Extracellular Polymeric Substances in Activated Sludge Flocs: Extraction, Identification, and Investigation of Their Link with Cations and Fate in Sludge Digestion

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ABSTRACT

Extracellular polymeric substances (EPS) in activated sludge are known to account for the flocculent nature of activated sludge. Extensive studies over the last few decades have attempted to extract and characterize activated sludge EPS, but a lack of agreement between studies has also been quite common. The molecular makeup of EPS has, however, remained nearly unexplored, leaving their identity, function, and fate over various stages in the activated sludge system mainly unknown. In spite of their critical involvement in bioflocculation and long history of related research, our understanding of EPS is still greatly limited and better elucidation of their composition and structure is needed.

The hypothesis of this research was that activated sludge floc contains different fractions of EPS that are distinguishable by their association with certain cations and that each fraction behaves differently when subjected to shear, aerobic digestion, anaerobic digestion and other processes. In order to examine this floc hypothesis, the research mainly consisted of three sections: 1) development of EPS extraction methods that target cations of interest (divalent cations, especially calcium and magnesium, iron, and aluminum) from activated sludge; 2) molecular investigations on activated sludge EPS using metaproteomic analyses, comprising sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein identification by liquid chromatography tandem mass spectrometry (LC/MS/MS), and hemaagglutination (HA)/HA inhibition assays; and 3) investigating the fate of EPS in sludge digestion using SDS-PAGE.

Evaluation of prior research and data from preliminary studies led to the development of the three extraction methods that were used to target specific cations from activated sludge and to release their associated EPS into solution. These methods are the cation exchange resin (CER) procedure for extracting Ca^{2+}+Mg^{2+}, sulfide extraction for removing Fe, and base treatment (pH 10.5) for dissolving Al. The cation selectivity in the three extraction methods, the composition of EPS (protein/polysaccharide), amino acid composition, and a series of sequential extraction data established initial research evidence that activated sludge EPS that are associated with different cations are not the same.

SDS-PAGE was successfully applied to study extracellular proteins from several sources of both full- and bench-scale activated sludges. The three extraction methods led to different SDS-PAGE profiles, providing direct evidence that proteins released by the three methods were indeed different sludge proteins. Another important outcome from this stage of

research was finding the similarity and differences of extracellular proteins between different sources of activated sludge. SDS-PAGE data showed that many of CER-extracted proteins were well conserved in all the sludges investigated, indicating that a significant fraction of Ca^{2+} and Mg^{2+}-bound proteins are universal in activated sludge. On the other hand, protein profiles resulting from sulfide and base extraction were more diverse for different sludges, indicating that Al and Fe and their associated proteins are quite dynamic in activated sludge systems. Protein bands at high densities were analyzed for identifications by LC/MS/MS and several bacterial proteins and polypeptides originating from influent sewage were identified in this study. This was also thought to be the first account of protein identification work for full-scale activated sludge.

The analysis of SDS-PAGE post sludge digestion revealed that CER-extracted proteins remained intact in anaerobic digestion while they were degraded in aerobic digestion. While the fate of sulfide-and base-extracted proteins in aerobic digestion was not as clearly resolved, their changes in anaerobic digestion were well determined in this research. Sulfide-extracted protein bands were reduced by anaerobic digestion, indicating that Fe-bound EPS were degraded under anaerobic conditions. While parts of base-extracted proteins disappeared after anaerobic digestion, others became more extractable along with the extraction of new proteins, indicating that the fate of base-extractable proteins, including Al-bound proteins, is more complex in anaerobic digestion than CER-extracted and sulfide-extracted proteins.

These results show that Ca^{2+}+Mg^{2+}, Fe^{3+}, and Al^{3+} play unique roles in floc formation and that each cation-associated EPS fraction imparts unique digestion characteristics to activated sludge. Finally, since a considerably different cation content is quite common for different wastewaters, it is postulated that this variability is one important factor that leads to different characteristics of activated sludge and sludge digestibility across facilities. The incorporation of the impact of cations and EPS on floc properties into an activated sludge model might be challenging but will assure a better engineering application of the activated sludge process.

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ATTRIBUTION

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Dr. Helm is the coauthor of Chapter 4. Protein identification work using liquid chromatography tandem mass spectrometry was performed at Dr. Helm's laboratory. He also assisted with analyzing protein identification results.

Dr. Ahn and Dr. Esen assisted with protein separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis in Chapter 4.

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Chapter 1. Executive Summary

Introduction

The interest in the role and fate of extracellular polymeric substances (EPS) in the activated sludge process has increased over the last few decades. EPS provide an essential polymeric matrix in which microorganisms are embedded and remain aggregated, accounting for the flocculent nature of activated sludge (Wingender et al., 1999). Since the successful operation of the activated sludge process depends primarily on efficient solid/liquid separation which can be achieved by efficient bioflocculation, the critical role EPS play in the activated sludge process cannot be overstated. EPS not only serve as flocculating agents, but are also believed to play a variety of critical functions for bacterial metabolism and maintenance in activated sludge. The enzymatic digestion of complex organic substrates to simple, small molecules (Frølund et al., 1995), protection of cells from harmful environmental conditions, and promotion of cell-cell recognition/communication (Wingender et al., 1999) should be all important processes for microbial communities residing in activated sludge.

The impact of EPS on solids handling processes in the activated sludge process has also received recent focus. There have been an increasing number of studies reporting that EPS account for the major organic fraction in activated sludge flocs (Frølund et al., 1996; Jahn and Nielsen, 1998; Münch and Pollard, 1997; Liu and Fang, 2002; Matias et al., 2003). Although EPS and active cell fractions will vary depending on the physiological characteristics of activated sludges, which are influenced by various factors (e.g., solids

retention time, the type of reactors, and influent feeding pattern), this information suggests that the fate of EPS in sludge digestion should be related to the sludge digestibility. In addition, the fate of EPS in sludge digestion should lead to further impact on the post digestion processes such as sludge conditioning, dewatering, and final disposal. As a consequence, the impact of EPS appears to span all treatment processes employed in the activated sludge process.

Because of these critical roles EPS play in the activated sludge process, a number of studies have attempted to extract and characterize EPS from activated sludge and a variety of extraction techniques such as the cation exchange resin (CER) procedure, cation chelation with EDTA or EGTA, sonication, base addition, and heat treatment have been employed for this purpose. However, it is not unusual to find a lack of agreement between studies as to the composition of EPS, contamination of EPS by cell lysis products, extraction efficiency and more importantly, the impact of EPS on various sludge characteristics. Generally, differing results have been attributed to the different extraction methods and types of sludges investigated.

It was recently proposed by Novak et al. (2003) that activated sludge flocs are comprised of different types of EPS with distinct metal ion binding characteristics. The authors proposed that the important exocellular biopolymer fractions in this floc model are: a) lectin-like proteins that are linked to polysaccharides and bridged by Ca^{2+} and Mg^{2+}, b) biopolymers that are bound to Fe, and c) organic material coagulated with Al. It was found that anaerobic and aerobic digestion of a single activated sludge resulted in remarkably different cation and biopolymer release and different degrees of volatile solids (VS)

destruction, leading to the suggestion that lectin-like proteins are mainly degraded under aerobic conditions while proteins associated with Fe are readily degraded under anaerobic conditions (Park et al., 2006b). It was shown that Al plays an important role in bioflocculation by improving effluent quality, but the impact of Al and the binding of organic matter on sludge digestion remains unclear (Park et al., 2006a,b) .

If the above floc model is valid, it begins to explain the differences seen in earlier EPS studies because several of the commonly used extraction methods are selective for the EPS associated with specific cations in floc (see below) and therefore, materials extracted by one method would be expected to differ from that extracted by others. It can be also inferred that previous approaches to extracting EPS may lead to significantly varying results based on the process operation and the influent wastewater cation content.

In order to elucidate the nature and impact of various EPS fractions, a more comprehensive approach to EPS extraction has been conducted in this research. Furthermore, molecular investigations of EPS structure have been employed since the molecular makeup of EPS has remained nearly unexplored, leaving their identity, function, and fate over various stages in the activated sludge system mainly unknown. The hypothesis of this research was that activated sludge floc contains different fractions of EPS that are distinguishable by their association with certain cations and that each fraction behaves differently when subjected to shear, aerobic digestion, anaerobic digestion and other processes. In order to examine this floc hypothesis, the research mainly consisted of three sections: 1) development of EPS extraction methods that target cations of interest (divalent cations, iron, and aluminum) from activated sludge; 2) molecular investigations

on activated sludge EPS using metaproteomic analyses, comprising sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein identification by tandem mass spectrometry, and hemaagglutination (HA)/HA inhibition assays; and 3) investigating the fate of EPS in sludge digestion using SDS-PAGE.

Characterization of Activated Sludge Exocellular Polymers Using Several Cation-Associated Extraction Methods (Chapter 3) [*Water Research* 41, 1679-1688]

Evaluation of prior research and preliminary investigations in our laboratory led to the development of an extraction strategy that can be used to target different cations in activated sludge floc and extract their associated extracellular polymeric substances (EPS). The methods we used were the cation exchange resin (CER) procedure, base extraction, and sulfide addition to extract EPS linked with divalent cations, Al, and Fe, respectively. A comparison of sludge cations before and after CER extraction revealed that most of Ca^{2+} and Mg^{2+} were removed while Fe and Al remained intact, suggesting that this method is highly selective for Ca^{2+} and Mg^{2+}-bound EPS. The correlation between sludge Fe and sulfide-extracted EPS was indicative of selectivity of this method for Fe-bound EPS. The base extraction was less specific than the other methods but it was the method releasing the largest amount of Al into the extract, indicating that the method extracted Al-bound EPS. Concomitantly, the composition of extracted EPS and the amino acid composition differed for the three methods, indicating that EPS associated with different metals were not the same. The change in EPS following anaerobic and aerobic digestion was also characterized

by the three extraction methods. CER-extracted EPS were reduced after aerobic digestion while they changed little by anaerobic digestion. On the other hand, anaerobic digestion was associated with the decrease in sulfide-extracted EPS. These results suggest that different types of cation-EPS binding mechanisms exist in activated sludge and that each cation-associated EPS fraction imparts unique digestion characteristics to activated sludge.

Evaluation of the Extracellular Proteins in Full-Scale Activated Sludges Using Cation-Specific Extraction Methods and Metaproteomic Analyses (Chapter 4) [Prepared for submission to *Environmental Microbiology*]

In order to study the role of cations in floc formation of activated sludge, extracellular polymeric substances (EPS) that were extracted using cation-targeted methods were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resultant protein profile was examined. Activated sludges were sampled from four full-scale wastewater treatment plants. The cation exchange resin (CER) extraction, sulfide extraction, and base extraction (pH 10.5) were applied to selectively extract EPS linked with Ca^{2+}+Mg^{2+}, Fe^{3+}, and Al^{3+}, respectively. Crude extracts were treated with sequential ammonium sulfate precipitation to separate EPS based on hydrophobicity and to enhance the separation of proteins in SDS-PAGE. Results showed that the three methods led to different SDS-PAGE profiles, suggesting that the extracted proteins are uniquely associated with specific cations and they have different hydrophobic characteristics. Protein bands at high intensities were analyzed for identifications by liquid chromatography tandem mass

spectrometry (LC/MS/MS). While many samples did not retrieve results from database searches indicating they are from unsequenced microorganisms in activated sludge, several bacterial proteins including extracellular enzymes, outer membrane proteins, adhesin proteins, flagella and polypeptides originating from influent sewage were identified in this study. The extraction efficiency of these proteins also differed between the methods. These results show that Ca^{2+}+Mg^{2+}, Fe^{3+}, and Al^{3+} play different roles in floc formation and that the metal content in influent wastewater is an important parameter influencing floc properties. Metaproteomic analyses were feasible for studying EPS in field sludges and show promise for monitoring the fate of sludge proteins at various stages in the activated sludge process.

Investigation of Lectins in Activated Sludge Flocs [Working paper; Appendix D]

Bacterial lectins are carbohydrate-binding proteins that are involved in bacterial infections and clumping of cells in biofilm formation. In order to investigate if lectins are involved in floc formation of activated sludge, hemaagglutination (HA) and HA inhibition assays were conducted on extracellular polymeric substances (EPS) extracted from activated sludges. Three extraction methods were used to extract EPS from activated sludge and sequential ammonium sulfate precipitation was applied to separate EPS and lectin activities based on hydrophobicity. The results showed that EPS harvested from several sources of activated sludges led to strong agglutination with trypsin-treated human red blood cells. While eleven monosaccharides tested failed to exhibit the inhibition on agglutination, several

glycoproteins such as asialofetuin, thyroglobulin, and mucin clearly reversed agglutination, indicating that these lectins are glycoprotein-specific. This inhibitory pattern was the same for both field activated sludges and laboratory-grown activated sludges, which were fed by synthetic chemicals, suggesting that these lectin activities are indigenous to activated sludge microorganisms not due to organics originating from influent sewage. The major lectin activities were found from a hydrophobic region of EPS. The activities remained unaffected after heat treatment of EPS at 55°C (0.5hr) but were abolished by EDTA treatment. These results shared similar properties with previously reported bacterial lectin proteins. Results found in this study suggest that lectin-carbohydrate interaction is one of the mechanisms responsible for bacterial aggregation in activated sludge. Exploiting general features of lectins such as requirement of divalent cations, heat stability, and deficiency of cysteine and methionine (precursors of volatile organic sulfurs) in their protein sequence may improve our knowledge of bioflocculation and sludge digestion of waste activated sludge.

Investigating the Fate of Activated Sludge Extracellular Proteins in Sludge Digestion Using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Chapter 5) [Proceeding paper for *WEFTEC 2007*]

The purpose of this study was to examine the fate of activated sludge extracellular proteins in batch anaerobic and aerobic digestion using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We were particularly interested in studying the fate of

proteins that are bound with different sludge cations. Extracellular polymeric substances (EPS) extracted by three cation-specific extraction methods, the cation exchange resin (CER) procedure for extracting Ca^{2+}+Mg^{2+}, sulfide extraction for removing Fe, and base treatment (pH 10.5) for dissolving Al, were subjected to SDS-PAGE and the resultant protein profiles were compared before and after sludge digestion. The initial SDS-PAGE on undigested activated sludge showed different protein profiles by the three methods, suggesting that Ca^{2+}+Mg^{2+}, Fe, and Al are bound with different characteristic EPS and play unique physio-biochemical roles in floc formation. The analysis of SDS-PAGE post sludge digestion revealed that CER-extracted proteins remained intact in anaerobic digestion while they were degraded in aerobic digestion. While the fate of sulfide-and base-extracted proteins in aerobic digestion was not clearly resolved, their changes in anaerobic digestion were well observed. Sulfide-extracted protein bands were reduced by anaerobic digestion, indicating that Fe-bound EPS were degraded under anaerobic conditions. While parts of base-extracted proteins disappeared after anaerobic digestion, others became more extractable along with the extraction of new proteins, indicating that base-extractable EPS are dynamic in anaerobic digestion. All these results suggest that the metal content in influent sewage have a significant impact on activated sludge characteristics and sludge digestibility. The potential application of SDS-PAGE and the three extraction methods for choosing a proper sludge pretreatment and digestion method are also discussed in this study.

Conclusions

Results found in this research supported a new floc hyopothesis that Al, Fe, and divalent cations play unique roles in floc formation of activated sludge. Moreover, each cation-associated organic fraction behaves differently to the different digestion environment. Consequently, the metal content in wastewaters should not be neglected in studying and modeling the activated sludge process. The new extraction strategy developed in this research not only was useful for studying different cation-bound EPS in activated sludge flocs but also could explain why previous EPS studies had led to differing results. The successful application of SDS-PAGE to full-scale activated sludge is thought to be of significance to both research and engineering implication in the field of biological wastewater treatment. Finally, the developed methods and findings from this research expect to conduce to developing fundamentally sound engineering application of biological wastewater treatment processes.

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Chapter 2. Literature Review

Activated Sludge Process and Floc Composition

The activated sludge process is the primary method used for treating municipal wastewater before its discharge into receiving waters. Bioflocculation is the central mechanism of the activated sludge process which results from the degradation of organic matter in influent sewage and the growth of new microbial cells in a flocculent mode. The effectiveness of bioflocculation directly influences the separation of sludge flocs from the treated liquid by gravity settling or more recently by membrane separation and the effectiveness of this separation determines the quality of the effluent. Although the activated sludge process has been used for nearly a century (Grady et al., 1999), the performance of the process still highly varies across facilities (Higgins and Novak, 1997b; Park et al., 2006a). Similarly, it is not unusual to find a wastewater treatment plant (WWTP) that does not meet regulatory effluent requirement due to ineffective bioflocculation and subsequent poor solid/liquid separation.

The activated sludge process also produces a byproduct, sludge, which needs to be properly treated prior to its ultimate disposal. While there are several choices to stabilize waste sludge, anaerobic digestion that utilizes complex anaerobic microbial consortia to convert sludge biomass to methane gas has been the most commonly practiced sludge stabilization method. However, the performance of anaerobic digestion is still hard to predict and its impact on subsequent sludge dewatering and post digestion odor generation

is largely unknown. Various sludge enhancement processes or digestion models have been developed to improve digestion efficiency (i.e., higher volatile solids (VS) reduction and pathogen inactivation) but the success of each process still appears to be sludge-dependent and hence requires a trial and error approach for design.

The need for better operation of the activated sludge process and digester performance has increased interest in developing a better, fundamental understanding of bioflocculation in the activated sludge process. Consequently, a great deal of research has been conducted to study bioflocculation and a traditional research focus has been to investigate the structure and composition of activated sludge flocs.

The structural composition of activated sludge flocs can be categorized as microorganisms (predominantly bacteria), organic matter in addition to microbial cells, and inorganic cations and anions (Eriksson and Alm, 1991; Jorand et al., 1995; Frølund et al., 1996; Higgins and Novak, 1997). From a microbiological standpoint, bioaggregates such as activated sludge flocs or biofilms are subdivided into cells and organic matter exterior to cells and the latter has been traditionally characterized as extracellular polymeric substances (EPS) or exocellular biopolymers. The term "biomass" which treats the bioaggregate as one unit is often used in wastewater engineering. In this case, both active and inactive fractions of biomass are used for kinetic determinations and this approach has been applied for mathematical modeling of the growth and decay of cell biomass and predicting effluent quality. Despite the benefits of a simplified floc model, this approach does not account for the origin and fate of EPS, which now appear to be a large organic sink for activated sludge (Frølund et al., 1996; Münch and Pollard, 1997; Liu and Fang,

2002; Matias et al., 2003). Furthermore, this numerical approach neglects the impact of various cations on floc formation. There have been numerous studies showing that the change in feed cations directly led to different characteristics of activated sludge and that effluent quality could be fairly well predicted with the concentration and content of cations in influent wastewater (Novak and Randall, 1986; Higgins and Novak, 1997b,c; Murthy et al., 1998; Holbrook et al., 2004; Li, 2005; Park et al., 2006a). Therefore, a better understanding of EPS and the cations that are intimately associated with these polyanionic biopolymers should be conducted for a more complete insight of floc structure and bioflocculation.

Extracellular Polymeric Substances (EPS)

EPS are organic polymers that are located at or outside the cell surface. EPS in activated sludge floc are responsible for forming microbial colonies and holding colonies, cells and other particulate materials together, leading to the flocculent characteristic of activated sludge (Wingender et al., 1999). The enzymatic digestion of polymeric organic substrates to simple, small molecules to be available for bacterial metabolism is another important characteristic associated with activated sludge EPS (Frølund et al., 1995; Guellil et al., 2001). EPS in activated sludges and biofilms are also known to promote cell-cell recognition/communication and protect cells against harmful environmental conditions such as turbulence, dehydration, antibiotics and biocides (Wingender et al., 1999). All these

show that EPS are a critical component in the formation of bioaggregates and in determining a variety of characteristics of microbial aggregates.

