Short Communication

Evaluation of surfactants on waste activated sludge fermentation by pyrosequencing analysis

Aijuan Zhou\textsuperscript{a}, Wenzong Liu\textsuperscript{b}, Cristiano Varrone\textsuperscript{c}, Youzhao Wang\textsuperscript{d}, Aijie Wang\textsuperscript{b,e}, Xiuping Yue\textsuperscript{a,*}

\textsuperscript{a} College of Environmental Science and Engineering, Taiyuan University of Technology, Taiyuan, China
\textsuperscript{b} Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China
\textsuperscript{c} Section for Sustainable Biotechnology, Aalborg University Copenhagen, Denmark
\textsuperscript{d} School of Mechanical Engineering and Automation, Northeastern University, Shenyang, China
\textsuperscript{e} State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology (SKLUWRE, HIT), Harbin, China

Highlights

• Evaluation of surfactants on WAS fermentation was analyzed by pyrosequencing.
• RL biosurfactant showed more positive effects over chemosynthetic surfactants.
• Dominant populations of different surfactant-treated WAS were diverse.
• Acid-producing bacteria detected in RL were more abundant.
• \textit{Megasphaera} and \textit{Oscillibacter} were dominant acidification species.

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Abstract

The effects of three widely-used surfactants on waste activated sludge (WAS) fermentation and microbial community structures were investigated. Rhamnolipid bio-surfactants (RL) showed more positive effects on WAS hydrolysis and acidification compared to chemosynthetic surfactants, such as sodium dodecyl-sulphate (SDS) and sodium dodecyl benzene sulfonate (SDBS). The highest SCOD and VFAs concentrations obtained with RL were 1.15-fold and 1.16-fold that of SDS, and up to 1.73 and 3.63 times higher than those obtained with SDBS. Pyrosequencing analysis showed that an evident reduction in bacterial diversity in surfactant-treated WAS. Moreover, acid-producing bacteria (such as \textit{Megasphaera} and \textit{Oscillibacter}), detected with RL, were (6.8\% and 6.4\% in proportion) more abundant than with SDS, and were rarely found in SDBS and the control. The results also revealed that RL allowed efficient hydrolysis enhancement and was favorable to functional microorganisms for further acidification during WAS fermentation.

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1. Introduction

Due to the widely used activated sludge processes for wastewater treatment, the disposal and treatment of waste activated sludge (WAS) has become an issue of particular concern. The rapid increasing energy demand, along with growing concerns for environmental protection, has suggested the use of WAS recycling as alternative energy source (Baek et al., 2014). Anaerobic digestion is acknowledged as the most cost-effective way for WAS treatment and the generation of bioenergy resources. As the organic compounds in WAS are mainly organized in the form of microbial cells, hydrolysis of microbial cell walls is considered as the rate-limiting step. WAS pretreatment is thus a prerequisite, in order to keep operations within an industrially acceptable time-frame. Many approaches involving physical, chemical or biological methods have therefore been developed for improving WAS solubilization (Appels et al., 2008).

Nowadays, surfactants assisted treatments are regarded as an alternative strategy for WAS pretreatment, due to their unique characteristics. Jiang et al. (2007a,b), for instance, investigated the enhancement of VFAs production from WAS fermentation by sodium dodecylsulphate (SDS) and sodium dodecyl benzene sulphonate (SDBS). Zhou et al. (2013) studied the feasibility of...
rhamnolipid biosurfactant (RL) on WAS pretreatment. Clearly, hydrolysis and acidification of WAS were both enhanced by surfactants. Based on previous report, surfactants can remove or break extracellular polymeric substances (EPS) to increase the effectiveness of subsequent bacterial treatment (Kavitha et al., 2014). However, little is known about the different effects on microbial community structure shifts during WAS fermentation, in the presence of various surfactants.

