Biodiesel production from granular sludge fed with sugar-containing wastewater

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Abstract
Utilizing excess sludge from a wastewater treatment plant to produce biodiesel has become an increasingly popular topic. However, the low lipid content in activated sludge has hindered biodiesel production from excess sludge. This study was designed to enhance biodiesel production from granular sludge fed with synthetic sugar-containing wastewater. The experiments were conducted with two identical 1.2-L SBRs, R1 and R2, using different sludge settling times. The SBRs were fed with a glucose-based synthetic sugar-containing wastewater at a chemical oxygen demand (COD) loading rate of 3.0 kg/(m^3·d). Sludge granulation was achieved in R2, whereas the sludge in R1 remained in the form of flocs. The result showed that the biodiesel yield of the granular sludge (30.14 ± 0.73 mg/g SS) was higher than that of the activated sludge (21.56 ± 0.95 mg/g SS) with the same feeding. The distributions of fatty acid methyl esters (FAMEs) were also different among the seed sludge, the cultured sludge flocs and the granular sludge. Methyl 14-methylpentadecanoate (14MeC15:0), methyl oleate (C18:1) and methyl linoleate (C18:2) were the three components of the biodiesel produced from the granular sludge that increased the fastest, which might promote the heating value and the low temperature fluidity of biodiesel. The analysis of the microbial community revealed a remarkable difference between the granular sludge and the activated sludge. Brown filamentous fungi *Phialophora* existed in the granular sludge instead of in the seed and cultured sludge flocs. The variations of the microbial community might elucidate the intrinsic change in the fatty acids produced from the granular sludge, which resulted in a higher yield and a better quality of biodiesel.

Keywords
Biodiesel; fatty acid methyl esters (FAMEs); granular sludge; sugar-containing wastewater; microbial population

INTRODUCTION
The massive amount of excess sludge from wastewater treatment plants (WWTPs) could be a potential feedstock for nutrient recycling and energy recovery. The resources recycled from excess sludge include nutrients (nitrogen and phosphorus), electricity, hydrogen, syngas, bio-oil and biodiesel (Tyagi and Lo, 2013). Biodiesel, or fatty acid methyl esters (FAMEs), is a renewable and environment-friendly energy source that could be directly used in traditional engines. Thus, the study of utilizing the microbial lipids in excess sludge to produce biodiesel has received a great amount of attention in recent years (Dufreche et al., 2007; Pastore et al., 2013; Olkiewicz et al., 2015).

However, the low biodiesel yield resulting from the low lipid content of wastewater sludge has indirectly increased the cost of biodiesel production. Therefore, researchers have tried to enhance the lipid accumulation of activated sludge by increasing the organic loading and the ratio of carbon and nitrogen, utilizing different carbon substances, and so on (Mondala et al., 2012, 2013, 2015; Sun et al., 2015). Research has shown that increasing the organic loading rate could promote the production of microbial lipids. Meanwhile, sugar-containing wastewater with its high concentration of organic matter is another difficult issue to address. Compared with conventional activated sludge, the dense structure and good settling properties of biogranules could enable high biomass retention and the toleration of high-strength wastewater and shock loadings (Liu and Tay, 2004; Su and Yu, 2005; Li et al., 2008; Yilmaz et al., 2008; Corsino et al., 2015). Aerobic granular sludge in an SBR (AGS-SBR) could be a competent small wastewater system for the degradation of high concentrations of industrial wastewaters (Schwarzenbeck, et al., 2005; Wang et al., 2007). Granular sludge could contain special microbial populations responsible for the biological treatment of sugar-containing wastewater. This microbial population possibly consists of some heterotrophic bacteria that utilize the organic content of the wastewater to produce microbial lipids for biodiesel production. Therefore, granular sludge for the treatment of sugar-containing wastewater may be a promising raw material for biodiesel production, which could also reduce the environmental contamination from wastewater discharge and convert excess granular sludge into valuable resources.

The purpose of this study was to investigate the feasibility and the mechanisms of biodiesel production from granular sludge during the treatment of sugar-containing wastewater compared with activated sludge with the same feeding condition. The relationships among sludge type, microbial population and biodiesel production were investigated. These research findings will help to establish a new technical approach to biodiesel production from wastewater sludge so that an alternative means of waste disposal can be achieved. Moreover, a new low-cost feedstock source of lipids for biodiesel production could be obtained.

