Microbial diversity and performance of an innovative micro-aerobic bioreactor

Huijun Li*, Jian Sun, Yunjun Yin, Shengliu Yuan, Ying Quan and Xiaofeng Li

State Key Laboratory of Water Environment Simulation, School of Environment, Beijing Normal University, Beijing, China.

Accepted 27 September, 2013

The treatment performance of two parallel continuous single stage activated sludge bioreactor: an aerobic bioreactor (ABR) operated at dissolved oxygen (DO) levels of 3-4 mg/L, and an innovative micro-aerobic bioreactor (MABR) operated at DO levels of 0.4-0.8 mg/L and the microbial diversity of MABR was investigated and compared. Under the similar conditions, at a hydraulic time (HRT) of 25 h, an organic loading rate (OLR) of 3.4±0.1 kg COD /m³·d, the influent total phosphate (TP) was 44±2 mg/L. The COD removal efficiencies of the MABR and the ABR were 93±3 and 90±3%, respectively, and no significant difference in performance was noted between the two systems (P=0.087>0.05). It was, however, noted that TP removal efficiency of the MABR (62±4%) was significantly higher than that of ABR (43±2%) (P=0.00). Phylogenetic analysis indicated that bacteria in the MABR was highly diverse. It is likely that facultative anaerobes, microaerophile and aerobes were able to coexist in the MABR. These findings might be helpful in developing an economic treatment system, which can be a better alternative for treatment of many pollutants requiring aerobic/anaerobic sequential treatment.

Key words: Biodiversity, micro-aerobic bioreactor, phosphorous removal, phylogenetic analysis.

INTRODUCTION

Many xenobiotic compounds which are refractory under aerobic conditions seem to be readily biotransformed anaerobically (Zitomer and Speece, 1993). In turn, partially degraded products which resist further anaerobic degradation can be completely mineralized by aerobic microorganisms (Long et al., 1993). These facts point out the limitation and complementary of aerobic and anaerobic treatments and lead to the development and application of anaerobic-aerobic sequence processes. In an anaerobic-aerobic sequence system, the anaerobic and aerobic bacteria function in separate units that complement each other.

An economical strategy of operating anaerobic-aerobic systems would be to construct micro-ecosystems integrating anaerobic and aerobic niches and creating synergism between reductive and oxidation catabolisms. The coexistence of anaerobes and aerobes in aerobic and anaerobic environments indicates the possibility of constructing microecosystems in which both anaerobes and aerobes are able to survive (Lens et al., 1995). Under O₂-limited conditions, aerobic respiring microorganisms can maintain very low dissolved oxygen concentrations in the aerobic/anaerobic cocultured system, and the eventual inhibition of anaerobes is avoided. Considerable research interest is currently being focused on investigations into micro-aerobic conditions in natural environ-

*Corresponding author. E-mail: lihuijunle@yahoo.com.cn. Tel: +86-15501138305.

Abbreviations: DO, dissolved oxygen; MABR, micro-aerobic bioreactor; ABR, aerobic bioreactor; OLR, organic loading rate; COD, chemistry oxygen demand; TN, total nitrogen; TP, total phosphate; PAOs, polyphosphate accumulating organisms; GAOs, glycogen accumulating organisms; EBPR, enhanced biological phosphorus removal; Inf., influent; Eff., effluent; Rem., removal rate.
ments (Pitcher et al., 2002), in the fields of wastewater treatment (Lasik and Nowak, 2007), biotech-nological fermentation (Li et al., 2010), bioremediation of toxic compounds (Franciscon et al., 2010) and cultivation of new bacterial species (Geelhoed et al., 2009). Micro-aerobic conditions may be defined as the transition condition, making use of aerobic and anaerobic respiration, or fermentation (Ju et al., 2005). This indicated the possibility of constructing micro-ecosystems integrating anaerobic and aerobic niches because of the anaerobes and aerobes that are able to survive in one reactor. 

