Effect of different seed sludge on start-up of EGSB reactor and Microbial community analysis by next generation sequencing

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**Abstract:** Parallel experiments were carried out on two identical EGSB reactors, and the use of seed sludge came from the anoxic sludge of the municipal Sewage Treatment Plant and sludge concentration pond of the automobile painting wastewater treatment station respectively. And the both reactor were successfully started up after nearly 60 days of operation. At the same time, using high-throughput sequencing analysis, the results showed that a total of 20 phyla were observed in four samples, and the most predominant phylum was *Proteobacteria* (23.3%), *Bacteroidetes* (30.4%), *Firmicutes* (47.1%) and *Proteobacteria* (70.5%) in FW, BW, CHW and CW, respectively. At the genus level, the superiority genera were *T78*, *Anaerovorax*, *vadinCA02*, *E6*, *Clostridium*, *WCHB1-05*, *Longilinea*, *Tsukamurella*, *Blvii28*, *Methanosarcina*, *HA73*, *W22* and *Fusibacter* in the B reactor, and the superiority genera were *HA73*, *Mycobacterium*, *Clostridium*, *Anaerovorax* and *Fusibacter* in the C reactor. It is expected to provide theoretical support for practical engineering application of EGSB reactor

**Introduction**

With the development of economy, all kinds of pollution became more and more serious. While among the various treatment technologies, anaerobic treatment systems are being encouraged because of several advantages, including low construction costs, small land requirements, low excess sludge production, plain operation and maintenance, energy generation in the form of biogas\textsuperscript{[1]} and robustness in terms of COD removal efficiency\textsuperscript{[2]}, pH stability and recovery time\textsuperscript{[3,4,5]}. A number of researchers have recommended anaerobic technology like expanded granular sludge bed (EGSB) reactor for the treatment of Urban sewage and industrial waste water\textsuperscript{[6-12]}. The start-up of EGSB reactors is a complicated process and a number of factors, including wastewater characteristics, acclimatization of seed sludge, pH, nutrients, presence of toxic compounds, loading rate, upflow velocity (Vup), hydraulic retention time (HRT), liquid mixing and reactor design affect the growth of sludge bed\textsuperscript{[7,13]}. In the present study, two identical EGSB reactors was investigated by the use of seed sludge came from the anoxic sludge of the municipal Sewage Treatment Plant and sludge concentration pond of the automobile painting wastewater treatment station respectively. At the same time, the microbial communities of seeding source (FW, CHW), sludge samples(BW, CW) were investigated with the next generation sequences (NGS) tool.
Material and methods

Bioreactor, inoculation and synthetic wastewater

Two parallel EGSB reactors were used in the experiment, which were named as B reactor and C reactor, respectively. The schematic diagram of the bench-scale EGSB reactor used in this study is shown in Fig. 1. The Plexiglas EGSB reactor was 60 mm in diameter and 1150 cm in height, giving a total volume of 4.34 L a working volume of 2.26 L and sediment volume of 1.57L. A peristaltic pump was used to introduce influent at the column bottom of the reactor. A gas-washing device was used to collect the generated CH$_4$ and CO$_2$ gas at the column top. A three-phase separator was installed at the reactor top to keep the biomass within the reactor. Excess sludge was discharged from the bottom of the EGSB reactor. The liquid up-flow velocity was controlled by inner recirculation. The EGSB reactor was operated under mesophilic conditions ($35 \pm 1^\circ C$) and its temperature was maintained by a water bath.

