Combined effects of enrichment procedure and non-fermentable or fermentable co-substrate on performance and bacterial community for pentachlorophenol degradation in microbial fuel cells

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**Highlights**

- Enrichment procedure and co-substrate had combined effects on system performance.
- Simultaneous-glucose was a strategy for efficient system performance.
- Diverse bacterial communities were developed in these different systems.

**Abstract**

Combined effects of enrichment procedure and non-fermentable acetate or fermentable glucose on system performance and bacterial community for pentachlorophenol (PCP) degradation in microbial fuel cells (MFCs) were determined in this study. Co-substrate and PCP were added into MFCs either simultaneously or sequentially. Simultaneous addition with glucose (simultaneous-glucose) achieved the shortest acclimation time and the most endurance to heavy PCP shock loads. Species of *Alphaproteobacteria* (simultaneous-acetate, 33.9%; sequential-acetate, 31.3%), *Gammaproteobacteria* (simultaneous-glucose, 44.1%) and *Firmicutes* (sequential-glucose, 31.8%) dominated the complex systems. The genus *Sedimentibacter* was found to exist in all the cases whereas *Spirochaetes* were merely developed in simultaneous-acetate and simultaneous-glucose. While *Epsilonproteobacteria* were only absent from sequential-acetate, simultaneous-glucose benefited to the evolution of *Lentisphaerae*. These results demonstrate simultaneous-glucose is a strategy for efficient system performance and the microbiological evidence can contribute to improving understanding of and optimizing PCP degradation in MFCs.

**1. Introduction**

The development of microbial fuel cells (MFCs) shows that producing electricity may not be the most important, short-term and practical application of this device, but one applicable field of MFCs is bioremediation of aquatic sediments and groundwater (Huang et al., 2011a). A number of factors should be addressed before this technology can be applied at larger scales. Exoelectrogentic bacteria on the anode are one of the most factors influencing system performance, and the factors that affect community structure and the importance of various microorganisms for improved system performance are just beginning to be understood (Kiely et al., 2011a; Logan, 2009). Enrichment procedure can play an important role in acclimation of efficient exoelectrogenic bacteria and various strategies have been used to enrich these microorganisms during the startup period for the reactor. Direct inoculation from domestic wastewaters or contaminated sites (Huang et al., 2011a; Liu et al., 2011; Pham et al., 2009), successive transfers from one MFC or microbial electrolysis cell to another MFC (Cao et al., 2010; Catal et al., 2008), placing the fresh electrode in the vicinity of an already active biofilm anode (Liu et al., 2008), using a serial dilution enrichment (Xing et al., 2010), setting suitable external resistances (Jung and Regan 2011; Lefebvre et al., 2011;
Rismani-Yazdi et al., 2011) or potentials (Huang et al., 2011b; Wei et al., 2010) have been extensively exploited to reduce startup time and improve system performance. In the case of congo red degradation in MFCs, simultaneous or sequential addition of congo red and glucose affected the composition of bacterial community and power production, demonstrating the importance of enrichment procedure to system performance (Hou et al., 2011). While non-fermentable acetate and fermentable glucose affect MFC system performance (Chae et al., 2009; Huang et al., 2011c; Lee et al., 2008), the reported explorations were limited to either the co-substrates of acetate and glucose (Chae et al., 2009; Huang et al., 2011c; Lee et al., 2008) or the enrichment procedure (Hou et al., 2011), neglecting the combined effects of co-substrate and enrichment procedure on system performance. This consideration of enrichment procedure together with co-substrate was thus desirable for improved MFC system performance.

Pentachlorophenol (PCP) is one recalcitrant compound and of concern due to its coexistence with other easily degradable organics in the environment. Conventional electrochemical/biological processes can achieve PCP degradation. Remaining challenges are electrochemically high operating cost and non-stable electrodes, and biologically much sludge generation (Huang et al., 2011a; Field and Sierra-Alvarez 2008). An MFC provides a low-cost and low-maintenance energy as well as a process that produces very little sludge (Logan, 2009, 2012). Bioanode was demonstrated to be an effective candidate for simultaneous removal of PCP and acetate or glucose in our early study (Huang et al., 2011c). However, proof of this demonstration demands direct microbiological evidence, which has not yet been reported. Moreover, little information is available about the combined effects of enrichment procedure and co-substrate on PCP degradation in MFCs.

