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Sludge age impacted the distribution, occurrence state and structure of organic compounds in activated sludge and affected the anaerobic degradability

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HIGHLIGHTS

- VS reduction showed exponential decrease as sludge age extended from 5 to 40 d.
- Decline in protein degradation accounted for 80.4% of the decrease of VS conversion.
- Changed amount and components of EPS correlated little to degradability of sludge.
- Increased molecular weight of EPS strongly correlated to the declined degradability.
- More stable secondary structure of protein in EPS determined decreased degradation.

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ABSTRACT

Long sludge age (10 ~ 30 d) is prevalent in the operation of wastewater treatment plants of China, leading to big challenge to subsequent anaerobic digestion of sewage sludge because of low degradability and poor methane production. In order to overcome the low sludge degradability and promote the directional enhancement, the effects of sludge age on anaerobic digestion performance, especially the corresponding influencing mechanism in terms of distribution, occurrence state and structure of organic compounds in sludge driven by different sludge age were investigated. It was found that when the sludge age during wastewater treatment extended from 5 d to 40 d, the VS reduction of sludge showed an "exponential" decrease, and the degradability of protein, polysaccharides and lipids in sludge all declined. Among them, the degradation of protein was the most affected (down-regulated by 35.8%), and the decrease in degradation of protein accounted for 80.4% of the decrease of VS conversion. Sludge age during wastewater treatment impact the distribution, occurrence state and structure of organic compounds in activated sludge. And it was clarified that instead of the changed amount and components of EPS, the increased molecular weight of EPS and more stable secondary structure of protein driven by α-helix and β-turn in EPS were mainly responsible for the declined degradability of protein and sludge.

1. Introduction

The large amounts of sludge generated by wastewater treatment plants in the 21st century makes the treatment and disposal of sewage sludge one of the most critical environmental issues. Anaerobic digestion (AD) process, which can recover energy (methane) from sludge, reduce substrate volume, destroy pathogens, and decrease odor problems of residual putrescible matters [1], has been widely applied in disposal of sewage sludge. However, in order to guarantee sufficient sludge age to remove nitrogen biologically in wastewater, the solids retention time (SRT), or sludge age, in conventional biological nutrient removal (BNR) processes is generally in the range of 10 ~ 20 days [2]. This leads to big challenges to subsequent AD of sewage sludge because of low degradability and poor methane production reported in lots of literatures [3245]. Among which, it has been reported that VS reduction during AD of sludge obtained from slaughterhouse wastewater treatment system decreased from 85% to 63% when the sludge age increased from 2 d to 4 d [2], and the specific gas production decreased from 0.18 to 0.07 m^3/kg VS_{fed} was found when the solid retention time in the wastewater treatment line increased from 8 to 35 days [3].

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Even though very short SRT(0.5 ~ 3 d days) during activated sludge stage has been reported to solve the inhibition caused by long sludge age and can improve sludge degradability[674], long sludge age (10 ~ 30 d) is still prevalent in the operation of wastewater treatment plants of China (Table S1) because of the original process design or low COD in influent driven by poor sewage collection system [8]. And this leads to the challenges of applying technical route of "thickening-anaerobic digestion-dewatering-land application" on sludge in China [8]. In order to overcome the low degradability of sludge, promote the AD performance and finally realize the environment friendly and economical disposal of sludge in China, it is the primary to clarify the inhibition mechanism of long sludge age on the subsequent AD performance.

Till now, some studies have investigated the influence of sludge age on the characteristics of sludge, and most of them focused on effects of floc structure, extracellular polymers and metabolites on hydrophobicity and flocculation properties of sludge. For the floc structure, Liao et al. [9] showed that under the SBR operating conditions, the morphology of the long sludge age sludge flocs was more regular than that of the short sludge age, and the floc size distribution was more stable. It was also reported that the shorter the sludge age, the rougher the surface of the flocs and the looser the structure [10]. For the flocculation performance, the total amount of extracellular polymeric substances (EPS), content of protein and polysaccharide in loose EPS and the surface potential of activated sludge decreased when sludge age was extended, resulting in increased hydrophobicity and then the flocculation ability [1112].

Referring to the influence of sludge age on AD performance, most of the studies limited to biogas production and degradation rate [313]. It is known that the degradability of sludge was decided by the characteristics of macromolecular organic components (MOCs) including protein, polysaccharides, lipids, cellulose, hemicellulose and lignin in it [14]. However, there was little research linking the distribution, occurrence and structure of MOCs driven by change of sludge age to the anaerobic conversion properties. The lack of this information limit the understanding of refractory organic matters in sludge driven by long sludge age, resulting in difficulties to promote the directional reinforcement of their degradability.

Therefore, the aim of the present study was to illustrate the influencing mechanism in terms of distribution, occurrence and structure of MOCs in sludge driven by sludge age, in order to promote the directional enhancement of degradability of long sludge age in the future, especially in China. Till now, except for sludge age, many factors in wastewater treatment process have been reported to influence the characteristics of MOCs, in which the organic-binding metals would restrict the molecular mobility and deteriorate the depolymerisation of biopolymers in sludge by bridging and hydrogen-bonding interactions [15], and the fine sand particles would bond with organic matters in sludge on the surface sites to form a larger bioinorganic-floc [16]. In order to avoid the influence of these factors, five completely mixed reactors with A/O process using the same simulating domestic sewage (with negligible fine sand particles and metal ion content) as influent were applied to cultivate activated sludge of differed sludge age. And biochemical methane potential (BMP) tests using flasks was then implemented to determine the effect of sludge age on the anaerobic degradation of them. The scanning electron microscope (SEM), EPS analysis, cell number analysis, circular dichroism (CD) spectra study and metagenomic approaches were combined to define the influencing mechanism of sludge age on anaerobic degradability of sludge.

