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Enhanced short-chain fatty acids production from waste activated sludge by alkaline-associated thermophilic *Geobacillus* sp. G1 pretreatment

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ABSTRACT

Thermophilic bacteria treatment, a typical efficient biological pretreatment method for complex organic waste degradation, was applied to hydrolyze waste activated sludge (WAS) to produce value-added products. An obvious enhancement in solubilization and acidification of WAS was obtained under alkaline-associated *Geobacillus* sp. G1 pretreatment. The maximal soluble carbohydrate and protein concentrations were 341 ± 15 and 1295 ± 45 mg COD/L, respectively, by alkaline-associated *Geobacillus* sp. G1 pretreatment at 60°C. The corresponding fluoresce in diacetate (FDA) hydrolase activity increased from 90 ± 5 to 206 ± 23 μg FDA/(mL h). The results of excitation emission matrix (EEM) fluorescence spectroscopy showed that WAS hydrolysis was enhanced by organics release through alkaline-associated *Geobacillus* sp. G1 pretreatment. The highest short-chain fatty acids (SCFAs) production was observed 3529 ± 150 mg COD/L, which was 1.5-fold and 1.4-fold of that only bysole alkaline (2330 ± 100 mg COD/L) or *Geobacillus* sp. G1 (2556 ± 95 mg COD/L) pretreatments. Expected SCFAs composition mainly consisted of acetic (HAc) and propionic (HPr), accounting for 63.5% of total SCFAs.

Keywords: Short-chain fatty acids (SCFAs); Waste activated sludge (WAS); Thermophilic bacteria; Pretreatment; Extracellular polymeric substances (EPS)

1. Introduction

Waste activated sludge (WAS) is one of the most complex environmental issues and cost problems for the wastewater treatment plants. An increasing attraction has been focused on sludge reduction and reutilization [1], such as biogas recovery or the production of short-chain fatty acids (SCFAs), which was critical to high added-value green chemicals and preferred substrates for many bioprocesses [2–5]. As an organic-enriched resource, WAS contained 70% biomass rich in proteins, which accounted for 35–61% of the total chemical oxygen demand (TCOD). The formation of SCFAs is firstly associated with the degradation of cell walls and the release of protein [6,7]. Cell lysis is the rate-limiting step for WAS fermentation, because of low level of microbial disruption in natural way by WAS itself, which increased the degradation time of consequent bioconversion or bioavailability [8]. In the past years, numerous efforts (alkaline, ultrasonic, free nitrous acids, and biological enzymes) have been dedicated to removing this barrier [9–11].

Efficient lysis enzymes or microorganisms are environmental friendly way to accelerate disrupting floc structure

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and cell walls, and then detaching extracellular polymeric substances (EPS) and intracellular materials into liquid phase. Thermophilic bacteria treatment has been considered to be an effective method for WAS disintegration among various cell lysis technologies [12–15]. During thermophilic bacteria treatment, nearly 98% of the mesophilic and psychrophilic bacteria died and lost the enzymatic activity, leaving 2% of specific thermophilic bacteria secreting protease and growing [16]. Meanwhile, the pretreatment temperature is around 60°C which is promising to enhance bacteria permeabilisation of WAS [17,18]. However, as one of microbial processes, WAS disintegration is always a limiting factor before enzyme or bacteria functions. Previous studies reported that alkaline could effectively solubilize particulate organic matter in WAS and improve its digestibility in a simple device, which could also prevent methanogens and promote SCFAs accumulation from WAS [19]. Since individual alkaline or thermophilic bacteria pretreatment method has been proven to be highly effective to WAS disintegration, their combination may lead to synergistic action and even higher efficiency.

In this study, the effects of alkaline-associated thermophilic *Geobacillus* sp. G1 pretreatment on WAS hydrolysis and SCFAs production were studied. The pretreatments of sole *Geobacillus* sp. G1 and pH 10 were taken as controls. The changes of sludge protein structure and microorganism activity were analyzed under the pretreatment of alkaline-associated *Geobacillus* sp. G1. The dissolved organic matter (DOM) and EPS were analyzed by excitation emission matrix (EEM) to evaluate the change of structural characteristics of WAS.

2. Material and methods

2.1. Source of WAS

WAS was collected from the secondary sedimentation tank of Taiping Municipal Wastewater Treatment Plant (Harbin City, Heilongjiang Province, China). The WAS was thickened by gravitational sedimentation for 24 h at 4°C, then screened with a 1 mm sieve to remove impurities, finally stored at 4°C prior for later use and test after adjusting volatile suspended solids (VSS) to 10000 mg/L. Its characteristics are mainly as follows: pH 6.72 ± 0.5, total suspended solids (TSS) 14630 ± 158 mg g/L, TCOD12350 ± 177 mg/L, soluble chemical oxygen demand (SCOD) 560 ± 52 mg/L, soluble protein 0 ± 6 mg COD/L, soluble carbohydrate 18 ± 2 mg COD/L and SCFAs 274 ± 78 mg COD/L.

