Augmentation of protein-derived acetic acid production by heat-alkaline-induced changes in protein structure and conformation

Xu Wang^{a, b, *}, Yanbo Li^a, Junxin Liu^{a, b, **}, Nan-Qi Ren^c, Jiuhui Qu^{a, b}

^a Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

^b State Key Joint Laboratory of Environment Simulation and Pollution Control, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

^c State Key Laboratory of Urban Water Resource and Environment, School of Municipal and Environmental Engineering, Harbin Institute of Technology, Harbin 150090, China

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ABSTRACT

Waste-derived acetic acid (HAc) is an attractive feedstock for microbe-mediated biofuel production. However, fermentative conversion of HAc from waste-activated sludge (WAS) has low yield because of the high concentration of proteins not readily utilizable by microorganisms without prior hydrolysis. We investigated a combined technology for HAc augmentation during sludge protein fermentation. The maximal HAc yield increased over two-fold, reaching 0.502 ± 0.021 g/g protein (0.36 ± 0.01 g COD/g COD, ~52% of the total volatile fatty acids) when synthetic sludge protein was heated at 120 °C for 30 min, treated at pH 12 for 24 h, and fermented at pH 9 for 72 h. Comprehensive analysis illustrated that the heat-alkaline pretreatment significantly induced protein fragmentation, simultaneously increasing the efficiency of protein biohydrolysis (from 35.5% to 85.9%) by inducing conformational changes indicative of protein unfolding. Consequently, the native α -helix content was decreased from 67.3% to 32.5% by conversion to an unordered shape, whose content increased from 27.5% to 45.5%; disulfide bonds were cleaved, whereas the main S–S stretching pattern was altered from gauche-gauche-gauche to gauche-gauche-trans, consequently causing increased protein susceptibility to proteolytic hydrolysis (76.3% vs. 47.0%). Economic analysis indicated that anaerobic fermentation with appropriate heat-alkaline pretreatment is a cost-effective approach for waste conversion to energy sources such as HAc.

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1. Introduction

As concerns over the depletion of oil reserves and the environmental and social impacts of oil combustion byproducts escalate, it is essential to develop alternative approaches to energy production and to diversify sustainable and economically viable energy sources in the commercial market (Dangerman and Schellnhuber, 2013). One of the attractive strategies to satisfy both needs is the utilization of waste-derived feedstock for the generation of different forms of energy via bioconversion (Nasidi et al., 2010). An example is the production of fermentative volatile fatty acids (VFA), an inexpensive energy source that can be used

to generate electricity and various valuable fuels such as biogas, hydrogen, and biodiesel (Lee et al., 2014). Waste-activated sludge (WAS) obtained through biological treatment of wastewater contains high quantities of organic matter, which can serve as a favorable feedstock for VFA synthesis through anaerobic fermentation (Li et al., 2011), thus minimizing disposable waste generation (Wang et al., 2015, 2012).

It is also increasingly recognized that the efficiency of downstream energy production is critically affected by VFA type, and acetic acid (HAc) has been identified as a promising feedstock (Wang et al., 2013). Previous studies indicate that HAc used as a substrate in a microbial fuel cell is preferable to other VFAs in terms of amount, coulombic efficiency, and electricity generation (Freguia et al., 2010; Chae et al., 2009). Another example is VFA-based biogas production under anaerobic conditions; among all VFAs tested, methanogenic bacteria had the highest tolerance to HAc (Wang et al., 2009). Hydrogen production is also much sensitive to VFAs used in fermentation and microbial electrolysis systems, and

* Corresponding author. Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China.

** Corresponding author. Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China.

E-mail address: xuwang@rcees.ac.cn (X. Wang).

hydrogen generation can be enhanced by elevating the HAc fraction in the feed (Uyar et al., 2009; Liu et al., 2012a). A study on VFA-driven lipid production showed that VFA feed containing higher ratios of HAc generated the highest lipid content (Fei et al., 2011). However, strategies to improve HAc yield during WAS dark fermentation have not been extensively investigated.

Proteins are essential components of microbial cells and extracellular polymeric substances and account for 50%–60% of the organic component in WAS (Lu et al., 2012). In anaerobic bioconversion, proteins are hydrolyzed by extracellular enzymes to short-chain polypeptides and amino acids and then fermented to VFAs that are metabolized by acetogenic and homoacetogenic microorganisms to generate HAc. However, naturally folded proteins are not susceptible to protease cleavage (Halabi et al., 2009), and because protein hydrolysis is the rate-limiting step, it retards HAc generation from WAS. Recently, protein hydrolysis has been found to be accelerated via manipulation of protein native conformation (Xiao et al., 2014); nevertheless, the process inhibited homoacetogenesis, emphasizing the need for enhanced hydrogen production from protein-containing wastewater.