2. Materials and methods

2.1. The cultivation of activated sludge with different sludge age

In this study, activated sludge were obtained by A/O process using simulating domestic sewage as influent by SBR systems. Five completely mixed reactors (Fig. S1) were used in the culture process. The effective operating volume of each reactor was 10 L. The operating scheme used was input, anoxic process (3.5 h,), aeration process (7.5 h), standing and output (1 h). The dissolved oxygen concentration in all the reactors were controlled by the daily adjustment of aeration and stirring, and it was controlled between 0.15 ~ 0.2 mg/L and 3.0 ~ 3.5 mg/L during anoxic and aeration processes, respectively. Each reactor ran 2 cycles per day, each cycle was 12 h (hydraulic residence time was 11 h), the amount of water in and out of each cycle was 8 L, and the system was continuously cultured for 100 days. The discharged sludge of R1, R2, R3, R4 and R5 was 2 L, 1 L, 0.5 L, 0.33 L and 0.25 L, respectively, so that the solid retention time (SRT), namely, sludge age, was 5 d, 10 d, 20 d, 30 d and 40 d, respectively.

The formulation and concentration of simulated domestic sewage for five reactors are shown in Table S2. The concentration of influent COD, NH_4^+-N and total phosphorus were 550 mg/L, 59 mg/L, and 16.7 mg/L, respectively. The sludge concentration (mixed liquid suspended solids, MLSS) was 1000 ± 80, 2000 ± 100, 2200 ± 90, 2500 ± 100 and 2600 ± 100 mg/L for R1, R2, R3, R4 and R5, respectively, during the operation, and the effluent properties were shown in Fig. S2. Five reactors were operated stably for 100 days, the concentration of COD, NH_4^+-N and total phosphorus in the effluent were below 20 mg/L, 10 ~ 25 mg/L and 5 ~ 10 mg/L, respectively, and the corresponding removal rate was above 96%, 57 ~ 83.1% and 40 ~ 70%, respectively.

2.2. The design of AD experiment

At the end of the operation of five reactors (day 100) in Section 2.1, the sludge with five different sludge ages was all obtained for the subsequent experiments. Biochemical methane potential (BMP) was conducted by AMPTS II-methane potential analysis tool (Bioprocess Control, Sweden). The inoculated sludge was taken from an AD reactor of sludge that no longer produced gas, with total solids content (TS, w/w %) of 0.90 ± 0.02% and volatile solids content (VS/TS, w/w %) of 56.99 ± 0.21%. The sludge with five different sludge ages were subjected to anaerobic bottles for AD batch test after the static settlement for 6 h, in order to remove water and increase the solid content. Each group was set up with 3 parallel samples for a total of 15 samples, with another two samples fed with only inoculated sludge as blank control samples. The test was carried out under conditions of inoculation ratio ($VS_{substrate}$: $VS_{inoculated\ sludge}$) of 2/1, the added mass of the inoculated sludge in each bottle was 200 g, and the total amount of $VS_{inoculated\ sludge}$ was 1.06 g. After the substrate and the inoculum were added, the mixture in all bottles was uniformly made up to 380 g with distilled water. At last, each test bottle was filled with nitrogen for 2 min and the

Table 1
The design and grouping of anaerobic digestion experiment.

Group	Substrate	Sludge age (d)	TS (%)	VS/TS (%)	Added VS (g)	Inoculation ratio
1	Sludge 1	5	3.02 ± 0.04	90.62 ± 0.11	2.00	1.9
2	Sludge 2	10	4.91 ± 0.03	88.91 ± 0.13	2.03	1.9
3	Sludge 3	20	3.64 ± 0.03	89.18 ± 0.15	2.05	1.9
4	Sludge 4	30	4.07 ± 0.07	89.46 ± 0.17	2.07	2.0
5	Sludge 5	40	3.50 ± 0.02	86.74 ± 0.19	2.02	1.9

bath temperature was set to 37 °C. The experiment design and grouping are shown in Table 1.

2.3. Analytical methods

2.3.1. SEM

The activated sludge of different sludge ages was observed on a scanning electron microscope (SEM, Hitachi S-4800, Japan). The gradient dehydration treatment was conducted before the observation on the machine, and the treatment process is shown in Table S3 in Supporting information.

2.3.2. Determination of total number of cells in sludge

The total number of cells in the sludge can be characterized by the sum of the number of dead cells and the number of viable cells. Bacteria are the most important microorganisms in the activated sludge, and the bacteria are single-celled microorganisms. Therefore, in this study, the total number of cells was calculated by measuring the number of dead and living bacteria in the activated sludge from five reactors by Flow Cytometer (BD FACSVerse). The method for determination of dead and live bacteria is described in the study by Foladori et al. [17]. The specific steps are shown in the Supporting information. And the extracellular materials content in unit VS could be estimated roughly according to formula (1).

$$\text{Extracellular materials content}\,[\%] = [1 - (\text{cell numbers} \times \text{cell weight})/1\,\text{g}] \times 100\% \quad (1)$$

In which the “cell numbers” means the cell numbers in 1 g VS, and the “cell weight” means the dry weight of a bacterial cell.

2.3.3. EPS extraction

In this paper, EPS is extracted for mass analysis and circular dichroism analysis to detect the secondary structure of protein substances, it is necessary to avoid destroying the secondary structure of proteins in EPS. The cation exchange resin (CER) method was chosen in this paper because resin causes low cell lysis and secondary structure change, and has become the widely accepted EPS extraction method [1819]. EPS extraction of activated sludge samples from five reactors using CER (Sinopharm, Beijing, China) was conducted in this study as previously reported [19]. The detailed procedures are listed in the Supporting Information.

2.3.4. CD spectra study

The extracted EPS was dissolved in deionized water and the protein concentration in the solution was brought to 50 μg/mL by dilution. According to Wu et al. [19], the CD spectra of the EPS solutions were recorded over the far-UV range of 190 ~ 240 nm with a CD spectropolarimeter (Jasco J-715, Jasco Corp., Tokyo, Japan) in a 0.1 cm quartz CD cuvette at 25 °C. The scan rate, response, bandwidth, and step resolution were 100 nm/min, 0.25 s, 1.0 nm, and 0.2 nm, respectively. Five scans were averaged to obtain one spectrum. The CD data are expressed in terms of the Mili-Degree (mdeg). The fractions of α-helix and β-turn in the secondary structure were obtained according to the methods of Perczel et al. [20] by CDNN2.1.