2.2. Isolation and hydrolysis test of Geobacillus sp. G1

The strain *Geobacillus* sp. G1 (Accession no. JX522538) is a thermophilic bacteria isolated from WAS [10]. It was grown at 60°C in LB liquid medium (pH 7.0). The effect of pH on the enzyme activity was examined, pH values ranging from 6.0 to 10.0. The strain was used as the inoculum at its exponential-growth phase (OD_{600} = 1.0–1.5), then centrifuged at a speed of 5000 rpm for 10 min and discharged the supernatant.

In this study, WAS was pretreated with *Geobacillus* sp. G1at 60°C shaken at 140 rpm. The experiments were conducted in 500 mL Erlenmeyer flasks with working volume of 300 mL. Different *Geobacillus* sp. G1 dosage ratio (V/V) (5%, 10%, 15%, 20%, 30%) and treatment time (0–24 h) were investigated in WAS hydrolysis to obtain the optimal dosage ratio and pre-treatment time. The blank test (thermal control) was conducted without *Geobacillus* sp. G1 inoculated. All the experiments were performed in triplicate.

2.3. Alkaline-associated Geobacillus sp. G1 pretreatment

Batch experiments were conducted in 500 mL serum bottles filled with 350 mL sludge each. The sole *Geobacillus* sp. G1 test was conducted with *Geobacillus* sp. G1 to prepared sludge (pH 7.0) at 60°C and kept for 6 h. The sole alkaline test was adjusted sludge to pH 10 using 6 M sodium hydroxide (NaOH). The alkaline-associated *Geobacillus* sp. G1 test was firstly adjusted sludge top H 10, then inoculated *Geobacillus* sp. G1 into the sludge at 60°C initially and kept for 6 h. The blank test was without *Geobacillus* sp. G1 or pH adjusting treatment, but maintained the same treatment temperature (60°C) and time (6 h) with previous *Geobacillus* sp. G1 test. After pretreatments, the pretreated sludge flushed with nitrogen gas for 20 min to remove oxygen, all flasks placed in a water-bath shaker at the speed of 100 rpm with temperature of 35°C. All the experiments were performed in triplicate.

2.4. Sludge sampling and analysis

Sludge samples were centrifuged at a speed of 10000 rpm after fermentation, then filtered through a 0.45 μm cellulose nitrate membrane filter and finally stored at 4°C prior to analysis. The filtrate was immediately used to analyze carbohydrate and protein. The determinations of TSS, VSS, SCFAs, SCOD, TCOD, carbohydrate and protein were the same as described in our previous publications [20,21]. The SCFAs were analysis by a gas chromatography (Agilent, 4890; J&W Scientific, USA) with a capillary column (19095Ne123HP-INNOWAX; 30 × 0.530 mm × 1.00 μm; J&W Scientific, USA), contained acetic (HAc), propionic (HPr), n-butyric (n-HBu), iso-butyric (iso-HBu), n-valeric (n-HVa), and iso-valeric acids (iso-HVa) [22]. Details of extraction processes of DOM and EPS in the WAS are conducted with Lu, Xing, Liu and Ren [6].

The DOM and EPS were detected in fluorescence EEM measured by fluorescence spectrometry (FP-6500, Jasco, Tokyo, Japan). Details of spectra scan and elimination of inner filtering effect and Raman scattering, and parallel factor analysis (PARAFAC) used to model EEM fluorescence data, are given in our previous publication [2]. Parallel factor analysis (PARAFAC) was used to model EEM fluorescence data to reduce negative effects of spectra overlap. Core consistency diagnostics were used to determine the appropriate number of components in the PARAFAC analysis [23]. The software Matlab 7.0.4 (MathWorks Inc., USA) and sigma plot 12.0 were employed to process the EEM data.

Fluorescein diacetate (FDA) hydrolase assays was used to measure enzyme activity produced by microbes, which was determined by a modification of the procedure of Sanchez-Monedero, Mondini, Cayuela, Roig, Contin and De Nobili [24]. One milliliter of sludge sample was shaken with 10 mL of 0.06 M potassium phosphate buffer (pH 7.6), and 0.2 mL FDA stock solution (2 mg/mL) for 1 h at 60°C.

