Microbiological aspects of thermophile pretreatment of activated sludge inhibiting electricity generation of microbial fuel cell

Yang-Guo Zhao, Yanhui Zhao, Yi Zhang, Liang Guo and Mengchun Gao

ABSTRACT

Thermophile pretreatment of activated sludge greatly improves the biodegradability of sludge, but whether the pretreated products are suitable for the electricity generation of microbial fuel cells (MFCs) is still little known. In this study, municipal activated sludge pretreated by a thermophilic bacterium and heating, respectively, was separately fed into the MFCs. The performance of MFCs was examined and changes of anodic microbial communities were investigated with scanning electron microscopy and 16S rRNA gene high-throughput sequencing on the Illumina Miseq platform. The results showed that MFCs fed with heating-pretreated sludge performed preferably and the power density reached 0.91–2.86 W/m^3. MFC anodes were covered with considerable *Geobacter* spp. However, the bioaugmentation of sludge with the thermophile was not able to support a high potential output although the pretreatment significantly increased the soluble chemical oxygen demand. The maximum power density approached 0.20 W/m^3 even when the anolyte was regularly changed. It was observed that amending pH did not improve the performance of MFC. Investigation on this anodic microbial community found that the relative abundance of *Lactobacillus* spp. exceeded 91%. Consequently, the thermophile-pretreated products stimulated the growth of non-exoelectrogens and finally the niches of anodic biofilm were completely occupied by *Lactobacillus* spp.

Key words | activated sludge, electricity generation, inhibition, *Lactobacillus*, microbial fuel cell, thermophilic bacteria

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INTRODUCTION

The activated sludge is a major byproduct of the biological treatment process in sewage treatment plants. It is estimated that activated sludge disposal accounts for 40% to 50% of the total wastewater treatment costs (Bala Subramanian *et al.* 2010). Therefore, development of cost-effective and high-efficiency disposal methods for activated sludge becomes an urgent requirement. Activated sludge contains abundant organic matter and has been widely used for the production of hydrogen (Wilson & Novak 2009) and methane, for composting and electricity generation in microbial fuel cells (MFCs) (Xiao *et al.* 2011). However, most of the organic matter in the activated sludge is inert and encapsulated by bacterial cell wall. This organic matter is poorly biodegradable and hard to produce energy from through biological systems. Hence, in order to improve the biodegradability of sludge, it is generally necessary to pretreat the sludge by the physical, chemical or biological techniques.

The biological pretreatment of activated sludge showed many advantages over others, such as low costs, energy conservation, and environmental friendly. It mainly employs the hydrolytic enzyme secreted by the microorganisms to dissolve the cell wall, and consequently the inert organics in the sludge are converted into the soluble chemical oxygen demand (SCOD). By this pretreatment, biodegradability of the sludge was greatly improved (Zheng *et al.* 2014). Solubilization by a thermophilic enzyme (S-TE) secreted by thermophilic microorganisms is a recently developed technique for the rapid digestion of organic matter in sewage sludge at high temperature (65 °C) (Tang *et al.* 2012). Compared with ultrasonic and ozone oxidation

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pretreatment, S-TE technique is able to hydrolyze the bacterial cell wall in a short time, release the soluble organic matter and extremely improve the biodegradability of the sludge (Tang *et al.* 2012). Guo *et al.* (2010, 2014) found that activated sludge pretreated by S-TE could improve hydrogen yield more efficiently than without pretreatment or pretreated by other microbial inoculants. To obtain high soluble protein, carbohydrate and SCOD, the optimized S-TE pretreatment conditions of sludge were set as 65 °C for 12–16 h, pH 7–9 and total suspended solids (TSS) at 4.31 g/L. For hydrogen yield by fermentation under the anaerobic condition, the optimum pretreatment condition was pH 6, 6.83 g/L of TSS and the treatment time for 8 h at 65 °C (Guo *et al.* 2012).

An MFC generates current during the anodic microorganisms degrading organic matter in the activated sludge; this consequently shows an encouraging application in waste treatment and resource recovery. Researches showed that the activated sludge pretreated by ozone, microwave (Mohd Zulkhairi *et al.* 2013), hot alkali (Oh *et al.* 2014), and ultrasound (Jiang *et al.* 2009) promoted the MFC electricity production. S-TE pretreatment of activated sludge also increased the SCOD and volatile fatty acids (VFAs) content (Guo *et al.* 2010) and provided favorable substrate for the anodic microorganisms of the MFC. This would be further beneficial to the generation of electricity and degradation of organic matter. However, whether S-TE-pretreated products of activated sludge could enhance MFC's electricity production has not been reported.

In this study, the products from the excess sludge pretreated by thermophile *Bacillus thermophilic* AT07-1 and temperatures (100 °C or 121 °C) were separately used in the MFC to produce power. By monitoring MFC electricity-generation efficiency, organic removal and microbial community composition, the present study will explore whether and how the pretreated sludge promotes MFC electricity production and explain the reason that leads to the results.

MATERIALS AND METHODS

MFCs setup

The MFCs consisted of two identical rectangular chambers, that is, the anodic and cathodic chambers, separated by a proton exchange membrane (Nafion 117, Dupont, USA). The effective area of the proton exchange membrane was 40 cm^2 and the total volume of the cell was 165 mL. The anode was made of a 6.5 cm × 10 cm carbon cloth and the cathode was a graphite carbon plate with a size of 5.0 cm × 7.5 cm. The proton exchange membrane, carbon cloth and graphite carbon plate were all pretreated by 1 mol/L NaOH for 5 h and followed by 1 mol/L HCl for 5 h. Finally, they were immersed in deionized water for 10 h to remove the surface chemicals. The anode and the cathode were connected by titanium wire over the external resistor of 1,000 Ω.

The voltage of MFCs across the cathode and anode was monitored by a data acquisition board (PIOS 813, Taiwan Hongge). The board was connected with the computer via an interface and the output voltage signal of the MFC was automatically recorded every 30 min.

