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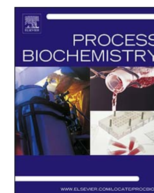

Microbial community response and SDS-PAGE reveal possible mechanism of waste activated sludge acidification enhanced by microaeration coupled thermophilic pretreatment

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ABSTRACT

Aiming to strengthen the performance of waste activated sludge digestion, pretreatment is a prerequisite for keeping operations within an industrially acceptable time- frame. In this study, the performance of micro-aeration coupled with thermophilic (MT) pretreatment on waste activated sludge solubilization and acidification was investigated. The results showed that the maximum soluble organics concentrations, including soluble proteins and carbohydrates, reached 2290 mg COD/L in 24 h when the ventilation rate and temperature were 0.05 vvm and 70 °C. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) test on pure-culture (*E.coli*) and scanning electron microscopy analysis indicated that MT pretreatment effectively destroyed microbial cell wall and resulted in an increase in soluble proteins. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis revealed that MT pretreatment reduced the diversity of the bacterial community during pretreatment. Along with the dissolution of a large quantity of organic matter, microbial species such as *Lactococcus* and *Methyloversatilis* bloomed at the end of acidification period, which contributed to SCFAs production. This study revealed that MT pretreatment facilitated the WAS hydrolysis efficiency and enhanced SCFAs (3149 mg COD/L with fermentation for 96 h) production, especially for acetic acid (50%) accumulation, which provides a new perspective for the application and recovery of sludge resources.

1. Introduction

The disposal of waste activated sludge (WAS) continues to be a major environmental concern worldwide [1]. Large amounts of WAS are produced from wastewater treatment plants (WWTPs), which contain high levels of organic matter, such as proteins and carbohydrates [2]. Taking the resource utilization of WAS into account, short-chain fatty acids (SCFAs) produced from the WAS fermentation process are attracting more interest as a crucial way to achieve biological carbon recovery [3,4]. In addition, the SCFAs are raw materials for the synthesis of biodegradable plastics polyhydroxyalkanoates [5]. Cost-effective microbial conversion to valuable products by anaerobic digestion (AD) is acknowledged as the most cost effective method for WAS treatment [6]. The hydrolysis of particulate organic matter (POM) into soluble substances is the first step and is believed to be the rate-limiting step, which has received more consideration [7,8]. WAS is composed of a multitude of microbial cells integrated with extracellular polymeric substance (EPS). This complex multi-component system makes the organic components in WAS in the form of solid particulates, which are rigid structures resistant to hydrolysis [9]. Limited sludge can be biodegraded unless the solid organic matter is adequately solubilized [10]. In fact, the mass of microbial cells in WAS are firstly autolyzed or hydrolyzed, and then their intracellular organics can be dissolved and converted into organic acids. Thus, pretreatment technologies are necessary to enhance the sludge solubilization and improve the production of soluble organic matter [11].

Microaeration, which is defined as the introduction of small amounts of oxygen into an anaerobic bioreactor to stimulate both anaerobic and aerobic biological activities, has the potential to accelerate the hydrolysis of complex organic matter [12,13]. Fermentative

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microorganisms, especially facultative anaerobes, were suggested to maintain low redox potential and supply more growth factors for the strict anaerobes [14]. Niu et al. revealed that microaeration improved the hydrolysis efficiency and enriched the fermentative and predatory bacteria responsible for sludge reduction [7]. In addition, the activities of synthesized hydrolytic enzymes are unaffected by aerobic, anoxic or anaerobic conditions [15]. Limited oxygen assisted anaerobic digestion can be an especially useful means of treating a variety of recalcitrant, persistent and eco-toxic organic wastes that are difficult to treat well by either aerobic or anaerobic treatments alone [16]. These findings suggest that hydrolysis can be improved by slight aeration. However, the pre-breaking of cells is still considered as limiting step.

With respect to the difficulty of breaking cells with microaeration, a strategy combining various pretreatments could overcome the constraint of releasing the dissolved organics [17]. Most of the studies focused on accelerating sludge solubilization and further biogas production by thermal, thermal-alkaline, ultrasonic and biological treatments [18]. Other methods, such as alkaline-mechanical [19], alkaline-microwave [20] or other pretreatment technologies have also been evaluated [21–23]. Of these methods, thermal treatment or a combination of methods was reported to be relatively effective strategies. The solubilization of organics in the WAS is known to occur through the thermal effect and enzymatic hydrolysis while thermophilic bacteria are subjected to optimal conditions for their growth [24]. Previous studies have shown that thermophilic microaerobic fermentation was able to enhance the biodegradation of WAS [25,26]. However, applying different aeration intensities, combined with diverse temperatures (< 100° C) for WAS pretreatment has not been completely explored. Incorporating the appropriate microaeration with the thermophilic pretreatment will hopefully increase the solubilization performance and thus have a positive effect on the sludge acidification. Otherwise, because protein is the main component of the complicated WAS, accounting for 50% to 60% of the total sludge composition [27], the analysis of the cell lysis, protein solubilization and microbial community transformation need to be explored in depth to clarify the enhancement mechanism.

