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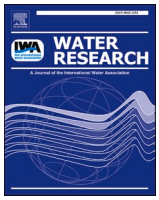

A novel strategy for waste activated sludge treatment: Recovery of structural extracellular polymeric substances and fermentative production of volatile fatty acids

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ABSTRACT

Structural extracellular polymeric substances (SEPS) as valuable biopolymers, can be extracted from waste activated sludge (WAS). However, the extraction yield is typically low, and detailed information on SEPS characterizations, as well as proper treatment of the sludge after SEPS extraction, remains limited. This study aimed to optimize the conditions of heating-Na_2CO_3 extraction process to increase the yield of SEPS extracted from WAS. Subsequently, SEPS were characterized, and, for the first time, insights into their protein composition were uncovered by using proteomics. A maximum SEPS yield of 209 mg g^{-1} volatile solid (VS) was obtained under optimal conditions: temperature of 90 °C, heating time of 60 min, Na^+ dosage of 8.0 mmol/g VS, and pH required to precipitation of 4.0, which was comparable to that from the aerobic granular sludge reported in literature. Proteomics analysis unveiled that the proteins in SEPS primarily originated from microorganisms involved in nitrogen fixation and organic matter degradation, including their intracellular and membrane-associated regions. These proteins exhibited various catalytic activities and played crucial roles in aggregation processes. Besides, the process of SEPS extraction significantly enhanced volatile fatty acid (VFA) production during the anaerobic fermentation of residual WAS after SEPS extraction. A maximum VFA yield of 420 ± 14 mg COD/g VS_{added} was observed in anaerobic fermentation of 10 d, which was 77.2 ± 0.1 % higher than that from raw sludge. Mechanism analysis revealed that SEPS extraction not only improved WAS disintegration and solubilization but also reduced the relative activity of methanogens during anaerobic fermentation. Moreover, SEPS extraction shifted the microbial population during anaerobic fermentation in the direction towards hydrolysis and acidification such as *Fermentimonas* sp. and *Soehngenia* sp. This study proposed a novel strategy based on SEPS extraction and VFA production for sludge treatment, offering potential benefits for resource recovery and improved process efficiency.

1. Introduction

Waste activated sludge (WAS) is the main by-product of conventional activated sludge process for municipal wastewater treatment plants (WWTPs). The management of this large volume of sludge presents a major operational cost challenge, accounting for up to 50 % of the overall expenses in WWTPs (Gonzalez et al., 2018). Some studies have proposed that recovery of a kind of bio-material from WAS, namely structural extracellular polymeric substances (SEPS), can offer promising opportunities to reduce the costs of sludge disposal (van Leeuwen et al. 2018). The SEPS exhibit unique properties that enable their extensive utilization in various industrial and agricultural applications (Boleij et al. 2018; Felz et al. 2016). Research has demonstrated that the presence of SEPS in fertilizers improved crop growth compared to fertilizers without SEPS (Lin et al. 2015). Furthermore, Kim et al. (2020) reported the SEPS recovered from aerobic granular sludge (AGS) and WAS had excellent self-extinguishing properties as good coating materials to increase flame retardancy of fiber. Currently, two scaled-up

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demonstrations to extract SEPS from AGS have been operated in the Netherlands (Bahgat et al. 2023; van Leeuwen et al. 2018).

The SEPS extraction process requires elevated temperatures (above 80 °C) and a pH level between 8 and 9 to effectively solubilize the hydrogel matrix, as documented in previous studies (Felz et al. 2016). According to existing methods, the SEPS yield from AGS typically ranges between 20 % and 35 % of VSS, whereas the yield from WAS is lower, ranging from 7 % to 19 % (Boleij et al. 2018; Li et al. 2021). In fact, WAS is much higher in production than AGS. If the SEPS can be efficiently recovered from WAS, the costs of sludge treatment can be significantly offset. Furthermore, knowledge of the exact composition of SEPS and its link to the function of individual components has not been fully investigated. For instance, proteins, the major component of SEPS, play significant roles in cell assembly and biofilm formation. The presence of proteins in SEPS facilitates the inclusion of diverse bacterial communities into a framework that promotes biofilm formation and enables crucial syntrophic relationships (Li et al. 2023). Kim et al. (2020) found that the proteins in SEPS contributed to flame retardant/suppressant features and significantly reduced the heat of fibers deposited with SEPS. However, most studies have only quantified protein concentrations in SEPS using colorimetric assays, and there are often some conflictive results in the literatures due to the interfering compounds and limitations of colorimetric assays (Adav and Lee 2008; Felz et al. 2019). Using advanced and insightful analysis into SEPS characterization is needed to better evaluate the specific property and favourably explore more potential applications of the SEPS (Chen et al. 2019; Kim et al. 2020). Inspired by researches related to sludge pretreatment for WAS solubilization, it is inferred that optimizing extraction conditions such as pH, heating time, and temperature could increase the SEPS yield by enhancing the solubilization of WAS matrix (Boleij et al. 2018; Lotti et al. 2019). Proteomics can provide detailed information on the types, functions and originations of isolated proteins from mammals, plants, and microbial communities (Chen et al. 2019), which can be expected highly promising for identifying and quantifying proteins in the SEPS. To our best knowledge, no study has reported the identification and in-depth characterization of proteins in SEPS by using proteomics analysis.

Following the SEPS extraction, a significant amount of organic matters still remain in the sludge. If these residues can be further converted into high-value products, it has the potential to enhance the efficiency of resource recovery from WAS. The alkaline-heating conditions employed during the SEPS extraction process have the potential to release organic matters into the liquid phase, thereby increasing its biodegradability (Kim et al. 2020). Therefore, it is reasonable to infer that anaerobic digestion could be integrated into the SEPS extraction process as a downstream technology to recover additional organic matters in the form of volatile fatty acids (VFA) from the alkaline sludge residuals produced during the EPS extraction process (Oliveira et al. 2021). However, it is currently unclear how the SEPS extraction process affects the efficiency of VFA production and the potential application of the "SEPS extraction + anaerobic fermentation" approach for WAS treatment.

Therefore, this study aimed to extract SEPS effectively from WAS and obtain a better understanding of its properties. This was accomplished by employing a modified heating-alkaline extraction method on WAS collected from a full-scale WWTP. The extracted SEPS was subjected to in-depth characterization using pyrolysis-gas chromatography-mass spectrometry (GC–MS) and proteomics techniques. Furthermore, the influence of the extraction process on downstream sludge treatment for VFA production was investigated and related mechanisms were comprehensively elucidated. The solubilization and biodegradability of WAS treated by SEPS extraction pretreatment were analyzed. The changes of microbial community during anaerobic fermentation were also identified to better understand the mechanisms. The findings have the potential to contribute to the development of more efficient and sustainable approaches for the treatment and resource recovery from WAS.

2. Materials and methods

2.1. Sludge source and characteristics

The sludge was collected from a secondary settler of a municipal WWTP with an A^2O process in Beijing, China. The collected sludge was concentrated by gravity thickening for 24 h and stored in a freezer at 4 °C. The main characteristics of concentrated sludge were as follows: total solids (TS) of 18.2 ± 0.1 g/L, volatile solids (VS) of 11.6 ± 0.1 g/L, pH of 6.7 ± 0.1, total chemical oxygen demand (COD) of 16,935 ± 158 g/L and soluble chemical oxygen demand (SCOD) of 94 ± 1.4 g/L. The inoculum for sludge fermentation has a TS of 53.6 ± 2.1 g/L and VS of 21.1 ± 0.7 g/L.