It is well known that heat and alkaline pretreatments are alternative approaches to facilitate solubilization of the extracellular polymeric material in the sludge and to break cell walls, resulting in the substantial release of intracellular organic matter for further VFA generation. However, the effects of these alternative pretreatment approaches on protein conformation and microbial conversion to HAc during anaerobic fermentation of WAS have not been fully investigated. In this study, we investigated a combined technology to improve the yield of HAc in dark fermentation of sludge protein; initial pretreatment of sewage sludge was applied at high temperature of 120 °C for 30 min, followed by incubation at pH 12 at room temperature for 24 h and fermentation at pH 9 for HAc production. This approach was first tested in the production of HAc from bovine serum albumin (BSA). Subsequently, the efficiency of BSA fragmentation and denaturation and the changes in its secondary structure were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, circular dichroism, dynamic light scattering, as well as excitation-emission matrix fluorescence spectroscopy and Raman spectroscopy among other techniques. Finally, the feasibility of the combined approach for the improvement of HAc yield was also verified using real sewage sludge along with an economic analysis.

2. Methodology

2.1. Sewage sludge and seed microorganisms

In this work, the sewage sludge was collected from a secondary clarifier of a WWTP in Northern China. The sludge was concentrated by allowing it to settle for approximately 24 h and was then air-dried for 10 days at 24 °C, ground in a hammer mill and screened with a 0.45 mm mesh, and finally stored at –20 °C for further tests. The main characteristics of the concentrated sludge are as follows: pH, 6.92 ± 0.05; total suspended solids (TSS), 16.8 ± 0.9 g/L; volatile suspended solids (VSS), 13.7 ± 0.6 g/L; total chemical oxygen demand (TCOD), 19.2 ± 1.4 g COD/L; soluble chemical oxygen demand (SCOD), 0.10 ± 0.01 g COD/L; total protein, 9.4 ± 0.5 g COD/L; total carbohydrate, 1.19 ± 0.05 g COD/L; and lipid, 0.28 ± 0.02 g COD/L. In addition, seeding sludge was obtained from the same site and then cultured prior to seeding microorganisms (see [Supplementary information](#)). Subsequently, the seeding sludge was centrifuged at 3000 ×g for 20 min to remove the soluble organics and washed twice with distilled water. The centrifuged sludge sediment was used as the seed inoculum for HAc production experiments.

2.2. Effects of heat and alkaline pretreatment on HAc production from synthetic sludge protein

Sewage sludge contains multiple types of proteins. To better investigate the effects of pretreatment on HAc production from sludge protein fermentation and to clearly understand the fate of the proteins in the process, pure protein, i.e., bovine serum albumin (BSA, Sigma-A7030) was used as a substrate model for mechanistic explorations. Specifically, the BSA sample was added into 500-mL serum vials at 1.80 g/L in 250 mL to test four different pretreatment conditions. The heat-pretreated sample (H-BSA) was autoclaved at 120 °C and 117.6 kPa (VARIOKLAV steam sterilizer) for 30 min and then cooled to room temperature (24 ± 1 °C). The alkaline-pretreated sample (A-BSA) was mixed with 6 M sodium hydroxide (NaOH) to adjust the pH to 12, stirred for 30 min, and placed in a temperature-controlled chamber at 24 °C for 24 h. The third sample (HA-BSA) was subjected to a combination of heat and alkaline pretreatment, i.e., 30-min heating at 120 °C and 24-h incubation at pH 12 at room temperature. The untreated raw sample (R-BSA) was used as the control. No microorganisms were added during the pretreatments of synthetic protein samples. After the pretreatments, 1.0 g of NaHCO₃ (buffer salt), 35 mg of K₂HPO₄·3H₂O (phosphorus source), 1.0 mL of trace element solution (g/L: EDTA-2Na, 2.0; FeSO₄·7H₂O, 2.0; H₃BO₃, 0.1; CoCl₂·6H₂O, 0.1; ZnCl₂, 0.1; Cu(NO₃)₂·5H₂O, 0.05; MnCl₂·4H₂O, 0.1; Na₂MoO₄, 0.75; NiCl₂·6H₂O, 0.02), and 25 mL of seed microorganisms were added to each vial. The mixture was adjusted to pH 7 by 6 M HCl or 6 M NaOH followed by nitrogen flush and rubber stopper sealing, and HAc fermentation was conducted at 34 ± 1 °C in an air-bath shaker (150 rpm).

Additionally, the effects of pH on HAc production from proteins were explored using HA-BSA as a substrate at pH 7 (control), 8, 9, 10, and 11; the pH was maintained by the addition of 6 M NaOH or 6 M HCl throughout the experiment.

2.3. Enzymatic protein hydrolysis

Protein accessibility to proteolysis after heat and alkaline pretreatment was evaluated as protein enzymolysis efficiency (E_{pro}), which can be calculated as follows:

$$E_{pro} = \frac{\Delta C_{pro}}{C_{pro,initial}} \quad (1)$$

where ΔC_{pro} is the difference in protein concentration (g/L) in the sample before and after the experiment, and $C_{pro,initial}$ is the initial protein concentration (g/L) in the sample.

Using eq. (1), ΔC_{pro} can be further determined as follows (Miron et al., 2000):

$$\Delta C_{pro} = \frac{C_{TN}}{\eta_{pro,n}} \quad (2)$$

where $\eta_{pro,n}$ is the average fraction of nitrogen content in protein (Miron et al., 2000), i.e., 0.16; and C_{TN} is the concentration of total nitrogen (TN) in the supernatant.

