Stereological estimation of structural change in floc matrix following weak ultrasonication

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Received 20 March 2007; accepted 25 April 2007

Abstract

Ultrasonication is an energy intensive process used to enhance organics' solubilisation and used in promoting methane yield from wastewater sludge. Applying limited ultrasound without full disruption of floc structure or cell walls can significantly enhance methane yield from sludge at reduced energy cost. This work probed the spatial distribution of extracellular polymeric substances, cells in the original wastewater flocs, and the yielded fragments following weak ultrasonication using a process of multiple staining and a confocal laser scanning microscope technique. The stereological information of the three-dimensional architecture of the original wastewater floc and ultrasonicated fragments was extracted and compared. The local structural changes following weak ultrasonication were presented for the first time, which should also correlate with the enhanced methanogenesis thereby produced.

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Keywords: Ultrasonication; Floc; Pre-treatment methods

1. Introduction

Anaerobic digestion is a very slow process due to the rate limiting step of hydrolysis (Neis *et al.*, 2000). Numerous pre-treatments have been reported to promote hydrolysis and to disintegrate sludge flocs, thereby enhancing the efficiency of the subsequent anaerobic digestion stage (Lee and Mueller, 2001; Mukherjee and Levine, 1992). Numerous methods have also been used to break up cell membranes in bioprocesses (Bougrier *et al.*, 2006; Hwang *et al.*, 2006; Kao *et al.*, 2006; Lai *et al.*, 2006; Liao, 2006; Show *et al.*, 2006; Tao *et al.*, 2006; Thongchul and Yang, 2006; Zhang *et al.*, 2007). Chu *et al.* (2001), along with Lee and Mueller (2001), commented on the application of pre-treatments to improve the biodegradability of sludge. High-power ultrasound at low frequencies could generate intensive cavitation which would result in sludge disintegration and the release of intracellular materials (Neis *et al.*, 2000). Show *et al.* (2006) experimentally proved that a

sonication treatment could improve the sludge properties in terms of soluble chemical oxygen demand (SCOD) increase and particle size reduction. Compared with other pre-treatment methods, *i.e.*, thermal treatment, ozone treatment, chemical treatment, etc., ultrasonication exhibits great potential of being less hazardous to the environment (Tiehm *et al.*, 1997, 2001).

Ultrasonication, however, requires a considerable amount of energy during treatment. Chu *et al.* (2002) examined how “weak” ultrasonic pre-treatment affects the anaerobic digestion of waste biosolids. The ultrasound applied was insufficient to fully disrupt the floc structure or the cell walls, but was still able to significantly enhance methane yield from biomass at reduced energy cost (Ding *et al.*, 2006). These authors claimed that, with gross structure intact, weak ultrasonication loosened the local structure of the floc matrix and created active sites for easy attack by microbes, hence producing an increased amount of methane. Up to the present, though, there exists no detailed study on how insufficient ultrasound on sludge flocs can enhance a subsequent methanogenesis process.

Extracellular polymeric substances (EPS) and cells form the bioaggregates, such as biofilm, sludge flocs, river snow, and others. Chen *et al.* (2007) demonstrated the distribution of EPS (proteins, α - and β -polysaccharides, and lipids) and cells in aerobic granules using a newly proposed multiple staining

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scheme and a confocal laser scanning microscope (CLSM) technique. This work probed the spatial distribution of EPS and cells in the original wastewater flocs and the yielded fragments following weak ultrasonication. With the stereological information collected in the scanned images, the authors of this research proved experimentally weak ultrasonication do yield local structural changes with gross architecture intact.

2. Experimental

2.1. Samples

Waste activated sludge was extracted from a wastewater treatment plant of the Presidential Enterprise Corp., Taiwan, which daily treats 250 tonnes of food-processing wastewater using primary, secondary, and tertiary treatments. The pH of the sludge was about 6.4. The chemical oxygen demand (COD) for the sludge was 9600 mg/L (TCOD), as determined by directly reading a spectrometer (DR/2000, HACH, U.S.A.). The elemental composition of the dried samples was C: 34.3%, H: 5.6% and N: 5.5%, according to an elemental analyzer (Perkin-Elmer, 2400 CHN).

2.2. Floc staining and CLSM test

In each test, one floc was carefully collected from the sample suspension with a wide-mouthed pipette. The collected floc was maintained in a fully hydrated state during staining. During staining, SYTO 63 (Molecular Probes, Carlsbad, CA, U.S.A.) solution (20 μ M, 100 μ L) was first added to the sample and the sample was placed on a shaker table for 30 min. Next, 0.1 M sodium bicarbonate buffer (100 μ L) was added to retain the amine group in a non-protonated form, which was followed by a fluorescein isothiocyanate (FITC) (Molecular Probes, Carlsbad, CA, U.S.A.) solution (10 g/L, 10 μ L), and the mixture was stirred at room temperature for 1 h. Subsequently, the fluorescently labeled lectin, concanavalin A (Con A) (Molecular Probes, Carlsbad, CA, U.S.A.), was conjugated with a tetramethylrhodamine solution (250 mg/L, 100 μ L) and incubated with the sample for an additional 30 min, followed successively by the addition of calcofluor white (fluorescent brightener 28, Sigma,

U.S.A.) solution (300 mg/L, 100 μ L) for 30 min. Following each of these five staining stages, the stained sample was washed twice with phosphate-buffered saline (pH 7.2) to remove excess stain, and the stained sample was stored at 4 °C. The stained flocs were then embedded for cryosectioning and frozen at –20 °C, after which 60 μ m sections were cut on a cryomicrotome and mounted onto microscopic slides for analysis.