2.2. Extraction procedure for SEPS

The SEPS was extracted using heating-Na_2CO_3 method according to the procedure with minor modifications (Felz et al. 2016). Moreover, the influence of heating temperature (50 to 90 °C), heating time (15 to 90 min), Na_2CO_3 dosage (2.5 to 9.5 mmol Na^+/g VS), and pH required to precipitation (1.5 to 5.0) was investigated to optimize the EPS extraction from the WAS used in this study, respectively. In each test, the WAS was placed in a water-bath. When the temperature increased to the set value, sodium carbonate was added and the mixture was stirred with a magnetic stirrer at 300 rpm at that temperature. After centrifugation at 10, 000 g for 10 min, the supernatant was dialyzed with a dialysis membrane (3.5 K MWCO, SnakeSkin, Thermo Fisher Scientific, USA) for 24 h against MiliQ water. Afterwards, the dialyzed extracts were precipitated by declining pH with 3.5 mol/L HCl. After harvesting by centrifugation at 4000 rpm for 10 min, the gel-like pellet was regarded as the SEPS.

2.3. Batch tests of anaerobic fermentation for VFA production

Batch tests for VFA production were conducted in identical serum bottles. Each bottle with a working volume of 400 mL was fed in different substrates. One serum bottle was fed with WAS without any treatment as a control group. One experimental test, named “extraction pretreatment”, was conducted using the remained sludge after SEPS extraction (mixture of solid fraction and liquid fraction). The other test, named “heat-alkali”, used the pretreated sludge under the same condition as the SEPS extraction (Temperature of 90 °C, Na_2CO_3 dosage of 8.0 mmol Na^+/g VS, heating time of 60 min). The tap water was added into bottles to the same working volume. All the above tests were carried out in triplicate.

Before the fermentation test, the pH of all bottles was adjusted with 2.0 mol/L HCl solution or NaOH solution to neutral, and 19 mmol/L 2-Bromoethanosulfophate was added to inhibit the methanogenesis (Fang et al. 2018). Nitrogen gas was flushed into the bottles for 5 min to remove oxygen and capped with rubber stoppers. Afterwards, the test bottles were placed in an air-bath shaker at a speed of 150 rpm at 37 ± 1 °C. Samples for analysis were taken from the bottles and analyzed daily.

2.4. Extracellular protein isolation and identification

The detailed protocol for protein isolation in SEPS was described in Supplementary Materials. In order to gain a thorough understanding of isolated proteins, the sequence database selected the dominant bacteria phyla found most commonly in sludge consortia. The data obtained in this study can therefore be maximized in terms of their scientific value.

2.5. Evaluation of the impacts of SEPS extraction on hydrolysis, acidogenesis and methanogenesis

Several batch tests were conducted to evaluate the impacts of SEPS

extraction on WAS anaerobic fermentation stages. For each test, 40 mL inoculated sludge collected from an anaerobic digester was added into four test bottles with a working volume of 200 mL, Bovine serum albumin (BSA), glucose and sodium acetate were used as a model protein, monosaccharide and VFA, and their degradation efficiencies were investigated to assess the impacts of extraction pretreatment on hydrolysis, acidogenesis and methanogenesis stages, respectively (Zhao et al. 2015). The concentration of protein, glucose and sodium acetate was chosen according to previous studies (Wan et al. 2020; Wu and Song 2019). The inoculum was prepared and the operational condition was the same as those described in Section 2.2. The detailed protocol of each test was described in our previous study (Wan et al. 2020).

2.6. Analytical methods

2.6.1. pyrolysis-GC–MS

The composition of extracted SEPS (freeze-dried solid) was analyzed by a pyrolysis- GC–MS following the procedures in Lin et al. (2015). Compounds in pyrolysates were identified based on their mass spectra and retention times through comparison to the NIST library and published literatures.

2.6.2. DNA extraction and microbial community analysis

The samples were harvested by centrifugation (12,000 rpm for 15 min at 4 °C) of raw sludge and samples collected from fermenters and stored at −20 °C for DNA extraction. The DNA was isolated using the DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany). The isolated DNA was confirmed by agarose gel electrophoresis. The quality of DNA was checked by Qubit3.0 DNA detection (dsDNA HS Assay Kit, Life Technology, USA) and Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The amplification and sequencing of bacterial (V3-V4) 16S rRNA genes were conducted using high throughput sequencing by the Illumina MiSeq platform. The high-quality sequences were analyzed at NCBI Sequence Read Archive following the procedure described by our previous study (Wan et al. 2020). The sequences were divided into operational taxonomic units (OTUs) at a 97 % similarity level. The Alpha diversity index of microbial communities was analyzed using Mothur version v.1.30.1. The relative abundances of microbial at phylum and genus levels were classified according to the taxonomic classification by a Ribosomal Database Project Classifier.

2.6.3. Other analysis

The measurement of TS and VS was according to the standard methods (APHA, 2005). pH was measured with a pH meter (Thermo Scientific Orion FE28, USA). The methane measurement was according