The chemical composition of activated sludge EPS reveals that these are very heterogeneous materials. Different extraction techniques often yield a variety of organic constituents, but it is agreed that activated sludge EPS consist of proteins, polysaccharides, humics, lipids and nucleic acids (Frølund et al., 1996; Higgins and Novak, 1997a; Dignac et al., 1998; Wingender et al., 1999; Liu and Fang, 2002). Earlier studies often indicated that polysaccharides were the most abundant and important EPS compound (Brown and Lester, 1980; Morgan et al., 1990; Horan and Eccles, 1999) but a number of recent studies have shown that the quantity of proteins is about two to three folds higher than polysaccharides in activated sludge EPS (Urbain et al., 1992; Frølund et al., 1996; Nielsen et al., 1996; Higgins and Novak, 1997a; Dignac et al., 1998; Wingender et al., 1999; Liu and Fang, 2002; Comte et al., 2007). It was also reported that glycoproteins are very likely present in activated sludge EPS so that part of the protein and carbohydrate content in EPS arises from the extraction of glycoproteins (Goodwin and Forster, 1985; Jorand et al., 1998; Horan and Eccles, 1999; Göner et al., 2003). The general characteristic of bacterial glycoprotein is interesting to note since it often exhibits both acidic characteristic (low isoelectric point) and hydrophobic characteristic (Jorand et al., 1998). Consequently, it can be involved in bacterial aggregation by both electrostatic bond (cation bridging) and hydrophobic interaction.

EPS from bioaggregates originate from a variety of sources, suggesting that activated sludge EPS are not only chemically heterogeneous but they are physiologically

different as well. EPS can be composed of a variety of biopolymers transported to the extracellular milieu by active secretion or export, lysed cellular components from the rupture of cell structure, hydrolyzed or digested exocellular substances, and materials adsorbed from the environment such as in wastewater being fed to an activated sludge system (Urbain et al., 1992; Dignac et al., 1998; Nielsen and Keiding, 1998; Wingender et al., 1999). However, it is mainly unknown how these different-origin EPS are distributed within the floc and contribute to the physiological property of activated sludge flocs. Furthermore, due to the scarcity of molecular investigation on activated sludge EPS, their identity, function, and fate in various stages of the activated sludge system remains veiled.

Extracellular Proteins and Metaproteomics

As stated above, proteins have been reported to be predominant organic matter in the extracellular matrix of activated sludge flocs, indicating their significant influence on both surface and bulk properties of activated sludge. The presence of acidic amino acids, glutamic acid (Glu) and aspartic acid (Asp), in extracellular proteins leads to the direct interaction (electrostatic bond) with cations in floc. Proteins also influence hydrophobicity of activated sludge due to the presence of hydrophobic amino acids, contributing to the flocculent nature of activated sludge in a hydrated environment. Due to its bulk quantity in activated sludge, the fate of extracellular proteins in sludge digestion should also be related to sludge digestibility. Consequently, a better understanding of the function and fate of

extracellular proteins in the activated sludge process is critical for improving our understanding of bioflocculation and the performance of sludge digestion.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a fundamental biochemical tool for the analysis of proteins or proteomes in biological system. In this method, anioic detergent SDS denatures proteins, with the aid of a reducing chemical and heat, and leads to the formation of single dimensional protein rods, which can be separated by electrophoresis according to their molecular weights (Laemmli, 1970). Technologies have been developed and in these days, proteins can also be separated based on both isoelectric points and molecular weights in two dimensional-PAGE (2D-PAGE) system. In spite of their fundamental use in biology and biochemistry, their use has been mainly limited for studying the pure culture system.

The application of SDS-PAGE into environmental samples was first demonstrated by Ogunseitan (1993). The samples studied by this author included raw and treated wastewater from the activated sludge system, but not the activated sludge biomass itself. Martínez et al. (2004) examined extracellular proteins in synthetic chemical-fed bench scale activated sludge using SDS-PAGE. They used the EDTA method to extract extracellular proteins and reported the observation of several protein bands with various molecular weights. However, SDS-PAGE was not provided in their publication, making it difficult to further examine their results.

More recently, a metaproteomic approach has been used in a very few studies to characterize complex microbiota (Wilmes and Bond, 2004; Ram et al., 2005; Klaassens et al., 2007). The term “metaproteomics” was first suggested by Wilmes and Bond (2004) for

characterizing the entire proteome of environmental microbiota at a given time. The approach usually comprises protein separation by gel electrophoresis and identification of selected proteins using mass spectrometry (MS). Wilmes and Bond (2004) also for the first time reported their metaproteomic investigation on a laboratory-scale activated sludge system designed for enhanced biological phosphorous removal (EBPR). The same authors also reported tentative identifications on three highly expressed proteins by MS-based de novo peptide sequencing. These proteins are outer membrane proteins (porins), acetyl coenzymeA acetyltransferase, and a protein component of an ABC-type branched-chain amino acid transport system stemming from uncultured ß-Proteobacteria *Rhodocyclus*-type polyphosphate accumulating organism. Due to the high expression of these proteins, the authors proposed that they are housekeeping proteins in EBPR sludge or directly pertain to EBPR biochemistry. Although the feasibility of the metaproteomic approach for activated sludge cultures was well demonstrated by Wilmes and Bond (2004), the study was based on a laboratory activated sludge grown on soluble synthetic chemical wastewater. Consequently, any ecological and physiological impact of full-scale influent on microbial communities in activated sludge cannot be included in such a synthetic chemical-fed system.

Collectively, a successful application of SDS-PAGE (or 2D-PAGE) to a full-scale activated sludge should be advantageous to a better engineering application of the activate sludge process: 1) it can pinpoint proteins that are critically involved in bioflocculation; 2) it might be useful for monitoring the fate of proteins in various stages of the activated

sludge process; and 3) it can also be used for analyzing physiological changes of microbial community in activated sludge to environmental change.

Cations and Their Links to EPS in Activated Sludge Floc

The polyanionic nature of cell surfaces and EPS has rendered the role of metallic ions in bioflocculation of great importance. Cations with multivalency are particularly important in this aspect since they are essential for the formation of ionic bridges for some EPS and maintain them in ordered structures, leading to the stability of bioaggregates. However, different cations also have different biochemical properties (charge valence, radius, red/ox characteristic, etc) and form unique hydrolysis products in aqueous environment, suggesting that different cations might have distinct biological and physiochemical roles in bioflocculation of activated sludge. This might be important because the major influent wastewater cations such as Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, $Fe^{2+/3+}$ and Al^{3+} vary considerably between different WWTPs (Park et al., 2006a) and this could be one major factor that accounts for differences between the operational characteristics of WWTPs and sludges they produce. Therefore, previously suggested cation-induced floc models and further literature dealing with the interaction of EPS with cations from pure and mixed cultures and in natural water which might pertain to activated sludge flocculation will be discussed below.

Divalent cation bridging (DCB) theory

The DCB theory has been proposed as one probable mechanism explaining the role of cations in bioflocculation of activated sludge (Tezuka, 1969). According to this theory, Ca^{2+} and Mg^{2+} are critical components of floc structure and both cations bridge equally, but nonspecifically, to anionic sites within the exocellular matrix, promoting microbial aggregation. Later studies (Kakii et al., 1985; Higgins and Novak, 1997a,b,c; Sobeck and Higgins, 2002) supported the DCB theory with the observation that external addition of monovalent cations such as Na^{+} and K^{+} into activated sludge resulted in the release of Ca^{2+} and Mg^{2+} into the sludge solution, which was accompanied by deterioration in settling and dewatering properties of sludge. Activated sludge deflocculation caused by electrophile-induced glutathione-gated K^{+} efflux (GGKE) stress response (Bott and Love., 2002) was also explained by this cation bridging theory. According to the authors, the release of cytoplasmic K^{+} into extracellular milieu of activated sludge by GGKE mechanism perturbs divalent cation-bridged floc structure, leading to the destabilization of floc. Higgins and Novak (1997b,c) proposed that the ratio of monovalent cations to divalent cations (M/D), on a charge equivalent basis, could be served as a rapid indicator of sludge characteristics. According to these authors, activated sludge with M/D exceeding 2 would encounter poor settleability and dewaterability. All these studies suggest that competition between monovalent cations and divalent cations exists for the same binding sites within activated sludge EPS.

EPS complexation with aluminum

Although Al is the most abundant metal in Earth's crust, little is known about its biological function in ecosystems. Neither genes nor proteins that are specific for Al in cellular transport have also been identified (Silver, 1998). Despite its poorly understood role in the biosphere, it is tempting to speculate that Al is a good candidate for binding of EPS in bioaggregates due to its high charge valence and low solubility. Our earlier field study also postulated that Al is an efficient scavenger of organic matter in activated sludges and hence improves bioflocculation (Park et al., 2006a). We obtained this result by investigating eight different WWTPs, none of which received artificial addition of Al salts during the process, and the study showed that higher level of Al in floc corresponded to better quality of plant effluents.

Some literature is available that describes an association between Al and polysaccharides produced from bacteria and higher organisms. Boult et al. (2001) investigated sixty two lentic waters in England and found that polysaccharides were negatively correlated with Al. No such correlation was found with Fe and Ca^{2+}. In addition, Al was not related to the total dissolved organic carbon where the other major carbon was humic and fulvic acids, indicating that there was specific affinity of Al for carbohydrate compounds in their water samples. Similarly, two studies (Vilge-Ritter et al., 1999; Masion et al., 2000) reported that polysaccharides found in lake and river water were preferentially coagulated by Al chemical salts. These results are in agreement with the study by Park et al. (2006a) who showed that the optimum alum dose for conditioning waste activated sludges was primarily dependent on soluble polysaccharides in each sludge sample. Murthy et al.

(2000) also showed that colloidal polysaccharides present in an autothermal thermophilic aerobic digester were better coagulated by alum than ferric chloride. Holbrook et al. (2004) also reported that the addition of alum into a submerged membrane activated sludge system led to more selective removal of soluble polysaccharides over proteins. Jugdaohsingh et al. (1998) showed that artificially added Al was deposited in exopolysaccharide biofilm that was secreted by the freshwater snail *Lymnaea stagnalis*. The binding of Al with EPS also occurred in the water column where the snail was grown but was removed prior to the addition of Al. From these results, the authors suggested that a strong sequestration of Al by exopolysaccharides produced by bacteria, algae, and higher organisms would be beneficial in protecting them from being directly exposed to Al.

It is likely from the above literature that there would be some higher affinity of Al for polysaccharides in activated sludge EPS. However, the reaction of Al with proteinaceous materials in activated sludge should not also be ruled out. According to Takara et al. (2007), many proteins from the cell extract of the cyanobacteria *Microcystis aeruginosa* bound to polyaluminum chloride immobilized in affinity chromatography column and some proteins, particularly proteins having molecular weights of ~34 kDa and between 50 ~ 60 kDa, were strongly chelated with Al, indicating their natural affinity on Al. Considering the usual presence and growth of cyanobacteria in semiclosed water, the authors claimed that these cyanobacterial proteins might be one important organic sink that inhibits aluminum coagulation in its water treatment for drinking water source.

Iron in activated sludge floc

Fe is an essential element for most microbial cells as a cofactor in various enzymes and its involvement in electron transfer system (Sticht and Rösch, 1998). For example, iron sulfur proteins in which cystenyl sulfur liagation with Fe is highly coordinated are ubiquitous in electron transfer systems and involved in various enzymatic catalyses (Czaja et al., 1995).

The oxidation-reduction characteristic of Fe is thought to make its impact in activated sludges considerably different from other cations. It was previously shown that the reduction of Fe in activated sludge either by Fe-reducing bacterium *Shewanella alga* (Caccavo et al., 1997) or by addition of sulfide (Nielsen and Keiding, 1998) led to a significant increase in solution turbidity, composed of organic colloids rather than free cells, which was responsible for the concomitant deterioration in sludge dewaterability. As a consequence, these studies suggested that oxidized Fe plays a critical role in floc stability and it might be a more important cation than Ca^{2+} and Mg^{2+}. Consistently, studies have also shown that Fe^{3+} has a better flocculating ability than Fe^{2+} (Rasmussen et al., 1994; Rasmussen and Nielsen, 1996; Muller, 2001). According to Novak et al. (2003), this iron property is particularly important for anaerobic digestion of activated sludge. They hypothesized that deflocculated activated sludge coupled to Fe reduction further undergoes destruction leading to volatile solids reduction in anaerobic digestion. Collectively, a recent study by Park et al. (2006b) demonstrated that the concentration of Fe in sludge floc was an important factor in determining digestibility of sludge by anaerobic digestion.

The selectivity of Fe (III) for proteins has been postulated by several researchers. Limitation in the availability of Fe for mammalian pathogens arises because most of the Fe

is complexed with proteins (Wooldridge and Williams, 1993). Caccavo et al. (1997) tested *S. alga* BrY and an adhesion-deficient strain of this species, *S. alga* RAD20 for their adhesion to amorphous Fe (III) oxide and found that inefficient binding of the latter to iron oxide was due to the high concentration of exopolysaccharides in this strain while the former was coated with hydrophobic surface proteins. Interestingly, once bound, the two strains showed identical Fe reduction rate, indicating that their binding to Fe^{3+} is a prerequisite for dissimilatory Fe reduction. It is also worthwhile to note that low Fe availability modulates the expression of *Corynebacterium diphtheriae* surface carbohydrate moieties for binding of host cells. Moreira et al. (2003) showed that *C. diphtheriae* grown under Fe limitation produces more neutral carbohydrates, in contrast to controls, that can bind with specific lectin probes. Conditioning experiments performed for wastewater sludges also suggested that there is higher affinity of ferric iron for proteins. It was demonstrated that Fe^{3+} salts selectively coagulated solution proteins produced from autothermal thermophilic aerobic digesters (Murthy et al., 2000; Abu-Orf et al., 2001). Similarly, Park et al. (2006b) reported that optimum the $FeCl_3$ dose required for conditioning of ten different waste activated sludges was determined by proteins in the sludge solution.

Adhesin-associated bacterial aggregation

Bacterial attachment to host cells is a crucial, initial step in bacterial infection and is often mediated by a variety of bacterial adhesins. Adhesins are molecules that enable bacteria to

stick to surfaces (White, 2000) and hence, play a pivotal role in bacterial aggregation and biofilm formation. Therefore, a knowledge of their biochemical properties such as hydrophobicity (amino acid composition), heat/pH stability, requirement of cations and their relation to cell physiology should be relevant to studying bioflocation of activated sludge.

Bacterial adhesins can vary from filamentous proteins such as fimbriae or fibrils to slimy exopolysaccharides (White, 2000). While the filamentous or mucous properties of these biopolymers are thought be very useful for bacteria to attach to a targeted surface or clump themselves, bacteria also use specific ligand molecules that can interact with a receptor localized on host cells (Touhami et al., 2003; Imberty et al., 2004). Lecins, carbohydrate-targeting proteins, are a good example of adhesins that are synthesized by cells for this purpose. Interestingly, lectins are often located on the tip or in the subunit of fimbriae or pili, rendering these appendages more mechanistically advantageous in bacterial adhesion (Heeb et al., 1982).

More detailed discussion of lectins and other adhesins that may be involved in the formation of activated sludge flocs are available in the following sections.

Lectins

Higgins and Novak (1997) suggested that extracellular proteins bridged with Ca^{2+} and Mg^{2+} in activated sludge floc are lectin-like proteins. Lectins are nonenzymatic protein adhesins that bind specifically and reversibly to carbohydrates and are found in a wide variety of organisms including animals, plants and bacteria (Weis and Drickamer, 1996). Lectins play

a crucial role in bacterial attachment to targeted cells such as in bacterial infections, colonization of bacteria, and clumping of cells in biofilm formation (Hoffman and Decho, 1999; Loris et al., 2003; Imberty et al., 2004) via specific sugar recognition that aids in the direct or indirect interaction with divalent cations (Weis and Drickamer, 1996; Imberty et al., 2004). It is also documented that lectin-mediated microbial aggregation occurs in the flocculation of yeasts in brewery processes (Touhami et al., 2003). For activated sludge, Higgins and Novak (1997) performed enzymatic digestion assay and carbohydrate binding site inhibition experiments and concluded that activated sludge proteins also possess lectin activity. These authors further proposed that lectins cross-linked to polysaccharides and bridged with divalent cations stabilize the entire EPS network of activated sludge flocs.

Since lectin proteins and carbohydrates bound to them appear to be one of the major activated sludge EPS, information about their structural and functional characteristics would be important to know. One of the most studied bacterial lectins are those produced from *Pseudomonas aeruginosa,* a Gram-negative bacteria usually found in biofilms and activated sludges. Some strains of *P. aeruginosa* are known to produce two types of lectins LecA and LecB (González-Castro et al., 1997; Cioci et al., 2003; Imberty et al., 2004). Both are tetrameric proteins with molecular weight of 12.8 kDa in the former and 11.7 kDa in the latter (Gilboa-Garber et al., 2000; Loris et al., 2003; Imberty et al., 2004). Both require Ca^{2+} and Mg^{2+} for carbohydrate binding (Imberty et al., 2004). LecA and LecB differ in terms of the specificity of carbohydrates. LecA is specific for galactose while a wider range of carbohydrates with higher affinity of fucose and mannose are bound to LecB (Cioci et al., 2003; Imberty et al., 2004). Avichezer et al. (1997) sequenced amino acids of

LecA and indicated that its composition shared high similarities with the lectins from other bacteria. One feature of these lectins is a deficiency in sulfur-containing amino acids, specifically cysteine (Cys) and methionie (Met). Gilboa-Garber et al. (2000) later revealed that LecB does not include any Cys and Met. This characteristic could be of specific interest in odor issues associated with anaerobically digested sludges because sulfur-possessing amino acids are precursors of volatile organic sulfur compounds that are key odor compounds produced from anaerobically digested sludge cake (Higgins et al., 2006; Muller et al., 2004).

The amino acid composition of lectins could also be important for analyzing their hydrophobic interacting capacity in bioflocculation. According to Avichezer et al. (1992), LecA has a predominant hydrophilic core with 35-55 residues located between two hydrophobic domains of amino acids, again similar to other lectins.

Fimbriae

Fimbriae, also often called pili, are filamentous proteins that are usually found in surfaces of Gram-negative bacteria (White, 2000). The involvement of fimbriae in bacterial infection was well documented in earlier studies (Young et al., 1985; Vesper, 1987) and it has long been believed that they play a crucial role in bacterial aggregation. Since these proteins usually exhibit hemaaglutinating capacity on erythrocytes (Young et al., 1985; and Heeb et al., 1982), the term *fimbriae* is often interchangeably used with lectins. Similar to lectins, there are common features of fimbriae shared by several groups of bacteria. They are acidic proteins (Young et al., 1985). They also lack Cys, Met, and tyrosine (Tyr)

(Korhonen et al., 1980; Young et al., 1985). On the other hand, a large number of glycine (Gly) and alanine (Ala) residues is often found in fimbriae, leading to the high hydrophobic characteristic. One interesting thing that can be learned from the literature is that their purification and characterization were greatly hampered by their polymeric nature and extremely stable characteristic. Consequently, many studies had to use unusually high dissociating conditions prior to gel permeation chromatography and SDS-PAGE for their isolation.

Alginate

The involvement of alginate in bioflocculation of activated sludge was suggested by Bruus et al. (1992). Alginates are a family of linear unbranched polysaccharides consisting of two repeating monosaccharide monomers, 1,4-linked α-L-guluronic acid and β-D-mannuronic acid (Christensen, 1999; Davies, 1999). Carboxylates from these copious acidic polysaccharides can directly bind to cations leading to a gel-like structure with mucoid properties (Lattner et al., 2003). Bruus et al. (1992) decided that alginates were important components of activated sludge from the observation that the addition of Na^+, K^+, Mg^{2+}, and EGTA resulted in the extraction of Ca^{2+} simultaneously with an increase in solution turbidity and deterioration in sludge filterability, while addition of Cu^{2+} improved the filterability of sludge. Although characterization of the turbidity-causing materials was not performed in this study, cation selectivity for Cu^{2+} and Ca^{2+} over other cations is a typical characteristic of alginate polysaccharides (Surtherland, 1999; Lattner et al., 2003).