Confocal laser scanning microscopy (CLSM) (Leica TCS SP2 Confocal Spectral Microscope Imaging System, Gmbh, Germany) was employed to visualize the distribution of cells and extracellular polymeric substances in the flocs. The flocs were imaged with a 10 $\times$ or 20 $\times$ objective and analyzed using Leica confocal software.

2.3. Ultrasonication test

After the CLSM test, the stained floc was transferred to a 200 mL vessel containing supernate of the sludge sample. Low frequency ultrasound was applied at an intensity of 56 W cm^{–2} for 2 s to disrupt the floc. As [Chu et al. \(2002\)](#) observed, the total energy input for the applied ultrasound was far from sufficient to completely disrupt the flocs and to release all soluble matters from sludge matrix.

Following ultrasonication, the floc was commonly disrupted into a few fragments. The fragments were carefully collected and then were tested again using CLSM, as stated above. A total of four sludge flocs were tested and analyzed.

3. Results and discussion

3.1. Floc disruption

[Fig. 1\(a\)](#) shows the fluorescent staining results probed at 360 μ m from the outer surface of floc #1. The floc has a round-shaped structure. According to the fluorescent intensity data presented in the figure, the protein was distributed over the entire floc. Lipids and β -polysaccharides were also distributed over the floc, but in a lower quantity. The cells (in red) and the α -polysaccharides (in light blue) were allocated most at the floc core area. The image shown in the lower-right corner was the combined image of the five individual stains.

Fig. 1. Fluorescent images of sludge floc. Green: proteins; red: cells; yellow: lipids; blue: β -polysaccharides; light blue: α -polysaccharides. Combined image: right-bottom corner. (a) Original floc #1; (b) fragment collected following ultrasonication (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Fig. 2. Combined fluorescent images of sludge floc and the yielded fragment.

Fig. 1(b) shows the fluorescent staining results probed at 360 μm from the outer surface of a fragment from floc #1. This fragment was located in the outer rim of floc #1, hence, was lacking cells and α -polysaccharide (Fig. 1(a)). However, the intensities of proteins and lipids were clearly probed, with most of the β -polysaccharide being lost in the fragment images. Restated, the present result contradicts those reported by Wang *et al.* (2006a,b) that the main component released from the

sludge matrix in ultrasonication was protein. Chen *et al.* (2007) noted that the β -polysaccharides were the main backbone for microbial aggregates such as aerobic granules. The removal of β -polysaccharide could significantly weaken the wastewater floc, hence yielding subsequent disintegration of structure under an ultrasonic field.

Based on the geometrical features of the original floc and the fragments, the disruption process is shown schematically in Fig. 2.

3.2. Structural change

The local architecture of the sampled flocs was quantified using the stereological method (Reed and Howard, 1999). First, the pixels of each fluorescent image for proteins, α - and β -polysaccharides, lipids, or cells were bileveled using Otsu's method, then the pair cross-correlation functions, $g(r)$, for each pair were evaluated. With $g(r) > 1$, the two substances were spatially attracting; while with $g(r) < 1$, they were spatially repulsing.

Fig. 3 shows some $g(r)$ functions enveloped with their 95% confidence intervals for the original flocs and their ultrasonicated fragments. All the $g(r)$ functions for original flocs revealed a mono-dispersed characteristic and peaked at around 2–10 μm in distance, indicating that the probed substances were largely associated with cell membranes and the peak was

Fig. 3. Pair cross-correlation functions $g(r)$ of original floc #1 (a, c and e) and fragments (b, d and f) in Fig. 1. β -Polysaccharides vs. β -polysaccharides (a and b); proteins vs. proteins (c and d); and lipids vs. α -polysaccharides (e and f).

correspondent to the distance between neighboring cells. Following ultrasonication, numerous minor peaks emerged on the $g(r)$ function over a wide range of distance, indicating disturbance of local architecture. However, the main peak of $g(r)$ remained unchanged, revealing that the gross structure of floc matrix had not deteriorated.

Hence, the present work for the first time proved that the application of weak ultrasound did yield changes in local structure, correlating with the hypothesis proposed by Chu *et al.* (2002). Apparently this change as well as the noted release of β -polysaccharides correspond to the enhanced methanogenesis reported in Chu *et al.* (2002) and Ding *et al.* (2006), and are probably attributable to the reduced internal mass transfer resistances of the fragments.

4. Conclusion

Applying ultrasound that is insufficient to fully disrupt floc structure or cell walls can significantly enhance the methane production yield from biomass. This work measured the spatial distribution of EPS and cells in individual wastewater flocs and their yielded fragments following weak ultrasonication, using a multiple staining and CLSM technique. The stereological information on how the EPS and cells are distributed in floc matrix was utilized to identify the corresponding local structural changes before and after weak ultrasonication.

Following ultrasonication, the β -polysaccharides were significantly lost from the floc matrix, probably correlating with the disruption of gross floc matrix under shear. The pair cross-correlation functions of the probed EPS and cells in the floc matrix had a mono-dispersed characteristic and peaked at around 2–10 μm in distance. Following weak ultrasonication, the gross architecture of the floc matrix remained intact, but the disturbances to the local architecture were probed by the reconstructed pair cross-correlation functions. Enhanced methane production from weakly ultrasonicated sludge may be attributable to the reduced interior mass transfer resistances associated with the noted disturbed structure by ultrasound.

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有限超音波處理下膠羽局部結構改變之空間測量

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摘 要

超音波程序為增強污泥有機物水解及提昇甲烷產率之耗能程序，在不破壞膠羽結構及細胞壁之前提下提供有限超音波能量仍可大幅增加甲烷產率。本研究使用多重染色及同軸共軛焦顯微技術探測膠羽在有限超音波處理前後之胞間高分子及細胞三維空間分佈，並首次報導有限超音波能量下膠羽局部結構改變之證據。