In the present work, the combined effects of enrichment procedure and co-substrate on system performance and microbial diversity in MFCs were examined. Two types of co-substrate of acetate and glucose, and two enrichment procedures, in which co-substrate and PCP were added into the MFCs simultaneously or sequentially, were tested in terms of startup time, electricity generation, PCP degradation rates, and quantification of PCP metabolites. The bacterial community on the anode was analyzed using a massively parallel sequencing technology of 454 pyrosequencing technique (Logan, 2012). Deeper insight into these aspects will enhance the performance of MFCs for efficient PCP bioremediation as discussed subsequently.

2. Methods

2.1. Reactor construction

Duplicate two-chamber MFCs were used, with the electrodes separated by a cation exchange membrane (CEM) (CMEMembranes International, Glen Rock, NJ) (2.5 cm in diameter) (Huang et al., 2011c). Graphite felt (Sanye Co., Beijing, China) was packed in each compartment and served as the anode and cathode. The net working volume of each chamber was 100 mL. A reference electrode (Ag/AgCl electrode, 195 mV versus standard hydrogen electrode, SHE) was used to obtain cathode and anode potentials, with all voltages reported here vs SHE. All of the reagents were wrapped with aluminum foil to avoid light illumination.

2.2. Inoculation and operation

Domestic wastewater collected from primary sedimentation tank of Lingshui Wastewater Treatment Plant in Dalian, China, was used as an inoculum. 50 mL of wastewater was inoculated into 50 mL of a nutrient solution which contained (per liter) (NH₄)₂SO₄ 0.386 g, K₂SO₄ 0.149 g, NaH₂PO₄·2H₂O (3.31 g), Na₂HPO₄·12H₂O (10.31 g), vitamins (12.5 mL/L) and minerals (12.5 mL/L) (Lovley and Phillips 1988). Acetate or glucose was added at a final concentration of 780 mg/L (COD basis). Prior to be refilled to the reactors, this medium was sparged with N₂ for 15 min. Two enrichment procedures were performed. One was the simultaneous addition of PCP and acetate or glucose together with the inoculum (simultaneous-acetate; simultaneous-glucose). In the other case, exoelectrogenic bacteria were firstly obtained using acetate or glucose as a substrate and wastewater as an inoculum. After the repeatable maximum power generation, the mature exoelectrogenic bacteria were then adopted by feeding PCP and acetate or glucose to evolve into the specific exoelectrogenic bacteria for co-metabolism of PCP (sequential-acetate; sequential-glucose). The replacement of anodic solution was carried out at the end of each fed-batch cycle (defined as a voltage of <20 mV). Unless otherwise stated, external resistance was set at 500 Ω. For the cathode chamber, the same NaH₂PO₄·Na₂HPO₄ buffer was used along with hexacyanoferrate (50 mM). All reactors were operated at room temperature of 22 ± 3 °C.

2.3. Chemical and electrochemical analyses

The voltage across an external resistor was recorded (30 min intervals) using a data acquisition board (PIS0813, Taiwan). Power density and polarization curve were obtained as previously described (Huang et al., 2011c). The bioelectrochemical behavior of anodic biofilms was examined using cyclic voltammetry (CV) and a three electrode configuration with a potentiostat (CHI 650A, Chenhua, Shanghai). The scanned potential between −0.6 and +0.6 V (vs. SHE) was performed at a scan rate of 1.0 mV/s under quiescent conditions.

Samples were periodically withdrawn from the MFCs and filtered through 0.22 μm pore diameter membrane filters. The analysis of PCP and metabolites (tetrachlorohydroquinone (TeCHQ), 2,3,4,5-tetrachlorophenol (TeCP), trichlorophenol, dichlorophenol and phenol) was performed using a high performance liquid chromatograph (HPLC Agilent 1100) equipped with a C₁₈ capillary column (ODS-2 Hypersil, Thermo) as previously described. PCP degradation rate (r, mg/L·h) was calculated as the net PCP decrease value divided by r: 
\[ r = \frac{C_i - C_f}{t} \]
where \( C_i \) is the initial PCP concentration (mg/L) and \( C_f \) (mg/L) is the PCP concentration at an operation time of t (h).

