comparison of degradation rates, 2 mM of free and iron complexed NTA was used. Fig. 4B and C shows the degradation of free NTA and ferric–NTA complex by the mixed species microbial granules. Free NTA was degraded completely in 14 h, at a specific rate of 0.7 mM (g MLSS)$^{-1}$ h^{-1}(Fig. 4B), while ferric–NTA was degraded completely in 40 h at a specific rate of 0.37 mM (g MLSS)$^{-1}$ h^{-1} (Fig. 4C). ferric–NTA degradation was not only slower compared to degradation of free NTA, but also associated with a longer lag phase between 10 and 20 h of the experiment. During the biodegradation of ferric–NTA, a gradual increase in dark orange-red precipitation (of iron released from the degraded Fe(III)–NTA complex) was observed in the glass cylinders.

4. Discussion

In general, aerobic microbial granules have been cultivated in laboratory-scale SBRs using easily degradable carbon sources such as glucose, acetate and ethanol as well as using real wastewaters such as municipal and food industry effluents (Bathe et al., 2005). The potential of mixed species microbial granules for significantly improving the process efficiency of aerobic wastewater treatment plants can be understood from

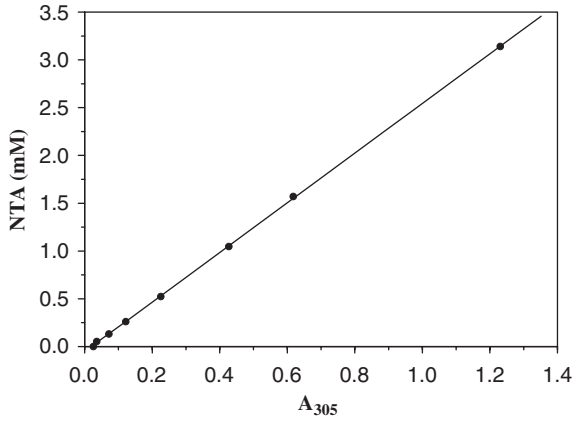

Fig. 2 – Photometric estimation of nitrilotriacetic acid (NTA) as copper–NTA complex at 305 nm. The calibration curve was prepared for quantifying NTA in synthetic wastewater.

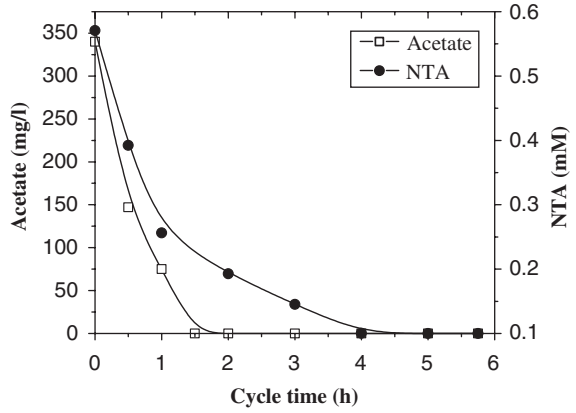

Fig. 3 – Substrate removal in single cycle period of SBR.

these investigations. Nevertheless, very little information is available on the applicability of these microbial granules for degradation of toxic chemicals or recalcitrant organic pollutants. Jiang et al. (2002) and Jiang et al. (2004) reported cultivation of aerobic microbial granules capable of phenol degradation. Aminopolycarboxylic acid type of chelating agents (e.g. NTA and EDTA) and their metal complexes are usually recalcitrant and resist microbial degradation (Witschel and Egli, 2001; White and Knowles, 2000). Nevertheless, biodegradation mechanisms of NTA and its metal complexes using both mixed and pure cultures have been the subject of research in recent years (Bolton et al., 1996; White and Knowles, 2003). NTA biodegradation has been reported in environments including soil, river water and activated sludge (Tiedje et al., 1973; Warren and Malec, 1972; Alder et al., 1990).

In the present study, aerobic microbial granules could be successfully cultivated in presence of NTA in a laboratory-scale SBR. The seed sludge used for cultivating granules had an average $SVI_{30\,min}$ value of 219 ml g^{-1}. Microscopic visualization revealed the dominance of filamentous microorganisms in the seed sludge (Fig. 1A). These observations are in accordance with the fact that SVI values of more than 150 ml g^{-1} are related to the presence of filamentous growth in activated sludge (bulking sludge). These filamentous microorganisms present in the seed sludge disappeared during the granulation process. Light and confocal microscopy showed that filaments were totally absent in mature granules (Fig 1C). NTA was included in the SWW during the granulation process in order to enrich the developing granules with NTA-degrading strains. Speciation of the chelating agent present in the experimental reactor was predicted with the help of CHEAQS, a freeware (provided by Wilko Verweij and downloadable from http://home.tiscali.nl/cheaqs/). It showed that at pH 7.0 and in presence of equimolar concentration of iron, 99% of NTA in distilled water would exist as ferric–NTA. Similar analysis also showed that in the SBR fed with SWW, NTA is likely to be present either as free NTA (47%) or as magnesium–NTA complex (49%).