The pretreatment of activated sludge

The bacterial strain used for pretreating activated sludge in this study was *Bacillus thermophilic* AT07-1 (CGMCC No. 5309), isolated from the garden soil of Hunan University, China, by Tang *et al.* (2012). AT07-1 is a Gram-positive bacterium; the optimum growth temperature and pH range are 65 °C and 6.8–7.5 respectively. Tang *et al.* (2012) and Guo *et al.* (2010, 2014) found that activated sludge pretreated by strain AT07-1 released more soluble organic matter and thus significantly improved hydrogen yield. Whether the pretreated products enhanced the electricity generation is still little known; thus this strain was used in this study. Before application, AT07-1 was activated by LB broth for 24 h at 65 °C. The activated sludge was obtained from the secondary sedimentation tank of Tuandao wastewater treatment plant, Qingdao China. As for the thermophile pretreatment, 10 mL of thermophilic bacterium AT07-1 culture with concentration of 1.05×10^9 CFU/mL was added to 100 mL of activated sludge, and the mixture was shaken in a water bath at 65 °C for 48 h. After that, the pretreated products were centrifuged at 5,000 *g* for 10 min and the supernatant was used as the substrate in MFC. The same activated sludge was pretreated by 100 °C heating for 60 min or 121 °C autoclaving for 20 min respectively. The heating-pretreated products were both centrifuged at 5,000 *g* for 10 min to obtain supernatant as MFC substrate.

To investigate whether the supernatants contained antibacterial substance, a disk diffusion antimicrobial susceptibility test was carried out with the antibiotic-sensitive Gram-negative bacterium *Escherichia coli* DH5α (Takara, Dalian, China) and Gram-positive bacterium *Bacillus subtilis* CGMCC 1.4255 according to the method described by Jorgensen & Turnidge (2015).

MFC start-up and operation

The inoculation sludge for MFCs was obtained from the Licunhe sewage treatment plant of Qingdao, China, and was acclimated anaerobically for 1 week at room temperature. Afterwards, 10 mL of anaerobic sludge was inoculated into the MFC anode chambers. The thermophile-pretreated supernatant (En), 100 °C-pretreated supernatant (He) and 121 °C-pretreated supernatant (Pr) were separately filled into the chamber for start-up of the bioreactor. Prior to addition, 2 mL of trace elements, 200 μL of amino acids and 200 μL of vitamin per liter (Bretschger *et al.* 2007) were added to the supernatant. The catholyte solution was prepared with 30 mmol/L potassium ferricyanide in deionized water. The MFCs were operated in batch mode; that is, the anolyte was changed every 2 days during MFC start-up whereas it was changed by the new supernatant once the voltage dropped to below 0.01 V during MFC operation. All experiments were carried out at room temperature (20–25 °C).

Monitoring of chemical and electrochemical parameters

The COD in the pretreated sludge supernatant and the MFC effluent was determined by potassium dichromate ($K_2Cr_2O_7$) rapid digestion method. The ammonia nitrogen was examined by Nessler's reagent spectrophotometry; protein was measured with phenol reagent method and polysaccharide was determined with a phenol method. VFAs and the ethanol in the MFC effluent were examined with a gas chromatograph as previously described by Guo *et al.* (2012).

Power density, polarization curve and the relating electrochemical parameters were measured and calculated as previously reported by Yang *et al.* (2016). When the open circuit potentials of the MFC were stable and reached the maximum, a resistance box (ZX21 type, Dongmao, Shanghai China) was used to obtain the power density and polarization curves by changing resistance from 9,000 to 20 Ω.

Microbial community structure and composition analysis

In order to reveal the differences of the microbial community in MFC anodes when feeding different substrates, the anodic biofilms of MFC En, He and Pr were subjected to scanning electron microscopy (SEM) observation. Briefly, a small piece of carbon cloth was cut from the anode and subjected to a series of pretreatments according to the method described by Chung & Okabe (2009). Afterwards, the microbial morphology on the MFC biofilm was observed by SEM (JEM-1200EX, JEOL, Japan). For the analysis of microbial community structure, total DNA of the biofilm sample was extracted with a Power Soil DNA extraction kit (Mobio, USA) followed by PCR (polymerase chain reaction) amplification using the bacterial 16S rRNA gene universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT -3′) as described by Qin *et al.* (2010). Afterwards, high-throughput sequencing was performed on the Illumina Miseq sequencing platform (Beijing, China). The diversity index, principal coordinates analysis (PCoA) and sequences classification were carried out according to a previous report (Gao *et al.* 2014).

Accession number of DNA sequence

The 16S rRNA gene sequencing reads by high-throughput sequencing were deposited in MG-RAST (Metagenomic Rapid Annotations using Subsystems Technology) with the IDs 311509–311512.

RESULTS

Electricity generation of MFCs with different substrates

The voltage and polarization curves of the MFCs with the thermophile-pretreated (En), 100 °C temperature-pretreated (He) and 121 °C sterilization-pretreated (Pr) activated sludges are shown in Figure 1.

For MFCs He and Pr, after three cycles of change of anolyte, the maximum output voltages reached 0.57 V and 0.64 V, respectively. Compared with that, the voltage of MFC En, in which the substrate was pretreated by the thermophilic bacterium *Bacillus thermophilic* AT07-1, reached a maximum voltage of 0.47 V at 470 h. Afterwards, it rapidly decreased to below 0.10 V even though the anolyte was continuously substituted with new solution. The voltage presented a slight increase once the fresh anolyte was added.

The maximum power of MFC En reached 0.20 W/m^3 with the current density of 1.18 A/m^3, while the maximum powers of MFCs He and Pr approached 2.86 and 0.91 W/m^3, respectively, when the current densities were 7.02 and 3.36 A/m^3. According to the linear slope of polarization curve fitting, the resistances of MFCs En, He, Pr were 117, 50 and 115 Ω, respectively.