2.3.5. Organic matters detection

The carbohydrates content in EPS was measured by the anthrone method with glucose as the standard. The protein and humic content in EPS were measured by the modified Lowry method using bovine serum albumin and humic acid as the respective standards [21]. The DNA content was measured by the Picogreen method (Molecular Probes, Eugene, OR) using calf thymus DNA as the standard. The molecular weight distribution of EPS is determined by high performance size exclusion chromatography (Agilent 1100 series, USA).

The methane yield was detected by the AMPTS II-methane potential analysis tool. The digestate in AD experiment was sampled when biogas production could not be detected. And the detection of TS, VS, total protein, total polysaccharides and total lipids in digestate were the same as which reported in Chen et al. [14].

2.3.6. Metagenomic sequencing analyses

The DNA extracted from activated sludge with sludge age of 5 days and 40 days was analyzed for methanogenic pathways via metagenomic approaches referred to Chen et al. [14] by Shanghai Biozeron Biotechnology Co., Ltd. (Shanghai, China). The raw datasets were deposited in the NCBI Short Read Archive (accession numbers: SRR9276153 and SRR9276154). The assembly of metagenomic datasets and open reading frame (ORF) annotation also referred to Chen et al. [14]. The cluster of orthologous groups of proteins (COG) for the ORFs was determined using the STRING database (http://stringdb.org/, version 8.3) with a BLASTP E-value cutoff of 1e-5 and in the eggNOG database (http://eggnog.embl.de/).

3. Results and discussions

3.1. The shifted AD performance of activated sludge with different sludge ages

In the present study, the cumulative amount of methane accumulation and final VS reduction during the BMP test of sludge 1 ~ 5 is shown in Fig. 1 (Table S4). It can be seen that the methane production for activated sludge lasted as long as 60 days (Fig. 1a). The results showed that as the sludge age changed from 5 d to 40 d, the methanogenic potential and final VS reduction of sludge gradually decreased from 406.5 ± 3.1 ml CH_4/g VS_{added} and 73.5 ± 0.5% to 265.3 ± 2.4 ml CH_4/g VS_{added} and 54.7 ± 0.4%, respectively. That was, the sludge age extension would inhibit the methanogenic potential and the final VS reduction of sludge.

It was found in Fig. 1b and 1c that the relationship between the value of sludge age and the methanogenic potential of sludge as well as final VS reduction could both be described as empirical relationship by the exponential function model (ExpDec1), with the determinate coefficients of 0.9594 and 0.8815, respectively. According to the fitting results, when the sludge age was 5 d, the AD performance of sludge was the best. When the sludge age was 10 d and 20 d, the AD performance was significantly inhibited, and the downward trend tended to be flat when sludge age changed between 20 and 40 days. The “exponential” decline was in accord with the empirical relationship reported by Bolzonella [3]. However, there was great deviation in specific parameters of the empirical relationship between the results in this study with Bolzonella’s study. This might be because that the data for fitting in Bolzonella’s study was obtained from either lab experiments, pilot tests or the large sludge treatment systems reported in literatures, in which the large differences in sludge characteristics and operational conditions would influence the fitting results. Under the same influent conditions, this study obtained ideal research matrix by controlling the sludge age, and it would be more systematic and reasonable in reflecting the influence of sludge age on sludge degradation performance.

3.2. The shifted degradation of MOCs in activated sludge with different sludge ages

The distribution of organic matter components of sludge 1 ~ 5 with different sludge ages, as well as the degradation amount and degradation percentage of protein, polysaccharides and lipids after BMP test are shown in Fig. 2. According to the results of Fig. 2a, the organic matter in the sludge cultivated by the simulated domestic sewage through the A/O process was mainly composed of proteins, polysaccharides and lipids substances, accounting for 88.4% to 94.7% of the total organic matters, of which protein substances were the main components (accounting for 53.5%~58.7%). In addition, it can be seen that sludge

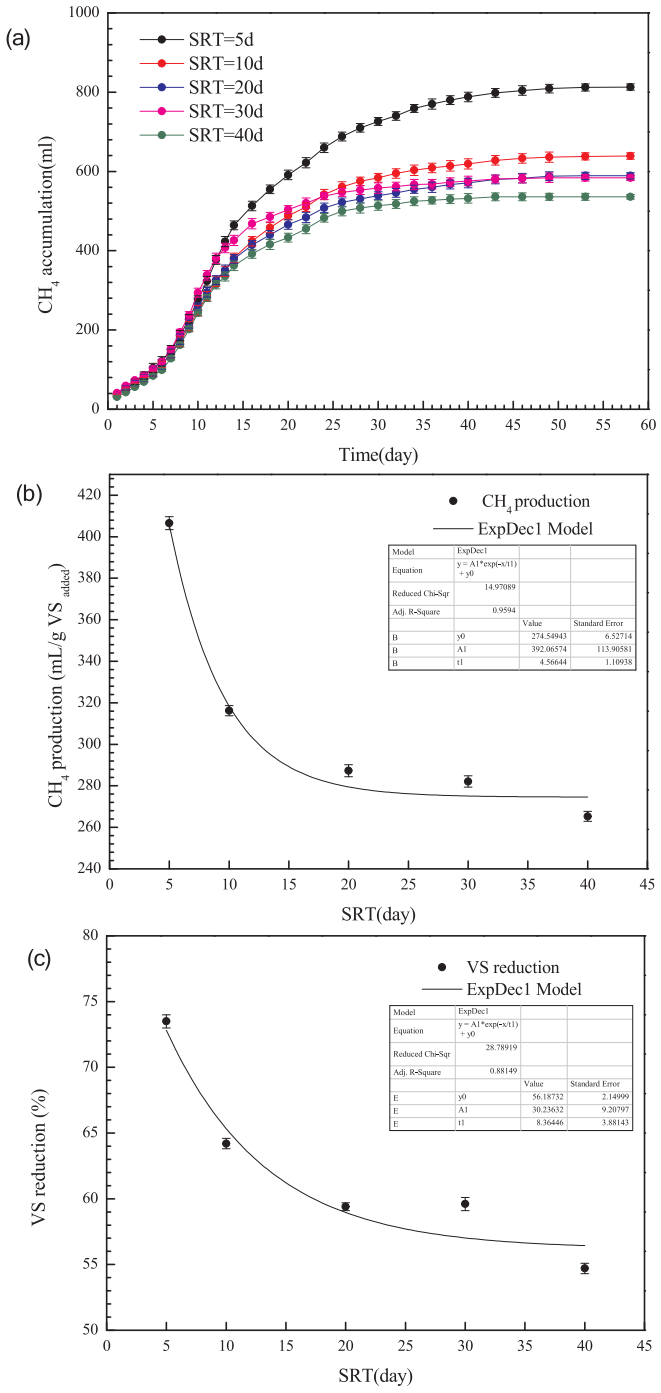

Fig. 1. The cumulative amount of methane accumulation in the BMP test of sludge 1 ~ 5 with different sludge age (a); The relationship between the value of sludge age and methanogenic potential of sludge (b) as well as the final VS reduction (c).