In SBR, all acetate was consumed during the anaerobic fill period and within the first 30 min of aeration. Most of the NTA was consumed after acetate removal (Fig 3). Acetate removal in the reactor is in accordance with the results of Beun et al. (2000) and DeKreuk et al. (2005) on aerobic granular sludge. The granules, once fully developed, were capable of completely degrading free NTA and ferric–NTA in batch experiments. Ferric–NTA complex was not degraded as readily as free NTA, as evidenced by the longer lag phase and slower rate of degradation (Figs 4B and C). From the experiments, specific degradation rates of 0.7 mM $(g\,MLSS)^{-1}\,h^{-1}$ for free NTA and 0.37 mM $(g\,MLSS)^{-1}\,h^{-1}$ for ferric–NTA were calculated. Interestingly, a two-stage degradation was observed during the Fe(III)–NTA experiment (Fig. 4C). As the degradation rate prior to the lag-phase and after the lag phase are identical, the following mechanism is suggested, which is further supported by the findings of other studies (Bolton et al., 1996). The degradation kinetics of Fe(III)–NTA is the result of the superposition of sorption processes and biological degradation processes. Obviously, sorption is the rate-limiting step and the enzymatic apparatus for Fe(III)–NTA degradation has to be induced. This means that during the first stage of removal, Fe(III)–NTA is sorbed onto the biomass until sorption capacity is exhausted. Only after degradation has started, Fe(III)–NTA can again be removed from the wastewater. Bolton et al. (1996) reported that removal of NTA–metal complex is limited by the transport of the complex through the cell membrane. Complete degradation of the chelating agent and its complex achieved in distilled water suggest that the treatment process reported here does not require addition of other essential nutrients necessary for supporting microbial growth. The granules could be used for up to four consecutive cycles of free NTA degradation, without losing degradative capability (data not shown). Visual and microscopic examination revealed that the granules retained their structural integrity after repeated use. Zhu and Wilderer (2003) have reported that granules could be stored without losing structural integrity by using extended idle conditions.

Literature reports on the degradation of xenobiotics by aerobic microbial granules are scanty. Laboratory culture of aerobic microbial granules for phenol degradation has been reported recently (Jiang et al., 2002). It appears that microbial granules cultivated using benign substrates can degrade such toxic or recalcitrant substances because of the diversity in their composition (Tay et al., 2005). In the present study, NTA