The objective of this work was to investigate the effect of microaeration and thermophilic (MT) pretreatment on the performance for organic solubilization and microbial community shifts during SCFAs accumulation. In this study, the effects of different ventilation rates and temperatures on WAS solubilization were studied. The effectiveness of MT pretreatment on WAS solubilization, hydrolysis and acidification was investigated by comparing it with single microaeration (M) or thermophilic (T) pretreatments under optimal conditions. Moreover, the dissolution of the proteins from *Escherichia coli (E. coli)* under MT pretreatment was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and scanning electron microscopy (SEM) analysis. To better understand the microbial response mechanism of the M-, T- and MT-treated WAS samples, the microbial community structure was examined using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis. Insights gained from this study are helpful in understanding the mechanism of pretreatment technology in enhancing sludge lysis and SCFAs accumulation, which provides theoretical support and a reference for subsequent resource utilization of WAS.

2. Materials and methods

2.1. Source of WAS

The WAS used in this study was withdrawn from the secondary sedimentation tank of the Taiping Municipal Wastewater Treatment Plant (Harbin City, Heilongjiang Province, China). The sludge was concentrated by allowing it to settle for 24 h at 4 °C. A summary of the main characteristics (average value plus standard deviation of three tests) of the WAS is listed as follows: pH 6.25 6.80; total suspended solids (TSS), 24,230 ± 158 mg/L; volatile suspended solids (VSS), 17,040 ± 178 mg/L; total chemical oxygen demand (TCOD), 23,730 ± 227 mg/L; soluble chemical oxygen demand (SCOD), 546 ± 47 mg/L; soluble protein, 112 ± 21 mg COD/L; soluble carbohydrates, 70 ± 5 mg COD/L; and total SCFAs, 212 ± 36 mg COD/L.

2.2. MT preteratment and batch acidification set-up

To study the effect of the microaeration rate on the solubilization of WAS under thermophilic conditions, pretreatment reactors (Fig. S1) were set up with ventilation rates of I-0, II-0.03 vvm (volume air per volume sludge per minute), III-0.05 vvm, IV-0.07 vvm, V-0.09 vvm, and VI-0.11 vvm (the dissolved oxygen contents were below 1 mg/L). The working volume of the WAS was 1.0 L, inoculated with 10% (v/v) seed sludge (cultured in semi-continuous reactors with a hydraulic retention time (HRT) of 10 d and operated for 30 d at 65 ± 1 °C; the SCOD, soluble carbohydrates and protein of the seed sludge were maintained at 4388 ± 70 mg/L, 375 ± 24 mg COD/L and 1015 ± 54 mg COD/L, respectively). The temperature was controlled at 65 ± 1 °C. Another five reactors as above described were then operated at 55, 60, 65, 70, and 75 °C, to investigate the effect of temperature on the hydrolysis of WAS.

Batch acidification experiments were carried out in four groups with an effective volume of 0.7 L each. One group was operated as the control (C) and was fed with untreated sludge. The other three groups were fed with pretreated sludge: MT group was pretreated with 0.05 vvm ventilation rate at 70 °C for 24 h, T group was pretreated at 70 °C for 24 h, and M group was pretreated under only microaeration with 0.05 vvm ventilation rate for 24 h (the pretreated groups were inoculated with 10% of the above-mentioned seed sludge during pretreatment stage). The reactors were placed on a magnetic stirrer (360 rpm) to ensure thorough mixing of the solutions and were operated anaerobically at 35 ± 1 °C. The sludge samples were collected every 24 h.

2.3. Analytical methods

Sludge samples collected from the reactors were immediately centrifuged at 10,000 rpm (9570 × *g*) for 10 min, then filtered through 0.45 μm cellulose nitrate membrane filters and finally stored at 4 °C prior to analysis. The samples were analysed for SCOD, SCFAs, solute carbohydrates and solute protein. The determinations of SCOD, TCOD, carbohydrates, protein, TSS and VSS were the same as described in previous publications [28]. An Agilent 7890 GC was utilized to analyze the composition of SCFAs [29]. The SCFAs production was calculated as the sum of the measured acetic (HAc), propionic (HPr), *n*-butyric (*n*-HBu), isobutyric (i-HBu), *n*-valeric (*n*-HVa) and isovaleric (i-HVa) acids. According to Grady et al., 1 g of protein, carbohydrates, HAc, HPr, HBu and HVa is equivalent to 1.50 g COD, 1.06 g COD, 1.07 g COD, 1.51 g COD, 1.82 g COD, and 2.04 g COD, respectively [30].

2.4. Scanning electron microscopy (SEM) characterization

The samples collected before and after MT pretreatment for scanning electron microscope observation (SEM, S4800 Hitachi, Ltd, Japan) were centrifuged at 6000 rpm (3445 × *g*) for 10 min, fixed overnight in 2.5% glutaraldehyde-amended PBS, dehydrated in a graded series of ethanol (50, 70, 80, 90, and 3 × 100%, with 15 min for each level), displaced by 50% isoamyl acetate in ethanol and 100% isoamyl acetate for 15 min each, and dried in a desiccator for 12 h [31]. Finally, the prepared samples were treated by spray-gold and observed by SEM operating at 15 kV.