MATERIALS AND METHODS

Cultivation of activated sludge and granular sludge

Two plexiglass columns (5 cm in diameter and 80 cm in height) with a working volume of 1.2 L were used as the SBR reactors. The seed sludge was obtained from a full-scale sewage treatment plant (Xiaojia River, Beijing, China), and the initial biomass concentrations were 2.8 g MLSS/L in the two reactors. They were operated in a fixed regular mode for a 4 h cycle with 5 min of feeding and 8 min of effluent withdrawal from the middle port of the column. For the cultivation of activated sludge, the settling time was kept at 30 min in R1, whereas the settlement time was gradually reduced from 30 min to 2 min in R2 to promote the granulation process. The aeration rate was 1.0 L/min in the two reactors. The reactors were fed with synthetic sugar-containing wastewater that consisted of glucose, ammonium chloride and other nutrients. The COD concentrations of the influent were both 1000 mg/L in R1 and R2, and the COD:N ratio was 50:1 in two bioreactors. The other nutrients contained the following components: 25 mg/L of Na_2HPO_4, 20 mg/L of KH_2PO_4 and 5 mL of trace mineral solution. $NaHCO_3$ was utilized to maintain the pH of the reactors in the range between 7.0 and 8.0.

In situ transesterification

The sludges, including the activated sludge (AS) and the granular sludge (GS), were treated for in situ transesterification using a modified method based on a previous research report (Mondala et al., 2009). The sludge samples were dewatered by centrifugation at 5000 r/min for 5 min (TSS: 6%). One gram of dewatered sludge, 7.5 mL of sulfuric acid-methanol (5%, v:v) and 10 mL of hexane were added to a 100-mL flask, and then the mixture was heated for 7 h at 75 °C for in situ transesterification. A condenser was used to minimize the loss of methanol and hexane due to evaporation with water at room temperature. After the transesterification was completed, the fatty acid methyl esters (FAMEs) were extracted, pooled, dried, re-dissolved and analysed by GC-FID based on the procedure presented in a previous study (Li et al., 2016).

Analytical methods

To determine the growth characteristics and the degradation ability of the activated sludge and the granular sludge, the sludge MLSS concentration, the sludge volume index (SVI), the particle size distribution, and the glucose and ammonium-nitrogen concentrations in the effluent were regularly examined. The sludge MLSS concentration and the sludge volume index (SVI) were measured according to the Standard Methods (APHA, 2005). The particle size distribution was measured using a laser particle size analyser (S3500, Microtrac, USA). The effluent glucose and ammonium-nitrogen concentrations were determined via the phenol-sulfuric acid method (Gerhardt et al., 1994) and Nessler's reagent colorimetry, respectively. The microbial population structure was analysed by high-throughput sequencing on an Illumina MiSeq PE300 platform (Li et al., 2016).

RESULTS AND DISCUSSION

Characteristics of activated sludge and granular sludge

The distinctions between activated sludge and granular sludge became increasingly apparent after cultivation under different settling times. Following granulation, the morphology of sludge from R2 completely changed, with granules becoming larger and denser and settling faster than those of the sludge flocs in R1. The mean size of the sludge increased to 300 and 640 μm for R1 and R2, respectively, after running for 84 days, compared with 60 μm for the seed sludge. Moreover, the value of SVI_{30} decreased from 111 mL/g to 47 and 40 mL/g for the sludge cultured from R1 and R2, respectively. In R2, the small and slow-settling sludge was gradually discharged by decreasing the settling time; thus, the granular sludge with a larger size and lower SVI value was achieved. The sludge from R1 showed a similar tendency in terms of size and settling ability, but it was still suspended and flocculent sludge (Figure 1). The sludge flocs in R1 were dominated by light-yellow bacteria with a little white filamentous fungus. However, the granules in R2 were formed by both light-yellow bacteria and brown filamentous fungus that tangled together and extended out of the surface. Although the activated sludge and the granular sludge had different morphologies, they both performed well in the degradation of glucose and ammonium-nitrogen, with removal rates higher than 90%.

