Comparison of the removal of COD by a hybrid bioreactor at low and room temperature and the associated microbial characteristics

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To improve the efficiency of wastewater treatment and characterize the microorganism communities, microorganisms were cultured and concentrated in hybrid bioreactors at a low temperature (\textdegree{}C, low-temperature hybrid bioreactor, LTHB) and room temperature (\textdegree{}C, room-temperature hybrid bioreactor, RTHB). The performance of the LTHB and RTHB in terms of COD removal efficiency, dehydrogenase activity and functional diversity of microbial communities were evaluated. The results show COD removal efficiency increased gradually over time from 39.76\% to 66.27\% for LTHB and fluctuated between 81.85\% and 94.78\% for RTHB. The dehydrogenase activity and microbial activity in LTHB was higher than those in RTHB, implying that microorganisms cultured at low temperature had higher activities and adaptabilities than those cultured at room temperature. This study suggests that hybrid bioreactors can treat wastewater at both low and room temperatures and provides valuable insight into the adaptation processes of the microorganisms during temperature changes.

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1. Introduction

Currently, aquatic ecosystems suffer from two severe problems, the limitation of water resources and water pollution. The latter is increasingly critical in many countries due to improper economic development patterns (Agrawal, 1999; Azizullah et al., 2011; Zhao et al., 2010). It is both urgent and necessary to establish environmentally benign and cost-effective measures to eliminate water pollution.

Biological wastewater treatment based on microorganisms or their aggregates is a widely applied environmental-friendly method in wastewater treatment and can be divided into aerobic and anaerobic treatment processes according to the relationship between microorganisms and oxygen (Chan et al., 2009). It can also be divided into activated sludge and biofilm treatment processes based on microbial suspension or fixation in treatment facilities (Fadi, 1999).

The pollution level of organic matter in water is often evaluated in terms of chemical oxygen demand (COD) removal efficiency. Similarly, COD removal efficiency is often used for reflecting the level of wastewater treatment (Wu et al., 2011b). To date, many treatment processes have high COD removal rates at room or high temperature (LaPara and Alleman, 1999; Wu et al., 2011a). The performance of most treatment processes however, is negatively affected by low temperature conditions which often result in a deterioration of process performance (Nachaiyasit and Stuckey, 1997).

In practice, the long-term studies of COD removal by biological wastewater treatment in low temperature conditions are often limited due to alternating seasons. This leads to the majority of these studies being conducted at \textdegree{}15\textdegree{}C conditions (Elmitwalli et al., 2002; Mahmoud et al., 2004). Wastewater is still however, discharged in winter when the temperature is often lower than \textdegree{}15\textdegree{}C. In addition, there has been more research focusing on low-temperature anaerobic biological wastewater treatments than aerobic biological wastewater treatments, which implies that studies of aerobic biotechnology to remove COD at less than \textdegree{}15\textdegree{}C are important.

It has been reported that the performance of most biological wastewater treatment measures based on microorganisms and their aggregates could be affected by temperature (Collins et al, 1978). Therefore, some studies have explored ways to improve the performance of biological wastewater treatment based on microorganisms and their aggregates. For example, some researchers have tried to change operating conditions like hydraulic retention time (HRT) or adding some measure of pre-treatment or post-treatment to improve COD removal efficiency at low temperature (Elmitwalli et al., 2002; Feng et al., 2008; Mahmoud et al.,...
It is well known that microbial properties such as microbial biomass and activities during the process of biological wastewater treatment are closely related to COD removal efficiency (LaPara et al., 2001). Therefore, in order to improve the COD removal efficiency at low temperatures, it is more practical to investigate microbial characteristics.

Most previous research has focused on either the enzyme activity or diversity of microbial communities to reflect microbial characteristics (Goel et al., 1998; Upton et al., 1990). Currently, dehydrogenase activity is often applied to assess microbial activity because the oxidation and decomposition of organic matter in treatment facilities often take advantage of microorganism dehydrogenation (Yang et al., 2002). Mature Biolog Microplate technology is also used for reflecting the diversity of microbial communities because it can reliably estimate and analyze diversity and characteristics of microbial communities (Kaiser et al., 1998). There have been some investigations into the relationship between removing pollution and microorganism characteristics at room temperature (Park and Lee, 2005), but few at low temperature such as 4 °C.