2.5. SDS-PAGE analysis

Previous studies showed that protein was the main component of the complicated WAS, accounting for 50–60% of the total sludge composition, and gram-negative bacteria are the main flora in WAS. Therefore, *E. coli* DH 5α (Takara, Dalian, China) was selected as the model strain. It was pure-cultured and pretreated under MT conditions to further confirm the validity of the pretreatment process on improving the dissolution of protein and destructiveness of cell wall by protein SDS-PAGE analysis. The pure culture *E. coli* was cultured overnight at 37 °C in 6 L of Luria-Bertani (LB) medium. The cells were collected after centrifugation at 1000 rpm (106 × *g*) for 10 min and washed three times with distilled water. The suspension was concentrated 5.5 times to obtain a high VSS bacterial suspension. The same MT pretreatment procedure was applied to the concentrated suspension. Protein SDS-PAGE was conducted to observe the changes in protein fractions in the *E. coli* suspension, supernatant and precipitate before and after pretreatment.

Both supernatant and cell precipitate samples were obtained from bacterial suspension (1 mL) by centrifugation at 10,000 rpm (9570 × *g*) for 5 min. After adding 100 μL of the loading buffer (5% mercaptoethanol and 2% SDS) to the samples and boiling water bath for 5 min, the proteins were analyzed in 12% polyacrylamide resolving gels and 5% polyacrylamide stack gels. All samples were electrophoresed with constant voltage until the dye front reached the bottom of the gel. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250, and were destained by methanol and acetic acid.

2.6. DNA (deoxyribonucleic acid) extraction, PCR, DGGE and sequence analysis

In this study, to evaluate how the pretreatment operation (MT, M and T) affected the microbial community, three samples were collected from MT, M and T at the end of pretreatment for 24 h and the acidification stage. The genomic DNA of the samples was extracted using an extraction kit (WATSON BIOTECHNOLOGIES Co. Ltd. Shanghai, China) according to the manufacturer's instructions [32]. DNA extracts were used as the template for PCR amplification of the 16S rRNA gene. The 16S rRNA gene was amplified for bacteria with a pair of universal primers (BSF101F-TGGCGGACGGGTGAGAA and BSF534R-ATTACCG-CGGCTGCTGG), and GC-clamp CGCCCGCCGCGCGCGGCGGGGCGGG-GGCACGGGGGG (Invitrogen, Co., Ltd., Shanghai, China) [33]. The 16S rRNA gene fragments were amplified using a 9700PCR meter (BioRad Laboratories, Hercules, USA) with the touchdown PCR method [34]. The PCR products obtained were applied in the DGGE analysis using a BioRad DCode universal mutation detection system (Bio-Rad Laboratories, Hercules, USA) with a linear denaturing gradient ranging from 30% to 60%. (100% denaturing gradient contains 7 M urea and 40% formamide (v/v)) Electrophoresis was conducted at 20 V for 30 min and at 150 V for 8 h at 60 °C. The obtained gels were then silver-stained and photographed. Selected DGGE bands were excised and re-amplified by PCR with the aforementioned primers without the GC clamp. The obtained sequences of PCR products were then compared with the reference microorganisms in the GenBank database using the BLAST (Basic Local Alignment Search Tool) program and EaTaxon server [35].

3. Results and discussion

3.1. Optimization of microaeration rate and temperature for enhancing WAS solubilization

Sludge solubilization can be expressed by the change of soluble proteins and carbohydrates, which are the two most common forms of organic matter and comprise a large proportion of COD in WAS [36]. Fig. S2 shows the effect of ventilation rates on the concentration of soluble protein (Fig. S2a) and carbohydrates (Fig. S2b). The cumulative yields increased rapidly in the first 24 h, without significantly increasing for the next 120 h. During the first 24 h, the concentration of total soluble proteins and carbohydrates was 1203, 1292, 1609, 1649, 1743 and 1734 mg COD/L with ventilation rates from I-0 to VI-0.11 vvm. Obviously, the microaerobic operation produced a positive impact on the release of organic matter. Nevertheless, further increasing the ventilation rate did not result in increasing the total soluble organics production apart from V-0.09 vvm. The reason might be that only the proper microaeration condition (~0.5 mg/L) can enrich fermentative and predatory bacteria in response to the hydrolysis of POM and lysis of biomass in sludge [7]. Higher aeration promoted the evolution of microbial community from facultative anaerobe to aerobes and decreased the functional bacteria for sludge reduction [13]. Considering energy demand in the long-term aeration conditions, III-0.05 vvm was selected as the optimal ventilation rate for the following experiments.

The effects of temperature on the soluble protein and carbohydrate production are shown in Fig. S2c and d, respectively. During the initial 24 h of hydrolysis, the yield of soluble organics improved rapidly, indicating that the hydrolysis of WAS is enhanced by the selection of thermophilic hydrolysis bacteria and thermal effects [26]. With the increase in operation time to 144 h, relatively stable production values were observed. The order of the various total concentrations at 24 h were as follows: 70 °C (2290 mg COD/L) > 75 °C (2176 mg COD/L) > 65 °C (1755 mg COD/L) > 60 °C (1709 mg COD/L) > 55 °C (1636 mg COD/L), suggesting that 70 °C provided more soluble substrates for next SCFAs accumulation. When the temperature was over 70° C, the microbial activity might be inhibited, [26] which resulted in the inhibition of sludge dissolution. Therefore, the hydrolysis of WAS within the initial 24 h at 70 °C could be assumed as the optimal conditions for coupling with microaeration (0.05 vvm) pretreatment process.