Although bacterial alginates have been found to be involved in biofilm formation by several genre of bacteria including *Azobacter* and *Pseudomonas* (Christensen, 1999), its dominance in activated sludge EPS is arguable. This might be because total uronic acids found in activated sludge are usually several folds lower than proteins and other polysaccharides (Frølund et al., 1996; Liu and Fang, 2002; Wilén et al., 2003b). Sobeck and Higgins (2002) also thought that alginates were unimportant in activated sludge based on reactor study results that showed that activated sludge fed with either Mg^{2+} or Ca^{2+} had similar sludge properties. However, considering the much higher Ca^{2+} (15 mM) than Mg^{2+} (2 mM) in the sludge studied by Bruus et al. (1992), and a study (Turakhia and Characklis, 1989) showing that the rate and extent of *P. aeruginosa* biofilm accumulation increased with increasing feed Ca^{2+} concentration, alginates could be of importance in some activated sludges.

Other adhesins

Adhesins rather than cell appendages may include hemagglutinin/protease (HAP) from *Vibrio cholerae* and outer membrane proteins (porins) from several Gram-negative bacteria. *V. cholerae* HAP is an interesting protein since it contains both cell-agglutinating ability and proteolytic acitivity (Häse and Finkelstein, 1990). It is a metalloenzyme that requires Ca^{2+} or Zn^{2+} for its full activity. It also shares an extensive homology with other metalloproteases such as *Pseudomonas aeruginosa* elastase (Häse and Finkelstein, 1990) and *Bacillus thermoproteolyticus* thermolysin (Kooi et al., 1997). Information from these

studies may suggest that a metalloprotease might be importantly associated with bacterial adhesion and colonization.

There have been an increasing number of studies showing that major outer membrane proteins, specifically porins, also act as an adhesin in bacterial colonization and infection. These include porins from *Pseudomonas fluorescens* (DeMot et al., 1992), *Rahnella aqutilis* (Achouak et al., 1998), *Azospirillum brasilense* (Burdman et al., 2000), and *Pseudomonas aeruginosa* (Azghania et al., 2002; Bodilis and Barray, 2006). This literature suggests that bacteria may have a variety of structures that can be potentially used for cell adhesion and aggregation.

Extraction of EPS and controversies in earlier studies of activated sludge EPS

Since higher yields of EPS are thought to be more desirable in studying the EPS content of activated sludge, a number of studies compared different extraction techniques and tried to find the most efficient method (Brown and Lester, 1980; Karapanagiotis et al., 1989; Frølund et al., 1996; Azeredo et al., 1998; Bura et al., 1998; Liu and Fang, 2002). However, the most common debate in EPS extraction studies has to do with the extraction efficiency of the different methods that have been used. For examples, Karapanagiotis et al. (1989) and Frølund et al. (1996) compared the CER procedure, base extraction, and thermal treatment and found that the CER procedure was the most effective method with the least amount of cell lysis. Liu and Fang (2002) concluded that base extraction with formaldehyde was much more efficient with little risk of cell lysis than other extraction methods using

CER and EDTA. On the other hand, Brown and Lester (1980) reported that the thermal treatment (steaming) was the most effective method among treatments tested including high speed centrifugation, chelation with EDTA, alkaline treatment, and ultrasonication. Similar contradiction can also be found in several other studies (Novak and Haugan, 1981; Azeredo et al., 1998; Bura et al., 1998).

Controversies in EPS extraction studies are also associated with the impact of extracted EPS on sludge characteristics. The quantity of EPS extracted by the CER procedure was negatively correlated to settling properties (Liao et al., 2001; Wilén et al., 2003a), but related to better dewatering characteristics of activated sludge (Jin et al., 2004; Mikkelsen and Keiding, 2002). However, EDTA-EPS and glutaraldehyde-EPS reported by Erikkson and Alm (1991) and Sponza (2002), respectively, showed negative correlations with both settling and dewatering properties of sludge. Results from the thermal treatment of sludge tended to show either no relationship (Shin et al., 2001) or positive relationship (Goodwin and Forster, 1985) between the amount of extracted EPS and settleablilty of sludge but accounted for poorer dewaterability of sludge (Kang et al., 1989).

Despite this confusing information from earlier studies, several important things can be noted. First, as Novak and Haugan (1981) suggested two decades ago that there is no universal method for providing quantitative extraction of exocellular biopolymers from sludge floc. Considerable disagreement regarding extraction efficiency between different methods and the low extractability of EPS, even from the best method designated in each study (typically, less than 100 mg EPS/g solids), supports this statement. Second, it is unlikely that the EPS extracted by a single method is representative of EPS in sludge floc.

Controversies about the impact of EPS on sludge characteristics have often been attributed to the different extraction methods with different experimental approaches (cultures, extraction time, shearing force, etc). However, the varying composition of EPS such as the quantity and ratio of proteins and polysaccharides associated with different extraction methods indicate that EPS extracted by different treatments could be qualitatively different and this is more likely the reason for the differences reported. Furthermore, it was seen from the reviewed literature that some types of EPS are highly selective for certain kind of cations over others. Since several extraction methods are specific for certain cations in floc, the extracted materials by different treatments should also be different.

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Chapter 3. Characterization of Activated Sludge Exocellular Polymers Using Several Cation-Associated Extraction Methods

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Chapter 4. Evaluation of the extracellular proteins in full-scale activated sludges using cation-specific extraction methods and metaproteomic analyses

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Evaluation of the extracellular proteins in full-scale activated sludges using cation-specific extraction methods and metaproteomic analyses

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Summary

In order to study the role of cations in floc formation of activated sludge, extracellular polymeric substances (EPS) that were extracted using cation-targeted methods were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resultant protein profile was examined. Activated sludges were sampled from four full-scale wastewater treatment plants. The cation exchange resin (CER) extraction, sulfide extraction, and base extraction (pH 10.5) were applied to selectively extract EPS linked with Ca^{2+}+Mg^{2+}, Fe^{3+}, and Al^{3+}, respectively. Crude extracts were treated with sequential ammonium sulfate precipitation to separate EPS based on hydrophobicity and to enhance the separation of proteins in SDS-PAGE. Results showed that the three methods led to different SDS-PAGE profiles, suggesting that the extracted proteins are uniquely associated with specific cations and they have different hydrophobic characteristics. Protein bands at

high densities were analyzed for identifications by liquid chromatography tandem mass spectrometry (LC/MS/MS). While many samples did not retrieve results from database searches indicating they are from unsequenced microorganisms in activated sludge, several bacterial proteins including extracellular enzymes, outer membrane proteins, adhesin proteins, flagella and polypeptides originating from influent sewage were identified in this study. The extraction efficiency of these proteins also differed between the methods. These results show that Ca^{2+}+Mg^{2+}, Fe^{3+}, and Al^{3+} play different roles in floc formation and that the metal content in influent wastewater is an important parameter influencing floc properties. Metaproteomic analyses were feasible for studying EPS in field sludges and show promise for monitoring the fate of sludge proteins at various stages in the activated sludge process.

Introduction

The activated sludge process, the world's largest use of biotechnology, is the primary method used for treating both domestic and industrial wastewater. Bioflocculation is the central mechanism of the activated sludge process which results from the degradation of organic matter in influent sewage and the growth of new microbial cells in a flocculent mode. The large, dense flocs formed in the process can then be separated from the treated liquid by gravity settling while the effectiveness of this separation determines the quality of facility effluent. Although the process has been used for nearly a century, an in-depth understanding of the bioflocculation that occurs in this process is limited and variations in

bioflocculation behavior are generally thought to explain the inconsistent performance of the process across facilities. Similarly, it is not unusual to find a wastewater treatment plant (WWTP) that does not meet regulatory effluent requirements due to ineffective bioflocculation and subsequent poor solid/liquid separation.

It is believed that extracellular polymeric substances (EPS) are responsible for forming microbial colonies and linking cells and other particulate materials together, leading to the flocculent characteristic of activated sludge (Wingender et al., 1999). Activated sludge EPS are thought to consist of various biopolymers secreted by activated sludge microorganisms, intracellular materials and cellular debris emerging from cell lysis, and organic matter adsorbed from influent sewage being fed to the system (Higgins and Novak, 1997; Dignac et al, 1998; Grady et al., 1999). Recent studies using visual and chemical examination (Frølund et al., 1996; Liu and Fang, 2002; Matias et al., 2003) have demonstrated that EPS are major organic components in activated sludge, indicating their critical role in bioflocculation and endogenous decay of activated sludge biomass (sludge digestion). Proper study of EPS in activated sludge is therefore necessary but several unfavorable factors associated with activated sludge such as the complexity of activated sludge microbiota, the influence of "dirty" influent wastewater on molecular investigations, and the lack of standardized EPS extraction methods have made it very difficult to characterize activated sludge EPS. Consequently, many disagreements are still prevalent, especially regarding the extraction method protocol and the impact of "extracted" EPS on sludge properties.

While various organic compounds constitute activated sludge EPS, proteins have been reported to be predominant organic component in this extracellular matrix (Urbain et al., 1993; Frølund et al., 1996), indicating their significant influence on both surface and bulk properties of activated sludge flocs. Proteins also influence hydrophobicity of activated sludge, contributing to the flocculent nature of activated sludge in a hydrated environment. The application of SDS-PAGE into environmental samples including raw and treated wastewater was first demonstrated by Ogunseitan (1993), although SDS-PAGE was not performed on the activated sludge itself. More recently, a metaproteomic approach, comprising separation of proteins by gel electrophoresis and identification of selected proteins using mass spectrometry, has been used in a very few studies to characterize complex microbiota (Wilmes and Bond, 2004; Ram et al., 2005; Klaassens et al., 2007). Wilmes and Bond (2004) for the first time reported their metaproteomic investigation on a laboratory-scale activated sludge system designed for enhanced biological phosphorous removal. Although the feasibility of the metaproteomic approach for activated sludge cultures was well demonstrated in this study, the study was based on a laboratory culture grown on soluble synthetic chemical wastewater. Consequently, any ecological and physiological impact of full-scale influent on microbial communities in activated sludge cannot be included in such a synthetic chemical-fed system.

The polyanionic nature of cell surface, extracellular proteins, and other EPS has rendered the role of metallic ions in bioflocculation of great importance. Cations with multivalency such as Ca^{2+}, Mg^{2+}, Fe^{3+}, and Al^{3+} are particularly important in this regard since they lead to overall structural stability of activated sludge bioaggregates (Keiding and

Nielsen, 1998; Park et al., 2006a) and some of these are also known to be key biochemical elements in adhesin and agglutinin that play an important role in the formation of biofilms (Imberty et al., 2004). We previously observed that major influent wastewater cations such as Na^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} varied considerably between WWTPs along with different effluent characteristics and sludge digestibility, leading us to suggest that function and fate of each of these cations and their associated EPS in activated sludge flocs are different (Park et al., 2006 a,b). This later motivated us to develop three different cation-specific extraction methods and to investigate EPS that are released when different cations are targeted (Park and Novak, 2007). The three methods are the CER extraction procedure, sulfide extraction, and base extraction to preferentially extract EPS linked with divalent cations, Fe^{3+}, and Al^{3+}, respectively. Briefly, the majority of Ca^{2+} and Mg^{2+} in sludge were ion-exchanged with the Na^+-form of CER while little Fe and Al was impacted by the same treatment, showing the selectivity of the CER procedure for divalent cations. The quantity of sulfide-extracted EPS was correlated with Fe in various sludges, indicating the ability of sulfide extraction to target Fe. Base extraction was the least selective method, but it was the method releasing the largest amount of Al into sludge solution, indicating that this method extracted Al-bound EPS.

In the present investigation, activated sludge EPS extracted under different cation-targeted conditions were subjected into SDS-PAGE in order to compare the protein pattern resulting from the three methods and to get a better insight of the role of cations in floc formation. The use of sequential ammonium sulfate ($(NH_4)_2SO_4$) precipitation prior to gel electrophoresis was useful to enhance the separation of activated sludge extracellular

proteins in SDS-PAGE, and therefore made it easy to compare SDS-PAGE profiles between the methods, and to characterize the hydrophobicity of extracted proteins. We also analyzed abundantly detected proteins in two different activated sludges using LC/MS/MS to search their identifications and putative roles in activated sludge flocs. We believe this is the first investigation on extracellular proteins recovered from several full-scale activated sludges using metaproteomic analyses.

Results

Background of WWTPs

Thickened activated sludge was collected from the return line of four full-scale WWTPs. Table 1 shows a general background of each WWTP and activated sludges sampled for this study. While domestic wastewater was a primary source of sewage to these facilities, different proportions of industrial wastewater were also included in these plant influents. Facilities A and B were operated using a conventional activated sludge process comprising a primary clarifier, an aeration basin (where bacterial degradation and flocculation occurs), and a secondary clarifier, while facilities C and D had an anoxic chamber prior to the aeration basin to enhance nitrogen removal. Facilities A, C, and D were similar regarding the daily load of wastewater, lower than 10 million gallons per day (MGD) while facility B treated a much larger wastewater, 350 MGD. Table 1 also shows concentrations of floc-associated (solid-bound) cations at different facilities. Iron (14.3 ± 13.7 mg/g dry solids)

was the most varied between different facilities, which was followed by calcium (8.0 ± 2.6 mg/g), aluminum (5.8 ± 1.8 mg/g), and then magnesium (3.6 ± 0.8 mg/g).

Quantity of EPS in crude extracts

Table 2 summarizes the quantity of proteins and polysaccharides and their compositional ratio in crude extracts from the CER procedure, base extraction, and sulfide treatment. The cation selectivity and quantitative analysis of the three extraction methods were described in detail in a previous study (Park and Novak, 2007). In general, the CER procedure led to the best extraction efficiency for both proteins and polysaccharides, followed by base extraction and sulfide treatment. Despite the difference seen in extraction efficiency, the ratio of protein to polysaccharide also differed between the methods and this served as a rapid indicator that EPS released by the three methods were different not only on a quantitative basis but on the qualitative basis as well.

Sequential $(NH_4)_2SO_4$ precipitation

The different characteristics of EPS between the three methods were additionally seen during the precipitation with $(NH_4)_2SO_4$. The detailed patterns of protein salting out in a sequential $(NH_4)_2SO_4$ precipitation for the three crude extracts are shown in Figure 1. As the data show, the final recovery of proteins at 90% $(NH_4)_2SO_4$ saturation was always the highest for CER extraction, which was about 10 to 30% more than that for base and sulfide

extraction, indicating that the gross composition of CER-extracted proteins is more hydrophobic than sulfide- and base-extracted proteins. The data also illustrate that the pattern of protein precipitation was different based on the type of sludge.

Interestingly, approximately 50% of extracellular polysaccharides extracted by any method were also precipitated out by 90% $(NH_4)_2SO_4$ saturation (data not shown). Furthermore, about a half of precipitated polysaccharides were salted out by early hydrophobic fractions, either below 20 or 30% of $(NH_4)_2SO_4$ (data not shown). These data indicate that half of total carbohydrate content in activated sludge EPS emerges from the presence of lipopolysaccharides, glycoproteins, and/or polysaccharides linked with other EPS such as extracellular proteins.

Molecular weight distribution of native activated sludge EPS

Prior to SDS-PAGE, molecular weight distribution of extracted proteins and polysaccharides were analyzed using EPS extracts from activated sludge D1. For this analysis, extracts from the three methods were individually filtered through six kinds of filters (1.5 μm, 0.45 μm, 500 kDa, 100 kDa, 30 kDa, and 3 kDa), proteins and polysaccharides were quantified from each filtrate, and finally their six size fractions were determined. Although the actual molecular weight of proteins and polysaccharides cannot be determined by this approach, their molecular weight distribution was thought to provide differences in EPS extracted by the three methods. In addition, size separation analysis might be useful for obtaining information regarding the native size of activated sludge EPS.

Both protein and polysaccharide data in Figure 2 show that the largest fraction of these biopolymers was present in the size between 0.45 μm and 500 kDa, independent of the extraction technique. The next largest quantitative fractions are EPS retained either in 1.5 μm-0.45 μm or in the size less than 3 kDa. There was no significant distribution of proteins and polysaccharides between 500 kDa and 30 kDa. The major fraction of native EPS in the size greater than 500 kDa is indicative of agglomeration property of EPS in activated sludge. The significant quantity of EPS less than 3 kDa indicates small fragments of organic matter are also retained in the extracellular matrix of activated sludge flocs.

Figure 2 also shows that the size distribution of extracellular proteins and polysaccharides was different between the three methods. A relatively large fraction of peptide-like proteins (less than 3 kDa) in base and sulfide extracts might be one of the reasons why a significant fraction of base and sulfide-extracted proteins did not recover even at high $(NH_4)_2SO_4$ saturation. The comparison of Figure 2A and 2B showed that the pattern of size distribution between proteins and polysaccharides also differed even for the same extraction technique. All these results suggest that organic pools linked with different metal ions in activated sludge flocs are also different with respect to their distribution between molecular sizes.

SDS-PAGE of activated sludge extracellular proteins

CER, base, and sulfide extracts from four different activated sludges were subjected to SDS-PAGE and their results are shown in the following section. It should be noted here

that proteins that did not precipitate out from $(NH_4)_2SO_4$ addition could not be loaded on to SDS-PAGE. Also, based on the property of single dimensional SDS-PAGE, proteins of same molecular weight but with different isoelectric points cannot be separated out in SDS-PAGE and therefore, the chance that more than one protein are present in a SDS-PAGE band cannot be ruled out.

Activated sludge from facility A. Figures 3 shows the SDS-PAGE of extracellular proteins in a full-scale activated sludge collected from facility A. Proteins extracted by the CER procedure, base extraction, and sulfide extraction are shown in Figure 3A, 3B, and 3C, respectively. Four lanes in each method are designated with different $(NH_4)_2SO_4$ saturation levels which resulted in fractionation of proteins based on hydrophobicity. Proteins in Lane 1 should be the most hydrophobic since they were salted out at low $(NH_4)_2SO_4$ saturation (0-20%) while proteins in Lane 4 were believed to be the least hydrophobic since they were precipitated out only at the highest salt saturation (60-90%). Some of the protein bands were present over the several lanes. The detection of same protein bands in a wide range of hydrophobicity indicates that not only hydrophobic interactions but other factors such as cation bridging, hydrogen bonding, or polymerization with other biopolymers are prevalent in EPS complex.

Overall images of SDS-PAGE show several distinct and identifiable protein bands, indicating that pure proteins were present in the extracellular matrix of field activated sludge and their separation was well resolved in SDS-PAGE. Proteins were mainly distributed between 10 kDa and 75 kDa. Some smear pattern of proteins, possibly from

degraded protein products or due to the presence of polysaccharides, were also present at lower molecular weights. Figure 3 also shows that many proteins appeared in all three extracts. However, their band intensities were clearly different based on the extraction method, indicating that some proteins were more preferentially extracted in one extraction condition. Good examples are band a1 (~13 kDa) which was a major hydrophobic protein in the sulfide extract, band a4 (~29 kDa) most clearly detected in the base extract, band a6 (~33 kDa) in the CER extract, and band a8 (~55 kDa) in the base extract. The fourth lane (60-90%) of the CER extract contained numerous proteins in the size between 20 kDa and 75 kDa. These hydrophilic proteins were the most substantially expressed in CER-extracted proteins, indicating that these proteins are intimately associated with divalent cations in activated sludge flocs and mainly extracted by the CER procedure.