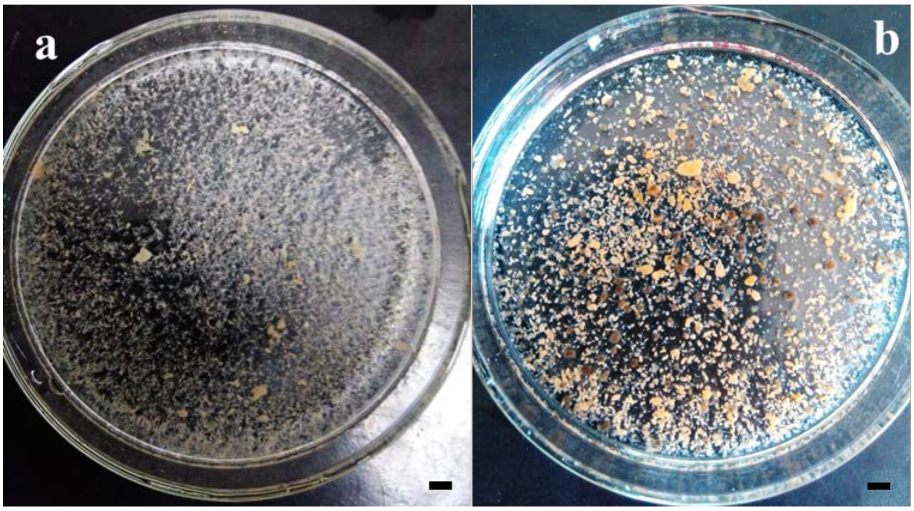

Figure 1. Morphologies of (a) activated sludge from R1 and (b) granular sludge from R2 after running for 84 days (bar = 5 mm).

Production of FAMEs from activated sludge and granular sludge

Differences concerning the yield and distribution of FAMEs were presented among the raw activate sludge (RAS), the cultured activated sludge (CAS) and the granular sludge (CGS) (Figures 2 and 3). Feeding with sugar-containing wastewater and the sludge granulation process could both increase the accumulation of microbial lipids. The total biodiesel yields from the CGS and the CAS were 30.14 ± 0.73 and 21.56 ± 0.95 mg/g SS, respectively, compared with 15.53 ± 0.87 mg/g SS for the RAS (Figure 2). Methyl palmitoleate (C16:1), methyl palmitate (C16:0), methyl oleate (C18:1) and methyl stearate (C18:0) were the dominant components in the biodiesel produced from seed sludge, and their yields were 2.08, 5.31, 3.23 and 1.58 mg/g SS, representing 13.4%, 34.2%, 20.8% and 10.2%, respectively (Figures 2 and 3). However, biodiesel produced from cultured sludge from both of the bioreactors contained less methyl palmitoleate (C16:1) and methyl palmitate (C16:0), but more methyl oleate (C18:1) compared with the seed sludge. The methyl palmitoleate (C16:1) from the CAS and CGS was reduced to approximately 1.6 mg/g SS. Compared to 2.61 mg/g SS methyl palmitate (C16:0) from the CAS, the CGS led to a higher relative yield, which was 3.99 mg/g SS. In addition, the yield of methyl oleate (C18:1) reached 9.98 and 14.79 mg/g SS for the CAS and the CGS, which accounted for 46.3% and 49.1% in the biodiesel. The changes in methyl palmitoleate (C16:1) and methyl oleate (C18:1) were consistent with the study of Mondala et al. (2012). Although more methyl oleate (C18:1) was found both in the CAS and the CGS, only the CGS led to more methyl linoleate (C18:2), which was as much as 3.5 times that of the RAS and the CAS. The percentages of FAMEs with 18 carbons in the biodiesel from the three types of sludge were 37.0% (RAS), 56.4% (CAS) and 66.8% (CGS), whereas the proportions of 16-carbon FAMEs were 47.6% (RAS), 19.6% (CAS) and 18.8% (CGS), respectively (Figure 3). The heating value would rise with the increase of the chain length (Pinzi et al., 2011); thus, the biodiesel produced from aerobic granular sludge may have a higher heating value. Apart from the increase of fatty acids with 18 carbons, more branched FAMEs were also found in the CAS and the CGS fed with synthetic sugar-containing wastewater. Branched FAMEs represented 16.6% and 9.6% of the biodiesel produced from the CAS and the CGS compared to 1.6% for the RAS. The CAS accumulated more methyl 12-methyltetradecanoate (12MeC14:0) and methyl 14-methylpentadecanoate (14MeC15:0), whereas only the CGS produced more methyl 14-methylpentadecanoate (14MeC15:0). Methyl branched FAMEs may improve the low temperature fluidity of biodiesel, which is one of the major problems associated with the use of biodiesel (Knothe, 2005).