3.2. Characterization of MT-pretreated E. coli pure- culture by SDS-PAGE and SEM

According to the electrophoretogram shown in Fig. 1, the variation of protein in different regions was remarkable, especially for supernatant protein. Prior to pretreatment, bands with different molecular weights were detected in the bacterial suspension and precipitates, whereas bare bands were detected in the supernatant. After MT pretreatment for 24 h, the protein bands were clearly detected in the supernatant. Many of the macromolecular protein bands in the suspension and precipitate disappeared, which might be caused by degradation and dissolution. *The E. coli* outer membrane proteins include OmpF, OmpC and OmpA, whose molecular weights are between 30 and 40 kD. After 24 h of MT pretreatment, the outer membrane protein bands (with molecular weights between 30 and 40 kD) were detected in the supernatant, indicating that the cell wall was likely to be ruptured and the proteins were released into the supernatant. This result indicated that

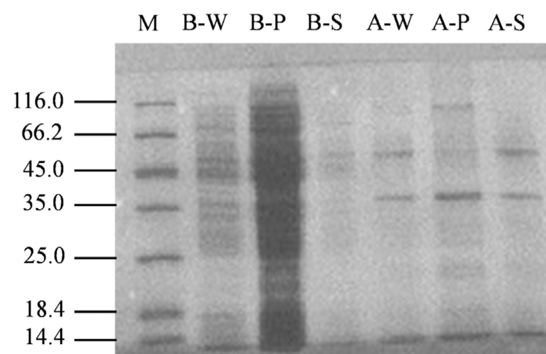

Fig. 1. SDS-PAGE electrophoresis. The analysis of total proteins existing in the *E. coli* suspension (B-W and A-W), precipitate (B-P and A-P) and supernatant (B-S and A-S) before and after MT pretreatment.

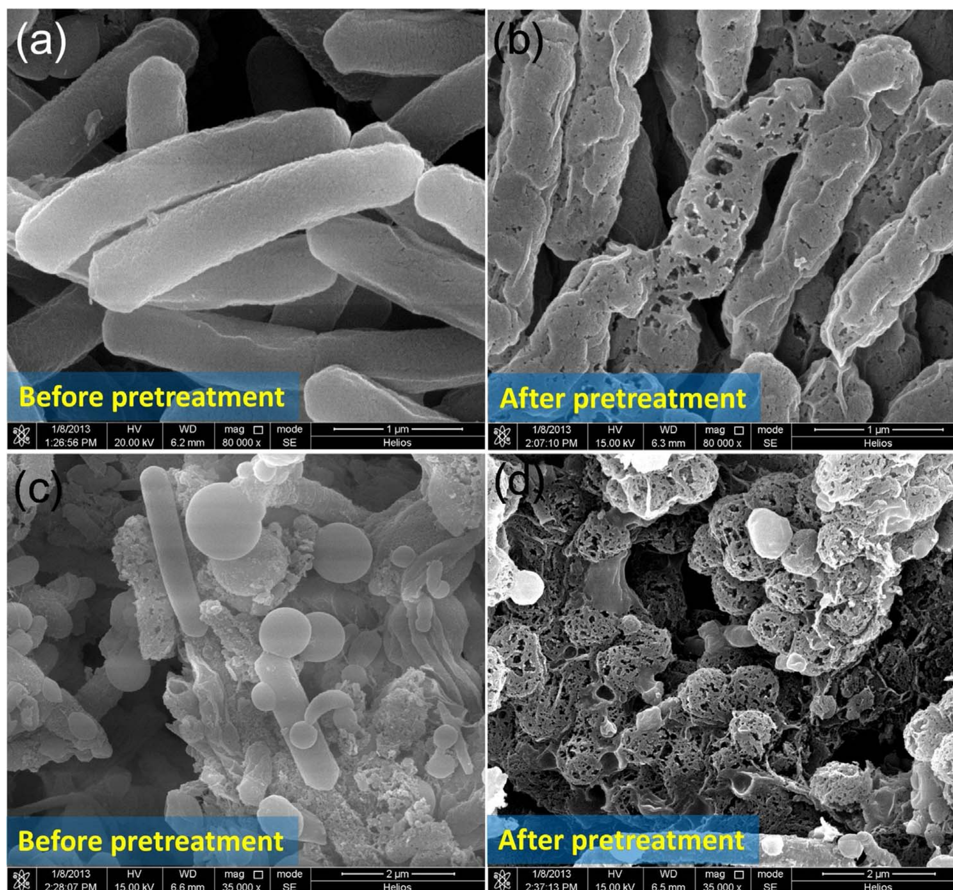

Fig. 2. SEM images of *E. coli* and waste activated sludge. *E. coli* (a) before and (b) after MT pretreatment; the sludge (c) before and (d) after MT pretreatment, respectively.

some proteins could be transferred from bacterial cells into the supernatant after MT pretreatment, which directly proves the effectiveness of the MT pretreatment procedure on the increase in protein dissolution.