B reactor: The seed sludge was domesticated from the anoxic sludge (mixed liquor volatile suspended solids (MLVSS):27.178 g/L) of the Fuliangxian municipal Sewage Treatment Plant, Jingdezhen, China, which was filtered through 0.2 mm Tyler mesh to eliminate most grit. At the same time, sludge samples were taken and stored for subsequent analysis, denoted as FW. The reactor was started up with the influent COD concentration at about 200 mg/L, and liquid up-flow velocity at 2.5 m/hr, hydraulic retention time (HRT) (22 h), and sodium acetate as carbon source. During 58 days of operation, the influent COD concentration of the synthetic wastewater gradually increased from 200 to 500, and 1000 mg/L. The simulated organic wastewater was simulated by using glucose, urea and potassium dihydrogen phosphate, which was prepared in proportion to COD: N: P=200:5:1. And the trace element solution was added to the synthetic wastewater at a volume ratio of 1:500, which consisted (g/L) of EDTA(5.0), CaCl$_2$$\cdot$2H$_2$O (5.5), FeSO$_4$$\cdot$7H$_2$O (5.0), ZnSO$_4$$\cdot$7H$_2$O (2.2), CoCl$_2$$\cdot$6H$_2$O (1.6), MnCl$_2$$\cdot$6H$_2$O (5.0), CuSO$_4$$\cdot$5H$_2$O (1.6), MgSO$_4$$\cdot$7H$_2$O (5.0), NiCl$_2$$\cdot$6H$_2$O (0.6), Na$_2$MoO$_4$$\cdot$2H$_2$O (5.0). Sludge samples were taken and stored for subsequent analysis at day 56, denoted as BW.

C reactor: The seed sludge was domesticated from sludge concentration pond (MLVSS:17.18 g/L) of the Changhe automobile painting wastewater treatment station, Jingdezhen, China. At the same time, sludge samples were taken and stored for subsequent analysis, denoted as CHW. The reactor was started up with the influent COD concentration at about 1000 mg/L, The simulated organic wastewater was used sodium acetate as carbon source, other conditions such as the B...
reactor. The EGSB reactor was successfully started during 62 days of operation, sludge samples were taken and stored for subsequent analysis at day 60, denoted as CW.

**Chemical analysis**

Wastewater parameters chemical oxygen demand (COD), Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS) were measured according to the Standard Method[14].

**Microbial community analysis**

The samples were subjected to total genomic DNA extraction using the Power Soil DNA extraction kit (Mo Bio, Carlsbad, CA) following the manufacture’s procedure. Preparation of 16S rRNA gene amplicon and subsequent Illumina sequencing were conducted by the DNA Services Facility at the University of Illinois at Chicago. Briefly, PCR amplification were conducted in triplicate reactions for all samples using the 515F/907R primer set that amplifies the V4-V5 region of the 16SrRNA gene[15,16]. Amplicons of the triplicate PCR reactions were pooled and then sequenced using an Illumina HiSeq 2000 instrument. De novo assembly of paired-end reads was performed with the software package CLC Genomics Workbench Version 6.0 (CLC bio, Cambridge, MA), and the resulting fastq files were trimmed using a cutoff quality score of Q15 and read length of larger than 200bp[17]. Only the forward reads were used for downstream analysis, as it was reported that including the reverse reads add little additional information[18].

After sequencing, the raw fastq files were demultiplexed and quality-filtered using QIIME (version 1.17). As the number of spurious phylotypes increases with sequencing effort, equal number of sequence reads should be used to compare microbial community among samples to minimize the sequencing artifact[19]. In this work, 40000 reads from every sample were randomly picked and grouped into operational units (OTUs) with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/). Chimeric sequences were identified and removed using UCHIME. Community diversity index (Chao1, PD, Simpson, Shannon diversity index) and rarefaction curves were generated using the MOTHUR program. The taxonomic assignment of OTUs was performed by RDP Classifier (http://rdp.cme.msu.edu/) against the silva (SSU115) 16S rRNA database at 70% threshold[20].

**Results and discussion**

**Start-up performance**

**Fig. 2. Removal efficiency of COD during the start-up and operation of an EGSB reactor**

The change of COD removal efficiency was shown in Figure 2 during the start-up process of the B/C EGSB reactor. As can be seen from the figure 2, the COD removal efficiency reached more
than 80% after the continuous operation of 15 days, which indicated that it was good adaptability for the simulated wastewater treatment in an B EGSB reactor. In the meantime, the COD removal efficiency can also achieve more than 80%, even reaching above 95%, when the COD concentration of influent was improved from 200, 500 to 1000 mg/L in the continuous operation of 58 days. At this time, it was successful that the EGSB reactor started up. Furthermore, compared with B reactor, the vaccinate sludge of C reactor was from industrial wastewater treatment sludge and the influent of carbon source was sodium acetate, and the COD concentration of the influent remained about 1000 mg/L. So the removal rate of COD was low, only about 50% at the beginning of the start-up C reactor, and with the improvement of domestication time, the COD removal rate increased gradually, and then stayed above 85%, at this time that a C reactor start-up success. The change trend of COD was similar results with the previously reported literature [21,22].

