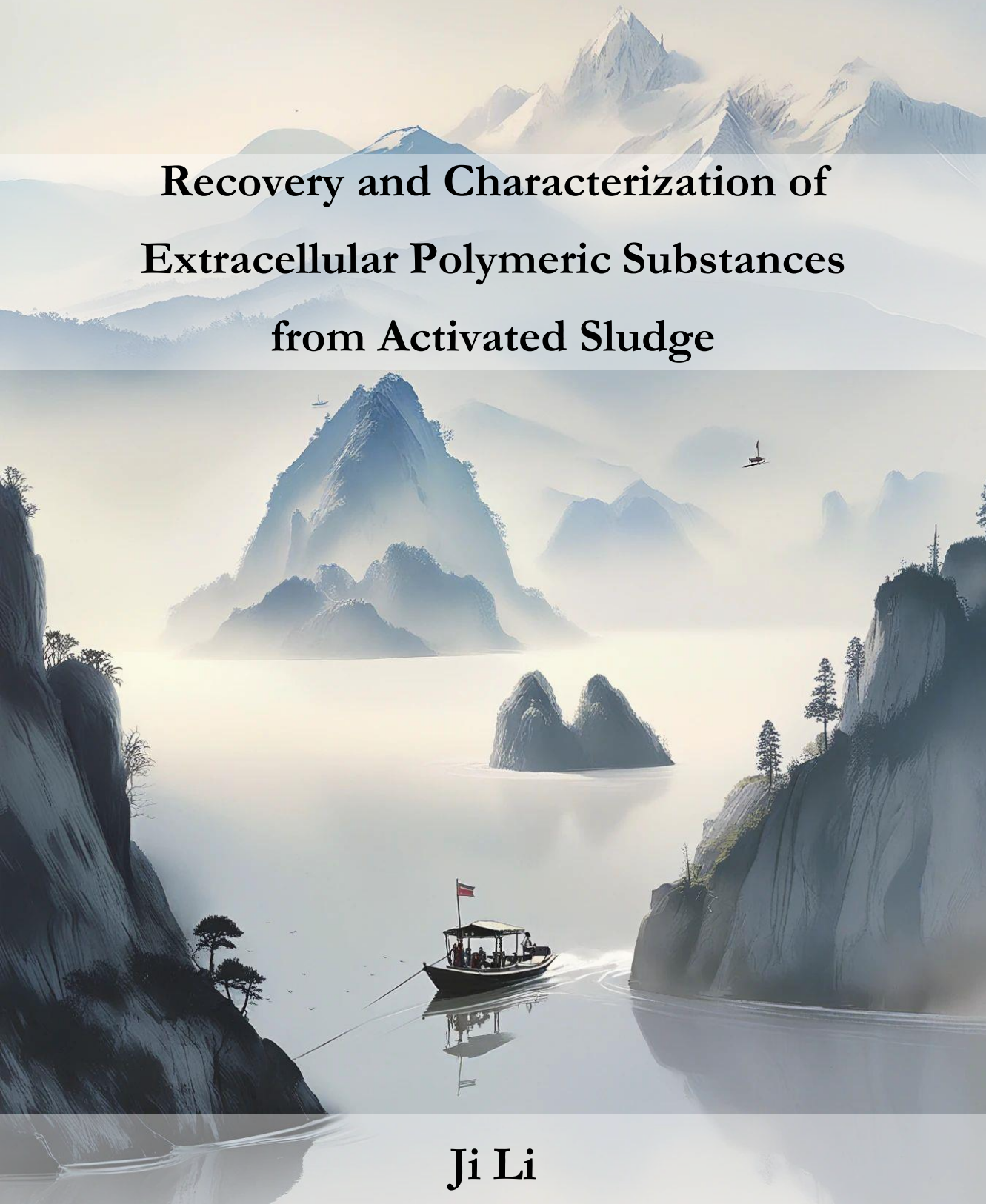
Recovery and Characterization of Extracellular Polymeric Substances from Activated Sludge



Ji Li

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Dissertation

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To my parents

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Summary

Sustainable wastewater treatment system have increasingly focused on resource recovery from wastewater. Excess sludge, primarily composed of a bacterial cell matrix embedded in extracellular polymeric substances (EPS), offers significant potential in this regard. accounts for approximately 10–40% of the total dry weight of sludge and is recognized as a promising bioresource for producing valuable bioproducts. However, despite the widespread use of flocculent sludge treatment plants, the recovery potential and properties of EPS in flocculent sludge have been largely overlooked.

This thesis focuses on the extracted from flocculent sludge, with the aim of exploring their extraction potential, structural characteristics, conformations, and properties. By analyzing EPS from various full-scale and lab-scale flocculent sludge systems, it examines the factors influencing EPS extraction potential and establishes correlations between these factors and EPS formation and properties. Further investigations into EPS composition and conformation provide a deeper understanding of its structure, shedding light on its role in sludge aggregation and potential applications. This thesis bridges engineering and fundamental perspectives to advance EPS research.

Chapter 1 provides a concise introduction to the growing interest in EPS recovery, highlighting its significance and potential. It also raises key questions about EPS derived from flocculent sludge, establishing a clear roadmap for the thesis and serving as a foundation for the experimental setups in this study.

In **Chapter 2**, the study focuses on evaluating the EPS recovery potential from flocculent sludge. Samples were collected from various full-scale wastewater treatment plants in China, and EPS was extracted for analysis. Influent characteristics, microbial community profiles and chemical characterizations of EPS were examined to assess their correlations. The EPS yield ranged from 9% to 19% of the organic fraction of raw sludge. The findings also revealed that EPS production is highly influenced by external environmental conditions and strongly linked to bacterial diversity and abundance. This chapter highlights the significant potential of flocculent sludge for EPS recovery.

Chapter 3 aims to explore the connections between various external factors and EPS formation. Lab-scale sequencing batch reactors (SBRs) were operated under controlled conditions, with specific operational and influent parameters designed to cultivate flocculent sludge. The results revealed that sludge fed with starch-rich influent showed significantly enhanced EPS formation, while low temperatures also

supported EPS synthesis. In contrast, organic loading rates and sludge retention time (SRT) had minimal impact on EPS yield. Furthermore, adaptations in EPS composition and properties indicated that both influent characteristics and operational conditions played a critical role in shaping EPS composition.

Recognizing the importance of understanding EPS structures, **Chapter 4** focuses on a detailed investigation of EPS composition and structure. Extracted EPS was fractionated into distinct components for analysis. Comparisons with commercial alginates revealed that typical alginate units—guluronic acid and mannuronic acid—were absent in all EPS fractions, indicating that EPS from flocculent sludge does not contain alginate structures. Further analysis of these fractions suggested the presence of glycolipid structures, specifically highlighting the significance of lipopolysaccharides (LPS), a type of glycolipid, in EPS. This chapter not only confirmed the absence of alginates but also underscored the critical role of glycolipids in EPS composition.

Chapter 5 delves into the structure of lipopolysaccharides (LPS) and their contributions to EPS properties by comparing EPS from flocculent and granular sludge. LPS was isolated from EPS and subsequently characterized. The study found that LPS comprised approximately 25% of the organic fraction of EPS in flocculent sludge, significantly higher than the 15% observed in granular sludge. LPS from flocculent sludge exhibited unique features, including lower glycan content, shorter glycan chains, lower molecular weight, and a higher prevalence of unsaturated lipids. These structural characteristics led to inverted crosslinks in calcium-bound LPS aggregates, contributing to the fluid-like hydrogel morphology of EPS. In contrast, LPS-Ca aggregates from granular sludge exhibited a bilaminar multilayered structure, resulting in the solid, self-standing hydrogel properties of EPS.

Chapter 6 summarizes the key findings of this thesis, highlighting the insights gained into EPS recovery, structure, and properties. Additionally, it proposes ideas for future research, including exploring bacterial activities involved in EPS biosynthesis, further investigation of LPS structures and their functions, and potential applications of EPS. These suggestions aim to advance the understanding and utilization of EPS in sustainable wastewater treatment and beyond.

Samenvatting

Het duurzame afvalwaterzuiveringssysteem richt zich op het terugwinnen van grondstoffen uit afvalwater. Overtollig slib bestaat voornamelijk uit een bacteriële celmatrix, ingebed in extracellulaire polymere stoffen (EPS). EPS vertegenwoordigt ongeveer 10% tot 40% van het totale droge gewicht van slib en wordt erkend als een veelbelovende bio-grondstof voor de productie van waardevolle bio-producten. Ondanks de prevalentie van vlokkig slib in zuiveringsinstallaties, is het terugwinningspotentieel en de eigenschappen van EPS in dit type slib nog onvoldoende onderzocht.

Dit proefschrift richt zich op het EPS dat wordt gewonnen uit vlokkig slib, met als doel de extractiemogelijkheden, structuren, conformaties en eigenschappen te onderzoeken. EPS-extractie uit verschillende grootschalige vlokkige slibmonsters heeft als doel het potentieel voor EPS-extractie vast te stellen. De correlaties tussen verschillende factoren die verband houden met EPS-vorming en -eigenschappen worden geëvalueerd. Verder onderzoek naar de samenstelling en formatie van EPS werpt licht op de structuren van EPS en biedt inzicht in de rol die het speelt in slibaggregaten en de toepassingen van EPS. Dit proefschrift presenteert een geïntegreerde benadering van EPS-onderzoek vanuit zowel een technisch als fundamenteel perspectief.

In **hoofdstuk 1** wordt een inleiding gepresenteerd om de huidige belangstelling voor het terugwinnen van EPS te benadrukken. Vervolgens worden verschillende onderzoeksvragen over EPS in vlokkig slib gepresenteerd, die dienen als leidraad voor het gehele proefschrift en voor de opzet van de experimenten in deze studie.

Hoofdstuk 2, richt zich op het EPS-terugwinningspotentieel van vlokkig slib. Hiervoor werden verschillende slibmonsters van grootschalige afvalwaterzuiveringsinstallaties in China verzameld om EPS te extraheren. Door middel van chemische karakterisering en microbiële gemeenschapsonderzoeken werden de invloedskenmerken en de correlaties tussen deze factoren geëvalueerd. Het EPS-rendement varieerde van 9% tot 19% van de organische fracties van ruw slib. De bevindingen geven aan dat de productie van EPS sterk afhankelijk is van externe omgevingen en een aanzienlijke relatie vertoont met de diversiteit en overvloed van bacteriën. Deze resultaten tonen aan dat vlokkig slib een waardevolle bron kan zijn voor EPS-terugwinning.

Het doel van **hoofdstuk 3** is om de verbanden tussen verschillende externe factoren met EPS te achterhalen. Op laboratoriumschaal werden sequencing batch

bioreactoren (SBR's) opgezet om onder gecontroleerde omstandigheden vlokkig slib te kweken (operationele en afvalwater kenmerken). De evaluatie van enkel de opbrengst, toonde dat voeding met afvalwater rijk in zetmeel opmerkelijk positieve effecten had op de vorming van EPS. Lage temperaturen ondersteunden ook de EPS-synthese. Organische belasting en slibretentietijd (SRT) bleken echter slechts geringe effecten te hebben. De verandering in de EPS-samenstelling en -eigenschappen toonde aan dat zowel de afvalwater kenmerken als de operationele omstandigheden de EPS-samenstellingen aanzienlijk veranderden.

Gezien het belang van kennis over EPS-structuren, verschuift **hoofdstuk 4** de focus naar de bestudering van EPS-samenstellingen en -structuren. EPS werd geëxtraheerd en gefractioneerd in verschillende delen. Vergelijking van de monosaccharidenanalyse en de identificatie van functionele groepen toonden aan dat EPS geen typische alginaatstructuren bevatte, zoals guluronzuren en mannuronzuren. Verdere analyse van de fracties suggereerde de aanwezigheid van glycolipidenstructuren, wat het belang van glycolipiden (zoals LPS, één type glycolipiden) in EPS benadrukt.

Hoofdstuk 5 richt zich op de rol van LPS-structuren en hun bijdrage aan de eigenschappen van EPS. Hierbij werd EPS uit vlokkig slib vergeleken met dat uit korrelslib. LPS werd eerst geïsoleerd uit EPS en daarna gekarakteriseerd. Het LPS-rendement van EPS in vlokkig slib bedroeg ongeveer 25% van de organische fractie van EPS, wat hoger was dan dat van korrelslib (15%). LPS uit vlokkig slib EPS vertoonde kenmerken zoals lagere hoeveelheden glycanen, kortere glycaanketenlengte, lager moleculair gewicht en een grotere kans op onverzadigde lipiden. Deze structuren resulteerden in de omgekeerde crosslinks van calciumgebonden LPS-aggregaten, wat bijdroeg aan de vloeistofachtige eigenschap van EPS-hydrogelmorfologie. LPS-Ca-aggregaten uit korrelig slib vertoonden daarentegen een bilaminaire meerlagige morfologie, wat bijdroeg aan de stevige, op zichzelf staande hydrogel van EPS.

Hoofdstuk 6 vat de belangrijkste bevindingen van dit proefschrift samen en biedt suggesties voor verder onderzoek. Hierbij worden onderwerpen zoals bacteriële activiteiten in verband met EPS-biosynthese, de functies van LPS-structuren, en de verkenning van EPS-toepassingen voor toekomstig onderzoek gepresenteerd.

Chapter 1

General introduction

1. Biological wastewater treatment process

The biological wastewater treatment process employs microorganisms to decompose and remove organic substances and nutrients (such as nitrogen and phosphorus) from wastewater. Most of the microorganisms exist in the form of microbial aggregates, such as sludge flocs and granules, which exhibit rich biodiversity and good sedimentation performance. Conventional biological processes utilize sludge flocs for treating industrial and municipal wastewater. In recent decades, newly developed aerobic granular sludge processes, characterized by dense and compact microbial aggregates, have also gradually been implemented in full-scale wastewater treatment plants.

These processes generate excess sludge as a byproduct of the transformation of pollutants into active biomass. Annually, millions of tons of excess sludge accumulate, posing significant pressures on treatment plants. The disposal of this excess sludge typically requires high investments, and improper treatments can lead to secondary environmental contamination, such as soil and water pollution. Furthermore, the efficient use of resources has become crucial, pushing sustainability efforts in wastewater treatment plants and making sludge disposal more challenging. Within this context, researchers are actively exploring methods to convert excess sludge into valuable resources.

2. Extracellular polymeric substances in sludge

The major components of sludge are bacteria and extracellular polymeric substances (EPS). EPS, secreted by microorganisms, plays an important role in the physical and chemical structures of flocs and granules, through a variety of physical and biochemical interactions such as electrostatic force, hydrogen bonding, and ionic bonds. EPS is a complex mixture of high molecular weight biopolymers and significantly impacts the properties and performance of sludge. It facilitates surface adhesion and provides a protective barrier to the microorganism against environmental stress, such as toxic substances and predatory organisms. Additionally, EPS mediates interactions between microbial communities and their environment, influencing the transport and bioavailability of organic substances and nutrients. This mediation enhances the overall efficiency of wastewater treatment processes (Sheng et al., 2010).

EPS consists of polysaccharides, proteins, lipids, humic substances, nucleic acids, and other biopolymers, along with some inorganic components. EPS can form a three-

dimensional architecture within the matrix (with pores, and channels) and carries numerous functional groups (e.g., carboxyl, phosphoric, amine, and hydroxyl), which significantly affect microbial aggregation (Yu, 2020)(Flemming and Wingender, 2010). The amount of EPS in sludge is affected by many factors, including influent characteristics and operational conditions. The complex interplays between these factors regulate the metabolic activities of microorganisms, thus influencing EPS synthesis. e.g., higher soluble organic but lower nutrient content (lower C/N and C/P ratios) in influent can promote EPS production. The low dissolved oxygen levels, low temperature, and base/acid conditions can stimulate EPS formation by altering bacterial metabolism (Sheng et al., 2010)(More et al., 2014). Additionally, the variability in EPS components and properties was influenced as well. High phosphate levels in wastewater might promote phosphate-rich EPS (Li et al., 2015) while extended solid retention time (SRT) can result in EPS with higher molecular weight (Duan et al., 2014). Deciphering the connection between the operational conditions and EPS composition and quantity is of great importance.

3. EPS Recovery from sludge

EPS not only imparts unique properties to sludge but is also a promising resource to be recovered as biomaterials. EPS recovery was initially conducted from granular sludge. In granular sludge, the robust EPS matrix holds microbial communities densely together, maintaining structural integrity and promoting sludge granulation. These EPS exhibit outstanding performances and have been manifested as highly valuable biomaterials. Kim et al. (2020) displayed that EPS extracted from aerobic granular sludge could be utilized as non-flammable surface coating materials. Numerous reports documented the capability of granular sludge EPS to bind heavy metal ions (Ni^{2+}, Pb^{2+}, Cd^{2+}) (Guibaud et al., 2012; Li et al., 2017) or organic pollutants (Wei et al., 2015). Chen et al. (2024) found that when the water-soluble EPS was employed to glue two polymethylmethacrylate (PMMA) slides together without additional chemicals, the pull-off shear strength ranged from 36 to 51 kPa across a broad pH range of 2 to 10, indicating a potential application of EPS as a bio-based adhesive.

EPS recovery from flocculent sludge has recently gained increasing attention. Compared to the small number of around 120 granular sludge plants worldwide, conventional flocculent sludge technologies are far more widely installed. e.g. in Europe alone, there are approximately 26000 flocculent sludge plants, and in China, there are over 4700 centralized flocculent sludge plants. This makes flocculent sludge

a more accessible raw material, which can provide a substantial supply for EPS recovery. The properties of flocculent sludge EPS, which might be dissimilar to those EPS from granular sludge, could lead to similar or different applications. For example, Zhang et al. (2014) demonstrated that EPS from flocculent sludge can be used as a novel biosorbent for Cu^{2+} removal from water. Go et al. (2019) investigated the corrosion inhibition efficiencies of EPS extracted from flocculent sludge by testing with carbon steel in 3.64% NaCl saturated with CO_2 at 25 ^{o}C, and found EPS inhibited metal corrosion on both anode and cathode sites. Shi et al. (2024) coated the EPS on lettuce and spinach seeds and observed that it protected them from high temperature and humidity. Therefore, EPS from flocculent sludge is a promising resource to be recovered, once its composition and properties are well studied, it is possible to develop useful bioproducts further.

4. EPS components

Due to the complexity of EPS composition, its characterization is significantly challenged. Although EPS has been a subject of intensive studies in the environmental field for decades, the existing knowledge is mostly based on a general study of EPS components such as polysaccharides and proteins without considering their high compositional and structural diversity. In fact, proteins are formed by amino acids with post-translational modifications (such as glycosylation, lipidation, and phosphorylation). In polysaccharides, the repeating units (i.e., monosaccharides) may be present in different sequences and incorporate various substitutions (e.g., methyl, acetyl, amino, phosphoric) (Flemming and Wingender, 2010; Yu, 2020). In addition to these complexities, there are conjugated macromolecules e.g. glycoconjugates (Bourven et al., 2015). In this respect, the word “glycans” might be appropriate to refer to the sugar-based EPS composition. Glycans include non-conjugated (free) polysaccharides and glycoconjugates.

Free polysaccharides are carbohydrates composed of monosaccharide units linked together by glycosidic bonds. Seviour et al. (2011) provided an evidence for the presence of “granulan” (the highly complex heteropolysaccharide) in the EPS of the *Competibacter*-enriched (>60%) granular sludge from an acetate-fed laboratory-scale reactor. Gonzalez-Gil et al. (2015) noticed that the ^{1}H NMR spectra of the EPS extracted from anaerobic granules displayed sugar ring resonances, typically referring to free polysaccharides such as gellan and xanthan. Additionally, Felz et al. (2020b) reported that glycosaminoglycans (linear heteropolysaccharides containing a derivative of an amino sugar) including hyaluronic acid-like and sulfated

glycosaminoglycans-like polymers, might be widespread in biofilms, contributing to the stability of these systems. Besides these, a significant amount of the free polysaccharides related to EPS research focused on “alginate-like exopolymers” (ALE). ALE was extracted from sludge according to the same protocol as alginates (which is a family of polysaccharides containing only mannuronic and guluronic acids as monomers) extracted from brown algae (Lin et al., 2010). Following the Food and Agriculture Organization (FAO) identification assays and based on the certain similarity with alginates, the extracted EPS was termed as “alginate-like exopolymers”. Subsequent research has often associated EPS with alginates to investigate their compositions, properties, and applications. However, some controversial results were noticed, e.g. some studies have detected mannuronic acids in EPS samples (Sam and Dulekgurgen, 2016; Schambeck et al., 2020), while others have not found any uronic acids (Zahra et al., 2023). Felz et al. (2020) also reported that EPS did not exhibit the same gel formation mechanisms as alginate typically did. These results raise doubt about the existence of alginate structures in EPS. Given these conflicting findings and uncertainties, there is a growing interest in answering the question of whether there is alginate or not in the EPS.

On the other hand, glycoconjugates are mainly glycoproteins and glycolipids. Both of them have carbohydrate (glycan part) components attached to proteins or lipids. A hemagglutination assay of lectin from flocculent sludge EPS indicated that glycoproteins and lectins constitute a group of proteins that are embedded in the biofilm network organization (Park and Novak, 2009). The presence of glycoproteins has also been confirmed in EPS extracted from flocculent sludge and granular sludge by size exclusion chromatography (SEC), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and a far western blotting method (Bourven et al., 2015, 2012). Boleij et al. (2018) applied high-performance liquid chromatography (HPLC), Mass Spectrometry (MS) and SDS-PAGE to analyze the protein component and clarify the structures of glycoproteins from anammox granular sludge and suggested that, the targeted glycoprotein was one type of surface layer (S-layer) proteins, which are often glycosylated and are one of the most commonly observed cell surface structures of prokaryotes. More recently, Chen et al., (2023) analyzed glycoproteins from seawater aerobic granular sludge and noticed that the glycans in these glycoproteins were always modified with N-linked glycosylation and O-linked glycosylation, indicating the glycan profile of glycoproteins is altered in response to environmental stimuli.

It is worth noting that besides glycoprotein, glycoconjugates consist of glycolipids as well. Lipopolysaccharide (LPS) is one type of glycolipid. LPS is a major component of the outer cell membrane of Gram-negative bacteria, contributing greatly to the structural integrity of biofilms. LPS increases the negative charge of the cell membrane and helps stabilize the overall structure. The hydrophilic glycans and the hydrophobic lipids in LPS confer amphiphilic features, which endow them with unique properties. Unlike glycoproteins, the characteristics of glycolipids (e.g. lipopolysaccharides) in EPS are still not recognized. Advances in understanding the structures and function of glycolipids from EPS could enhance our knowledge of EPS molecules and open new avenues for their recovery and application in various industries.

To summarize, due to the large amount of wastewater treatment plants worldwide using activated sludge process, EPS from flocculent sludge is a promising resource to be recovered. In order to develop EPS-based products, it is significantly important to evaluate the recovery potential of EPS, the influencing factors, and unravel the exact chemical composition of the extracted EPS.

5. Scope and outline of the thesis

This thesis focused on the study of EPS recovered from flocculent sludge. For this work, EPS was extracted from flocculent sludge following the sodium carbonate extraction method and characterized afterwards. Originally, the targeted EPS component for characterization was alginate-like exopolymers due to its interesting gel-forming properties. However, driven by the doubt about the existence of alginate in the extracted EPS, the presence of alginate was reinvestigated. Interestingly, besides clearing this doubt, new findings pop up, leading to an exciting research direction. Therefore, to keep the footprint of this evolvement, the transition from alginate-like exopolymers (**Chapter 2** and **Chapter 3**) to the doubt (**Chapter 4**) and the findings of lipopolysaccharides (**Chapter 4** and **Chapter 5**) was maintained. Two main aspects explored in this thesis were listed as follows, and the scheme of the thesis is shown in Figure 1:

1. The recovery potential of EPS from flocculent sludge at full-scale wastewater treatment plants by taking China as a case study country (**Chapter 2**) and the correlation between the operational conditions and the EPS property by using lab-scale reactors operated under well-defined conditions (**Chapter 3**).

2. The exact EPS composition by clarifying the presence or absence of alginate (free polysaccharides) in the extracted EPS (**Chapter 4**) and the findings of lipopolysaccharides (one type of glycolipids) in the EPS extracted from both flocculent and granular sludge (**Chapter 4** and **Chapter 5**).

In Chapter 2, aiming to evaluate the EPS recovery potential from flocculent sludge, the EPS was extracted from sludge samples collected at eight full-scale wastewater treatment plants in China. The connection between the extraction yield and the microbial community was explored.

In order to search for the factors influencing the EPS extraction yield and its property, lab reactors were operated with well-defined synthetic media and operational conditions for the research in **Chapter 3.**

The focus of **Chapter 4** was to clarify the doubt of whether alginate was present in the extracted EPS or not. To achieve this, direct monosaccharides analysis by HPAEC-PAD of the EPS in comparison to commercial alginate was performed.

Due to the findings of lipopolysaccharides in the extracted EPS in **Chapter 4**, with the aim of studying the differences and similarities of the LPS from flocculent sludge and granular sludge, the LPS was extracted from both types of sludge, the structures were investigated and compared in **Chapter 5**.

Chapter 6 summarized the findings generated from this thesis, discussed the limitations, and provided perspectives for future EPS research.