Pronase E, also called actinase E, is a protease derived from *Streptomyces griseus* and is widely used to achieve complete enzymatic hydrolysis of a protein (e.g., in the analysis of amino acid composition) because it contains more than 10 effective proteolytic components (Bermejo-Barrera et al., 1999). Moreover, Pronase E was also applied recently to decrease solid content and enhance anaerobic digestion of sewage sludge (Roman et al., 2006). Accordingly, Pronase E was used as a model protease and added at the concentration of 50 mg/L to the reactors containing R-BSA and

HA-BSA, and the test was performed under the conditions described above. At set intervals, 5 mL of the mixture was sampled and immediately treated at 100 °C for 10 min to inhibit enzyme activity. After cooling to room temperature, the residual protein in sample was precipitated by adding 5 mL of 15% trichloroacetic acid, and the mixture was centrifuged at 3000 ×g for 20 min. Subsequently, the supernatant was collected for determination of C_{TN} using a TOC/TN analyzer (Shimadzu TOC-VCPH/TNM-1).

2.4. Protein structure determination

To determine protein secondary structure, circular dichroism (CD) spectra were acquired using an automatic recording spectropolarimeter (JASCO J-715). The secondary structure content in the samples was estimated using the curve-fitting method of the far-UV CD spectrum and the JASCO secondary structure manager (Greenfield, 2006). The spectra were acquired at 25 ± 0.1 °C with a scan speed of 50 nm/min and spectral bandwidth of 1 nm; each spectrum represented the average of four scans. The secondary structure was measured over the wavelength range of 200–250 nm using a cuvette with a path length of 1 cm against distilled water as a blank and estimated by the mean residue ellipticity (MRE, deg·cm²/dmol):

$$MRE = \frac{\theta_{obs} M}{n l C} \quad (3)$$

where θ_{obs} is CD in millidegrees (m deg), M is protein molecular weight (g/dmol), n is the number of amino acids (583 in BSA), l is cuvette path length (1 cm), and C is protein concentration (g/L).

The intensity-weighted average protein hydrodynamic diameter (D_h , nm) was determined by the dynamic light scattering (DLS) technique using a Zetasizer system (Malvern Nano ZS90) with a He–Ne laser operated at a wavelength of 633 nm and a scattering angle of 90°. The size was estimated as the mean value of three runs. The data were analyzed using Dynamics 6.10.0.10 software at the optimized resolution. D_h was estimated by autocorrelation analysis of scattered light intensity using the translational diffusion coefficient based on the Stokes–Einstein equation:

$$D_h = \frac{kT}{3\pi\eta D_w^{25^\circ C}} \quad (4)$$

where k is Boltzmann's constant, T is absolute temperature, η is water viscosity, and $D_w^{25^\circ C}$ is the translational diffusion coefficient.

Three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy (JASCO FP-6500) was used to further analyze the R-BSA and HA-BSA composition. The sample was filtered through a 0.50-μm hydrophilic membrane and diluted to an appropriate concentration prior to EEM analysis. The spectra were obtained with the excitation and emission wavelengths gradually increased from 200 to 500 nm at 1 nm step to obtain the EEM fluorescence.

Raman spectroscopy (Jobin Yvon HR-800) was used to characterize the conformation of protein disulfide bridges. Raman spectra were acquired in the range of 100–1600 cm^{−1} using a He–Ne laser beam and irradiation at a wavelength of 632.8 nm; each spectrum was determined as the accumulated average of three exposures at room temperature. To quantify protein structural composition, the spectra were normalized to the intensity of the phenylalanine band, i.e., 1000 at 1004 cm^{−1} (Tuma, 2005). The Raman spectra were fitted to the Gaussian model using the Origin Pro 9.1 software.

2.5. HAc production by real WAS fermentation

The sewage sludge (details in Section 2.1) was diluted and then

used for fermentative HAc production. The HAc yield of the untreated (R-WAS) and heat-alkaline pretreated (HA-WAS) sludge samples was compared in two identical reactors, where fermentation pH was set at 7 and 9, respectively, using 6 M HCl or 6 M NaOH. The seed microbes and other operational and analytical parameters were as described above.

2.6. Other analytical methods

Determinations of pH, protein, carbohydrate, lipid, VFAs, COD, TSS, and VSS content were performed as previously described (Cai et al., 2004). Moreover, the conversion factor of protein to COD was set as 1.5 g COD/g protein, according to a previous report (Miron et al., 2000). The detailed analytical procedures for biogas component analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fluorescence in situ hybridization (FISH), and acid-forming enzyme activity analysis are provided in the Supplementary information.