The reaction samples were centrifuged at a speed of 8000 rpm after stopping with 10 mL of acetone. The absorbance of the supernatant solution was measured at 490 nm using a fluorescence spectrometry (FP-6500, Jasco, Tokyo, Japan) and expressed as μg FDA/(mL h) of sludge. The calibration curve was conducted with Sanchez-Monedero, Mondini, Cayuela, Roig, Contin and De Nobili [24].

3. Results and discussion

3.1. The optimization of Geobacillus sp. G1 dosage on WAS solubilization

Different dosage of *Geobacillus* sp. G1 was inoculated into WAS to investigate the effects on WAS solubilization, followed by analysis of the FAD activity to gain insight into the hydrolases activity. Considering the effect of temperature and the lysis performance of *Geobacillus* sp. G1 on WAS, the thermal pretreatment (60°C) was used as the control test (blank). As shown in Fig. 1, five dosages (5%~30%, V_{G1}:V_{WAS}) had different effects on FDA hydrolase activity and soluble organic substance accumulation. *Geobacillus* sp.G1 pretreatment led to a significant increase in the FDA hydrolase activity over the blank (thermal control) (Fig. 1a). With the increase of pretreatment time, the FDA hydrolase activity sharply increased from 6 h onward, and the maximal activity was obtained at dosage of 10% (403.4 ± 4 μg FDA/(mL·h), 6 h), which was 1.5-fold of that obtained in the blank (thermal control) (272.8 ± 3μg FDA/(mL·h), 6 h).

In the current study, the change of SCOD, soluble protein and carbohydrate concentrations were applied to express the solubilization effect of *Geobacillus* sp. G1 pretreatment. Obviously, the optimal *Geobacillus* sp. G1 dosage for WAS solubilization was 10% (Fig. 1b). The specific concentrations of SCOD, soluble protein and carbohydrate for 6 h were 4130 ± 170, 1063 ± 15 and 213 ± 6 mg COD/L, respectively, which were 1.5-fold, 1.7-fold and 1.1-fold of that obtained without *Geobacillus* sp.G1 treatment. However, when *Geobacillus* sp.G1 dosage rate is keep increasing > 15%, the concentrations of SCOD, soluble protein and carbohydrate did not further increase with the increase of *Geobacillus* sp. G1 dosage. Taking SCOD as an example, the concentrations were 3990 ± 180, 3390 ± 280 and 3020 ± 120 mg/L at *Geobacillus* sp.G1 dosage rate of 15%, 20% and 30%, respectively. Under the thermal condition, it is proved that the inoculated thermophilic strains can rapidly develop into the predominant microbes in the digestion system [25]. The variation of soluble organics was a composite result of hydrolysis of organic substrates and further metabolic process. More inoculated thermophilic bacteria and other acclimated microorganisms resulted in more consumed soluble organics [26]. Thus, a high accumulation of soluble organics can be achieved at an optimal *Geobacillus* sp. G1 dosage in WAS, and improved floc disruption of WAS can further increase the bioavailability.

3.2. Enhanced WAS hydrolysis by alkaline-associated Geobacillus sp. G1 pretreatment

The effects of alkaline (pH 10)-associated *Geobacillus* sp. G1 pretreatment on WAS hydrolysis were further examined (Fig. 2). Previous studies showed that the dissolution of protein and carbohydrate was mainly through the break of sludge matrix [27]. A large amount of soluble carbohydrate and protein were observed after alkaline-associated *Geobacillus* sp. G1 pretreated. The highest concentrations of 340 ± 15 mg COD/L for soluble carbohydrate and 1295 ± 45 mg COD/L for soluble protein were appeared under the alkaline-associated *Geobacillus* sp. G1 pretreatment, which were 1.6-fold and 1.2-fold of that obtained in sole *Geobacillus* sp. G1. The concentrations of soluble carbohydrate and protein in the black test (thermal control) were only 202 ± 13 and 783 ± 30 mg COD/L.

It has been reported that sludge flocs could be divided into four layers, dissolved organic matter (DOM), dissolved organic matter (LB-EPS), tightly bound extracellular polymeric substances (TB-EPS) and pellet [27,28]. The distribution of enzymes activity in the different layers of sludge flocs is shown in Fig. 2a. The FDA hydrolase activity under alkaline-associated *Geobacillus* sp. G1 pretreatment in DOM was