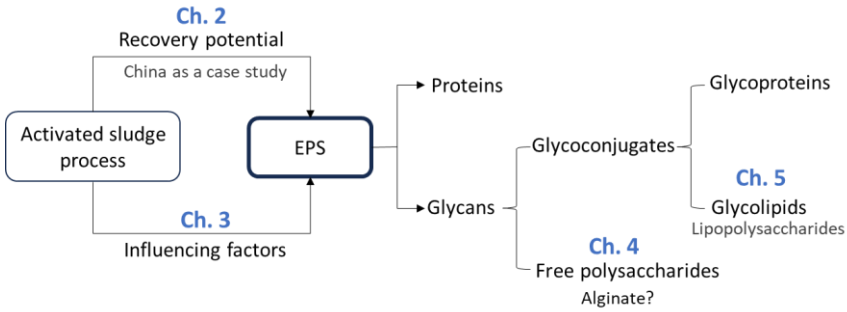

Figure 1 Scheme of the different chapters in this research focusing on EPS recovery and characterization from activated sludge.

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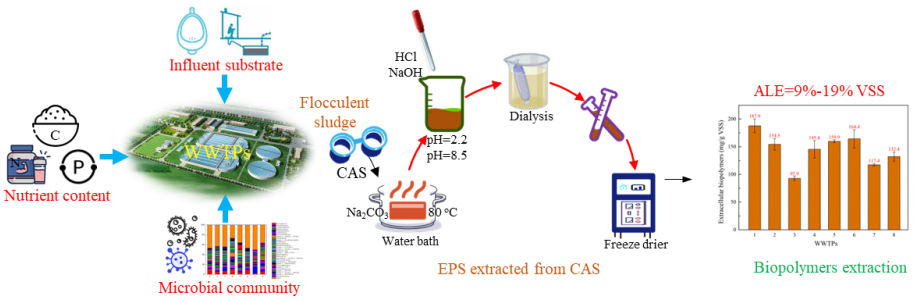
Chapter 2

Recovery of extracellular biopolymers from activated sludge: potential, characteristics and limitation



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Abstract

Extracellular polymeric substances (EPS) are biopolymers that can be recovered from waste sludge, which could contribute to more sustainable operation of wastewater treatment plants (WWTPs). EPS extraction from granules sludge has already been well studied and applied in the Netherlands. An example is alginate-like exopolymers contained in the EPS could be a potential resource with a highly-added value. On the other hand, there is little attention to the recovery of EPS from activated sludge. In this study, activated sludge samples from eight conventional biological process plants in China were collected and then EPS were extracted to investigate their recovery potential, chemical properties and limiting factors. The results revealed that the EPS extracted d from activated sludge ranged from 90 to 190 mg/g VSS. The compositional characteristics of the EPS were observed by FTIR, 3D-EEM and UV-visible spectra, demonstrating some differences in the composition and properties of EPS from different WWTPs. The EPS had a similarity of about 60% to commercial alginate concerning chemical functional groups and the alginate equivalent was over 400 mg per gram of EPS. Moreover, the EPS consisted of poly-(guluronic acid) blocks (20%-30%) and poly-(guluronic acid-mannuronic acid) blocks (8%-28%), and the hydrogel formation tests indicated that condensed beads were immediately formed once the drops of the biopolymers came in contact with $CaCl_2$ solution. These results demonstrated that the EPS had a relatively high gel-forming capacity and might also have a potential application as commercial alginates. Furthermore, the factors influencing EPS formation such as influent substrate, nutrient content and microbial community and the related mechanisms were investigated. Among them, increasing soluble organics and low nutrients (C/N/P) in the influent could promote EPS formation. Also, different bacteria might have positive or negative effects on EPS formation. The diversity and abundance of bacteria were identified as a crucial and decisive factor controlling EPS production and compositions.

1. Introduction

Resource recovery from wastewater is currently emphasized to develop a sustainable wastewater treatment system. In this context, excess sludge can be considered a potential resource for recovering highly valuable products such as phosphate, cellulose, bioplastics, biopolymers, *etc*. (Li et al., 2015; Loosdrecht and Brdjanovic, 2014). Activated sludge is a matrix of bacterial cells embedded in the extracellular polymeric substance (EPS). The EPS accounts for about 10%-40% of the total dry weight of activated sludge (Basuvaraj et al., 2015). EPS mainly consists of polysaccharides, proteins, lipids, nucleic acids and humic substances (Boltz et al., 2017; Felz et al., 2016). The EPS substances embed the microcolonies and form a matrix in which these microcolonies and single cells are embedded by flocculation. Various physical and biochemical interactions such as electrostatic force, hydrogen bonding, or ionic bonds form the mechanisms for the floc formation and stability (Basuvaraj et al., 2015; Mahendran et al., 2012). EPS is considered as the protective matrix for environmental pressures, mechanical stability, external heavy metals, and toxic compounds and also as the storage of carbon or water (Schambeck et al., 2020; Sheng et al., 2010). The biopolymers in the matrix form a unique structure that can be recovered for highly valuable biomaterials (T.T. More et al., 2014; Sheng et al., 2010).

Much attention on recovering the biopolymers has focussed on EPS from granular sludge (Lin et al., 2018, 2015; Xue et al., 2019). EPS was identified as the substitute for alginates that can be used in the food, paper, textile, medical, and construction industries and also in agriculture and horticulture (Felz et al., 2019; Kim et al., 2020; Lin et al., 2018, 2015; Rehm, 2010; Xue et al., 2019). Lin et al. (2015) have evaluated the performance of EPS extracted and purified from granular sludge as non-flammable surface coating and Kim et al. (2020) showed that EPS could comply with the aviation requirements for aircraft interiors by the Federal Aviation Regulations (FAR). Currently, EPS extraction from granular sludge has been scaled up to a demonstration scale in an installation in Zutphen (the Netherlands). Because of its unique properties, the EPS has been branded Kaumera®.

Kaumera recovery from granular sludge is moving towards a commercial market introduction. However, according to the current studies, the content of EPS from activated sludge is only 7%-19% VSS (Lin et al., 2010, 2013; Schambeck et al., 2020; Yang et al., 2014), which is lower than that of granular sludge (35.1%±1.9% SS) (Kim et al., 2020; Lin et al., 2010). On the other hand, the total amount of excess sludge in

conventional WWTPs is huge. For example, there were more than 60 million t/a (80% moisture content) of excess sludge in China in 2019. Handling excess sludge accounts for nearly half of the total investment and operation costs of WWTPs. If the EPS recovery from activated sludge could turn out to be high-value biopolymers like EPS from granular sludge, it could offset some of the costs of handling sludge and also make WWTPs more sustainable towards the circular (Hao et al., 2019; Li et al., 2015; Loosdrecht and Brdjanovic, 2014). Conversely, there have been relatively few studies on recovering EPS from activated sludge, while the existing studies on EPS around activated sludge mostly focused on identifying and evaluating their roles in biological nutrient removal and sludge disposal processes (Seviour et al., 2019; Sheng et al., 2010). The economic value of EPS from activated sludge was largely ignored.

For this reason, this study was initiated to investigate EPS extracted from activated sludge. Activated sludge from eight plants from different regions of China was collected, and then EPS was extracted in accordance with the previous methods (Felz et al., 2019, 2016). The amounts and compositions of EPS were investigated and evaluated by comparing them with EPS from granular sludge. Next, the physical and chemical properties of the EPS were analyzed to clarify their performance gaps relative to commercial alginate. Finally, the influences and related mechanisms of such factors as substrate and nutrient content in influent were summarized to deeply understand the EPS formation in activated sludge.

2. Materials and methods

2.1. Sludge characteristics

Activated sludge samples were collected from eight WWTPs (WWTP 1-8, the conventional biological nutrient removal processes with municipal wastewater) in different regions of China. The locations and activated sludge characteristics are listed in Table S1. The average influent quality and other detailed information are listed in Table 1. The activated sludge was concentrated by a 0.15-mm filter, sieved, and then stored in a refrigerator (4 ^{o}C) for use.

2.2. Extraction protocols

EPS were extracted with the method described by (Felz et al., 2019, 2016). The washed and centrifuged activated sludge was put into baffled flasks. Na_2CO_3 in the demineralized water (up to the concentration of 0.5% (w/v)) was added to the sludge samples. Hence, the total volume of the mixture was kept at 400 mL with MLSS=30 g/L. The flasks were then stirred for 35 min at 80 °C in a water bath and finally

centrifuged at 4000×g and 4 °C for 20 min. Supernatants were collected and pellets (for baffling) were removed respectively. pH of the supernatants was adjusted to 2.2 with 1 M HCl for purification. Then, the acidified supernatants were centrifuged at 4000×g and 4 °C for 20 min again. The pellet was collected and re-dissolved with 1 M NaOH at pH 8.5. The dissolved biopolymers were dialyzed for 24 hours in a dialysis bag with a molecular weight cut-off of 3.5 kDa to remove the solubilized ions. Finally, the samples were frozen at -50°C and lyophilized (Lin et al., 2010). In this study, EPS extracted from eight different activated sludge were labeled as EPS-1 to EPS-8.

Table 1 Average influent qualities and biological nutrient removal processes of eight WWTPs.

WWTPs	Process	TCOD (mg/L)	SCOD (mg/L)	TN (mg N/L)	NH_4^+ (mg N/L)	TP (mg P/L)	PO_4^{3-} (mg P/L)
1	AAO/SBR	115.9±8.6	53.2±1.2	36.4±3.6	29.5±0.2	2.8±0.0	2.37±0.0
2	SBR	315.1±3.9	88.4±3.6	65.6±0.1	49.5±0.1	5.3±0.0	4.06±0.1
3 [A]	AAO	345.3±5.1	95.5±2.1	38.7±1.7	19.3±0.1	4.7±0.2	1.72±0.0
4	AAO	78.1±15.9	28.5±8.5	20.5±1.4	16.0±0.3	1.6±0.1	1.74±0.0
5	AAO	191.8±18.6	79.9±0.7	48.8±0.2	33.1±0.4	3.6±0.1	3.27±0.1
6	AAO	186.3±15.8	88.1±30.3	40.4±1.6	27.6±0.2	3.3±0.0	2.42±0.0
7	AAO	108.4±2.8	27.7±0.2	21.7±0.9	15.8±2.7	1.5±0.0	1.76±0.4
8	AAO	195.5±1.5	58.5±2.8	40.7±2.2	35.4±1.4	3.5±0.3	NA [B]

[A]: Municipal wastewater: industrial wastewater=1:1. [B]: NA-not analysis.

2.3. Analytical methods

2.3.1. Chemical analysis

Total polysaccharide measurements were based on the phenol-sulfuric acid assay proposed by (Dubois et al., 1951) with D-glucose as the standard. The commercial alginate (extracted from brown algae) was chosen as the standard to evaluate the amount of alginate equivalents in EPS with the phenol-sulfuric acid assay as well (Felz et al., 2019; Lin et al., 2010). Total proteins were prepared according to the procedure of Lorry assay with bovine serum albumin used as the standard (Lowry et al., 1951). TS (total solid), VS (volatile solids), MLSS (mixed liquor suspended solids), MLVSS (mixed liquor volatile suspended solids), and pH were detected, according to the standard methods (APHA, 2012). The fractionations of the EPS, including a family of copolymers comprised of mannuronic acid (M) and guluronic acid (G) units arranged in an irregular block pattern of varying proportions of GG, MG, and MM blocks, were detected by the methods of partial acid hydrolysis as described in (Lin et al., 2013). Moreover, ionic hydrogel formation was tested with 2.5% (w/v) $CaCl_2$ following the procedure described by (Lin et al., 2013) and (Felz et al., 2016).

A one-way ANOVA was used to estimate the difference between activated sludge from different WWTPs. The significance level of 95% was based on the data obtained from the duplicated experiments. The correlation coefficient (R^2) between different independent variables was also analyzed in this study.

2.3.2. Spectroscopic analysis

Other characterizations of EPS were detected by the UV-visible absorbance measurements from 800 to 200 nm using an Agilent Cary 5,000 UV–Vis–NIR spectrophotometer (resolution 1.0 nm). $SUVA_{254}$ and $SUVA_{260}$ were the normalized values of UV-Vis absorbance at 254 nm and 260 nm per mg TOC of the sample, respectively (Weishaar et al., 2003). E_2/E_3, E_2/E_4, and E_4/E_6 refer to the ratio of different UV-visible absorbance at 250 and 365 nm, at 240 and 420, and at 465 and 665 nm, respectively. Among them, $SUVA_{254}$ has a positive correlation with the aromaticity and $SUVA_{260}$ has a positive correlation with the hydrophobicity, while the indexes of E_2/E_3, E_2/E_4, and E_4/E_6 have a negative correlation with the humification degree, molecular size, and condensation (Liu et al., 2019; Polak et al., 2007).

The functional groups were analyzed by the Thermo Fisher Fourier transform infrared spectrometer (FTIR) at the wavenumber of 4,000-400 cm^{-1}. The spectra of commercial sodium alginate (extracted from brown algae, viscosity 4-12 cP, 1% in H_2O) were also recorded as the standard and the similarity between different EPS and the alginate was estimated with the help of FTIR software (Li et al., 2019).

Three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy was applied to identify fluorescent compounds contained in EPS (Sheng and Yu, 2006). In this study, 3D-EEM spectra were collected with subsequent scanning emission (*Em*) spectra from 220 to 550 nm at 1 nm increments by varying the excitation (*Ex*) wavelength from 200 to 500 nm at 5 nm increments. *Ex* and *Em* slits were maintained at 5 nm and the scanning speed was set at 1,200 nm/min for all the measurements. The spectrum of distilled water was recorded as the blank. The statistical analysis of 3D-EEM data was performed using the MATLAB software.

3. Results and discussion

3.1. Extraction yields and compositions

The amounts of EPS extracted from different activated sludge (WWTP 1-8) are shown in Table 2. WWTP 3 had the lowest yield of the biopolymers (92.9±3.3 mg/g VSS) about half as compared to the highest yield from WWTP 1 (187.9±12.3 mg/g VSS).

The amounts of the biopolymers ranged from 90 to 190 mg/g VSS (9%-19% VSS). Interestingly, the EPS yields are comparable to those analyzed in the previous studies with the same extraction methods, e.g., 187±94 mg EPS/g VSS with municipal wastewater (Schambeck et al., 2020); 100-150 mg EPS/g VSS with only propionate (Yang et al., 2014); 72±6 mg EPS/g VSS from aerobic activated sludge (Lin et al., 2013). There is a certain gap in the EPS amount between activated sludge and granular sludge cultured with sodium acetate (31±1.6% SS, (Lin et al., 2018), but the difference becomes less when compared with granular sludge fed with mixed wastewater (approximately 25% of slaughterhouse wastewater discharged in the sewage) (160±4 mg/g VSS, (Lin et al., 2010). In a word, EPS extracted from activated sludge seems to offer a comparative potential to these from granular sludge in a quantitative sense.

Table 2 Yields and proportions (total proteins and total polysaccharides) of extracellular polymeric substances (EPS).

Samples	EPS yields (mg/g VSS sludge)	Total polysaccharides (PS, mg/g EPS)	Total proteins (PN, mg/g EPS)	Ratio of PN/PS
EPS-1	187.9±12.3	130.6±3.0	366.2±7.0	2.8
EPS-2	154.5±10.4	119.8±9.8	383.3±6.3	3.2
EPS-3	92.9±3.3	123.5±11.5	258.2±15.3	2.1
EPS-4	145.4±15.6	112.5±5.9	376.1±20.8	3.3
EPS-5	159.9±2.3	121.8±9.5	464.7±56.3	3.8
EPS-6	164.4±16.4	109.6±2.5	467.6±10.5	4.3
EPS-7	117.4±2.1	106.9±6.0	358.5±13.9	3.4
EPS-8	132.4±8.3	75.1±8.8	337.8±26.6	4.5

Further characterization of the composition was conducted to evaluate the specific properties of the EPS. As shown in Table 2, the total polysaccharides in EPS-8 were the lowest, around 75.1±8.8 mg/g EPS, while EPS-1 showed the highest fraction, around 130.6±3.0 mg/g EPS. Moreover, total polysaccharides in the other EPS ranged from 105 to 120 mg/g EPS. The total proteins are also shown in Table 2. They varied significantly between different EPS samples. Some studies have noted the importance of the ratio of PN to PS (PN/PS) for evaluating the composition and properties of organics (Mahendran et al., 2012; Zhu et al., 2015). The PN/PS ratio of EPS extracted from AGS was at around 3.3 while anaerobic granules are reported to have a PN/PS ratio of 4.2. These values are higher than those reported for floc (1.6) and biofilms (1.2) (Mahendran et al., 2012; Zhu et al., 2015). A higher PN/PS ratio denotes more extracellular protein substances, which has been suggested as important for sludge granulation and stability maintenance (Park et al., 2008; Zhu et

al., 2012). In this study, PN/PS were also analyzed and the results are shown in Table 2. EPS-8 and EPS-6 had the highest PN/PS, at nearly 4.5. The PN/PS ratio of the EPS extracted from activated sludge ranged from 3.0 to 4.5, except for EPS-3 (1.9). The ratios were clearly higher than that of EPS reported before for activated sludge, but they are close to that of the EPS extracted from granular sludge (Mahendran et al., 2012; Zhu et al., 2015). The higher PN/PS ratio might be attributed to the different extraction protocols. The extraction protocol for EPS in this study has been developed to solubilize the structural EPS (Felz et al., 2016), whereas many previously used extraction methods did not really solubilize all EPS (Seviour et al., 2019).

3.2. Evaluation of extracellular biopolymers

3.2.1. Alginate equivalents and fractionations

EPS was considered as a potential substitute for alginates. In this study, commercial alginate was chosen as the standard to evaluate the amount of alginate equivalents in EPS (Lin et al., 2010). EPS-3 and EPS-8 exhibited the highest alginate equivalents, around 525 mg/g EPS, while EPS-6 was the lowest one, about 420.4±5.7 mg/g EPS. The alginate equivalents relative to the commercial alginate (42%-52%) for the EPS from activated sludge is similar as reported to the EPS extracted from granular sludge ranging from 48%-53% (Lin et al., 2010).

Alginate is able to form a gel matrix due to three different kinds of blocks including GG, MG, and MM blocks. They are distributed randomly in the molecular chain of alginate, which has specific properties and results in different fine chemical structures. Their proportion, distribution, and length determine the chemical and physical properties of alginate molecules. GG blocks have a higher affinity towards divalent cations than the other two blocks and the order of gel-forming capability of the three blocks is MM blocks ≤ MG blocks << GG blocks. In this study, the poly-mannuronic acid (M) and guluronic acid (G) in the EPS were isolated and measured aiming to evaluate the gel-forming capacity (Felz et al., 2016; Lin et al., 2013). The results (Table 3) indicated that the recovery yield of blocks from the biopolymers was at 40%-60%, with about 20%-30% GG blocks and 8%-28% MG blocks in the chemical structure of all the EPS. Moreover, the monomer ratios of G:M from different EPS were similar at about 2.0-3.5. Ionic hydrogel formation tests revealed that Ca^{2+}-EPS beads displayed good and similar hydrogel, which demonstrated that EPS was able to interact with divalent ions and formed a similar hydrogel as alginate did. These results indicated that alginate-like structures were one part of the important

structural EPS.

Table 3 Fractions of different building blocks obtained from extracellular polymeric substances.

Samples	Poly-G	Poly-MG	Poly-M	Recovery efficiency	Ratio G/M
EPS-1	21.9%±1.2%	16.9%±0.9%	6.5%±0.2%	45.2%	2.0
EPS-2	28.3%±1.5%	22.3%±0.1%	6.4%±0.1%	57.1%	2.2
EPS-3	20.3%±1.0%	16.4%±0.5%	1.2%±1.0%	38.0%	3.0
EPS-4	30.5%±2.3%	18.3%±1.5%	7.3%±0.3%	56.0%	2.4
EPS-5	23.2%±1.3%	15.4%±2.3%	3.3%±0.4%	41.9%	2.8
EPS-6	24.3%±0.3%	28.5%±0.5%	2.5%±1.0%	55.2%	2.3
EPS-7	23.3%±0.9%	13.4%±0.1%	3.3%±0.1%	40.1%	3.0
EPS-8	25.9%±1.3%	8.7%±0.3%	4.8%±0.1%	39.4%	3.3

3.2.2. Similarity of functional groups identification

The functional groups of EPS are the critical part reflecting the primary chemical properties of organics and also the effective parameters to evaluate the possible applications. Therefore, functional groups were measured and the obvious difference in band assignments and characters of functional groups contained in the biopolymers are listed in Table S2 (Jia et al., 2017; Niu et al., 2016; Yin et al., 2015; Zhang et al., 2016). The main structures of EPS varied from different WWTPs. Among them, EPS-3, 4, and 7 should belong to polysaccharide-dominated structures; in contrast, EPS-2, 5, 6, and 8 had more protein-like chemical substances. Both functional groups for polysaccharides and proteins corresponded to the indication of total proteins and polysaccharides analysis. These results demonstrated that different environmental or operational parameters (like influent quality, substrate, temperature, solid retention time, etc.) might have a strong influence on the composition of the biopolymers from activated sludge. The different structures and fractions of proteins and polysaccharides contribute to varied chemical properties and functions in EPS.

Moreover, commercial alginate was used as the standard substance to evaluate the similarity of functional groups by FT-IR software, and the results are shown in Table 4. No significant difference in the spectra was found and the quantitative similarity of chemical functional groups reached up to 60%-83%. Above all, these results provided some important insights in evaluating EPS extracted from activated sludge and comparing them with the EPS extracted from granular sludge in composition characterization and properties. It can be concluded that the EPS extracted from the

activated sludge seems to have similar properties as compared to the commercial alginate.

Table 4 Similarity of functional groups between extracellular polymeric substances (EPS) by the quantitative analysis via FTIR spectrometer identification software (Li et al., 2019).

Sample	EPS-1	EPS-2	EPS-3	EPS-4	EPS-5	EPS-6	EPS-7	EPS-8
Similarity	82.9%	79.5%	65.9%	71.8%	76.1%	64.3%	80.6%	62.0%

3.3. Spectral analysis for physical and chemical properties

3.3.1. UV-Visible spectroscopy

The special wavelengths in the UV-visible spectra are generally seen as the parameter strongly related to the degree of humification, aliphatic and aromatic compounds, which reflects the chemical structure of biopolymers stability (Liu et al., 2019; Polak et al., 2007; Weishaar et al., 2003). The results of the UV spectra of the EPS are shown in Figure S3. Based on the descriptions of these indexes in *Section 2.3.3*, differences among these EPS were obvious, as analyzed below: i) Analysis of humification shows that EPS-4 contained more stable compounds with higher molecular weight and also a higher degree of humification and condensation. In other EPS, the indexes of E_2/E_3=4.5-5.0, $E_4/E_6\approx$6.0, and $E_2/E_4>$5.8 indicate that the content of aliphatic compounds was relatively high. ii) Results of aromatics suggest that EPS-1 and EPS-3 contained more aromatic substances with higher unsaturation degree, while EPS-8 contained less aromatics. iii) Evaluation of hydrophobicity indicates that EPS-1 had a higher proportion of hydrophobic components, followed by EPS-3, EPS-4 and EPS-5 and EPS-8. Lower hydrophobicity is strongly associated with higher solubilities and instabilities of gel-forming in water. In summary, the chemical structures of the EPS-4 showed the most humified structure. On the other hand, EPS-1, EPS-2, EPS-7, and EPS-8 demonstrated different humified or aromatized structures but did not differ significantly from each other.

3.3.2. 3D-EEM spectroscopy

Three-dimensional excitation-emission matrix (3D-EEM) spectroscopy was used to further characterize the composition of EPS and the results are shown in Figure S4. According to the corresponding excitation (*Ex*) and emission (*Em*) wavelengths, the fluorescence regions are divided into Flu I-Flu V areas. Among them, Flu I and Flu II indicate aromatic protein-like substances (aromatic protein I and II), respectively. Flu IV represents soluble microbial by-product-like substances (SMPs). Flu III and Flu V include fulvic acid-like and humic acid-like organics (Dai et al., 2018). There are clear

peaks of fluorescent substances at Flu IV for EPS-2~8, which indicates that biopolymers contained more SMPs. In contrast, EPS-1 revealed fewer SMPs, which was basically consistent with the relatively higher alginate equivalent in EPS-1, as concluded in *Section 3.2*. EPS-4 meant obvious absorbance peaks at Flu III and Flu V, which indicates that it contained higher contents of fulvic acid-like and humic acid-like substances. Moreover, the quantitative analysis of fluorescence density indicated the main fluorescent substances contained in the biopolymers were varied from others, which implies that the chemical properties of the biopolymers are relatively different.

4. Factors limiting EPS formation

Obviously, EPS from activated sludge is attractive as the potential substitute for commercial alginates. However, as before mentioned, there were still differences in EPS amount and their physical and chemical properties from activated sludge compared with EPS from granular sludge. Thus, it is necessary to further analyze the factors including influent substrates, nutrient content, and microbial community, to figure out the connections between EPS formation and associated properties.