In this study, the effect of three widely used surfactants (SDS, SDBS and RL) on WAS fermentation was investigated. VFAs yields, composition and particulate organic matter solubilization were monitored. It is well known that before bacteria can assimilate high molecular weight compounds, the compounds usually need to be hydrolyzed by extracellular enzymes (Cadoret et al., 2002). Therefore, hydrolytic enzyme activities were also studied, to elucidate the mechanisms. In recent years, high-throughput sequencing technologies such as 454 pyrosequencing have been developed to fully explore the microbial diversity in the environment, which can generate 400,000 sequences (100–400 bp in length), compared with the 192 sequences (700 bp) produced by one run of Sanger sequencing (Zhang et al., 2005). Therefore, the microbial community structure was examined using high-throughput pyrosequencing of the 16S rRNA gene in this study, which can provide important information to better understand the microbial response mechanism to surfactant-treated WAS.

2. Methods

2.1. Source of waste activated sludge

WAS was collected from Wenchang municipal wastewater treatment plant (Harbin City, China) and concentrated by settling at 4 °C for 24 h, prior to tests. Its characteristics (average value plus standard deviation of triplicates) were as follows: pH 6.49 ± 0.27, total suspended solids (TSS) 23,056 ± 151 mg/L, volatile suspended solids (VSS) 14,000 ± 127 mg/L, total chemical oxygen demand (TCOD) 22,063 ± 379 mg/L, soluble chemical oxygen demand (SCOD) 445 ± 18 mg COD/L, total protein 12,476 ± 278 mg COD/L, total carbohydrate 1611 ± 79 mg COD/L, solute carbohydrate 55 ± 17 mg COD/L and VFAs 75 ± 8 mg COD/L.

2.2. Experimental setup and operations

WAS fermentation was conducted in 500 ml serum bottles filled with 350 ml sludge each. Based on the results of previous study (Zhou et al., 2013), a surfactant dosage of 0.04 g/g TSS was applied to the sludge. Nitrogen gas was flushed to remove oxygen; all bottles were capped, sealed, and stirred in an air-bath shaker (100 rpm) at 35 ± 1°C for 192 h. All the fermentation experiments were carried out in triplicate.

2.3. DNA extraction and pyrosequencing

Before DNA extraction, sludge samples were centrifuged at 8000 g to remove supernatant. DNA was extracted from sludge sediments of three replicate reactors using EZNA® Soil DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA), and then pooled together. The primers 27F (5′-AGAGTTTGATCCTGTCAG3′) and 533R (5′-TTCACGGCAGCTGTCCGAC3′) were used for the 454 pyrosequencing. To achieve the sample multiplexing during pyrosequencing, barcodes were incorporated between the 454 adaptor and forward primer. The barcodes primers used in this study were RL (AGTAGACGTC), SDS (AGTACTACTA), SDBS (AGTAGAGAG) and the control (AGACTCGAGG), respectively; the corresponding forward primer was TTACCGCGGTGTCCGAC. After being purified and quantified, the PCR amplicon was used for pyrosequencing on a Roche 454 GS FLX, and the raw sequences have been deposited in the NCBI Short Read Archive (SRA) database under the accession numbers SRR2045673. The adapters, barcodes, and primers in all raw sequences were trimmed to minimize the effects of random sequencing errors. Sequences shorter than 350 bp, or containing any ambiguous base calls, were removed.

The remaining sequences were clustered into operational taxonomic units (OTUs), using the 97% identity threshold (3% dissimilarity level). Rarefaction curves and Chao1 were generated and assigned to taxonomic classifications. Principal component analysis (PCA) was also conducted. Finally, a Venn diagram with OTUs was applied for depicting the similarity and difference between the different sludge fermentation systems.