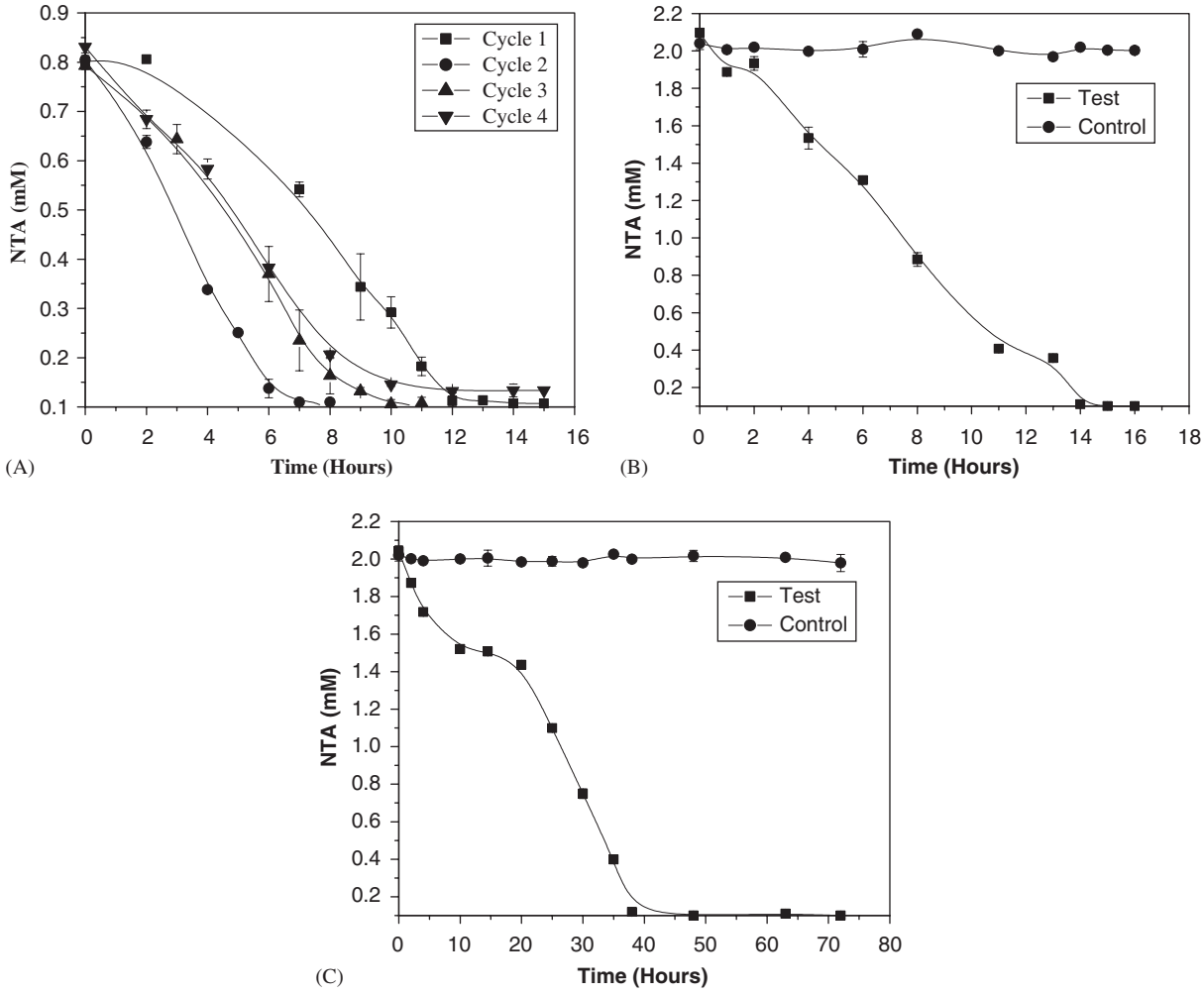

Fig. 4 – Biodegradation of free NTA and Fe(III)–NTA by aerobic microbial granules. (A) Repeated cycles of free NTA degradation. Fresh granules were used for degradation of 0.8 mM of free NTA. Experiment was terminated at the end of cycle 4 and granules were put in SWW for 1 day. (B) Degradation profile of free NTA, (C) degradation profile of Fe(III)–NTA. Experiments (B) and (C) were performed twice and the data represent the average of triplicate samples taken at each time point. Error bars represent the standard deviation of triplicate samples.

present in the SWW used for cultivation of the granules very likely acted as selective pressure for strain enrichment. Apart from NTA, only acetate was present as a carbon source. As a carbon source, acetate is readily degradable and would be degraded in the first 30 min of aeration phase (Beun et al., 2000). This would mean that for the major part of the aeration period, only NTA would be available as carbon source. The present data clearly show that presence of the chelating agent does in no way interfere with the microbial granulation process. In this context, it is interesting to note that Taweechaisupapong and Doyle (2000) observed that in an in vitro coaggregation assay, chelating agents interfered with the aggregation of microorganisms. However, the concentrations which they employed were much higher than the ones used in the present experiment. In co-aggregation experiments carried out using strains isolated from the aerobic granules, we could not observe any effect of chelating agents (data not presented). Apparently, more focused research needs to be carried out to understand the mechanisms involved in microbial granulation process.

Granule microstructure and microbial diversity are strongly dependent on the carbon source available during granulation process (Jiang et al., 2004) and especially the degradation kinetics of the carbon source in terms of feast and famine conditions (Bathe et al., 2005; McSwain et al., 2004). Confocal laser scanning microscopy of granules revealed that surface as well as the interior of granules were dominated by rod or cocci-shaped bacteria (Fig 1C). Complete degradation of chelating agents and their metal complexes may be mediated by one or more bacterial strains. Due to the compact granular nature of the biomass, large number of microorganisms could be densely packed in the bioreactor. This in turn means that pollutant transformation is rapid and large volumes of waste can be rapidly treated using a compact bioreactor. Moreover, the relatively large size (Fig 1B) and high density (specific gravity of granules in the present case was about 1.5) of

granules cause relatively fast settling and easy separation of treated effluent from the biomass.

Efficient degradation rates of NTA and ferric–NTA by pre-cultivated aerobic microbial granules reported in the present study for the first time points to the possibility of developing useful technologies for rapid biodegradation of industrial liquid wastes using compact bioreactors. However, there is a need for further exploration of degradation capability to handle other metal–NTA complexes and elucidation of the degradative strain diversity in the granules. It also needs to be examined whether aerobic microbial granules are amenable to bioaugmentation, whereby natural isolates with useful capabilities or engineered catabolic strains could be integrated into pre-cultured microbial granules for further enhancement of substrate conversion rates. Preliminary observations using GFP-labelled *Pseudomonas putida* cells and subsequent microscopic investigation showed that externally introduced cells quickly integrate into mature aerobic microbial granules (Schwarzenbeck, unpublished).

5. Conclusions

Stable mixed species microbial granules were developed for the first time in SBRs fed with acetate–NTA. Most of the influent NTA in the reactor was removed during the aeration period, following acetate consumption.

Complete removal of different initial concentrations of free NTA and Fe–NTA was demonstrated in batch experiments using distilled water as medium. Pre-cultivated granules completely degraded 2 mM of free NTA and Fe(III)–NTA in 14 and 40 h, respectively. The respective specific degradation rates were 0.7 and 0.37 mM $(g\,MLSS)^{-1}\,h^{-1}$.

Efficient degradation of a recalcitrant synthetic chelating agent by aerobic microbial granules suggests their potential application in situations where heavy metals are co-disposed with metal chelating agents.

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