Activated sludge from facility B. Figure 4 shows the SDS-PAGE of extracellular proteins harvested from the field activated sludge B. It can be seen that overall SDS-PAGE patterns between the three methods are also different for this sludge set. Some of the most distinctive protein bands are indicated in Figure 4. Band b1 which is about 130 kDa in its molecular size was a unique base-extracted protein. The same band was weakly seen in the sulfide extract but omitted in the CER extract. On the other hand, band b2 (~55 kDa) and b3 (~52 kDa) were the most strongly detected in the CER-extracted proteins while band b4 (~22 kDa) and b5 (~16 kDa) was very clearly observed from the sulfide extract. Protein bands in the fourth lane of CER-extracted proteins again mainly belong to the CER extract although a protein band at about 37 kDa is also relatively strongly observed in the base

extract. These results suggest that organic pools targeted by the three methods are not the same and they are possibly spatially distant within activated sludge flocs.

The visual comparison of Figure 3 and 4 based on observing protein's molecular weight, appearance in the extraction method, and hydrophobic property allows describing that there are several common extracellular proteins between these two very different activated sludges. The 29 kDa base-extracted protein and CER-hydrophilic proteins, designated as proteins in 20 kDa ~ 55 kDa in the fourth lane of CER extract, are good examples. Indeed, these protein bands also appeared in activated sludges from other facilities (see below), indicating that they are ubiquitous proteins in activated sludge flocs.

Activated sludge from facility C and D. SDS-PAGE of extracellular proteins collected from activated sludge C and D are shown in Figure 5 and 6, respectively. Crude extracts were separated in three parts for these sludge sets. Sulfide extraction for these two sludges resulted in very low extractability of proteins (Table 2) and their resolution in SDS-PAGE was also relatively poor. It was speculated that this is because of the presence of an anoxic chamber prior to the aeration basin in these WWTPs, which might have affected the fate of iron-associated organic matter. Nevertheless, different SDS-PAGE patterns between the three methods, especially between CER extraction and base extraction, can be again observed in these sludge sets. A couple of protein bands worth for separate notes are band c2 (~34 kDa), c3 (~52 kDa), and c4 (~55 kDa) in activated sludge C. Band c2 was the most strongly observed in the base extract followed by the sulfide and then the CER extract. On the other hand, band c3 and c4 were mainly shown in base-extracted proteins. These three

bands did not appear appreciably in counterparts of activated sludge D, implying that these proteins are unique functional products of microbiota in activated sludge C. In contrast, 29 kDa protein and a group of CER hydrophilic proteins were well conserved in both activated sludges.

Identification of proteins using LC/MS/MS

During the analysis of SDS-PAGE, protein bands with strong intensities and universality were of interest and prompted further study to seek for their identification. Information that can be derived from their putative identification would be useful in understanding their physiological roles in activated sludge flocs and their fate in various stages of activated sludge system. A total of twelve protein spots from activated sludge A and C were excised, trypsin-digested, and subjected to LC/MS/MS. The resultant MS/MS spectra were used for sequencing peptides and identifying proteins using the national center for biotechnology information (NCBI) non-redundant database.

Tables 3 summarize the results of LC/MS/MS and identification of selected protein bands in activated sludge A and C. As expected, many protein bands did not get any results from the database searches or returned with a relatively low confidence (i.e., number of peptides sequenced are lower than three). A review of raw mass spec data on those undetermined proteins revealed that a great deal of good MS/MS spectra were obtained from LC/MS/MS but did not retrieve any results. This indicates that the origin of these proteins is most likely from unsequenced microorganisms in activated sludge.

Although about half of the samples could not be determined by LC/MS/MS, the remaining samples led to positive hits on protein identification in this study. First, protein in band a3 was tentatively identified to be superoxide dismutase (SOD) from several ß-Proteobacteria including *Polaromonas sp. JS666*, *Rubrivivax gelatinous PM1*, *Dechloromonas aromatica RCB*, and *Thiobacillus denitrificans ATCC 25259*. SOD is a key enzyme that is involved in antioxidant defense system in nearly all aerobic organisms by catalyzing the dismutation of superoxide radical into oxygen and hydrogen peroxide. The band a3 was clearly detected in all CER extracts (60-90%) from four activated sludges. The hydrophilic characteristic of SODs is often used in purifying these enzymes from bacterial cultures (Kang et al., 1998). In adition, nominal molecular weights of proteins identified (21 kDa) demonstrate a consistency with the position of protein band in SDS-PAGE. These observations support that protein in band a3 is bacterial SOD.

Band a6 and a5 from the CER extract were matched with outer membrane protein (porin) from *Rubrivivax gelatinous PM1* and five other ß-Proteobacteria and root adhesin from *Pseudomonas fluorescens,* respectively. Molecular weights of these two proteins are also matched fairly well with the bands in SDS-PAGE. While only a single peptide (RDQSTLAIAGAGVKY) was found for band a5, the major y-ion peaks of the MS/MS spectra were 100% matched with the sequence STLAIAG and a BLASTP search of this sequence also led to root adhesin and its homologs including protein F and the outer membrane porin F precursor from the same organism. This indicates that the protein in the band a5 is possibly a root adhesin protein or its homolog. Several outer membrane porins from Gram-negative bacteria have been reported to be involved in bacterial colonization

and attachment to host cells (DeMot et al., 1992; Achouak et al., 1998; Azghania et al., 2002; Holm et al.,2004; Bodilis and Barray, 2006). Hence, these two outer membrane proteins might be associated with the formation of bacterial colonies in activated sludge flocs, possibly with the aid of divalent cations.

The band a7, which was also detected in the CER extracts from other sludges, was a putative leucine-specific binding protein precursor from *Sinorhizobium meliloti*. This protein is a component of ABC transporter for the branched-chain amino acid and is a periplasmic component. Considering its subcellular location, this protein appears to be a product of cell lysis. However, it could not be determined at this stage whether the protein was from auto cellular lysis or extraction-associated lysis and why this protein was mainly extracted by the CER procedure. Nevertheless, it has been reported that ABC transporter is also required for bacterial attachment and is a virulence factor (Sauer and Camper, 2001), indicating its potential role in bioflocculation of activated sludge.

One interesting result from LC/MS/MS analysis was the finding of polypeptides associated with human products in activated sludge EPS. As shown in Table 3, the protein sampled from band a4 was identified to be Elastase III A from *Homo sapiens*. Mass spec also worked best on this band sample so that 24 peptides were sequenced with 52% sequence coverage, strongly supporting its identification. Elastase III A is a serine protease produced in the human pancreas. A review of MS/MS data confirmed that this polypeptide was not from the trypsin used in the digestion procedure. Its detection in full-scale activated sludge indicates that this human gut protein adsorbed onto flocs and survived through the activated sludge system. Band c1, the band at a similar position in the base extract from

activated sludge C, was also identified as Elastase III A, indicating this protein is universal in full-scale activated sludge and a meaningful component of activated sludge EPS.

Finally, the protein in band c2 from the base extract from activated sludge C was shown to be flagellin from spirochaetes *Treponema pallidum*. The finding of a flagellin, a protein subunit comprising flagella polymer, in the major base extract was also unexpected since flagella are associated with the motility of planktonic cultures and the genes responsible for flagellar synthesis and maintenance are usually down-regulated in biofilm cultures, although flagella was involved in initial cell-to-surface in biofilm formation (Garrett et al., 1999; Sauer and Camper, 2001).

Discussion

In order to better understand the role of cations in floc formation and to characterize extracellular polymers in activated sludge flocs, we used three different cation-specific extraction methods and metaproteomic analyses on the extracted EPS in the current investigation. The results seen from the gross composition of EPS, their size distribution in native state, protein salting out in sequential $(NH_4)_2SO_4$ precipitation, and SDS-PAGE provided strong evidence that activated sludge EPS that are released under different cation-targeted conditions are not the same materials. Although the specific role of cations in activated sludge flocs could not be identified in this study, these results suggested that divalent cations, Fe, and Al are associated with different characteristic EPS and play different roles in the formation of 3-dimensional structure of activated sludge flocs.

It was also found in this study that CER-extracted hydrophilic proteins were well conserved in all activated sludge flocs while base- and sulfide-extracted proteins were much more variable between the sludge source, indicating that the fate of Fe and Al and their bound organic matter are quite dynamic in activate sludge systems. Interestingly, CER-extracted proteins also displayed more hydrophobic characteristic than two other methods so that the role of Ca^{2+} and Mg^{2+} and their associated EPS in forming cell colonies can be assumed. In this micro-region of flocs, outer membrane proteins, adhesins, pili, and lectin-like adhesive biopolymers are likely to be involved in agglutinating cells with the aid of divalent cations. Indeed, a great deal of literature is available showing the direct involvement of divalent cations and aforementioned extracellular materials in cell agglutination in pure cultures (Young et al., 1985; Swart et al., 1994; Touhami et al., 2003; Imberty et al., 2004). Also, it was observed in another study that lectin activity, which was evidenced by hemagglutinating activity and specificity for sugar moieties on glycoproteins, was concentrated in a hydrophobic fraction of activated sludge EPS (Park et al., in preparation).

We also confirmed in this study that quantitative comparison between different extraction methods is not an appropriate approach because different methods released different kinds of proteins and presumably other EPS from flocs. This might be an important result since dealing with EPS in multicultural bioaggregates such as activated sludge flocs and biofilms, the proper choice of extraction method has been always an important issue (Nielsen and Jahn, 1999) and the extraction efficiency of the method is one important parameter when comparing methods.

The SDS-PAGE was useful for characterizing extracellular proteins from field activated sludge. It has been generally perceived that separating the proteins from a full-scale activated sludge by SDS-PAGE is a challenge due to the complex microbial consortia in activated sludge matrix and to the impact of unknown sources of proteins and other organic matter coming from a sewer line. We tried several clean-up and concentration methods and found that sequential $(NH_4)_2SO_4$ precipitation was very efficient in preparing the activated sludge samples for SDS-PAGE. In addition, this approach allowed investigating the general hydrophobicity of proteins. However, we also admit that, due to the limit of single dimensional characteristic of SDS-PAGE, two dimensional analysis will provide a better opportunity to characterize proteins and will be a better approach, especially when de novo sequencing is needed.

While limited, the data available from LC/MS/MS analysis in this study allowed categorization of extracellular proteins in full-scale activated sludge into five different groups: (1) enzyme associated with bacterial defense; (2) cell appendage; (3) outer membrane proteins; (4) intracellular materials; and (5) influent sewage. The finding of polypeptides associated with human products in activated sludge flocs was a surprising result. It can be thought that the presence of a foreign protease might induce a response from the native activated sludge microorganisms. Furthermore, a possible entrance of these human protein enzymes into receiving waters via the facility effluent might be an important ecological issue. Consequently, their impact on activated sludge and their fate in the activated sludge process may worth for further study.

Finally, since a considerably different cation content is quite common for different wastewaters, we postulate that this variability is one important factor that leads to different characteristics of activated sludge across facilities. The incorporation of cations and EPS into activated sludge engineering models, which are used for constructing and operating the process, might be challenging but will assure a better engineering application of the activated sludge process.

Experimental procedures

Sampling of activated sludge

Activated sludge cultures were collected from four full-scale WWTPs as listed in Table 1. Facilities A, C, and D are located within 1hr driving distance from our laboratory. The sample from facility B was contained in a cooler and shipped to the laboratory using overnight service. All the sludge samples were kept at 4°C during analysis and extraction of EPS.

Chemical analysis of activated sludge

Metals of interest, Ca^{2+}, Mg^{2+}, Fe and Al in activated sludge were measured using EPA method 3050B (Acid digestion for metals analysis of soils, sediments, and sludges, 1996). For this analysis, the sludge pellet obtained from centrifuge (15,000g x 15 min) was dried

in a 105°C oven and the recorded mass of solids underwent acid digestion. The metal concentrations in the acid-digested samples were quantified using Atomic Absorption Spectrometer. Total solids (TS) and total volatile solids (VS) in activated sludge were measured according to APHA Standard Methods (1995).

EPS Extraction

Choice of EPS extraction methods. In order to characterize EPS that are released when different cations are targeted from activated sludge, three different extraction methods were used as proposed by Park and Novak (2007). These are: (1) the cation exchange resin (CER) procedure to extract Ca^{2+} and Mg^{2+}-bound EPS by exploiting high affinity of Na^{+} form of resin for Ca^{2+} and Mg^{2+}; (2) sulfide addition to release Fe-bound EPS by forming precipitate of FeS; and (3) base extraction (at pH ~10.5) to extract Al-coagulated EPS by dissolving Al at alkaline conditions.

Outlines of extraction of EPS. All the sludge samples (0.4 L) were initially centrifuged at 15,000 g for 15 minutes to remove a liquid fraction of sludge. The resultant pellet was resuspended in 0.2 L of extraction solution and underwent one of the three extraction methods described below. All the extractions were performed in a 4°C constant temperature room. In order to enhance extraction, each extraction method included stirring provided by a mixing paddle in a four baffled plastic extraction beaker. At completion of extraction, the disturbed sludge was centrifuged at 15,000 g for 15 minutes at 4°C and the supernatant was

further passed through 1.5 μm filter to produce crude extract. CER extraction followed the same procedure as that of Frølund et al. (1996). Briefly, the dose of CER (Dowex 50 x 8, Na^+ form, 20-50 mesh) was adjusted to 60 g resin/g VS. The extraction buffer was a low strength of phosphate buffer saline (PBS) (2 mM KH_2PO_4, 6 mM Na_2HPO_4, and 10 mM NaCl) and the extraction was performed for 1 hr at 600 rpm in a four-baffled extraction beaker. Following digestion, the resin beads were separated by filtering through a nylon wire mesh (250 μm). The procedure for sulfide extraction was modified from the method of Nielsen and Keiding (1998) and consisted of two extraction steps: an iron-reducing step and physical extraction step. The sludge pellet from the prior centrifugation was resuspended in 10 mM NaCl solution, transferred to 0.5 L flask, and purged with N_2 gas for 30 minutes. The dose of sulfide was determined to achieve a molar ratio of S^{2-}/Fe at 1.5 using the stock solution of $Na_2S•9H_2O$ (600 mM, pH ~7.5). Sulfide-added sludge gradually turned black with gentle mixing on a wrist action shaker over six hours (at 4°C). The sludge was then transferred to a general extraction beaker under N_2 gas flow and sheared for 15 minutes at 900 rpm. For base extraction, the sludge pellet was resuspended with 10 mM NaCl and the pH was raised to 10.5 using 1N NaOH. The pH adjusted sample was then transferred to an extraction beaker and underwent a 1 hr extraction (600 rpm). The extraction was performed in the presence of N_2 to minimize pH drop and auto-oxidation of extracted organic matter.

Size separation of EPS. Size separation was performed as previously described by Park et al. (2006a). Ultrafiltration was performed by filtering at 60 psi through Amicon YM500

(500 kDa), YM100 (100 kDa), YM30 (30 kDa), and YM3 (3 kDa) membranes (Amicon, Massachusetts).

Quantification of protein and polysaccharide. The extracted protein was determined by Frølund et al. (1996) modification of the Lowry et al. (1951) method to account for the interference of phenolic compounds and humic substances in protein quantification. To minimize the influence of different extraction solution and residual sulfide on protein quantification, protein was also measured following precipitation with 80 % (v/v) acetone and resuspension with PBS. The standard was bovine serum albumin. Polysaccharide was measured by phenol-sulfuric acid method of Dubois et al. (1956) using glucose as the standard.

Sequential $(NH_4)_2SO_4$ precipitation

The crude extract was precipitated by sequential addition of $(NH_4)_2SO_4$ to separate EPS based on different hydrophobicity. The four $(NH_4)_2SO_4$ saturation levels (0/20, 20/40, 40/60, 60/90) were used for activated sludge A and B while three fractions (0/30, 30/60, 60/90) were prepared for activated sludge C and D. Each precipitation step was performed on ice for more than six hours and completed by centrifuging at 20,000g for 30 minutes. The collected precipitate was resuspended in PBS and dialyzed extensively in same PBS with multiple changes at 4°C. The dialysis tubing was 6~8 kDa cellulose membrane (Fisher).

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970). A 4-15% gradient gel (Bio-rad) was mainly used for SDS-PAGE. For LC/MS/MS analysis, either 10% or 15% of SDS-PAGE gel was used to improve the separation of proteins in the region of molecular weights of interest. The sample was mixed with 4-time SDS-PAGE sample buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 2% bromophenol blue, and 20% ß-mercaptoethanol) and heated in 95°C for at least 15 minutes. Following a heating process, the sample was centrifuged at 12,000 rpm for 5 minutes and the supernatant was used for SDS-PAGE. The gel was stained with Coomassie Brilliant Blue-R 250.

LC/MS/MS and protein identification

Protein identification work was conducted in four stages: 1) decision of protein bands to be identified; 2) sample preparation for MS analysis; 3) sample analysis by LC/MS/MS; and 4) database searching and protein identification. Once proteins of interest are decided, four spots ($1mm^3$) from each protein band were excised from SDS-PAGE gel. Samples were reduced with dithiothreitol, alkylated with iodoacetamide, and digested overnight with trypsin (37°C). The digestion was halted by adding acetic acid (pH~4) and peptides were vortexed out of the gel for 15 minutes followed by sonication for 15 minutes. The digested samples were analyzed using a Tempo nano MDLC system (Applied Biosystems)

interfaced with 4000 QTRAP mass spectrometer in NSI mode. An autosampler was utilized to load 2 μL of sample onto a New Objective IntegraFrit ProteoPep II column (IFC50-PR2-5). Peptides were eluted and the three most abundant precursors were collected and fragmented and then MS/MS spectra were processed by Analyst version 1.4.1 software. Finally, MASCOT software (http://www.matrixscience.com) was used to analyze MS/MS spectra and identify proteins. The NCBI nonredundant (nr) database was searched once for all organisms and once for just bacteria. Protein identifications were considered significant and correct with three peptides identified each having a probability based Mowse score≥55 for the nrdb with all organisms and a probability based Mowse score≥50 for the nrdb with only bacteria where the individual ion score had a $p<0.05$ for random matches.

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Table 4-1. General background of four WWTPs and activated sludges sampled in this study[a].

Plant	Influent source	Daily load (MGD)	TS (g/L)	VS (g/L)	Fe (mg/g)	Al (mg/g)	Ca (mg/g)	Mg (mg/g)
A	80% domestic	6	8.4	6.7	7.0	6.3	7.1	3.7
B	~40% industrial	350	22.4	17.0	38.7	6.4	7.3	2.4
C	Mainly domestic	3	9.2	7.3	7.6	3.1	4.7	4.5
D1	75% domestic	6	10.4	8.0	8.0	5.3	9.5	4.0
D2	75% domestic	6	12.8	9.5	10.4	8.1	11.5	3.4

[a]The metal concentration is based on 1 gram dry solids of activated sludge

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Table 4-2. Summary of activated sludge EPS extracted by the three extraction methods.

	Protein (mg/g VS)			Polysaccharide (mg/g VS)			Protein/Polysaccharide		
Sludge	CER	Base	Sulfide	CER	Base	Sulfide	CER	Base	Sulfide
A	74	69	22	33	22	11	2.2	3.1	2.1
B	70	42	33	37	13	10	1.9	3.3	3.4
C	59	41	11	23	12	3	2.6	3.4	3.8
D1	73	47	16	26	11	4	2.8	4.2	4.2
D2	70	31	13	22	8	3	3.2	3.7	3.7

Table 4-3. Results of LC/MS/MS database searches and protein identification.