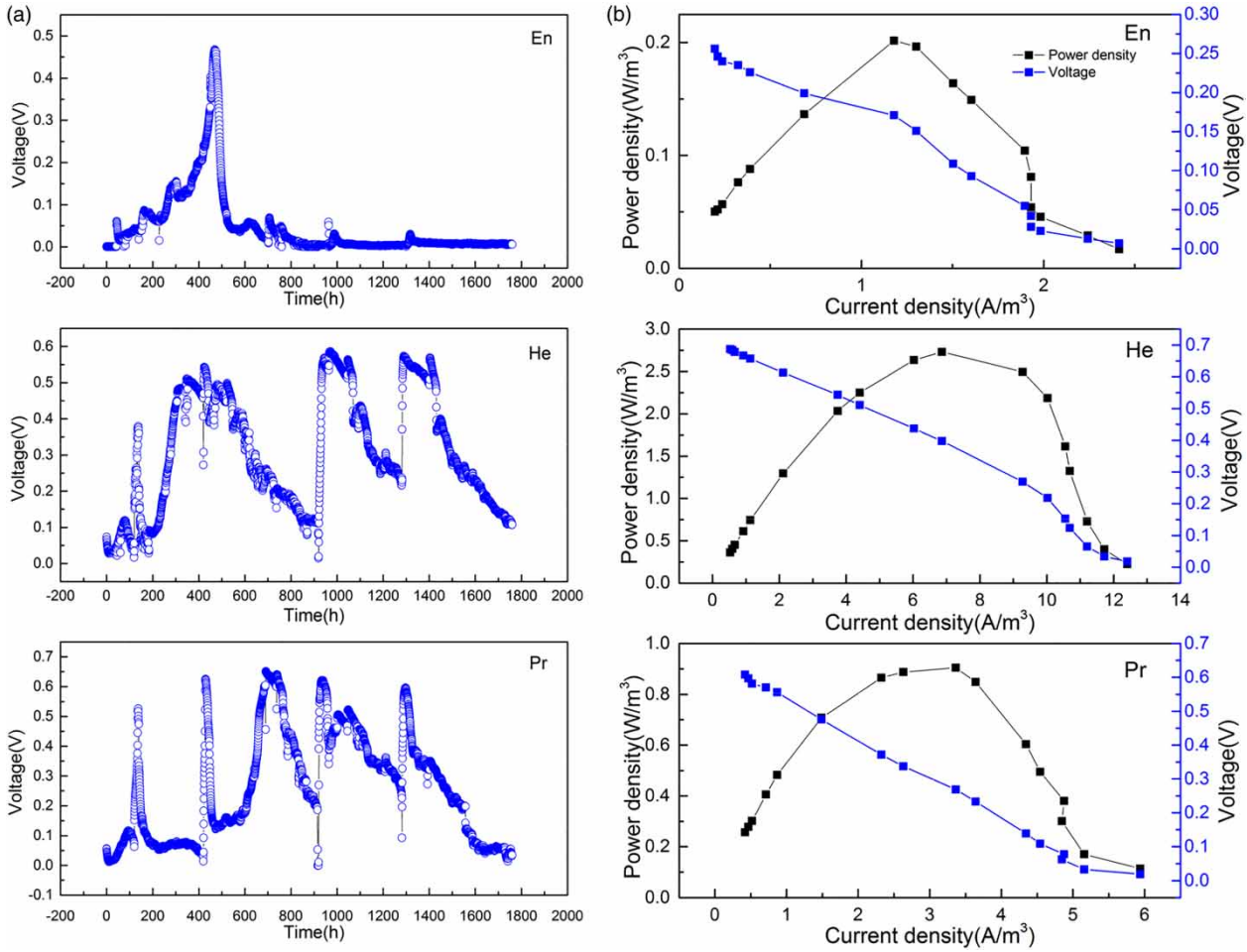

Figure 1 | Voltage changes (a) and power density and polarization curves (b) in MFCs fed with the activated sludge pretreated by thermophile (En), 100 °C heating (He) and 121 °C heating (Pr).

Removal of organic pollutants in MFCs

The changes of SCOD in the anolyte were monitored within a steady cycle of MFCs En, He and Pr and results are shown in Figure 2. The SCOD of sludge was well released by pretreatment. It was found that the concentration of SCOD in thermophile-pretreated sludge was above 20,000 mg/L, and 100 °C- and 121 °C-pretreated sludges contained about 5,000 mg/L of SCOD. This suggested that the performance of pretreatment with thermophilic bacteria AT07-1 was better than the other two heating methods. Regarding the variations of SCOD in the MFCs, the organic matter in the supernatant continued to be degraded by the anaerobic microbes in the MFC anodic chambers and released more SCOD at the beginning of the cycle. Hence, the SCOD in MFCs En, He and Pr increased to 24,040, 5,890 and 5,440 mg/L on about the 50th hour of operation. According to Figure 2, MFCs En and He reached the maximum voltage within 50 h, which indicated that the SCOD has provided abundant substrate for the microorganisms in electricity production. In addition, it was found that the output voltage of MFC Pr rose rapidly in 100–200 h, while the SCOD concentration decreased greatly during 100–200 h. The increase of voltage presented a lag time with the SCOD.

The concentration of SCOD in the three groups of MFCs showed a decreasing trend, but the MFC En output voltage was low and the electricity-production efficiency was poor. At the end of the cycle, the removal rates of SCOD of MFC En, He and Pr were 41.67%, 67.26% and 56.15%, respectively.