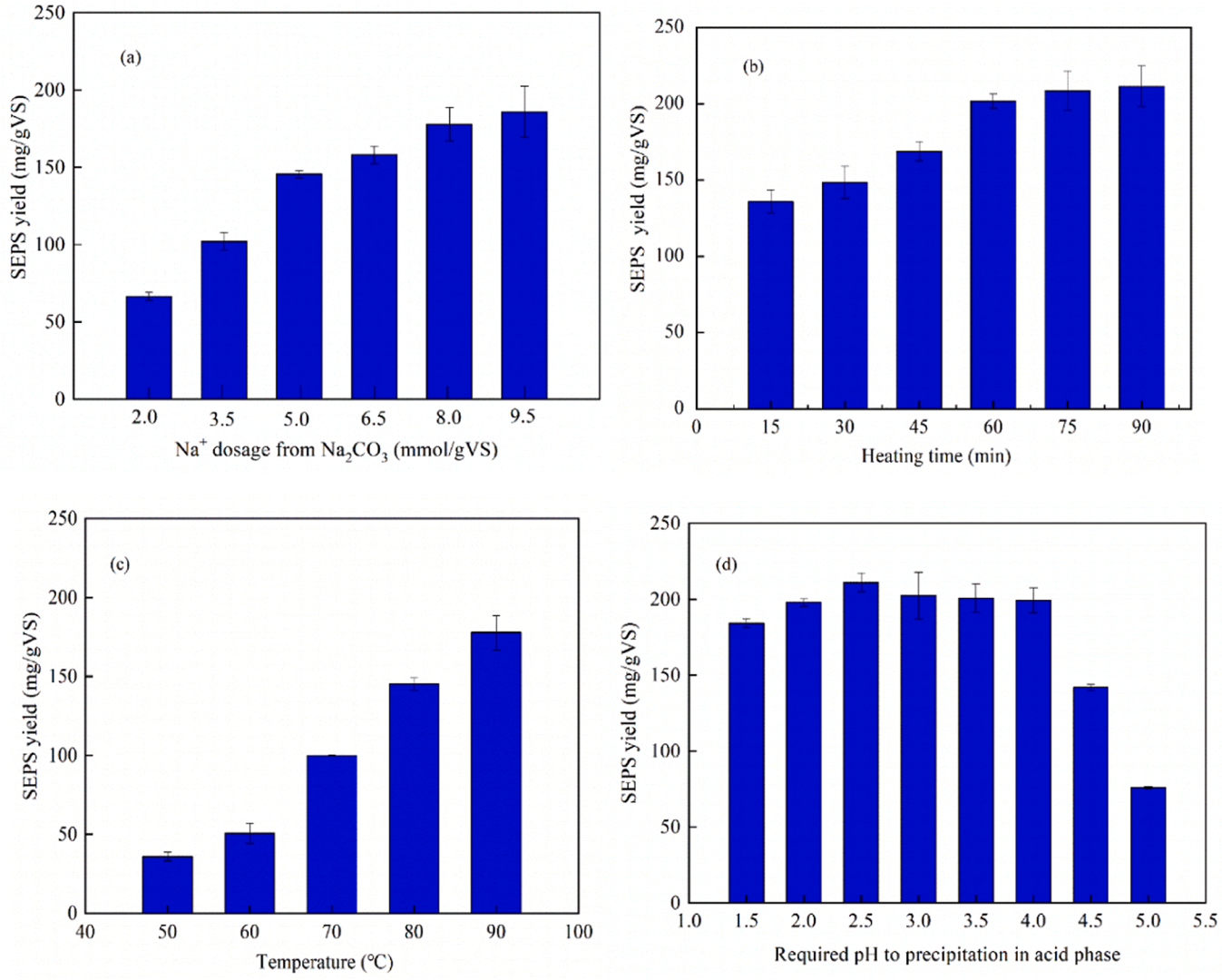

Fig. 1. Influence of different parameters on recovery yield of SEPS from WAS: (a) heating temperature, (b) heating time, (c) Na^+ dosage, and (d) pH required to precipitation.

to Fang et al. (2014). Samples for analysis of soluble COD, VFA and dissolved organic matter (DOM) were firstly centrifuged at 12,000 g for 10 min, and then the supernatant was filtered by syringe membrane with 0.45 μm pore size. The soluble COD concentration was measured using Hach kits. The VFA concentration was measured with a gas chromatograph with a flame ionization detector and an Agilent 19091F-112 column (25 m × 320 mm × 0.5 mm). Helium was employed as the carrier gas with a flow rate of 1.8 mL/min. The temperatures for the injection port, column, and detector were set at 240, 240, and 250 °C, respectively (Fang et al. 2018). The distribution of major DOMs in terms of molecular weight in the sludge was determined with liquid chromatography-organic carbon detection-organic nitrogen detection (LC—OCD-OND, DOC-LABOR, Karlsruhe, Germany). The detailed procedures were according to Xiao et al. (2016).

3. Results and discussion

3.1. SEPS yield and characterization

Fig. 1 shows the influence of heating temperature, heating time, Na^+ dosage and pH required to precipitation on SEPS yield. The increased Na^+ dosage, heating temperature and heating time resulted in higher SEPS yield. Considering the SEPS yield and cost of heating and chemicals for the extraction process, the optimized parameters in this study were chosen as follows: the heating temperature of 90 °C, the heating time of 60 min, Na^+ dosage of 8.0 mmol/g VS and the pH required to precipitation of 4.0. In comparison with the results of Lin et al. (2010), the extraction method used in this study prolonged and increased the heating temperature, heating time and Na^+ dosage (Felz et al. 2016; Li et al. 2021; Schambeck et al. 2020). These variations promoted solubilization of the hydrogel matrix of WAS, which was the first step and essence of SEPS recovery, and contributed to the SEPS yield (Seviour et al. 2009). On the other hand, the pH required to precipitation increased from 2.2 to 4.0. An ionic hydrogel test showed that the fluffy beads were immediately generated once the drops of SEPS were in contact with 2.5 % (w/v) $CaCl_2$ solution. It implied that the recovered SEPS had a relatively good gel-forming capacity and application potential. Note that the extreme complexity of sludge makes SEPS extraction challenging, and it is extremely difficult to extract all SEPS components without certain degree of cell wall breakage (Chen et al. 2019; Felz et al. 2016). Our results showed that the release of DNA within the extracted SEPS was low with 1.19 mg g^{-1} VSS compared to previous reports in range of 1.7 − 4.1 mg g^{-1} VSS (Chen et al. 2019; Pellicer-Nàcher et al. 2013; Wu and Xi 2009). The contribution of extracellular DNA from autolysis or cell death should be taken into account. Our findings supported the cell integrity of various biofilms maintained without substantial leakage of intracellular substrates. Consequently, the balance between high extraction yield and minimal biomass lysis was satisfactory and superior to those documented in previous studies.

The SEPS has irregular, small aggregated patches and rough surfaces (Supplementary Materials). Elemental mapping shows that carbon, nitrogen, and oxygen elements overlapped with each, accounting for about 74 % of the total composition. The calcium content is only 0.45 %, which partly indicated that the Ca^{2+} displacement into Na^+ during SESP extraction was sufficient. Besides, 1.64 % of sulfur and 4.07 % of phosphorus were also detected on the surface of extracted SEPS, which were likely derived from lipid and phosphoproteins, phosphorylated carbohydrates or phosphates minerals (Schulz and Schulz 2005). The results by pyrolysis-GC–MS analysis shows that polysaccharides-derived products (such as 5-methylfurfurdhyde), the combination of amino acids (such as indole and toluene) and some lipid-derived products (such as C_{12}–C_{16} fatty acid fractions and esters) were observed (Puyol et al. 2017). FTIR spectroscopy analysis (in Fig. 2b and Table S1) further demonstrated that most identified functional groups were assigned to polysaccharides, lipids and proteins. All these results suggest that the extracted SEPS was a complex mixture, mostly composed of proteins, polysaccharides and lipids, which were in line with previously reported studies (Felz et al. 2019; Kim et al. 2020).

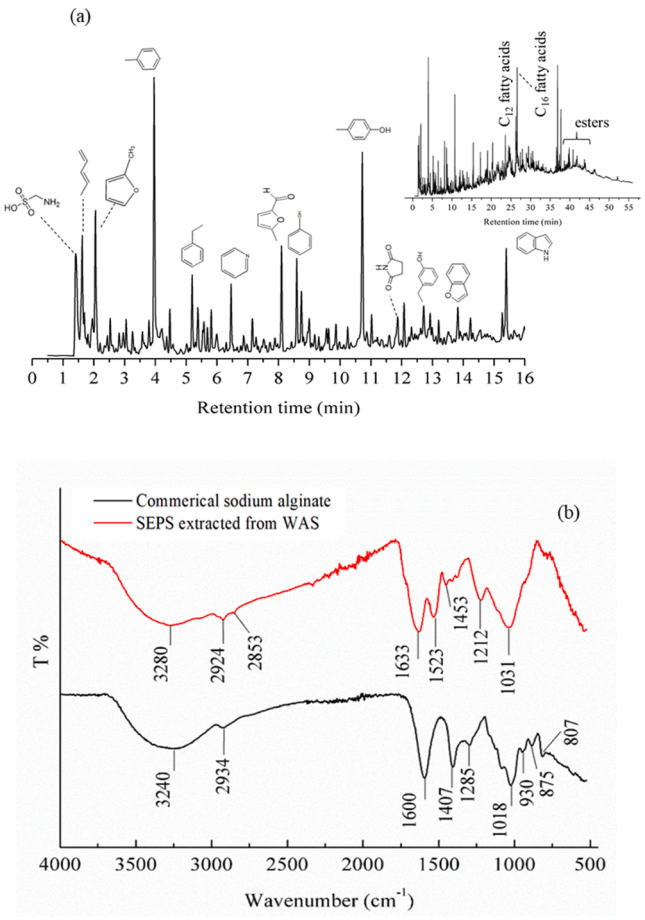

Fig. 2. Characterization of SEPS components: (a) pyrolysis-GC–MS of SEPS; (b) FTIR spectra.