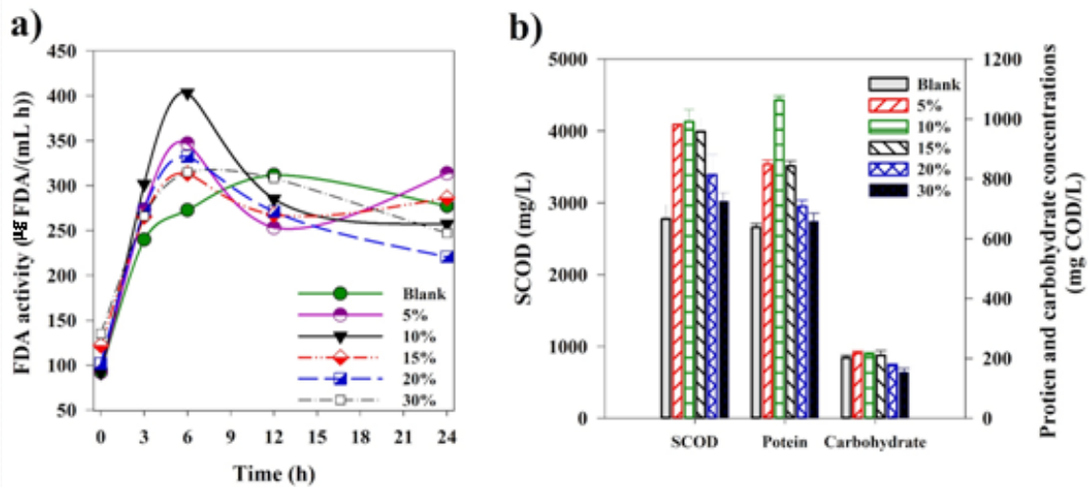

Fig. 1. Hydrolytic enzyme activity and organic release after *Geobacillus* sp. G1 pretreated.

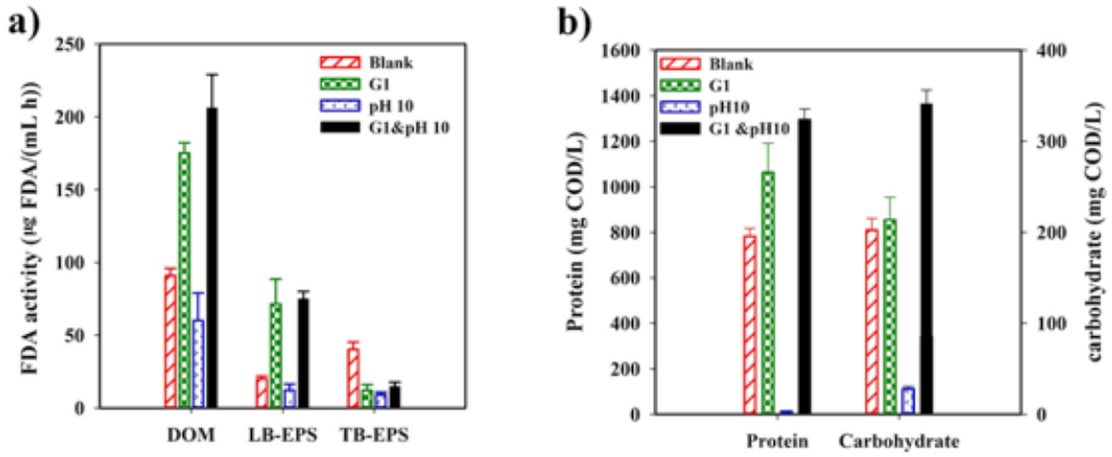

Fig. 2. FDA hydrolase activity in DOM, LB-EPS and TB-EPS (a) and Effects of different pretreatments on organic release (b).

the highest (210 ± 20 μg FDA/(mL·h)), which was 1.1-fold, 4.3-fold and 1.5-fold of that obtained in the sole *Geobacillus* sp G1, pH 10 and the blank (thermal control), respectively. The corresponding FDA hydrolase activity under alkaline-associated *Geobacillus* sp. G1 pretreatment in EPS was 90 ± 10 μg FDA/(mL·h). The results showed that alkaline-associated *Geobacillus* sp. G1-treated WAS had higher extracellular hydrolytic enzyme activity both in the DOM and EPS layers compared with the sole pretreatment of *Geobacillus* sp. G1. A constant strong alkaline condition will inhibit microbial activities, including enzyme activities [29,30]. But, in this study, the combined method was initially to adjust pH 10 for hydrolysis enhancement. In previous studies, sludge alkaline (pH 10) fermentation has been reported to achieve highly efficient hydrolysis and acidification, and the alkaline condition will be neutralized by acid release during organic degradation [31,32]. In this study, the dissolution of protein and carbohydrate was mainly through the break of sludge matrix during 6 h pretreatment, in the meanwhile, intracellular enzymes released from disrupted cells. Therefore, FDA hydrolase activity would higher after appropriate pretreated. The higher FDA hydrolase activity in different sludge layer indicated the higher disintegration effect to EPS matrix, leading to the release of extracellular organics into the liquid phase, especially soluble protein.