Shen and Guiot (1996) investigated the impact of influent dissolved oxygen (DO) on the characteristics of anaerobic granules at various DO concentrations (0.5-8.1 ppm) in 1-L and 5-L laboratory-scale modified upflow anaerobic sludge bed (UASB) reactors. These results show that the anaerobic/aerobic coupled reactor can be successfully operated under O$_2$-limited conditions and could function as an ideal engineered ecosystem that integrates aerobic and anaerobic niches. Compared to the conventional aerobic process, micro-aerobic systems are more energy-efficient requiring less energy for aeration. If such a treatment process could be induced to concurrent removal of organic substances and nutrients, an important step in wastewater treatment appears to be possible. A novel, micro-aerobic bioreactor characterized by a lower DO level (0.4-0.8 mg/L) and higher OLR (3.3-3.5 kg COD/m$^3$·d) than those in conventional activated sludge reactor is proposed here to achieve lower energy consumption and lower capital costs.

Cloning and sequencing of PCR-amplified 16S rRNA gene fragments have been successfully applied for the analysis of bacterial community structure in a wide range of environmental samples (Calheiros et al., 2010). In this study, this approach was used to determine bacterial community structure and biodiversity in the MABR.

Research into the operational characteristics of wastewater treatment facilities, operating at low DO levels, is of great importance in terms of energy saving and the development of novel processes. More work should be done to obtain a clear understanding for this system. The main objective of this research is to investigate the impact of DO concentration on the performance of two activated sludge bed reactors treating high organic wastewater. Special attention was paid to the operating characteristics under limited aeration condition. It is expected that this study will provide some fundamental information for the recognition and application of micro-aerobic treatment processes.

MATERIALS AND METHODS

Synthetic wastewater composition and seed sludge

Glucose was used as the sole organic source of synthetic wastewater. The synthetic wastewater with the following composition was used: glucose, 3.5 g/L; (NH$_4$)$_2$SO$_4$, 0.7 g/L; KH$_2$PO$_4$, 0.2 g/L; NaCl, 0.1 g/L; CaCl$_2$, 0.03 g/L; MgSO$_4$·7H$_2$O, 0.1 g/L; NaHCO$_3$, 0.2 g/L; tap water.

Reactor set-up and operation

We used two parallel identical laboratory-scale column-type continuous flow reactors comprising a plastic feed tank and a lucite aerated column. The two reactors were provided with a hopper bottom with the following dimensions: length of 90 cm, internal diameter of 10 cm and working volume of 6.3 L. The metering pump transferred wastewater from the feed tank to the bottom of the aerated column at a controlled rate. The effluent overflowed from the top of the aerated column and was subsequently discharged. Air was introduced via a porous stone diffuser at the bottom of the reactor. A heating rod was used to maintain the temperature of the reactor at 25±1°C. The scheme of the reactor is shown in Figure 1.

![Figure 1. The scheme of the reactor.](image-url)
Table 1. Performance of the MABR and ABR at steady phase.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>COD (mg/l)</th>
<th>TN (mg/l)</th>
<th>TP (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABR</td>
<td>3427±58</td>
<td>291±56</td>
<td>90±3</td>
</tr>
<tr>
<td>ABR</td>
<td>3427±58</td>
<td>185±84</td>
<td>93±3</td>
</tr>
</tbody>
</table>

MABR, micro-aerobic bioreactor; ABR, aerobic bioreactor; COD, chemistry oxygen demand; TN, total nitrogen; TP, total phosphate; Inf., influent; Eff., effluent; Rem., removal rate.

A 2 g sample of activated sludge and soil was placed in the aerated column containing 6.3 L of synthetic wastewater. The mixture was incubated in batch mode for two days to obtain enough biomass prior to continuous operation. The synthetic wastewater was prepared daily, after which it was continuously pumped into the aeration column; water sampling and situ DO and pH measurements were conducted daily in the upper part of the reactor. The system had a hydraulic retention time (HRT) of 25 h and an organic loading rate (OLR) of 3.3-3.5 kg COD/ m³·d. The DO level of the aeration column was controlled manually and maintained at 3-4 mg/L and 0.4-0.8 mg/L in the ABR and MABR, respectively. The pH was adjusted to 7.0±0.5 by adding 1 M NaHCO₃ or 1 M HCl.

Analytical methods

Chemistry oxygen demand (COD); total nitrogen (TN) and TP were measured according to standard methods (APHA, 2005). DO was determined by DO meter (HANNA HI 9145); pH was determined by pH meter (HANNA HI 8124). The average values and standard deviations of the consecutive measurement sets collected under the quasi-steady-state conditions were considered to represent the corresponding treatment and were individually compared with a one-way analysis of variance (ANOVA) using IBM SPSS statistics 20.0. P<0.05 was considered significant difference.