4.1. Influent substrate

As shown in Table 1, ratios of SCOD/TCOD were different ($p<0.05$) from different WWTPs. WWTP 3 had a low SCOD/TCOD ratio (about 27.7%) as 50% of influents were deprived of industrial wastewater. Interestingly, WWTP 3 exhibited the lowest EPS production at around 92.9±3.3 mg/g VSS. The same phenomenon also happened in WWTP 7, in which the influent SCOD/TCOD was 25.2%, resulting in a relatively low level of EPS extraction (115.3 mg/g VSS). On the other hand, WWTP 1 had the highest EPS production, up to 187.9 mg/g VSS, corresponding to the highest influent SCOD/TCOD ratio, 45.9%. It could be concluded that the soluble organics (SCOD) content in the influent influenced the EPS formation to an extent; a high SCOD content would enhance the EPS formation. These relationships may partly be explained by the fact that higher soluble organics could benefit microbes by promoting metabolisms and thus enhance the secretion of metabolites (the main origins of EPS). This explanation was also supported by (Yang et al., 2014), who claimed that organic loading was related to the formation of biopolymers. Increasing soluble organics would stimulate granules to secrete more extracellular cyclic diguanylate monophosphate (c-di-GMP, a well-conserved second messenger in bacteria that promotes granules formation and regulates high-glycans formation)

and contribute to further secretion of the biopolymers (Ross et al., 1987; Yang et al., 2014). In addition, (Peng et al., 2012) also held that increasing the influent COD led to a decrease in the polysaccharides content in EPS. An exceptional case occurred for WWTP 2, in which the influent SCOD/TCOD was as low as 28.1%, but its EPS extraction was as high as 154.5 mg/g VSS. It is thus hypothesized that there might be other factors affecting the EPS information. Moreover, the high degree of humification of EPS-4 was caused by the low influent COD concentration and a long SRT in WWTP 4, which resulted in the serious endogenous metabolism of flocs and thus produced more humic acid-like substances and soluble microbial by-product-like substances.

4.2. Nutrient content (C/N/P)

It has been confirmed that the yield of EPS depends heavily on the influent nutrient content (C/N/P) (Hoa et al., 2003; Liu et al., 2006; Sanin and Durmaz, 2003; Wang et al., 2014). Therefore, the linear correlations between EPS yields and influent C/N ($p<0.05$) & C/P ($p<0.05$) were calculated and the results are shown in Figure 1. The EPS production gradually increased along with decreasing C/N and/or C/P and the correlation coefficients between C/N and EPS were at R^2=(-)0.71 and C/P at R^2=(-)0.72. There are several possible explanations: i) external unfavorable surroundings (low C/N or C/P, high N & P load) for bacteria leading to low metabolisms activities but promoting more EPS secretion including exoenzymes as protective layers; ii) cell disintegration and autolysis also releasing intracellular substances from cells and thus increase the content of proteins and polysaccharides in EPS (Hoa et al., 2003; Liu et al., 2006; Sanin and Durmaz, 2003; Wang et al., 2014). Shortly, reducing the carbon source or increasing the nutrient content (N or P) in influent (lower ratio of C/N/P) could be speculated to increase the EPS formation in activated sludge.

On the other hand, some research revealed that C/N>20 resulted in a high content of polysaccharides and thus contributed to EPS increase (Hoa et al., 2003; Liu et al., 2006; Sanin and Durmaz, 2003; Wang et al., 2014), which might be due to that excessive carbon sources could not be fully transformed by microorganisms and accumulated for formatting polysaccharides in sludge matrixes. It may be also related to the highly soluble organics resulting in a high value of C/N and promoting the EPS production as described above. As for the overloading carbon source (C/N>40), bacteria would be in an abnormal condition with a lack of N and P elements for cell proliferation and growth. Thus, extracellular substances are likely to be endogenously consumed to supply N and P. Under this circumstance, the

relationships between EPS and nutrient content (C/N/P) are still unclear and need to be further studied.

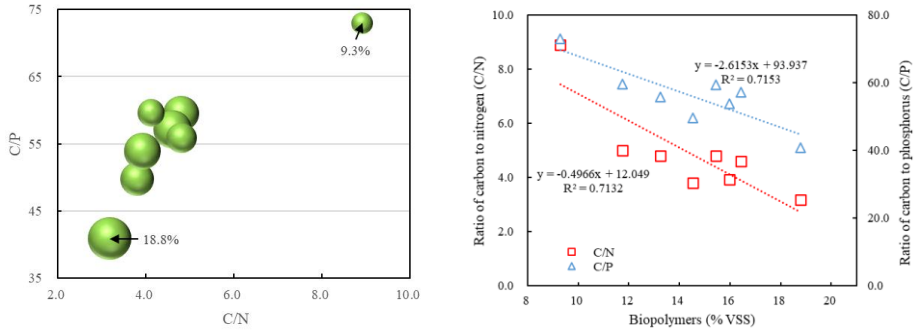

Figure 1 Linear fitting of relationships between extracellular polymeric substances (EPS) and nutrient content (C/N/P).

4.3. Microbial community

Biopolymers are produced by the microbial community in the activated sludge. Would microbial abundance and activity take key roles in regulating and formatting EPS? With this question, bacterial diversity and abundance were checked to investigate the inextricable relationship between microbes and EPS. The linear correlation coefficients (R^2) showed that the EPS tended to be negatively correlated with the bacterial abundance (Ace index, R^2=(-)0.30), followed by a negative correlation with the diversity index (Shannon, R^2=(-)0.24), whereas almost no correlation with the bacterial homogeneity in diversity (Simpson index, R^2=0.03). These results indicate that the dominant species and higher abundance of bacteria would decrease the EPS, which might be attributed to that high EPS yields could be expected with the enrichment of pure bacterial strains. On the contrary, some studies suggested that there were synergistic effects between different microorganisms, which would increase the utilization efficiency of the substrate and thus result in the formation of EPS (T. T. More et al., 2014). This discrepancy could be caused by some special enzymes secreted by different microorganisms which could help other microorganisms to obtain sufficient nutrient requirements in the complex environment and increase the formation of EPS. Anyway, there is no convincing conclusion on the effect of dominant species and their abundance associated with EPS formation.

4.3.1. Phylum levels

A sequential analysis of the microbial community from the different WWTPs was

annotated by the phylum levels of genus species using the RDP Classifier. The abundance of microorganisms at the phylum levels is shown in Figure 2.

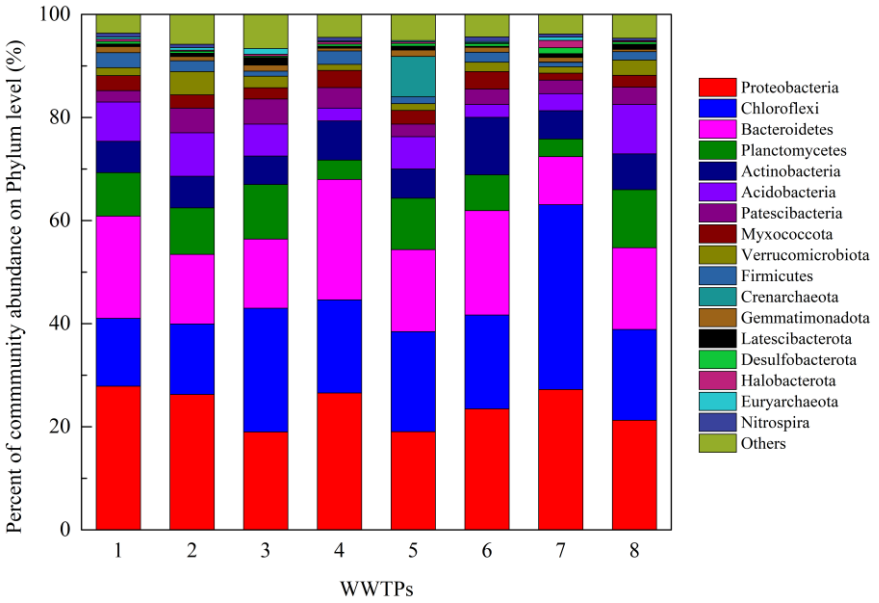

Figure 2 Abundance of microorganisms at the phylum levels from the different wastewater treatment plants (WWTPs).

The correlation coefficients (R^2) between the microbial community at the phylum level and the EPS are shown in Table S5. As shown in Figure 2, the phylum, *Proteobacteria*, *Chloroflexi*, *Bacteroidetes,* and *Planctomycetes* were at a higher level, ranging from 10% to 30%. In addition, the abundances of *Proteobacteria*, *Bacteroidetes,* and *Chloroflexi* were significantly correlated with EPS formation. For example, the abundance of *Proteobacteria* and *Bacteroidetes* in WWTP 3 was the lowest one, around 19.0% and 13.4%, respectively, and *Chloroflexi* was the highest one, at 24.0%, whereas the EPS extraction from WWTP 3 was at the low level (92.9±3.3 mg/g VSS). A similar tendency also occurred in WWTP 7. Moreover, the abundance of *Proteobacteria* and *Chloroflexi* in WWTP 1 was at 27.9% (highest) and 13.2% (lowest), respectively, but the EPS extraction was the highest one, 187.9±12.3 mg/g VSS. Above all, it can be confirmed that *Proteobacteria* and *Chloroflexi* phylum took a critical role in producing EPS, as also found in some previous studies (Lee and Mooney, 2012; Meng et al., 2019; Zhang et al., 2016). Furthermore, the phylums, *Firmicutes*, *Myxococcota*, *Bacteroidetes,* and *Nitrospira,* had a strong positive correlation (R^2>0.5), while *Latescibacterota, Chloroflexi,* and *Zixibacteria* tended to be a significantly negative correlation (R^2>(-)0.6). It could be concluded that different

microbial communities at the phylum level had an important effect on the EPS formation.

4.3.2. Family levels

The abundance of microorganisms at the family levels is shown in Figure 3. The relationship between family levels and the EPS was analyzed and shown in Table 5. Different families significantly affected EPS formation, as EPS mainly originates from cellular secretion and is closely associated with bacteria metabolism. These results also indicated that EPS formation and characteristics varied with synergetic interactions between different bacteria rather than only depending on one specific bacteria. Hence, the high correlation coefficient (0.73) of one kind of microorganism means significant effects on EPS formation, while low coefficient (such as 0.31 and 0.15) means a minor effect.

The family, *Saprospiraceae* (mainly functioned as denitrification in the biological nutrient removal process), indicated a positive correlation with the EPS extraction (R^2=0.50). In WWTP 3, there was a relatively low abundance of Family *Saprospiraceae*, at 6.6%, and the EPS was also at the lowest level, with a further low protein content at 258.2±15.3 mg/g EPS. Similarly, WWTP 1 and WWTP 6 took the trend that the higher abundance of Family *Saprospiraceae* had the higher EPS extraction. (Xia et al., 2008) have indicated that Family *Saprospiraceae* degraded organics by secreting associated enzymes (extracellular proteins) in the flocs matrix and gave an increase to protein content in EPS. A strong negative correlation (R^2=(-)0.73) between Family *Anaerolineaceae* and EPS was also found. (Narihiro et al., 2012) concluded that Family *Anaerolineaceae* played a main role in determining the acidification and catabolism of polysaccharides and thus promoting the conversion of large molecules into small molecules with easy absorbability and usability for bacteria. Thus, extracellular polysaccharides would also be degraded by the higher abundance of Family Anaerolineaceae resulting in the lower EPS production. There was also a positive correlation (R^2=0.31) for Family *Comamonadaceae*, which was already reported as putative PAOs (Ge et al., 2015) and mainly associated with the production of zoogloeal (Sadaie et al., 2007). The low abundance (less than 2%) of Family *Comamonadaceae* in WWTP 3 and WWTP 7 resulted in the insufficient formation of zoogloeal and thus a lower EPS extraction. There was a significant positive correlation (R^2=0.50) between EPS extraction and Family *Nitrosomonadaceae*, which is regarded as the dominant AOB. Besides, other microbes also showed a correlation with EPS extraction.

In conclusion, the results confirm that the microbial community correlates with EPS formation. In other words, both influent parameters and operating conditions could ultimately affect the dominant bacteria in the biological process and eventually lead to the difference in the formation of EPS.

Table 5 Relationships between the microbial community at the family levels and extracellular biopolymers extraction.

Family Genera		*Saprospiraceae*	*Anaerolineaceae*	*Comamonadaceae*	*Rhodocyclaceae*	*Chitinophagaceae*	*Nitrosomonadaceae*
WWTPs	1	11.58%	1.90%	3.55%	4.48%	2.56%	2.43%
	2	5.83%	2.52%	3.99%	7.11%	1.25%	2.77%
	3	7.05%	8.00%	2.11%	1.81%	1.80%	1.28%
	4	10.87%	2.01%	6.63%	5.27%	5.33%	2.68%
	5	4.35%	3.23%	3.04%	3.32%	4.12%	1.94%
	6	9.32%	2.58%	3.30%	2.30%	1.13%	2.53%
	7	2.23%	13.68%	1.14%	2.24%	0.80%	2.75%
	8	6.11%	4.49%	5.34%	1.65%	4.07%	1.72%
R^2		0.50	(-)0.73	0.31	0.49	0.15	0.50

Key functions of ***Saprospiraceae***: denitrification, degrade protein; ***Anaerolineaceae***: Denitrification, decompose carbohydrates via fermentation; ***Comamonadaceae***: Produce zoogloeal, with a reduced increase in cell mass; ***Rhodocyclaceae***: denitrifying rod-shaped bacteria; ***Chitinophagaceae***: Denitrification; ***Nitrosomonadaceae***: Nitrosation/conversion of organic nitrogen to nitrate.

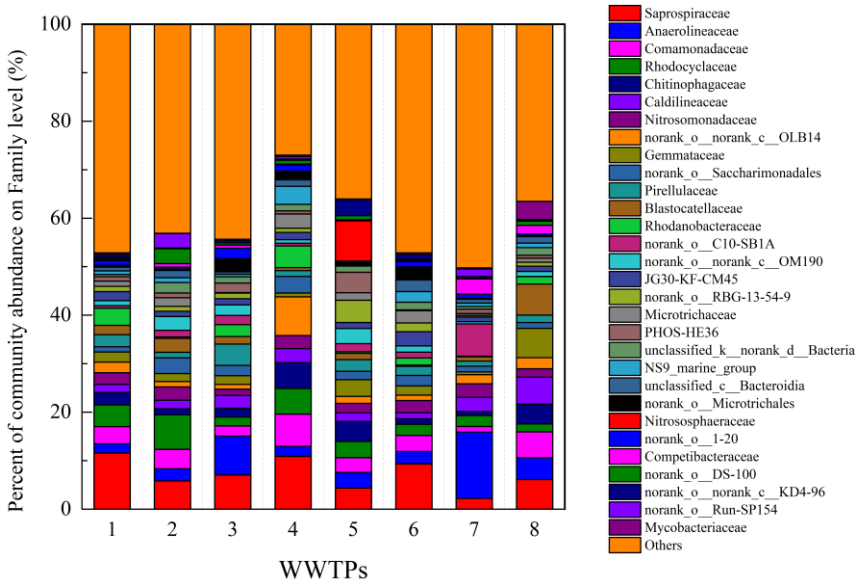

Figure 3 Abundance of microorganisms at the family levels from the different wastewater treatment plants (WWTPs).

5. Conclusions

The yield and the chemical characteristics of extracellular biopolymers extracted from the flocs of activated sludge were analyzed and evaluated, and the properties of the biopolymers were also elucidated. Moreover, the influencing factors and related mechanisms of EPS formation were identified. Based on the results, the main points can be addressed as follows:

i) EPS extraction from activated sludge (eight wastewater treatment plants, WWTPs) ranged from 90 to 190 mg/g VSS, which is comparable to the lower levels reported for the production of the EPS extracted from granular sludge.

ii) The chemical structure evaluation of the EPS from activated sludge revealed a similarity of >60% to commercial alginate concerning functional groups.

iii) Alginate equivalent of >400 mg/g EPS and 40%-60% of the mannuronic acid (M) and guluronic acid (G) units in the biopolymers as well as the good performances of the ionic hydrogel formation mean a potential application of the EPS from activated sludge as a promising substitute for commercial alginates.

iv) The general composition of the biopolymers extracted from the eight WWTPs was quite similar, such as proteins, polysaccharides, and humic acids. However, there were also some differences in the component content, which could result in some different chemical properties of the biopolymers.

v) Both influent substrate and nutrient content affected EPS formation, which was mainly associated with substance degradation and cellular metabolism.

vi) The dominant bacterial involved in sludge was a decisive factor affecting EPS formation, behaving differently with either positive or negative effects. Moreover, EPS formation was mostly associated with nitrification and denitrification processes.

In conclusion, EPS extracted from activated sludge was also confirmed as a sustainable alternative for some existing chemical materials. However, EPS production from activated sludge was as low as 9%-19%. Therefore, it is meaningful to improve the formation of the biopolymers in activated sludge by some effective strategies, such as modifying the influent and optimizing the operational parameters as well as the extracted protocols. Moreover, the enrichment of the dominant bacteria in the biological nutrient removal processes without affecting the effluent quality of WWTPs would be also the optimal measurement for maximizing EPS production.

Supporting information

Table S1 Locations and characteristics of different flocculent excess sludge.

WWTPs	Locations	MLSS	MLVSS	MLVSS/MLSS
1	Huludao city, Liaoning province, in Northeast China	30.0±0.1	13.4±0.3	44.6%
2	Dingzhou city, Hebei province, in Northern China	24.0±0.2	10.9±0.4	45.5%
3	Linyi city, Shandong, province, in Eastern China	14.9±0.4	8.7±0.5	57.9%
4	Anyang city, Henan province, in Central China	27.4±3.1	14.1±1.2	51.5%
5	Shenzhen city, Guangdong province, in Southern China	32.9±0.01	15.9±0.01	48.2%
6	Fushun city, Sichuan province, in Southwest China	64.4±0.1	13.4±0.6	20.9%
7	Haining city, Zhejiang province, in Eastern China	34.9±0.01	14.4±0.4	40.2%
8	Chaoyang Gaobeidian district, Beijing, in Northern China	28.1±0.8	15.1±0.9	53.6%

Table S2 Different band assignments for the FTIR spectral features (cm^{-1}) of different extracellular biopolymers.

Wavenumber (cm^{-1})	Vibration types	Corresponding groups
950	O-P-O stretching	Nucleic acids
1,072	C-H in-plane bending	Polysaccharides
1,233	C-N stretching; C-OH stretching	Amide III; Polysaccharides
1,393	C-H stretching in $-CH_3$	Amines and Lipids
1,450	CH_3 bending, CH_2 stretching	Methyl, Methylene
1,530	C-H stretching, N-H bending	Amide II
1,655	C=O stretching	Amide I
2,930	C-H stretching (CH_2 groups)	carbohydrates and lipids
2,960	C-H stretching (CH_3 groups)	carbohydrates and lipids

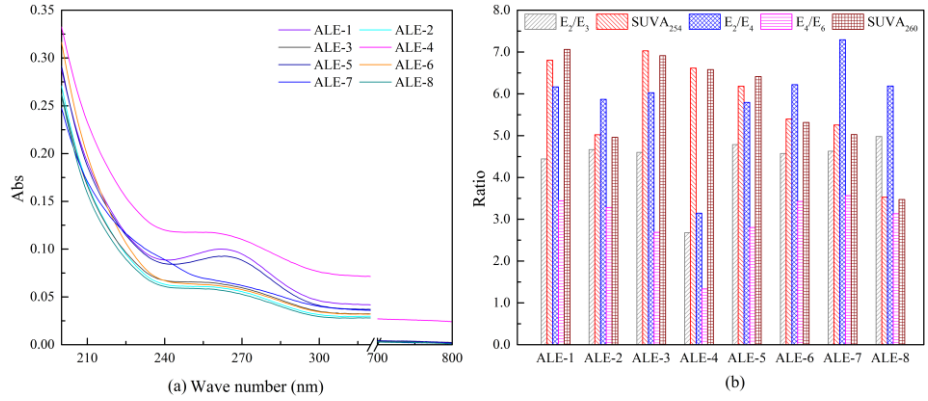

Figure S3 UV-visible spectra of extracellular biopolymers.

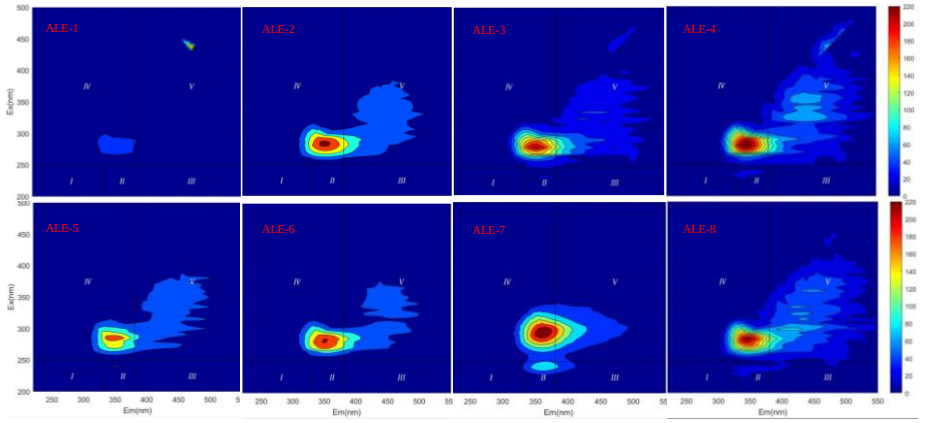

Figure S4 Three-dimensional excitation-emission matrix (3D-EEM) of extracellular biopolymers. (EEM spectra were collected with subsequent emission (Em) spectra from 220 to 550 nm at 1 nm increment by varying the excitation (Ex) wavelength from 200 to 500 nm at 5 nm increments. Ex and Em slits were maintained at 5 nm and the scanning speed was set at 1,200 nm/min for all the measurements.)

Table S5 Correlation coefficients (R^2) between the microbial community at the phylum level and the EPS extraction.

Phylum levels	R^2	Phylum levels	R^2	Phylum levels	R^2
Firmicutes	0.76	*Crenarchaeota*	0.21	*Halobacterota*	(-)0.34
Myxococcota	0.66	*Gemmatimonadota*	0.11	*Patescibacteria*	(-)0.53
Bacteroidetes	0.58	*Acidobacteria*	0.08	*Zixibacteria*	(-)0.64
Nitrospira	0.54	*Planctomycetes*	(-)0.03	*Chloroflexi*	(-)0.67
Proteobacteria	0.41	*Verrucomicrobiota*	(-)0.06	*Latescibacterota*	(-)0.76
Actinobacteria	0.34	*Desulfobacterota*	(-)0.10		

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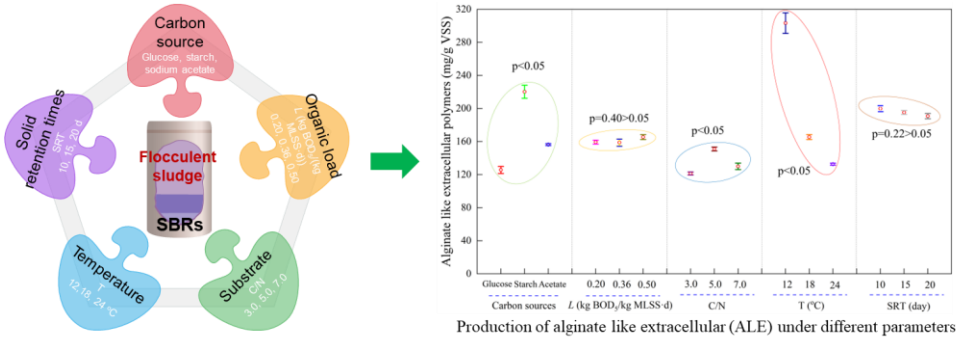
Chapter 3

Factors affecting the formation of extracellular polymeric substances in activated sludge



Production of alginate like extracellular (ALE) under different parameters

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Abstract

Extracellular polymeric substances (EPS) recovered from excess sludge have been evaluated as an eco-friendly, cost-effective, and sustainable alternative to highly valued materials. However, the EPS extraction from activated sludge ranges normally from 90 to 190 mg/g VSS, which is only equivalent to the lowest edge of the EPS production from aerobic granular sludge. But activated sludge is much higher in production than granular sludge and thus a further investigation was expected on key factors and associated mechanisms controlling EPS formation of activated sludge. The investigation was conducted by lab-scale sequencing batch bioreactors. The experiments revealed that activated sludge with starch used as an influent substrate contained the highest EPS production (220.3±8.0 mg/g VSS). The low temperature was favorable to enriching EPS, up to 303.3±21.5 mg/g VSS at 12 ^{o}C. Moreover, EPS reached up to 137.8±13.2 mg/g VSS at C:N=5:1 and slightly declined with increased or decreased C/N ratio. The specific EPS yield was 63.7 mg EPS/(g BOD_5) at a low organic load, which was twice as high as that with high organic loads. However, SRT had a minor effect on EPS formation. Obviously, such scenarios as starch-rich and low temperature could promote EPS production. Furthermore, the characteristic analysis among different EPS, confirmed, that different working conditions would exert a significant influence on the composition and chemical properties of EPS, which implies that controlling some parameters could be an approach to directionally cultivating EPS for their unique structures and potential applications.

1. Introduction

As a complex high-molecular-weight mixture of biopolymers in aggregates in biological wastewater treatment systems, such biopolymers as alginate-like exopolymers and sulfated polysaccharides have been identified as highly valuable raw biomaterials (Felz et al., 2020; Kim et al., 2020; Lin et al., 2018, 2015; Xue et al., 2019). For example, many researchers (Cao et al., 2019; Guibaud et al., 2003; Inbaraj et al., 2008) reported the potential applicability of the EPS as bio-sorbents for heavy metal and toxic organic compounds removal. In addition, the combination of the biopolymers with $Fe_2(SO_4)_3$ is promising to be applied in raw drinking water treatment (Ma et al., 2008). For this reason, biopolymers are being considered as a potential alternative to conventional chemical polymers because of their biodegradability, non-toxicity, and high efficiency.