The objectives of this study are to (1) develop a highly effective and environmentally benign aerobic biotechnology to remove COD from wastewater, (2) compare the COD removal efficiency of the proposed hybrid bioreactor at low (4 °C) and room (25 °C) temperature conditions, and (3) explore the microorganism characteristics (dehydrogenase activity and functional diversity of microbial communities) during low and room temperature wastewater treatment. The results of this study will provide a promising biotechnology to remove COD matters at low (4 °C) and room (25 °C) temperature conditions, present valuable information about microbial characteristics during low temperature wastewater treatment processes as well as provide technical support for culturing and fostering microorganisms in low temperature wastewater biological treatment systems.

2. Methods

2.1. Experimental design

The process flow of the hybrid bioreactor is described in Fig. 1. The experimental system consisted of three parallel cylinder-shaped hybrid bioreactors (total and useful volume: 2.0 and 1.8 L, respectively), operated at either 4 or 25 °C. The volume of original activated sludge (which was collected from the aeration tank of a domestic sewage treatment plant) was 600 mL in each bioreactor. A refrigerator was used for storing the synthetic wastewater at 4 °C. The chemical composition of the synthetic wastewater was as follows: CH3COONa (0.4670 g/L), NH4Cl (0.1528 g/L), KH2PO4 (0.0300 g/L), MgSO4·7H2O (0.0226 g/L), CaCl2·2H2O (0.0082 g/L), FeCl3·6H2O (0.0002 g/L).

This study began in November 2010 and ran until April 2011. The hybrid bioreactors were put into thermostat incubators, and kept at one of two constant temperatures (4 ± 1 and 25 ± 1 °C for LTHB and RTHB respectively). Each hybrid bioreactor was operated at a cyclic time of 12 h with a COD feed of 500 ± 50 mg/L. The times for filling, reaction, settling and discharging were 15, 600, 90 and 15 min, respectively. One liter of the synthetic wastewater was added during each cycle. The dissolved oxygen (DO) was controlled at more 2 mg/L to maintain the activity of the activated sludge. To avoid sludge bulking, the volume of activated sludge was kept between 600–700 mL and the excess sludge removed before the next filling. The activated sludge in the LTHB was obtained through cooling the activated sludge gradually from 25 to 15 °C (over 14 days) and from 15 °C to 4 °C (over 30 days).

2.2. Analytical methods

The pH and DO of the synthetic wastewater was determined using a pH meter (PHS-3CT, Q/SMSB1-2005) and DO analyzer (JPJB-608, Q/YXLG155), respectively. The COD was determined according to the standard potassium dichromate digestion method (GB11914-89) provided by the Environmental Protection Administration of China (Wei, 2002). The dehydrogenase activity was measured using a modified triphenyl tetrazolium chloride (TTC) method (Klapwuk et al., 1974) where the sodium sulfide was the reducing agent and toluene the extracting agent. One enzyme activity unit was expressed as the amount of enzyme required to oxidize 1 mL of activated sludge suspension (33 g/L) to 1 μg triphenyl tetrazolium formazan (TPF) in 1 h.

The process to determine the functional diversity of microbial communities was as follows. Community-level substrate utilization was assayed at three temperatures (4, 15, 25 °C) using the commercially available Biolog™ ECO Microplates (Hayward, CA, USA). The plate contained an array of 96 wells and 31 types of carbon sources. The wells contained a redox-sensitive tetrazolium dye (oxidation indicator) which would turn purple as a result of respiratory electron transport in metabolically active cells (Balser and Wixon, 2009). Therefore, plate color was directly proportional to respiratory activity. For all samples, 50 μL aliquots were used for each Biolog and 150 μL aliquots were added into each well of every Biolog™ ECO Microplate, and analyzed according to Guckert (Guckert et al., 1996). Plates were incubated at 4, 15 and 25 °C and color development (590 nm) was evaluated using a Biolog Microplate Reader every 24 h for seven days (168 h).