Band no.	Extraction method	Number of peptides sequenced	Sequence coverage (%)	Proteins matched	Best matching species	Nominal mass (kDa)
a1	Sulfide	–	–	No proteins matched	–	–
a2	Sulfide	–	–	No proteins matched	–	–
a3	CER	5	30	Superoxide dismutase (SOD)	*Polaromonas sp. JS666*	21
a4	Base	24	52	Elastase III A	*Homosapiens*	30
a5	CER	1	3	Root adhesin	*Pseudomonas fluorescens*	34
a6	CER	7	4	Outer membrane protein (porin)	*Rubrivivax gelatinous PM1*	36
a7	CER	4	5	Putative leucine-specific binding protein precursor	*Sinorhizobium melioti*	39
a8	Base	–	–	No proteins matched	–	–
c1	Base	2	7	Elastase III A	*Homosapiens*	30
c2	Base	2	7	Flagella filament 33 kDa core protein	*Treponema pallidum*	33
c3	Base	–	–	No proteins matched	–	–
c4	Base	–	–	No proteins matched	–	–

*Same peptide sequence may be present multiple times if it had more than one LC/MS/MS scan.

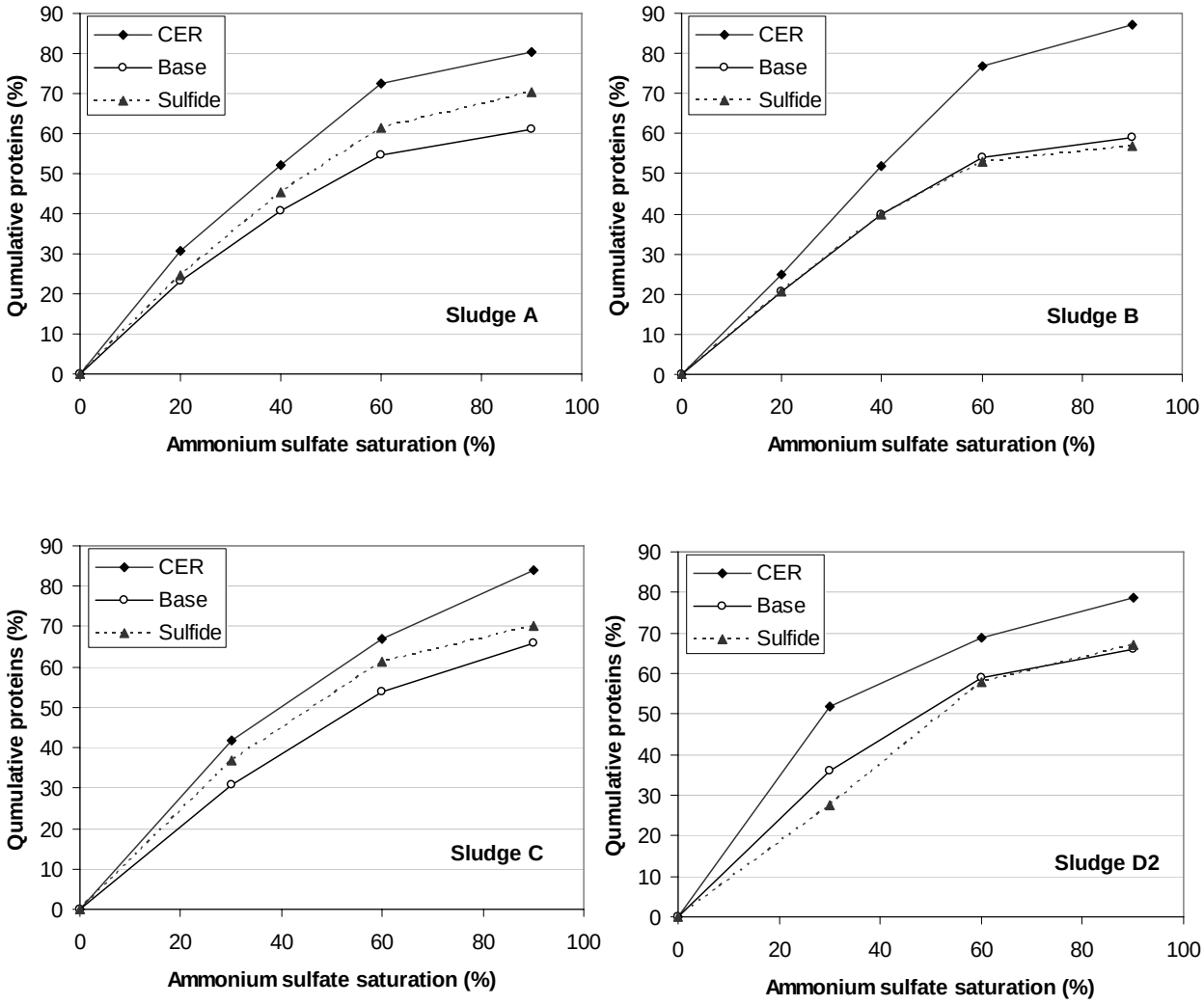

Figure 4-1. Cumulative curve of proteins precipitated during sequential ammonium sulfate precipitation.

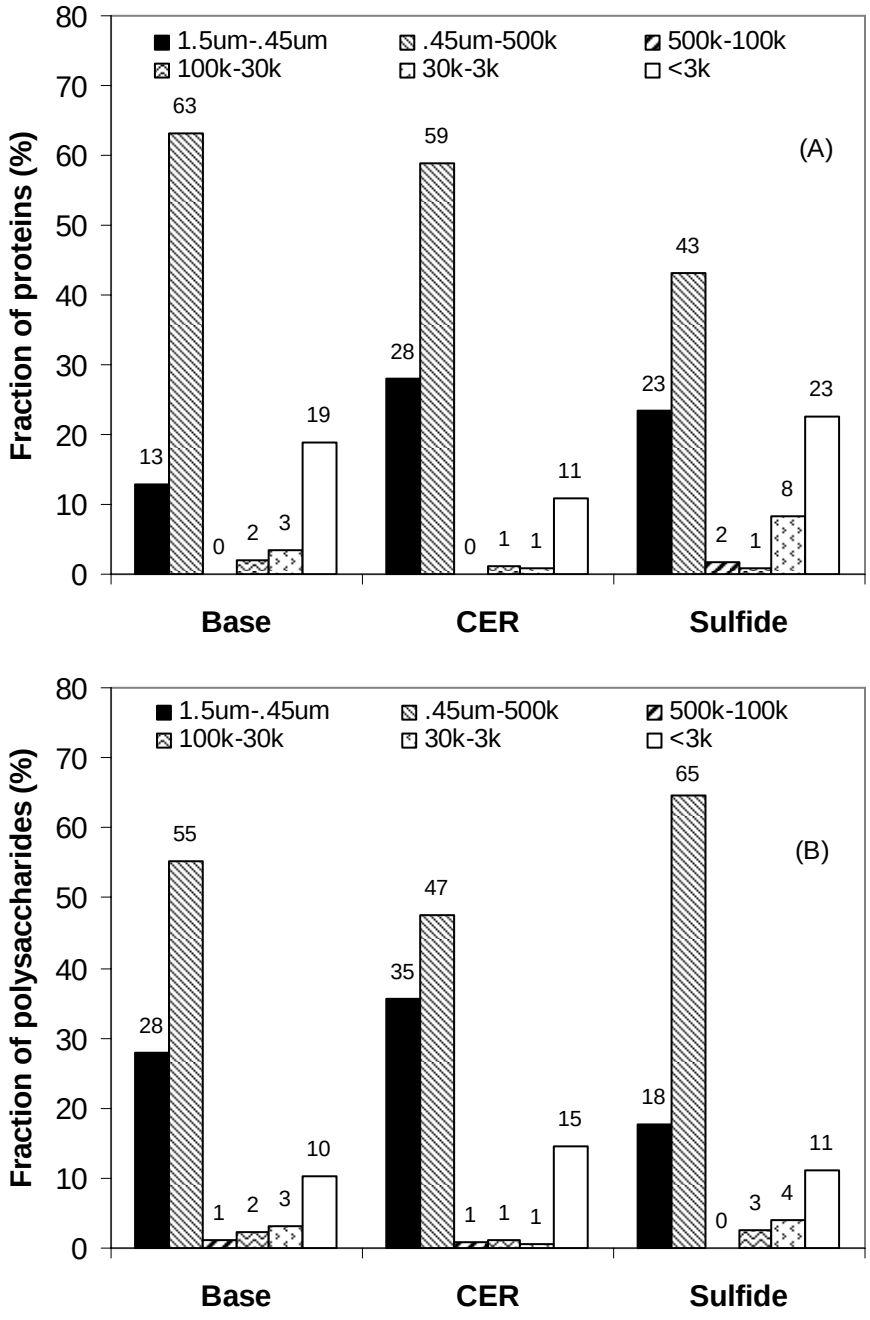

Figure 4-2. Molecular weight distribution of (A) proteins and (B) polysaccharides extracted from activated sludge D1. The values of crude extracts are listed in Table 4-2.

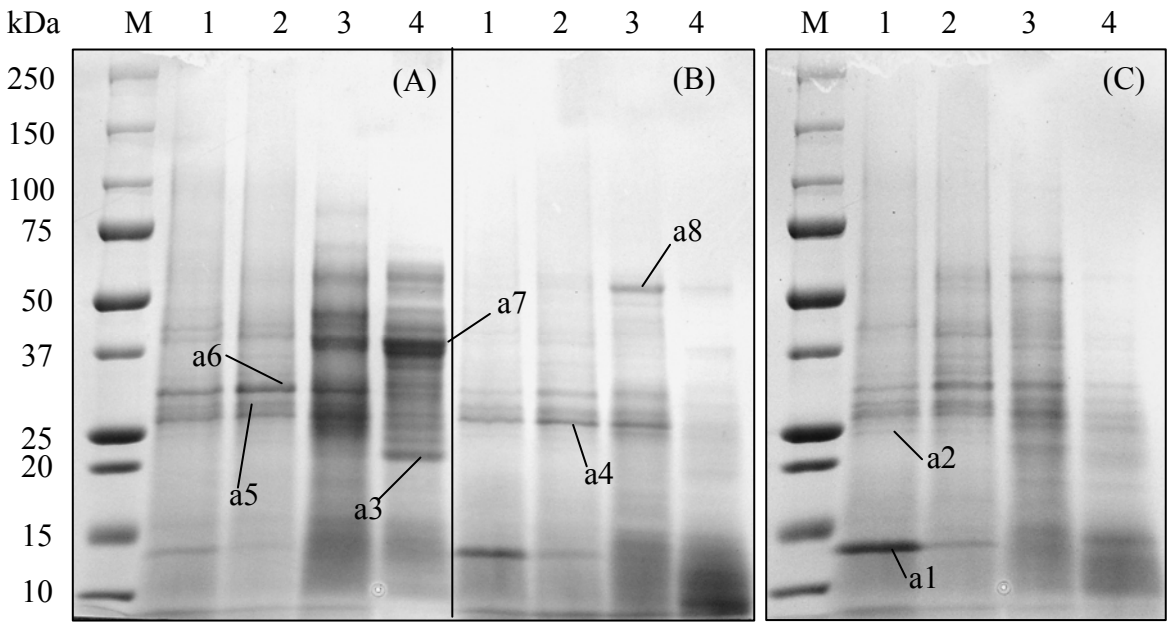

Figure 4-3. SDS-PAGE of extracellular proteins in activated sludge A extracted by (A) the CER procedure, (B) base extraction, and (C) sulfide treatment. Lane M: molecular weight markers, Lane 1: proteins separated with 20 % $(NH_4)_2SO_4$ saturation, Lane 2: 20 ~ 40 %, Lane 3: 40 ~ 60 %, and Lane 4: 60 ~ 90 %. Proteins loaded are 40 μg for lanes 1 to 3 and 20 μg for lane 4.

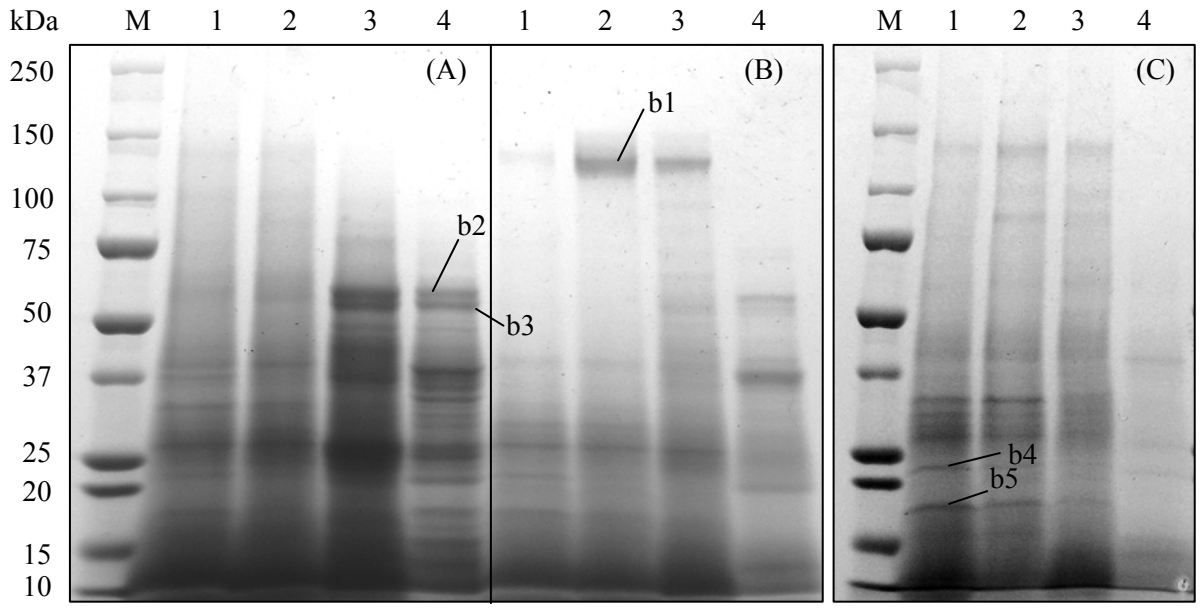

Figure 4-4. SDS-PAGE of extracellular proteins in activated sludge B extracted by (A) the CER procedure, (B) base extraction, and (C) sulfide treatment. Lane M: molecular weight markers, Lane 1: proteins separated with 20 % $(NH_4)_2SO_4$ saturation, Lane 2: 20 ~ 40 %, Lane 3: 40 ~ 60 %, and Lane 4: 60 ~ 90 %. Proteins loaded are 40 μg for lanes 1 to 3 and 20 μg for lane 4.

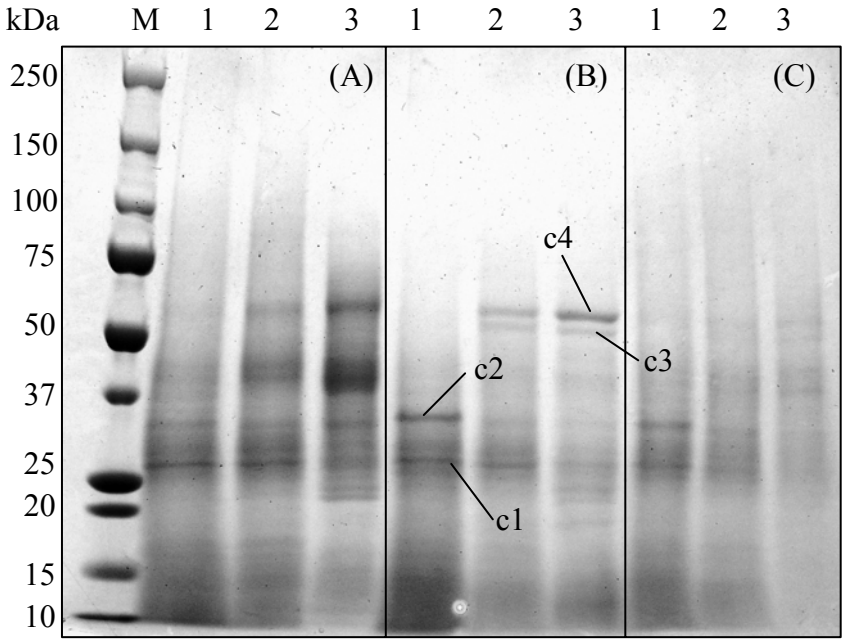

Figure 4-5. SDS-PAGE of extracellular proteins in activated sludge C extracted by (A) the CER procedure, (B) base extraction, and (C) sulfide treatment. Lane M: molecular weight markers, Lane 1: proteins separated with 30 % $(NH_4)_2SO_4$ saturation, Lane 2: 30 ~ 60 %, and Lane 3: 60 ~ 90 %. Proteins loaded are 40 μg for lanes 1 and 2 and 20 μg for lane 3.

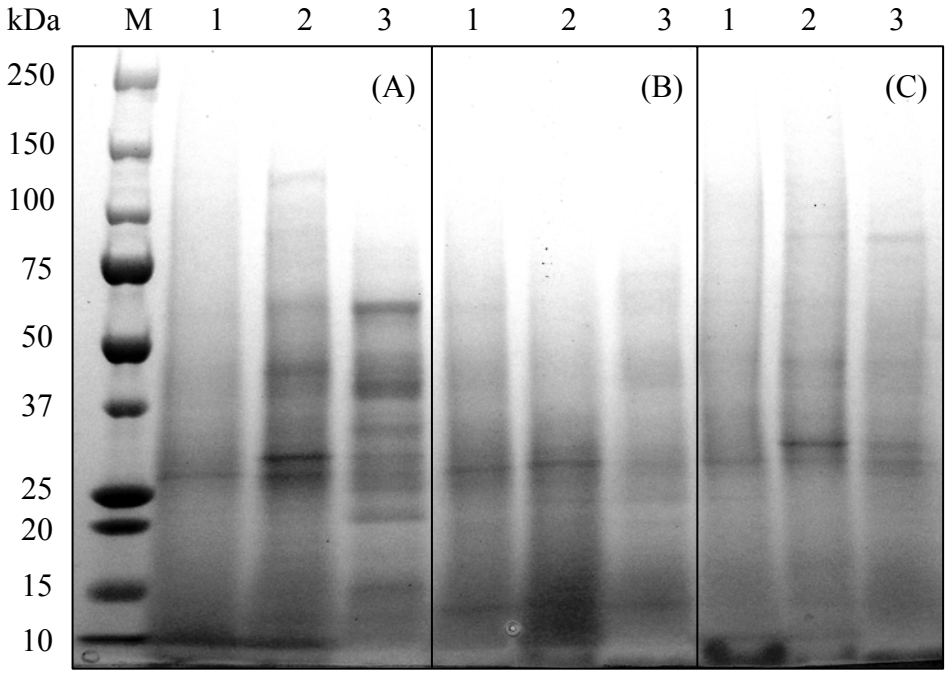

Figure 4-6. SDS-PAGE of extracellular proteins in activated sludge D2 extracted by (A) the CER procedure, (B) base extraction, and (C) sulfide treatment. Lane M: molecular weight markers, Lane 1: proteins separated with 30 % $(NH_4)_2SO_4$ saturation, Lane 2: 30 ~ 60 %, and Lane 3: 60 ~ 90 %. Proteins loaded are 40 μg for lanes 1 and 2 and 20 μg for lane 3.

Chapter 5. Investigating The Fate of Activated Sludge Extracellular Proteins in Sludge Digestion Using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

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ABSTRACT

The purpose of this study was to examine the fate of activated sludge extracellular proteins in batch anaerobic and aerobic digestion using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We were particularly interested in studying the fate of proteins that are bound with different sludge cations. Extracellular polymeric substances (EPS) extracted by three cation-specific extraction methods, the cation exchange resin (CER) procedure for extracting Ca^{2+}+Mg^{2+}, sulfide extraction for removing Fe, and base treatment (pH 10.5) for dissolving Al, were subjected to SDS-PAGE and the resultant protein profiles were compared before and after sludge digestion. The initial SDS-PAGE on undigested activated sludge showed different protein profiles by the three methods, suggesting that Ca^{2+}+Mg^{2+}, Fe, and Al are bound with different characteristic EPS and play unique physio-biochemical roles in floc formation. The analysis of SDS-PAGE post sludge digestion revealed that CER-extracted proteins remained intact in anaerobic digestion while they were degraded in aerobic digestion. While the fate of sulfide-and base-extracted

proteins in aerobic digestion was not clearly resolved, their changes in anaerobic digestion were well observed. Sulfide-extracted protein bands were reduced by anaerobic digestion, indicating that Fe-bound EPS were degraded under anaerobic condition. While parts of base-extracted proteins disappeared after anaerobic digestion, others became more extractable along with extraction of new proteins, indicating that base-extractable EPS are dynamic in anaerobic digestion. All these results suggest that the metal content in influent sewage have a significant impact on activated sludge characteristics and sludge digestibility. The potential application of SDS-PAGE and the three extraction methods for choosing a proper sludge pretreatment and digestion method are also discussed in this study.