DNA extraction and PCR amplification

0.5 g of wet sludge was washed three times using phosphate-buffered saline (PBS, pH 7.0), followed by centrifugation (5000 rpm) at 4°C, for 5 min. The genomic DNA was then extracted using the method of Tsai and Olson (1991). Approximately 10 μg DNA g⁻¹ of wet sludge was obtained using electrophoresis on 1% (w/v) agarose gel and visually compared with a molecular mass ladder. To acquire suitable amplicons, 10-100-fold dilutions of crude DNA were used as templates for subsequent PCRs.

To minimize PCR bias, three separate reactions were run for each sample and pooled. PCR amplification of the 16S rRNA gene fragments was carried out using the forward primer 27f (5'- AGA GTT TGA TCC TGG CTC AG-3') and the reverse primer 1492r (5'- GGT TAC CTT GGG CTC AG-3'). The conditions for the PCR amplification were as follows: initial denaturation for 5 min at 95°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 52°C, extension for 1.5 min at 72°C, and final extension for 8 min at 72°C. PCR products were purified using TaKaRa purification kits.

Cloning and sequencing of 16S rRNA gene

The cloning of amplified 16S rRNA gene fragments into the TA cloning vector PMD18-T was performed following the manufacturer's instructions. Then, DH5α chemically competent E. coli was transformed with the plasmids according to manufacturer's instructions. Transformants were selected by blue-white selection on Luria-Bertani agar plates containing ampicillin (150 μg/mL). Cloned inserts were amplified from lysed colonies by PCR with plasmid-vector specific primers M13F (5'- GTT AAA CGA CGG CCA G-3') and M13R (5'- CAG GAA ACA GCT ATG AC-3'). Clones were sequenced by Beijing Sunbiotech Co., Ltd.

Phylogenetic analysis

All the nucleotide sequences were searched against the Ribosomal Database Project (RDP, release 10.0) and the GenBank database using the BLAST program. Phylogenetic trees were constructed using MEGA version 4.0 by the neighbor-joining algorithm, and the Jukes-Cantor distance estimation method with bootstrap analyses for 1000 replicates was performed. The possible chimeras in the obtained sequences were checked using the BELLEROPHON prior to phylogenetic analysis (Huber et al., 2004).

Nucleotide sequence accession numbers

The particle 16S rRNA gene sequences that were determined have been deposited in the GenBank, nucleotide sequence databases under accession nos. JN620460-JN620494.

RESULTS

Operation performance of ABR and MABR

ABR and MABR were operated for 60 days. Table 1 presents the pollutant concentration in the influent, effluent and pollutant removal efficiency at the steady stage. These results indicate that although the DO concentrations were very different in the ABR and MABR, both of these systems indicated good performance in terms of COD removal. The concentration of COD in the influent was 3427±58 mg/L, after 20 days of acclimation, and the effluent COD of the MABR and ABR stabilized at levels of 291±56 and 185±84 mg/L, respectively. The COD removal efficiency stabilized to levels of 93±3 and 90±3% respectively; both reactors had high COD removal efficiency and there was no significant difference (P=0.087>0.05).

Nutrients removal and conversion process

During the 60 days operating period, influent TN and TP concentrations were measured at 167±5 and 44±2 mg/L
respectively. After 22 days of acclimation, the effluent TN of ABR and MABR were 22±3 and 46±6 mg/L respectively, and the TN removal efficiency of the ABR (86±3%) was higher than that of the MABR(73±3%). At the steady stage, TP concentrations in the effluents of ABR and MABR were 23±3 and 17±2 mg/L, respectively and the TP removal efficiency of the MABR (62±4%) was thus significantly higher than that of ABR(43±2%) (P=0.00).