3.2. Protein characteristics: origin, localization and molecular function

To better understand the proteins in the SEPS, the identification and quantification of proteins were conducted by using proteomics. In total, 19,905 spectra, 450 peptides and 198 identified proteins were detected from the SEPS. As shown in Fig. 3a, a majority of these identified proteins were originally associated with the phylum *Proteobacteria* (92, 46.5 %), followed by those from the phyla *Nitrospirae* (52, 26.3 %) and *Bacteriodetes* (38, 19.2 %). Other microbes including the phyla *Actinobacteria* (15, 7.6 %) and *Firmicutes* (1, 0.5 %) contributed a small fraction of the proteins. Note that some detected bacteria had low relative abundances, but they might play more important roles on the formation of SEPS. For example, the *Nitrospirae* contributed 26.3 % of proteins to the SEPS, although it only accounted for 1.4 % of the total bacterial community. On the other hand, as the most top dominant bacteria, the *Actinobacteria* accounted for 43.4 % of the total bacterial community, but only 15 identified proteins in SEPS were contributed by *Actinobacteria*. Above all, it can be confirmed that the diversity and abundance of bacteria were crucial and decisive factors controlling biopolymers' extraction and composition (Flemming and Wingender 2010). Moreover, many of these specific microbes are responsible for nitrogen fixation and degradation of organic matters, as also mentioned by some previous studies (Thomas et al. 2011).

The results based on GO-Slim subcellular localization indicate that 306 proteins were annotated for cellular components (as shown in

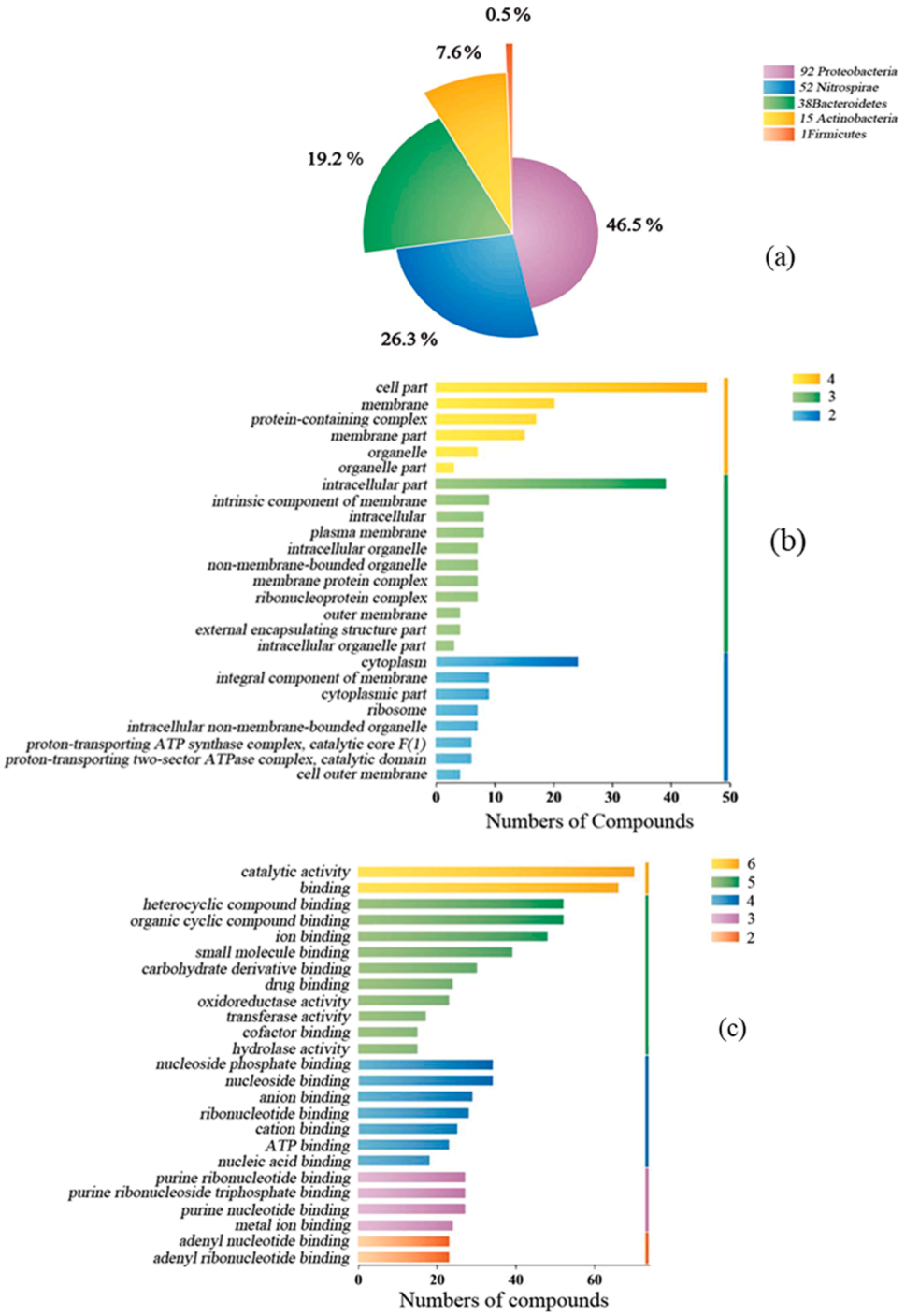

Fig. 3. Overview of proteomics, distribution and functions of the SEPS proteins (a) complete constitution of proteins in SEPS at phylum level; (b) protein classifications according to subcellular localization; (c) protein classifications according to molecular function (Note: Number on the right side of coordinate axis represents the hierarchical levels).

Fig. 3b). Among them, approximately 71 % of the annotated proteins were derived from the membrane-related regions and intracellular. 39 intracellular part, 24 cytoplasm, 20 membrane and 9 intrinsic components of membrane, as well as 8 plasma membrane was identified, respectively. According to Fig. 3c, the GO-Slim molecular function analysis indicates that most annotated proteins were involved in functions of various binding like heterocyclic compound binding, organic cyclic compound binding, ion binding, carbohydrate derivative binding and small molecule binding. Among them, a total of 31 annotated proteins were responsible for carbohydrate derivative binding. This result supported the study of Boleij et al. (2018), who identified a high abundant glycoprotein exited in the SEPS extracted from anammox granular sludge. Despite its preliminary character, this result inspired us to investigate the proteins and carbohydrates as integrated structures in the EPS research field in future. Moreover, some annotated proteins in the SEPS were associated with metal ion binding (e.g., Fe^{3+}-, Zn^{2+}-, Mg^{2+}-, and Ca^{2+}-binding). This finding can explain why the extracted SEPS had good behaviour on the ionic hydrogel. It is the specific proteins in the SEPS that bund with metal ions in sludge. And the proteins may also play crucial roles in floc aggregation and biofilm formation. As reported by Chen et al. (2019), the number of metal-binding proteins in EPS during the development of anammox biofilm from the initial to the

mature biofilm increased by approximately 2-fold.

3.3. Influence of SEPS extraction on the performance of anaerobic fermentation

Fig. 4a shows the evolution of VFA yield during anaerobic fermentation of sludge. The VFA yields increased rapidly at the beginning and then gradually reached a plateau in each case. The SEPS extraction significantly improved VFA yield all over the fermentation. A maximal VFA yield from sludge with SEPS extraction was 420 ± 14 mg COD/g VS_{added}, which was 77.2 % higher than that of raw sludge without pretreatment. A summarized comparison of VFA yields from sludge with different pretreatment methods is provided in Table 1. As shown in Table 1, the obtained VFA yield was comparable to the results in relevant studies, where various pretreatment methods were applied to treat WAS for enhancement of fermentative VFA production. Note that there was a minor difference in VFA yield between sludge with heat-alkali pretreatment (428 ± 25 mg COD/g VS_{added}) and sludge with SEPS extraction pretreatment (420 ± 14 mg COD/g VS_{added}), although the former sludge was with higher VS than the latter due to SEPS recovery.