KEYWORDS

Activated sludge, cations, EPS, extraction, proteins, SDS-PAGE, sludge digestion

INTRODUCTION

The activated sludge process is the primary method used for treating municipal wastewater before its discharge into receiving waters. Despite its usefulness in treating a large, variety source of wastewater, the process also produces byproduct, sludge, which needs to be properly treated prior to its ultimate disposal. A significant cost demand in the activated sludge process also lies on solids handling processes downstream of the activated sludge

system (Davis and Masten, 2002), rendering proper and efficient treatment of sludge of great importance.

While there are several choices to stabilize waste sludge, anaerobic digestion that utilizes a complex anaerobic microbial consortia to convert sludge biomass to methane gas has been the most commonly practiced sludge stabilization method. To assure various options for final disposal, the digested sludge product needs to meet a certain regulatory requirement. One parameter often used in evaluating the stability of digested sludge, especially with respect to vector attraction reduction, is the efficiency in reducing the volatile fraction of waste sludge. Currently, reduction of volatiles solids (VS) at a minimum of 38% is the one of the criteria required for biosolids (digested sludge) to be land-applied (USEPA, 2003). In order to enhance sludge digestion, to achieve a higher VS reduction and pathogen inactivation, various sludge pretreatments employing mechanical shearing, sonication, ozone oxidation, base treatment, etc have been introduced. However, the success of imparting these sludge disintegration techniques into anaerobic digestion has not always been guaranteed (Muller, 2006). Similarly, anaerobic digesters with various configurations such as digesters in series with different temperature and/or solids retention time have been designed but the variability in the performance of such processes is not also unusual. Although anaerobic digestion is the most popular sludge stabilization method in the US, the success of the process still appears to require trial and error approach for design.

The need for better digester performance has increased interest in developing a better, fundamental understanding of bioflocculation in the activated sludge process.

Bioflocculation is the central mechanism of the activated sludge process which results from the degradation of organic matter in influent sewage and the growth of new microbial cells in a flocculent mode. The effectiveness of bioflocculation directly influences the separation of sludge flocs from the treated liquid by gravity settling or by membrane separation and the effectiveness of this separation determines the quality of the effluent. Consequently, a great deal of research has been conducted to better understand bioflocculation and a traditional research focus has been to investigate the structure and composition of activated sludge flocs.

Activated sludge flocs can be thought of as a mix of EPS, microorganisms, cations and other debris that is bound into a single structural unit. Activated sludge EPS originate from microbial metabolism, cell lysis, and organic matter adsorbed from influent wastewater (Urbain et al., 1993; Dignac et al., 1998, Nielsen and Keiding, 1998; Park et al., 2007), providing a polymeric matrix in which microorganisms are embedded and remain aggregated. Hence, EPS play a central role in bioflocculation and account for the flocculent nature of activated sludge. In addition, there have been a number of studies reporting that EPS account for the major organic fraction in activated sludge flocs (Frølund et al., 1996; Münch and Pollard, 1997; Liu and Fang, 2002; Matias et al., 2003). Although EPS and active cell fractions will vary depending on the physiological characteristics of sludges investigated, this information suggests that the impact of EPS on floc stability and treatment processes such as sludge conditioning and dewatering could be significant. Furthermore, it may be a reasonable assumption that the fate of EPS in sludge digestion is related to the sludge digestibility.

Due to the polyanionic nature of EPS and cell surfaces, cations become an important structural component as a binding agent within the biopolymeric matrix (Bruus et al., 1992; Urbain et al., 1993; Higgins and Novak, 1997). Cations with multivalency are traditionally considered important since they are essential for the formation of ionic bridges for some EPS and maintain them in ordered structures, leading to the stability of activated sludge bioaggregates. However, the importance of both content and concentration of cations also needs to be stressed since different cations also have different biochemical properties (charge valence, radius, red/ox characteristic, etc) and form unique hydrolysis products in aqueous environment. Similarly, Park et al. (2006) showed that the digestibility of waste activated sludge under anaerobic conditions was greatly influenced by Fe concentration in sludge while the digestibility under aerobic conditions was closely associated with the degradation of Ca^{2+} and Mg^{2+}-linked materials, suggesting that different group of cations participate in floc formation in different ways with distinct biological and physiochemical roles in activated sludge.

While various organic compounds constitute activated sludge EPS, proteins are known to be the most abundant organic matter in this extracellular matrix (Urbain et al., 1993; Frølund et al., 1996; Nielsen et al., 1996; Higgins and Novak, 1997). Consequently, better understanding of the function and fate of extracellular proteins in the activated sludge process should be critical for improving our understanding of bioflocculation and the performance of sludge digestion. We previously demonstrated the use of metaproteomic analyses comprising protein separation using SDS-PAGE and identification of selected proteins by liquid chromatography tandem mass spectrometry in studying extracellular

proteins for several full-scale activated sludges (Park et al., in preparation). This led us to conduct an investigation into the fate of activated sludge extracellular proteins in sludge digestion.

The objective of this study was to investigate the fate of activated sludge proteins in anaerobic and aerobic digestion using SDS-PAGE. A parallel study of both anaerobic and aerobic digestion was performed for a single activated sludge to compare the fate of designated extracellular proteins in different digestion environments. The extraction/purification protocol consisted of three cation-specific EPS extraction methods, ammonium sulfate precipitation, and SDS-PAGE (Park et al., 2007), which made it feasible to study different cation-bound proteins in activated sludge with respect to their indigenous molecular weights, their general hydrophobic characteristics, and finally their fate in sludge digestion.

MATERIALS AND METHODS

Sludge samples

Waste activated sludge was collected from a local wastewater treatment plant and used for extracting EPS. Two 4L reactors were used to batch digest activated sludge under both anaerobic and aerobic conditions. The detailed reactor configuration is described in Park et al. (2006). Anaerobic digestion was performed at 37°C while room temperature was used to digest sludge under aerobic conditions. Mixing was continuously provided by magnetic

stirrers for both anaerobic and aerobic digestion. To minimize the pH decrease in aerobic digestion due to nitrification, the aeration was turned on and off at every 4hr interval while the mixing was on all the time. The digestion was performed for 30 days and digested sludges were immediately subjected to EPS extraction as described below.

Three EPS extraction methods

The extraction methods used were the cation exchange resin (CER) procedure, sulfide addition, and base treatment (pH 10.5) to extract EPS bound with Ca^{2+}+Mg^{2+}, Fe, and Al, respectively. The rationale behind choosing these methods to target cations of interest is described in detail in Park and Novak (2007). All the extractions followed the same procedures as shown in Park et al. (in preparation). In brief, 0.4L of both undigested and digested sludges were centrifuged at 15,000 g for 15 minutes to produce a cake pellet. The precipitate was resuspended in 0.2 L of extraction solution and underwent one of the three extraction methods described below. When the extraction was complete, the disturbed sludge was centrifuged at 15,000 g for 15 minutes at 4°C and further filtered using 1.5 μm and 0.45 μm filters. The crude EPS extract in this study was designated as EPS present in 1.5 μm filtrate. Dowex resin (50 x 8, Na^+ form, 20-50 mesh) was used in CER extraction with dose of 60g resin/g VS in undigested activated sludge. The same mass (160g) of the resin was also applied to digested sludges so that the dose based on VS became higher for digested sludges. This approach was considered correct since the CER is to target sludge cations, whose total mass do not change in sludge digestion. The extraction buffer was a

phosphate buffer (2 mM KH_2PO_4, 6 mM Na_2HPO_4, and 10 mM NaCl) and extraction was performed for 1 hr at 600 rpm in a four-baffled extraction beaker. Following extraction, the resin beads were separated by filtering through a nylon wire mesh (250 μm). Sulfide extraction consisted of two extraction steps: an iron-reducing step and a physical extraction step. The initial pellet was resuspended in 10 mM NaCl solution, transferred to 0.5 L flask, and purged with N_2 gas for 30 minutes. The dose of sulfide was determined to achieve molar ratio of S^{2-}/Fe at 1.5 using the stock solution of $Na_2S•9H_2O$ (600 mM, pH ~7.5). Sulfide-added sludge gradually turned black with gentle mixing on a wrist action shaker over six hours (at 4°C). The sludge was then transferred to a general extraction beaker under N_2 gas flow and sheared for 15 minutes at 900 rpm. For base extraction, the sludge pellet was resuspended with 10 mM NaCl and the pH was raised to ~10.5 using 1N NaOH. The sample was then transferred to an extraction beaker and underwent a 1 hr extraction (600 rpm). Base extraction was conducted under N_2 headspace to minimize pH drop and auto-oxidation of extracted organic matter.

Ammonium sulfate ($(NH_4)_2SO_4$) precipitation

The $(NH_4)_2SO_4$ precipitation for activated sludge EPS followed the same procedure described in Park et al. (in preparation). The crude extract was primarily used for $(NH_4)_2SO_4$ precipitation but 0.45 μm filtrate was also used for some experiments. A 30 mL of extract was placed in a centrifuge tube and a designated amount of $(NH_4)_2SO_4$ was added either in one step or four sequential steps to provide $(NH_4)_2SO_4$ saturation levels at 90%

(0/90) and (0/20, 20/40, 40/60, 60/90), respectively. Each precipitation procedure was conducted on ice for more than six hours and completed by centrifuging at 20,000 g for 30 minutes. The collected precipitate was resuspended in a phosphate buffer and dialyzed extensively in the same buffer with multiple changes at 4°C. The dialysis tubing was 6~8 kDa cellulose membrane (Fisher).

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970). A 4-15% gradient gel (Bio-Rad) was used for SDS-PAGE. The sample was mixed with 4-time SDS-PAGE sample buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 2% bromophenol blue, and 20% ß-mercaptoethanol) and heated in 95°C for at least 15 minutes. Following a heating process, the sample was centrifuged at 12,000 rpm for 5 minutes to remove debris and the supernatant was used for SDS-PAGE. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue-R 250.

Chemical analysis

Protein was quantified by Frølund et al. (1996) modification of the Lowry et al. (1951) method to account for the interference of phenolic compounds and humic substances in original Lowry et al. method. In order to further minimize the influence of different extraction solution and residual sulfide on protein quantification, protein was also measured

following precipitation with 80 % (v/v) acetone and resuspension with a phosphate buffer. Bovine serum albumin was used as a standard. Metals of interest, Ca^{2+}, Mg^{2+}, Fe and Al in activated sludge were measured using EPA method 3050B (Acid digestion for metals analysis of soils, sediments, and sludges, 1996). The metal concentrations in the acid-digested samples were quantified using Atomic Absorption Spectrometer. The soluble cations (in 0.45 μm filtrate) were analyzed using a Dionex (Sunnyvale, California) ion chromatograph. Total solids (TS) and total volatile solids (VS) in activated sludge were measured according to APHA Standard Methods (1995).

RESULTS

SDS-PAGE of activated sludge EPS from undigested activated sludge

Activated sludge EPS were extracted from a full-scale activated sludge using the three extraction methods as previously shown by Park and Novak (2007). The quantities of proteins extracted by the three methods are listed in Table 1. It can be seen that both the CER procedure and base extraction resulted in a similar quantity of proteins from activated sludge prior to digestion, which was about three times higher than the amount of proteins extracted by sulfide treatment. This result tends to be interpreted as base and CER extractions are much more effective methods than sulfide treatment but the SDS-PAGE data, shown below, provided direct evidence that proteins released by the three methods are

indeed different sludge proteins, suggesting that a quantitative comparison of EPS for the three methods is not an appropriate approach.

Figure 1 shows the SDS-PAGE of CER, base and sulfide extracts in 1.5 μm and 0.45 μm filtrates. The usual sample preparation for SDS-PAGE requires clean-up and concentration methods and we used $(NH_4)_2SO_4$ precipitation with both sequential (0/20, 20/40, 40/60, 60/90) and one step (0/90) precipitation in this study. SDS-PAGE profiles shown in Figure 1 are following (0/90) $(NH_4)_2SO_4$ precipitation. Several important observations were made from the results shown in Figure 1.

First, numerous distinct protein bands were obtained, indicating that extracellular proteins present in activated sludge were well separated by SDS-PAGE. The success of SDS-PAGE for full-scale activated sludge might itself be a meaningful result since the application of SDS-PAGE into complex environmental samples such as activated sludge flocs is generally known as a difficult task. It can be further seen from Figure 1 that some proteins are abundant as shown with a strong band intensity indicating that they may play an important role in the physiology of activated sludge flocs. Identifying these strongly expressed proteins and understanding their biochemical roles in activated sludge may contribute to a better engineering application of the activated sludge process. Currently, identification of proteins from field activated sludge is a challenge (Park et al., in preparation) since the genomes of most of activated sludge microorganisms have not yet been sequenced. However, the development of metagenomic analysis and mass spec technologies may bring meaningful protein identification work in near future (Martin et al., 2006; Klaassens et al., 2007).

Second, protein profiles were different between the three extracts, indicating that extracellular proteins that were released under cation-targeted conditions were not the same. Although there were some common proteins shown in all three extracts, their band intensities were clearly different between the three methods, indicating that some proteins were more favorably extracted under a certain extraction condition. Good examples of this are seen in the protein bands indicated with Arabic numerals, from 1 to 8. While bands of 1 to 3 were mainly observed in CER extracts, the ~13 kDa protein (indicated by bands 6 and 8) was mainly found in base and sulfide extracts. On the other hand, bands 4 and 5 were strongly expressed in base extracts while band 7 was the unique sulfide-extracted protein. As described by Park et al (in preparation), the difference of protein profiles between the three methods was even more clearly seen when sequential $(NH_4)_2SO_4$ precipitation was used. Figures 2A, 3A, and 4A, respectively, show the SDS-PAGE of CER, sulfide, and base extracts that underwent sequential $(NH_4)_2SO_4$ precipitation. The sequential $(NH_4)_2SO_4$ approach also revealed a general hydrophobic property of extracellular proteins in activated sludge flocs. Four different lanes (Lane 1 to 4) in these figures are designated for different ammonium sulfate saturations resulting in the separation of proteins with different hydrophobicity. Proteins in Lane 1 are the most hydrophobic while proteins in Lane 4 are the least hydrophobic (Park et al., in preparation).

Third, there was not a significant difference between protein profiles in 1.5 μm and 0.45 μm filtrates of the three extracts. Figure 1 shows that both EPS passing 1.5 μm and 0.45 μm filters led to almost identical SDS-PAGE results. This result was somewhat unexpected since preparing 0.45 μm filtrate by passing raw extracts through 0.45 μm filter

was significantly more difficult than preparing with 1.5 μm filter. The 0.45 μm protein values for base and CER extracts accounted for about 80% of their 1.5 μm extracts while 90% for the sulfide extract (Table 1). Although limited, this observation may become useful for better understanding polymerization of EPS in activated sludge flocs and the size distribution of EPS in the native state.

Extraction of EPS and their SDS-PAGE following batch anaerobic and aerobic digestion

The quantities of proteins extracted by the three methods post anaerobic and aerobic digestion are also shown in Table 1. Nitrification can decrease the pH of sludge during aerobic digestion and therefore in order to minimize a significant pH drop in aerobic digestion, the air was turned on and off every 4hr while mixing was provided all the time. The final pH of aerobically digested sludge was found to be 5.4 while the pH of anaerobic sludge was 7.1. The %VS reductions achieved by 30 day anaerobic digestion and aerobic digestion were 39% and 48%, respectively.

CER extraction. The overall digestion and extraction data were consistent with the previous report by Park and Novak (2007) who used a different source of activated sludge for both anaerobic and aerobic digestion and the three extraction methods. As shown in Table 1, the crude CER-extractable proteins was reduced by 19% in anaerobic digestion but underwent significant decrease in aerobic digestion leading to a decrease of CER-

extractable proteins by 61%. A large concentration of Ca^{2+} and Mg^{2+} were found in the solution phase of sludge following aerobic digestion (Table 2) indicating the degradation of organic matter bound with these cations under aerobic conditions. However, this was not the case in anaerobic digestion. Collectively, both EPS and divalent cation data suggested that divalent cation-bound EPS were not degraded in anaerobic digestion. The results obtained from the analysis of SDS-PAGE further strengthened this finding. The SDS-PAGE of CER-extracts following anaerobic and aerobic digestion is shown in Figure 2B and 2C, respectively. The resultant SDS-PAGE clearly demonstrated that most of CER-extracted proteins remained intact in sludge flocs even after 30 day anaerobic digestion while a significant fraction of the same proteins underwent degradation by aerobic digestion. Some of the most distinct protein bands from anaerobically digested sludge were band c1 and a group of proteins in Lane 3 and Lane 4. It seems obvious from this result that these sludge proteins were not available for biodegradation in anaerobic digestion. Figure 2 also shows that there was a new protein band that emerged after anaerobic digestion. The band c2 was a unique CER-extracted protein from anaerobically digested sludge. Furthermore, this protein band was not detected at all in two other extracts, indicating that this protein is possibly associated with anaerobic metabolism and tightly bound with divalent cations. The degradation of CER-extracted proteins in aerobic digestion but not in anaerobic digestion indicates that these materials are indeed “biodegradable” but “not bioavailable” in anaerobic digestion. This further raised a question why the divalent cation-bound EPS were not available in anaerobic digestion. One possible assumption is that these extracellular materials are tightly bound with divalent cations, possibly involved in cell

agglutination (forming cell colonies) and normal anaerobic digestion does not have an efficient mechanism that induces the dissociation of this structure. In aerobic digestion, on the other hand, the reduction of pH and/or over-expression of several proteolytic enzymes in microorganisms might impact this structure, rendering the associated EPS bioavailable. Further research is planned to verify this hypothesis.

Sulfide extraction. The quantitative difference for sulfide-extracted proteins for anaerobic and aerobic digestion was not as clear as for CER-extracted proteins. About 6% of crude sulfide-extracted proteins were removed by anaerobic digestion while 16% of proteins were consumed by aerobic digestion. The difficulty in conducting a quantitative analysis on sulfide-EPS before and after digestion was already observed in the earlier study (Park and Novak, 2007). It was also observed in the same study that sulfide extraction for activated sludge was more efficient following disturbance or breakup of the sludge, which indicated the influence of sludge strength or shearing condition on sulfide extraction. Consequently, considering the difference in floc strength of undigested and digested sludges, the comparison of sulfide-extractable EPS before and after digestion on a quantitative basis may not provide useful information. Nevertheless, the fate of sulfide-extracted proteins in anaerobic and aerobic digestion was examined by comparing the changes in SDS-PAGE before and after digestion. The comparison of Figure 3A and 3B shows the changes in sulfide-proteins in anaerobic digestion. It can be seen from these figures that the major sulfide-extracted protein, s1, was partially degraded in anaerobic digestion. Moreover, most of proteins present in Lane 3 and Lane 4 from undigested sludge (Figure 3A) were not

detected after anaerobic digestion (Figure 3B). These results suggest that much of organic matter that can be extracted by addition of sulfide (organic matter bound with Fe^{3+} and Fe^{2+} in sludge) was bioavailable and degraded during anaerobic digestion. This observation is in accordance with Park et al. (2006) who showed that the Fe concentration was the primary factor determining VS reduction efficiency in eight different waste activated sludges. It appears that under anaerobic conditions, Fe-associated material is made bioavailable, likely by reduction of Fe(III) to Fe(II), thereby releasing organic matter into solution where they are then degraded.