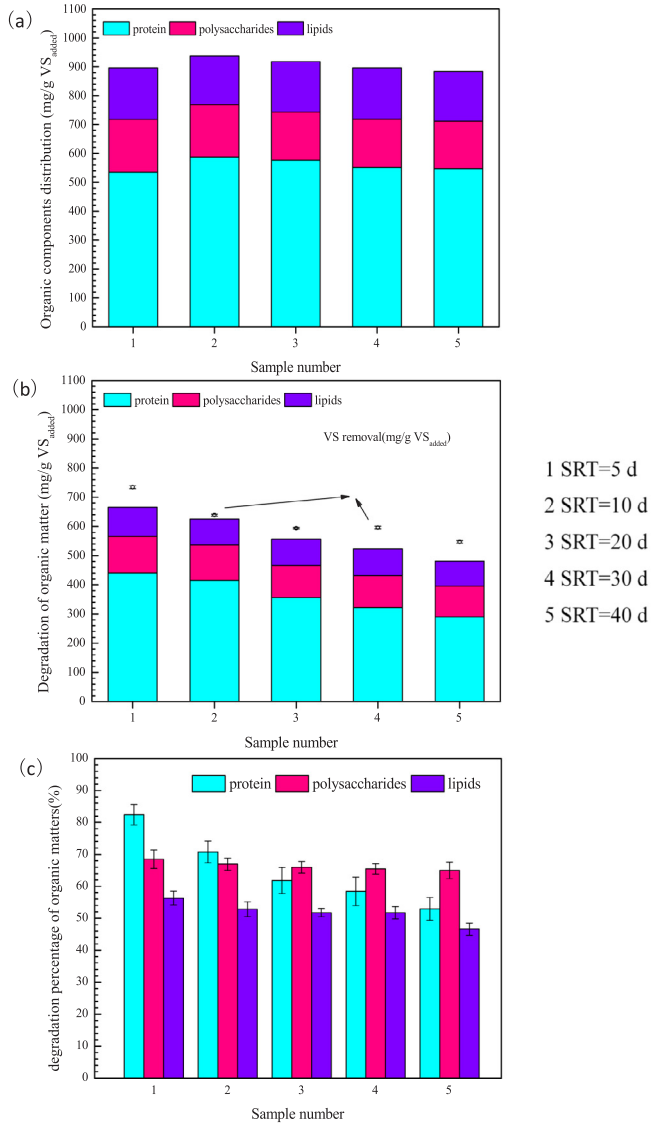

Fig. 2. (a) Distribution of organic matter components before anaerobic digestion of sludge 1 ~ 5; (b) Degradation amount of organic matters after anaerobic digestion; (c) Degradation percentage of organic matters.

cultivated in this study was almost free of lignocellulosic substances, which was because that lignocellulosic substances were exogenous organic matter in sewage instead of microbial components and microbial secretions [2223]. With the increase of sludge age, the content of polysaccharides in sludge showed a downward trend, and the changes of other organic components were not obvious.

Except for the composition of MOCs, the extension of sludge age also affected the degradation of MOCs during BMP text (Fig. 2b and 2c). Firstly, it was found that the degradation of proteins was the main contributor to VS reduction, and its contribution was between 53% and 65% in the five groups of samples, which indicated the dominating roles of protein in the anaerobic transformation of sludge [2414]. When the sludge age was 5 d, the degradation percentage of various organic matters in the sludge was relatively high. However, when the sludge age was extended from 5 d to 20 d, the degradation percentage of protein, polysaccharides and lipids in the sludge decreased rapidly from 82.4%, 68.5% and 61.8% to 61.8%, 65.9% and 51.8%, respectively. When the sludge age continued to extend to 40 d, the degradation percentage of protein, polysaccharides and lipids continued to drop to 52.9%, 65.0% and 46.6%, and it can be seen that the declining trend of the degradation percentage of these three types of organic matters tended to be flat. This result further explained the changes in methane production and final VS reduction.

In addition, when the sludge age was extended from 5 d to 40 d, the degradation percentage of protein decreased by 35.8%, which was much higher than the descent rates of degradation percentage of polysaccharides (5.0%) and lipids (17.2%). Moreover, the decrease in the degradation amount of protein accounted for 80.4% of the decrease in the VS removal amount. Therefore, it can be seen that the degradation of protein in sludge was the most affected when the sludge age was extended. And the decrease of protein degradation was the