2.4. Bacterial community analysis

Samples for microbial consortia analysis were collected from MFCs (simultaneous-acetate; simultaneous-glucose; sequential-acetate; sequential-glucose) at the final of PCP (15 mg/L) consumed with mature exoelectrogenic bacteria. Electrodes were fragmented using sterile scissors. Cells attached on the electrodes were rinsed three times using sterile water. The suspended cells were collected by centrifugation. Total genomic DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories Inc.) according to the manufacturer’s instructions. 16S rRNA gene fragments containing V1–V3 regions were amplified from the extracted DNA with primer sets, 8F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 533R (5’-TACGCGGCTGCTGGCAC-3’) with unique barcode to sort each sample from the mixed pyrosequencing outcomes. The amplicon concentration was measured and a mixture of all amplicon libraries was used for pyrosequencing on a Roche massively parallel 454 GS-FLX. Operational taxonomic units (OTUs) and rarefaction curves were determined based on a threshold of 97% similarity using the MOTHUR program (http://www.mothur.org). All sequences were phylogenetically assigned.
with taxonomic classifications obtained from the SILVA 106 databases (http://www.arb-silva.de/).

3. Results and discussion

3.1. Startup

Following inoculation, simultaneous-acetate had a total acclimation period of 489 ± 44 h, shorter than 589 ± 54 h in sequential-acetate (Table 1). Similarly, simultaneous-glucose presented a total acclimation period of 475 ± 46 h, outnumbering 509 ± 48 h for sequential-glucose. These results demonstrate the benefit of simultaneous enrichment to the availability of exoelectrogenic bacteria. After four cycle acclimations, the difference in power production between the two enrichment procedures disappeared, exhibiting around 2.0 W/m³ in PCP-acetate MFCs and 1.3 W/m³ in PCP-glucose reactors (Table 1). This result illustrates the importance of co-substrate type and the negligible effect of enrichment procedure on power production.

3.2. Polarization curves

There was no appreciable effect on the maximum power density when the two enrichment procedures were applied to both acetate (Fig. 1A and C) and glucose (Fig. 1B and D) reactors, with a maximum power production of around 2.0 W/m³ at 6.3 A/m³ from acetate reactors (Fig. 1A) and 1.3 W/m³ at 5.4 A/m³ from glucose MFCs (Fig. 1B). This result implies the maximum power was mainly affected by co-substrate. For glucose MFCs, anode potentials under the two enrichment procedures showed negligible change with the increase of current density (Fig. 1D). In the case of acetate reactors, however, the variety of anode potentials with an increase of current density was different, in which simultaneous addition presented more stable than sequential addition (Fig. 1C), implying that the anodic biofilms of sequential addition suffered from a more severe polarization. It is therefore reasonable to expect that the composition of microbial structure may depend on not only the type of co-substrate but also the enrichment procedure, which will be demonstrated later.

3.3. PCP degradation and metabolites

The trend of PCP degradation was similar for all the cases, in which PCP degradation rate increased with the increase in initial PCP concentrations ranging from 5 to 15 mg/L (Fig. 2A). At an initial PCP of 15 mg/L, sequential-acetate reached a quicker rate of 0.16 ± 0.01 mg/L-h than 0.057 ± 0.004 mg/L-h with simultaneous-acetate. In contrast, at a PCP range of 5–15 mg/L, there was less difference in PCP degradation rates between sequential-glucose and simultaneous-glucose (Fig. 2A). In the absence of current generation in the open circuit controls, PCP degradation rates in the four cases were low, ranging from 0.031 mg/L-h to 0.055 mg/L-h. These results were consistent with the reported (Hou et al., 2011) in which sequential and simultaneous additions had no effect on congener degradation with the presence of glucose. However, at an initial PCP level higher than 20 mg/L, simultaneous-glucose achieved the highest PCP degradation rate of 0.13 ± 0.01 mg/L-h among the four cases, much higher than the reported 0.039–0.051 mg/L-h (Li et al., 2010; Mun et al., 2008). This result implies the benefit of simultaneous-glucose mode to heavy PCP shock loads.