In contrast to SDS-PAGE from anaerobically digested sludge, there were not identifiable protein bands following aerobic digestion. Rather, strong smears over a low molecular weight region comprised sulfide-extracted proteins from aerobically digested sludge. Although the chance of degradation of sulfide-extracted proteins in aerobic digestion should not be ruled out, the data also need to be carefully interpreted since aerobic digestion in this study contained both air on/off conditions. Furthermore, the change in proteins during sample preparation might also account for this unusual protein pattern. It was observed that concentrating and heating processes for sulfide- and base-extracted EPS from aerobically digested sludge led to formation of some gel-like materials that might have possibly affected the performance of SDS-PAGE. Further study is therefore needed to correctly verify the fate of sulfide-extractable proteins in aerobic digestion.

Base extraction. The reduction of base-extractable proteins in aerobic and anaerobic digestion was similar so that 21% and 25% reduction of base-extracted proteins was achieved by aerobic and anaerobic digestion, respectively. The series of SDS-PAGE for base-extracts before and after sludge digestion are shown in Figure 4. For anaerobically digested sludge, band b1, b2, b3, and b4 were distinctly expressed after anaerobic digestion. While band b1 was also best extracted from undigested activated sludge under basic conditions, bands 2 to 4 became quite extractable following anaerobic digestion. This result might be associated with the change in floc properties (e.g., floc morphology) that occurred in anaerobic digestion so that base conditions could efficiently reach and release these materials. Another possible explanation is that aluminum in sludge has collected organic materials that became loose from floc during anaerobic digestion, possibly from cell lysis. As a result, these newly collected organics can then be extracted by base.

Some identifiable proteins at ~15 kDa and ~27 kDa were observed from aerobically digested sludge but the majority of base-extracted proteins also appeared as smears, similar to the sulfide-extractable proteins. Consequently, the fate of base-extractable proteins in aerobic digestion could not be clearly resolved.

DISCUSSION

Along with the increase in public's concerns on land application of sludge, regulations and costs pertaining to biosolids handling processes expect to be more stringent in future, making proper sludge digestion of great importance. A higher reduction of VS in sludge

digestion is always desirable in this aspect since it will reduce the mass of sludge that needs to be handled and reduce the potential for odor generation and vector attraction that are directly associated with public health and aesthetic issues. Various physical and chemical pretreatment processes have been suggested to enhance sludge digestion and various digestion models with different reactor configurations have also been developed to achieve producing a better class of biosolids. However, the success of those technologies and models has not been always guaranteed, indicating that the performance of digestion is quite sludge-dependent.

The current investigation was undertaken as a part of research for studying floc composition in activated sludge using SDS-PAGE and three cation-specific extraction methods. The results found in this study showed that floc materials that degrade in one digestion environment are limited to a subset of EPS materials. These results are in a good agreement with a previous finding that floc materials that degrade in anaerobic and aerobic conditions are not the same materials. Park et al. (2006) showed that VS reduction in anaerobic digestion was primarily determined by Fe concentration in sludge while the aerobic digestion was associated with the solubilization of Ca^{2+} and Mg^{2+} along with the increase in soluble nitrogen. The SDS-PAGE data obtained in this study provided strong evidence that the degradation of CER-extracted proteins, Ca^{2+} and Mg^{2+}-bound proteins, was minimal in anaerobic digestion but substantial in aerobic digestion. An explanation of why this EPS pool is not bioavailable in normal mesophilic anaerobic digestion is not known but this observation might also be related to the survival of microbial colonies in feed sludge through anaerobic digestion. Higgins et al. (2007) demonstrated that a

significant portion of pathogen-indicating organisms in feed sludge remained viable through anaerobic digestion, supported by a quantitative polymerase chain reaction method, although they were not culturable by standard culturing methods. Since divalent cations and many adhesive biopolymers such as lectins, fimbriae, and pili that show high affinity for divalent cations have been shown to be directly associated with cell agglutination and microbial colonization (Touhami et al., 2003; Imberty et al., 2004), this divalent cation-bound EPS might also help survive them in anaerobic digestion. In terms of their removal in anaerobic digestion, placing an aerobic reactor following anaerobic digester might provide a condition that is able to dissociate this organic pool, leading to further VS reduction. Also, since divalent cations are fairly extractable under weak acidic conditions (Kakii et al., 1985), a digestion model that employs a fluctuation in pH, such as an acid/gas system, might also be able to target this organic pool. The approach using a combination of SDS-PAGE and the three extraction methods for different digestion schemes is planned for future research.

In contrast to divalent cation-bound proteins, Fe-bound proteins were shown to be degraded in anaerobic digestion. However, there were still materials remaining, which could be extracted by the addition of sulfide, indicating that the complete Fe-associated organic pool was not degraded. Nevertheless, the result suggests that the analysis of concentration of Fe in the feed sludge might be useful in predicting the sludge digestibility by anaerobic digestion. The fate of base-extractable proteins, presumably including Al-bound EPS, appeared to be complex in anaerobic digestion. Base treatment of feed sludge is one of the methods often used in the enhanced sludge digestion (Chiu et al., 1997).

Consequently, knowing the cation levels in feed sludge and the analysis of SDS-PAGE following various treatments before and after digestion might provide useful information that may help choose a proper treatment method and operation for the designated sludge.

Finally, the success of SDS-PAGE in isolating proteins from full-scale activated sludge and following digestion under anaerobic and aerobic conditions shows promises for its use in monitoring the fate of proteins in various stages of activated sludge process and other biological wastewater treatment processes. A knowledge of the biochemical function and fate of system proteins may lead to scientifically sound engineering application of biological processes in wastewater treatment.

CONCLUSION

Based on the data collected in the study, the specific following conclusions are drawn:

- The extraction methods targeting specific cation-associated materials extract distinct proteins
- CER-extractable proteins, or divalent cation-bound proteins, are mainly degraded in aerobic digestion but not appreciably in typical mesophilic anaerobic digestion
- Sulfide-extractable proteins, or iron-associated proteins, are degraded by anaerobic digestion.
- The fate of base-extractable proteins in anaerobic digestion appeared to be complex. The collection of organics by aluminum and its solubilization during base extraction

might partly account for this.

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Table 5-1. Summary of extracellular proteins extracted from undigested activated sludge, anaerobically digested sludge, and aerobically digested sludge (Unit of proteins: mg/g VS)

Extraction methods	Size	Undigested sludge	Anaerobically digested sludge	Aerobically digested sludge
CER	1.5 μm	74.0	59.8 (19%)	29.0 (61%)
	0.45 μm	59.5	36.3 (39%)	18.0 (70%)
Base	1.5 μm	68.7	51.6 (25%)	54.7 (21%)
	0.45 μm	55.2	34.2 (38%)	48.5 (12%)
Sulfide	1.5 μm	22.2	20.8 (6%)	18.8 (15%)
	0.45 μm	20.3	13.5 (33%)	17.0 (16%)

* Values in parenthesis indicate the percentile reduction by digestion

Table 5-2. Changes in solids, solution cations, and solution proteins by 30 day batch anaerobic and aerobic digestion.

Sludge	TS (g/L)	VS (g/L)	Na^+ (mg/L)	K^+ (mg/L)	NH_4^+ (mg/L)	Mg^{2+} (mg/L)	Ca^{2+} (mg/L)	protein (mg/L)
Before	8.38	6.74	73.8	21.9	-	25.2	57.0	21.0
Anaerobic	5.75	4.09	87.0	71.6	260	18.3	29.4	182
Aerobic	5.12	3.49	72.1	69.5	33.6	45.2	166	29.0

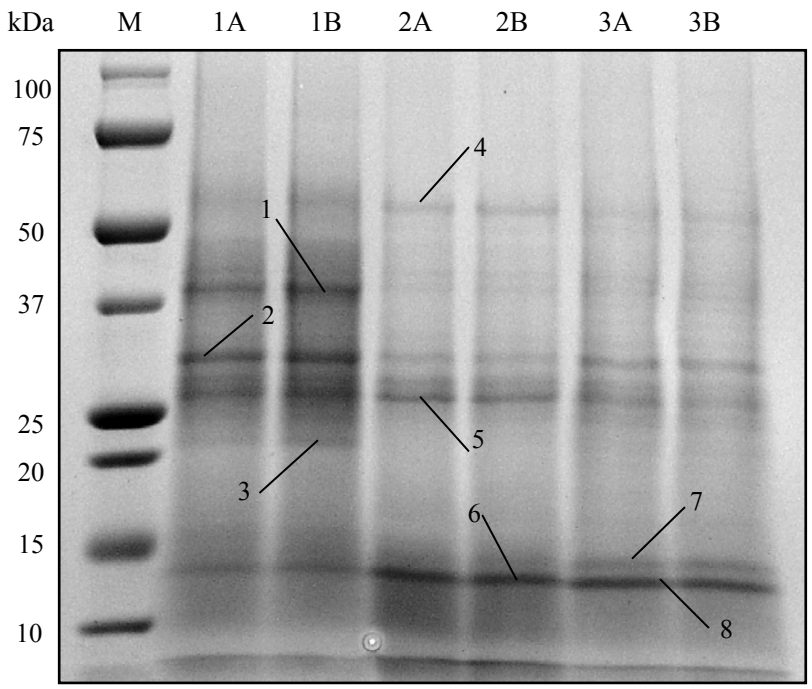

Figure 5-1. SDS-PAGE of activated sludge extracellular proteins in 1.5 μm and 0.45μm filtrates. Each extract was treated with 90% $(NH_4)_2SO_4$ precipitation. Lane M: molecular weight markers; Lane 1A: 1.5 μm filtrate of the CER extract, Lane 1B: 0.45 μm filtrate of the CER extract; Lane 2A: 1.5 μm filtrate of the base extract; Lane 2B: 0.45 μm filtrate of the base extract; Lane 3A: 1.5 μm filtrate of the sulfide extract; Lane 3B: 0.45 μm filtrate of the sulfide extract. 50 μg of proteins were loaded in each lane.

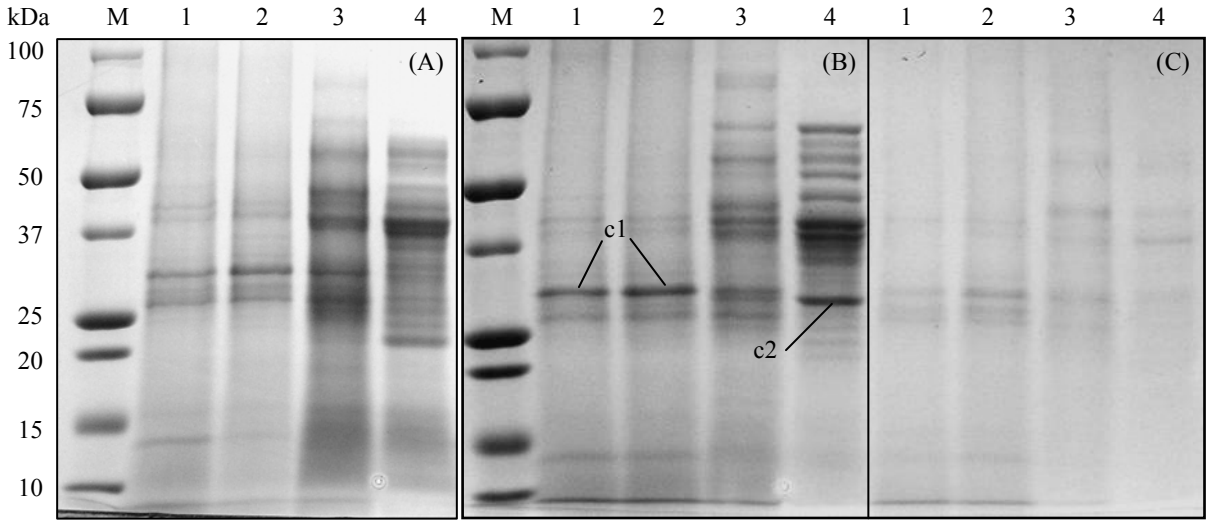

Figure 5-2. SDS-PAGE of CER-extracted proteins from (A) undigested activated sludge, (B) anaerobically digested sludge, and (C) aerobically digested sludge. Lane M: molecular weight markers, Lane 1: proteins separated with 20 % $(NH_4)_2SO_4$ saturation, Lane 2: 20 ~ 40 %, Lane 3: 40 ~ 60 %, and Lane 4: 60 ~ 90 %. For undigested activated sludge, 40 µg of proteins were loaded onto Lanes 1 to 3 and 20 µg onto Lane 4. For anaerobically digested sludge, 33 µg, 32 µg, 25 µg, and 20 µg of proteins were loaded onto Lane 1 to 4, respectively. For aerobically digested sludge, 14 µg, 19 µg, 11 µg, and 5 µg of proteins were loaded onto Lane 1 to 4, respectively. The mass of proteins loaded after digestion was determined based on % reduction of proteins in each $(NH_4)_2SO_4$ fraction before and after digestion.

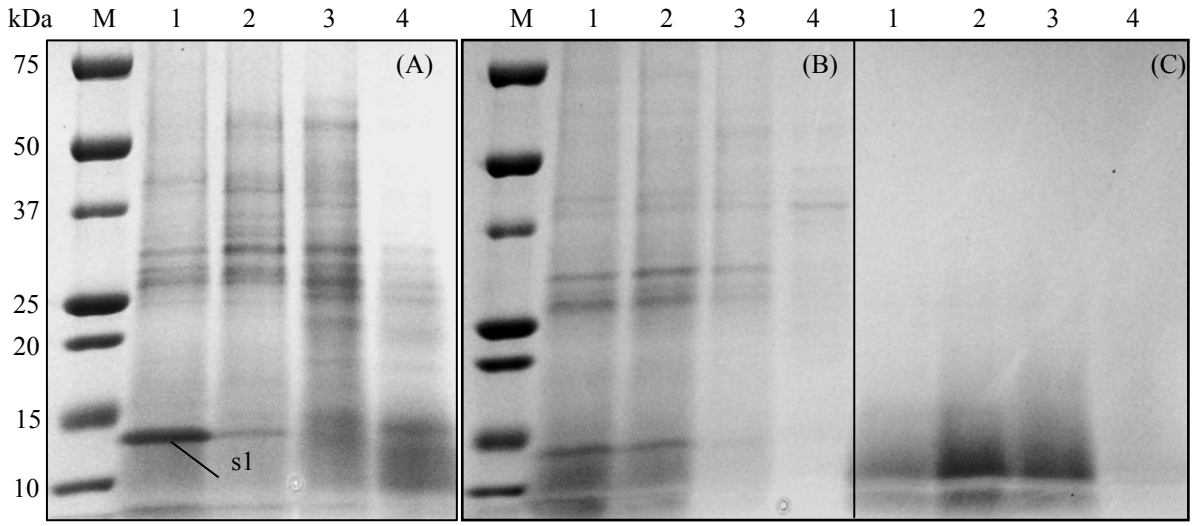

Figure 5-3. SDS-PAGE of sulfide-extracted proteins from (A) undigested activated sludge, (B) anaerobically digested sludge, and (C) aerobically digested sludge. Lane M: molecular weight markers, Lane 1: proteins separated with 20 % $(NH_4)_2SO_4$ saturation, Lane 2: 20 ~ 40 %, Lane 3: 40 ~ 60 %, and Lane 4: 60 ~ 90 %. For undigested activated sludge, 40 µg of proteins were loaded onto Lanes 1 to 3 and 20 µg onto Lane 4. For anaerobically digested sludge, 40 µg, 39 µg, 20 µg, and 8 µg of proteins were loaded onto Lane 1 to 4, respectively. For aerobically digested sludge, 28 µg, 40 µg, 37 µg, and 17 µg of proteins were loaded onto Lane 1 to 4, respectively. The mass of proteins loaded after digestion was determined based on % reduction of proteins in each $(NH_4)_2SO_4$ fraction before and after digestion.

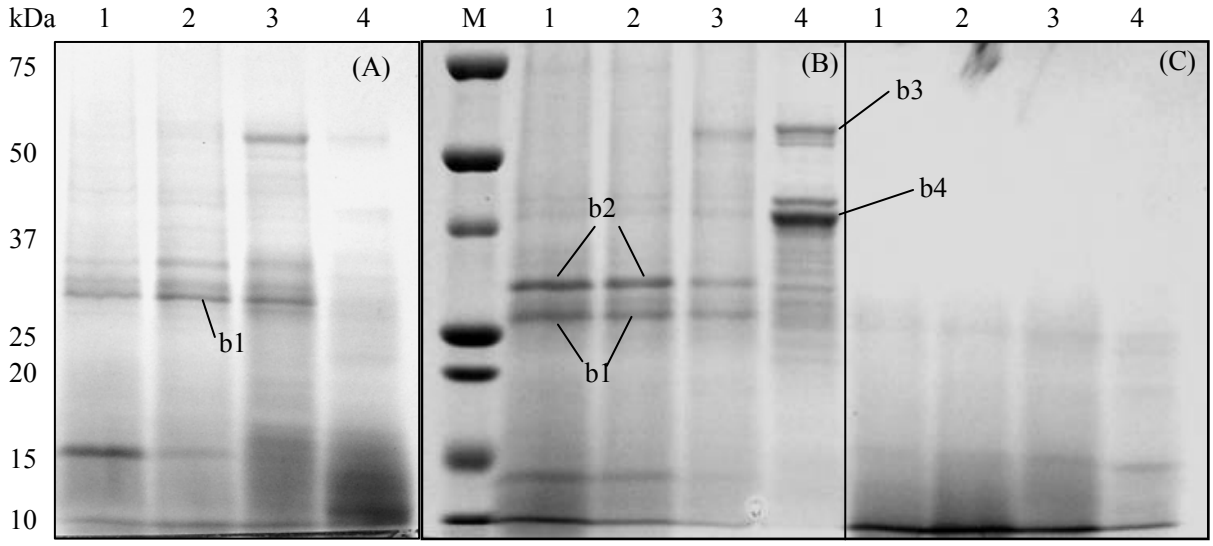

Figure 5-4. SDS-PAGE of base-extracted proteins from (A) undigested activated sludge, (B) anaerobically digested sludge, and (C) aerobically digested sludge. Lane M: molecular weight markers, Lane 1: proteins separated with 20 % $(NH_4)_2SO_4$ saturation, Lane 2: 20 ~ 40 %, Lane 3: 40 ~ 60 %, and Lane 4: 60 ~ 90 %. For undigested activated sludge, 40 µg of proteins were loaded onto Lanes 1 to 3 and 20 µg onto Lane 4. For anaerobically digested sludge, 40 µg, 32 µg, 25 µg, and 17 µg of proteins were loaded onto Lane 1 to 4, respectively. For aerobically digested sludge, 37 µg, 39 µg, 35 µg, and 20 µg of proteins were loaded onto Lane 1 to 4, respectively. The mass of proteins loaded after digestion was determined based on % reduction of proteins in each $(NH_4)_2SO_4$ fraction before and after digestion.

Chapter 6. Engineering Significance

Extracellular polymeric substances (EPS) have significant impacts on all treatment processes employed in the activated sludge process. First, EPS are essential components that are required for aggregating microorganisms, accounting for the flocculent nature of activated sludge. Since the success of the activated sludge process depends on the effectiveness of bioflocculation and subsequent solid/liquid separation, the role of EPS in floc formation in activated sludge is clearly important. In addition, since EPS comprise a large organic fraction in activated sludge, a better understanding of EPS is also expected to lead to better operation and control of sludge digestion and post digestion processes such as sludge conditioning, dewatering, and final disposal. Currently, it is largely unknown why some sludges digest better than others; why certain sludges are more odorous than others; which disintegration technology works best for enhancing sludge digestion, which dewatering method and polymer are best to use. All these questions might be potentially answered by identifying the interaction and fate of EPS in each process.