The evaluation of EPS properties and structures in the previous studies indicated that EPS could be applied in the industries of food, paper, textile, medical, construction, and even in agriculture and horticulture (Felz et al., 2020; Kim et al., 2020; Lin et al., 2018, 2015; Xue et al., 2019). Lin et al. (2015) and Kim et al. (2020) concluded that EPS extracted from aerobic granular sludge was suitable as a substitute for the biomaterials for non-flammable surface coating. Much attention to recovering the biopolymers has focussed on EPS from granular sludge. EPS extraction from granular sludge has been scaled up to a demonstration-scale plant in Zutphen, the Netherlands. The recent study also revealed that there was a similarity of the biopolymers extracted from activated sludge, up to 60% concerning functional groups by FTIR spectra compared to the commercial alginate; the alginate equivalent of the extracted EPS was also over 400 mg/g EPS (40%-60% of the mannuronic acid and guluronic acid units); moreover. These results imply that the EPS extracted from activated sludge was also promising as a potential substitute for commercial alginates (Li et al., 2021). However, a lower EPS level was normally associated with activated sludge (ranging from 90 to 190 mg/g VSS) (Li et al., 2021), which is only at the lowest edge of EPS from granular sludge (200 to 350 mg/g VSS (Adav and Lee, 2008; Boleij et al., 2019; Felz et al., 2016; Zhu et al., 2015)). In practice, there is a larger amount of activated sludge produced in wastewater treatment plants (WWTPs) compared with granular sludge. There were relatively few studies on recovering EPS from activated sludge and the existing studies on EPS from activated sludge mostly focused on identifying and evaluating their roles in biological nutrient removal and sludge disposal processes. The economic value of EPS from activated

sludge seems largely ignored.

Under the circumstances, improving the EPS formation in activated sludge for recovering the biopolymers is significant and expected for a highly valuable biomaterials application. Among them, the EPS is usually associated with the substrate concentration and operational conditions, such as influent substrate, organic load, dissolved oxygen (DO), temperature, pH, sludge retention time (SRT), etc., which governs the substrate utilization rate and accordingly the formation rate of the biopolymers in aggregates. For example, a high DO concentration could promote microbial metabolisms and thus result in the high production of biopolymers (Sheng et al., 2010; Shin et al., 2000). Also, temperature and pH directly affected the metabolic process and thus changed the production and compositions of the biopolymers (More et al., 2014; Sheng and Yu, 2006). Furthermore, the biopolymers would be consumed or converted by the hydrolysis and fermentation processes as a result of the disintegration of sludge flocs under limited oxygen or depletion conditions (Shin et al., 2000). Finally, the changes of dominant microbial in the system under different SRTs would also affect the biopolymer formation (More et al., 2014; Sheng et al., 2010). Based on the findings in our previous study (Li et al., 2021), increasing soluble organics (SCOD) content and lowering nutrient content (C/N and C/P ratios) in the influent could be favorable to the EPS formation (Li et al., 2021). Different bacteria in biological nutrient removal (BNR) processes had either a positive or a negative effect on EPS formation (Li et al., 2021). However, it is still unknown the deeply associated mechanisms and the optimum working conditions for EPS formation in activated sludge. To recover highly valued biomaterials, further ascertaining the associated mechanisms and the key factors controlling the EPS formation in activated sludge should be necessarily effective in improving the potential of biomaterials' recovery.

Within this context, activated sludge in this study was cultured in the lab-scale sequencing batch bioreactors (SBRs) with different working conditions, including different carbon sources, organic load, substrate (C/N), temperature, and solid retention times (SRTs). The study focused on the nature, production, and recovery of EPS associated with their characteristics and properties. The involved mechanisms of different parameters affecting the EPS formation were a top priority. Principal component analyses (PCA) were also applied to determine the significant variables controlling EPS formation of activated sludge. Thus, the possible measurement and/or optimization for the EPS improvement were finally summarized for the

sustainable operation of WWTPs.

2. Materials and methods

2.1. Cultivation and collection of activated sludge

Activated sludge was cultured in lab-scale aerobic/anoxic sequencing batch reactors (SBRs) with a working volume of 5.0 L. The bioreactors were conducted daily at three consecutive cycles. As shown in Figure S1, each anaerobic/aerobic cycle comprised 8.0 hours including 4.5 hours aerobic, 2.5 hours aerobic, and 1.0 hours settling. After settling, one-third of the supernatant liquid was discharged (about 10 min) and fed with fresh influent (about 8 min in the aerobic phase). Finally, five minutes before the end of the aerobic phase, the designed volume of mixed liquor with biomass was withdrawn to maintain SRT for approximately 15 days. DO was kept at 3.0-4.0 mg/L in the aerobic phase. Stirring speed was 150 rpm/min in the aerobic and anoxic phases.

2.2. Experimental set-up

As shown in Table 1, five groups (I-V) were conducted with different influent parameters and operational conditions for activated sludge cultivation. Each group was controlled with various carbon sources (glucose, starch, and sodium acetate), organic load (*L*=0.20, 0.36, and 0.50 kg BOD_5/kg MLSS·d), influent substrate (C/N=3.0, 5.0, and 7.0), temperature (T=12, 18 and 24 ^oC) or solid retention time (SRT=10, 15 and 20 d), respectively. The other designed substrates are shown in Table 1. Other substrate and trace elements are shown in Table S2. Moreover, two parallel bioreactors were set up for each different operational parameter.

In this study, the removal performances of COD, nitrogen, and phosphate of bioreactors were measured. The average removal efficiency of COD, TN, NH_4^+-N, TP and PO_4^{3-}-P were stable at the high levels of around 88.6%-93.8%, 70.4%-84.4%, 96.0%-99.8%, 79.2%-83.2% and 93.4%-97.6%, respectively. After lasting for at least 15 days with stable nutrient removal, sludge samples were collected from the different bioreactors every day and afterwards concentrated by a 0.15-mm filter and stored in a refrigerator (4 ^oC) for EPS extraction.

2.3. EPS extraction protocols

EPS was extracted by the method of heating+Na_2CO_3 in accordance with the previous study. The mixture contained dry sludge (g), demineralized water (mL), and Na_2CO_3 (g) with a ratio of 1:50:0.25 and then was heated at 80 ^oC for 35 min in the water

bath. By adjusting the pH of the supernatant to 2.2, the biopolymers could gel for EPS further purification. Extraction of EPS from every different activated sludge was conducted in duplicate.

Table 1 Influent parameters and operational conditions in different bioreactors for cultivating flocculent sludge.

	COD (mg/L)	NH_4Cl (mg N/L)	KH_2PO_4 (mg P/L)	Carbon source	Organic load [A]	C/N [B]	T (°C) [C]	SRT (d) [D]
I	500	25	5.0	**Glucose, Starch, Sodium acetate**	0.36	20	24	15
	400	20	4.0		**0.20**			
II	700	35	7.0	Sodium acetate	**0.36**	20	24	15
	1,000	50	10		**0.50**			
	150					**3.0**		
III	250	50	5.0	Sodium acetate	0.36	**5.0**	24	15
	350					**7.0**		
							12	
IV	500	25	5.0	Sodium acetate	0.36	20	**18**	15
							24	
								10
V	500	25	5.0	Sodium acetate	0.36	20	24	**15**
								20

[A]: Organic load (*L*), kg BOD_5/(kg MLSS·d); [B]: C/N, BOD_5/TN; [C]: T, temperature; [D]: SRT, solid retention time.

2.4. Analytical methods of characterizations

Colorimetric assays were chosen to provide relevant insights about the composition contents of EPS. Polysaccharides (PS) and proteins (PN) analyses were detected by a phenol-sulfuric acid assay with D-glucose and the Lorry assay with bovine serum albumin respectively, based on the conceptual framework proposed by Dubois et al. (Dubois et al., 1951) and Lowry et al. (Lowry et al., 1951). One commercial alginate (extracted from brown algae, viscosity 4-12 cP, 1% in H_2O) was chosen as the standard with the phenol-sulfuric acid assay to evaluate alginate equivalents (Felz et al., 2019; Li et al., 2021; Lin et al., 2010).

The chemical functional groups of the EPS were analyzed by a Thermo Fisher Fourier transform infrared spectrometer (FTIR). The FTIR spectra in KBr pellets (98 mg KBr+2 mg sample) were recorded at the wavenumber of 4,000-400 cm^{-1}. Moreover, three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy was also applied to identify fluorescent compounds (Sheng and Yu, 2006). 3D-EEM spectra were collected with subsequent scanning emission (*Em*) spectra from 220 to 550 nm at 1 nm increments by varying the excitation (*Ex*) wavelength from 200 to 500 nm at 5 nm increments (Li et al., 2021). UV-visible absorbance measurements were used for detecting other characterizations such as the humification and

aromaticity degree from 800 to 200 nm using an Agilent Cary 5000 UV-Vis-NIR spectrophotometer (resolution 1.0 nm) (Li et al., 2021; Liu et al., 2019).

The fractions of mannuronic acid (M) and guluronic acid (G) poly-units in the biopolymers were isolated by partial acid hydrolysis (Felz et al., 2016; Lin et al., 2013). Ionic hydrogel formation was tested with 2.5% (w/v) $CaCl_2$ solution to evaluate the ionic hydrogel properties according to the procedures described by Lin et al. (Lin et al., 2013) and Felz et al. (Felz et al., 2019). The other parameters including COD, BOD_5, TN, NH_4^+-N, TP, PO_4^{3-}-P, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and pH were detected according to the standard methods (APHA, 2012).

2.5. Statistical analysis

In Group II with different organic loads, it is more meaningful to evaluate the specific EPS yield as calculated by *Eq. 1*.

$$Y_{ALE/BOD_5} = \frac{E_{ALE}}{BOD_{5,Inf.} - BOD_{5,Eff.}} \quad (1)$$

Where, $Y_{EPS/BOD5}$, the specific EPS yields, which means the EPS production with per gram organics (BOD_5) consumption; E_{EPS}, the EPS extraction; $BOD_{5,Inf.}$ and $BOD_{5,Eff.}$, the BOD_5 concentration in influent and effluent, respectively.

Analysis of variance (ANOVA) was also used to discuss the difference of EPS extracted from activated sludge with different working conditions based on the data obtained from the duplicated bioreactors. For each solution and each method, samples were taken in duplicate to ensure the accuracy of experimental data. Furthermore, PCA could reduce the dimensionality of the data set and thus transform the results into useful knowledge that could be easily interpreted. Therefore, it was applied to evaluate the cause-effect relationship correlations between EPS production and operational factors and thus to identify the decisive factors affecting EPS formation. In this study, relationships were observed by retaining the first two principal components (PC1 and PC2) and plotting in two dimensions where the cases and loading were set in the biplot.

3. Results

3.1. EPS extraction and compositions

The amount of EPS extracted from different activated sludge is shown in

Supplementary Information Table S3. The average extractions were in the range of 120-300 mg/g VSS, which were comparable to those EPS extracted from full-scale activated sludge (90-190 mg/g VSS) (Li et al., 2021) and were also similar to those results reported in the previous studies (Schambeck et al., 2020; Yang et al., 2014).

To an extent, therefore, activated sludge cultured by synthetic wastewater under different operational conditions in lab-scale SBRs was presentative of excess sludge from full-scale WWTPs. Moreover, Table S3 also indicated that some factors significantly affected the EPS formation in activated sludge ($p<0.05$). Among others, the EPS production in the sludge with starch being a carbon source reached 220.3±7.9 mg/g VSS, which was much higher than those fed with glucose and sodium acetate. Also, low temperature (12 ^{o}C) could be favorable to the EPS formation, up to 303.3±12.5 mg/g VSS. In contrast, organic loads and SRT had minor effects on the EPS production ($p>0.05$).

The alginate equivalent is the vague but important parameter for evaluating the relative purity of alginate, which represents the highly valuable products in the biopolymers (Li et al., 2021; Lin et al., 2010). Table S3 indicates that the average alginate equivalent of different EPS was as high as 550 mg/g EPS. Moreover, with starch as a carbon source, or at C/N=5.0, or temperature≥18 ^{o}C, the alginate equivalent of EPS considerably reached the highest level, up to 650 mg/g EPS.

Total polysaccharides and proteins were measured and the results are also listed in Table S3. Polysaccharides and proteins were the major constituents in EPS and the corresponding amounts were in the range of 300-470 mg/g EPS and 100-230 mg/g EPS, respectively. Proteins were significantly enriched in the biopolymers, characterized by the high PN/PS ratios of 2.2-4.1 except for the activated sludge fed with starch as a carbon source (PN/PS=1.5) and temperature at 24 ^{o}C (PN/PS=1.8).

3.2. Different fractions by partial acid hydrolysis

A family of copolymers comprising mannuronic acid (M) and guluronic acid (G) units were in an irregular block pattern of varying proportions of GG, MG, and MM blocks in alginates. Their proportion, distribution, and length determined the chemical and physical properties of alginate molecules (Lin et al., 2013). As shown in Supplementary Information Table S4 , the recovery yields of different blocks in EPS were at 40%-65%, with about 20%-35% GG blocks and 12%-28% MG blocks. Moreover, the monomer ratios of G:M of the biopolymers from different activated sludge were in the range of 2.0-3.0. The GG-rich EPS flocs benefited the activated

sludge that formed a compact gel structure due to its higher affinity towards divalent cations than the other two blocks (Lin et al., 2013; Seviour et al., 2009). These results were consistent with the above analysis of alginate equivalent. According to Lee and Mooney (2012) and Hay et al. (2013), furthermore, the ionic hydrogel properties of EPS were also tested by dripping in Ca^{2+} solution to evaluate the capacity of forming hydrogel. The results revealed that Ca^{2+}-EPS beads (cross-linked through Ca^{2+} ions) displayed a great ionic hydrogel-forming property among different EPS, which demonstrated that there was a hydrogel being stabilized by divalent ions and represented a similar gel formation to alginate.

In summary, the above experimental results indicated that the compositions and characteristics of EPS were reformed by different operational parameters, but the special physical and chemical properties indicated that EPS in activated sludge were a promising substitute for commercial alginate. On the other hand, the EPS yield was relatively low in activated sludge, but the results demonstrated that adjusting or optimizing some parameters in biological systems might be favorable to EPS formation. Therefore, the associated mechanisms of different factors involved in the EPS formation were analyzed in the following sections to figure out the optimal strategies for the high production or special properties of EPS.

4. Discussion

4.1. Factors influencing EPS formation and properties

4.1.1. Carbon sources

The influent with starch as a carbon source had a noticeable effect on forming EPS in activated sludge and the production reached the highest level of 220.3±8.0 mg/g VSS, followed by sodium acetate (156.4±1.2 mg/g VSS) and glucose (125.7±4.3 mg/g VSS). Similarly, González-García et al. (González-García et al., 2015) also found that starch as a carbon source contributed to biosynthesizing more extracellular polymers than glucose, galactose and xylose for the marine bacterium. Moreover, Wang et al. (H. Wang et al., 2014) demonstrated that the biopolymers were slightly higher in sludge samples cultivated with starch than that of glucose.

These results indicated that large molecule organics (such as starch) were more favorable for EPS synthesis than small molecule organics (like glucose and sodium acetate). It might be due to that starch could not only serve as a carbon source but also play a key role in adsorption bridging for bacterial proliferation (Liu and Fang, 2003). According to the extended DLVO theory, an aggregation matrix forms when

the distance between bacteria is shortened enough to overcome the maximum repulsive force (Xu et al., 2016). Macromolecular organic matters could directly shorten the energy potential barrier and rapidly form stable sludge flocs (Xu et al., 2016), which was proved by better sludge flocculation performance of starch-rich sludge than glucose and sodium acetate in settlement phases. Moreover, the evaluated parameters based on the UV-visible spectra are listed in Table 2. The E_2/E_3, $SUVA_{254}$ and E_2/E_4 values of the starch-rich EPS were the lowest, at 4.30, 2.75 and 5.00, respectively, which means the low content of aromaticity and aliphatic compounds. Also, E_4/E_6 of starch-rich EPS was the highest, at 2.38, which indicated that it was at a low degree of humification and aromaticity. In all, the starch-rich EPS performed higher adsorption bridging capacities and had more large molecular organics in the extracellular biopolymers than other EPS (Liu et al., 2019; Polak et al., 2007; Weishaar et al., 2003).

Table 2 Results of UV-visible spectra (Liu et al., 2019; Polak et al., 2007; Weishaar et al., 2003).

Parameters	Glucose	Starch	Sodium acetate	Functions
E_2/E_3 [A]	5.05	4.30	5.12	Molecular weight of dissolved organic materials (DOM)
$SUVA_{254}$ [B]	4.73	2.75	3.34	Unsaturated structural components
E_2/E_4 [C]	5.89	5.00	5.27	Condensation degree of humic substances
E_4/E_6 [D]	2.25	2.38	2.32	Humification and aromaticity degree

[A]: E_2/E_3, the UV-visible absorbance ratio at 250 and 365 nm, which reflects the molecular weight of dissolved organic materials (DOM) contained in EPS extraction.

[B]: $SUVA_{254}$, the value of the UV-Vis absorbance at 254 nm normalized by per mg TOC of EPS extraction, which indicates that the absorbance of unit TOC (mg/L) at 254 nm reflects the unsaturated structural components in EPS extraction.

[C]: E_2/E_4, the ratio of absorbance at wavelengths of 240 and 420 nm, which is inversely proportional to the condensation degree of humic substances.

[D]: E_4/E_6, the absorbance ratio at 465 and 665 nm, which represents the information of humification and aromaticity degree.

As for small molecular organics such as sodium acetate and glucose, the sludge fed with sodium acetate was more favorable than glucose for the EPS formation (F. X. Ye et al., 2011). More easily biodegradable organics such as sodium acetate were involved in the electron transfer and energy metabolism processes and could promote a higher level of extracellular enzymes in the extracellular matrix (Laspidou and Rittmann, 2002). Moreover, readily biodegradable organic substrates like sodium acetate could directly cross the cell membrane and enter the cell interior to be easily converted into acetyl Co-A and thus participate in the tricarboxylic acid (TCA) cycle. However, complex organic substances (starch, sucrose, etc.) need to be hydrolyzed

and broken into small molecules by multi-step bioreactions (F. X. Ye et al., 2011). Therefore, a high level of enzymes including extracellular enzymes were likely involved in the complex organic metabolism (Li and Yang, 2007), which was also confirmed by the higher proteins in EPS.

4.1.2. Organic loads

With different organic loads, EPS production was about 160 mg/g VSS, which implied that the influent organic load seemed nothing to do with EPS formation. Indeed, there was also no obvious difference in biomass produced between different influent organics by measuring the observed biomass yield (Y_{obs}) in Corsino et al. (2017). Furthermore, the specific EPS yields ($Y_{EPS/BOD5}$) were calculated to evaluate the EPS production for per gram organics (BOD_5) consumption. It showed that $Y_{EPS/BOD5}$=63.7 mg EPS/(g BOD_5) at L=0.20 kg BOD_5/kg VSS·d was twice as high as at L=0.36 and 0.50 kg BOD_5/(kg VSS·d), 36.3 and 26.5 mg EPS/(g BOD_5), respectively. The possible mechanism might be attributed to that a large part of organic matter would be oxidized and decomposed to carbon dioxide to provide the energy for cellular metabolism and microbial proliferation rather than biosynthesize biopolymers. In contrast, the utilized efficiency of organics at low organic load was higher and promoted extracellular structural biopolymer formation (Rusanowska et al., 2019). Moreover, the protein content decreased from approximately 400 mg/g EPS to 320 mg/g EPS with the increase of organic load, while polysaccharides were almost identical, at about 150 mg/g EPS. It was due to that the microorganisms would produce more exoenzymes or exoproteins outside the cells to maintain the stable metabolism under the conditions of lower organic deprivation and chronic starvation. Furthermore, the low organic load could promote the high protein content in the EPS matrix (Rusanowska et al., 2019).

4.1.3. Substrate (carbon over nitrogen, C/N)

The EPS extraction reached a peak of 137.8±13.2 mg/g VSS at C/N=5.0. On the contrary, the production declined slightly with increasing or decreasing C/N. A profound emphasis in several reports was given that C/N was associated with EPS production (Liu et al., 2010; More et al., 2014; F. Ye et al., 2011). However, there were no fixed favorable C/N ratios, for example, an optimum C/N ratio was at 0.5 mentioned by Liu et al. (2010), but it was at 20 suggested by F. Ye et al. (2011)and even at 40 reported by Durmaz and Sanin (2001). The proteins and polysaccharides content showed that the C/N ratio had significant effects on the components of EPS.

With decreasing C/N, the proteins content increased, whereas the polysaccharides content decreased, which resulted in a rise in the PN/PS ratio.

The functional groups of EPS were detected by FTIR. Amide I region (1,700-1,600 cm^{-1}) of EPS suggested their protein compositions were markedly different, which indicated that EPS formation at a low C/N ratio had more protein-like substances. Moreover, the band near 1,136 cm^{-1} (C–O–C stretching vibrations belonging to carbohydrate) and 1,072 cm^{-1} (C-H in-plane bending belonging to polysaccharides) also represented more polysaccharide-dominated structures in the biopolymers at a high ratio of C/N. Moreover, there are also significant differences in the bands, like C-H stretching at 2,930 cm^{-1} (CH_2 groups) and 2,960 cm^{-1} (CH_3 groups). Increasing nitrogen concentration (a low C/N ratio) favored protein synthesis in the sludge matrix and decreased the extracellular polysaccharide accumulation that almost all carbon sources would be consumed for microbial proliferation and metabolism (Geyik and Çeçen, 2016; Z. Wang et al., 2014; F. Ye et al., 2011). Moreover, a shortage of carbon sources could result in cellular autolysis and endogenous respiration and thus promote more exoenzyme protein.

4.1.4. Temperature

Previous studies have revealed that temperature was one of the most important parameters influencing EPS formation in sludge (More et al., 2014; Sheng and Yu, 2006). The experimental results in this study also showed that there was a sharp decline in EPS production along with the increasing temperature. At T=12 ^oC, EPS extraction was at 303.3±21.5 mg/g VSS, and rapidly decreased to 165.5±3.0 mg/g VSS at 18 ^oC and 132.7±1.2 mg/g VSS at 24 ^oC, respectively. However, Sutherland (2001) and Nichols et al. (2005) reported that the decrease in temperature could cause a decline in growth rate and the synthesis of cell wall polymer, which made more precursors available for biopolymer synthesis. Similarly, Gao et al. (2010) also supported that the biopolymers in flocs gradually decreased with increasing temperature, while the soluble biopolymers in supernatants gradually increased. It might be due to that the transferring capacities of nutrients and oxygen were significantly low and the viscosity of sludge also increased at low temperatures, which caused the flocs to gather in clusters tightly, and extracellular substances were wrapped to form a dense colloidal network. Thus, enzymatic activities, electron transfer capacities as well as substance conversion capacities would be hindered seriously, resulting in low metabolites including structural extracellular biopolymers (Sutherland, 2001). Meanwhile, 3D-EEM fluorescence spectroscopies showed a

higher fluorescence intensity of solubility-like microbial metabolites and protein-derived compounds fractions (Region Flu IV) at 24 °C than that at 12 °C, while fulvic acid-like and humic acid-like substances (Region Flu V) were lower at high temperature. It implies that bacterial metabolism was indeed disrupted at low temperatures, as described above.

Results of ionic hydrogel-forming properties reveal that Ca^{2+}-EPS beads displayed different hydrogel-forming properties between different EPS at different temperatures. Ionic hydrogel properties of EPS at 12 °C were also of poor performance (loosely hydrogel at about 24 mm) than EPS at 18 °C (about 13 mm) and 24 °C (about 11 mm), which implies that the temperature would affect the hydrogel abilities of EPS. There were also significant changes in proteins and polysaccharides content. Proteins decreased from 468.6±1.6 mg/g EPS to 351.0±34.1 mg/g EPS with temperature increase, while polysaccharides showed an increase from 114.3±6.3 mg/g EPS to 149.3±11.0 mg/g EPS.

4.1.5. Solid retention times (SRT)

The results at different SRTs showed that EPS extraction was all in the range of 190-200 mg/g VSS. PN and PS were in the range of 400-420 mg/g EPS and 100-120 mg/g EPS, respectively, which indicated that SRT had a minor effect on EPS formation and their compositions. By contrast, Duan et al. (2014) gave a totally different idea; they concluded that a short SRT implied a relatively faster and higher active microbial metabolism and a shift of dominant bacterial populations, resulting in more extracellular carbohydrates and protein accumulated in the biopolymers. The settleability and compressibility of the sludge with different SRTs were associated with the extracellular biopolymers and the flocculation performance. A long SRT apparently realized better sludge flocculation and a better settling capacity (Li et al., 2007; Liao et al., 2001; More et al., 2014; Sheng and Yu, 2006). In this study, however, there was no difference between sludge flocculation and the settling capacity at different SRTs. Anyway, different SRTs still need to be further investigated.

4.2. Significant variables identification by principal component analyses (PCA)

Since the PCA is based on the correlation matrix, the results can be interpreted as the correlations of the original correlated environmental/operational factors with each uncorrelated variable (principal components, PCs) and the behaviors of each variable are ascribed to each loading value (Vasilaki et al., 2018). The biplot of the first 2 PCs to visualize the combined behaviors of significant parameters that affect

EPS formation (in Supplementary Information Figure S5), revealed that PCA reduced the dimensionality into two significant principal components (PCs) that represented around 80.2% of the total variance (PC1=42.7%, PC2=37.5%). Based on the highest total variance of 42.7% in PC1, carbon sources and C/N presented high negative loading values, which indicated that PC1 decreased with increasing carbon sources and C/N. Moreover, temperature had a relatively highly positive loading in PC1 (0.60). Therefore, the parameters in PC1 could be effective in describing the behaviors of carbon/nitrogen sources and temperature performance. PC2 explained about 37.5% of the total variance, and the effects of carbon sources on PC2 were diminished since it had very low absolute loadings (0.31). The PC3 was largely correlated to organic load rate (0.83) and SRT (0.51) although presenting only 19.5% of the explained variance.