2.4. Analytical methods

Sludge samples were centrifuged at 10,000 rpm after anaerobic fermentation, then filtered through a 0.45 μm cellulose nitrate membrane filter and finally stored at 4 °C, prior to analysis. The determination of TSS, VSS, carbohydrates, proteins, VFAs, α-glucosidase and protease activities was performed as previously described (Zhou et al., 2013). VFAs production was calculated as the sum of the measured acetic (HAc), propionic (HPr), n-butyric (n-HBu), iso-butyric (iso-HBu), n-valeric (n-HVa) and iso-valeric (iso-HVa) acids. The COD conversion factors are 1.50 g COD/g protein (assumed as (C6H12O6 N)x), 1.06 g COD/g carbohydrate (assumed as C6H12O6), 1.07 g COD/g HAc, 1.51 g COD/g HPr, 1.82 g COD/g HBu, and 2.04 g COD/g HVa.

3. Results and discussion

3.1. Effect of surfactants on WAS fermentation

Effects of the three surfactants on WAS fermentation are shown in Fig. 1. Both, organics solubilization (Fig. 1A) and hydrolytic enzymes activity (Fig. 1B) were enhanced by all the surfactant treatments. After 48 h, the maximum SCOD concentration obtained with RL reached 2252 ± 118 mg COD/L, which was 1.15-fold and 1.73-fold higher than that with SDS and SDBS, respectively; control test only obtained 902 ± 78 mg COD/L. As proteins and carbohydrates are the main constituents of WAS, the solubilization of WAS organics can be expressed (with a reasonable approximation) by that of soluble proteins and carbohydrates. Consequently, the release of soluble proteins and carbohydrates was also strengthened by the RL treatment, with a concentration of 1171 ± 103 and 263 ± 8 mg COD/L in 48 h, respectively, which was ~1.50-fold and ~2.38-fold over that obtained with SDS and SDBS. These findings were in accordance with the results of the corresponding activities of protease (117 ± 7 versus 59 ± 2 and 18 ± 13 Eu) and α-glucosidase (29 ± 4 versus 11 ± 1 and 13 ± 2 Eu), with RL, SDS and SDBS treatments, respectively. Cadoret et al. (2002) reported that a variable fraction of enzyme activities were associated to the easily extractable extracellular polymeric substances (EPS) from sludge flocks (Cadoret et al., 2002). The reason for this might be that surfactants facilitate the detachment of aggregated cells and of EPS, while releasing immobilized enzymes, then resulting in the elevated organics solubilization. Seemingly, RL had a positive effect on the solubilization of particulate organics from sludge flocks over that by chemical synthetic surfactants.

Fig. 1C shows the time-course profile of VFAs production. VFAs yield sharply increased in all tests, from 72 h onward, and then...
decreased with the further increase of fermentation time. The maximum VFAs concentration was 5844 ± 77, 5036 ± 11, 1611 ± 61 mg COD/L with the RL, SDS and SDBS tests, respectively, while it only reached 836 ± 189 mg COD/L in the control test. Clearly, the surfactant-treated tests led to an increase in VFAs yield over the control test. This was particularly evident in the case of RL treatment, which led to a VFAs yield 1.16-fold and 3.63-fold higher than that obtained with SDS and SDBS.

VFAs composition is considered crucial when the WAS hydrolysate is used as external carbon source. HAc, for instance, was the favorite substrate for nutrients removal. As shown in Fig. 1D, and in accordance with a previous study by Yang et al. (2014), HAc formed the major part of total VFAs in SDS and RL tests (2063 ± 10 and 1520 ± 207 mg COD/L); HPr and n-HBu represented the second and third most abundant metabolites, respectively. These results seemed to be in agreement with Wang et al. (1999), which showed that the top two VFAs were HAc and HPr, no matter which type of sludge pretreatment method was used (ultrasonic, thermal and freezing). On the contrary, HPr, HAc and iso-HVa were the most abundant VFAs in the case of SDBS, as well as in the control tests. This would suggest that the treatment methods can, indeed, influence the acidification product spectrum. Furthermore, considering HAc concentration, it seems that SDS was better than RL, for the production of external carbon source from WAS fermentation. However, HPr is also considered an important component for biological phosphorus removal (Lopez-Vazquez et al., 2009). In the RL test, HPr concentration reached 1434 ± 38 mg COD/L, while it was only 867 ± 8 mg COD/L in the SDS test.