2.7. Statistical analysis

The results are presented as the mean ± standard deviation of triplicate experiments performed in the same conditions. The statistical differences between samples were analyzed using IBM SPSS statistics 21.0 software; $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Comparison of pretreatment and fermentation approaches to HAc production

First, the effect of different pretreatments of synthetic sludge protein on the yield of HAc produced by dark fermentation was examined at pH 7 (Fig. 1a). HAc production started after 6 h and the yield increased with fermentation time in all the tested samples; however, after 72 h, the increase was not significant ($p > 0.05$). Therefore, the effects of alternative pretreatments on HAc yield were comparatively evaluated at the fermentation time of 72 h. HA-BSA fermentation demonstrated 1.11-, 1.47-, 2.24-fold higher HAc yield (0.416 ± 0.016 g/g protein) than A-BSA (0.376 ± 0.015 g/g protein), H-BSA (0.283 ± 0.025 g/g protein), and R-BSA (control; 0.186 ± 0.018 g/g protein), respectively. Therefore, the combination of heat (120 °C) and alkaline (pH 12) pretreatment caused a significant increase in HAc production.

Consequently, the HA-BSA was used to further test the effect of fermentation pH on HAc generation (Fig. 1b). Increases in pH significantly stimulated HAc production over time, and the maximal HAc yield (0.502 ± 0.021 g/g protein)—which was approximately 2.70-fold higher than that of the control (Fig. 1a)—was observed at pH 9 after 72 h of fermentation. In Fig. S1, HA-BSA fermentation also represented a much higher total VFA yield (0.790 ± 0.031 g/g protein, or 0.70 ± 0.03 g COD/g COD) than did by R-BSA fermentation (0.346 ± 0.014 g/g protein, or 0.32 ± 0.01 g COD/g COD) and other recent literature (Morgan-Sagastume et al., 2015; 2011; Jie et al., 2014). Despite the findings indicating that fermentation at pH 9 is the optimum condition for VFA accumulation while feeding with heat-alkaline pretreated substrates, additional studies also report that maximal VFA yield was achieved in the untreated-substrate fed reactor when fermentation was carried out at pH 10 (Yuan et al., 2006). Indeed, the specific ranges of the optimum pH conditions for VFA production are dependent on the types of substrate used. Thus far, the reported optimal pH values are in the range of 8–11 for VFA accumulation during the anaerobic fermentation of sewage sludge (Lee et al., 2014). Overall,

Fig. 1. Effects of pretreatment approaches and fermentation pH on HAC production. (a) Bovine serum albumin (BSA) was used as a model sludge protein and heat-pretreated at 120 °C for 30 min (H-BSA), incubated at pH 12 at 24 °C for 24 h (A-BSA), or subjected to combined heat-alkaline pretreatment (HA-BSA); the untreated raw sample (R-BSA) was used as the control. The samples were then mixed with real sewage (a source of seed microorganisms) and phosphate-buffered nutrient solution and fermented at pH 7 for the indicated times. (b) HA-BSA was fermented at different pH for the indicated times. The data are expressed as the mean $\pm$ standard deviation of triplicate tests.

the heat-alkaline pretreatment combined with anaerobic fermentation at pH 9 for 72 h was considered as an effective approach to augment HAC production in this work.

Subsequently, the FISH assay was performed for a better understanding of the augmentation of HAC yield at fermentation pH 9 (see Fig. S2 for details). Further images analysis demonstrated that the average ratio of bacteria to archaea in the reactors was 101:1 (pH 9) and 27:1 (control, pH 7). The ratio of bacteria to archaea in these two reactors was aligned with their observed HAC yields, implying the increased proportion of bacteria probably contributed to the augmented HAC yield. Many bacteria have been reported to be capable of producing HAC under anaerobic conditions (Mitchell, 1998). Some species of *Clostridia*, which are closely related to *Clostridium spp.*, have been shown to generate HAC under obligate alkaliphilic (pH 8–12) conditions (Zhilina et al., 2005). Additional species of *Clostridia*, which were similar to the genus *Anaerobranca*, have been documented to be the predominant microorganism in alkaline fermentation conditions (Zhang et al., 2010). As previously reported (Prowe and Antranikian, 2001; Gorlenko et al., 2004), the genus *Anaerobranca* comprises only a few species among anaerobic microorganisms that have the capacity to convert proteins to HAC as key final product at pH 6–10 and temperature 30–70 °C. Additionally, the requirement of *Anaerobranca* needs sodium ions (Engle et al., 1995). In this work, 6 M sodium hydroxide was applied to ensure alkaline conditions, and substantial amounts of sodium ions were present in the reactor, which may explain why HAC accumulated at pH 9. Since an alkaline environment is not conducive to methanogenesis, the hydrolysis and the susceptibility of the available substrates for the acidogenic bacteria might be key factors to augment HAC production, rather than the competition between the acidogenic bacteria and the methanogens. Thus, future efforts should be devoted to decipher the effect of heat-alkaline stimulation on the susceptibility of substrates to acidogenic bacteria responsible for improved HAC generation.

3.2. Effect of heat-alkaline stimulation on protein susceptibility to hydrolysis

In dark fermentation, hydrolysis is a critical step for HAC production from protein. Thus, we subsequently focused on investigating the effects of the heat-alkaline pretreatment on protein hydrolysis and susceptibility to proteolytic degradation.