2.3. Data analysis

Statistical Package for the Social Sciences (SPSS) Version 16.0 was used for Principal Component Analysis (PCA) and variance analysis (ANOVA); p was set at 0.05 for all analyses.
3. Results and discussion

3.1. Comparison of LTHB and RTHB COD removal performances

The COD removal efficiencies of LTHB and RTHB during November 2010 to April 2011 were investigated (Fig. 2). The initial COD concentration was kept around 500 mg/L. In LTHB, the average COD removal efficiency was 55.70% and was the lowest during the first month (Fig. 2a). This implied that the microbial aggregates (activated sludge) collected at room temperature (~25 °C) in the LTHB could not rapidly adapt to the sudden change in environment. After the first month, there was significant growth (from 39.76% to 66.27%) in COD removal efficiency. This growth might be due to the microorganisms adapting to the low temperature conditions over time with cryophilic microorganisms becoming the dominant species under rich organic matter conditions. The increased cryophilic microorganism mass increasingly demanded the dominant species under rich organic matter conditions. The increased cryophilic microorganism mass increasingly demanded more organic matter, resulting in the gradual enhancement of COD removal efficiency in LTHB. In comparison, RTHB performed well at all times with an average COD removal rate of 88.45% and reaching a peak at 94.78% (Fig. 2a). This may be because the dominant species in LTHB were cryophilic microorganisms and the cold-adapted enzyme secreted by these microorganisms still had catalytic activity at low temperatures, as seen in a previous similar study (Han et al., 2006). Moreover, it was found that the suitable LTHB range of temperature was marked wider than that of RTHB. The dehydrogenase activity determined at optimal temperature in LTHB was higher than that in RTHB. There was still dehydrogenase activity below 15 °C in LTHB but not in RTHB (Fig. 2b). This may be because the dominant species in LTHB were cryophilic microorganisms and the cold-adapted enzyme secreted by these microorganisms still had catalytic activity at low temperatures, as seen in a previous similar study (Han et al., 2006). Moreover, it was found that the suitable LTHB range of temperature was markedly wider than that of RTHB. The dehydrogenase activity determined at optimal temperature in LTHB was 27.5% higher than in RTHB, implying that the microorganisms cultured and concentrated at low temperature had more robust adaptabilities.

3.2. Dehydrogenase activity

The hybrid bioreactors became gradually steady after 6 months and the dehydrogenase activity was determined in April 2011 (Fig. 2b). The optimal dehydrogenase activity temperature was 35 °C for LTHB and 45 °C for RTHB. The dehydrogenase activity in both LTHB and RTHB dropped rapidly after reaching optimal temperatures. This may be because the lipids of the membrane became stiff below optimum temperatures, leading to the reduced efficiency of transport proteins embedded in the membrane (Nedwell, 1999).

When the temperature was below 40 °C, the dehydrogenase activity in LTHB was higher than that in RTHB. There was still dehydrogenase activity below 15 °C in LTHB but not in RTHB (Fig. 2b). This may be because the dominant species in LTHB were cryophilic microorganisms and the cold-adapted enzyme secreted by these microorganisms still had catalytic activity at low temperatures, as seen in a previous similar study (Han et al., 2006). Moreover, it was found that the suitable LTHB range of temperature was markedly wider than that of RTHB. The dehydrogenase activity determined at optimal temperature in LTHB was 27.5% higher than in RTHB, implying that the microorganisms cultured and concentrated at low temperature had more robust adaptabilities.
As the optimal dehydrogenase activity temperature was 35 °C for LTHB, the effects of incubation time on dehydrogenase activity at 35 and 4 °C for LTHB were investigated (Fig. 2c and d). The dehydrogenase activity at 4 °C increased with time up to 2 h after which time it was replaced by a persistent decrease. The reaction rate at 4 °C demonstrated a moderate decline and reached a peak (0.56 µg/mL h) after 1 h. The optimal incubation time was considered to be 2 h for LTHB at 4 °C. When the temperature was 35 °C, the dehydrogenase activity increased over time and reached a peak (24.7 µg/mL) after 4 h of incubation. The reaction rate reached a peak of 8.14 µg/mL h after 2 h. Therefore, the optimal incubation time was 2 h for LTHB at 35 °C.

The reasons for the pattern observed in dehydrogenase activity at 4 °C might be as follows. At the beginning of the experiment, the experimental conditions were aerobic allowing the aerobic microorganisms to utilize sufficient oxygen and demonstrate a high level of dehydrogenase activity. At low temperature the oxygen may have decreased with time due to the increasing aerobic microorganism biomass. These microorganisms gradually depleted the oxygen resulting in anoxic conditions and weakening the microbial activity. Consequently, the dehydrogenase activity decreased with time. Moreover, the increasing amount of TPF limited the formation of more TPF. Over time, the TPF would also combine with the sludge at low temperature, making the extraction difficult.