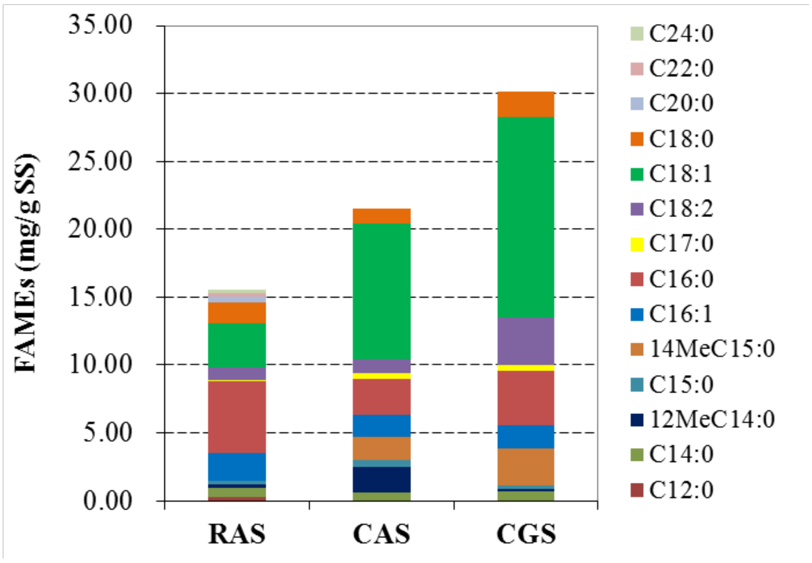

Figure 2. Yield of each FAME based on the weight of dry sludge. RAS – raw activated sludge, CAS – cultured activated sludge, CGS – cultured granular sludge.

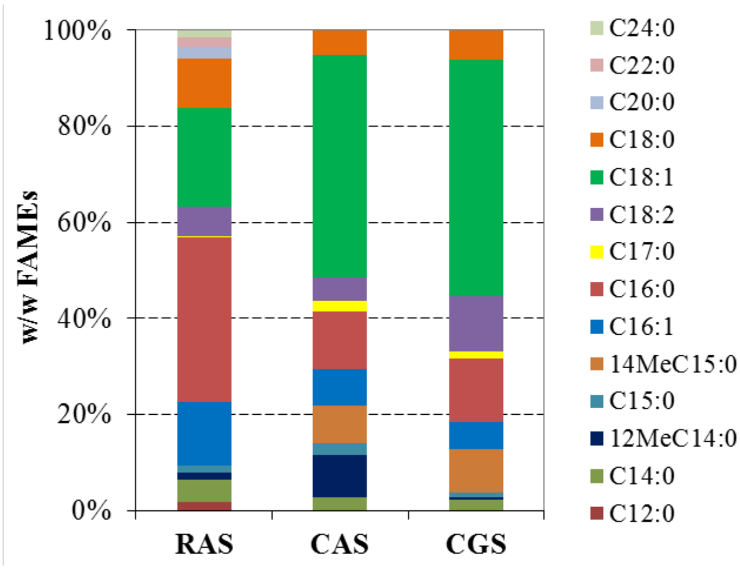

Figure 3. Distribution of FAMEs in the biodiesel produced from the sludge. RAS – raw activated sludge, CAS – cultured activated sludge, CGS – cultured granular sludge.