Microbial community

To reveal the shift in the microbial community structure during the start-up process of the B\C EGSB reactor, The sampling BW and CW were prepared as a mixture of 10 mL mixed liquor at three ports (60 mm, 210mm, and 360 mm from the bottom). Employing 454-pyrosequencing, 358009 sequences in total were generated from 4 samples (FW, BW, CHW and CW). After filtering the low quality reads using the RDP Initial Process in Pyrosequencing Pipeline (PP) and trimming the adapters, barcodes and primers, denoising, filtering out chimeras, 270403 effective sequences, nearly 76% of the total sequences remained. This number of sequences was comparable to other studies that also adopted 454-pyrosequencing[23-26]. The multiple a-diversity indices were shown in Table 1.

As can be seen from Table 1, the OTUs number of CHW from industrial wastewater was less than half that of FW from urban sewage treatment plants. And that variational trend of Shannon index, Chao1, and PD was the same as OTUs from the Table 1. These indicated that the bacterial richness and microbial diversity of urban sewage treatment plants was significantly higher than that of industrial wastewater. At the same time, After FW was domesticated by the laboratory in the B EGSB reactor, the values of OTUs, Chao1 and PD have decreased, which indicated that the bacterial richness and microbial diversity had decreased. However, after CHW was domesticated by the laboratory in the C EGSB reactor, the values of OTUs, Chao1 and PD had increased, indicating that the bacterial richness had become more abundant. The Shannon index (H’) for four samples ranged from 3.1 to 8.4, and showed a downward trend. And that variational trend of Simpson diversity index was the same as Shannon index from the Table 1. Which indicated the community richness was decreased.

Table 1. Raw, effective bacterial reads, and plus numbers of OTUs, Chao1, PD, Shannon and Simpson of four activated sludge samples at level of 3% cutoff.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Raw reads</th>
<th>Effective reads</th>
<th>OTUs</th>
<th>Chao1</th>
<th>PD</th>
<th>Shannon</th>
<th>Simpson</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW</td>
<td>72707</td>
<td>41635</td>
<td>1546</td>
<td>1770</td>
<td>108</td>
<td>8.4</td>
<td>0.99</td>
</tr>
<tr>
<td>BW</td>
<td>94172</td>
<td>65768</td>
<td>1158</td>
<td>1489</td>
<td>86</td>
<td>6.6</td>
<td>0.97</td>
</tr>
<tr>
<td>CHW</td>
<td>75695</td>
<td>67879</td>
<td>627</td>
<td>1023</td>
<td>55</td>
<td>5.9</td>
<td>0.95</td>
</tr>
<tr>
<td>CW</td>
<td>115435</td>
<td>95121</td>
<td>714</td>
<td>1106</td>
<td>60</td>
<td>3.1</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Note: OTUs: operational taxonomic units; Shannon: Shannon's diversity index; Chao1 -community richness. A higher number represents more richness; Simpson - community diversity. A higher number represents more diversity; The biodiversity indices (OTUs, Chao1, PD, Shannon and Simpson) were calculated based on normalization of the number of the filtered sequences from each sludge sample to 40000.
As shown in Fig. 3, for B reactor and C reactor systems, 20 phyla were detected by high-throughput sequencing, the microbial community structures on phylum level were different, in terms of either the most predominant phylum or the content of each phylum. In FW sample, the most predominant phylum was Proteobacteria (23.3%), which was consistent with the results in other studies\cite{23}, followed by Firmicutes (19.6%), Chloroflexi (19.4%) and Bacteroidetes (10.8%); while in BW sample after successful start-up of B reactor, the most predominant phylum was Bacteroidetes (30.4%), followed by Chloroflexi (22.2%), Firmicutes (15.9%), Synergistetes (9.9%), Actinobacteria (4.2%) and Proteobacteria (4.1%); The variation trend of inoculated samples and domesticated samples was in accordance with the previous work of Chen et al.\cite{15}. Furthermore, in the C reactor systems, the most predominant phylum were Firmicutes (47.1%) and Proteobacteria (70.5%) in CHW and CW, respectively, followed by Proteobacteria (38.9%), Actinobacteria (5.2%) and Bacteroidetes (4.5%) in CHW sample, and followed by Bacteroidetes (13.1%), Firmicutes (10.7%), Synergistetes (1.8%) and Actinobacteria (1.8%) in CW sample, which was not consistent with the results in other studies\cite{26,27,28}. This may be related to the fact that the inoculated sludge came from automotive coating wastewater.