The PCA results revealed that biopolymer content correlated with carbon sources, substrate and temperature. Among them, temperature was a key factor that affected EPS formation, followed by carbon sources and substrate (C/N). By contrast, the organic loads had little effect on EPS production.

4.3. Highly valuable biopolymer recovery

In summary, it is important to figure out the optimal conditions for the biopolymers/EPS formation from the perspective of highly valuable biopolymer recovery from activated sludge. In practice, it should also be considered that the environmental/operational conditions are suitable for a high efficiency of the biological removal process. Starch and temperature are two significant contributory factors to improving EPS production. However, some scenarios with starch-rich could only enhance the EPS production. Moreover, the low temperature only occurs in the winter but is not favorable to the biological removal process. Adjusting the influent characteristics, including C/N and organic load in the BNR processes without affecting the effluent quality of WWTPs, would be also an approach to maximizing the EPS production. Moreover, the results also imply that it would be possible to achieve directional cultivation of the EPS with different proportions of polysaccharides or proteins and even with some special chemical/physical properties. Therefore, the feasible applications or economic benefits for highly valuable biomaterials recovery could be improved.

On the other hand, all of these parameters are working together on forming EPS in practice, thus the multi factors instead of independent variables should be paid more

attention to for ascertaining the optimal strategies for EPS formation and characteristics in activated sludge in future studies.

5. Conclusions

Extracellular polymeric substance (EPS) recovered from activated sludge has been identified as a kind of highly valuable biomaterial for potential applications. However, EPS extraction from activated sludge is often limited by the low level of EPS formation in flocs matrixes. Within this study, the key factors controlling the EPS formation were experimentally identified for activated sludge to improve the EPS production from activated sludge. The results indicate that carbon sources and temperature had the major effects on EPS formation. The EPS production could reach 220.3±8.0 mg/g VSS with starch being the carbon source, which was higher than sodium acetate and glucose. In addition, the low temperature was more favorable to enriching EPS, and it reached 303.3±21.5 mg/g VSS at 12 °C. Moreover, a higher organic load could decrease the specific EPS yields ($Y_{EPS/BOD5}$). C:N=5:1 in the influent could contribute the highest EPS production among different C/N ratios, up to 137.8±13.2 mg/g VSS. Interestingly, SRT had a minor effect on EPS production, always ranging from 190 to 200 mg/g VSS under different SRTs. The PCA analysis confirmed that temperature was a key factor affecting EPS formation, followed by carbon sources and substrate (C/N). On the other hand, there were noticeable differences in the chemical properties of the EPS under different working conditions. In summary, adjusting or optimizing operational parameters could improve the EPS formation in activated sludge in theory. More importantly, this study concludes that the directional cultivation of the biopolymers/EPS with some special chemical/physical properties could be an approach to improving the value of recovered biomaterials and expanding the potential applications.

Supporting information

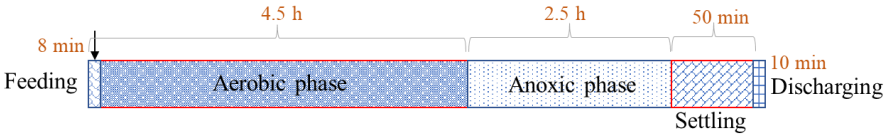

Figure S1 Operation of sequencing batch reactors (SBRs) in one cycle.

Table S2 Other compounds and the trace elements in bioreactors.

Compounds	Unit (mg/L)	Compounds	Unit (mg/L)
$CaCl_2$	200	KI	180
$MgSO_4{\cdot}7H_2O$	320	$CoCl_2{\cdot}6H_2O$	150
$FeCl_3{\cdot}6H_2O$	1,500	$CuSO_4{\cdot}5H_2O$	30
H_3BO_4	150	$MnCl_2{\cdot}4H_2O$	120
$Na_2MoO_4{\cdot}2H_2O$	60	EDTA	10,000
$ZnSO_4{\cdot}7H_2O$	120		

*1.0 L substrate solution contains 1.0 mL trace elements solution.

Table S3 Extracellular polymeric substances (EPS) yields and compositions under different operational parameters.

Groups	Parameters	EPS yields (mg/g VSS)	Alginate equivalents (mg/g EPS)	Proteins (mg/g EPS)	Polysaccharides (mg/g EPS)	PN/PS
Carbon sources	Glucose	125.7±4.3	540.2±33.9	321.0±31.9	141.4±7.8	2.3
	Starch	220.3±7.9	648.8±10.2	338.0±43.9	225.6±15.7	1.5
	Acetate	156.4±1.2	544.7±44.6	346.6±24.2	162.8±16.1	2.3
Organic loads (kg BOD5/kg MLSS·d)	0.2	159.3±2.4	634.2±3.0	397.8±14.4	154.1±2.1	2.6
	0.36	158.9±4.2	611.2±6.4	382.3±11.0	152.7±4.9	2.5
	0.5	165.5±3.0	572.5±10.3	321.0±10.9	143.3±6.3	2.2
Ratios of carbon to nitrogen	3.0	121.4±1.9	598.2±6.5	446.8±5.6	121.8±2.1	3.7
	5.0	151.0±2.3	687.1±9.6	430.5±14.2	125.4±3.3	3.4
	7.0	130.0±3.9	584.0±4.7	377.7±16.7	146.2±4.5	2.6
Temperature (°C)	12	303.3±12.5	567.6±12.3	468.6±1.6	114.3±6.3	4.1
	18	165.5±3.0	677.3±20.5	351.0±34.1	149.3±10.9	2.4
	24	132.7±1.2	684.3±15.3	300.2±25.4	163.9±2.7	1.8
Solid retention time (days)	10	199.8±4.1	625.9±23.6	420.7±11.9	104.1±3.1	4.0
	15	195.3±2.4	623.1±25.5	413.0±2.3	121.0±0.4	3.4
	20	190.8±3.2	607.7±19.9	405.0±15.3	116.8±3.6	3.5

Table S4 Fractions of different building blocks obtained from extracellular polymeric substances.

Groups	Samples	Poly-G	Poly-MG	Poly-M	Recovery efficiency	Ratio G/M
Carbon sources	Glucose	22.9%±1.5%	18.9%±0.6%	6.5%±0.2%	48.2%	2.0
	Starch	33.3%±2.3%	25.3%±2.4%	6.4%±0.1%	65.1%	2.5
	Acetate	20.3%±0.5%	17.4%±1.6%	1.2%±1.0%	39.0%%	2.9
Organic loads (kg BOD5/kg MLSS·d)	0.2	30.5%±1.0%	21.3%±0.9%	7.3%±0.3%	59.0%	2.3
	0.36	27.2%±1.3%	19.4%±0.1%	3.3%±0.4%	49.9%	2.8
	0.5	24.3%±2.4%	28.5%±0.5%	2.5%±1.0%	55.2%	2.3
Ratios of carbon to nitrogen	3.0	23.3%±0.9%	13.4%±0.1%	3.3%±0.1%	40.1%	3.0
	5.0	30.9%±1.3%	23.7%±0.3%	4.8%±0.1%	59.4%	2.6
	7.0	21.8%±2.1%	16.6%±0.4%	5.4%±0.3%	43.8%	2.2
Temperature (°C)	12	27.4%±2.3%	12.3%±1.2%	4.8%±0.3%	44.5%	3.1
	18	29.3%±2.4%	28.5%±0.5%	2.5%±1.0%	60.2%	2.6
	24	30.3%±0.3%	25.3%±1.0%	3.2%±0.5%	58.9%	2.7
Solid retention time (days)	10	28.5%±1.8%	23.5%±2.1%	7.4%±0.5%	59.5%	2.1
	15	25.6%±1.4%	24.5%±3.2%	4.4%±0.4%	54.6%	2.3
	20	24.4%±2.5%	23.2%±0.2%	3.4%±0.6%	51.1%	2.4

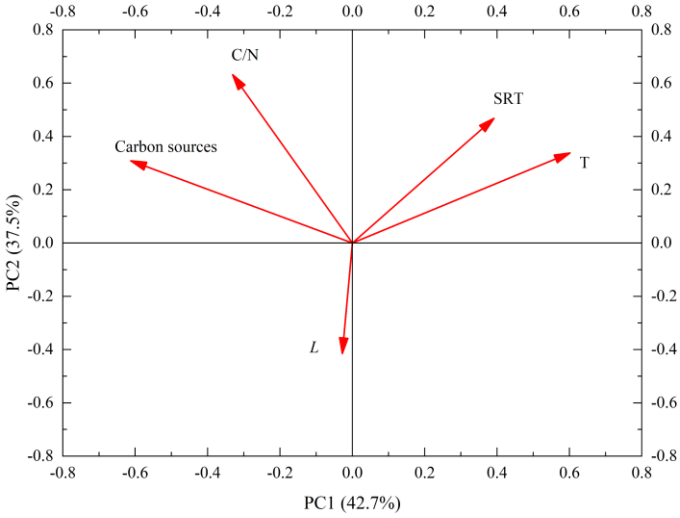

Figure S5 Loading biplot for principal component analysis (PCA) for the correlations of operational parameters with the alginate like extracellular polymers (ALE) formation.

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Chapter 4

Reinvestigating the composition of alginate-like exopolymers extracted from activated sludge

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Abstract: Over the past decade, a significant amount of research work on extracellular polymeric substances has been done on the “alginate-like exopolysaccharides” (ALE, also called “alginate-like exopolymers”). The term was used based on the Food and Agriculture Organization (FAO) biopolymer identification test. Although various chemical analyses have been conducted to characterize extracted ALE, it remained unclear whether ALE contains the two sugar monomers of alginates. Aiming to obtain a direct answer to the question: are there alginates in the ALE extracted from sludge, activated sludge was collected from two wastewater treatment plants in two different countries, where the ALE was previously extracted, characterized and reported in the literature. The extracellular polymers were extracted from these sludges and fractionated according to the standard protocol. The sugar monomer composition of each fraction was analyzed, with special attention to the presence of mannuronic acid (M) and guluronic acid (G) which compose alginate biopolymers. None of these monomers were found in the extracted EPS, indicating there are no alginates resembling polymers extracted from the sludges. The possibility of the presence of other glycan components, such as lipopolysaccharides in EPS was investigated and confirmed.

1. Introduction

Extracellular polymeric substances (EPS) form a complex matrix in sludge, providing structural support to sludge flocs, and playing a crucial role in biological wastewater treatment processes. To identify the diverse components of EPS, analyses of its composition and chemical structures have been widely conducted in many studies. In 2010, Lin et al. extracted the EPS from sludge according to the same protocol as used for alginate extraction from brown seaweed (Lin et al., 2010). Following the FAO/WHO biopolymer identification, together with further analysis of fractionation and spectroscopic measurement, it was suggested that the EPS resembled alginates, leading to the proposal that the EPS might be considered a potential substitute for commercial alginates (Felz et al., 2016; Lin et al., 2010, 2013, 2015). Consequently, the EPS extracted with such a method was termed "alginate-like exopolysaccharides" (ALE, also as “alginate-like exopolymers” afterwards). Gradually, the “ALE” gained increasing attention. As of 2024, there are about 70 studies associating the EPS investigation with ALE extraction and characterization (search in *Web of Science* with keywords, sludge and alginate-like-). These studies covered a broad range of fields, including the production and functions of ALE in sludge (Rollemberg et al., 2021; Sarvajith and Nancharaiah, 2023; Yang et al., 2014; Zahra et al., 2022), as well as their chemical/physical properties and potential applications (Cydzik-Kwiatkowska et al., 2022; Dall’Agnol et al., 2022; Lin et al., 2015; Schambeck et al., 2020). Among them, over 40 papers directly considered the ALE as exactly the same as alginates in the chemical composition.

Alginates are mainly produced by seaweed and also by two bacterial genera, *Azotobacter vinelandii* and *Pseudomonas spp*. (Clementi, 1997; Rehm, 2010). They are linear polysaccharides composed of mannuronic acid (M) and guluronic acid (G). Although ALE was frequently considered as alginates, until now there was hardly any direct evidence showing the presence of the two sugar monomers in literature. Instead, analytical methods, such as colorimetric assays for total carbohydrates and proteins (Felz et al., 2019), spectroscopic techniques like Fourier Transform Infrared (FTIR) (Sam and Dulekgurgen, 2016; Schambeck et al., 2020) and Nuclear Magnetic Resonance (NMR) (Gonzalez-Gil et al., 2015) related to functional groups, along with fractionation by partial acid hydrolysis (Meng et al., 2019; Sarvajith and Nancharaiah, 2023) and the hydrogel formation tests (Dall’Agnol et al., 2022; Felz et al., 2016; Zahra et al., 2022), have been employed to characterize the ALE. Based on the results from these indirect analyses, conclusions about the resemblance to alginates were drawn.

Moreover, there is an increasing number of studies that simply assumed that ALE was identical to alginates and used it as the basis for further investigation. It is worth pointing out that alginates are a family of polysaccharides that have a specific chemical composition and properties; taking ALE as alginates for granted may hinder the EPS research in unravelling the new components, understanding its physical and chemical properties, recovering it as valuable biomaterials and further developing the EPS-based sustainable products. Hence, before such a general conclusion is drawn, it is significantly important to obtain a direct answer to the following question: are there always alginates in the ALE extracted from sludge?

Aiming at reinvestigating the composition of ALE, in current research, the activated sludge was collected from two wastewater treatment plants from two different countries, where the ALE was previously extracted, characterized and reported in the literature (Li et al., 2021; Lin et al., 2013). The ALE was extracted from these sludges and fractionated according to the previously reported protocol (Li et al., 2021; Lin et al., 2013). The sugar monomer composition of each fraction was analyzed, and a direct search for the two sugar monomers, mannuronic acid (M) and guluronic acid (G), was performed, in comparison to the commercial alginates. Furthermore, the presence of other carbohydrate components in the ALE was investigated. Research results will shed light on clarifying the composition of ALE and identifying the EPS components.

2. Materials and methods

2.1. Sludge sample collection and the extracellular polymeric substances extraction

Activated sludge was collected from two municipal wastewater treatment plants (WWTP): Delft (Harnaschpolder) WWTP located in the Netherlands (with a VS/TS ratio of sludge at 77.8%±0.7%) and Beijing (Gaobeidian) WWTP in China (60.1%±0.3%). Sludge samples were centrifuged at 3,900 rpm for 15 min first, and the pellets were lyophilized for further analysis.

EPS extraction was done according to the previous protocol (Felz et al., 2016; Li et al., 2021). Lyophilized sludge samples (3.0 g from each WWTP) were heated at 80 ^{o}C in 100 mL of a sodium carbonate solution (0.5% w/v) for 30 min. Afterwards, the mixture was centrifuged at 3,900 rpm for 15 min. The supernatant was collected and its pH was adjusted to 2.2 with 1.0 M HCl. After centrifuging at 3,900 rpm for 15 min, the pellet was solubilized and adjusted pH to 8.5 with 1.0 M NaOH before being

dialyzed in the dialysis bag (MWCO 3.5 K) against demi water overnight. The dialyzed EPS solution was lyophilized for further analysis.

The organic and ash content of both the sludge and EPS samples were determined according to the standard methods as specified by APHA (APHA, 2012).

2.2. Partial acid hydrolysis of the extracted extracellular polymeric substances

Partial acid hydrolysis of the extracted EPS samples was performed according to Lin et al. (Lin et al., 2010). EPS (0.25 g) was dissolved into 9 mL of demi water. After the addition of 1 mL of 3.0 M HCl, it was heated at 100 °C for 0.5 h. Once cooled, the mixture was centrifuged at 3,900 rpm for 30 min. The supernatant was collected, neutralized with 1.0 M NaOH and poured into an ethanol solution with 80 % (vol/vol) for being precipitated overnight. After centrifugation (3,900 rpm for 30min), both the precipitate and the ethanol supernatant were collected and lyophilized, which were referred to as Fraction I and Ethanol supernatant I, respectively. The previous non-soluble material after heating at 100 °C in a HCl solution was dissolved in 1.0 M NaOH; the pH was decreased to 2.85 by the addition of 1.0 M HCl. The mixture was centrifuged at 3,900 rpm for 30 min. The supernatant was collected, neutralized with 1.0 M NaOH and precipitated by the addition of ethanol to a final concentration of 80% (v/v). After centrifugation (3,900 rpm for 30min), both the precipitate and the ethanol supernatant were collected and lyophilized, which were referred to as Fraction II and Ethanol supernatant II, respectively. Finally, the non-soluble material at pH 2.85 was dissolved with 1.0 M NaOH and adjusted to pH 7. It was poured into ethanol solution with 80% (v/v). Both the precipitation and the ethanol supernatant were collected and lyophilized, which were referred to as Fraction III and Ethanol supernatant III. The diagram of partial acid hydrolysis was included in the Supporting Information Figure. S1.

2.3. Sugar monomers detection by HPAEC-PAD

To analyze the sugar monomers, complete acid hydrolysis was performed according to Felz et al. (Felz et al., 2019)and Rhein-Knudsen et al.(Rhein-Knudsen et al., 2017). All samples (1.0 mg of EPS, Fraction I-III, and Ethanol supernatant I-III) were hydrolyzed in 1 mL of 1 M HCl in an oven (105°C) for 8 hours. After centrifugation, the supernatant was neutralized with 1 M NaOH and filtered through 0.22 µm PVDF filters. Filtrates were diluted with Milli-Q water in accordance with the concentration of the standard sugar monomers. High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD, a Dionex ICS-

5000+, a CarboPac PA20 column, and an AminoTrap pre-column) was applied to analyze the sugar monomers. The standard compounds contained seven neutral sugars (fucose, rhamnose, galactose, glucose, xylose, mannose, and ribose), two amino sugars (galactosamine and glucosamine), and two uronic acids (galacturonic acid and glucuronic acid). Peak identifications were done by overlaying the chromatograms of EPS with standards spiked EPS samples (EPS with the addition of sugar monomer standards). In addition, three commercial sodium alginates (Protaweld® RF 6650 alginate, food grade; Protaweld® S 120 and Scogin® LDH alginate, industrial grade, FMC BioPolymer) were used as the control, following the same procedure as described above.

In order to get information about what kind of glycans could contribute to the carbohydrates in the extracted EPS and its fractions, the molar ratios between the sugar monomers in each sample were used to search the hits in the database of "Bacterial carbohydrate database" (http://csdb.glycoscience.ru/database/).

2.4. Determination of the total proteins and carbohydrates content of the extracted EPS and its fractions

The total proteins and carbohydrates content of the extracted EPS and its fractions were determined by the BCA assay (with BSA as standard) (Smith et al., 1985), and the phenol sulfuric acid method (with glucose as the standard) (Dubois et al., 1951), respectively. All samples were dissolved in 0.1 M NaOH at a concentration of 1000 mg/L overnight before the analysis. Analysis was done in triplicates for each sample.

2.5. Functional groups analysis by the FTIR spectroscopy

The Fourier Transform Infra-Red (FTIR) spectra of the extracted EPS, all fractions and commercial sodium alginates were recorded in the 4,000–600 cm^{-1} region at room temperature on a FTIR Spectrometer (Perkin Elmer, Shelton, USA).

2.6. Lipopolysaccharides isolation and staining

Lipopolysaccharides (LPS) extraction from the EPS was conducted using the hot phenol-water protocol (Davis and Goldberg, 2012; DeLeo and Otto, 2008). 10 mg of the EPS extract was dissolved in 1 mL of a 1×PBS solution first. In order to eliminate contaminating protein, treatment with proteinase K was performed prior to the extraction step. For this purpose, 10 μL of proteinase K (10 mg/mL) was added to 200 μL of an EPS solution and the tubes were incubated at 65 °C and 1,000 rpm for 3 hours. In the next step, an equal volume (200 μL) of water-saturated phenol solution

(preheat at 68 °C) was added to the mixtures followed by vigorous shaking at 68 °C for 15 min. Suspensions were then cooled on ice and poured in 200 μL of Milli-Q water before centrifugation at 12,000 rpm for 15 min. Gently, the tube was taken out and the top aqueous layer was transferred to 1.5 mL polypropylene tubes. The bottom layer of the phenol phase was re-extracted twice by an equal volume of Milli-Q water. All of the top layer solutions were mixed together and dialyzed against demi water in the dialysis tubing (MWCO 3.5K) and lyophilized afterwards.

The lyophilized samples were solubilized in sample buffer to the desired concentration (1 mg/mL) and prepared with loading buffer for the SDS gel running according to the previous study. Around 16.7 μg of each sample or 2.0 μg of lipopolysaccharides standard (provided by the kit, *Escherichia coli* serotype 055:B5) was separated on 12% SDS gel with a 4% stacking gel under reducing conditions at 200 V for 35 min. Coomassie blue and Pro-Q Emerald 300 staining of the gels were performed according to the protocol provided by Thermo-Fisher Scientific (Fomsgaard et al., 1990; Kittelberger and Hilbink, 1993).

3. Results

3.1. EPS yields and its total proteins and carbohydrates content

In order to reinvestigate the composition of “alginate-like exopolymers” extracted from activated sludge and compare it with the data in the literature, the sludge was collected from one WWTP in the Netherlands and one in China, where the sludge was studied and the EPS analysis was reported (Li et al., 2021; Lin et al., 2013). Based on the same extraction protocol as that described in the literature, “ALE” was extracted. To avoid confusion, the extracted “ALE” will be termed as the extracted EPS in the following text.

Table 1 The yield and total proteins and carbohydrates content of the EPS extracted from activated sludge collected in the Netherlands and China.

Sludge	EPS yield (mg/g VSS sludge)	Total carbohydrates (mg glucose equivalent/ g VS EPS)	Total proteins (mg BSA equivalent/ g VS EPS)
Delft, the Netherlands	167.6±18.4	183.8±0.3	386.5±3.3
Beijing, China	179.5±1.5	159.5±2.0	480.0±4.4

The yields of the extracted EPS were 167.6±18.4 and 179.5±1.5 mg/g (VS ratio), respectively. These results are in line with the yield range reported by Li et al.(Li et al., 2021) (90-190 mg/g VS). In addition, the total content of proteins and

carbohydrates of the two extracted EPS is relatively similar (Table 1).

3.2. EPS fractionation and sugar monomers analysis

Alginate is the salt of alginic acid with a linear copolymer consisting only of mannuronic acid (M) and guluronic acid (G) residues, arranged in heteropolymeric blocks (i.e. MG blocks) and homopolymeric blocks (i.e. GG blocks and MM blocks). Partial acid hydrolysis has been used to fractionate alginic acid to separate those blocks, with the aim of characterizing different alginates. Due to the assumption that the "alginate-like exopolymers" have similarities with alginates, this method was introduced by Lin et al. (Lin et al., 2010) to characterize the EPS extracted from sludge collected from different wastewater treatment plants.

In the current research, both extracted EPS samples were fractionated according to the same protocol. The percentages of each fraction are listed in Table 2. It is interesting to note that despite the slight variation in the percentage of each fraction, the obvious similarities between the two EPS samples are: that all of the collected EPS fractions contain a high amount of proteins. As alginates are carbohydrates, the higher protein content in the different fractions indicated that chemically, the EPS fractions generated by partial acid hydrolysis are different from the building blocks of alginates.

Table 2 Percentage of each fraction and their chemical characterization of the total proteins and carbohydrates.

Sludge	Contents	Fractions (precipitated in ethanol solution) [C]			Ethanol supernatants (not precipitated in ethanol solution)		
		Fraction I	Fraction II	Fraction III	Ethanol supernatant I	Ethanol supernatant II	Ethanol supernatant III
Delft, the Netherlands	Fractions [A]	121.3±6.3	27.5±2.7	273.2±14.0	324.5±2.2	31.6±6.9	107.7±9.3
	Total carbohydrates [B]	255.9±7.7	245.5±14.5	82.1±1.1	97.8±34.8	50.0±5.6	120.9±10.2
	Total proteins [B]	377.2±13.8	290.6±4.8	638.6±4.2	422.5±9.3	258.2±9.5	444.9±3.1
Beijing, China	Fractions [A]	82.1±0.4	48.1±8.3	359.5±1.5	189.6±1.1	67.4±16.0	116.2±4.0
	Total carbohydrates [B]	257.2±3.5	320.1±26.8	124.7±3.6	119.5±13.0	152.2±5.4	122.0±1.5
	Total proteins [B]	557.1±4.1	336.1±7.9	693.3±5.8	596.2±0.6	626.6±1.0	594.4±3.8

[A:] Fraction content was presented based on the volatile solid of fractions to the volatile solid of EPS (mg/g VS ratio).

[B:] Total proteins and carbohydrates were calculated based on the volatile solids of each fraction. (total carbohydrates unit: mg glucose equivalent/g VS of the EPS fraction; total proteins unit: mg BSA equivalent /g VS of the EPS fraction).

[C]: Final concentration of the ethanol solution was 80% vol/vol.