Diversity and phylogenetic analysis

To obtain more detailed information on microbial community in the MABR, partial 16S rDNA fragments were PCR-amplified from the extracted DNA using bacterial specific primers sets, 35 bacterial clones were obtained from MABR. The phylogenetic tree (Figure 2) indicated that bacteria in the MABR was highly diverse. Analyzed clones represented four different phyla of the domain bacteria. These belonged to the Firmicutes (54.3%), Actinobacteria (20%), Proteobacteria (17.1%) and Bacteroidetes (8.6%). In the phylum of Firmicutes, Bacilli (48.6%) was the most abundant class followed by Clostridia (5.7%), suggesting that the Bacilli was the dominant group in the bacterial population. All the clones in the class of Bacilli belong to the Lactobacillales order that comprise the lactic acid bacteria according to RDP analysis. Lactic acid bacteria are Gram-positive, facultative anaerobic organisms that can convert sugars to lactic acid. Clones of W4, W13, W36, W37, W40 and W54 clustered together and had the highest identity score of 99% with Leuconostoc pseudomesenteroides, a lactic acid bacteria isolated from Tibetan Qula cheese (Duan et al., 2008). The abundance of sequences related to lactic acid bacteria clearly indicates the glucose fermentation and facultative anaerobes might exist in the MABR. Clones of W12, W21, W47, W51, and W46 clustered together and had the highest identity with an uncultured bacterium clone PeH08 isolated from Midgut and Hindgut of the Humus-Feeding Larva of Pachnoda ephippiata (Egert et al., 2003). Clones of W3, W24, W42 had the highest identity score of less than 96% with uncultured bacterium clone isolated from gut. There were seven clones clustered together in Actinobacteria, six of them were classified into Bifidobacterium spp. and showed a relationship with Bifidobacterium minimum, Bifidobacterium psychraerophilum and Bifidobacterium subtile. Bifidobacteria are anaerobic or facultatively anaerobic bacteria that are typically found in the intestinal tract of humans and animals, also from environmental materials such as sewage and anaerobic digester (Watanabe et al., 2009). Wertz and Breznak (2007) reported that there were low concentrations of DO in the gut and most of the microbes isolated from gut were microaerophile. This indicated that there might be anaerobes and microaerophile that existed in the MABR. According to RDP analysis, sequences of the phylum Proteobacteria were classified into Alphaproteobacteria (5.7%) and Gammaproteobacteria (11.4%). All the sequences in the cluster of Alphaproteobacteria and Gammaproteobacteria were classified in the family of Rhodobacterales and Enterobacteriaceae respectively. Members of the Enterobacteriaceae are facultative anaerobic organisms fermenting sugars to lactate and various other end products. The phylogenetic analysis results show that anaerobes and microaerophiles were able to survive in the MABR. The coexistence of facultative anaerobes and microaerophile suggests that micro-aerobic and anaerobic niches might coexist in the MABR and that the MABR might function in a similar way to an aerobic/-anaerobic sequencing bioreactor.

DISCUSSION

He et al. (2009) reported that organic substances could be significantly biodegraded in the reactor at DO in the range of 0.8-5 mg/L. Peng et al. (2001) reported that under the condition of OLR of 2.4 kg COD /m²·d, and DO of 0.8 mg/L, the COD removal efficiency reached to 95%. In the present study, both reactors had high COD removal efficiencies; the results were consistent with the findings listed above. ABR and MABR have similar COD removal efficiency, however, compared with the ABR (48 L/h), the air flow rate of the MABR was 21 L/h, resulting in 56% saving in aeration costs. From the operational point of costs, operation at low DO reduces energy consumption.