Fig. 3. Relative abundance of phylum in all samples

More detailed analyses at genus level were also carried out to identify microorganism involved in the start-up process (Fig. 4). A total of 45 genera (each accounts for more than 0.5% of the total) were observed in four samples. Results showed diversity of microbes among samples, the relatively abundant bacterial genera were Clostridium (7.80%), Longilinea (1.44%), Methylosinus (1.17%), WCHB1-05 (1.03%) and Caldilinea (0.97%) in FW, T78 (4.11%), Anaerovorax (4.00%), vadinCA02 (3.85%), E6 (3.46%), Clostridium (3.44%), WCHB1-05 (3.06%), Longilinea (3.04%), Tsukamurella (3.00%), Blvii28 (2.88%), Methanosarcina (2.67%), HA73 (2.63%), W22 (1.97%) and Fusibacter (1.03%) in BW, Bacillus (30.63%), Dechloromonas (3.13%), Hyphomicrobium
(2.57%), Microbacterium (2.23%), Sporomusa (1.88%), Proteiniclasticum (1.59%), Sporosarcina (1.47%), Acinetobacter (1.43%), Novosphingobium (1.40%), Azospira (1.34%), Faecalibacterium (1.31%), Coprococcus (1.05%) and Propionibacterium (1.02%) in CHW, HA73 (1.59%), Mycobacterium (1.18%), Clostridium (0.69%), Anaerovorax (0.63%) and Fusibacter (0.50%) in CW. In B reactor system, the relative abundance of Methanosarcina and Methanosaeta were 0.05% and 0.04% in FW sample, respectively. While the relative abundance of Methanosarcina (2.67%) and Methanosaeta (0.58%) significant increased after acclimation, and the relative abundance of unclassified genus decrease from 72.6% to 49.6%. However, in C reactor system, the relative abundance of unclassified genus was both about 30% after acclimation, and the relative abundance of other genus significant increased from 6.4% to 61.4%. The above analysis deduced that the community richness and microbial diversity in municipal wastewater treatment plants was higher than that in industrial sewage treatment station, and the community richness and microbial diversity after acclimation in an EGSB reaction may increase or decrease depending on the sludge inocula.

**Fig. 4. Distribution of dominant microorganism (>0.5%) in all samples (at genus level)**

**Conclusions**

Parallel start-up experiments were carried out on two identical EGSB reactors using different sludge inoculation, and they were successfully started up after nearly 60 days. At the same time, the changes of microbial flora before and after the start-up of the EGSB reactor were analyzed by high-throughput sequencing. The results showed that a total of 20 phyla were observed in all samples, and there was different the most predominant phylum, which was Proteobacteria (23.3%), Bacteroidetes (30.4%), Firmicutes (47.1%) and Proteobacteria (70.5%) in FW, BW, CHW and CW, respectively. At the genus level, the relatively abundant bacterial genera were Clostridium, Longilinea, Methylosinus and WCHB1-05 in FW, T78, Anaerovorax, vadinCA02, E6, Clostridium, WCHB1-05, Longilinea, Tsukamurella, Blvii28, Methanosarcina, HA73, W22 and Fusibacter in BW, Bacillus, Dechloromonas, Hyphomicrobium, Microbacterium, Sporomusa, Proteiniclasticum,
Sporosarcina, Acinetobacter, Novosphingobium, Azospira, Faecalibacterium, Coprococcus and Propionibacterium in CHW, HA73, Mycobacterium, Clostridium, Anaerovorax and Fusibacter in CW.

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