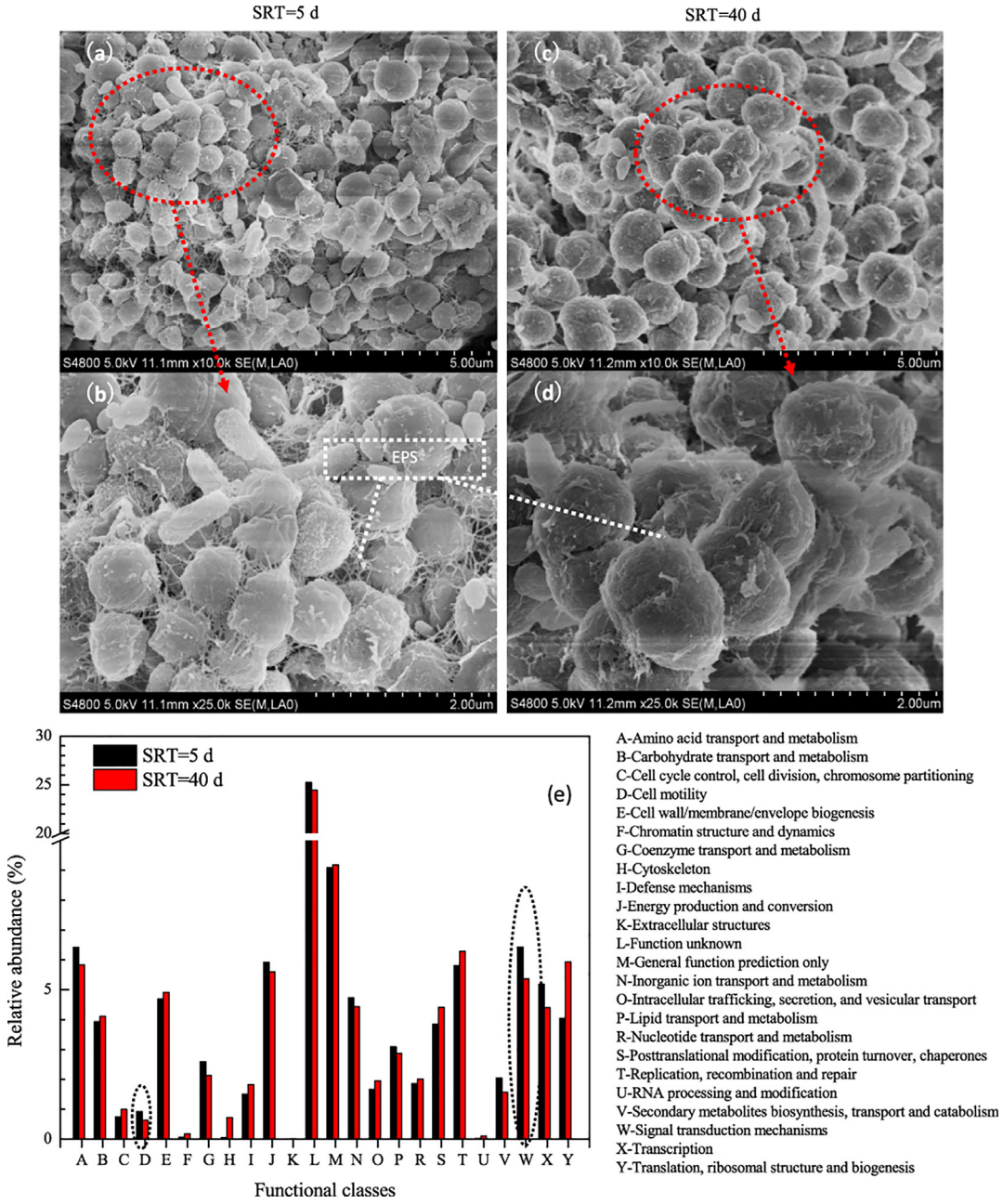

Fig. 3. (a-d) The scanning electron micrographs of activated sludge with sludge age of 5 d and 40 d; (e) The relative abundance of major metabolically related functional genes (COG functional annotation) of activated sludge with differed sludge age.

most important reason for the decline in AD performance of sludge.

3.3. Influencing mechanism of sludge age on anaerobic conversion of sludge

3.3.1. Effect of sludge age on sludge growth morphology

In this study, SEM were performed on activated sludge samples of different sludge ages. As shown in Fig. 3a ~ 3d, the microorganisms in the sludge were present in the form of bacterial micelles. There was a certain difference in the size between the cells ranging from 0.5 to 1 μm in diameter, and each cell surface was wrapped in large amount of fibrous materials, that was EPS [2526]. At the same time, it can be seen that when the sludge age was 5 d, the EPS between the cells was loose, and the cells or colonies were loosely connected (Fig. 3a and 3b). When the sludge age was 40 d, the EPS between the sludge cells was relatively denser, and the cells or colonies were more closely connected to became an organic whole (Fig. 3c and 3d). The compaction of the EPS when the sludge age was extended, which was in accord with previous study [9], may affect the access for enzymes and microbes to the organic matters in and outside the cells [1027], thus inhibiting the subsequent AD performance.

In order to further analyze the effect of sludge age on sludge growth, metagenomic sequencing analyses [28] were conducted on activated sludge samples with sludge ages of 5 d and 40 d. According to the results of metagenomic sequencing, the relative abundance of the major metabolically related functional genes (COG functional annotation) of the two groups of sludge was shown in Fig. 3e. It can be clearly seen that the relative abundance of genes associated with the D-cell motility and W-Signal transduction mechanism in sludge microbes was significantly reduced when sludge age extended from 5 d to 40 d (from 0.93% and 6.43% to 0.64% and 5.37%, respectively), suggesting the weaker cell motility and signal transmission between cells. The results further support the denser EPS structure of activated sludge with longer