Effect of the microbial community on the production of FAMEs

The microbial lipids in sludge were the only lipid feedstock for biodiesel production in this study. Thus, the variation in the microbial community may reveal the intrinsic reason for the distinctions of biodiesel in terms of yield and the distribution of FAMEs. Figures 4 and 5 illustrate the differences in the microbial communities between the CAS and the CGS, including fungi and bacteria at the genus level. The results showed that *Phialophora* was the dominant fungi in granular sludge, representing 73.1%, followed by *Hypocreales_unclassified* (23.2%). Liu et al. (2013) reported a new species of *Phialophora*, which was characterized by a brown colony. This is consistent with the previously mentioned existence of brown filamentous cells in the granular sludge (Figure 1). The concentration of oleic acid (C18:1) was very high (69.9-73.6% of the total fatty acids) in *Phialophora dermatitidis*, and the yield of oleic acid (C18:1) could reach 236 mg/g cells (Richard, 1976). The growth of *Phialophora* may be one of the reasons for the rapid increase in methyl oleate (C18:1) in biodiesel produced from the granular sludge of R2. Unfortunately, being limited to the current information, 72.6% of the fungi in the cultured activated sludge were still incertae sedis, whereas *Hypocreales_unclassified* and *Dipodascus* were identified as the other two main fungi from the CAS, representing 11.5% and 10.1%, respectively. *Dipodascus* were members of the family of yeasts in the order *Saccharomycetales*, and the main fatty acids were oleic acid (C18:1) and linoleic acid (C18:2) from many species of *Dipodascus* (Sajbidor et al., 1994). Therefore, the existence of fungi (such as *Dipodascus* and *Phialophora*) may cause the increase of unsaturated FAMEs with 18 carbons in the biodiesel from the cultured sludge in the two bioreactors.

As for the bacterial population, the CAS and the CGS were quite different. *Saccharibacteria_norank*, *Zoogloea* and *Photobacterium* were the main members in the CAS at 26.4%, 13.0% and 8.3%, respectively (Figure 5). *Saccharibacteria*, also known as *TM7*, is a highly ubiquitous phylum in soils, sediments, wastewater and animals (Ferrari et al., 2014). It was reported to be one of the glucose-utilizing species in a full-scale anaerobic sludge digester (Ariesyady et al., 2007). *Zoogloea* was found to be one of the genera that showed a high positive correlation with branched fatty acids (Ma et al., 2016). The growth of *Zoogloea* in the CAS could result in the increase of branched FAMEs in the biodiesel product (Figure 2). Additionally, bacteria from the CAS also contained *Comamonadaceae_unclassified* (5.8%), *Cytophagaceae_uncultured* (4.5%), *Saprospiraceae_uncultured* (3.8%), *Tolumonas* (3.2%), *OM27_clade* (3.0%) and

Roseobacter_clade_CHAB-I-5_lineage (3.0%). However, *Photobacterium*, *Cytophagaceae_uncultured* and *Flavobacterium* became the most dominant species in the CGS, and their proportions were 26.6%, 10.4% and 11.4%, respectively. Palmitic acid (C16:0) was reported to be a major fatty acid from *Photobacterium* (Rivas et al., 2006; Srinivas et al., 2013; Yoon et al., 2005), and the yield of methyl palmitate (C16:0) was higher in the CGS than in the CAS (Figure 2). It was reported that the *Cytophaga-Flexibacter* group preferentially accumulated iso-branched fatty acid, such as *Cytophaga johnsonae* and *Cytophaga sp. strain samoa* (Fautz et al., 1979). The occurrence of *Cytophagaceae_uncultured* may also be one of the donors of branched fatty acids in the CAS and the CGS. There were also some other species in the CGS, including *Comamonadaceae_unclassified* (4.6%), *Nakamurella* (4.3%), *Saccharibacteria_norank* (2.7%), *Janthinobacterium* (2.3%), *Leptothrix* (2.2%), *Xanthomonas* (2.0%) and *Zoogloea* (1.9%). Obviously, the variations of the microbial community resulted in corresponding distinctions of biodiesel yield and the distribution of FAMEs between the cultured sludge in the two bioreactors.