Fig. 4b shows that acetate, propionate, isobutyrate, n-butyrate, isovalerate and n-valerate were detectable in each test, and there were no obvious differences in VFA composition between different tests. The acetate and propionate were the dominant types of VFAs in all tests, with a total range of 60 %–65 %. The sum of isobutyrate and n-butyrate represented under 15 % of the total VFAs, and the amount of valerate accounted for 20 %–24 % in all cases. The mixed VFA products can be concentrated from fermentative broth and/or recovered as high-valued and easily separated biopolymers like medium fatty acids and polyhydroxyalkanoates (PHA) via secondary bioconversion (Lee et al. 2014). The fermentative broth rich in acetate and propionate are also a good carbon source for biological nutrient removal (Chen et al. 2007).

Fig. 4c shows that pH rapidly decreased on the first two days of fermentation, and then gradually increased probably due to abundant ammonia released from protein degradation (Wan et al. 2020). The pH remained within the range of 6.2 to 7.5 without the addition of a pH buffer, thus ensuring suitable conditions for anaerobic fermentation. During anaerobic fermentation, methane production was largely avoided (Fig. 4d). The maximum cumulative methane production of 12.3 mL/g VSS was obtained in the control, which was 5.6 and 3.2-folds higher than that from sludge with SEPS extraction and heat-alkali pretreatment, respectively. Obviously, suppression of the consumption of

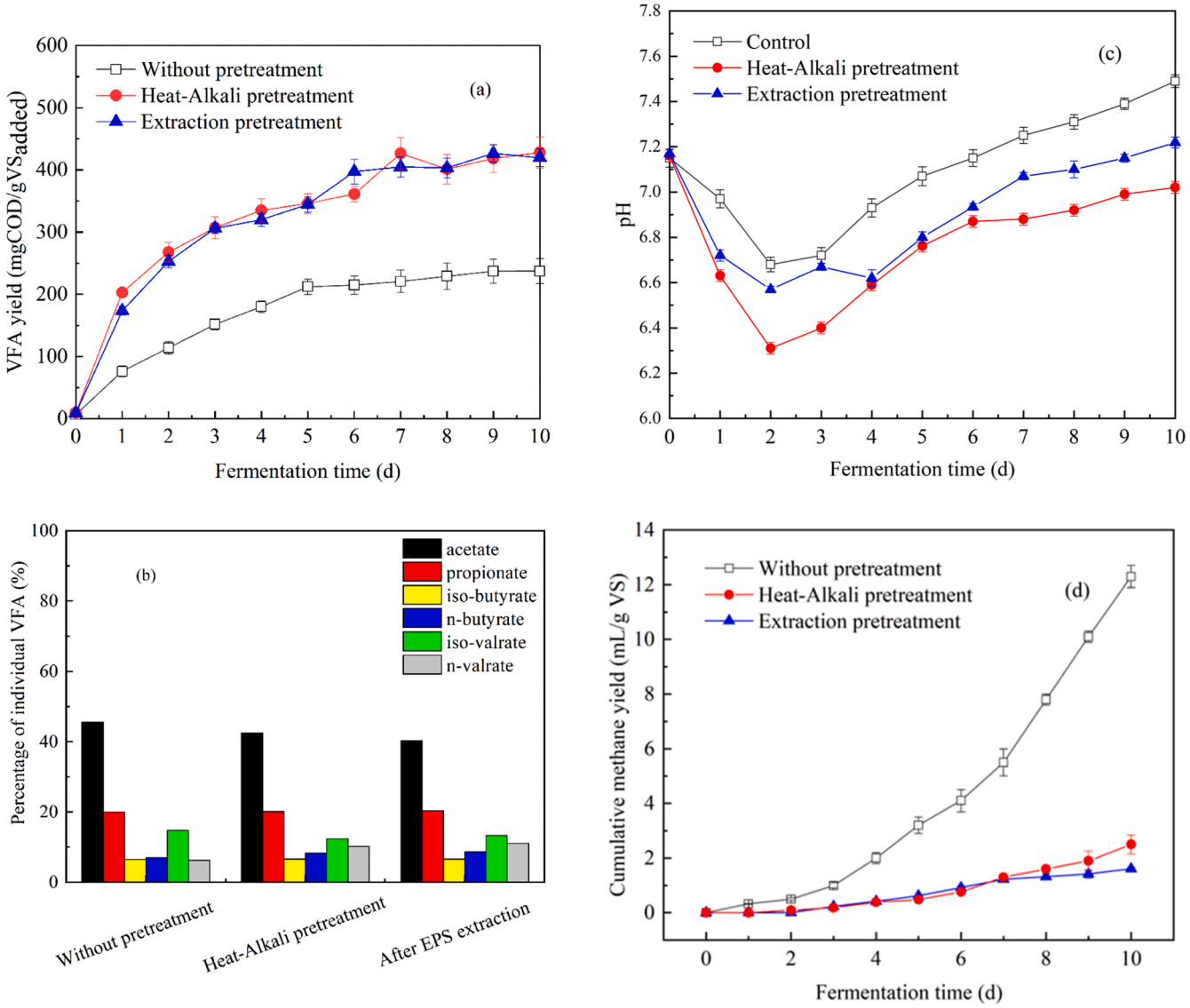

Fig. 4. Influence of pretreatments on (a) VFA production, (b) percentage of individual VFA under maximum production, (c) pH and (d) cumulative methane yield during anaerobic fermentation.

Table 1
Comparison of VFA yields from sludge with SEPS extraction in this study and other pretreatment methods in previous studies.

Pretreatment methods and conditions	Operation condition of anaerobic fermentation	Maximum VFA yield	Reference
150 mg N/L of nitrate photolysis, UV lamp (254 nm, 28 W), pH=5.5	33 °C, 120 rpm	370.11 mg COD/g VS_{added}	(Liu et al. 2024)
0.3 g Sodium citrate/g TSS, 121 °C, 30 min	37 °C, without pH control	354.5 mg COD/g VS_{added}	(Fang et al. 2022)
0.046 g $FeCl_3$/g TSS	35 °C, without pH control	235 mg COD/g VS_{added}	(Zhan et al. 2021)
0.08 g Citric acid/g TSS	35 °C, without pH control	384.4 mg COD/g VS_{added}	(Zhang et al. 2023)
pH=6.0, 3-cycle freezing at −20 °C for 6 h and subsequent thawing at 25 °C for 2 h, 0.15 g CaO_2/g VSS	36 °C, initial pH=6	438.5 mg COD/g VS_{added}	(Zhao et al. 2023)
90 °C, 8.0 mmol Na^+/g VS, 60 min	35 °C, without pH control	420 mg COD/g VS_{added}	This study

VFA for methane production contributed to the enhanced VFA production.