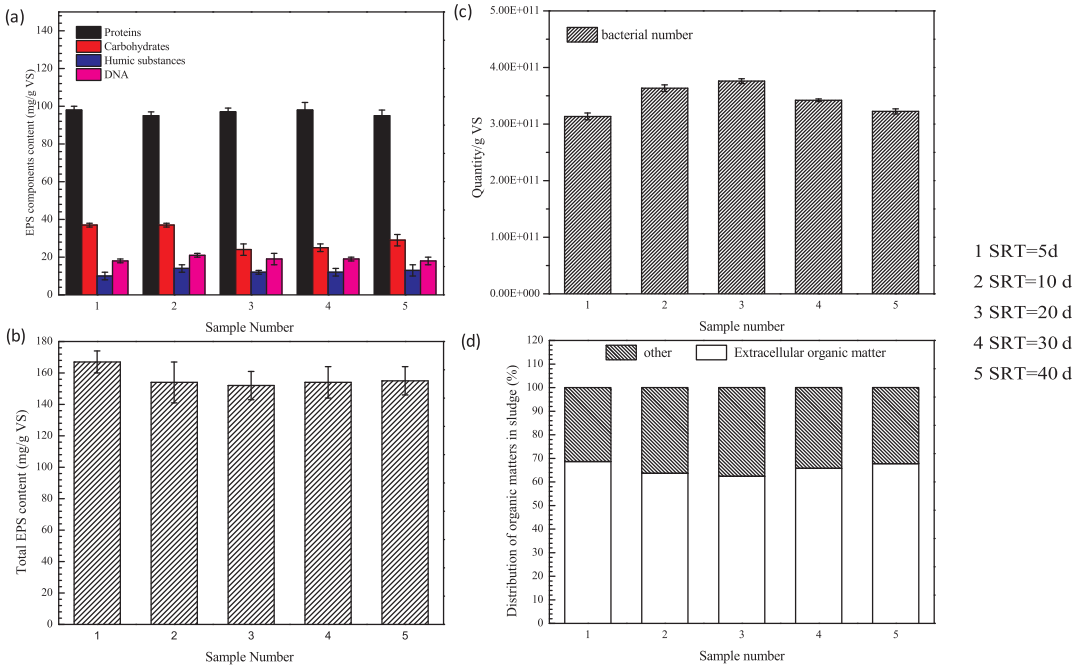

Fig. 4. (a) The components distribution of EPS extracted from activated sludge; (b) The total amounts of EPS extracted from activated sludge; (c) The number of cells in the activated sludge based on VS; (d) The concentration of extracellular organic matter in VS of activated sludge.

sludge age.

3.3.2. *Effect of sludge age on the extraction amount and components of EPS*

According to the results shown in Fig. 4a and 4b, the total EPS decreased as the sludge age extended and it was mainly caused by the decreased extracellular carbohydrates as little change was found in the content of extracellular protein and humic substances extracted from five activated sludge samples. The EPS amount and extracellular carbohydrates extracted from the sludge was the highest at sludge age of 5 days (167 ± 7 and 37 ± 1 mg/g VS, respectively). When the sludge age was 5 d, the sludge concentration in R1 was low (MLSS = 1000 ± 80 mg/L) and could not consume all the carbon sources available for growth, which could be supported by the highest COD (Fig. S2) in the effluent in reactor R1. Under this condition, it has been reported that excess substrate could be stored by microorganisms as intracellular substances and extracellular polymers, resulting in the high EPS content of activated sludge [2912]. When the sludge age were 10 d and 20 d, the sludge concentration in R2 and R3 increased to 2000 ± 100 and 2200 ± 90 mg/L, respectively. As the COD in effluent of R2 ~ R3 were similar to R1 (Fig. S2), the nutrients available to the microorganisms and the carbon source storage of the microorganisms would be correspondingly reduced compared to R1 [30], leading to the decreased content of EPS as well as extracellular carbohydrates (152 ± 9 and 24 ± 3 mg/g VS). The growth rate of microbial cells was reported to decrease significantly when the sludge age was too long (30 ~ 40 d) because of lack in nutrients, and the microbial metabolic capacity and intake of extracellular organic matter would also decreased [12], therefore the content of EPS and extracellular carbohydrates slightly picked up when the sludge age was 30 ~ 40 d compared to 20 d, which was in accord with the previous study [30].

Since the extracting EPS was limited by the extraction rate of methods [31], the extraction amount of EPS had certain limitations in reflecting the real amount of extracellular EPS. The organic matters in sludge has been reported to be composed of microbial cells and EPS [32], in order to further verify the distribution of extracellular organic matters, the number of cells in the sludge was analyzed by a flow cytometry and the results were converted into the number of cells in the unit VS, as shown in Fig. 4c. It can be seen that when the sludge age was prolonged, the number of cells in the unit VS of the sludge first increased and then decreased. The dry weight of each bacterial cell is about 10^{-12} g [48], the proportion of extracellular organic matters in VS was estimated by formula (1) and the result is shown in Fig. 4d. It was estimated that that the ratio of extracellular organic matters in VS was about 68.7% when the sludge age was 5 d. The proportion decreased to about 62.4% when the sludge age was extended to 20 d, and then increased slightly at 30 d and 40 d. These results further verified the change in the amount of EPS content in sludge after the extension of the sludge age from 5 d to 40 d (firstly decreased and then slightly picked up).

Referring to the subsequent AD performance, on the one hand, the reduction of extracellular organic matters, especially extracellular carbohydrates in the unit VS, might limit the rate of degradation of polysaccharides. This was because that the intracellular matters or cell structures were more difficult than extracellular matters to be accessed by enzymes and microbes as the sufficient intrinsic strength of cell envelope [32]. On the other hand, as the sludge age was extended, there might also be change in the molecular structure of the organic matters and then affect anaerobic conversion performance of the sludge.

3.3.3. *Effect of sludge age on the molecular weight of EPS*

The organic molecular weight (MW) distribution of EPS extracted from the activated sludge with differed sludge age is shown in Fig. 5. The results showed that the organic molecules of 10000 ~ 100000 Da and 100000 ~ 1000000 Da occupied the main components in the EPS extracted from the five kinds of activated sludge. The sums of the proportions of these two components were 79.4%, 84.0%, 84.2%, 87.4% and 85.2% in the sludge samples with sludge age of 5 d, 10 d, 20 d, 30 d and 40 d, respectively. And with the sludge age increasing, the proportion of organic molecules of < 3500 Da and 10000 ~ 100000 Da gradually decreased, and the proportion of organic molecules of 100000 ~ 1000000 Da gradually increased. This indicated that as the sludge age was extended, the molecular weight of organic matters in EPS was larger. It has been well known that in the biodegradation process of organic molecules, the smaller the molecular weight, the more easily biodegraded, and conversely, the larger the molecular

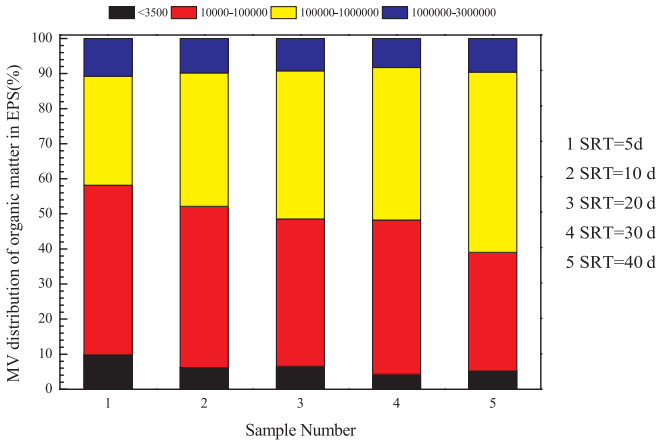

Fig. 5. Organic molecular weight (MW) distribution in EPS extracted from activated sludge with differed sludge age.

weight, the more difficult it was to be biodegraded [3334]. Therefore, the degradation performance of activated sludge might be inhibited with the extension of sludge age.