Protein fragmentation is the process of breaking up proteins into smaller fragments, making them more accessible for further anaerobic conversions (Kim et al., 2003). Consequently, the

fragmentation degree of HA-BSA was indirectly determined by SDS-PAGE (see Fig. 2). BSA without pretreatment (R-BSA, lane b) was used as a negative control for protein fragmentation; one thicker band at 50–60 kDa and faint bands below 200 kDa were observed, which agreed with previous findings (Ajandouz et al., 2008), notwithstanding the characteristic bands vary slightly among different commercial BSA products. However, the characteristic bands of native BSA had noticeably disappeared for the HA-BSA, and smeared bands were shown in the right side (lane c) of Fig. 2, indicating that heat-alkaline treatment may have successfully fragmented BSA into smaller pieces.

Moreover, E_{pro} measurements also indicated critical effects of the heat-alkaline pretreatment on protein susceptibility to proteolysis (Fig. 3a). After 1 h, the E_{pro} of the HA-pretreated sample was significantly higher than that of the control (76.3% vs. 47.0%). Protease activity was also enhanced by the heat-alkaline pretreatment (Fig. 3b), and the protein hydrolysis efficiency in HA-BSA increased to 85.5% compared to 35.9% in R-BSA.

Protein secondary structure directly influences the susceptibility to enzymatic hydrolysis, which can be substantially enhanced after protein unfolding (Renard et al., 2014). Protein conformation is determined by hydrogen bonding-mediated folding of the peptide backbone into α -helices and β -sheets. We therefore used CD to compare conformational transitions of protein molecules in R-BSA and HA-BSA samples. As shown in Fig. 4a, the R-BSA spectrum had two negative peaks at 208 and 222 nm, generally characteristic of an α -helical structure (Greenfield, 2006). However, the CD signal of HA-BSA decreased significantly, suggesting that the pretreatment induced changes in the secondary structure of the protein molecules. The protein structural content displayed as tree maps (Fig. 4b) further indicates that R-BSA consisted of $67.3 \pm 1.1\%$ α -helices, $15.9 \pm 0.2\%$ β -sheets, $7.4 \pm 0.6\%$ β -turns, and $9.3 \pm 1.5\%$ unordered structure, which is consistent with previous reports (Reed et al., 1976). After heat-alkaline pretreatment, the α -helix content decreased to 33.3%, which is almost half of that in the control. These data demonstrate that the heat-alkaline stimulation decreased ordered protein folding, consequently increasing the amount of unordered structure (Fig. 4b) and suggesting that the heat-alkaline stimulation approach significantly facilitates protein denaturation (Fig. 5a). Moreover, DLS is another useful technique to explore protein folding based on size (Mitra et al., 2007). Fig. 5b shows denaturation of HA-pretreated proteins, as evidenced by a 2.04-fold increase in average hydrodynamic diameter compared to that of the untreated protein.

EEM spectroscopy enables comprehensive profiling of protein

Fig. 2. SDS-PAGE images indicating the degree of protein fragmentation following heat-alkaline pretreatment. (a) Protein markers (10–200 kDa), (b) R-BSA, and (c) HA-BSA.

fluorescence in complex mixtures over a range of excitation and emission wavelengths, thus providing superior analytical potential for fluorescence measurements (Sheng and Yu, 2006). Here, we acquired EEM spectra to reveal the structural transitions in the HA-BSA sample, represented by transformations in the tryptophan microenvironment induced by protein denaturation (Bhattacharya et al., 2011). In Fig. 5c, the maximum peak fluorescence intensity of the HA-treated sample was substantially lower than that of R-BSA (2.6×10^6 vs. 4.7×10^6 a.u.), whereas the maximum excitation and emission wavelengths demonstrated a simultaneous shift (excitation: 270 nm $\rightarrow$ 262 nm; emission: 350 nm $\rightarrow$ 357 nm), revealing that the decrease of fluorescence intensity caused by the heat-alkaline pretreatment was mediated by the quenching of aromatic residue fluorescence (Christen et al., 2013).

Protein secondary structure is stabilized by the formation of disulfide bonds. Raman spectroscopy has been used to probe the conformation of protein disulfide bonds based on the frequency of S–S stretching mode (Paris et al., 2012). We first used additional experiments performed on amino acids and short peptides in solution to verify that the contribution of the low-wave-number aromatic Raman lines to the considered spectral region was negligible. Upon band decomposition, as shown in the left chart of

Fig. 3. Effects of pretreatment on BSA enzymatic hydrolysis. (a) Heat-alkaline pretreated (HA-BSA) and untreated (R-BSA) synthetic sludge protein samples were incubated with 50 mg/L pronase E for 1 h and analyzed for their susceptibility to proteolytic hydrolysis expressed as enzymolysis efficiency. (b) Protein hydrolysis efficiency during fermentative conversion to HAC at pH 9 for 72 h. The data are expressed as the mean $\pm$ standard deviation of triplicate tests.