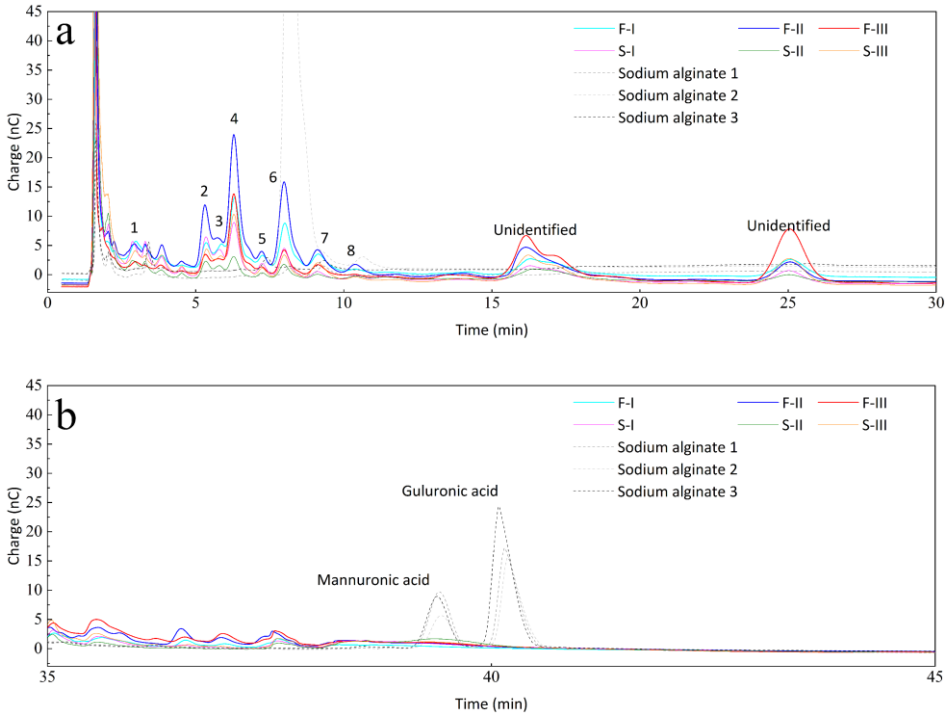

Figure 1 HPAEC-PAD chromatograms of standards, different EPS fractions and three commercial alginates after complete acid hydrolysis. Sugar monomer speaks: 1-fucose, 2-galactosamine, 3-rhamnose, 4-glucosamine, 5-galactose, 6-glucose, 7-xylose and 8-mannose. Sodium alginates concentration was at 50 mg/L. **(a)** retention time at 0-30 min with the eluent of 2 mM sodium hydroxide, **(b)** retention time at 35-45 min with the eluent of 50 mM sodium acetate plus 200 mM sodium hydroxide. The baseline drifted at 30-35 min periods as the eluent was changed. F I-III and S I-III represent Fractions I~III and Ethanol supernatants I~III, respectively.

Despite the fact that EPS and its fractions are protein-dominated, there is still the possibility that the carbohydrates contain alginates. In order to investigate if the carbohydrates in the EPS fractions contain the two sugar monomers of alginates (guluronic acid and mannuronic acid), HPAEC-PAD detection and analysis were performed. The chromatograms are shown in Figure 1. Compared to the standards, within the neutral sugar and amino sugar region (Figure 1a), only one commercial alginate contained glucose and/or xylose, while all EPS fractions held eight different sugar monomers. Moreover, within the acidic sugar region (Figure 1b), all commercial alginates exhibited two sugar monomer peaks, which are the peaks of guluronic acid and mannuronic acid. However, no such peaks appeared in the chromatogram of EPS fractions. This strongly indicates that the extracted EPS chemically has no similarity

with alginates. Interestingly, all of the collected EPS fractions contain fucose, galactosamine, rhamnose, glucosamine, glucose, galactose, xylose and mannose, but the amount of these sugar monomers is different. E.g. glucose was the dominant sugar monomer in Fraction I and II, while rhamnose was dominant in Ethanol supernatant I and glucosamine was dominant in Ethanol supernatant III.

To further explore what kind of carbohydrate polymers could be present in the extracted EPS, the molar ratios of the sugar monomers (as shown in Figure 2) were used to search in the "bacterial carbohydrate database", most of the hits for Fraction I were O-antigens of lipopolysaccharides, while for ethanol soluble parts, most were the core of lipopolysaccharides. Clearly, no alginate was found in the extracted EPS and it seemed that lipopolysaccharides might be one of the components of the extracted EPS.

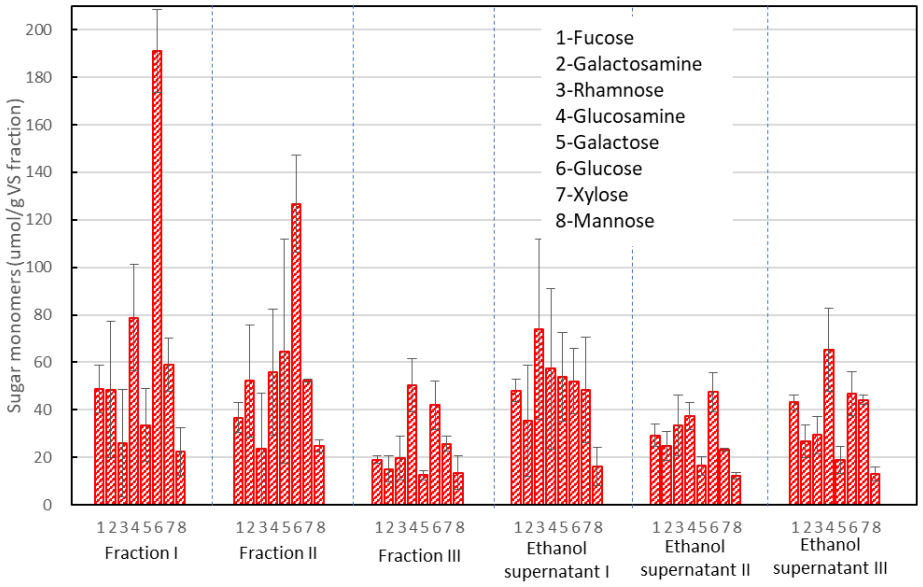

Figure 2 Quantitative analysis of sugar monomers in different EPS fractions determined by HPAEC-PAD. The molar mass of different standard sugar monomers is listed in Supporting Information Table S2.

3.3. FTIR spectra

In order to study whether there were any functional groups related to lipopolysaccharides, FTIR spectroscopy was performed. The FTIR spectra of the EPS fractions extracted from the sludge of Delft (the Netherlands) are shown in Figure 3,

while those from Beijing (China) are shown in the Supporting Information Figure S3.

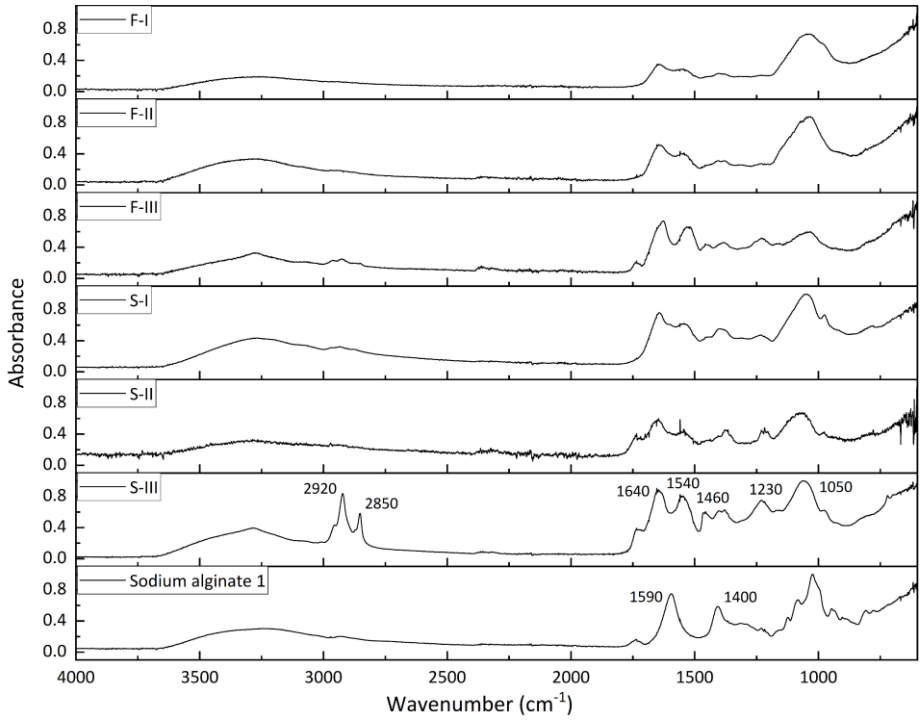

Figure 3 Comparisons of FTIR spectra between different EPS fractions (from Delft, the Netherlands) and commercial sodium alginates. F I-III and S I-III represent Fractions I-III and Ethanol supernatants I-III, respectively. Three commercial sodium alginates (another two are depicted in Supporting Information Figure S5) exhibited strong-sharp asymmetric stretching bands near 1,590 cm^{-1} and 1,400 cm^{-1}, which were the typical bands of carboxylate functional groups of both guluronic and mannuronic acids units in alginates (Ramos et al., 2018; Shi et al., 2008). On the contrary, all EPS fractions did not show these peaks, supporting the conclusion that the EPS has no chemical connection with alginates.

Detailed information about peak wavenumbers is listed in Supporting Information Table S4. It was observed that, similar bands appeared for both EPS, i.e., the broad band between 3,400 cm^{-1} and 3,100 cm^{-1} might be related to the hydroxyl groups. The peaks at around 1,640 cm^{-1} and 1,540 cm^{-1} corresponded to N-H bending which may be ascribed to amide I and amide II of nitrogen compounds, respectively (Xu et al., 2023). The peaks at around 1,230 cm^{-1} could be attributed to phosphate and/or C-N stretching vibrations of amide III. The stretching vibrations of both C-H (2,820 to 2,940 cm^{-1}; 1,460 to 1,470 cm^{-1}) and phosphate (1,200 to 1,265 cm^{-1}, 1,106 cm^{-1}, and 960 to 983 cm^{-1}) might indicate the presence of lipid A in the lipopolysaccharides.

The band at 1,050-1,085 cm^{-1} could be assigned to the C-O-C stretching of polysaccharide bonds.

It was noted that there was almost no lipid-related peak in Fraction I, while in ethanol supernatant III, the peaks at 2,920 cm^{-1} and 2,850 cm^{-1} were much stronger compared to other fractions. These two peaks were attributed to the stretching vibration of the $-CH_2$ group, where a significant contribution may come from the lipid (Krishna et al., 2020; Lee and Mooney, 2012). In addition, as there were bands at 1,460 cm^{-1} and 1,230 cm^{-1} which indicated the presence of lipid A, and bands at 1,050 cm^{-1} which implied the presence of polysaccharides, there was a high possibility for the existence of lipopolysaccharides.

3.4. LPS extraction and validation

As there was an indication from the FTIR spectra on the presence of lipopolysaccharides (LPS), the hot phenol-water extraction method, specific for LPS extraction, was performed on the EPS (DeLeo and Otto, 2008). The yields of LPS extraction from the EPS of the Netherlands (Delft) and China (Beijing) was about 20.5±0.8% and 23.5%±1.0% VS of the extracted EPS, respectively. FTIR spectra were obtained to compare the difference between the EPS and the LPS (Figure 4a) The extracted LPS samples exhibited a broad and smooth band between 3,400 cm^{-1} and 3,100 cm^{-1} which was in accordance with the hydroxyl group of the carbohydrates, and a strong band at 1,050 cm^{-1}, which can be attributed to the C-O-C stretching of polysaccharides. The stretching vibrations of C-H (2,820 to 2,940 cm^{-1}; 1,460-1,470 cm^{-1}), and phosphate (1,200 to 1,265 cm^{-1}) are indicative of lipid A presence. Additionally, the amide I peak at around 1640 cm^{-1} was maintained while amide II at 1,540 cm^{-1} disappeared. This was probably due to the proteinase K digestion followed by phenol isolation of proteins from EPS, while other compounds containing a -NH group (such as the N-acetyl glucosamine from lipid A) still conferred the amide I peak at 1,640 cm^{-1}. Apparently, the FTIR spectra indicate that there were lipopolysaccharides in the extracted EPS.

Separation over SDS-PAGE gel followed by Pro-Q Emerald 300 staining was used to validate the presence of LPS structures in these extracts (Fomsgaard et al., 1990; Kittelberger and Hilbink, 1993). LPS is a large glycolipid composed of three structural domains: lipid A, the core oligosaccharide, and the O-antigen (Figure 5). Lipid A is the hydrophobic portion of the LPS. The core oligosaccharide is a non-repeating oligosaccharide that is linked to the N-acetyl glucosamines of lipid A. The O-antigen

is an extended polysaccharide that is attached to the core oligosaccharide. It is composed of a repeating oligosaccharide made of two to eight sugars (as shown in Figure 5). As shown in Figure 4b, LPS from both the two EPS gave a characteristic staircase (ladder-like) pattern of bands, indicating the presence of O-antigens composed of repeating units (as shown in Figure 4b within the blue rectangle). On the other hand, Coomassie blue staining of the gels showed no band indicating the absence of contaminating proteins (Figure 4b). Therefore, with the specific LPS extraction and staining, it was verified that a significant amount of the extracted EPS was LPS.

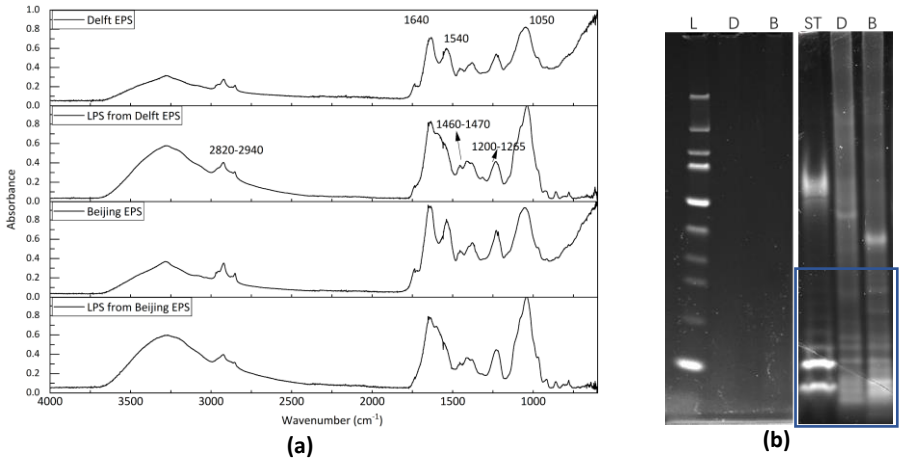

Figure 4 Characteristics and analysis of lipopolysaccharides (LPS) extraction. **(a)** FI-TR spectra of EPS from Delft (the Netherlands) and Beijing (China), and LPS extraction from these EPS. **(b)** Images of LPS polyacrylamide-gel after staining (4-12% acrylamide gel). Left: Coomassie blue staining was used for proteins. Right: Pro-Q Emerald 300 staining was used for the LPS. L: protein ladder; D: LPS was extracted from Delft EPS; and B: LPS extracted from Beijing EPS. ST, lipopolysaccharides standard from *Escherichia coli* serotype 055:B5.

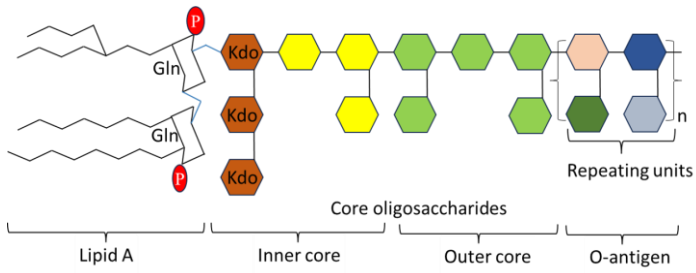

Figure 5 Schematic of the basic structure of lipopolysaccharides (LPS). Gln: N-Acetyl glucosamine. Kdo: keto-deoxyoctulosonate, and P: phosphate

4. Discussion

4.1. Alginate monomers are not present in the EPS extracts from activated sludge

In the field of activated sludge research, over the past decade, a significant increasing amount of EPS research has focused on "alginate-like exopolysaccharides" (ALE, also called "alginate-like exopolymers" in some works of the literature), which is heavily associated with alginate. Although various chemical analyses have been performed to characterize the extracted ALE, and it was assumed that ALE contained the building blocks (GG, MG, MM blocks) made from the two typical sugar monomers mannuronic acid (M) and guluronic acid (G) of alginates, there was no direct proof that these two sugar monomers have ever been detected in the ALE. In order to search for a direct proof, in the current research, activated sludge EPS was extracted by following the same protocol for alginates extraction from algae (also the same as ALE) and fractionated to obtain the different "building blocks". The sugar monomers of all of the EPS fractions were identified based on the standards and compared with the commercial alginates. Interestingly, no mannuronic acid and guluronic acid were found in the extracted EPS at all, which clearly suggested that there is no alginate in the extracted EPS from the activated sludge collected.

When looking back at the literature on ALE, there are a few reports describing the dissimilarity of ALE with alginates. Some studies focused on identifying the presence of mannuronic acids and guluronic acids by FTIR, but only mannuronic acid was claimed to be detected (Sam and Dulekgurgen, 2016; Schambeck et al., 2020), while others concluded that their EPS samples did not contain guluronic or mannuronic acid (Zahra et al., 2023). In addition, despite the fact that the ALE extracted from aerobic granular sludge could form a hydrogel with metal ions, which was considered one of the similarities between ALE and alginates, the stiffness of EPS hydrogels formed was significantly weaker than that of alginate hydrogels (Felz et al., 2020). Especially, the integrity of the EPS hydrogel was still maintained after the addition of EDTA, while the alginate ionic gels could be completely solubilized by the EDTA.

Besides the comparison between ALE and alginate, recent investigations related to the genetic potential of alginate biosynthesis in sludge raised a similar doubt. Based on metagenome-assembled genomes (MAGs) of 23 Danish activated sludge samples, it was observed that the genetic potential for alginates production was restricted to a few low-abundant bacteria, leading to the conclusion that it is unlikely for alginates to be a dominant EPS in the sludge of WWTPs (Dueholm et al., 2023). Doloman et

al.(Doloman et al., 2024) examined the MAGs that revolved around the alginate metabolism cluster in anaerobic granular sludge, but only found one sludge sample having the full operons for the synthesis of alginates while the other two sludge samples were lacking over five important operons out of a total of 12 operons.

Based on current research and the literature, it can be concluded, there is a low possibility for the existence of alginates not only in the activated sludge but also in other types of sludge such as aerobic granular sludge and anaerobic granular sludge. Regarding the term "alginate-like exopolysaccharides" (ALE, also as “alginate-like exopolymers”), it is recommended to keep in mind that ALE is definitely not identical to alginates. It mostly reflects that the polymers form a gel with calcium ions.

4.2. There are Lipopolysaccharides in the EPS

It is well known that bacteria can synthesize a diverse array of glycans, often attached to proteins (glycoproteins) and lipids (glycolipids), or as loosely associated polysaccharides (Reid et al., 2012). If alginate is not the component of the glycans in the EPS, what could be the possible components is an interesting topic to be investigated. Intrigued by the strong indication of lipopolysaccharides from the FTIR spectrum of Ethanol supernatant III, and phenomena that half of the extracted EPS did not precipitate out from the 80% ethanol-water solution after partial acid hydrolysis, a specific LPS extraction was performed. With the characteristic staircase (ladder-like) pattern of bands shown in the SDS-PAGE gel after staining, it was verified that there was LPS in the extracted EPS.

LPS are glycolipids. It is an amphiphilic molecule with a hydrophobic lipid A region embedded in the outer membrane of Gram-negative bacteria (Seltmann and Holst, 2002). Beyond lipid A, there is a core sugar region and the O-antigen. The O-antigen is composed of 20-70 repeating units of 3-5 sugar monomers (Seltmann and Holst, 2002). As cell turnover and lysis result in the presence of both “cell-bound” and “free” LPS in natural aquatic systems (Rietschel et al., 1993), it can be assumed that there are both “cell-bound” and “free” LPS in the EPS of activated sludge. Since LPS is anionic due to phosphate and acid groups in lipid A, it can be solubilized with alkaline extraction and end up in the extracted EPS. During partial acid hydrolysis, the O-antigens were cleaved from the LPS (Hase and Rietschel, 1976). As the O-antigens are polysaccharides and hydrophilic, they form a precipitate in ethanol, and thus can be collected in Fraction I and II, whereas the leftover (lipid A + core region of LPS) is hydrophobic, it may form micelles in the 80% of ethanol solution without

precipitating out and enriching in Ethanol supernatant III. Therefore, the FTIR spectrum of Ethanol supernatant III showed the strongest bands for lipids.

As shown in Figure 5, LPS is an anionic polymer. Each LPS molecule has multiple negative charges from phosphate and carboxylic acid groups in lipid A and the core region. It was suggested by Adams et al.(Adams et al., 2014) that there are at least six negatively charged groups per LPS. Probably, this anionic property could result in gelling with calcium and has led to a misinterpretation of certain resemblances between the extracted EPS (ALE) and alginates.

LPS is the major component of the surface of Gram-negative bacteria and its glycan portion is situated at the outermost region (Nakao et al., 2012). If there are both “cell-bound” and “free” LPS in the EPS of activated sludge, its anionic and amphiphilic properties certainly influence the property of the EPS. Regarding carbohydrates research in the EPS, most of the studies were focused on free polysaccharides. However, besides free polysaccharides, glycoproteins and glycolipids contain carbohydrates as well. Both of them were found in the EPS of the sludge (Chen et al., 2023; Conrad et al., 2003). Therefore, to obtain a complete overview of the glycans in EPS, it is necessary to include the structure and function of glycoproteins and glycolipids (e.g. LPS) in addition to free polysaccharides.

5. Conclusions

By reinvestigating the sugar monomer composition of EPS extracted as alginate-like exopolymers (ALE) from activated sludge samples, it was revealed that no mannuronic acid and guluronic acid were found in the extracted EPS. If present alginates would likely be a rare feature in extracellular polymers from wastewater sludges, further characterization of the EPS suggested that lipopolysaccharides (LPS) could be a significant fraction of the carbohydrates in the EPS. Therefore, direct proof of the composition by chemical analysis is significantly important for EPS research.

Supporting information

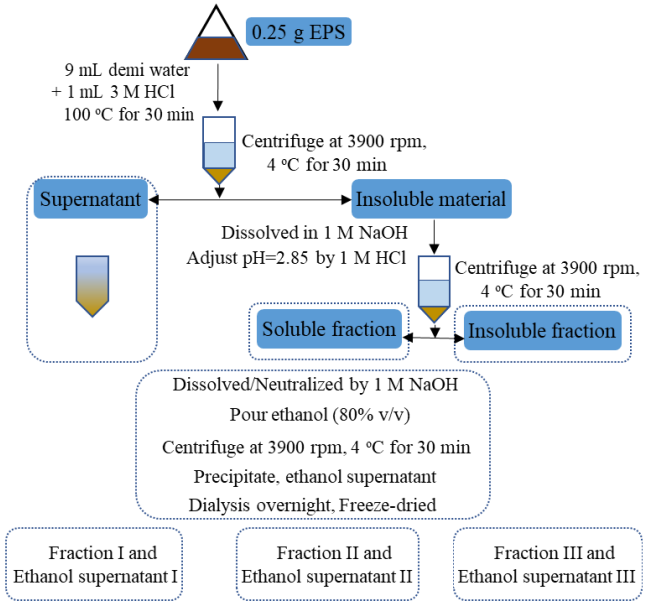

Figure S1 Diagram of EPS fractionation according to partial acid hydrolysis (Lin et al., 2010).

Table S2 Molar mass of different sugar monomers.

Sugars M (g/mol)	Fucose	Galactosamine	Rhamnose	Glucosamine	Galactose	Glucose
	164.16	215.64	164.16	179.17	180.16	180.16
	Xylose	Mannose	Ribose	Galacturonic acid	Glucuronic acid	
	150.13	180.16	150.13	194.14	194.14	

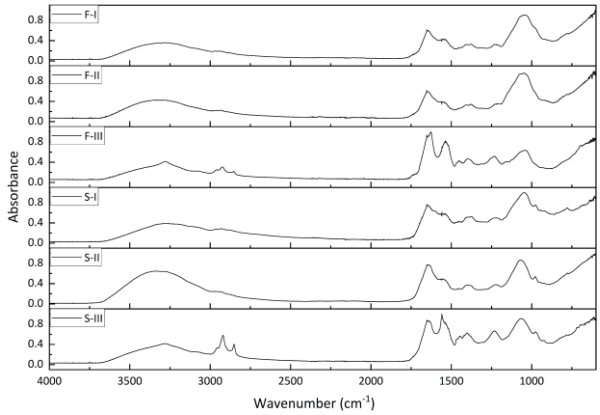

Figure S3 Comparisons of FTIR spectrum between different EPS fractions (from Beijing, China).

Table S4 Summary of the peak wavenumbers in the FTIR spectrum.

Name of the fractions	Wavenumbers in the FTIR spectrum (cm^{-1})
Fraction I	3285, 1652, 1558, 1408, 1056
Ethanol supernatant I	3290, 3090, 2930, 2892, 1638, 1558, 1458, 1405, 1342, 1243, 1059, 971, 788
Fraction II	3200, 2958, 1643, 1557, 1541, 1398, 1383, 1306, 1237, 1047
Ethanol supernatant II	3288, 2306, 1740, 1643, 1543, 1447, 1369, 1231, 1217, 1068, 975
Fraction III	3287, 3081, 2969, 2931, 2897, 2869, 2357, 1729, 1623, 1541, 1456, 1387, 1228, 1163, 1048
Ethanol supernatant III	3296, 2925, 2854, 2371, 1738, 1652, 1548, 1454, 1397, 1382, 1234, 1193, 1067, 994
EPS	3285, 2932, 2854, 1736, 1643, 1545, 1458, 1387, 1236, 1050, 979, 931

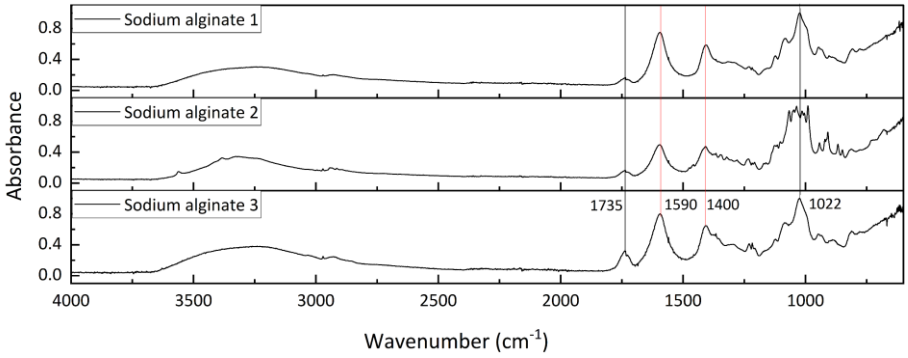

Figure S5 FTIR spectrum of three commercial sodium alginates.