3.3.4. Effect of sludge age on the secondary structure of protein in EPS

The secondary structure of the protein is maintained by the hydrogen bond formed between the carbonyl group and the amide group on the backbone, and the hydrogen bond is the main force for stabilizing the secondary structure [19]. The secondary structure would directly affect the stability and flocculation characteristics of the protein, therefore influencing its anaerobic degradability. And the far-UV CD spectrum of protein is a direct reflection of its secondary structure.

Fig. 6 shows the CD spectra of proteins in EPS extracted from activated sludge with differed sludge age. It can be seen that the EPS extracted from each sample of activated sludge had a positive peak at 190–195 nm, and a negative peak near 205 nm, which as close to the structure of the α-helix. A negative peak was shown around 198 nm, a small and broad positive peak was around 220, indicating the random coiled structure of protein in the EPS. Furthermore, the percentages of α-helix and β-sheet in the secondary structure are listed in Table 2. It can be seen that the proteins in the EPS were mainly β-sheet (including anti-parallel structure and parallel structure) and random coil conformation, which was consistent with the results of Wu et al. [19].

Fig. 6 and Table 2 also showed that after the sludge age was

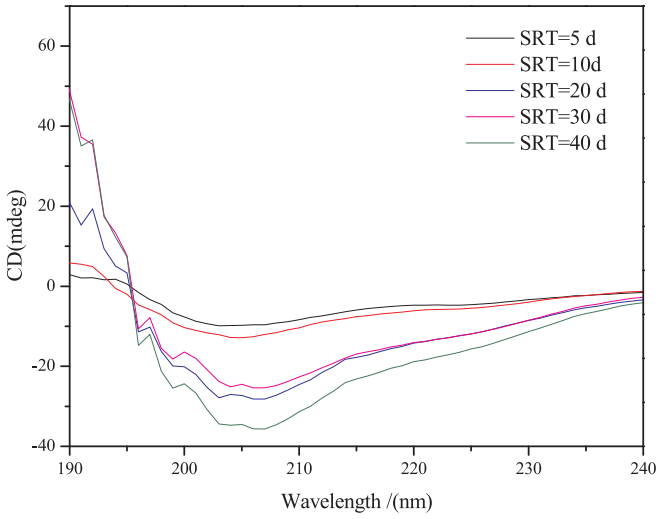

Fig. 6. CD spectra of proteins in EPS extracted from activated sludge with differed sludge age.

Table 2
The percentages of α-helix and β-sheet in the secondary structure in EPS extracted from sludge 1 ~ 5.

Substrate	Sludge age (d)	α-Helix (%)	β-sheet Antiparallel (%)	β-sheet Parallel (%)	β-Turn (%)	Unordered (%)
Sludge 1	5	13.8	30.9	9.2	15.8	30.4
Sludge 2	10	14.6	32.6	8.7	16.2	27.8
Sludge 3	20	16.5	29.9	8.7	16.7	28.2
Sludge 4	30	18.4	27.2	8.6	17.0	28.8
Sludge 5	40	18.9	28.0	8.4	17.2	27.6

extended, the secondary structure of the protein in the EPS changed significantly. When the sludge age was extended from 5 d to 20 d, the ratio of α-helix and β-turn structure of protein in EPS increased significantly from 13.8% and 15.8% to 16.5% and 16.7%, respectively. At the same time, the proportion of the β-sheet and random coil structures was significantly reduced from 40.1% and 30.4% to 38.6% and 28.2%. When the sludge age was extended from 20 d to 40 d, the further secondary structure change of protein in EPS was not obvious. The α-helix and β-turn were reported to induce protein stability, promote the aggregation and flocculation of activated sludge [35]. However, the large presence of antiparallel β-sheet and random coil structures would weaken the stability and aggregation of activated sludge [363537]. In addition, α-helix of protein were found more resistant to oxidation the than the β-sheets [19]. Therefore, it can be suggested that when the sludge age was extended from 5 d to 40 d, the structure of the extracellular protein in the sludge changed rapidly toward a more stable structure driven by stronger internal hydrogen bonding force of α-helix and β-turn, which would increase the difficulty of enzymes or microbes to hydrolyze. As the content of extracellular protein differed little between five activated sludge samples, the decreased degradability of protein with the extension of sludge age during the subsequent AD was more likely to be determined by the change of MW and secondary structure.

3.3.5. Systematic analysis of influencing mechanism

The correlation between the main parameters relating to the occurrence state of organic compounds in sludge and the anaerobic degradation properties is shown in Fig. 7. The long sludge age was found to affect the AD performance in this study, and this phenomenon has also been reported in the previous study, which was explained by that the long sludge age would cause high degree in degradation of organic matters and cell hydrolysis residues encapsulated in EPS [3]. However, the amount of EPS, extracellular protein as well as extracellular carbohydrates driven by differed sludge age were all not correlated with the degradability of sludge ($P > 0.05$). It suggested that although the change in amount and components of EPS might have impacts, it was not the main reason for the declined degradability of sludge. The proportion of organic molecules of 100000 ~ 1000000 Da in EPS (representing the macromolecular organic matter in this study) was found to strongly negatively correlated to VS reduction, degradation percentage of protein, polysaccharides and lipids in sludge ($P < 0.01$). Even though lipids were undetected in EPS, the larger molecular weight of EPS still affected their degradation. And this could be explained that the lipids was the main components of cell membrane, and the inhibited degradation of EPS would resist the access of microbes and enzymes to lipids contained in cell membrane[3839]. Moreover, the ratio of α-helix and β-turn structure of protein in EPS were also found to negatively correlated to the VS reduction, degradation percentage of protein ($P < 0.05$), verifying that the stronger internal hydrogen bonding force of α-helix and β-turn would inhibit the degradation of protein. In addition, as an increase in the extent of protein secondary structure was companied by the occurrence of interaction between the protein and the polysaccharides [40], the degradation percentage of