Changes of the protein, polysaccharide, ammonia and VFAs in the anolyte are shown in Figure 3. The concentration of protein in the three groups showed the same decreasing trend with the MFC running. The protein showed the highest degradation rate at the beginning of the cycle, and dropped from 2,500–3,000 mg/L to

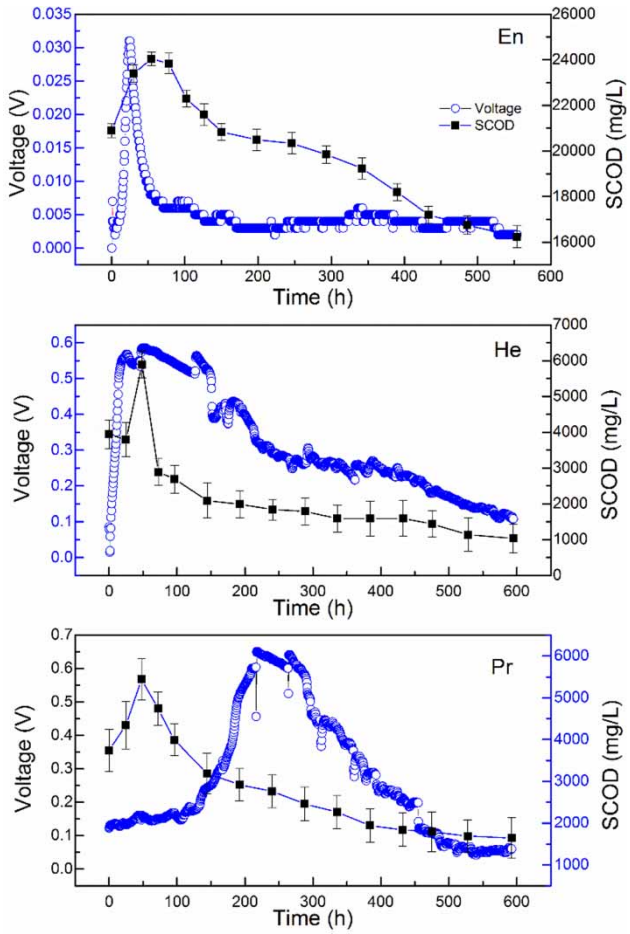

Figure 2 | Voltage and SCOD changes in MFCs fed with the activated sludge pretreated by thermophile (En), 100 °C heating (He) and 121 °C heating (Pr) in one feeding cycle.

1,100–1,700 mg/L within 30 h. Hence, MFC possessed enough available organic matter to produce electricity at the beginning of operation and consequently the initial voltage showed a rapid increase. At the end of the cycle, the average degradation rates of protein in MFC En, He and Pr were 55.46%, 84.99% and 78.95% respectively.

The polysaccharide from the activated sludge is also one of the main energy sources for the anodic microorganisms in MFCs. The 121 °C pretreatment of sludge contained higher polysaccharide than the other two pretreatments. At the beginning of 50 h, the polysaccharide in this MFC quickly dropped from 766.29 to 379.14 mg/L. After that, the polysaccharide degradation came into a stable status and tended to have the same trend as MFCs En and He. Interestingly, at the beginning of 30 h, the polysaccharide in MFC En was slightly increased from 484.86 to 523.43 mg/L and then declined steadily like that of MFC He. The polysaccharide in MFC He rapidly degraded to 306.29 mg/L in 50 h and then decreased steadily. The accumulative degradation rates of polysaccharide in MFC En, He and Pr at the end of the cycle were 34.92%, 58.89%, and 65.25% respectively.

Ammonia was also removed by the MFC operation. Ammonia was 125.80 and 102.91 mg/L in supernatant of 100 °C- and 121 °C-pretreated sludges, respectively. However, it approached 751.16 mg/L in thermophile-pretreated sludge supernatant. The ammonia in MFC He and Pr showed the same trends, increasing to 272.99 mg/L and 362.86 mg/L, respectively, at 25 h, and then decreasing rapidly. After 288 h of MFC operation, the ammonia came into a stable status. Compared with that, the ammonia in MFC En decreased during the whole operation cycle. Especially, during the first 30 h, the ammonia quickly dropped from 751.16 to 347.40 mg/L. Finally, at the end of cycle, it deceased to about 50 mg/L and consequently showed the highest ammonia removal rate with 93%.

Changes of volatile fatty acids and pH

The VFAs originating from the anaerobic fermentation of macromolecular organic matter are a relatively efficient carbon source for MFCs. The content of VFAs determines the MFC electricity-production performance. In this study, changes of VFAs concentrations in the three MFCs within one feeding cycle are shown in Figure 3(d). Compared with Figure 2, the correlation between the VFAs content and power generation efficiency is clearly observed.

In one substrate feeding cycle, VFAs content in MFC En rapidly increased from 1,762.94 to 5,046.46 mg/L and MFC En power generation reached the maximum output voltage at the same time. After 100 h of operation, the VFA in MFC En decreased gradually and the output voltage came into a low stage. The VFAs content in the MFC He reactor increased from 400.51 to 1,596.31 mg/L in 48.5 h and the corresponding output voltage also reached the maximum. The VFAs content kept stable in 48.50–120.50 h, and the corresponding voltage also tended to be steady at this time. After 120.50 h, output voltage of MFC He gradually decreased with the VFAs content reducing. The VFA content and voltage of MFC He were positively correlated. The VFAs curve in MFC Pr anode fluctuated greatly during the whole feeding cycle. Similar to the MFC He, the VFAs gradually increased from 604.42 to 2,826.04 mg/L within 120 h. During this time, the output voltage of MFC Pr also increased gradually at the beginning of the electricity production. The VFAs showed a little fluctuation in 120–240 h, which might be ascribed to the fact that degradation of the substrate was accompanied by the production of VFAs.