One metabolite of tetrachlorohydroquinone (TeCHQ) through PCP hydroxylation in para position, a common reaction in the pathways for this compound degradation in conventional biological processes (Field and Sierra-Alvarez, 2008), was found to be accumulated in both simultaneous-acetate and simultaneous-glucose reactors (Fig. 2B). Higher quantities of TeCHQ in simultaneous-glucose than simultaneous-acetate implies the importance of simultaneous-glucose mode to this degradation pathway. However, this simultaneous mode was different from the sequential mode, in which TeCHQ was only found in acetate reactors compared to its un-detection in glucose MFCs (Fig. 2B) (Huang et al., 2011c). Besides TeCHQ, 2,3,4,5-tetrachlorophenol (TeCP), another common metabolite released from PCP ortho-dechlorination in conventional biological processes (Field and Sierra-Alvarez 2008), was accumulated gradually in all the four cases (Fig. 2C). Any trichlorophenol, dichlorophenol and phenol were un-detectable in all of the four modes, suggesting the primary de-chlorination of PCP in the present MFCs. Higher TeCP accumulation in sequential situation than simultaneous mode demonstrates the more preferable formation of TeCP to sequential mode. The differences in PCP degradation rate (Fig. 2A) and accumulation of metabolites (TeCHQ and TeCP) (Fig. 2B and C) in the four cases reflect that both enrichment procedures of simultaneous and sequential modes, and co-substrates of acetate and glucose affected PCP degradation rate and pathway.

3.4. Biocatalytic activity

Cyclic voltammograms were obtained to reveal the biocatalytic activities of the anodic biofilms enriched with simultaneous-acetate, simultaneous-glucose, sequential-acetate, and sequential-glucose modes. For sequential-acetate, one-oxidation peak of 0.75 mA at a potential of 0.36 V and two-reduction peaks of −0.22 mA at 0.15 V and −0.54 mA at −0.23 V were observed in the biofilms (Fig. 3A). No peaks were observed in the abiotic controls (Fig. 3A). Comparatively, two-oxidation peaks of 1.10 mA at 0.17 V and 0.46 mA at −0.075 V, and one-reduction peak of −1.00 mA at −0.30 V appeared with simultaneous-acetate, exhibiting narrower shifts from acetate controls with no presence of PCP (Fig. 3A). This result illustrates the more benefit of simultaneous mode than sequential situation to the activities of exoelectrogenic bacteria in acetate MFCs. In the case of glucose, biofilms acclimated with sequential mode exhibited a lack of a larger difference from

| Table 1 | Acclimation of microbial consortia enriched with simultaneous or sequential addition of PCP and co-substrate of acetate or glucose (initial PCP concentration: 15 mg/L. The initial three cycles in sequential addition did not contain PCP). |
|---|---|---|---|---|
| Lag period (h) | Simultaneous-acetate | Simultaneous-glucose | Sequential-acetate | Sequential-glucose |
| 1st cycle | 30 ± 4 | 10 ± 2 | 90 ± 4 | 10 ± 3 |
| Maximum power (W/m³) | 0.7 ± 0.1 | 0.5 ± 0.1 | 1.3 ± 0.2 | 0.8 ± 0.1 |
| Lasting time (h) | 145 ± 12 | 75 ± 8 | 160 ± 15 | 105 ± 12 |
| 2nd cycle | 1.0 ± 0.1 | 0.9 ± 0.2 | 2.0 ± 0.1 | 1.7 ± 0.3 |
| Maximum power (W/m³) | 1.5 ± 0.2 | 1.3 ± 0.3 | 2.0 ± 0.1 | 1.7 ± 0.2 |
| Lasting time (h) | 157 ± 11 | 238 ± 19 | 130 ± 16 | 105 ± 13 |
| 3rd cycle | 2.0 ± 0.2 | 1.3 ± 0.3 | 2.0 ± 0.1 | 1.7 ± 0.2 |
| Maximum power (W/m³) | 2.0 ± 0.1 | 1.3 ± 0.2 | 2.0 ± 0.2 | 1.3 ± 0.1 |
| Lasting time (h) | 84 ± 9 | 52 ± 6 | 135 ± 12 | 80 ± 7 |
| 4th cycle | 2.0 ± 0.2 | 1.3 ± 0.2 | 2.0 ± 0.2 | 1.3 ± 0.1 |
| Maximum power (W/m³) | 2.0 ± 0.1 | 1.3 ± 0.2 | 2.0 ± 0.2 | 1.3 ± 0.1 |
| Lasting time (h) | 74 ± 7 | 100 ± 11 | 75 ± 8 | 150 ± 13 |
| Total acclimation time (h) | 489 ± 44 | 475 ± 46 | 589 ± 54 | 509 ± 48 |
those with simultaneous mode (Fig. 3B), demonstrating the less effect of enrichment procedure on the activities of exoelectrogenic bacteria with the presence of co-substrate of glucose.