Corresponding SEM images of *E. coli* and WAS with and without MT pretreatment are shown in Fig. 2. Pure-cultured *E. coli* was approximately 2–3 μm in length, intact with no damage to the cell wall (Fig. 2a). After pretreatment, the *E. coli cells* were broken and wrinkled, the cell wall was almost cracked (Fig. 2b). Peptidoglycan is the most important unit of strength in the cell wall structure, which is a network structure formed by the cross-linking of the glycans and peptides. Lysozyme release and temperature effects are both likely to disrupt glycosidic bonds, peptide bonds or amide bonds, thus destroying peptidoglycan structures, leading to the dissolution of the cell wall of bacteria. Further investigation was conducted on WAS, the typical morphology of initial WAS constructed by intact cells, particulates and flocs (shown in Fig. 2c). The microbial cells in the sludge were closely arranged and the cell surface was smooth without defects. It was clearly observed that the microbial cells and flocs in the sludge were damaged, and showed the characteristic of porous rupture after 24 h of MT pretreatment (Fig. 2d). It was further proven that the MT pretreatment had a significant effect on the microbial cell lysis, which provided more favorable organic matter for the acidification process of WAS.

3.3. Time-courses of soluble organics changes under M, T and MT pretreatments

To verify the effects of MT pretreatment on the performance of WAS acidification, the changes in SCOD during digestion were determined and shown in Fig. 3a. The concentration of SCOD increased in the early 24 h pretreatment stage and fluctuated during the following digestion phase. Generally, the SCOD content of the treated sludge increased compared to the untreated sludge at the end of the pretreatment period. The SCOD of M, T, MT and control groups were 1571 ± 111, 3686 ± 156, 4886 ± 109 and 1300 ± 162 mg/L at 24 h, respectively (Fig. 3b). The increased SCOD concentration in M and T groups indicated that microaerobic conditions or thermophilic operation both enhanced the sludge lysis and promoted the dissolution of organic matter. The combined MT group achieved the maximum concentration. This might be accessible for the dual function of M and T. On one hand, Fu et al. found that limited oxygen introduced to the anaerobic system could accelerate the hydrolysis process compared to extremely anaerobic conditions [37]. The relative abundances of microbial community associated with hydrolysis process of AD were raised under microaerobic conditions [37]. On the other hand, the inoculation of seed sludge helped to improve the activity of the thermophilic bacteria during the pretreatment process, which was more beneficial to improving the dissolution of sludge through the thermal effect and enzymatic hydrolysis. The lytic enzymes excreted by bacteria enhanced the sludge reduction rate during anaerobic digestion [38]. Furthermore, some recalcitrant fraction of biological flocs and compounds could only be degraded under aerobic conditions [39]. Because the improved SCOD was mainly caused by sludge destruction and the release of intracellular matter, MT pretreatment would therefore increase the concentration of dissolved organic matter.

Fig. 3c and d shows the influence of MT pretreatment on protein and carbohydrate production. With MT pretreatment, the concentrations of protein and carbohydrate drastically increased to 1346 and 615 mg COD/L at 24 h, respectively. These results were in accordance with previous results for increasing SCOD (Fig. 3a). Additionally, the results indicated that under the MT pretreatment, the amounts of total protein and carbohydrates released from sludge destruction displayed 1.5 and 6.5 times higher than T and M groups at 24 h, respectively. As Mata-Alvarez et al. showed the hydrolysis of protein was more dependent on the biological activity than the hydrolysis of carbohydrates, while the

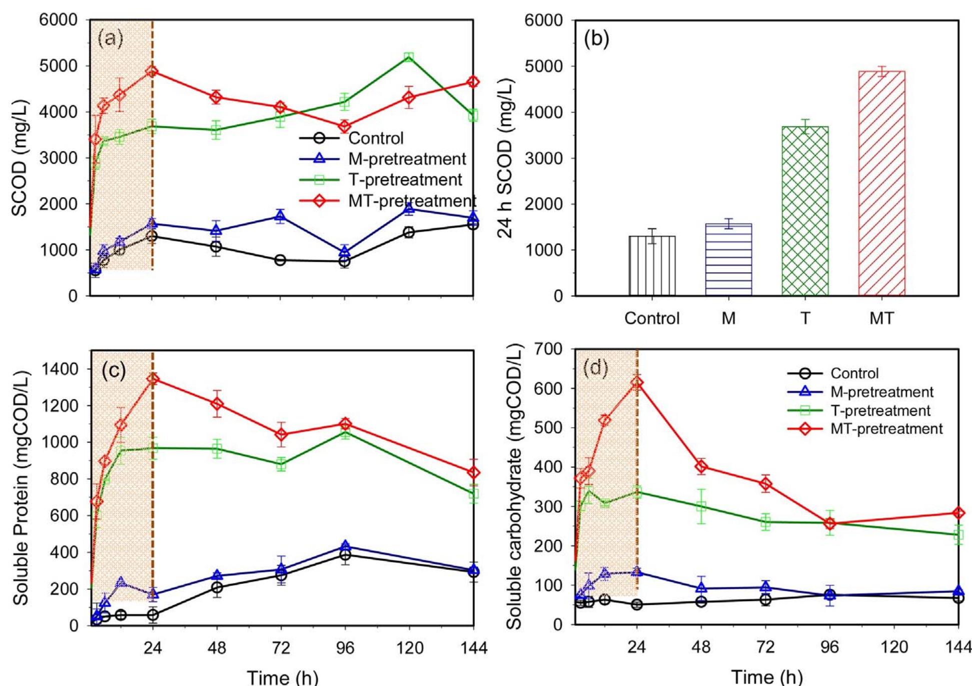

Fig. 3. The concentration of soluble organic matter change in WAS under M, T, MT pretreatments and untreated control during fermentation. (a) SCOD; (b) corresponding SCOD concentrations of the control and M-, T- and MT-treated sludge at 24 h; (c) soluble protein; and (d) soluble carbohydrates.