In the last decades, enhanced biological phosphorus removal (EBPR) in activated sludge systems has become a widely applied wastewater treatment process for the removal of P without the use of chemical precipitation. EBPR can be achieved through the activated sludge process by re-circulating sludge through anaerobic and aerobic conditions (Oehmen et al., 2007). Ju et al. (2005) reported that under micro-aerobic conditions, the organisms might simultaneously perform aerobic and anaerobic respiration, or the fermentation and the aerobic and anaerobic niches might coexist, since the anaerobes and aerobes are able to survive in such systems. This means that the MABR might function like the aerobic/anaerobic sequential process and has the higher P removal efficiency than ABR. In our study, the air flow rate of the MABR was only 21 L/h, which was much less than ABR (48 L/h). Brdjanovic et al. (1998) reported that excessive aeration clearly negatively affects the biological phosphorus removal (BPR) processes. Our result is consistent with the finding listed above. There were a group of microorganisms that are largely responsible for P removal in the EBPR, which are known as the polyphosphate accumulating organisms (PAOs). The most well known PAO group was Candidatus Accumulibacter.
Figure 2. Phylogenetic trees constructed by NJ method and showing the phylogenetic positions of all the clones. Bootstrap replication was 1000. Scale bar estimated difference in nucleotide sequences positions.
phosphatis (Accumulibacter), closely related to Rhodococcus in the Betaproteobacteria. Results illustrated in Figure 2 indicate that none of the sequences detected from the MABR belonged to the known PAOs. There were numerous factors affecting the proliferation of PAOs, among them, the microbial competition of PAOs and glycogen accumulating organisms (GAOs) was the focus of many studies. The proliferation of GAOs, which compete with PAOs for the carbon sources without contributing to the EBPR process, results in reduced biological P removal efficiency (Oehmen et al., 2010). Application of different carbon source and ratios of organic carbon to P in the influent have been shown to have significant impacts on the competition between the PAOs and GAOs (Ahn and Speece, 2006). Numerous studies have indicated that COD/P ratio (for example >50 mgCOD/mgP) in the wastewater feed tends to favor the growth of GAOs instead of PAOs. A low COD/P ratio (10-20 mgCOD/mgP) should be more favorable to the growth of PAOs (Mino et al., 1998). Beside, Mino et al. (1998) also noted that competition for the carbon source was the deciding factor affecting the predominance of particular organism. Zengin et al. (2010) reported that continued glucose feeding favored preferential growth of GAOs over PAOs through direct glycogen metabolism. In this research, glucose was the sole carbon source and the COD/P ratio in the influent was as high as 74-85 mg COD/mgP, which was not suitable for the growth of PAOs. This might be the reason why no known PAOs were detected from the MABR. However, there were 28.6% of the sequences belonging to the cluster of Actinobacteria, Flavobacteria, which were commonly detected in EBPR processes (Zengin et al., 2011). This indicated that MABR might function like a conventional EBPR process.

EBPR-based treatment can be extremely efficient, but its requirement for anaerobic pretreatment zones may be problematic when retrofitting existing activated sludge installations. Moreover, the EBPR process may display inconsistencies in performance, since for optimal results, relatively high concentrations of VFAs need to be present in the influent wastewater (Mullan et al., 2005). For these reasons, the availability of effective yet economical P removal strategies is thus of great importance to the water industry. Mullan et al. (2005) reported that a single-stage aerobic P removal system might have advantages over conventional EBPR technology in terms of (a) rate of throughput, (b) tolerance of high nitrate levels (since the anaerobic phase of EBPR is sensitive to nitrate), (c) reduced dependence upon wastewater strength, and (d) operation at low VFA concentrations.

In our research, the P removal efficiency of the MABR was higher than that of the ABR, thus indicating that the EBPR process could function in a single-stage activated sludge bioreactor operating under micro aerobic conditions. Expect for P, the micro-aerobic treatment system could be better alternative for treatment of many pollutants requiring aerobic/anaerobic sequential treatment. Meanwhile, the MABR used less oxygen which means energy saving and lower initial and operating costs than conventional EBPR process.

According to our knowledge, researches regarding micro-aerobic technology were focused on performance (Chu et al., 2006; Díaz et al., 2010). The present study may therefore represent the first report of cultivation-independent molecular approaches for elucidating the phylogenetic composition, and the diversity of the microbial communities in an microaerobic bioreactor. Microaerobic technology required advances concerning not only the identity and biodiversity of the microorganisms involved in the systems, but also their biochemistry and metabolism. Beyond the taxonomy of organisms, which was now routinely studied using rDNA and rRNA-based methods, the next challenge is to obtain more information about the genetics and gene expression of the microaerobic-related enzymes.

Conclusions

The results of this research indicate that it is possible to remove organic substances and phosphorus simultaneously in a single stage activated sludge bioreactor under oxygen-limited conditions. The findings are important in terms of recognizing and supplementing the EBPR process.

Operation at low DO means energy saving, as well as lower initial costs and operating costs. Phylogenetic analysis showed that anaerobes and microerophile were able to survive in the MABR, so the micro-aerobic treatment system might be a better alternative to the aerobic/anaerobic sequential processes. More work is necessary to understand the fundamental biochemical and micro-biological mechanisms of micro-aerobic treatment systems.

ACKNOWLEDGEMENT

Our acknowledgements go to the State Key Laboratory of Water Environment Simulation, School of Environment, Beijing Normal University.

REFERENCES