### 3.2. Overall analysis of pyrosequencing

From the results of 454 pyrosequencing, 11,650, 13,542, 11,849 and 16,859 high-quality reads (average length of 477 bp) were obtained for the control, SDBS, SDS and RL tests, respectively. Furthermore, OTUs at 3% distance were also detected (2023 for the Control, 1840 for SDBS, 1577 for SDS and 1607 for RL). However, new bacterial phylotypes continued to emerge even after 12,000 reads sampling with pyrosequencing (Fig. 2A). The maximum theoretical OTUs, based on the Chao1 index, were 3109, 2913, 2709 and 2663, respectively, which means the community species richness of untreated sludge was higher, while a reduction in bacterial diversity occurred after surfactants pretreatment. Based on the Shannon index, the RL and SDS also had relatively lower diversity (5.89 and 5.87) than that of the SDBS and Control (6.27 and 6.51). The reason behind this may be that some bacteria in the raw WAS could not survive in the presence of RL and SDS, and the number of bacterial communities thus decreased. That is, the proportions of functional microorganisms involved in the hydrolysis and acidification were higher, which resulted in the elevated organic solubilization and hydrolytic enzyme activity.

PCA was used to identify the differences among bacterial community structures (Fig. 2B). Principal components 1 and 2 explained 54.0% and 32.8% of the total community variations. SDBS and Control samples clustered together and were well separated from SDS and RL, while there was a clear distinction between SDS and RL. This was supported by hierarchical cluster analysis (Fig. 3A), which also revealed three distinct clusters (the first cluster was represented by Control and SDBS, and was separated from...
SDS and RL). That is, different surfactants treatments substantially changed bacterial community structure, despite the fact that the same initial source of microbial consortia was shared.

The sum of total classified OTUs (for all four communities) was 4706, but only 251 OTUs (5.3% of the total OTUs) were shared by them (Fig. 2 C). The majority of the shared OTUs were Proteobacteria (45.0%), Planctomycetes (13.5%) and Actinobacteria (11.1%). SDBS and Control shared more OTUs (807, 17.1% of total) than SDS and RL (539, 11.4%), SDBS and SDS (624, 13.3%) and SDBS and RL (522, 11.1%). OTUs that were unique to each community numbered 1002 for Control, 675 for SDBS, 737 for SDS and 783 for RL, and all together accounted for 67.9% of the total number of observed OTUs.

3.3. Microbial diversity and distribution analysis

To further investigate the diversity of microbial community, phylogenetic analysis of the 16S rRNA gene sequences was performed in phyla, class and genus level. Clear changes were observed in microbial community structure, after different surfactants pretreatments (Fig. 3).

Bacteroidetes, Firmicutes, Proteobacteria and Chloroflexi were the dominant phyla for all four communities (accounting for 77.0% in Control, 70.0% in SDBS, 70.4% in SDS and 73.3% in RL of the total bacterial sequences), however their distribution was quite different (Fig. 3B). Sun et al. (2014) also found that Proteobacteria, Bacteroidetes, Firmicutes and Chloroflexi were the main bacterial phyla in conventional anaerobic digesters (Sun et al., 2014). In the present study, the percentage of Proteobacteria decreased after surfactants treatments, reaching 46.1% (Control), 35.6% (SDBS), 34.7% (SDS) and 43.2% (RL), respectively. Likewise, the same trends were observed for Bacteroidetes and Chloroflexi. On the other hand, Firmicutes, which are known to play a critical role in the anaerobic hydrolysis and acidification process (Bertin et al., 2012), were highest (in relative abundance) in SDS (26.9%) higher in RL (24.4%), and were found to be lower in SDBS (6.7%) and lowest in Control (4.2%).