3.4. Influence of SEPS extraction on WAS solubilization and biodegradability

Hydrolyzing WAS to make them soluble is the first and rate-limiting step for anaerobic fermentation. In this work, the SCOD concentration in WAS increased from 94 ± 3.4 mg/L to 4815 ± 18 mg/L after SEPS extraction. In order to better distinguish the compositions of dissolved substances, the specific fractions of DOMs were investigated by using LC—OCD-OND. According to different molecular weights, the observed DOMs are mainly classified into biopolymers (>2000 kDa, corresponding to high molecular weight protein, polysaccharides and amino-sugars), humic substances (~1000 Da), building blocks (300–500 Da, corresponding to breakup products of humic substances), low molecular weight (LMW) neutrals (< 350 Da, corresponding to alcohols, aldehydes, ketones and mono-oligosaccharides) and LMW acids (< 350 Da) (Lu et al. 2018). As shown in Table 2, bio-polymers and LMW-neutrals were the most dominant components in DOMs from sludge after SEPS extraction, and their concentrations increased about 37 and 83 times compared with that from WAS, respectively. It implies that the SEPS extraction promoted the release of readily biodegradable substances like proteins and carbohydrates, which increased the biodegradation rate and biodegradability in the follow-up anaerobic fermentation (Fang et al. 2019; Zhen et al. 2017).

The concentration of building blocks (68.6 mg C/L), the breakdown products of humic substances, was 22 times higher than that in raw sludge. According to Kipton et al. (1992), even though humic substances were stable compounds, they underwent considerable changes in high pH solutions, and their solubility increased with increasing pH. The presence of humic substances is challenging for anaerobic digestion as they affect enzymatic activity by immobilizing enzymes, but some humic-like substances such as quinones in sludge can improve the efficiency of electron transfer and acidification. However, the influence of concentration variation of building blocks and humic substance due to SEPS extraction on VFA production is unclear, and it needs to be further investigated.

Table 2
Concentrations of dissolved organic matter (DOM) in raw sludge and sludge with SEPS extraction.

Test groups	Bio-polymers (mg C/L)	Humic substance (mg C/L)	Building block (mg C/L)	LMW-neutrals (mg C/L)	LMW-acids (mg C/L)
Raw sludge without pretreatment	9.78	10.72	2.93	2.93	0
Sludge after SEPS extraction	374.8	110.4	68.6	247.1	0

3.5. Influence of SEPS extraction on hydrolysis, acidogenesis and methanogenesis

To investigate the influence of extraction pretreatment on each step of sludge fermentation, a series of batch tests were carried out by using model compounds (BSA, glucose and sodium acetate) as substrates. As shown in Fig. 5 and Table 3, the specific degradation rate of BSA and glucose decreased from 29.11 ± 0.12 and 35.87 ± 0.11 mg g^{-1} VS·h in the control to 31.16 ± 0.09 and 33.52 ± 0.12 mg g^{-1} VS·h in the extraction pretreatment test, respectively. These results indicated that the SEPS extraction pretreatment had a slightly adverse effect on hydrolysis and acidogenesis. In addition, the specific degradation rate of sodium acetate in the extraction pretreatment test was 2.83 ± 0.01 mg g^{-1} VS·h, which indicated that the relative activity of methanogens (i.e., SCFA consumers) reduced by 52.27 %. Thus, it reveals that the suppression induced by extraction pretreatment on methanogenesis was much more severe than that on hydrolysis and acidogenesis. This may partially explain why less methane was produced in the extraction pretreatment test. Considering the results of enhancing WAS solubilization and VFA production, it can be concluded that the extraction pretreatment had a much greater positive effect on WAS hydrolysis and acidogenesis than its negative effects. Relevant studies have also observed that some pretreatment methods would inhibited the processes of hydrolysis, acidogenesis and methanogenesis, but increased the performances of anaerobic fermentation. It was possibly ascribed to the production of inhibitors during pretreatment (Chen et al. 2008; Gonzalez et al. 2018). The detailed mechanisms behind the phenomenon are still not fully clear and should be studied more thoroughly in future.

3.6. Influence of SEPS extraction on microbial community and diversity

The number of operational taxonomic units (OTUs) was 901 in the control digester and 645 in the pretreated digester, with 410 sharing. In addition, the Chao and Shannon index in the control digester and pretreated digester was 822.1, 1112, 3.55 and 3.79, respectively. The results of Alpha diversity revealed that the extraction pretreatment decreased the diversity, and abundance of the microbial community did not largely change the microbial structure.

Fig. 6a presents the foremost bacteria in the control and experimental digesters including phylum *Firmicutes, Actinobacteria, Bacteroidetes, Patescibacteria, Chlorofiexl*, and *Proteobacteria*. All of them have been documented to be capable of converting organic matters (e.g., proteins and carbohydrates) into VFA under anaerobic conditions (Li et al. 2018). The relative abundance of *Firmicutes* was remarkably enriched in the pretreated digester (28.21 % versus 12.40 % in the control digester). A lot of microbes belonging to the *Firmicutes* have been reported to excrete extracellular hydrolytic enzymes such as protease and cellulose. Other dominant phyla, *Bacteroidetes* and *Patescibacteria* reported to play crucial roles in hydrolysis and acidification steps also showed higher abundances in the pretreated digester as well, and their relative abundances increased from 14.34 % and 15.01 % in the control digester to 18.38 % and 21.72 % in the pretreated digester, respectively. The results were in accordance with the observed increased efficiency of hydrolysis and acidification. In contrast, the relative abundance of *Actinobacteria* reduced from 21.73 % in the control digester to 17.16 % in the pretreated digester, while *Chloroflesi* did not change much (7.8 %

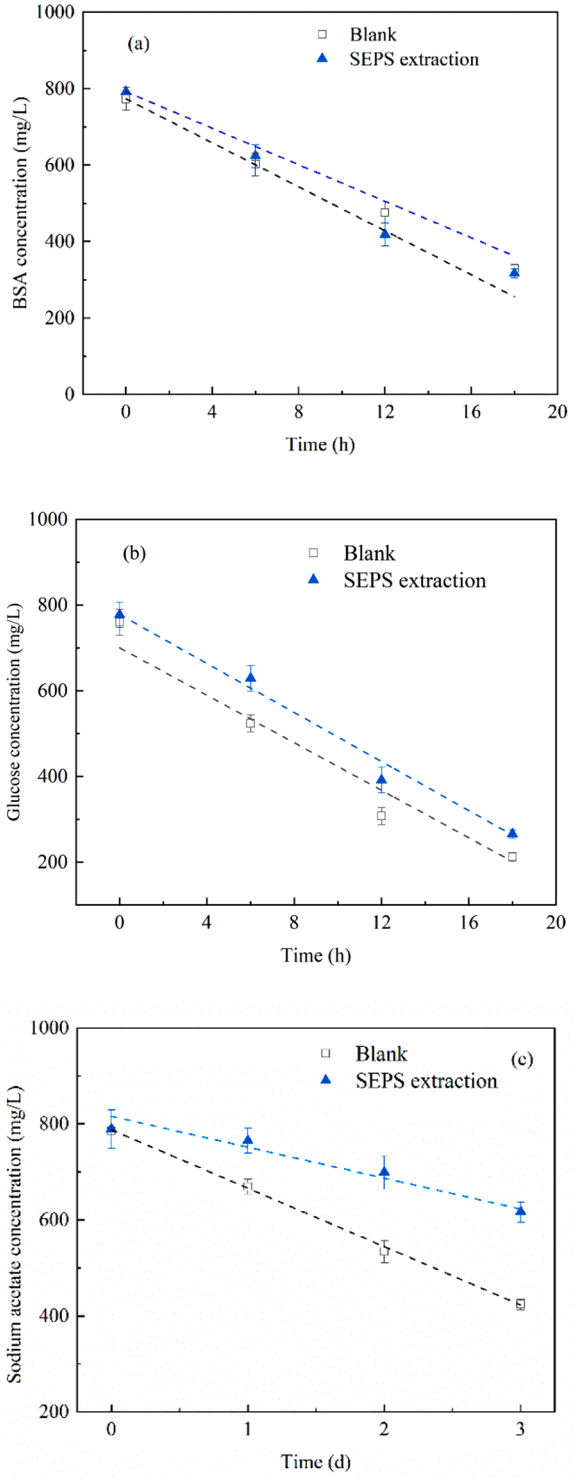

Fig. 5. Influence of SEPS extraction on the degradation performance of (a) BSA, (b) glucose, and (c) sodium acetate. Error bars represent standard deviations of triplicate tests.

in the pretreated digester versus 9.9 % in the control digester).