3.3. Characterization of pretreated WAS by EEM spectra with PARAFAC

Three-dimensional EEM spectra was applied to comprehensively reveal the component changes of WAS under different pretreatment. The typical EEM fluorescence spectra of DOM and EPS samples extracted from sludge are illustrated in Fig. 3. The correct number of components was determined by using PARAFAC [33]. Four main peaks were observed at the excitation/emission wavelengths (Ex/Em) and identified as: 275/350nm (tryptophan protein-like substances, Com.1), 275/305 nm (tyrosine protein-like substances, Com.2), 350/450 nm (nicotinamide adenine dinucleotide (NADH), Com.3) and 340/400 nm (humicacid-like substances, Com.4) [6,34].

The fluorescence intensities could provide additional information about the chemical nature of the macromolecules and further imply the structural differences [28]. The fluorescence intensities of four main substances in the DOM and EPS are shown in Fig. 4. The EEM fluorescence spectra were similar in the peak locations, but had different fluorescence intensities (FI). The intensities of DOM were used to evaluate the effect of sludge destruction after pretreated, which were apparently enhanced by alkaline-associated *Geobacillus* sp. G1 pretreatment. Tyrosine protein-like substances was the primary component with a FI of 847, 670 and 246 by alkaline-associated *Geobacillus* sp. G1, sole *Geobacillus* sp. G1 and pH 10 pretreatments, respectively, compared to 500 in the blank (thermal control). Tryptophan protein-like substances represented the second most abundant compounds in all samples, which was highest with a FI of 590 by alkaline-associated *Geobacillus* sp. G1. It was reported that the humic acid-like substances mainly existed in the LB-EPS [35]. The FI of humic acid-like substances in DOM layer increased from 8 to 74 by alkaline-associated *Geobacillus* sp. G1 pretreatment, which indicated that the extracellular polymeric substances were released to the supernatant, under the alkaline-associated *Geobacillus* sp. G1 pretreatment, accompanying the sludge matrix destruction. Moreover, the compositions of the DOM and EPS were similar for all the sludge samples, indicating similar structural characteristics of these substances treated by both individual and alkaline-associated *Geobacillus* sp. G1 sludge pretreatment methods. As either thermophilic bacteria or alkaline could cause the break-up of sludge matrix [19,25], the protein-like substances in the DOM layer after pretreated (Fig. 4), including tryptophan and tyrosine protein-like substances, was ordered as pH 10 < blank (thermal control) < *Geobacillus* sp. G1 < alkaline-associated *Geobacillus* sp. G1. The FI of EPS peaks in EEM spectra were dramatically changed by alkaline-associated *Geobacillus* sp. G1 pretreatment. Taking tyrosine-like substance as example, in the TB-EPS layer, the concentration (FI) was significantly decreased from 445 to 127. The same phenomenon was also observed for the other three substrates. Conversely, in the LB-EPS layer, the FI of tyrosine-like substance was increased from 135 to 211. The reason was that the TB-EPS layer was