The specific enzyme activities did not change significantly in either anaerobic or aerobic sequencing batch reactors (SBR) and their specific process parameters (Goel et al., 1998; Nybroe et al., 1992) but were affected by temperature (Droste and Sanchez, 1983). The effects of temperature on dehydrogenase activity in this study were similar to that of Han et al. (2006) on low-temperature biofilms with a few exceptions. For example, this study was conducted at 4 and 35 °C while the other study was carried out at 4 and 30 °C. In addition, the subject of this study was low-temperature microbial aggregates (activated sludge culture at low temperature) while it was low-temperature biofilm in Han’s study. Finally, the compositions of the experimental wastewaters were also different. The results of these two studies demonstrate that dehydrogenase activity of microbial aggregates is positively affected by low temperature in both activated sludge and biofilm treatment processes.

3.3. Functional diversity of microbial communities

3.3.1. Changes in average well color development (AWCD) and metabolic intensity of carbon sources

AWCD is an important index to indicate the utilization of carbon sources and its rate of change can reflect microbial activity. AWCD is also closely related to the numbers and types of microorganisms who can use the single carbon source (Zak et al., 1994). The AWCD increased with time at 15 and 25 °C for both LTHB and RTHB (Fig. 3a). Neither LTHB nor RTHB changed significantly when the incubation temperature was 4 °C. It might be that, firstly, the carbon sources existing in Biolog Microplate were pulvorous, was and therefore hard to dissolve in the microbial suspension at 4 °C. Secondly, the inoculums concentration was small, resulting in less cryophilic microorganisms in the inoculums. As a result, little of the carbon source could be utilized by microbial aggregates at 4 °C. Furthermore, neither LTHB nor RTHB showed visible changes before 24 h, but both experienced a moderate rise after 24 h, which was replaced by a slight growth after 120 h (Fig. 3a). The maximum rate change appeared at 96 h.

The results of analysis of variance of AWCD at 96 h indicated as follows. When the temperature was 4 °C, both LTHB and RTHB could use little of the carbon source. When the temperature was 15 and 25 °C, the AWCD in LTHB was higher than that in RTHB, i.e., the microbial activity in LTHB was higher than that in RTHB.

These results were similar to the results of the dehydrogenase activity, further indicating that the microorganisms (microbial aggregates) cultured and concentrated at low temperature had higher activities and adaptabilities than those cultured and concentrated at room temperature. These results demonstrate that temperature was a significant factor affecting the ability of the microbial aggregate to utilize the carbon source.

3.3.2. Principal component analysis on diversity of utilization of carbon sources

The 96 h Biolog data were used for the Principal Component Analysis (PCA). According to the variance, the contribution rate of the accumulative total of the principal components should more than 85% (Hao et al., 2003). There were four principal components extracted with a contribution rate of 61.74%, 15.28%, 7.77% and 4.11%, respectively. Among these, the total of principal component 1 (PC1) and principal component 2 (PC2) was 77.02%, which meant that it was feasible to explain the microbial communities utilizing the carbon sources using PC1 and PC2.

The results of the PCA on microbial community in LTHB and RTHB at different temperature are summarized in Fig. 3b with the correlation coefficients between the main carbon source group and PC1 or PC2 in Table 1. When the temperature was 4 °C, there was no significant difference between samples of LTHB and RTHB. This was attributed to the activity of some of the microorganisms being very low under cold conditions (Chazarenc et al., 2010), which leaded to a decrease in the rate of utilization of carbon sources. When the temperature was 15 °C, there were small
differences between the samples of LTHB and RTHB. When the incubation temperature was 25 °C, the differences were highly significant ($p < 0.05$, Fig. 4). The samples in the LTHB tended to the positive direction of both PC1 and PC2 but the samples in the RTHB tended to the positive direction of PC1 and the negative direction of PC2. It can be seen in Table 1 that there were 11 carbon sources (contain four groups: phenolic acid, carboxylic, amino acid and polymer) correlated with PC1 and three carbon sources (amine, amino acid, carbohydrate) correlated with PC2. These 14 carbon sources were combined into six different carbon source groups.

<table>
<thead>
<tr>
<th>Principal component 1 (PC1)</th>
<th>Correlation coefficient</th>
<th>Principal component 2 (PC2)</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic acid</td>
<td>4-Hydroxy benzoic acid</td>
<td>0.98</td>
<td>Amino Acid</td>
</tr>
<tr>
<td></td>
<td>Itaconic acid</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>o-glucoside acid</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Carboxylic acid</td>
<td>Pyruvic acid methyl ester</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>o-galacturonic acid</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>t-asparagine</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Amino Acid</td>
<td>t-Serine</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>t-arginine</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tween 40</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Polymer</td>
<td>Tween 80</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A-cyclodextrin</td>
<td>0.90</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. AWCD for different carbon source groups of microbial communities in LTHB and RTHB at different temperatures (Different letters represent significant differences at $p < 0.05$, error bars are standard deviation.)
(Table 1) which could be utilized by microorganisms in the Biolog Microplate. It also illustrated that PC1 and PC2 could completely reflect all microbial communities utilizing the carbon sources.