Detailed information about bacterial communities was presented in the genus level distributions. As shown in Fig. 6b, the microbial communities in the two digesters consisted of various bacteria associated with hydrolysis (e.g., *Petrimonas* sp., *Sedunebtuvacter* sp.) and acidogenesis (e.g., *Soehngenia* sp., *Acetiabaerobium* sp; *Fermentimonas* sp., *Petrimonas* sp). *Petrimonas* sp. and *Sedimentibacter* sp. are known for breaking down complex organic matters into simpler compounds, which

Table 3
Influence of SEPS extraction on degradation rates of model substrates during anaerobic fermentation.

Test groups	Specific degradation rate (mg g^{-1}·VS·h)		
	BSA	Glucose	Sodium acetate
Raw sludge without pretreatment	29.11 ± 0.12	35.87 ± 0.11	5.93 ± 0.02
Sludge with SEPS extraction	31.16 ± 0.09	33.52 ± 0.12	2.83 ± 0.01

can increase the availability of complex substrates. The higher abundance of *Petrimonas* sp. in the pretreated digester (1.82 %) compared to the control digester (0.38 %) could lead to more efficient breakdown of complex organic matters into simpler sugars, facilitating the subsequent stages of anaerobic fermentation. On the other hand, the presence of *Mycobacterium*, typically involved in the degradation of complex organic compounds, decreased from 19.09 % to 13.00 %. This decrease might indicate that the pretreatment favored other bacteria over *Mycobacterium* for the degradation of available substrates due to changes in the availability or type of organic matters after pretreatment. Moreover, enrichment in abundances of certain genera related to acidogenesis was also observed in the pretreated digester. *Fermentimonas* sp., known for fermenting organic substrates to acetate, significantly increased from 0.20 % in the control to 7.42 % in the pretreated digester. Similarly, *Soehngenia* sp. and *Clostridium_sensu_stricto_13*, which are typical fermenting bacteria, also showed enrichment in the pretreated digester (7.62 % and 0.38 % compared to 0.35 % and 0.01 % in the control digester). According to the above findings, it indicated that the SEPS extraction pretreatment significantly influenced the microbial community, favoring the growth of certain hydrolytic and acidogenic bacteria. The percentage of hydrolytic and acidogenic bacteria increased from 5.21 % to 18.15 % in the pretreated digester. This demonstrated the effectiveness of the pretreatment process in creating a more favorable microbial community for both hydrolysis and acidogenesis. Therefore, it is reasonable to speculate that the pretreatment improved the performance of VFA production by enriching bacteria closely related to hydrolysis and VFA production.

3.7. Overall understanding and implications

Maximizing the utilization of sludge resources is crucial for the sustainable development of WWTPs. For this purpose, this study focused on the improvement of SEPS extraction and further anaerobic fermentation to produce VFA for WAS treatment. This work showed that this joint process can extract nearly 209 mg g^{-1} VS and subsequently obtain a maximum VFA production of 420 ± 14 mg COD/g VS_{added}, and the VFA production was 77.2 ± 0.1 % higher than that from raw sludge. The SEPS yield achieved in this study surpassed the range of 90 to 190 mg g^{-1} extracted from WAS reported in previous literatures, and was even comparable to the range of 200 to 300 mg g^{-1} observed from AGS (Li et al. 2021; Lin et al. 2013; Schambeck et al. 2020). From the specific gel-forming properties and functional groups identified by FT-IR, the extracted SEPS can act as a biosorbent for the removal of heavy metals and organic pollutants (Feng et al. 2021). Moreover, advanced purification techniques can be utilized to eliminate impurities, or blending SEPS with other biopolymers create hybrid materials to enhance the performances. In addition to gel-forming abilities, other functional extracellular biopolymers are gradually being isolated, characterized, and applied. One possible application of SEPS was already shown by recombining with guluronic acid/guluronic acid, mannuronic acid/-guluronic acid and mannuronic acid/mannuronic acid blocks at varying ratios in metal adsorption, flame retardation, and seed coating (Shi et al. 2023).

The observed minor difference in VFA yield between sludge with

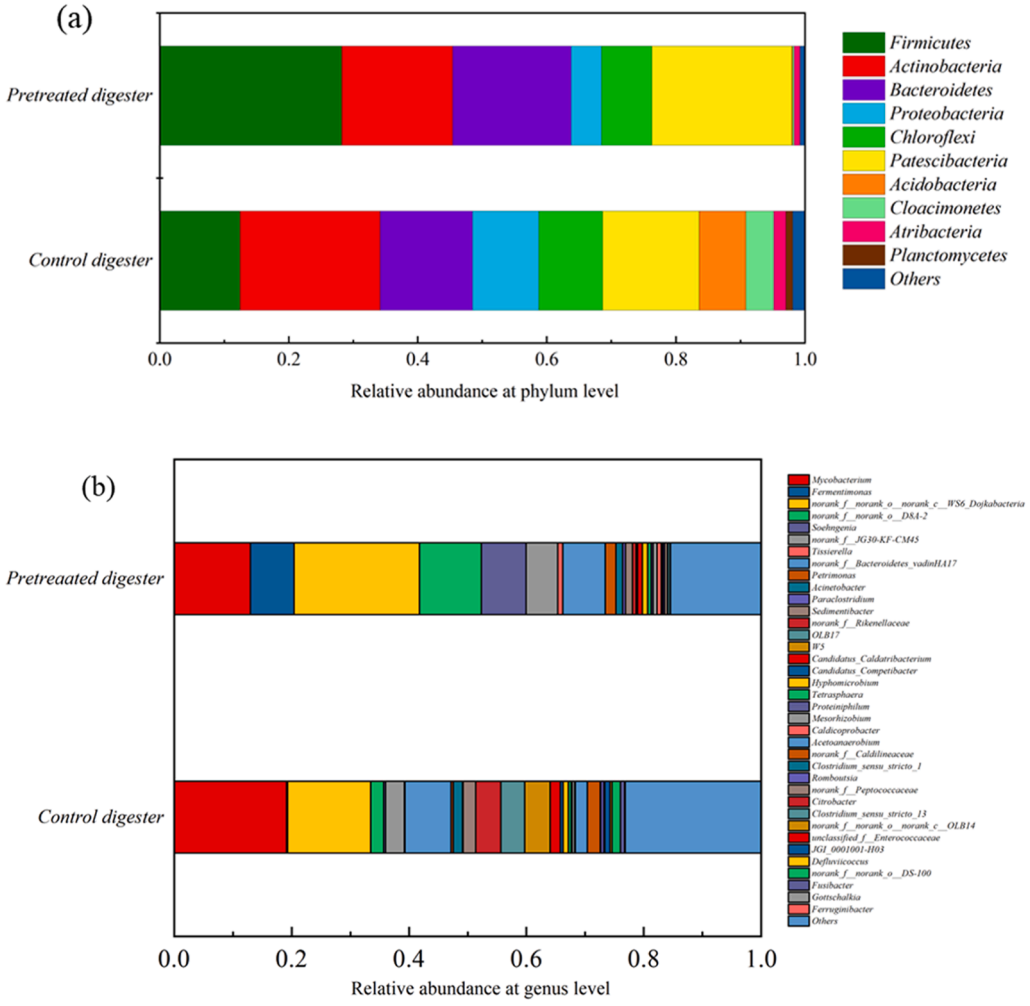

Fig. 6. Relative abundances of microbial community (a) at phylum level, (b) at genus level.