hydrolysis of carbohydrates was more dependent on the thermal effect [40]. In this study, when the pretreatment procedure was controlled at 70 °C coupled with 0.05 vvm ventilation rate, large percentage of bacterial cell wall might be cracked and lysed, which could cause the concentration of soluble proteins (1346 mg COD/L) and carbohydrates (615 mg COD/L) to increase significantly. The concentrations of protein and carbohydrates were then reduced, which can be explained by the anaerobic fermentation process in which the acidogenic bacteria produced a large amount of organic acid substances by using the dissolved organic matter.

3.4. SCFAs production and composition

Figs. 4 a and Fig. S3 show the concentrations of total SCFAs and each component, following pretreatment strategy. It was shown that the total SCFAs accumulated rapidly after the switch from the MT pretreatment to anaerobic fermentation. The concentrations increased to 3149 mg COD/L for group MT until 96 h (Fig. 4b), which was 2.5 and 1.1 times higher than that obtained in groups M and T, respectively. After 96 h, there was only a small change. This stable SCFAs accumulation was mainly due to its relatively balanced rate of generation and consumption in the sludge fermentation period. In contrast, the yield of group T began to increase significantly from the second day, and the highest yield of SCFAs made little difference with MT group. It can be concluded that the total amount of SCFAs did not change much when limited oxygen was added into the anaerobic digester. It is likely that the total SCFAs concentration mainly depended on temperature effect. In addition, the limited oxygen concentration had a significant effect on the component accumulation of SCFAs. As shown in Fig. 4b, acetate represented the highest portion of SCFAs, which could be subsequently used as substrate by the aceticlastic methanogenic archaea. However, the production of HPr was unfavored in anaerobic environments with higher redox conditions [41]. Importantly, the proportion of HAc (Fig. 4b) in MT (50%) group was higher in comparison with the value of corresponding treated sludge without microaeration (40% for T group). Its production increased significantly from 383 mg COD/L at 24 h to 1577 mg COD/L at 96 h for MT treated sludge (Fig. S3a). This was in agreement with previous research revealing that the application of microaeration in an anaerobic digestion process was able to enhance

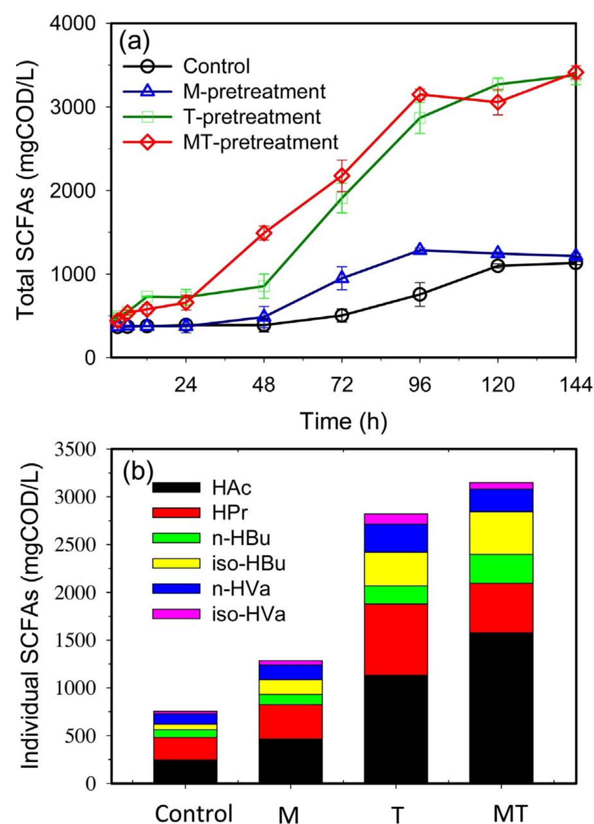

Fig. 4. Time-course profiles of total SCFAs and individual SCFAs concentrations. (a) Total SCFAs change in WAS with the M, T, MT pretreatments and in the untreated control; (b) the concentration of individual SCFAs at 96 h in acidification stage.

HAc formation and decrease propionate accumulation [42,43]. It was validated that limited oxygen promoted an increase in HAc yields and enhanced the breakdown or oxidation of the less favorable SCFAs form. Therefore, there was a clear indication that MT pretreatment could pose a positive effect on anaerobic acidification performance.