Fig. 5d, the presence of components in both R-BSA and HA-BSA reveals the conformational variability of disulfide linkages. Significant differences in both the wavenumbers and relative intensities of these components were also identified. First, although HA-BSA presented a large and asymmetric profile in the 480–520 cm^{-1} interval, fitted by two components at 505 and 516 cm^{-1} , R-BSA displayed a symmetric monocomponent-shape peaking at 503 cm^{-1} in the same region. Second, although both samples presented one component at $\sim 530 \text{ cm}^{-1}$, R-BSA provided an additional component at a wavenumber as high as 542 cm^{-1} . In a previous study, the 500–550 cm^{-1} region in the Raman spectrum was attributed to S–S stretching vibration mode, and peaks detected around 500–510, 515–525, and 535–545 cm^{-1} were characteristic of the gauche-gauche-gauche (g-g-g), gauche-gauche-trans (g-g-t), and trans-gauche-trans (t-g-t) rotamers, respectively (Paris et al., 2012). In line with the curve fitting of the band, the conformation of disulfide bridges was determined (Fig. 5d, right panel). In the untreated protein (R-BSA), 17 disulfide bridges were detected (11 g-g-g, 3 g-g-t, and 3 t-g-t). However, heat-alkaline pretreatment decreased the number of disulfide bridges in the sample proteins to

Fig. 4. Protein secondary structure before and after heat-alkaline pretreatment. (a) The far UV-CD spectra (200–250 nm) of untreated synthetic protein (R-BSA) and heat-alkaline-treated protein (HA-BSA). Each spectrum is the average of four scans. (b) Tree map of protein structural composition based on decoding of CD spectra. The rectangles in individual plots represent protein secondary structure (α -helix, β -sheet, β -turn, or unordered shape); rectangle sizes and color intensities correspond to average mass fractions of protein structures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Protein structural transition induced by heat-alkaline pretreatment. (a) Dominant structures in control sample (R-BSA) and heat-alkaline-pretreated sample (HA-BSA). (b) Distribution profiles of R-BSA and HA-BSA hydrodynamic diameter determined by dynamic light scattering (DLS). The average hydrodynamic diameter was 7.9 nm for R-BSA and 16.1 nm for HA-BSA. (c) Three-dimensional excitation-emission matrix (EEM) fluorescence spectra of R-BSA and HA-BSA. (d) Curve fitting of the S–S Raman band of R-BSA and HA-BSA (left panel), and the composition of S–S bridge conformations based on analysis of the 490–560 cm^{-1} Raman regions (right panel). Green and red lines indicate the individual Gaussian-fitted components and peaks, respectively, and dotted lines represent the experimental data. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

9 while simultaneously increasing the g-g-t conformation (2 g-g-g, 5 g-g-t, and 2 t-g-t), illustrating that heat-alkaline pretreatment not only destroyed protein disulfide bonds but also altered the protein conformation.

It is well known that hydrolysis is the rate-limiting step because the cell wall and extracellular polymeric substances in sludge will impose physico-chemical barriers to the hydrolysis of intracellular organic matters. Numerous studies have reported that heat and alkaline pretreatment among other methods play a significantly beneficial role in sludge acidification since they can facilitate

extensive solubilization of organic matters and then increase bioavailability of substrates for subsequent VFAs formation (Kim et al., 2003; Vlyssides and Karlis, 2004; Dogan and Sanin, 2009; Chen et al., 2007). However, since soluble protein (BSA) was used here as the main substrate to explore the effects of the heat-alkaline stimulation in relation to HAC production (see Sub Section 3.1), the findings implied that there should be a new insight into the mechanisms in relation to HAC augmentation beyond the well-known solubilization. With reference to the above multiple analyses, now it is quite evident that the heat-alkaline

pretreatment results in the simultaneous occurrence of both protein fragmentation and denaturation, which has additive effects on protein hydrolysis and susceptibility to proteolytic degradation and therefore, the increment of HAC yield in the process.