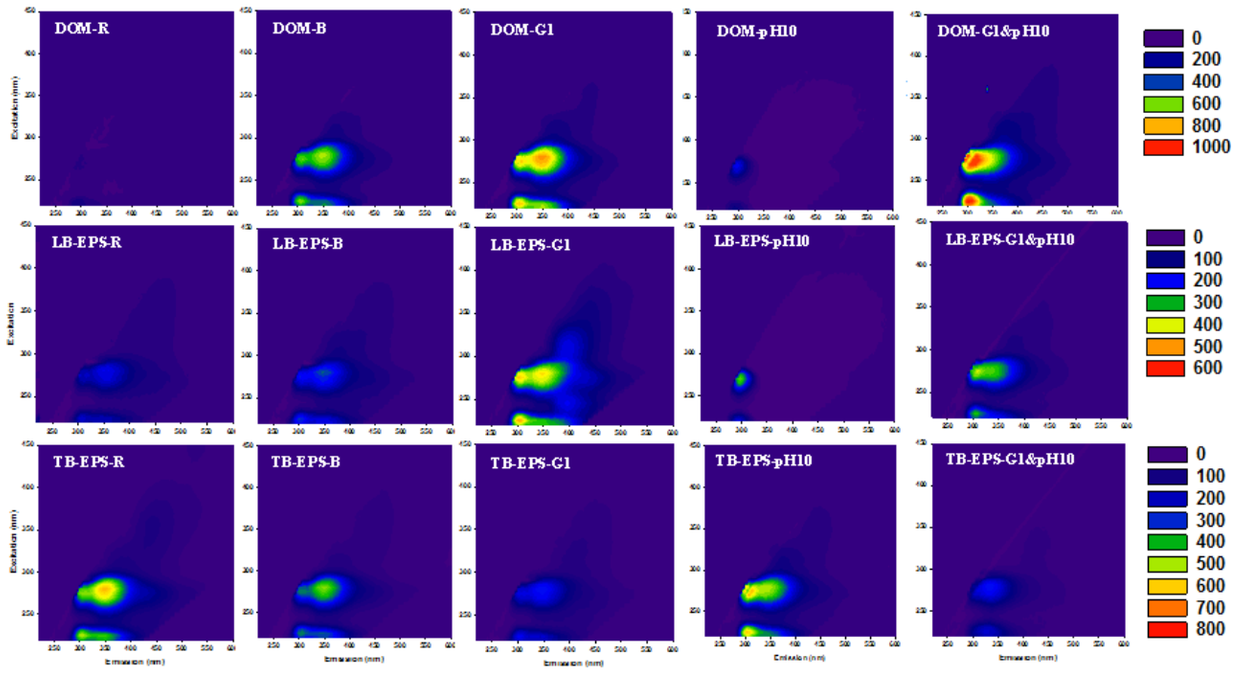

Fig. 3 .EEM fluorescence spectra of DOM, LB-EPS and TB-EPS. Raw WAS as controls, Blank of thermal-pretreated WAS (thermal control), *Geobacillus* sp. G1-pretreated WAS and alkaline-associated *Geobacillus* sp. G1 pretreated WAS

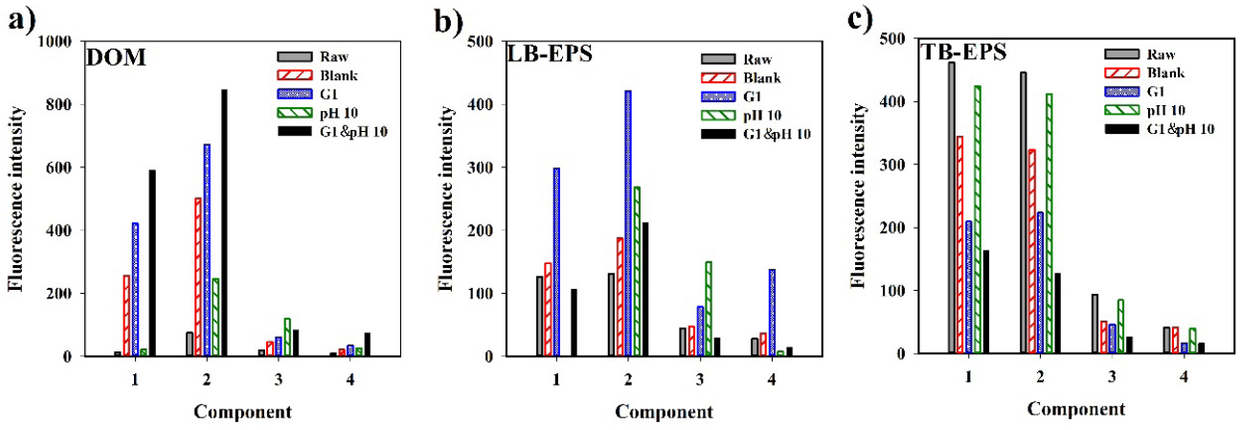

Fig. 4. Fluorescence spectral intensity of components in the DOM, LB-EPS and TB-EPS samples Raw WAS as controls, Blank of thermal-pretreated WAS (thermal control), *Geobacillus* sp. G1-pretreated WAS and alkaline-associated *Geobacillus* sp. G1 pretreated WAS

tightly attached to the cell surface, owning to the disintegration effect of the combined pretreatment, extracellular polymeric organics in this layer were released; while the LB-EPS layer was characterized by loose structure and rheological property, which was less affected by the pretreatment, but easily adsorbed the released organics from the intracellular and TB-EPS layer [20]. Previous study proposed that sludge hydrolysis rates depended on the diffusion of enzyme surface active site into sludge matrix particles [36]. Thus, the enzymes released by pretreatment adsorbed by, or bound to EPS released into solution through the disruption of the EPS matrix and microorganism, which inevitably led to the enhanced sludge solubilization. The detaching of EPS from WAS was effectively decomposed by *Geobacillus* sp. G1 pretreatment, and further improved by the alkaline-associated *Geobacillus* sp. G1 pretreatment.