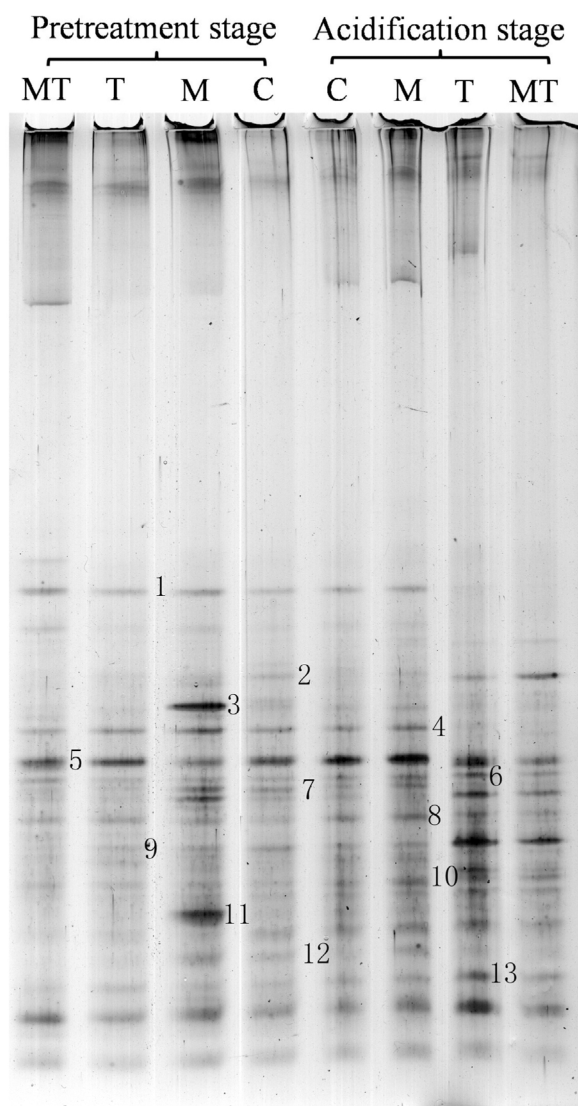

Fig. 5. PCR-DGGE profiles of bacterial 16S rRNA genes from WAS. The profiles were gathered from the samples of WAS under different pretreatment conditions (MT, T, M and C the unpretreated sludge) for 24 h and at the end of acidification stage of each group. The gels with band were collected from the DGGE gel and labeled bands 1–13.

3.5. Microbial community distribution and diversity analysis

The DGGE profiles of microbial community from the WAS after different pretreatments (M, T and MT) and at the end of acidification stage are shown in Fig. 5. Representative bands were selected from the DGGE gel for sequencing to identify the species presented in the reactors (Table 1). DGGE analysis in T and MT pretreated sludge showed a downward trend of the band number, which showed reduced and similar patterns of microbial diversity. The microbial diversity in unpretreated sludge was abundant and evenly distributed, whereas higher bacterial diversity was observed in the group M. This confirmed that limited oxygen introduced to the anaerobic system could increase the relative abundance of microbial community. Although T or MT pretreatment could reduce the diversity of bacterial communities, it was likely to stimulate the growth of favored competitive advantage species and thus enhance the hydrolysis performance. After the acidification period, reorganization of the community was observed due to the rapid adjustment to the fermentation environment. The changes in the T and MT pretreated groups were significant. At the pretreatment stage, the microbial species, represented by three dominant bands (bands 1, 4 and 5), were detectable. According to the GenBank database comparison

Table 1
Phylogenetic affiliation of bacterial 16S rRNA gene sequences retrieved from DGGE bands.

Band	Closest relatives in GenBank	Similarity	Accession
Band 1	*Exiguobacterium sibiricum*	95%	CP001022
	Exiguobacterium artemiae	90%	AM072763
Band 2	*Lactococcus raffinolactis*	95%	EF694030
	Lactococcus piscium	95%	DQ343754
Band 3	*Burkholderia mallei*	97%	CP000011
	Burkholderia pseudomallei K96243	97%	DQ108392
Band 4	*Arthrobacter oxydans*	100%	X83408
	Arthrobacter nitroguajacolicus G2-1	100%	AJ512504
Band 5	*Candidatus Saccharimonas aalborgensis*	92%	CP005957
	Pontibacter ramchanderi LP43	90%	JQ806111
Band 6	*Thermomonas koreensis*	99%	DQ154906
	Thermomonas brevis	99%	AJ519989
Band 7	*Desulfovibrio alaskensis*	90%	Y11984
	Desulfovibrio vietnamensis	90%	X93994
Band 8	*Arthrobacter uratoxydans*	98%	X83410
	Arthrobacter methylotrophus	97%	AF235090
Band 9	*Methyloversatilis thermotolerans* 3t	95%	KC782839
	Thiobacillus thioparus	94%	ARDU01000017X80
Band 10	*Arthrobacter Protophormiae*	100%	X80745
	Arthrobacter oxydans	99%	X83408
Band 11	*Arthrobacter Protophormiae*	100%	X80745
	Arthrobacter soli	99%	EF660748
Band 12	*Chryseolinea serpens*	93%	FR774778
	Echinicola jeungdonensis	93%	GU339180
Band 13	*Thermaerobacter subterraneus*	63%	AENY01000079
	Thermaerobacter nagasakiensis	63%	AB061441