3.3. Verification of the combined technology using HAC yield from real WAS

After mechanistic investigations, the feasibility of using heat-alkaline pretreatment combined with fermentation at pH 9 for augmentation of HAC production was tested in the reactor fed with real WAS. As indicated in Table 1, the HAC production of the control (no pretreatment, pH 7 fermentation) was only 0.08 ± 0.02 g COD/g COD after 72 h of fermentation, but heat-alkaline pretreatment combined with the fermentation at alkaline pH increased the yield by more than three-fold (0.31 ± 0.04 g COD/g COD). Sludge hydrolysis was also observed in the tests, and the SCOD at alkaline fermentation (pH 9) was significantly higher than that at neutral fermentation (7.93 ± 0.22 vs. 2.55 ± 0.17 g COD/L, respectively), as observed by other researchers (Liu et al., 2012b). As further demonstrated in Table 1, a much higher total VFA production (0.58 ± 0.03 vs. 0.17 ± 0.01 g COD/g COD) was found in the HA-WAS reactor with fermentation at pH 9, which was in line with greater values of protein utilization (79.5 ± 3.2 vs. $20.1 \pm 2.2\%$, respectively) and ammonia concentration (291 ± 18 vs. 65 ± 3 mg N/L, respectively) in the HA-WAS fed reactor than those in the R-WAS fed reactor. This is because the ammonia was produced during the conversion of amino acids to VFAs by the deamination reaction. Moreover, heat treatment of the seeding sludge significantly reduced the CH_4 yield during the fermentative processes (see Fig. S3). Nevertheless, the CH_4 yield of the R-WAS reactor at pH 7 was much higher than that of the HA-WAS reactor at pH 9. It is well known that the optimum pH range for the growth of methanogens is 6.8–7.2, and the CH_4 yield with the microbiology results above are consistent with this range. On the contrary, more H_2 was produced in the HA-WAS reactor at pH 9, which agreed with previous outcomes (Cai et al., 2004). In the tests, HA-WAS included a variety of organic matter such as protein, carbohydrate and lipids and presented higher substrate concentrations than HA-BSA; it was therefore expected the HA-WAS-fed reactor would exhibit a higher HAC yield in comparison to the reactor fed with HA-BSA. However, as shown in Fig. 1, the HAC generation from HA-BSA was 0.36 ± 0.01 g COD/g COD (or 0.502 ± 0.021 g/g protein), which was slightly higher than that from HA-WAS. The activity of the two key enzymes relevant to HAC production with HA-BSA was higher than that with HA-WAS (Table S1), suggesting that more organic matter was converted to HAC in the reactor fed with HA-BSA. This finding also implies that the difference in HAC production between HA-WAS and HA-BSA resulted from different microbe-mediated reactions rather than from different availability of substrates. In addition, it is clear that HAC originates from the amino acids conversion during protein fermentation, whereas amino acids exhibit significantly different size and structure and are mediated by different metabolic pathways (Ramsay and Pullammanappallil, 2001). In this work, HA-WAS contained multiple proteins and was different from BSA in protein type and amino acid composition,

which also probably contributed to the difference in HAC generation. Further studies are required to elucidate these findings. Consistent with the increase in HAC yield, the heat-alkaline pretreatment also increased protein utilization efficiency, which was three-fold higher than that of the untreated control.

Overall, the yields of total VFAs and individual HAC produced from the real WAS (i.e., 0.58 g COD/g COD and 0.31 g COD/g COD, respectively) were found to be significantly higher than that reported in previous studies (Morgan-Sagastume et al., 2011; Yuan et al., 2006; Tan et al., 2012; Feng et al., 2009), suggesting the use of the combined technology to augment sludge-derived HAC yield was feasible. Furthermore, although the present work provided detailed evidence to verify the mechanism by which the proteins change (fragmentation and denaturation) under the condition of the heat-alkaline pretreatment, and presented information on its effects on HAC accumulation, comparative studies with an expanded coverage of pretreatment approaches are still essential to identify the most efficient option and to decipher the mechanisms in relation to augmented HAC generation from a multidisciplinary perspective.

3.4. Economic assessment

In Table 2, the economic potential of the combined strategy for HAC production was further assessed by means of a scenario analysis based on the experimental results and literature data obtained. In the focused scenario, the sewage sludge is initially fermented to HAC by utilizing the combined strategy (heat-alkaline pretreatment plus fermentation at pH 9), and the dewatered residue is then disposed of at an off-plant landfill, while the produced HAC is assumed to be used to hybrid power generation for revenue estimation. In addition, a baseline scenario in which the sewage sludge is converted to HAC using the control method (no pretreatment plus fermentation at pH 7), and the other processes were the same as described in the focused scenario. It should be noted that, in the former experimental parts, 6 M NaOH was applied to ensure alkaline conditions and substantial amounts of sodium ions present in the reactors might have effects on the bioprocess of HAC formation. However, it also has been observed in the literature that the separation of the solid with the liquid in the dewatering process after alkaline fermentation might not be much easier than the traditional fermentation processes because of the existing of the NaOH in the fermented sludge (Lee et al., 2014). Furthermore, the use of $\text{Ca}(\text{OH})_2$ in adjusting alkaline conditions was found to exhibit nearly the same effect on VFA production and sludge reduction as that of NaOH, along with additional benefits on improved sludge dewatering, decreased chemical costs, and greater fermentation liquid recovery efficiency (Li et al., 2011). In this respect, $\text{Ca}(\text{OH})_2$ seems to be more feasible than NaOH in the applications of tomorrow and therefore, it was used to assess the cost of alkaline pretreatment and fermentation in the focused scenario. Table 2 indicates that the benefit of the focused scenario arises from the substantial reduction of for-disposal sludge and the increased revenue from HAC-based bio-energy production, despite the unavoidable chemical and energy consumption costs of the combination of sludge pretreatment and

Table 1

Profiles of protein utilization, VFAs, HAC, TCOD, SCOD, soluble phosphorus ($\text{PO}_4^{3-}\text{--P}$) and ammonia in the R-WAS and HA-WAS fed reactors with a fermentation time of 72 h^a.

	Protein utilization (%)	VFA yield (g COD/g COD)	HAC yield (g COD/g COD)	TCOD (g COD/L)	SCOD (g COD/L)	$\text{NH}_4\text{--N}$ (mg N/L)	$\text{PO}_4^{3-}\text{--P}$ (mg P/L)
R-WAS at pH 7	20.1 ± 2.2	0.17 ± 0.01	0.08 ± 0.02	11.35 ± 0.80	2.55 ± 0.17	65 ± 3	53 ± 4
HA-WAS at pH 9	79.5 ± 3.2	0.58 ± 0.03	0.31 ± 0.04	12.20 ± 0.85	7.93 ± 0.22	291 ± 18	289 ± 20

^a The initial concentrations of SCOD and TCOD loaded to the reactors are 0.09 ± 0.01 g COD/L and 13.05 ± 0.91 g COD/L, respectively.