3.5. Microbial community diversity structure and phylogenetic analysis

Pyrosequencing produced 299 OTUs at 6490 (simultaneous-acetate), 324 OTUs at 7257 (simultaneous-glucose), 402 OTUs at 7129 (sequential-acetate) and 449 OTUs at 6416 (sequential-glucose) high-quality reads (average length of 455 bp), respectively based on a threshold of 97% similarity. There were highly diverse phyla in the communities of the anodic biofilms amended with PCP-acetate or PCP-glucose and enriched with simultaneous or sequential addition (Fig. 4).

Fig. 1. Polarization curves of (A and C) acetate and (B and D) glucose MFCs with the simultaneous addition (triangle symbol) or sequential addition (circle symbol) of 15 mg/L PCP (△ and ○ in (A) and (B); voltage; △ and ○ in (A) and (B); power; △ and ○ in (C) and (D); anode potential; △ and ○ in (C) and (D); cathode potential).

Fig. 2. (A) Effect of initial PCP concentrations on PCP degradation rate, time course of PCP metabolites of (B) TeCHQ and (C) TeCP with enrichment procedures of simultaneous addition of PCP and (△) acetate or (○) glucose, and sequential addition of PCP and (△) acetate or (○) glucose at an initial PCP concentration of 15 mg/L.

Fig. 3. Comparison of CVs in (A) acetate-PCP and (B) glucose-PCP fed MFCs with an enrichment procedure of (△) sequential or (○) simultaneous addition (----- acetate or glucose fed control; --- abiotic control).