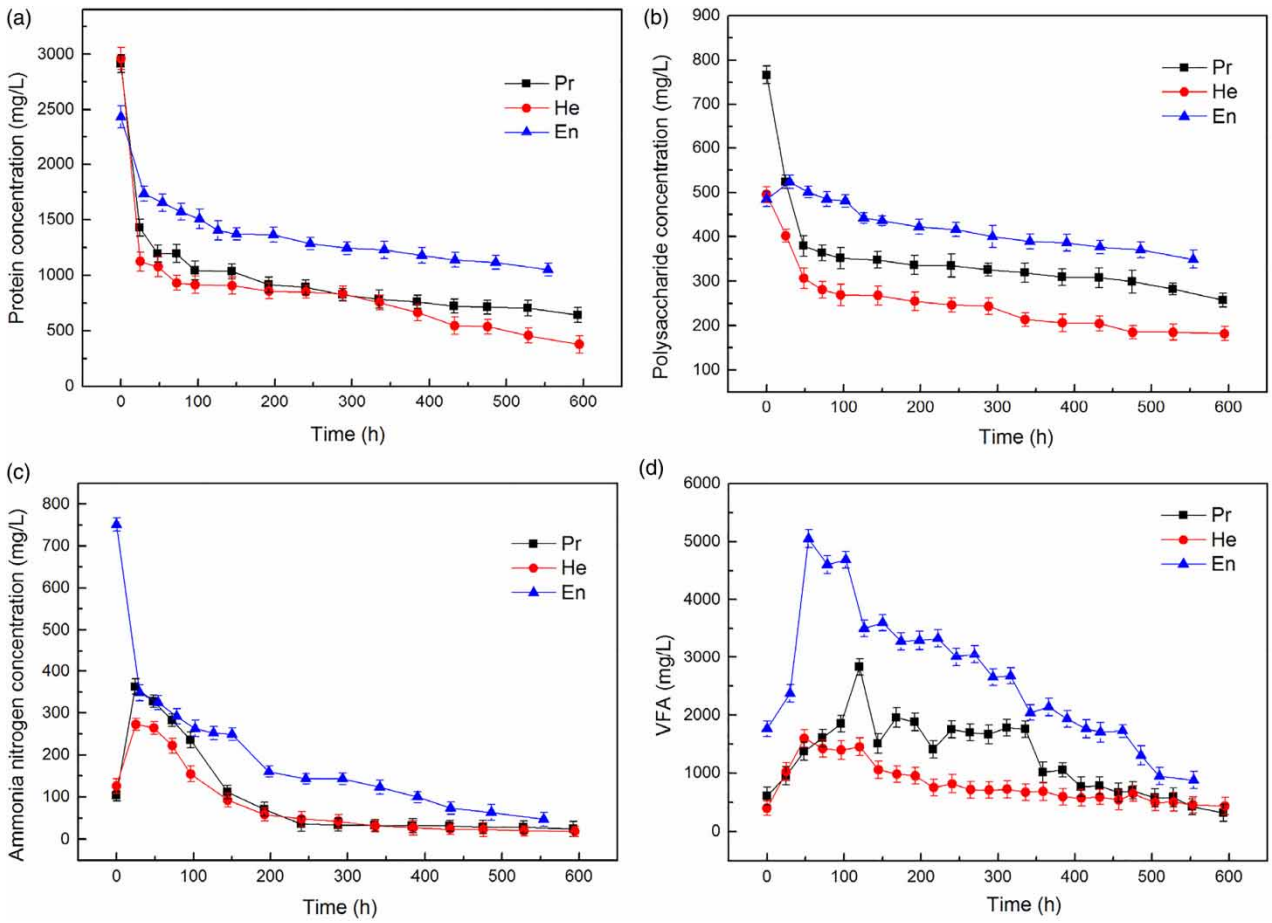

Figure 3 | Changes of protein (a), polysaccharide (b), ammonia nitrogen (c) and volatile fatty acids (VFAs) (d) in the anode chambers of MFCs fed with the activated sludge pretreated by thermophile (En), 100 °C heating (He) and 121 °C heating (Pr).

The pH in MFC En feeding solution was 6.26, which rapidly declined to pH 5 or less and then came into a stable state. For most of the time in one feeding cycle, the pH was between 4 and 5. This acidic environment did not benefit the growth of the exoelectrogens and the electrode chemical reaction, resulting in a low power generation. Compared with that, the pH value in the MFC He and Pr seemed better. In MFC He, the pH of feeding solution was 7, it decreased rapidly to 6.44 within 48.50 h and then fluctuated drastically, finally decreased to pH 5.23. The influent pH of MFC Pr was 7.30 and it presented the same changing trend as the MFC He. However, it showed more gentle decline and finally approached pH 5.5.

Performance improvement of MFC with thermophile-pretreated activated sludge

To improve the performance of MFC feeding with thermophile-pretreated activated sludge, the anodic solution pH was controlled by adding sodium hydroxide or buffer solution. During one feeding cycle of MFC En, the pH of the anodic solution was adjusted to above pH 7 once it deceased to below pH 6. The output voltage revealed the same changes with the pH value, that is, high pH led to high voltage. First, improvement of pH to 7.38 led to an increase of voltage to 0.15 V, followed by a natural drop of pH value and voltage within 25 h. Second, improvement of pH to 8.43 led to a slight increase of voltage to 0.12 V, followed by a natural drop of pH value to 5.64 within 117 h. The pH value was thirdly improved to 7.19, followed by an increase of output voltage from 0.098 to 0.11 V. It was found that the concentration of SCOD in the MFC En anode chamber was high; thus acidogenic capability was strong and the pH value decreased rapidly.

The anolyte in the MFC En was replaced by thermophile-pretreated activated sludge plus phosphate-buffered saline (PBS) solution to examine whether the buffer solution benefited the MFC electricity generation. It was found that

MFC En with the PBS buffer reached maximum voltage of 0.11 V when the PBS was added. This result indicated that the pH amendment by adding PBS buffer also benefited the exoelectrogens.

Analysis of anodic microbial community

The morphology of MFC anodic biofilm was observed and it was found that most microbes on MFCs Pr and He anodic biofilm were bacilli and vibrio. Compared with that, most microbes on MFC En were also bacilli, but the cell size was relatively larger, obviously different from that of MFCs Pr and He (Figure 4).

The microbial communities of inoculated sludge (In) and MFCs En, He and Pr anodic biofilm were analyzed by high-throughput sequencing technology. A total of 40,000 target sequences per samples were finally obtained. Rarefaction curves (Figure 5(a)) showed that the inoculated sludge (In) contained the most possible species and the observed species approached 1,200. However, the MFC En fed with thermophile-pretreated sludge possessed a low-diverse microbial community and revealed only 300 observed species. The diversity of microbial communities of MFCs fed with heating-pretreated sludge lay between the samples In and En.