Table 2

Preliminary economic analysis of the combined strategy for sludge-derived HAC production.

General parameters		Values
Annual sludge production (million ton wet solids/y)		30 ^a
Sludge concentration (g TSS/L)		20 ^b
Ratio of TCOD to TSS		1.14
Initial sludge temperature (°C)		25 ^b
Initial sludge pH		7 ^b
Environmental temperature (°C)		25 ^b
Yield coefficient of HAC from HA-WAS (g COD/g COD)		0.31
Yield coefficient of HAC from R-WAS (g COD/g COD)		0.08
Yield coefficient of hydrogen from wastes (m ³ /kg COD)		1.34 ^c
Cost of dewatering, transportation, and landfill (\$/ton dry solids)		250 ^d
Price of Ca(OH) ₂ (\$/ton)		100 ^e
Specific heat value of sludge (kJ/kg °C)		4.18 ^d
Lower heat value of hydrogen (kWh/m ³)		3 ^f
CHP efficiency for electricity		0.35 ^f
CHP efficiency for heat		0.50 ^f
Electricity price (\$/kWh)		0.106 ^g
Heat price (\$/Mcal)		0.07 ^g
Baseline scenario	Sludge reduction (%)	15
	Annualized cost of fermentation (\$ million/y)	21.1 ^j
	Annualized sludge disposal cost (\$ million/y)	127.5
	Revenue of HAC-converted energy (\$ million/y)	15.9 ^k
	Annual net cost (\$ billion/y)	132.7
Focused scenario	Sludge reduction (%)	48
	Annualized cost of alkaline pretreatment (\$ million/y)	1.8 ^h
	Annualized cost of heat pretreatment (\$ million/y)	49.8 ⁱ
	Annualized cost of fermentation (\$ million/y)	21.1 ^j
	Annualized cost of sludge disposal (\$ million/y)	78.0
	Revenue of HAC-converted energy (\$ million/y)	61.5 ^k
	Annual net cost (\$ million/y)	89.2
	Annual savings (\$ million/y)	43.5
Comparison		

^a Estimated data for China (Khan et al., 2013).^b Based on our previous field survey.^c The maximum value reported (Lu et al., 2009).^d Literature values (Dhar et al., 2012).^e Data from <http://www.alibaba.com>.^f Combined heat and power (CHP) system was used (Monlau et al., 2015).^g Historical prices (Cho et al., 2014).^h Based on the Ca(OH)₂ usage for alkaline treatment of sludge at pH 12.ⁱ Estimated from the difference in the energy needed to heat the sludge to the pretreatment temperature and the recovery of heat from the thermal pretreated sludge (Dhar et al., 2012).^j Estimated from the energy costs to operate the fermentation systems (Metcalf and Eddy 2003) and the heat capture from the fermented residue (Dhar et al., 2012).^k Hydrogen generation using HAC as substrate; the biohydrogen was converted via CHP, and the revenue was based on the price of hybrid power.

fermentation. Overall, this strategy would result in approximately \$43.5 million savings per annum with added benefits such as waste reduction and greater biofuel production in comparison to the baseline scenario. Nevertheless, it should be also noted that the cost of the heat pretreatment was much significantly higher than that of the alkaline stimulation in the focused scenario (49.8 \$ million/y vs. 1.8 \$ million/y), which may diminish the beneficial role of the pretreatment component in the whole strategy since the yield of HAC from HA-WAS might not be extremely greater than that from A-WAS, even if further verification is still needed. Therefore, feasibility studies with a broader range of pretreatment methods are necessary to complete the picture and to identify key aspects that require further cost-benefit improvement. Further, this preliminary economic analysis was conducted mainly based on the laboratory-scale parameters in the present study, and some uncertainties might exist in the expected results since the full-scale system would behave differently in terms of HAC production, sludge reduction, and downstream applications. Considering that the sludge-derived HAC production and its downstream applications is an emerging research field with several unexplored facets, it is understandable that the current analysis conducted in the work only addressed some of the components needed for a comprehensive evaluation. Therefore, larger-scale trials are required to evaluate the economic feasibility of this combined technology.

4. Conclusions

We investigated a combined strategy to enhance HAC yield from WAS fermentation through structural manipulation of the sludge protein component. Specifically, the heat-alkaline pretreatment caused protein fragmentation and simultaneously, increased the efficiency of protein denaturation by destroying disulfide bonds and inducing protein unfolding, which subsequently improved protein susceptibility to proteolytic cleavage and hydrolysis, resulting in augmentation of HAC yields. In addition, the validation of the combined strategy for sludge-derived HAC production and the economic evaluation both confirm the potential feasibility of this a priori technology for waste-to-energy applications.

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Appendix A. Supplementary information

Supplementary information related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2015.10.055>.

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