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Chapter 5

Understanding the ionic hydrogel-forming property of extracellular polymeric substances: differences in lipopolysaccharides between flocculent and granular sludge

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Abstract:

An interesting and potential property of extracellular polymeric substances (EPS) is the hydrogel formation with calcium ions. Aiming at understanding the significant difference in the hydrogel formed between EPS from flocculent and granular sludge, a targeted investigation of the lipopolysaccharides (LPS), one of the important EPS components, was performed. LPS was isolated from the EPS of flocculent and granular sludge, and both the glycan and the lipid A parts of LPS were characterized and compared. The morphology of LPS-calcium (LPS-Ca) aggregates were visualized by the polymyxin B-based fluorescent probe. The LPS constituted about 25% and 15% of the EPS from flocculent and granular sludge, respectively. The flocculent sludge LPS showed a lower amount of glycans, shorter glycan chain length, lower molecular weight, and higher possibility of containing unsaturated lipids than the granular sludge EPS. The flocculent sludge LPS-Ca aggregates demonstrated invert structures with the water phase in between, contributing to the fluid-like property of the respect EPS-Ca. In contrast, with the remarkably different chemical structure, LPS-Ca aggregates from granular sludge displayed bilaminar multilayered morphology, contributing to the solid, self-standing hydrogel of EPS-Ca.

1. Introduction

Extracellular polymeric substances (EPS) have been considered essential for the biofilm structure. They are also the important matrix to form the three-dimensional microbial structure of flocs and granules. The role of EPS in different biofilms has currently attracted increasing attention. Great efforts have been made to distinguish the distinction between EPS from flocculent and granular sludge (Lv et al., 2014; McSwain et al., 2005; Zhu et al., 2015). The general approach is that, after EPS extraction from flocculent or granular sludge, the EPS composition and properties are analyzed, and differences as well as similarities are compared.

An interesting EPS property is the gel-forming property (Lin et al., 2013; Schambeck et al., 2020; Seviour et al., 2009). Seviour et al. (2009) studied the EPS sol-gel transition at different pH, observing that strong gel was formed by EPS from granules while not by any of the EPS samples from flocs. The ionic gel formation property of EPS with $CaCl_2$ solution was studied by Lin et al. (2013) afterward, it was reported that the mechanical property of EPS-Ca hydrogel from granular sludge was much stronger than that from flocculent sludge. The proposed explanations for gel formation were mainly based on the interactions between proteins and polysaccharides with calcium (Goh et al., 2014). It was believed that certain amino acid residues present in EPS could interact with calcium ions (Khalili et al., 2004; Leonard, 2013). On the other hand, Seviour et al. (2009) suggested that glycans were the dominant agents for forming a strong gel instead of proteins. Glycans contain functional groups such as hydroxyl and carboxylate groups, which can interact with calcium ions through ion-dipole or ion-ion interactions (Mierczyńska et al., 2015; Wang et al., 2018). It is noted that glycans include free polysaccharides and glycoconjugates such as glycoproteins and glycolipids. Different types of glycans might interact with calcium ions in a dissimilar way. Therefore, to understand the exact contribution of glycans to EPS hydrogel formation, it is necessary to investigate each type of glycans separately.

Very recently, it was reported that lipopolysaccharides (LPS, one type of glycolipids) were one of the important glycoconjugates in EPS extracted from flocculent sludge (Li et al., 2024). Previous studies indicated that LPS could be the key component for aggregate formation (Parikh and Chorover, 2007; Pazol et al., 2022; Rahnamoun et al., 2020). LPS is amphiphilic, with lipid A as the hydrophobic part and glycans as the hydrophilic part (including core oligosaccharides and O-antigen). Lipid A is relatively conservative. It interacts with calcium ions through the coordination of phosphate

and/or carboxylate groups. In addition, the glycan part in LPS is extremely variable and could be crosslinked forming dimensional structures via calcium ions (Balantič et al., 2022; Leonard, 2013; Valentine et al., 2020). These findings indicate that there are active sites in LPS which can interact with calcium ions. What is the difference and similarity between the LPS from flocculent sludge and granular sludge and how does the LPS and calcium interaction influence the EPS and calcium hydrogel? These are important research questions to be answered for a better understanding of the gel-forming property of the EPS. To study this, LPS was isolated from the EPS of flocculent and granular sludge. Both the glycan part and the lipid A part were characterized and compared. The interaction of LPS with calcium ions was visualized by the specific staining. This study sheds light on the possible connections between LPS structures and composition with the EPS gel-forming property.

2. Material and methods

2.1. Extracellular polymeric substances (EPS) extraction from flocculent and granular sludge

The flocculent sludge was collected from two municipal wastewater treatment plants using the activated sludge process in Delft and Rotterdam, the Netherlands; and the granular sludge was collected from two municipal wastewater treatment plants using the aerobic granular sludge process (Nerada®) in Utrecht and Garmerwolde, the Netherlands. The collected raw sludge samples were first frozen and lyophilized afterwards for storage. The EPS was extracted from both the flocculent and granular sludge according to the previously reported extraction protocol (Li et al., 2021), i.e. 3.0 grams of dried sludge was resuspended in 100 mL demi-water with adding 0.5 grams of sodium carbonate. The mixtures were then heated at 80 ^{o}C for 30 min. After centrifuge, the supernatant was adjusted to pH 2.2 with 1 M HCl. The precipitates were collected, with a part of them being saved for the ionic hydrogel formation tests (the details refer to section 2.3), and the rest being dissolved in 1 M NaOH till pH 8.5 and dialysed in the dialysis bag with the molecular weight cut-off (MWCO) of 3.5 KDa before lyophilization.

These lyophilized EPS samples were dissolved in 0.1 M NaOH at a concentration of 1,000 mg/L overnight before the determination of total carbohydrates content using the phenol-sulfuric acid method (Dubois et al., 1951) with glucose as the standard and the total proteins content using BCA assay with BSA as the standard (Smith et al., 1985).

2.2. Lipopolysaccharides (LPS) isolation from EPS

The LPS was extracted from those lyophilized EPS samples using the hot phenol-water method in accordance with the previous studies (Davis and Goldberg, 2012; DeLeo and Otto, 2008; Li et al., 2024). 10 mg of EPS samples was completely dissolved in 1 mL of a 1xPBS solution (pH=7.0). Then, 50 uL of proteinase K solution (10 mg/mL) was added and the mixture was heated at 65 ^{o}C for 3 hours to cleave the peptides. An equal volume (1mL) of water-saturated phenol solution (pre-heat at 68 °C) was added to the mixtures followed by vigorously shaking at 68 °C for 15 min. Suspensions were then cooled on ice with the addition of 1mL of Milli-Q water before centrifugation at 12,000 rpm for 15 min. The tube was gently taken out from the centrifuge and the top aqueous layer was transferred into 10 mL of polypropylene tubes. The bottom layer of the phenol phase was re-extracted twice by an equal volume of Milli-Q water. All of the top layer solutions were mixed together and dialyzed against demi water in the dialysis tubing (MWCO 3.5 KDa) and lyophilized afterwards.

2.3. Interaction of both EPS and LPS samples with calcium ions

EPS-Ca hydrogel formation tests were conducted to assess the hydrogel formation capacity of EPS extracted from both flocculent and granular sludge (Felz et al., 2016). Part of the EPS acidic precipitates formed at pH 2.2 (the details refer to section 2.1) were dissolved by adding 1.0 M NaOH till pH 8.5. The final concentration of the four EPS samples was at around 1.5% (w/v). Afterwards, those EPS solutions were transferred into plastic cylinders respectively. The size of the plastic cylinders was 23x15 mm (Diameter x Height), with the two ends sealed by the dialysis bag (MWCO 3.5 kDa). The plastic cylinders filled with EPS were put into a glass beaker containing 2.5% calcium chloride solution (w/v) for 48 hours with slow stirring. The calcium solution was changed every 12 hours. After the gel formation tests finished, the EPS-Ca gel samples were collected for microscopic study.

Regarding the interaction of LPS with calcium ions, instead of dissolving the sample in 1.0 M NaOH, LPS was first dissolved in demi-water. The LPS solution was filled in the plastic cylinders with the size of Diameter x Height=1.0x1.0 mm, with the two ends sealed by the dialysis bag (MWCO 3.5 kDa). The plastic cylinders filled with LPS were put into a glass beaker containing 2.5% calcium chloride solution(w/v) for 48 hours with slow stirring. The LPS-Ca aggregates were collected for further microscopic study.

2.4. LPS characterisation and visualization

2.4.1. LPS detection by SDS-PAGE with Pro-Q Emerald 300 staining

In this study, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to visualize the possible length of the glycan part in LPS according to Li et al. (2024), i.e., the lyophilized LPS samples were solubilized in miliQ water (1 mg/mL) before mixing with the loading buffer. 10 uL of LPS solutions and 8 uL of LPS standard (from *Escherichia coli* serotype 055:B5, 0.25 mg/mL) were loaded into the wells and separated on 12% SDS gel with a 4% stacking gel under reducing conditions at 200 V for 40 min. Afterwards, Pro-Q Emerald 300 staining of the gels was performed according to the protocol provided by Thermo-Fisher Scientific. The image of the gel was taken by a ChemiDoc MP imager (Bio-Rad, Hercules, CA).

2.4.2. Sugar monomers detection by HPAEC-PAD

Sugar monomers in the four LPS samples were analysed using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) following the procedure described in (Felz et al., 2019). 1.0 mg of LPS samples were first hydrolysed in 1.0 mL of 1 M HCl in a 105°C oven for 8 hours. After centrifugation, the supernatant was neutralized with 1 M NaOH and filtered through 0.22 µm PVDF filters. Filtrates were diluted with Milli-Q water in accordance with the concentration of the standard sugar monomers before being injected into the HPAEC-PAD. Seven neutral sugars (fucose, rhamnose, galactose, glucose, xylose, mannose, and ribose) and two amino sugars (galactosamine and glucosamine) were selected as standards in this study.

2.4.3. Functional groups identification by FTIR

The functional groups of LPS were analyzed by Fourier Transform Infra-Red (FTIR) spectroscopy over a wavenumber range from 4,000 cm^{-1} to 600 cm^{-1}.

2.4.4. Morphology of LPS-Ca aggregates visualisation using the polymyxin B-based fluorescent probe

Polymyxin B labelled with Rhodamine B was used to visualise the structures and morphology of LPS-Ca aggregates. The mechanism of the staining is that polymyxin B interacts with LPS molecules via their cationic L-α-γ-diaminobutyric acid (Dab) side chains and the hydrophobic residues LPS-Ca aggregates which were obtained as described in Section 2.3 were washed with 1xPBS twice, followed by the incubation with polymyxin B labelled with Rhodamine B solution (1.1 ug/mL) at room

temperature for about 2 min. After centrifugation, the pellet was washed 4 times with 1xPBS to remove the residual probes. Finally, the samples were resuspended in 100 uL of 1xPBS for acquiring fluorescence imaging immediately. The images of polymyxin B with fluorescent probe Rhodamine B were captured with a CY3 filter. For each samples, various spots were chosen examined to observe the main features of these aggregations.

3. Results

3.1. The ionic hydrogel formation property of EPS from flocculent sludge and granular sludge

EPS was extracted from two flocculent sludge samples and two aerobic granular sludge samples and their yields are listed in Table 1. The contents of total carbohydrates and proteins of each EPS are listed in the Supplementary Materials Table S1. It is noticed that the EPS yield from aerobic granular sludge is relatively higher than that from flocculent sludge. While looking at the hydrogel-forming property of EPS with calcium ions, the difference was apparent. Although both EPS from flocculent sludge and granular sludge exhibited the ability to form viscous hydrogels, the EPS-Ca hydrogel from flocculent sludge still looked like a fluid and was not able to self-stand at all (Figure 1). In contrast, the EPS-Ca hydrogel from granular sludge became a solid piece reflecting the shape of the mould and able to self-stand completely.

Figure 1 EPS ionic hydrogel formation test by immerging EPS solution in calcium chloride solution ($CaCl_2$, 2.5%, w/v). EPS concentrations were at around 1.5%±0.3%.

Table 1 Yields of EPS and LPS from both flocculant and granular sludge.

Sludge type	Wastewater treatment plant	EPS yield (mg EPS/g VS sludge)	LPS yield (mg LPS/g VS EPS)
Flocculent sludge	Delft	164.3±11.8	248.4±4.4
	Rotterdam	299.8±2.4	256.7±7.3
Aerobic granular sludge	Utrecht	245.2±11.9	146.4±14.6
	Garmerwolde	223.7±1.7	154.1±13.7

3.2. LPS isolation and characterisation

To understand the dissimilarity between the EPS-Ca hydrogels, a more targeted investigation by studying the specific EPS components was conducted. Lipopolysaccharides, which are an important type of glycolipids in the EPS, were selected for the investigation.

3.2.1. Yield of LPS isolation

The yield of LPS is listed in Table 1. It was interesting to see that the amount of LPS extracted from the EPS of granular sludge was much lower than that from flocculent sludge. Moreover, the total carbohydrates content of the extracted EPSs varied at 16%-22% (see Supporting Information Table S1), while the amount of LPS was already at 15%-25% of the extracted EPS. This implies that the amount of LPS might contribute significantly to the amount of total carbohydrates within the EPS. Additionally, their structure and property may influence the characteristics of EPS.

3.2.2. Glycan chain length distribution of LPS

LPS consists of lipid A and glycans (including core oligosaccharides and O-antigen, as shown in Supporting Information Figure S2). SDS-PAGE with Pro-Q Emerald staining was applied to investigate the chain length distribution of O-antigen which influences the molecular weight of LPS samples. O-antigen in LPS varies in their sugar monomer composition and the number of repeating units. The more repeating units in the O-antigen, the longer the chain length is, and the higher molecular weight the LPS is. This can be displayed in the SDS-PAGE with the well-known “ladder-like” bands (Jann et al., 1975). Each band corresponds to the O-antigen with different repeating units. The fluorescence image is presented in Figure 2. Clearly, the ladder-like pattern was displayed among each LPS sample, indicating that all of the LPS samples contained the O-antigen part. As the bands at the upper gel represent higher molecular weight O-antigen with longer sugar chains (Jann et al., 1975; Whitfield et al., 2020), it seems that the LPS from granular sludge should have relatively longer sugar chains.

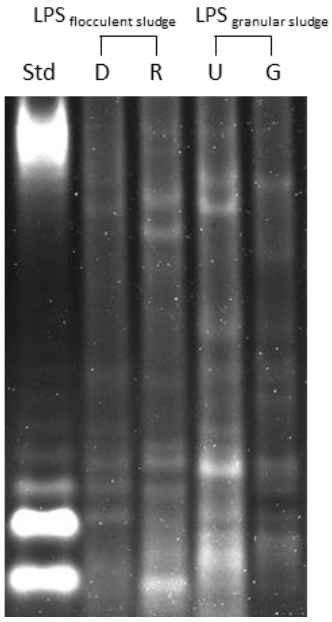

Figure 2 SDS-PAGE with Pro-Q Emerald kit staining for lipopolysaccharides (LPS). Std, LPS standard from *Escherichia coli* serotype 055:B5. D, R, U and G: LPS samples were extracted from EPS in Delft, Rotterdam, Utrecht and Garmerwolde plants, respectively.

3.2.3. Sugar monomers composition of glycans in LPS

Quantitative analysis of monosaccharides in the LPS was conducted by HPAEC-PAD and the results were summarised in Table 2. In general, the total amount of sugar monomers in the LPS from granular sludge was higher than that from flocculent sludge, meaning that there is a higher amount of glycans in the LPS from granular sludge. This is in agreement with the findings from the SDS-PAGE analysis that the LPS from granular sludge has a longer sugar chain. Interestingly, for the LPS from flocculent sludge, the difference between the two samples is relatively big, i.e. the total amount of sugar monomers in the LPS from the sludge collected at the Rotterdam wastewater treatment plant is much higher and close to that of granular sludge. Whether it is due to the properties of the sludge in this specific wastewater treatment plant, or the change of the amount of sugar monomers between flocculent sludge and granular sludge was in a broad range, is an interesting topic to follow up.

To look at the detailed composition, the relative mole percentages of different sugar monomers were calculated and listed in Table 2. Firstly, there were a few sugar monomers that were more than 20% (relative mole percentage) of the total amount of sugar monomers in the LPS. i.e. fucose, mannose and ribose in the LPS from

flocculent sludge; fucose and mannose in the LPS from aerobic granular sludge. Judging from this bigger amount, probably, those sugars were part of the components in the O-antigen repeating units. Secondly, the significant difference between LPS from flocculent sludge and granular sludge was the amount of mannose and ribose: the LPS from granular sludge was rich in mannose while the LPS from flocculent sludge was rich in ribose. Mannose is C6 sugar while ribose is C5 sugar. Although mannose and ribose were found in both the core regions and the O-antigen of LPS (Samuel and Reeves, 2003), with more -OH groups and bigger molecule size, a higher mole percentage of mannose might increase the hydrophilic property and the glycan size, providing more opportunities for hydrogen bonding and intermolecular interactions. In fact, it was reported that the variations of sugar monomer composition in different polysaccharides affect the aggregate's morphology (Charchoghlyan and Park, 2013; Miyoshi et al., 1998).

Table 2 Relative mole percentages of sugar monomers and the total amount in weight of lipopolysaccharides.

Sludge type	Plant	Total (mg/g VS LPS)	Fucose (%)	Galactosamine (%)	Glucosamine (%)	Galactose (%)	Glucose (%)	Mannose (%)	Ribose (%)
Flocculent sludge	Delft	435.3	22.9	3.4	6.2	2.4	9.7	27.5	27.9
	Rotterdam	620.9	17.0	3.1	7.5	4.7	8.2	28.4	31.2
Aerobic granular sludge	Utrecht	693.6	24.0	4.2	7.7	4.6	10.2	40.4	9.0
	Garmerwolde	801.7	20.5	4.4	7.7	4.7	11.8	42.1	8.8

3.2.4. Functional groups related to the lipid A in LPS

The functional groups of LPS were investigated by FTIR spectroscopy. The spectra are shown in Figure 3, the peaks at the wavenumber of around 2,920 and 2,860 cm^{-1} were assigned to the vibration of the methylene ($-CH_2$), while peaks at around 2,960 cm^{-1} and 1,380 cm^{-1} were assigned to methyl ($-CH_3$) vibration. The hydrocarbon chains of fatty acids in lipid A are the main contributors to these peaks (Liu, 2021). The typical 1,550 cm^{-1} peaks of the amide II groups (-NH) which were strong in EPS samples (EPS FTIR spectra were shown in Supporting information Figure S2) disappeared in the LPS spectra, indicating that there was little protein present due to the pretreatment of protease before the LPS extraction. Thus, the vibrations at 1,630 cm^{-1} were mainly from the amide I group (O=C-NH) of the N-acetylglucosamine parts in lipid A. The wavenumbers at around 1,230 and 1,217 cm^{-1} were from the phosphate groups (-P-O) (1,230 cm^{-1} is metal ions bond phosphate and 1217 cm^{-1} is hydrogen bond phosphate), either attaching to N-acetylglucosamine in lipid A or Kdo (3-deoxy-d-manno-oct-2-ulopyranosonic acid) in the inner core.

Notably, there were some different peaks observed in the FTIR spectra between the LPS from flocculent sludge and granular sludge: firstly, the relative peak intensity between the peak at 1,040 cm^{-1} (the typical C-O-C bond of glycans) and the peak at 1,630 cm^{-1} (amide I group of N-acetylglucosamine) is higher for the LPS from granular sludge than that of flocculent sludge (2 versus 1.6), suggesting that the LPS from granular sludge contain more glycans, which aligned with the aforementioned analysis.

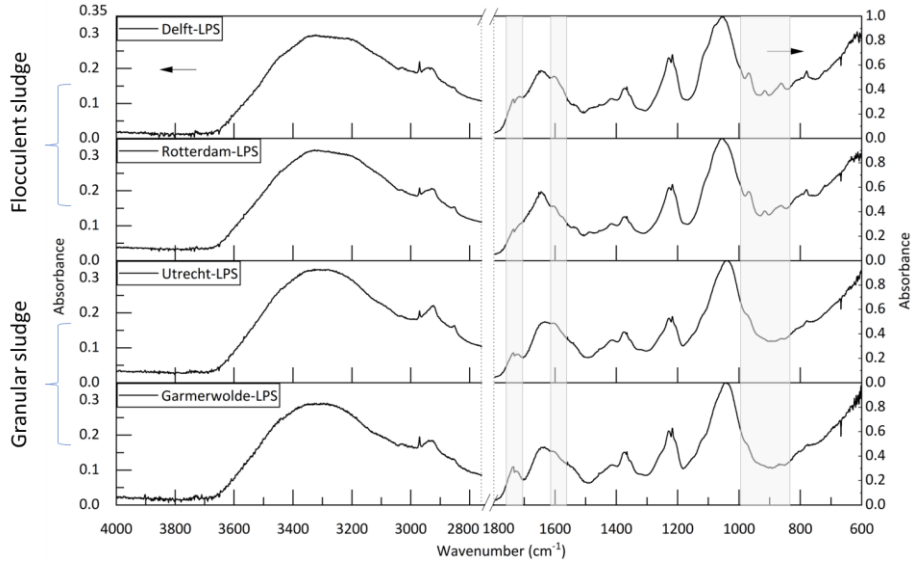

Figure 3 FT-IR spectra of lipopolysaccharides (LPS) isolated from flocculent sludge (Delft and Rotterdam) and granular sludges (Utrecht and Garmerwolde) (wavenumber from 4000-2800 and 1800-600 cm^{-1}).

Secondly, the wavenumber region of 1,700-1,750 cm^{-1} corresponds to the ester carbonyl (-C=O) absorption in lipid A (Valentine et al., 2020). Two peaks are presenting at 1,735 cm^{-1} and 1,718 cm^{-1} in the spectra of the LPS from granular sludge, with the absorbance intensity at 1,735 cm^{-1} stronger than that at 1,718 cm^{-1}. In comparison, these peaks are hardly seen in the spectra of the LPS from flocculent sludge. Given that the C=O groups can participate in hydrogen bonding interactions with water molecules or neighbouring polar groups, (Brandenburg et al., 1997), the position and intensity of these carbonyl bands reflect the hydrogen bonding state of the ester carbonyl groups (Dreier et al., 2019), i.e. the peak at 1,718 cm^{-1} attributed to hydrogen-bonded carbonyl groups, while the peak at 1,735 cm^{-1} attributed to the non-hydrogen bonded (free) carbonyl groups (Urakawa et al., 2017). A stronger peak at 1,735 cm^{-1} in granular sludge implies that the lipid exhibits higher density and/or

the lipid layer is more densely packed, so that the carbonyl groups have little chance of being involved in the hydrogen bonding (Dicko et al., 1998).

Lastly, there are a few bands present in the spectrum of LPS from flocculent sludge below the wavenumber of 1,000 cm^{-1} (bands with the peak at around 780, 865, 920 and 965 cm^{-1}), while this region in the spectrum of LPS from granular sludge is rather smooth. Interestingly, these bands correspond to the characteristic vibrations of trans-disubstituted alkenes (-CH_2) in unsaturated fatty acid chains. Compared to the saturated fatty acids, the unsaturated fatty acids with trans-disubstituted -CH_2 do not pack densely (Lewis and McElhaney, 2013). This could also explain why no strong peaks are appearing at 1,735 cm^{-1} in the spectrum of the LPS from flocculent sludge.

In summary, the FTIR spectroscopy analysis demonstrates that in comparison to the LPS from granular sludge, the LPS from flocculent sludge contains fewer glycans and the possible presence of unsaturated fatty acids in lipid A which limits dense packing.

3.3. LPS-Ca aggregates visualization

To investigate the role of LPS in EPS-Ca hydrogel formation, the interaction between the LPS samples and calcium ions was investigated. The phase contrast microscopic images of LPS-Ca aggregates are shown in Figure 4a. All of the LPS samples interact with calcium forming aggregates. However, the size of the aggregates is significantly different. The majority of the LPS-Ca aggregates from flocculent sludge is about 10-20 µm, distributing evenly in the aqueous solution. While the size of the LPS-Ca aggregates from granular sludge is much bigger, falls in the range of 100-400 µm.