PCoA for four microbial communities is shown in Figure 5(b). PC1 is the more important coordinate and its cumulative explained variance ratio accounts for 75.61%. At this coordinate, the distance between samples He and Pr was 0.02, while the sample En was far away with distance of 0.7. Although sample In revealed a distance of 0.02 with samples He and Pr on PC1, it was separated by another principal coordinate, PC2. Hence, microbial community structure of samples He and Pr is more similar than for other samples.

The composition and cluster analysis for the microbial communities of the four samples at the phylum level is shown in Figure 5(c). The relative abundances of phylum Proteobacteria in samples In, Pr, He and En were 40.15%, 63.55%, 43.87% and 2.85% of total sequences, respectively. The relative abundances of phylum Firmicutes were 5.94%, 20.59%, 22.81% and 96.17%. The sample En was interesting and it contained the highest proportion of phylum Firmicutes with 96.17%. The third highest proportion phylum is Bacteroidetes, the relative abundances were 18.22%, 10.35%, 23.99% and 0.25% in sample In, Pr, He and En, respectively. According to the cluster analysis, the microbial community composition of samples He and Pr was very similar and sample En separated further from the group. This result accorded well with the PCoA.

Table 1 shows the results of microbial community composition and abundance in samples In, En, He and Pr at the genus level. It is noted that genera *Clostridium*, *Geobacter*, *Pseudomonas*, *Lactobacillus* and *Azospirillum* were significantly different between samples. The relative abundance of *Clostridium* increased from 1.57% in sample In to 10.30%, 13.09% and 3.37%, in samples Pr, He and En respectively. *Geobacter* species are typical electricity-producing microorganisms. The relative abundance of *Geobacter* was highest in sample Pr with 2.02%, followed by sample He with 0.56% and In with 0.42%. Sample En contained only 0.02% of *Geobacter* species. The relative abundance of *Lactobacillus* species in sample En approached 91.45%. *Lactobacillus* species ferment sugars to produce lactic acid. They are acidophilic flora, the optimum pH is 5.5–6.0 and they tolerate the pH of 3.0–4.5.

DISCUSSION

Heating is a simple but effective pretreatment option for activated sludge to generate electricity in MFCs. During the heating treatment, inert organic matter in the sludge was released and further hydrolyzed into simpler organic compounds (Shanableh & Jomaa 2001). Research found

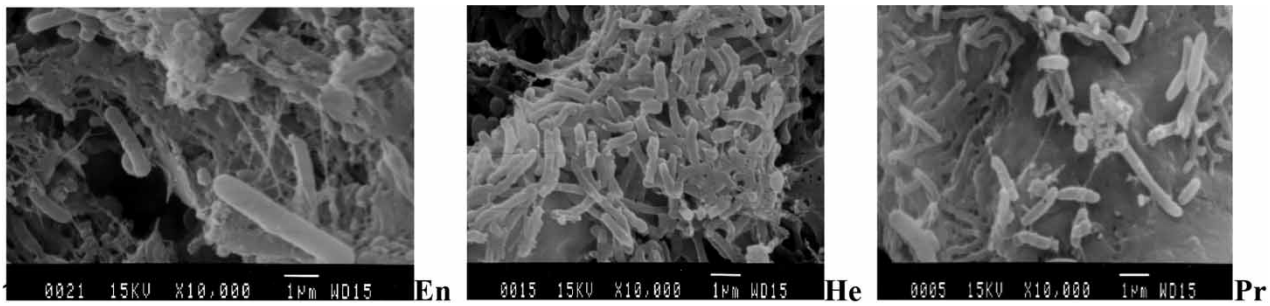

Figure 4 | Morphology of anodic films of MFCs fed with the activated sludge pretreated by thermophile (En), 100 °C heating (He) and 121 °C autoclaving (Pr).