Gammaproteobacteria, producing low amount of power and highly existing in anodic bacterial clumps (Logan, 2009; Rabaey et al., 2004) or polyaromatic hydrocarbon polluted sites (Alonso-Gutiérrez et al., 2009), were abundantly found at 44.0% with simultaneous-glucose compared to 26.3% for sequential-glucose, both of which were much higher than 11.5% for simultaneous-acetate and 4.1% with sequential-acetate (Fig. 4). This result highlights the importance of simultaneous-glucose mode to the abundance of Gammaproteobacteria. Differently, Alphaproteobacteria, rapidly degrading petroleum in conventional biological processes (Alonso-Gutiérrez et al., 2009),
The existence of this strain here was probably due to oxygen diffusion from the cathode to the anode as well as the residual oxygen after nitrogen sparging (still 1.2–1.8 mg/L oxygen left in the medium after 15 min N₂ sparging), which might support aerobic or facultative growth (Chae et al., 2009). Considering the quick PCP degradation (0.16 ± 0.01 mg/L-h) with sequential-acetate mode at an initial PCP concentration of 15 mg/L (Fig. 2A), the high relative abundance of Mycobacterium here may be positively related with this efficient PCP degradation. In addition, sulfate-reducing and de-chlorinating bacteria Desulfovibrio, producing hydrogen in the presence of sulfate, outcompeting methanogenic archaea and scavenging hydrogen formed by itself (Field and Sierra-Alvarez, 2008), achieved abundances of 1.5% (simultaneous-glucose) and 1.1% (sequential-glucose), higher than 0.02–0.07% in simultaneous-acetate or sequential-acetate mode (Fig. 5). Similarly, Pseudomonas strains, co-metabolically degrading chlorinated phenols in conventional biological processes (Field and Sierra-Alvarez, 2008) and predominant in glucose MFCs in the absence of PCP (Kiely et al., 2011b,c; Ren et al., 2011), reached higher abundances of 3.2–3.3% in PCP-glucose MFCs than 0.3–1.9% in PCP-acetate reactors (Fig. 5). These results highlight the importance of glucose to the abundance of Desulfovibrio and Pseudomonas, consistent with previously reported in the absence of PCP (Chae et al., 2009; Kiely et al., 2011b,c). Proteiniphilum, isolated from a conventional anaerobic reactor treating brewery wastewater (Chen and Dong, 2005), was absent from sequential-glucose MFCs compared to the others of 2.3% (sequential-acetate), 2.8% (simultaneous-glucose) and 11.0% (simultaneous-acetate). In addition, Sedimentibacter (formerly Clostridium), capable of reductively dechlorinating beta-hexachlorocyclohexane in a conventional coculture with a Dehalobacter species (van Doesburg et al., 2005), was found for the first time present at the highest abundance of 17.4% (sequential-glucose) among the 35 dominant genera, Mycobacterium strain was the most prevailing with simultaneous-acetate (14.1%) and sequential-acetate (26.5%) MFCs compared to the 1.5–3.5% for both simultaneous-glucose and sequential-glucose MFCs (Fig. 5), highlighting the beneficial acetate to the abundance of this microorganism. Mycobacterium has been extensively studied for PCP degradation in conventional aerobic processes (Field and Sierra-Alvarez, 2008). The existence of this strain here was probably due to oxygen...
reason for the presence of many other bacteria such as Spirochaetes in simultaneous-acetate and simultaneous-glucose reactors, Lentisphaerae with simultaneous-glucose mode and Sedimentibacter in all the four tested cases was unclear. It is also unknown whether the bacteria exist as exoelectrogenic bacteria among faster-dechlorination competitors, or a low level of current generation provides some benefits for PCP dechlorination through interactions. In fact, the numerical abundance of bacteria in biofilms is not a priori to correlate to capacities of the predominant species for high power production in the absence of PCP (Kiely et al., 2011b,c; Wrighton et al., 2011). The diverse species for simultaneous-glucose mode here might be important for short acclimation time (Table 1) and endurance of heavy PCP shock loads (Fig. 2A).

Geobacter and Shewanella families, model microbes for exoelectrogenic studies (Kiely et al., 2011a; Logan 2009), were not observed in all of the four cases, presumably due to the presence of recalcitrant PCP. This also reflects that the diversities of exoelectrogenic and/or dechlorinated bacteria in the present systems might extend beyond the commonly studied Geobacter and Shewanella sp.

Considering that microorganisms in the aggregates or biofilms are likely to experience syntrophic metabolism of the added substrates in the absence of PCP (Kiely et al., 2011b,c), syntrophic processes among exoelectrogenic bacteria, de-chlorination bacteria, and exoelectrogenic de-chlorination bacteria cannot be excluded, which enabled the biofilms or aggregates to successfully convert PCP-acetate or PCP-glucose into electrical current coupled with PCP degradation. Further investigations in this direction are warranted.

4. Conclusions

Enrichment procedure (simultaneous or sequential) and co-substrate (acetate or glucose) had combined effects on system performance for PCP degradation in MFCs. Simultaneous-glucose was a strategy for bacterial acclimation and endurance of heavy PCP shock loads. There was approximately 20% improvement in the acclimation time when simultaneous-glucose was used compared to sequential-acetate. PCP metabolites of TeCHQ and TeCP were quantitatively different in the four cases. There were substantial differences and highly diverse phyla in communities of anode biofilms under the four modes, contributing to improving understanding of and optimizing PCP degradation in MFCs.

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