3.4. SCFAs and soluble organics conversion during fermentation

SCFAs are the important high added-value green chemicals and preferred substrates for many bioprocess [20]. The concentrations of SCFAs production and composition are illustrated in Fig. 5. The total SCFAs concentration was increased with the fermentation time till 96 h, and further

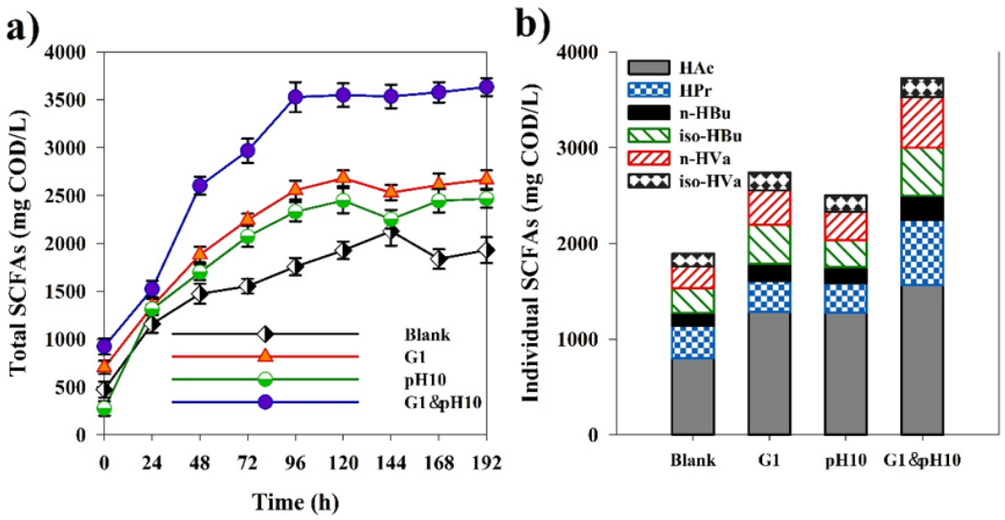

Fig. 5. Total SCFAs concentration (a) and ratio of individual SCFAs

increasing the fermentation time could not improve the SCFAs production. The maximum SCFAs concentration was 3529 ±150 mg COD/L (352.9 ± 15 mg COD/g VSS) in the alkaline-associated *Geobacillus* sp. G1 test at a fermentation time of 96 h, which was 1.5-fold and 1.4-fold of that obtained under sole alkaline (2330 ± 100 mg COD/L) and *Geobacillus* sp. G1 (2556 ± 95 mg COD/L) pretreatments. It is worth noting that only 1324 ± 45 mg COD/L (132.4 ± 4.5 mg COD/g VSS) was obtained in the blank (thermal control). This result was better than the previous research using other strains on WAS treatment. It was showed that about 3000 mg COD/L of VFAs obtained from WAS fermentation by inoculated with alkali-tolerant strain (pH 10) at the fermentation time of 96 h [37]. Moreover, the SCFAs concentrations showed a quadratic increase with fermentation time with alkaline-associated *Geobacillus* sp. G1 pretreatment ($Y_{SCFAs} = -0.1X^2 + 38X + 896$, $R^2 = 0.98$, $p < 0.05$). Fig. 5b showed the percentage of individual SCFAs under different pretreatment conditions at 96 h. HAc was the most abundant product, which was the most suitable substrate for many bioprocess, such as biogas and biopolymer production [38,39]. The concentration of HAc in alkaline-associated *Geobacillus* sp. G1 pretreatment was 1563 ± 37 mg COD/L (156.3 ± 3.7 mg COD/g VSS), which was 1.2-fold, 1.3-fold and 1.9-fold of those obtained in *Geobacillus* sp. G1, pH 10 and blank (thermal control) tests, respectively. HPr and n-HVa were the other two top SCFAs. However, the order of the three SCFAs was different among these pretreatments. The order was HAc > HPr > n-HVa in alkaline-associated *Geobacillus* sp. G1 and pH 10 tests, while it was changed to HAc > iso-HBu > n-HVa in the *Geobacillus* sp. G1 and the blank (thermal control) tests. The sum of HAc, HPr and n-HVa was approximately 80% of total SCFAs in alkaline-associated *Geobacillus* sp. G1 pretreatment.