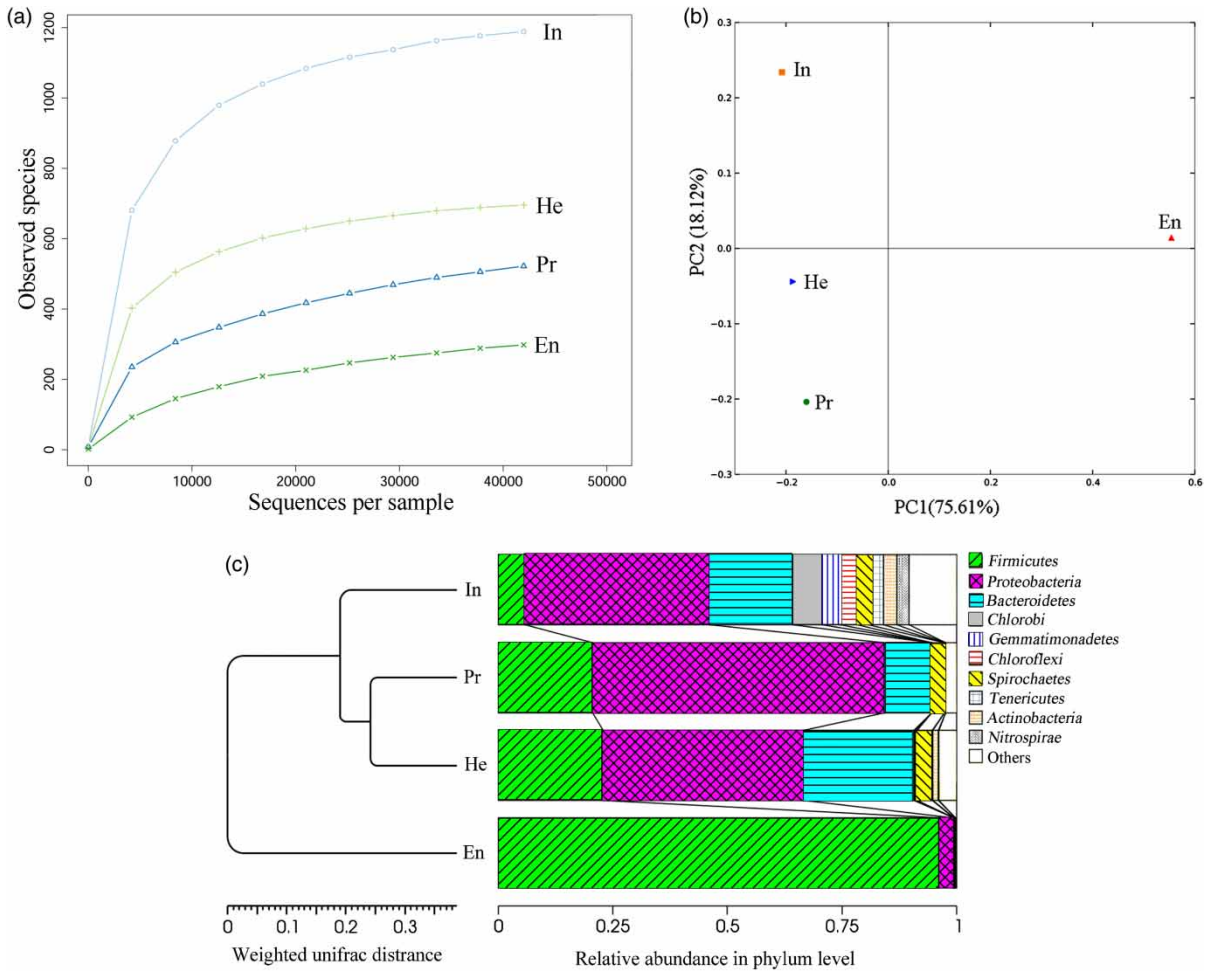

Figure 5 | Rarefaction curve (a), principal coordinates analysis (b) and composition (c) of microbial communities for inoculated sludge (In), MFCs En, He and Pr anodic biofilms.

that 30%–40% of the released SCOD in the sludge was gradually hydrolyzed to VFAs as the temperature increased (Zheng *et al.* 1998). VFAs were the favorable carbon source for MFCs and the suitable VFAs content was able to promote electricity production (Liu *et al.* 2005). In this study, the activated sludge pretreated at 100 °C and 121 °C heating produced about 1,000 mg/L of VFAs. These VFAs are enough to sustain the output voltage of MFCs at 0.57 and 0.64 V, respectively, and MFCs consequently performed well.

Some researchers used different techniques to pretreat the activated sludge for MFC electricity generation. Mohd Zulkhairi *et al.* (2013) pretreated activated sludge by ozone and microwave and found that microwave treatment released about 600 mg/L of SCOD. Consequently, they used the product as substrate and MFC reached maximum voltage and output power density of 0.17 V and 42 mW/m^2, respectively. Oh *et al.* (2014) pretreated activated sludge with ultrasound and hot alkali. They showed that the ultrasound pretreatment released up to 6,000 mg/L of SCOD. MFC maximum voltage approached 0.25 V and the maximum power density was 12.67 mW/m^2. These studies achieved a relatively high SCOD, but the power generation efficiency was generally limited. It is very likely that some substances that inhibited the electricity production were introduced during the pretreating process, such as pH imbalance caused by alkali, and the generation of oxidizing chemicals in microwave, ozone and ultrasound treatment. In the present study, inefficient power generation of MFC En might be due to the thermophile-pretreated sludge products which were not suitable for the survival of exoelectrogens. Thus, after long-term operation of the MFC, the exoelectrogens were reduced or eliminated.

Table 1 | Relative abundance of bacterial genera in inoculated sludge and MFC anodic microbial communities

		Relative abundance[b]			
Phyla or Classes	**Genera[a]**	**In**	**Pr**	**He**	**En**
Bacteroidetes	*Chitinophaga*	0.00	0.42	0.83	0.01
	Flavobacterium	0.01	0.02	1.57	0.00
	Others	18.21	9.91	21.59	0.24
	Subtotal	18.22	10.35	23.99	0.25
Firmicutes	*Lactobacillus*	0.12	0.05	0.06	91.45
	Clostridium	1.57	10.30	13.09	3.37
	Ruminococcus	0.00	0.54	0.14	0.04
	Others	4.25	9.70	9.52	1.31
	Subtotal	5.94	20.59	22.81	96.17
Nitrospirae	*Nitrospira*	2.60	0.01	0.01	0.02
	Others	0.01	0.00	0.00	0.00
	Subtotal	2.61	0.01	0.01	0.02
Alphaproteobacteria	*Brevundimonas*	0.03	0.20	0.67	0.02
	Devosia	0.10	0.07	0.50	0.01
	Azospirillum	0.30	5.88	7.59	0.12
	Telmatospirillum	0.02	0.51	0.50	0.02
	Sphingomonas	0.08	0.36	0.86	0.01
	Sphingopyxis	0.12	0.15	0.62	0.02
	Others	5.06	3.25	5.51	0.52
	Subtotal	5.71	10.42	16.25	0.72
Betaproteobacteria	*Achromobacter*	0.03	0.59	0.48	0.32
	Pandoraea	0.04	2.39	0.43	0.04
	Alicycliphilus	0.00	0.70	0.17	0.01
	Hydrogenophaga	0.08	0.04	0.55	0.01
	Rubrivivax	0.02	0.89	0.43	0.00
	Gallionella	0.97	0.00	0.00	0.00
	Dechloromonas	1.12	4.71	0.56	0.07
	Sterolibacterium	0.63	0.00	0.00	0.00
	Others	12.97	32.19	11.9	0.41
	Subtotal	15.86	41.51	14.52	0.86
Deltaproteobacteria	*Desulfobulbus*	0.24	0.03	3.32	0.01
	Desulfovibrio	0.23	0.57	1.31	0.01
	Geobacter	0.42	2.02	0.56	0.02
	Syntrophus	2.34	0.00	0.00	0.01
	Others	6.78	0.11	0.74	0.04
	Subtotal	10.01	2.73	5.93	0.09
Gammaproteobacteria	*Crenothrix*	0.62	0.00	0.00	0.00
	Pseudomonas	0.24	4.78	2.76	0.11
	Vibrio	0.07	0.07	0.04	0.78
	Dokdonella	0.07	0.72	0.74	0.01
	Stenotrophomonas	0.06	1.88	0.83	0.07
	Others	7.38	1.06	1.55	0.17
	Subtotal	8.44	8.51	5.92	1.14
Spirochaetes	*Treponema*	0.62	2.71	3.13	0.02
	Others	1.77	0.00	0.20	0.01
	Subtotal	2.39	2.71	3.33	0.03

[a]Only established genera are shown.
[b]Only the genera more than 0.5% in relative abundance are shown.