In addition, the variation in the PC scores for LTHB and RTHB at different incubation temperatures were significantly different between PC1 scores of every sample ($p < 0.05$) but not significant for PC2. This was because the PC1 accounted for the majority of the variance (61.74%) and had many carbon sources closely associated with it. This led to differences between the microbial communities utilizing carbon in the different samples being easily identified. Conversely, the variance explained by PC2 was small (15.28%), with only a few types of associated carbon sources such as amino acid and amine which were not main substrates used by microorganisms.

3.3.3. Analysis of the metabolic capability of different types of carbon sources

There were 31 types of carbon source in the Biolog ECO Microplate which could be classified into six main classes (4 types of polymer, 10 types of carbohydrate, 7 types of carboxylic acid, 6 types of amino acid, 2 types of amine, 2 types of phenolic acid).

This study used the 96 h Biolog data to estimate the metabolic capability of the microorganisms in LTHB and RTHB on different types of carbon sources. The microorganisms in both LTHB and RTHB showed similar tendencies to first utilize the amino acid and polytypes of carbon sources. The microorganisms in both LTHB and RTHB had high metabolic capabilities when utilizing carbon sources than those in RTHB at both low and room temperature, which again was similar to the results of the dehydrogenase activity and AWCD tests.

3.3.4. Comparison on functional diversity indexes

Shannon index, Simpson index and McIntosh index were used to reflect the species richness, common species and homogeneity of microbial communities, respectively. This study used the 96 h Biolog data to analyze the microbial functional diversity in LTHB and RTHB at different temperatures.

Species richness and common species in LTHB and RTHB were similar but the homogeneity of microbial communities was significantly different ($p < 0.05$) at the same incubation temperature (Table 2). This was perhaps affected by both temperature and the composition of the synthetic wastewaters, i.e., using the same wastewater resulted in similar microbial species in LTHB than RTHB ($p < 0.05$). This implies that the microbial aggregates in LTHB had higher metabolic capabilities when utilizing carbon sources than those in RTHB at both low and room temperature, which again was similar to the results of the dehydrogenase activity and AWCD tests.

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shannon index</th>
<th>Simpson index</th>
<th>McIntosh index</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTHB in 25°C</td>
<td>3.02 ± 0.06a</td>
<td>17.37 ± 1.03a</td>
<td>2.66 ± 0.07a</td>
</tr>
<tr>
<td>RTHB in 25°C</td>
<td>2.94 ± 0.13a</td>
<td>15.72 ± 2.07a</td>
<td>1.80 ± 0.10b</td>
</tr>
<tr>
<td>LTHB in 15°C</td>
<td>2.73 ± 0.05ab</td>
<td>13.15 ± 0.69ab</td>
<td>2.23 ± 0.05c</td>
</tr>
<tr>
<td>RTHB in 15°C</td>
<td>2.90 ± 0.23ab</td>
<td>9.69 ± 2.67bc</td>
<td>0.76 ± 0.21d</td>
</tr>
<tr>
<td>LTHB in 4°C</td>
<td>2.29 ± 0.48ab</td>
<td>6.99 ± 3.84ab</td>
<td>0.04 ± 0.01e</td>
</tr>
<tr>
<td>RTHB in 4°C</td>
<td>2.02 ± 0.74b</td>
<td>7.01 ± 5.41bc</td>
<td>0.03 ± 0.01e</td>
</tr>
</tbody>
</table>

(Different letters represent significant differences at $p < 0.05$).

4. Conclusion

The proposed hybrid bioreactors could efficiently remove organic matters (COD) from wastewater at both low and room temperatures. The activity and adaptability of microorganisms in the LTHB was higher than those in the RTHB when the temperature of wastewater was below 40°C. This study provides an environmentally benign, highly effective and practical bio-measure to remove organic matter during different seasons and even under special conditions such as temperatures at 4°C. This study also compares dehydrogenase activity and microbial function diversity of microorganisms between low and room temperature, potentially providing technical guidance for culturing microorganisms.

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