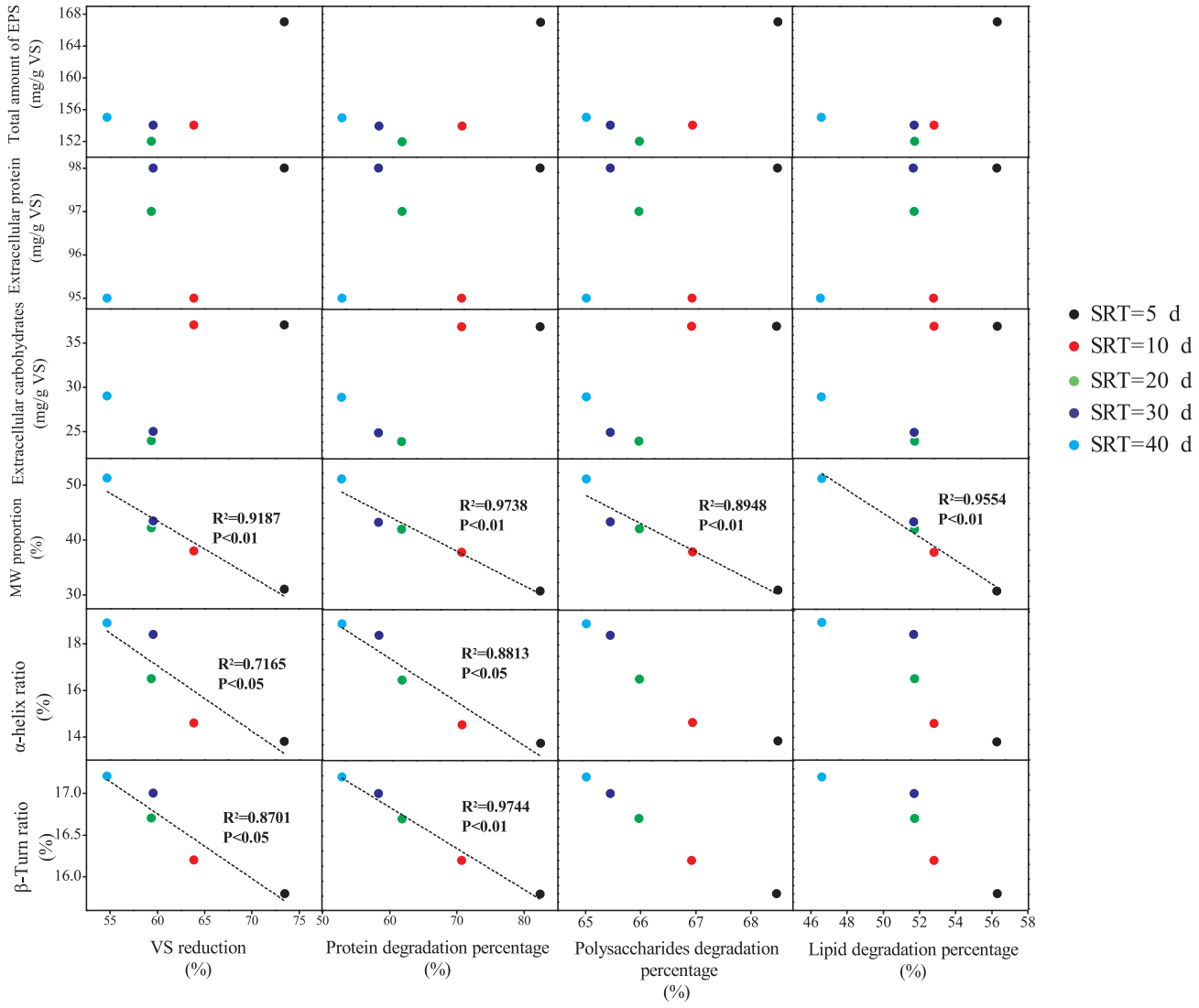

Fig. 7. The correlation between the main parameters relating to the occurrence state of organic compounds in sludge and the anaerobic degradation properties, in which the MW proportion was the proportion of organic molecules of 100000 ~ 1000000 Da in EPS.

polysaccharides would also decrease with the increase in ratio of α-helix and β-turn structure of protein in EPS.

In summary, the prolong of sludge age during wastewater treatment would significantly influence the distribution, occurrence state and structure of organic compounds in sludge. However, when refer to the inhibition mechanism of long sludge age on the AD of sludge, it was the increased MW of EPS and more stable structure driven by stronger internal hydrogen bonding force of α-helix and β-turn of protein in EPS, instead of the changed amount and components of EPS, that mainly determined the declined degradability. This result suggested that the methods able to reduce the molecular weight of organic matters and destroy the secondary structure of protein, especially α-helix and β-turn structure, such as thermal hydrolysis [41 42]and alkaline treatment [4344], which has been reported to enhance the AD performance of organic matters [454647], might be solutions for efficient AD of sewage sludge with long sludge age.

4. Conclusion

When the sludge age during wastewater treatment extended from 5 d to 40 d, the VS reduction of the sludge showed an “exponential” decrease, and the degradability of protein, polysaccharides and lipids in sludge all declined. Among them, the degradation of protein was the most affected (down-regulated by 35.8%), and the decrease in degradation of protein accounted for 80.4% of the decrease of VS conversion. The decrease of protein degradability was the most important factor for the decline in anaerobic degradability of sludge.

When the sludge age during wastewater treatment extended from 5 d to 40 d, the occurrence of organic compounds in sludge changed significantly. The floc structure of the sludge was more compact, the relative abundances of genes related to signal transduction and cell motility were weakened, the amount of EPS and extracellular carbohydrates decreased, the MW of EPS was larger and the second structure of the extracellular protein changed rapidly. It was the increased MW of EPS and more stable secondary structure of protein driven by α-helix and β-turn in EPS, instead of the changed amount and components of EPS, that mainly determined the declined degradability.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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