In this study, Betaproteobacteria microorganisms were extremely dominant with 41.51% in abundance in the anodic microbial community of MFC Pr. Chae *et al.* (2009) also found a large proportion of Betaproteobacteria in the microbial community in MFCs with different substrates. In addition, some microorganisms in classes

Alphaproteobacteria, Gammaproteobacteria, and Deltaproteobacteria were also proved to be efficient electricity-production bacteria (Kim *et al.* 2002; Holmes *et al.* 2004; Song *et al.* 2015). Further analysis showed that the microbial community of MFC Pr and He contained high abundance of *Geobacter* species in class Deltaproteobacteria and their proportions were up to 2.02% and 0.56%, respectively. Studies have shown that *Geobacter* are obligate anaerobes capable of utilizing acetate as a carbon source and they usually performed anaerobic respiration using Fe^{3+} and Mn^{4+} as electron acceptors (Sharma & Kundu 2010). As the typical exoelectrogens in MFCs, *Geobacter* species were an important factor that determined the electricity-production efficiency of MFCs (Shehab *et al.* 2013; Lesnik & Liu 2014). The higher biomass and activity of *Geobacter* often led to a better electricity production of MFCs (Dunaj *et al.* 2012). Thus, the high abundance of *Geobacter* is consistent with the performance of MFCs Pr and He.

In contrast, the relative abundance of phylum Firmicutes in MFC En reached 96.17%, which is far more than MFC Pr and He. Further analysis showed that *Lactobacillus* in Firmicutes was the predominant genus in MFC En anodic biofilm with a relative abundance of 91.45%. As the name shows, the main product of *Lactobacillus* during degrading of SCOD is lactic acid, which thus significantly decreases the pH of the MFC anodic solution. The low pH environment was not suitable for the survival of exoelectrogens. In this study, pH in MFC En went down to below 4.5 for most of the time in one feeding cycle and the exoelectrogens survive with difficulty under this kind of condition. However, *Lactobacillus* favored the low pH and consequently became the main flora in MFC En (Hammes & Vogel 1995). The SEM results (Figure 4) showed MFC En anodic biofilm was covered by larger bacteria than other MFCs, which accorded well with the morphology of *Lactobacillus*. Thus, from the microbiological points of view, it was further confirmed why the MFC En performed worse in electricity production than other MFCs. The spatial niche on the anodic biofilm was completely occupied by *Lactobacillus* and the serious acidic environment further squeezed the nutritional niche. The significant changes of niches largely restricted the growth and multiplication of other microorganisms, including exoelectrogens. In addition, it was reported that thermophilic bacterium AT07-1 was able to secrete extracellular enzymes, which contain polypeptide antibiotics (Tang *et al.* 2012). Peptide antibiotics are biologically active polypeptides, which attack the cell membrane and destroy the integrity of the membrane, and finally leads to bacteria cells lysis. Researchers reported that extracellular enzymes secreted by thermophilic bacteria were capable of efficiently dissolving other microorganisms (Desai & Dhala 1969; Dean & Ward 1991). However, in the present study, the thermophile-pretreated activated sludge products did not show the inhibitory effect on Gram-negative bacteria *Escherichia coli* and Gram-positive bacteria *Bacillus subtilis* (data not shown).

Anolyte pH is an important factor affecting the electrical properties of MFCs. Behera *et al.* (2010) reported MFCs with a slightly alkaline anolyte (about 7.5) resulted in better electricity generation and organic removal. Jiang *et al.* (2009) found that the removal efficiency of total COD was the best at the pH value of 7.2. When the pH value was higher or lower than 7.2, the MFC removal efficiency of COD was heavily impacted. In this study, the MFC En anodic chamber was an acidic environment which inhibited the normal metabolic activity of exoelectrogens and other microbes, resulting in acidogenic non-exoelectrogens' absolute dominance. In the present study, pH adjustment was performed in the anode in order to make a suitable environment for the survival of exoelectrogens. However, the *Lactobacillus* had formed a complete biofilm; the exoelectrogens find it hard to survive in such a system and thus could not improve electricity-production efficiency.

Compared with the other pretreatments, thermophile pretreatment of sewage sludge resulted in higher SCOD and VFAs and the biodegradability of sludge was improved significantly. However, due to the system pH decrease, a large number of *Lactobacillus* inhibited the subsequent electricity production process. To resolve this problem, an anaerobic fermentation tank (equivalent to the acidogenic phase of a two-phase anaerobic treatment system) can be set up before the MFCs, where the SCOD can be further degraded to VFAs and ethanol. The VFAs and ethanol with a suitable pH value would be favored by MFCs.

In conclusion, thermophilic bacterium pretreatment of activated sludge (En) released more SCOD than heating pretreatments (Pr/He). However, MFCs fed with products of Pr/He performed better in organic matter removal and electricity generation than MFC En, where considerable VFA accumulated, pH value seriously decreased and the role of supplied alkali and buffer was limited. The high-efficiency electricity production bacteria *Geobacter* spp. predominated in anodic biofilm of MFCs Pr and He. However, the absolute dominant bacteria in anodic biofilm of MFC En were *Lactobacillus* spp. with an abundance of 91.45%. *Lactobacillus* spp. occupying the spatial niche of the anode was the key reason for the inefficiency of MFC.

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