A proposed metabolic pathway for SCFAs production, especially for the HAc production, from protein and carbohydrate can be found in the previous study [40]. Different amounts of soluble organics in the fermentation system can directly reflect the substrate utilization and metabolite production. Fig. 6 shows that with the increase of SCFAs concentration, the concentrations of soluble protein and carbohydrate were linearly decreased, and reached minimal concentration at 96 h. As to alkaline-associated *Geobacillus* sp. G1 test, the concentration of soluble protein decreased rapidly from 1238 ± 22 to 876 ± 12 mg COD/L during the initial 96 h, while the soluble carbohydrate decreased from 340 ± 15 to 203 ± 5 mg COD/L. Correspondingly, the soluble protein and carbohydrate concentrations at 96 h were 502 ± 54 and 154 ± 12 mg COD/L in *Geobacillus* sp. G1 test, 418 ± 23 and 155 ± 8 mg COD/L in pH 10 test, and 641.3 ± 24 and 116.9 ± 8 mg COD/L in the blank (thermal control), respectively. In our previous study, the inoculated thermophilic strains can rapidly develop into predominant microbes in the digestion system, favoring degradation of the organic substrates and changing the microbial community [10,13,41]. Alkaline pretreatment is used to enrich Clostridia sp. and Delta proteobacteria sp. when connected to anaerobic fermentation of pretreated WAS [42]. While thermophilic pretreatment led to typical species enriched, including Caloramator and Clostridium, belonging to class of Clostridia and Phyla of Firmicutes [43]. Thus, the usage of alkaline associated *Geobacillus* sp. G1 affect the microbial communities under alkaline and thermophilic process. The Clostridia species, including Clostridia and *Geobacillus* sp. may be dominant as the main functional communities on hydrolysis and acidification [10,43]. The results were consistent with the previous research that the soluble organics were consumed for SCFAs accumulation at the presence of microorganisms in the fermentation system [28].

Considering the treatment efficiency and costs as the major bottleneck for WAS utilization, alkaline, thermal and acid pretreatments were commonly used for WAS solubilization [44]. Additional costs were still entailed, including chemicals, electricity input and specific reactors for different conditions. As a result, the feasibility of combined pretreatment was proved to shorten the time of hydrolysis and acidification for the SCFAs production from WAS fermentation. This introduction of pretreatment method could help reducing the pretreatment time and costs, due to better per-

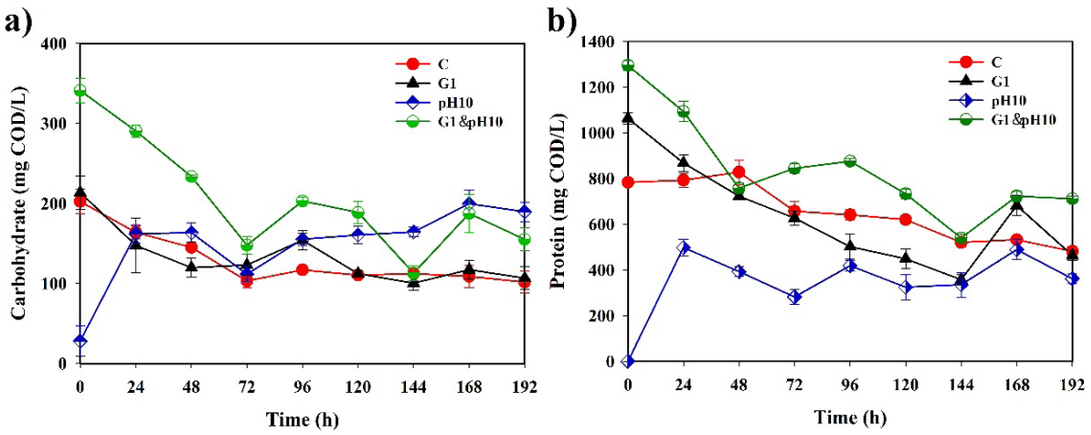

Fig. 6. Soluble protein (a) and soluble carbohydrate (b) concentration change in fermentation time

formance obtained after a short thermal pretreatment time and a small amount of additional chemicals.

4. Conclusions

The effects of alkaline-associated *Geobacillus* sp. G1 on WAS hydrolysis and SCFAs accumulation were investigated. The optimal *Geobacillus* sp.G1 dosage rate for WAS treatment was 10% (V_{G1}:V_{WAS}), which improved the hydrolysis of organic matter. The combined pH adjustment (10) further improved both FDA hydrolase activity and particulates organics solubilization in the WAS. From EEM spectra analysis, the dominant compositions were tryptophan and tyrosine protein-like substances by the alkaline-associated *Geobacillus* sp. G1 pretreatment. The detaching of EPS from WAS was obviously improved by this pretreatment method. SCFAs was also achieved the highest concentration at the fermentation time of 96 h. The results indicated that pH adjustment (10) could further enhanced WAS hydrolysis and SCFAs accumulation on the basis of *Geobacillus* sp. G1 pretreatment, which was more effective than sole *Geobacillus* sp. G1 and alkaline (pH 10) pretreatments.

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