results, phylogenetic tree of DGGE band clones and relative isolated bacteria clones were built and shown in Fig. S4. It was found that band1 was close (95%) to *Exiguobacterium,* a facultative anaerobic bacterium that can secrete hydrolytic enzymes such as lignolytic enzyme andβ-glucosidase, which are closely related to the hydrolysis step [44]. Bands 4 and 5 were closely related (above 90%) to *Arthrobacter* and *Saccharibacteria*, which were commonly found in WAS and showed the ability to degrade various organic compounds, as well as sugar compounds [45,46]. The species represented by bands 2 and 9 in the DGGE profile were close to fermentative bacteria *Lactococcus* and *Methyloversatilis.* They were consistently found in the reactor T and MT groups at the end of acidification stage, suggesting a strong adaptability to the changing conditions. These bacteria, detected from T and MT pretreated cultures, were found to have an intimate relationship with sludge anaerobic digestion. *Lactococcus* was known to metabolize glucose to SCFAs and presented functions in organic compound biotransformation [47]. Such bacterial groups generally dominated in fermentation stage [48]. It can be inferred that the process of community change was mainly caused by the increase of dissolved organic matter, which could be used by acid-producing microorganisms after pretreatment. They accelerated the growth and reproduction of bacteria in anaerobic acidification stage. On the other hand, the microbes with high competitive advantages were gradually enriched and dominated (bands 2, 5 and 9) and were likely to play an important role in the process of SCFAs production. With the disappearance of the inferior species, the diversity of the community was reduced. However, no significant differences were observed in the community organization of T and MT pretreated microcosms at acidification stage, indicating that the microaeration conditions did not have a significant effect on the community structure after the pretreatment process. Therefore, the probable reason for its high proportion of HAc production in MT group can be attributed to the high microbial activity as the stimulation from microaeration pretreatment.

The pH of the T and MT groups was tested at 144 h and was found to be below 6.5 (6.3 and 6.4), which was not suitable to the growth of methanogens. Methanogenesis is known to be strongly pH-dependent, and most methanogenic bacteria function in a pH range of 6.5–7.2, with

an optimum pH near 7.0 [49]. The slight decrease in pH, which was caused by the SCFAs accumulation, and such a short fermentation time (144 h) of sludge, were detrimental to the growth and enrichment of the methanogens. Therefore, the community changes of methanogenic archaea and the methane production were not explored in this study.

3.6. Significance and potential implementation

The relatively high-costs, external addition of chemical reagents and substantial energy input were required for the current pretreatments, which make them impractical for potential implementation. This study demonstrated the effect of MT pretreatment on enhancing WAS acidification through a process assessment with SDS-PAGE and microbial community response analysis. The experimental results showed that MT pretreatment greatly improved the disintegration and acidification performance of WAS under mild aeration (0.05 vvm) and thermophilic (70 °C) conditions for 24 h. Optimized aeration and temperature conditions further reduced energy consumption and therefore facilitated the practical application of the pretreatment method. More SCFAs were produced by MT pretreatment with shorter fermentation time compared with control tests. Especially for the microaeration pretreatment process, outstanding advantages for the HAc accumulation were shown. VFAs composition is considered to be crucial when the WAS hydrolysate is used as an external carbon source. Among the six VFAs, HAc was regarded as the preferred substrate for many bioprocesses, such as nutrient removal and co-polymer production [22]. In this study, HAc accumulation was strongly dependent on microaeration conditions. The group MT yielded more HAc (~50%), followed by T (~40%) and M (~36%) groups. The promotion of POM dissolution and the increase in acetic acid proportion in SCFAs would enhance the carbon recovery from WAS.

SDS-PAGE analysis was used to display the relationship between cell lysis (outer membrane protein dissolution) and the measured organic matter dissolution, which indicated the mechanism of MT pretreatment on the solubilization and hydrolysis efficiency of WAS. Further investigation by DGGE revealed that the abundance of anaerobic functional microorganisms was significantly advantageous to the SCFAs accumulation in MT pretreatment procedure. Adjusting the aeration during the pretreatment stage played an important role in structuring the innate microbial community in WAS but did not influence the community structure during the process of acidification. Insights gained from this study were helpful to understand the mechanism of pretreatment technology in enhancing sludge lysis and SCFAs (particularly HAc) accumulation, which will provide theoretical support and reference for the subsequent resource utilization of WAS and may have crucial implications for the operation of WWTPs.

4. Conclusions

The following conclusions were made from the investigation of the effects of MT pretreatment on WAS solubilization and subsequent acidification processes. (I) Hydrolysis of WAS for 24 h at 0.05 vvm and 70 °C constituted the optimal conditions for MT pretreatment process, with which 1609 and 2290 mg COD/L of total soluble proteins and carbohydrates were obtained, respectively. (II) SDS-PAGE and SEM analyses indicated that MT pretreatment could effectively destroy *E. coli* cells and result in an increase in protein content in supernatant under optimal conditions. Floc disintegration and cell lysis in the pretreatment process were important reasons for sludge solubilization and organic matter dissolution. (III) The acidification of WAS showed that MT pretreatment led to positive impacts on the production of SCFAs, especially for acetic acid accumulation, due to the microaerobic pretreatment, which enhanced organic matter dissolution and the stimulated growth of microbial species such as *Lactococcus* and *Methanogenesis*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2017.09.010.

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