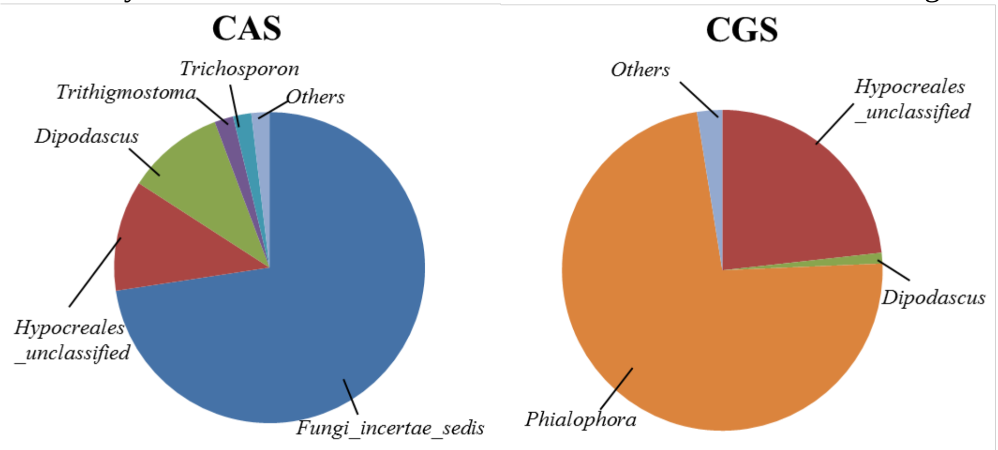

Figure 4. Distribution of fungi in cultured activated sludge (CAS) and granular sludge (CGS) at the genus level.

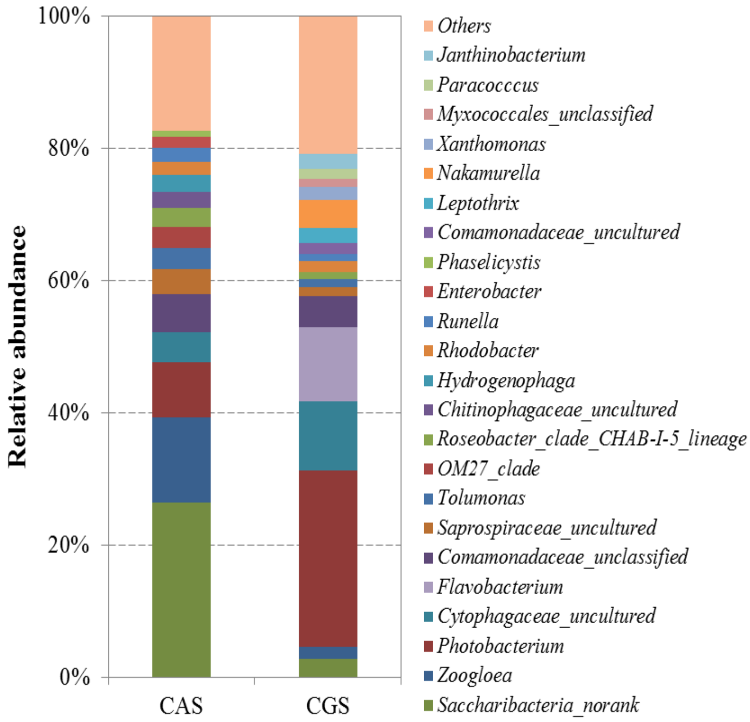

Figure 5. Distribution of bacteria in the cultured activated sludge (CAS) and the granular sludge (CGS) at the genus level.

CONCLUSIONS

Feeding with sugar-containing wastewater promoted lipid accumulation in sludge. Under the same feeding strategy, the granular sludge showed an advantage in biodiesel production both in yield and in quality. Compared with the seed sludge (biodiesel yield of 15.53 ± 0.87 mg/g SS), the granular sludge achieved a higher biodiesel yield (30.14 ± 0.73 mg/g SS) than the cultured activated sludge (21.56 ± 0.95 mg/g SS). Biodiesel produced from the granular sludge contained more methyl oleate (C18:1) and methyl linoleate (C18:2). FAMEs with longer carbon chains released more heat energy; thus, the heating value of biodiesel from the granular sludge would increase. Unsaturated fatty acids also increased in the granular sludge, which may improve the low temperature fluidity of biodiesel. The above variations may be attributed to the change in the microbial community, including the growth of fungi (*Phialophora*) and a change in the bacterial population.

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