To achieve targeted visualization of LPS, the polymyxin B based fluorescence probe was applied. Polymyxin B specifically binds to the lipid A in LPS. By labelling with rhodamine B as the fluorescence probe, the specific morphologies of LPS-Ca aggregates were clearly visualized with the fluorescence signal. There are some similar morphologies observed in all LPS-Ca samples (supplemental material Figure S3), however, significant differences were observed in these LPS-Ca aggregates as shown in Figure 4b. LPS-Ca from flocculent sludge displayed the loosely packed inverted structure with water phase (black holes) in between the aggregated structure, with LPS-Ca $_{Delft}$ as inverted micelle and LPS-Ca $_{Rotterdam}$ as inverted rod, specifically. In comparison, LPS-Ca from granular sludge demonstrated the densely packed structure, with LPS-Ca $_{Utrecht}$ as bilaminar multilayered vesicles (onion-like) and LPS-Ca $_{Garmowolde}$ as spheres.

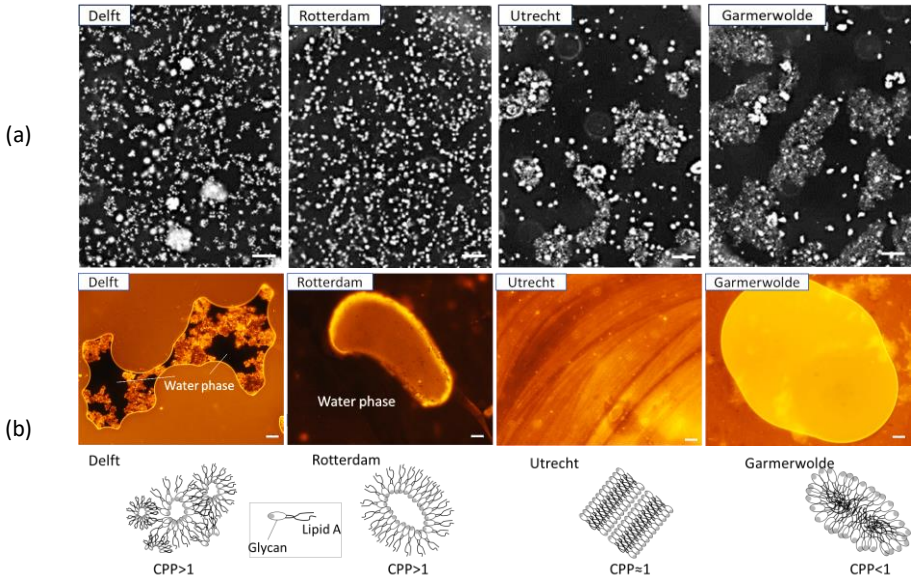

Figure 4 The interaction between LPS and calcium ions (calcium chloride). a). phase contrast images showing the LPS-Ca aggregates. The size of LPS-Ca aggregates from flocs (Delft and Rotterdam) is much smaller than that from granules (Utrecht and Garmerwolde) (20-40 µm versus 200 µm). Scale bar: 100 µm. b). Visualization of LPS-Ca aggregates using polymyxin B labelled with Rhodamine B staining. The fluorescence images were captured with a CY3 filter. The fluorescent signal rich region indicate that binding of the lipid A in the LPS. Scale bar: 20 µm. The schematic diagram underneath the figures gives the possible formed structure and the concept of critical packing parameter (CPP) (more detailed information about CPP was discussed in section 4.1).

4. Discussion

4.1. Differences in LPS between flocculent and granular sludge influence the morphology of LPS-Ca aggregates

Lipopolysaccharides (LPS), one of the important EPS components, were specifically extracted from the EPS isolated from flocculent and granular sludge, characterized and compared. LPS consists of the glycan part and lipid A. Looking at the glycan part, the LPS from granular sludge contained more glycans, with longer glycan chain lengths and higher molecular weights. Regarding the lipid A part, it was more packed in the LPS from granular sludge. Moreover, there was almost no visible signal of unsaturated lipids observed in the FTIR spectrum of the LPS from granular sludge. In comparison, there were clear signals of unsaturated lipids with trans configuration in the LPS from flocculent sludge. By applying the staining with polymyxin B-based fluorescent probe, bilaminar multilayered structures were observed in the LPS-Ca aggregates from granular sludge, while the inverted structures were observed in the

LPS-Ca aggregates from flocculent sludge.

LPS are amphiphilic molecules, which can form a variety of aggregated structures in aqueous solutions. The type of those structures can be connected with the critical packing parameter (CPP) (Dopazo et al., 2021). This parameter is calculated as $CPP = \frac{V}{a_0 l_c}$, where *V* is the volume of the hydrophobic part of the molecule (in the current research it is the volume of the lipid A chains); a_0 is the area of the head group (here it is the optimal area of the glycan part); *lc* is the critical length of the hydrophobic tail (the critical length of the lipids).

According to the literature, the length of lipids in the LPS is relatively conservative (Wolny et al., 2011). Thus, *lc* can be assumed the same for LPS from both types of sludge. At this aspect, the *CPP* comparison can be simplified as $\frac{V_{lipidA}}{a_{glycans}}$, implying that the difference in the characterization of lipid A and glycans could influence the morphology of the LPS-Ca aggregates. In fact, there was a strong indication that the LPS from flocculent sludge has unsaturated fatty acid chains (with trans configuration). These chains introduce kinks in their structure due to double bonds, thus increasing the volume of the lipid A chains (Casares et al., 2019; Lewis and McElhaney, 2013). Furthermore, at the glycan part, the relatively shorter glycan chain length and lower molecular weight of the LPS from flocculent sludge might induce a lower optimal area *a*. Thus, with a higher volume (*V*) of lipid A and a lower optimal area (*a*) of glycans, $CPP_{\text{LPS from flocculent sludge}}$ is higher than $CPP_{\text{LPS from granular sludge}}$, favouring a less packed structure. Interestingly, according to (Israelachvili, 2011; Parikh and Chorover, 2007) if the aggregation structure is lamellar-like, CPP$\approx$1 (e.g. Figure 4b LPS-Ca Utrecht granules); if it is an invert structure, CPP>1 (e.g. Figure 4b LPS-Ca Delft and Rotterdam flocs); if it is sphere-like, CPP<1 (e.g. Figure 4b LPS-Ca Garmerwolde granules). When the different morphology of LPS-Ca in Figure 2 is considered, the same conclusion that $CPP_{\text{LPS from flocculent sludge}} > CPP_{\text{LPS from granular sludge}}$ can be drawn as well. Therefore, the difference in the morphology of LPS-Ca aggregates is indeed in agreement with the dissimilarity of the chemical structure and composition between the LPSs.

4.2. LPS serving as an important component contributes to EPS properties

LPS accounted for about 15%-25% of the total organic mass of the extracted EPS which is a significant fraction. LPS are amphiphilic molecules, forming a variety of

aggregated structures in aqueous solutions. These aggregated structures could be stabilized by calcium ions through bridging adjacent phosphate groups in lipid A. The chemical structure and morphology of LPS-Ca might contribute to the property of EPS-Ca. Apparently, the compact bilaminar multilayered structure favors the formation of tightly packed structures that can self-stand, such as the EPS-Ca from granular sludge demonstrated. In contrast, the invert structure with the water phase in between the aggregation hinders the packing of the molecule, resulting in a fluid-like structure that is impossible to self-stand, such as the EPS-Ca from flocculent sludge displayed. Moreover, fatty acids are the components of lipids. Usually, unsaturated fatty acids have lower melting points and exist as a liquid at room temperature (Caliph et al., 2000; Jannin et al., 2008). Saturated fatty acids are comparatively more tightly packed together than unsaturated fatty acids, they have higher melting points (Siram et al., 2019). As another influential fact, the high chance of unsaturated fatty acids in the LPS from flocculent sludge might increase the fluidity of EPS-Ca.

It is worth pointing out that, in the current discussion, assumptions and simplifications have been made to get a general overview of the connection between the characteristics of LPS and the structure of the EPS-Ca aggregates. To further understand the exact correlation, future research is needed to determine the chemical structure of lipids by GC-MS and the molecular formula of the glycan chains by NMR and MS. In addition, the conformation of amphiphilic molecules such as LPS are extremely sensitive to environmental factors such as the ionic strength, type of ions in the solution, the pH and temperature (Israelachvili, 2011), which are necessary conditions to be considered when studying the structure of EPS hydrogel.

5. Conclusions

Lipopolysaccharides (LPS), which is an important glycolipid component in the extracellular polymeric substances (EPS), constituted about 15%-25% of the EPS from flocculent and granular sludge. With a lower amount of glycans, shorter glycan chain length, lower molecular weight and high possibility of containing unsaturated lipids in the LPS from flocculent sludge, the LPS-Ca demonstrated an invert structure, contributing to the fluid-like property of the EPS-Ca. In contrast, with the remarkably different chemical structure, LPS-Ca from granular sludge displayed bilaminar multilayered morphology, contributing to the solid self-standing gel of EPS-Ca. The study of LPS sheds light on the properties of EPS and further developing EPS-based products.

Supporting Information

Table S1 Total proteins and carbohydrates of EPS from different sludge.

Sludge samples	EPS yield (mg/g VS sludge)	Total proteins (mg BSA equivalent/g VS EPS)	Total carbohydrates (mg glucose-equivalent/g VS EPS)	PN/PS
Delft flocs	164.3±11.8	395.6±0.0	163.1±4.4	2.39
Rotterdam flocs	299.8±2.4	380.9±3.7	166.4±5.0	2.44
Utrecht granules	245.2±11.9	410.7±6.0	159.6±0.8	2.96
Garmerwolde granules	223.7±1.7	388.9±6.1	216.1±1.4	1.79

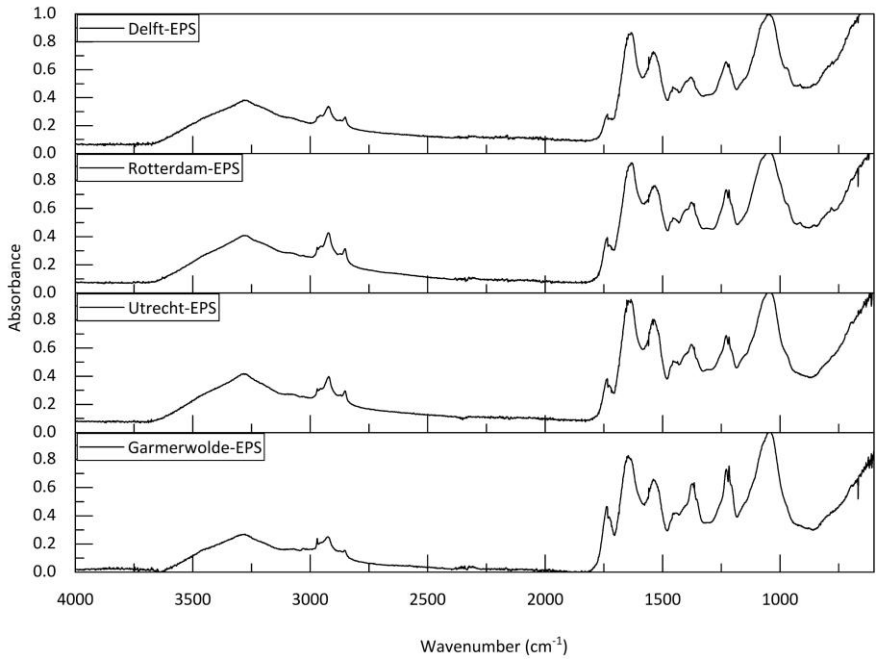

Figure S2 FTIR spectra of EPS samples from different sludge.

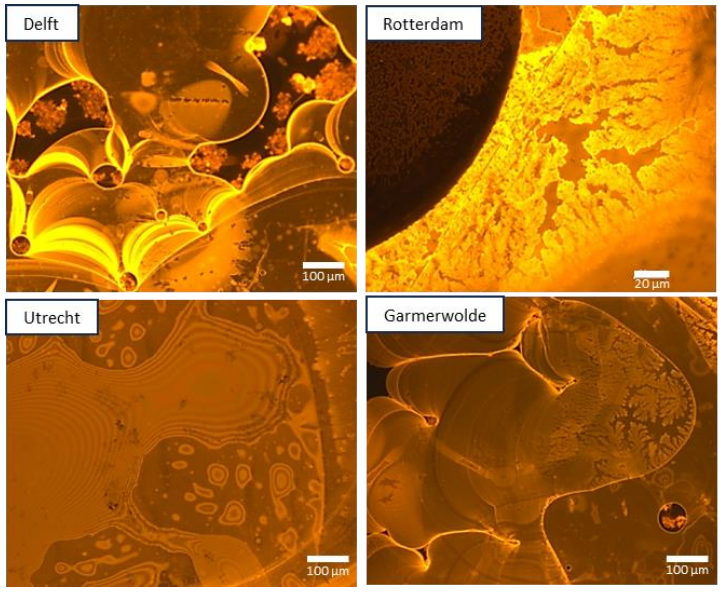

Figure S3 Overview of LPS-Ca morphology using polymyxin B labelled with rhodamine B staining. Microscopy images were captured with a CY3 filter.

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Chapter 6

Outlook

During the biological wastewater treatment process, a large amount of excess sludge is generated. In most cases, the excess sludge is considered as a waste and the handling cost can reach 50% of the total wastewater treatment costs (Kroiss, 2004). However, this excess sludge is a potential resource where useful materials, such as extracellular polymeric substances (EPS), can be recovered and upcycled back into the material flow of the society, contributing to the circular economy. Inspired by the EPS recovery from aerobic granular sludge, aiming at searching for a larger resource, the feasibility of EPS recovery from activated sludge was investigated.

Through the chapters, the EPS recovery potential from activated sludge collected at full-scale wastewater treatment plants and its connections with the microbial community were evaluated. The influence of the operational conditions was explored with lab-scale reactors under well-designed conditions. The specific EPS component was uncovered and characterized further. During the journey, new research questions have emerged, which will be addressed in this section.

1. EPS recovery potential and its influencing factors

Activated sludge was collected from full-scale plants from different regions in China. It was found that the EPS yield ranged from 9%-19% of total organic fractions of raw sludge (Chapter 2). High EPS yields happened along with the low microbial diversity. The presence of Family *Saprospiraceae* and *Nitrosomonadaceae* had a positive correlation with EPS production while Family *Anaerolineaceae* had a negative correlation. Well-defined lab-scale reactors were initiated for operation to study the individual factors influencing EPS production and compositions (Chapter 3). With starch as the carbon source, the amount of the extracted EPS reached at around 22% of the organic fraction of the sludge, and the low temperature (12 ^{o}C) was favorable to enhancing EPS production to around 30% of the organic fraction of the raw sludge. On the other hand, organic load and solid retention time (SRT) had almost no correlation with EPS production.

Based on these findings, one of the suggestions for future work is to generate an EPS model, which can be used to predict the amount of EPS and its composition in flocculent sludge and even in granular sludge. Two modules can be included in this model. For the module on EPS production, the understanding of EPS synthesis pathways involved with bacteria metabolisms, and the connections of EPS production with different factors (such as operational conditions and influent composition), need to be further explored. Secondly, stoichiometric equations and

variables associated with EPS formation need to be figured out by investigating the secretion of intermediate metabolites, shedding of the cell surface structure to the extracellular matrix, and the impact of cell lysis. For the module on EPS characterization, given the importance of the EPS properties in exploring their application, compositions such as proteins and polysaccharides, important monosaccharides (e.g. sialic acids), and glycoconjugates (glycoproteins and glycolipids) can be included. Finally, to validate and optimize the model, the yield of more EPS samples extracted from different wastewater treatment plants, together with the input of detailed operational parameters, and the EPS characterization data are needed.

2. Specific EPS components

To develop EPS-based products, it is extremely important to understand their composition. To continue sorting out the EPS components, an examination of the presence of alginate in the extracted EPS was performed (Chapter 4). Neither mannuronic acid (M) nor guluronic acid (G) was found in the extracted EPS confirming the absence of alginates. On the other hand, this research led to the findings of lipopolysaccharides (LPS, one type of glycolipids) in the extracted EPS. Furthermore, in Chapter 5, it was found that both the EPS from flocculent sludge and granular sludge contain LPS with a comparable content to total carbohydrates and proteins. LPS in EPS from activated sludge exhibited characteristics such as lower amounts of glycans, shorter glycan chain length, lower molecular weight, and a higher possibility of containing unsaturated lipids than that of granular sludge, determining the interaction of LPS with calcium and further affecting the properties of EPS ionic hydrogel.

The doubt about the existence of alginate in the extracted EPS was clarified, which led to the next question: what about its presence in the sludge biomass? Alginate can be produced by two bacterial species, *Azotobacter vinelandii and Pseudomonas spp.* These bacteria were found in both flocculent sludge and granular sludge, with low abundance (Dueholm et al., 2023). To validate the existence of alginate in sludge, direct analysis of the monosaccharides: mannuronic acid (M) and guluronic acid by HPAEC-PAD and NMR in raw sludge after acid hydrolysis should be done. This analysis should be carried out directly on the raw sludge, which can avoid uncertainty such as the loss of alginate molecules during the extraction and pretreatment processes.

Secondly, it was found that one of the differences in the LPS between flocculent

sludge and granular sludge was related to lipid A. It was suggested by the FTIR spectrum that there was a higher chance of the presence of unsaturated fatty acids in the lipid A of LPS from flocculent sludge than that from granular sludge. To further investigate the fatty acid structure in the lipid A, ^{13}C NMR and GC/MS-based analysis of the LPS can be conducted to characterize further the hydrocarbon chain length as well as the numbers of the double carbon bond and their positions in fatty acid residues (Chen et al., 2024; Chiu and Kuo, 2020).

It was also noticed that there were differences within the LPS from the same type of sludge (flocculent sludge or granular sludge) e.g. LPS from the activated sludge collected at the wastewater treatment plant in Rotterdam exhibited a longer sugar chain and higher molecular weight than that from the activated sludge collected at Delft, which was more similar to the LPS from granular sludge to some extent. This raised the questions: what is the range of the variability in LPS chemical structure within one type of sludge? Is there any overlap between this range of flocculent sludge and granular sludge? Increasing the number of LPS samples from wastewater treatment plants for the analysis of the chemical structures and generating this range can be the starting point.

3. EPS-based products

Different from the EPS-Ca hydrogel from granular sludge, the EPS-Ca from flocculent sludge cannot form the self-standing hydrogel. One of the explanations could be the dissimilar chemical structure of the LPS. LPS is an amphiphilic molecule. It forms hydrogel when the concentration is higher than its critical micelle concentration (CMC, also called critical aggregation concentration) (Aurell and Wistrom, 1998). Thus, there might be the possibility that due to the different chemical structures, a higher concentration is needed for the EPS-Ca from flocculent sludge to form a firm hydrogel. In this respect, future work can be done on the study of the CMC of the LPS and EPS from different types of sludge with the help of rheological measurement, and select appropriate EPS concentrations for further tests.

Glycans and lipid parts involved in LPS make it amphiphilic. The degrees of hydrophilic and hydrophilic parts significantly affect LPS properties. This amphiphilic structure might contribute to the development of the adhesive material, which can be another topic in future research.

4. Limitations in this study

In the current discussion, the critical packing parameter (CPP) was connected to the

aggregated structures of LPS. In the parameters for CPP calculation, the length of the lipids was assumed to be the relatively conservative part and set as the same for both flocculent sludge and granular sludge. This assumption is reasonable if the variability of lipid A is compared with that of the glycan part in LPS. However, there are still differences between lipid A, e.g, Chen et al. (2024) measured fatty acid residues from the water-soluble fraction of EPS, and found there were variable carbon chain lengths of lipids with different relative amounts. Secondly, the volume of lipids was simplified as only considering the conformation of fatty acids, since unsaturated fatty acids occupy a larger space, thus building a higher volume than those with saturated fatty acids. However, the conformation of amphiphilic molecules such as LPS is extremely sensitive to environmental factors such as the ionic strength, type of ions in the solution, pH, and temperature (Israelachvili, 2011), which were not taken into account in this thesis.

Polymyxin B labeled with Rhodamine B as the fluorescence probe was applied to stain and visualize the structure of LPS-Ca aggregates. Polymyxin B is a small molecular polypeptide with positive charges and hydrophobic tails, and can specifically interact with LPS. Thus, once it binds with the LPS, it gives the fluorescent signal. However, it was observed that when alginate was chosen as the negative control, there were fluorescent signals present. This could be attributed to the absorption of the Rhodamine B by alginate (Kaushal and Tiwari, 2010). It is difficult to be washed out completely. Looking at the mechanisms of polymyxin staining, lipopolysaccharides display different structures like crystalline, branched, and terrace morphologies. Thus, the microscopic observation focused only on the morphology instead of the intensity.

The hot-phenol method was selected to isolate LPS from EPS. This procedure is designed theoretically for the isolation of smooth LPS (with entire parts of lipid A, oligosaccharides cores, and O-antigens) from biomass. The lipooligosaccharide (LOP), which only contains lipid A and oligosaccharide cores, could be co-extracted. Longer lipids in LOP (compared to glycans) can largely change the structures and aggregate morphology, interfering with the interaction of LPS with calcium as well as their behaviors in EPS. Moreover, there might be the chance that other hydrophilic components in EPS such as polysaccharides, dissolved in the water phase and co-extracted as well. The possible presence of LOP and polysaccharides might affect the ladder-like pattern in SDS-PAGE and the amount and type of monosaccharides in HPAEC-PAD analysis.

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Curriculum vitae

Ji Li was born on February 15th, 1993, in the picturesque countryside of Xiaogan, China.

As a child in his hometown, he witnessed significant environmental deterioration, which deeply impacted him. After completing high school in 2011, he was admitted to Wuhan University of Science and Technology in Wuhan, China. His growing interest in biology and chemistry inspired him to study water and wastewater treatment techniques to protect and improve the environment. During his bachelor's studies, Ji developed a particular passion for water purification, with a focus on biological nutrient removal technologies.

After completing his bachelor's degree in 2015, he moved to Beijing to continue his "wastewater journey" by pursuing a Master of Science in the School of Environment and Energy Engineering at Beijing University of Civil Engineering and Architecture (BUCEA) under the supervision of prof. Xiaodi Hao. During this time, his academic curiosity broadened to include resource and energy recovery from wastewater.

In 2018, he completed his master's project and joined a membrane manufacturing company. However, after a year of repetitive and uninspiring work, he decided to return to wastewater research and pursue a PhD. Through the collaboration between his supervisors, prof. Xiaodi Hao (BUCEA) and prof. Mark van Loosdrecht (TU Delft), the *Sino-Dutch R&D Centre for Future Wastewater Treatment Technologies* was established in 2016. As a PhD student in this centre, he was fortunate to be accepted into the 2 (BUCEA) + 2 (TU Delft) year PhD cultivation program. In 2019, he was awarded a scholarship from the China Scholarship Council (CSC), providing financial support to study abroad. After three years of study in Beijing, and with encouragement from prof. Hao, he moved to TU Delft in 2022. There, he conducted extensive research on extracellular polymeric substances from activated sludge for another two years. He successfully completed his PhD project in the Environmental Biotechnology (EBT) section of TU Delft in 2024.

Currently, he is continuing his research in the EBT section at TU Delft as a postdoctoral researcher, with a focus on the isolation and characterization of lipopolysaccharides.

List of publications

1. **Li, J**., Hao, X.D., van Loosdrecht, M. C. M., & Lin, Y.M. (2025). Understanding the ionic hydrogel-forming property of extracellular polymeric substances: Differences in lipopolysaccharides between flocculent and granular sludge. *Water Research*, 268, 122707. https://doi.org/10.1016/j.watres.2024.122707. (**Chapter 5**)

2. **Li J**., Hao X.-D., Persiani P., van Loosdrecht M.C.M., Lin Y.M. (2024). Reinvestigating the composition of alginate-like exopolymers extracted from activated sludge. *ACS ES&T Water,* 4, 3007–3015. https://doi.org/10.1021/acsestwater.4c00217. (**Chapter 4**)

3. **Li, J**., Hao, X.-D., Gan, W., van Loosdrecht, M.C.M., Wu, Y.Y. (2022) Controlling factors and involved mechanisms on forming alginate like extracellular polymers in flocculent sludge. *Chemical Engineering Journal,* 439, 135792. https://doi.org/https://doi.org/10.1016/j.cej.2022.135792. (**Chapter 3**)

4. **Li J**., Hao X.-D., Gan W., van Loosdrecht M.C.M., Wu, Y.Y. (2021) Recovery of extracellular biopolymers from conventional activated sludge: potential, characteristics and limitation. *Water Research,* 205, 117706. https://doi.org/10.1016/j.watres.2021.117706. (**Chapter 2**)

5. **Li, J**., Yang, W.B., Hao, X.D., Lin, Y.M., van Loosdrecht, M.C.M., (2024). Little alginates synthesized in EPS: Evidences from high-throughput community and metagenes. *Water Research*, 265. https://doi.org/10.1016/j.watres.2024.122211.

6. **Li, J**., Hao, X., Shen, Z., Wu, Y., van Loosdrecht, M.C.M. (2023). Low-temperature drying of waste activated sludge enhanced by agricultural biomass towards self-supporting incineration. *Science of the Total Environment*. 888, 164200. https://doi.org/10.1016/j.scitotenv.2023.164200.

7. **Li J**., Hao X.-D., Gan W., van Loosdrecht M.C.M., Wu, Y. (2022) Enhancing extraction of alginate like extracellular polymers (ALE) from flocculent sludge by surfactants. *Science of the Total Environment*. 837, 155673. https://doi.org/https://doi.org/10.1016/j.scitotenv.2022.155673.

8. Hao X.-D., **Li J.,** Liu R.-B., van Loosdrecht M.C.M. (2024). Resource Recovery

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Conference contributions

1. 14th IWA Specialized Conference on the Design, Operation and Economics of Large Wastewater Treatment Plants, Budapest Hungary (2024). The differences between lipopolysaccharides from flocculent and granular sludge influence the ionic hydrogel formation of extracellular polymeric substances. *Poster presentation*