On the class level, the majority of sequences belonged to 14 classes, among which α-proteobacteria, β-proteobacteria, Clostridia and Sphingobacteria were the dominant ones (Fig. 3C). α-proteobacteria are reported to be responsible for the utilization of carbohydrate and production of HBu (Lenin Babu et al., 2013), while Clostridia are capable of producing VFAs by utilizing soluble organics (Kato et al., 2004), which were all greatly enhanced in the presence of SDS and RL (~25% and ~23% versus ~15% and ~5% of SDBS and Control).

Further investigation on the genus level provided more detailed information about microbial communities (Fig. 3D). Megasphaera, Oscillibacter and Clostridium belong to the phylum Clostridia. Megasphaera, which is known to be a fermentative acidogenic bacterium and reported to have the ability of degrading carbohydrate and lactic acid to form HAc, HPr and HBu (Weimer and Digman, 2013), took up the largest proportion in RL (6.8%). Oscillibacter (6.4%), commonly associated with the metabolism of α-glucose and a few pentoses, such as D-ribose and D-xylose, and α-HVa was the major final product (Iino et al., 2007), also clearly dominated in the RL community. Interestingly, these two genera were not detected in the other three experimental conditions. This suggests that RL probably created more favorable conditions for these
bacteria during WAS fermentation. *Clostridium* is known to produce VFAs through Stickland reaction, which was considered to be the main pathway for the accumulation of VFAs in WAS fermented in alkaline conditions (Liu et al., 2012), and was mainly detected in SDS (1.3%). *Planctomyces* and *Pirellula*, which belong to the phylum *Planctomycetia*, commonly associated with the decomposition of complex organic matters (Kulichevskaya et al., 2012), also reached the highest abundance with RL (2.1% and 2.6%) and SDS (1.6% and 2.6%). The applied molecular ecology proved to be a useful tool to better understand the phenomenon of elevated organics solubilization. The genus *Pseudomonas* (belonging to the phylum γ-proteobacteria) can metabolize glucose.
and xylene for producing VFAs, and was mainly detected in RL (0.35% versus 0.24% and 0.28% in SDSBS and SDS) as well. Moreover, *Pseudomonas* is also known to oxidize simple carbohydrates to produce RL (Van Rijn et al., 1996); this was confirmed by the observation of in-situ RL generation during WAS fermentation in our previous study (Zhou et al., 2013). In fact, RL concentration increased from initial 880 ± 92 mg/L to 1312 ± 7 mg/L (at 96 h) with an initial RL dosage of 0.04 g/g TSS.

Overall, the functional microorganisms in surfactant-treated WAS fermentation process resulted in a more rapid hydrolysis and acidification of organic matter. The results obtained using RL were more favorable than those obtained with chemosynthetic surfactants. It is well known that the economic viability is the major bottleneck for surfactants to be used for WAS treatment. The positive feedback of in situ RL production during WAS fermentation (in the presence of small amounts of RL) could thus provide a distinct advantage over synthetic surfactants and promote the implementation of RL use for WAS treatment. Detailed investigation on the synthesis ability (and microbial communities evolution) with decreasing RL concentrations, as well as the optimization of sludge retention time and minimum RL dosage in semi-continuous mode, would provide valuable insight into the viable utilization of RL for WAS treatments. Therefore, further research is advisable.

4. Conclusions

Surfactants treatment led to an evident enhancement of WAS hydrolysis and acidification. Moreover, RL showed more positive effects over chemosynthetic surfactants (SDS and SDSBS). Using high-throughput pyrosequencing, we demonstrated an evident reduction in bacterial diversity in surfactant-treated WAS. RL surfactants presented a positive effects on acid-producing bacteria (such as *Megasphaera* and *Oscillibacter*), which became more abundant than with chemosynthetic surfactants. Moreover, *Pseudomonas* took the highest proportion in RL, which significantly supported the phenomenon of in situ RL generation during WAS fermentation, a distinct advantage over chemical synthetic surfactants, in view of applied molecular ecology.

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References