heat-alkali pretreatment and sludge with extraction pretreatment suggested that SEPS extraction may be a promising alternative to conventional heat-alkali pretreatment for enhancing VFA production (Liu et al. 2009; Wang et al. 2016). The SEPS extraction enhanced WAS solubilization and the production of biodegradable substances for acidification, which contributed to an increase in the biodegradation rate and VFA production in subsequent anaerobic fermentation. During SEPS extraction, the elevated pH can modify the structure, surface properties, and electrostatic charge of EPS (Gonzalez et al. 2018). This alteration can neutralize the positively charged sites on EPS molecules, reducing the electrostatic attraction between EPS components and enhancing its solubility. Also, alkali environment can induce saponification of the cell membrane's lipid bilayer, leading to the release of intracellular contents and protein denaturation (Cai et al. 2004; Toutian et al. 2021). Meanwhile, the elevated temperature contributed to the solubilization of carbohydrates and proteins. The protein denaturation can occur above temperatures of 75 °C, which makes proteins more prone to biodegradation, so an increase in biodegradation would be expected (Gonzalez et al. 2018).

Moreover, as a typical component in WAS, SEPS plays an important role in the formation of a tertiary network structure. After the SEPS extraction, the flocs sludge lost their structural integrity, resulting in a decrease in network strength and cohesive force. As a result, the flocs became more susceptible to fracturing, and there was an increase in the surface area available for hydrolytic and acidifying enzymes (Felz et al. 2016; Hu et al. 2023).

The SEPS has been considered as a non-easily biodegradable biopolymer. Reported studies clearly demonstrated that the presence of SEPS limited the efficiency of methane production in anaerobic digestion (Hu et al., 2023; Zhang et al., 2019). It was observed that during anaerobic digestion, the alginate-like exopolysaccharides in EPS were not effectively broken down. However, the degradation of these alginate-like exopolysaccharides by a specific enriched bacterial community can enhance methane production from WAS by 115 %–185 % (Zhang et al. 2021). We conducted anaerobic fermentation experiments using the extracted SEPS as the sole substrate and observed that only less 3.1 ± 0.12 mg COD/g VS_{added} of VFA was produced after 10 days of fermentation. Guo et al. (2020) observed that only 12.2 % and 9.4 % of SEPS were degraded in anaerobic digestion of 10 d from WAS and AGS, respectively. Thus, the VFA production of WAS after SEPS extraction was comparable to that with a pretreatment of alkaline and heating method.

A treatment strategy based on "SEPS extraction + anaerobic fermentation" was proposed, as depicted in Fig 7. Initially, the thickened WAS in the WWTPs undergoes SEPS recovery, while the remained sludge is subjected to anaerobic fermentation to produce VFAs. The SEPS recovery process not only improves the efficiency of sludge treatment but also opens up avenues for the valorization of SEPS as a valuable resource. During the SEPS extraction and anaerobic fermentation processes, nitrogen and phosphorus will be released and accumulated in the broth, potentially increasing the nitrogen and phosphorus load in wastewater treatment. To address this challenge, the proposed treatment strategy suggests transferring the VFAs-rich broth to a recovery and separation system, where nitrogen and phosphorus can

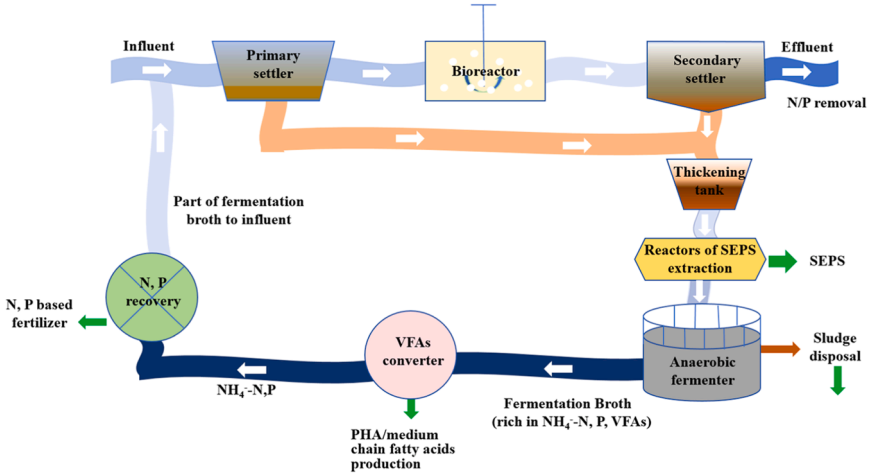

Fig. 7. An innovative operation concept of a WWTP by integrating the SEPS recovery and VFA production and other technologies reported previously.

be selectively recovered through struvite precipitation. Consequently, the treated broth was directed to the biochemical reaction tank to enhance biological nitrogen removal as carbon source. Overall, our study demonstrated the proof-of-concept of SEPS extraction and anaerobic digestion for WAS treatment, which shows promise in improving resource utilization, and nutrient management in wastewater treatment.

In this study, the coupling of SEPS extraction and VFA production presents a novel and promising approach to WAS treatment, but much research is still needed. For practical applications, beyond laboratory scales, it is promising to improve quality control and market prospects of valuable products from larger-scale operations. The quality control of extracted SEPS depends on its intended application. A better focus on a clearer roadmap for the practical implementation of SEPS production from WAS is necessary. Moreover, future research should aim to identify the optimal conditions for integrating SEPS extraction with VFA recovery from WAS, to minimize environmental and economic impacts while maximizing resource recovery benefits.

4. Conclusions

In this work, we demonstrated the promising application potential of "SEPS extraction + anaerobic fermentation" for the treatment of WAS. The SEPS was successfully and efficiently extracted from WAS under optimized conditions, including a heating temperature of 90 °C, a heating time of 60 min, a Na^+ dosage of 8.0 mmol/g VS, and a pH required to precipitation of 4.0. The maximum yield of SEPS reached up to 209 ± 13 mg g^{-1} VS, and the SEPS from WAS showed a similar potential as that from AGS in a quantitative sense and relatively good granulation capacity. The improved extraction efficiency can be mainly attributed to efficient ion exchange and EPS solubilization. For the first time, this study utilized proteomics to uncover that the proteins in SEPS predominantly originated from the intracellular and membrane-associated regions of microbes involved in nitrogen fixation and organic matter degradation. These proteins exhibited catalytic activity and binding functions. Moreover, the sludge after SEPS extraction yielded a remarkable 77.2 ± 0.1 % increase in VFA production compared to the control. The SEPS extraction significantly promoted the disintegration and solubilization of sludge and removed non-easily biodegradable EPS polymers from sludge. Besides, the extraction pretreatment had more severe inhibitory effects on methanogenesis than hydrolysis and acidogenesis during anaerobic fermentation. Moreover, the SEPS extraction enriched the microbial community related to hydrolysis and acidification, which was responsible for VFA production.

CRediT authorship contribution statement

Wei Fang: Writing – review & editing, Writing – original draft, Conceptualization. **Ru Zhang:** Formal analysis. **Wenjing Yang:** Methodology. **Henri Spanjers:** Writing – review & editing. **Panyue Zhang:** Resources.

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122421.

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