In studying EPS in activated sludge, this research developed a useful extraction strategy to characterize extracellular polymeric substances (EPS) in activated sludge flocs and used, for the first time, metaproteomic analyses to investigate extracellular proteomes of full-scale activated sludge. A particular outcome of this research was the successful application of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to full-scale activated sludge. Its further success in isolating proteins from various digested sludges shows promises for its use in monitoring the fate of proteins in various stages of the

activated sludge process and other biological wastewater treatment processes. Furthermore, it has potential applications for monitoring metabolic footprints (such as gene and protein expression) of microbial community to certain events (e.g., toxicity) in biological wastewater treatment processes. Both the developed methods and findings from this research are thought to be of significance to the current wastewater engineering community and contribute to developing scientifically sound engineering application of biological processes in wastewater treatment. Several important findings that can be considered for direct engineering implication are described below.

This research supported previous findings from Novak et al. (2003) and Park et al. (2006 a,b) that Al, Fe, and divalent cations participate in floc formation in different ways by binding different EPS to flocs. Hence, cation content in influent sewage has a significant influence on activated sludge characteristics and sludge digestibility. Since a considerably different cation content is very common for different wastewaters, it was concluded that the variability in cations is one important factor that leads to different characteristics of activated sludge (effluent quality and sludge digestibility) across facilities. Consequently, the analysis of metal content in influent sewage and activated sludge, which is a fairly simple laboratory analysis, should reveal important information about the properties of sludge being generated. Also, wastewater treatment plants (WWTPs) may practice adding certain cations to adjust for an imbalance in cations. In this aspect, the cation content and concentrations suggested by Higgins and Novak (1997 a,b) and Park et al. (2006a) may be good examples for WWTPs to target.

The approach using a combination of the three extraction methods and SDS-PAGE before and after sludge digestion led to important, scientific findings that parts of EPS in activated sludge, particularly that are bound with divalent cations, do not degrade in a typical mesophilic anaerobic digestion. Since higher volatile solids (VS) reduction is always desirable with respect to final solids handling processes, further VS reduction by destroying this divalent cation-associated EPS should be beneficial in a WWTP. For the removal of this organic pool, aerobic digester following anaerobic digestion might provide a condition that is able to dissociate and degrade divalent cation-associated organics. Also, since divalent cations are fairly easily extractable under weak acidic conditions (Kakii et al., 1985), a digestion model that employs a fluctuation in pH, such as an acid/gas system, can be also considered for destroying this organic pool. Continuing from the report of Park et al. (2006b), the degradation of Fe-bound proteins in anaerobic digestion was further proved by SDS-PAGE in this research. Consequently, knowing the Fe level in feed sludge may be useful for predicting anaerobic digestion efficiency. With respect to the enhanced sludge digestion, the cation analysis of feed sludge and SDS-PAGE following various treatments (e.g., mechanical shearing, sonication, ozone treatment, base treatment, and thermal treatment) before and after digestion might provide useful information that may help choose a proper treatment method and operation for the designated sludge.

Finally, it was found in this research that activated sludge microorganisms use lectin-mediated aggregation as one of the mechanisms for bioflocculation. Extensive literature is available showing that these particular adhesive biopolymers are mainly produced in later growth stages (late log phase and stationary state) or low nutrient medium conditions

(Sharon et al., 1981; Heeb et al., 1982; Vesper, 1987; Touhami et al., 2003), indicating that expression of these biopolymers is related to cell physiology. The substrate and nutrient availability can be dictated by influent sewage characteristics but also significantly influenced by a feeding pattern in a WWTP. As a consequence, a change in reactor configuration (such as utilization of selectors or baffles) or varying the feeding pattern may alter physiological characteristics of activated sludge microorganisms and hence expression of these biopolymers. It was observed in this research that specific lectin activity increased after both anaerobic and aerobic digestion, suggesting that these biopolymers are extremely stable and resistant to biological attack. Consequently, it will be important to determine if sludges with a low quantity of lectin-like biopolymers are beneficial for sludge digestion (i.e., higher VS reduction) with acceptable effluent quality.

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Appendix A (Chapter 3)

Summary of solids, metals, and EPS from activated sludges used in this study

	TS	VS	Fe	Al	Protein (mg/L)			Polysaccharide (mg/L)		
Plant	(g/L)	(g/L)	(mg/g TS)	(mg/g TS)	Sulfide	CER	Base	Sulfide	CER	Base
A	6.4	5.0	3.1	1.7	58	247	205	14	117	80
B1	14.4	10.7	16.7	5.3	202	549	-	54	223	-
B2	13.8	10.8	10.1	8.1	147	699	438	30	275	104
B3	10.4	8.0	8.0	5.3	124	586	379	30	208	91
B4	11.2	8.7	9.8	7.8	108	356	368	25	185	144
C	6.9	5.5	7.6	10.8	87	200	170	25	71	62
D1	37.9	28.1	43.9	3.4	955	1152	1397	273	599	430
D2	36.5	28.1	47.7	4.9	1043	1649	1205	360	821	503

	Proteins (mg/g VS)			Polysaccharides (mg/g VS)			Protein/polysaccharide		
Plant	Sulfide	CER	Base	Sulfide	CER	Base	Sulfide	CER	Base
A	11.6	49.1	40.7	2.7	23.2	15.9	4.2	2.1	2.6
B1	18.9	51.3	-	5	20.8	-	3.8	2.5	-
B2	13.6	64.5	40.4	2.8	25.4	9.6	4.9	2.5	4.2
B3	15.5	73.4	47.4	3.7	26.1	11.4	4.2	2.8	4.2
B4	12.4	41	42.4	2.9	21.3	16.6	4.3	1.9	2.6
C	16	36.7	31.1	4.6	13	11.4	3.5	2.8	2.7
D1	34	41	49.7	9.7	21.3	15.3	3.5	1.9	3.2
D2	37.1	58.7	42.9	12.8	29.2	17.9	2.9	2.0	2.4

Statistical analysis using paired t-test on Protein/Polysaccharide

	Sulfide	*CER*
Mean	3.9	2.3
Variance	0.4	0.1
Observations	8	8
Pearson Correlation	0.3	
Hypothesized Mean Difference	0	
df	7	
t Stat	7.13704	
P(T<=t) one-tail	0.00009	
t Critical one-tail	2.99795	
P(T<=t) two-tail	0.00019	
t Critical two-tail	3.49948	

Statistical analysis using paired t-test on Protein/Polysaccharide

	CER	*Base*
Mean	2.3	3.1
Variance	0.2	0.6
Observations	7	7
Pearson Correlation	0.6	
Hypothesized Mean Difference	0	
df	6	
t Stat	-3.43058	
P(T<=t) one-tail	0.00698	
t Critical one-tail	3.14267	
P(T<=t) two-tail	0.01396	
t Critical two-tail	3.70743	

Analysis without using B1 data set

Statistical analysis using paired t-test on Protein/Polysaccharide

	Sulfide	*Base*
Mean	3.9	3.1
Variance	0.4	0.6
Observations	7	7
Pearson Correlation	0.6	
Hypothesized Mean Difference	0	
df	6	
t Stat	3.26685	
P(T<=t) one-tail	0.00855	
t Critical one-tail	3.14267	
P(T<=t) two-tail	0.01710	
t Critical two-tail	3.70743	

Analysis without using B1 data set

Data associated with Figure 1

Changes in cations by the CER procedure

Plant A

	TS (%)	Ca (mg/L)	Mg (mg/L)	Fe (mg/L)	Al (mg/L)	Ca (mg/gTS)	Mg (mg/gTS)	Fe (mg/gTS)	Al (mg/gTS)
Before[1]	1.007	71.6	42.0	67.9	26.0	7.1	4.2	6.7	2.6
After[2]	0.958	8.7	13.8	63.9	24.2	0.9	1.4	6.7	2.5
Centrate[3]	-	1.4	0.7	6.1	2.2	-	-	-	-

Plant B

	TS (%)	Ca (mg/L)	Mg (mg/L)	Fe (mg/L)	Al (mg/L)	Ca (mg/gTS)	Mg (mg/gTS)	Fe (mg/gTS)	Al (mg/gTS)
Before[1]	1.165	157.3	31.4	116.0	89.0	13.5	2.7	10.0	7.6
After[2]	1.052	34.1	13.5	103.3	82.4	3.2	1.3	9.8	7.8
Centrate[3]	-	2.6	0.5	10.2	4.6	-	-	-	-

Plant E

	TS (%)	Ca (mg/L)	Mg (mg/L)	Fe (mg/L)	Al (mg/L)	Ca (mg/gTS)	Mg (mg/gTS)	Fe (mg/gTS)	Al (mg/gTS)
Before[1]	0.940	45.2	23.4	35.1	10.4	4.8	2.5	3.7	1.1
After[2]	0.890	5.5	8.3	31.3	10.5	0.6	0.9	3.5	1.2
Centrate[3]	-	0.5	0.2	3.0	0.8	-	-	-	-

Activated sludges were collected from three different wastewater treatment plants for testing changes in cations during CER extraction.

1) Total solids and cations in sludge before CER extraction
2) Total solids and cations in sludge after CER extraction
3) Total cations found in the centrate of the CER-extracted sludge (12,000g x 15 min)

Data associated with Figure 2

Cations found in crude extracts following the three extraction methods (activated sludge B4)

In	Ca (mg/L)	Mg (mg/L)	Fe (mg/L)	Al (mg/L)	protein (mg/L)	poly-saccharide (mg/L)
Whole sludge before extraction	157.3	31.4	116.0	89.0	-	-
CER-extract	2.6	0.5	10.2	4.6	356	185
Base-extract	13.4	3.8	10.1	13.4	368	144
Sulfide-extract	5.2	1.9	0.6	0.0	108	25

Cations and EPS released by sonication from the same activate sludge

In	Ca (mg/L)	Mg (mg/L)	Fe (mg/L)	Al (mg/L)	protein (mg/L)	poly-saccharide (mg/L)
Sonication-extract	18.7	2.3	5.5	2.4	257	98

Data associated with Figure 3

Sulfide-extracted protein vs. Fe

Regression statistics	
Multiple R	0.987554
R square	0.975262
Adjustste R square	0.971139
Standard Error	1.6928
Observation	8

Analysis of Variance					
	DF	SS	MS	F	P
Regression	1	677.8548	677.8548	236.5439	<0.0001
Residual	6	17.194	2.8657		
Total	7	695.0488	99.2927		

	Coefficient	standard Error	t stat	P-value
y0	9.4916	0.9028	10.5132	<0.0001
a	0.5662	0.0368	15.38	<0.0001

Sulfide-extracted polysaccharide vs. Fe

Regression statistics	
Multiple R	0.966574
R square	0.934266
Adjustste R square	0.92331
Standard Error	1.0315
Observation	8

Analysis of Variance					
	DF	SS	MS	F	P
Regression	1	90.7312	90.7312	85.2766	<0.0001
Residual	6	6.3838	1.064		
Total	7	97.115	13.8736		

	Coefficient	standard Error	t stat	P-value
y0	1.7216	0.5501	3.1295	0.0203
a	0.2072	0.0224	9.2345	<0.0001

Sulfide-extracted EPS (protein+polysaccharide) vs. Fe

Regression statistics	
Multiple R	0.984358
R square	0.968961
Adjustste R square	0.963788
Standard Error	2.5983
Observation	8

Analysis of Variance					
	DF	SS	MS	F	P
Regression	1	1264.58	1264.58	187.3062	<0.0001
Residual	6	40.5084	6.7514		
Total	7	1305.089	186.4413		

	Coefficient	standard Error	t stat	P-value
y0	11.2132	1.3858	8.0917	0.0002
a	0.7734	0.0565	13.686	<0.0001

CER-extracted EPS (protein+polysaccharide) vs. Fe

Regression statistics	
Multiple R	0.051543
R square	0.002657
Adjustste R square	0
Standard Error	18.0731
Observation	8

Analysis of Variance					
	DF	SS	MS	F	P
Regression	1	5.2205	5.2205	0.016	0.9035
Residual	6	1959.82	326.6366		
Total	7	1965.04	280.72		

	Coefficient	standard Error	t stat	P-value
y0	73.5877	9.6388	7.6345	0.0003
a	0.0497	0.3931	0.1264	0.9035

Base-extracted EPS (protein+polysaccharide) vs. Fe

Regression statistics	
Multiple R	0.592508
R square	0.351066
Adjustste R square	0.221279
Standard Error of Estimate = 6.6372	18.0731
Observation	8

Analysis of Variance					
	DF	SS	MS	F	P
Regression	1	119.1589	119.1589	2.7049	0.161
Residual	5	220.2611	44.0522		
Total	6	339.42	56.57		

	Coefficient	standard Error	t stat	P-value
y0	51.6817	3.6756	14.0608	<0.0001
a	0.2376	0.1445	1.6447	0.161

Data associated with Figure 4

Analysis of amino acids in EPS from three different sludges

Amino acid	Sludge A1			Sludge B2			Sludge B3		
(mg/L)	CER	Base	Sulfide	CER	Base	Sulfide	CER	Base	Sulfide
Aspartic Acid	58	92	10	116	63	11	101	53	9
Glutamic Acid	59	98	12	128	63	8	102	53	8
Cysteine	6	12	3	13	9	3	12	6	2
Lysine	15	25	3	42	28	3	32	14	2
Threonine	30	46	5	71	34	6	49	25	4
Arginine	18	30	2	46	19	3	35	15	2
Serine	22	36	3	48	25	4	28	16	3
Tyrosine	11	31	3	27	21	4	24	16	3
Histidine	9	16	2	20	11	2	14	7	1
Proline	21	35	3	49	25	4	41	20	3
Glycine	31	46	7	71	35	7	65	28	5
Alanine	35	56	7	92	42	8	71	33	5
Valine	33	54	6	78	37	7	66	31	5
Methionine	7	12	1	20	9	1	17	9	1
Isoleucine	27	45	5	58	29	5	47	23	4
Leucine	39	66	6	92	44	8	73	36	5
Phenylalanine	22	39	3	55	28	5	41	22	3
Ornithine	1	8	1	3	6	1	3	3	0
Taurine	0	1	1	1	1	0	1	0	0
Hydroxyproline	0	0	0	0	0	0	0	0	0
Lanthionine	1	1	0	3	1	0	0	0	0
Hydroxylysine	0	0	0	0	0	0	0	0	0
Sum	446	749	84	1031	528	88	820	411	66

Analysis of amino acids in EPS from three different sludges (Compositional)

Amino acid Composition	Sludge A1			Sludge B2			Sludge B3		
(%)	CER	Base	Sulfide	CER	Base	Sulfide	CER	Base	Sulfide
Aspartic Acid	13.1	12.3	12.1	11.3	11.8	12.3	12.3	12.9	13.5
Glutamic Acid	13.2	13.0	14.3	12.4	11.9	9.6	12.4	12.9	12.1
Cysteine	1.3	1.6	3.2	1.3	1.7	3.8	1.4	1.5	2.7
Lysine	3.4	3.3	3.8	4.0	5.3	3.2	3.9	3.5	3.4
Threonine	6.7	6.1	6.1	6.8	6.5	7.1	6.0	6.0	6.5
Arginine	4.1	4.0	2.8	4.5	3.6	2.9	4.2	3.7	2.7
Serine	5.0	4.8	4.1	4.6	4.8	4.3	3.4	3.8	4.3
Tyrosine	2.5	4.2	4.0	2.6	4.1	4.3	2.9	3.9	4.5
Histidine	2.1	2.1	1.9	1.9	2.0	1.8	1.8	1.8	1.8
Proline	4.6	4.7	3.6	4.8	4.8	4.1	5.0	4.8	4.6
Glycine	7.0	6.2	8.1	6.9	6.5	7.5	7.9	6.9	7.5
Alanine	8.0	7.5	8.1	8.9	7.9	8.7	8.7	8.1	7.8
Valine	7.5	7.2	7.5	7.6	7.1	7.5	8.1	7.6	7.6
Methionine	1.5	1.7	1.0	1.9	1.7	1.5	2.0	2.1	1.6
Isoleucine	6.0	6.0	6.1	5.6	5.5	5.9	5.8	5.7	5.6
Leucine	8.8	8.8	7.0	8.9	8.3	8.9	8.9	8.7	7.8
Phenylalanine	5.0	5.2	3.5	5.4	5.3	5.4	5.1	5.3	5.2
Ornithine	0.3	1.1	1.2	0.3	1.1	0.9	0.3	0.8	0.4
Taurine	0.1	0.1	1.2	0.1	0.1	0.1	0.1	0.1	0.2
Hydroxyproline	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lanthionine	0.1	0.1	0.3	0.3	0.1	0.3	0.0	0.0	0.0
Hydroxylysine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sum	100	100	100	100	100	100	100	100	100

Ratio of base and sulfide-extracted amino acid composition to CER-extracted amino acid composition.

Amino acid Compositional Ratio	Sludge A1		Sludge B2		Sludge B3	
	Base	Sulfide	Base	Sulfide	Base	Sulfide
Aspartic Acid	0.9	0.9	1.1	1.1	1.1	1.1
Glutamic Acid	1.0	1.1	1.0	0.8	1.0	1.0
Cysteine	1.2	2.5	1.3	2.9	1.0	1.9
Lysine	1.0	1.1	1.3	0.8	0.9	0.9
Threonine	0.9	0.9	0.9	1.0	1.0	1.1
Arginine	1.0	0.7	0.8	0.6	0.9	0.7
Serine	1.0	0.8	1.0	0.9	1.1	1.3
Tyrosine	1.7	1.6	1.6	1.7	1.3	1.5
Histidine	1.0	0.9	1.1	1.0	1.0	1.0
Proline	1.0	0.8	1.0	0.9	1.0	0.9
Glycine	0.9	1.2	1.0	1.1	0.9	1.0
Alanine	0.9	1.0	0.9	1.0	0.9	0.9
Valine	1.0	1.0	0.9	1.0	0.9	0.9
Methionine	1.1	0.7	0.9	0.8	1.0	0.8
Isoleucine	1.0	1.0	1.0	1.1	1.0	1.0
Leucine	1.0	0.8	0.9	1.0	1.0	0.9
Phenylalanine	1.0	0.7	1.0	1.0	1.0	1.0
Ornithine	4.0	4.3	3.7	3.0	2.4	1.2
Taurine	1.6	17.0	1.2	1.3	0.9	1.9
Hydroxyproline	-	-	-	-	-	-
Lanthionine	1.0	2.4	0.5	1.3	-	-
Hydroxylysine	-	-	-	-	2.0	2.8

Data associated with Figure 5

EPS Protein and polysaccharide at different sizes of filtrates following sequential extractions (Activated sludge B3)

Protein (mg/L)							
Size	Base	CER	Sulfide	Sulfide after CER	Sulfide after sulfide	CER after CER	CER after sulfide
1.5 µm	378	586	124	344	124	408	543
0.45 µm	330	422	95	252	101	283	376
500 kDa	91	76	42	76	49	73	85
100 kDa	92	75	40	78	51	76	89
30 kDa	84	69	38	72	46	61	84
3 kDa	72	64	28	70	31	48	75

Polysaccharide (mg/L)							
Size	Base	CER	Sulfide	Sulfide after CER	Sulfide after sulfide	CER after CER	CER after sulfide
1.5 µm	91	208	30	79	30	140	196
0.45 µm	65	134	24	54	22	98	131
500 kDa	15	35	5	11	5	41	38
100 kDa	14	34	5	10	5	40	39
30 kDa	12	32	4	9	4	37	37
3 kDa	9	31	3	7	3	31	33

Data associated with Figure 7 *(also Figure 1 in Chapter 4)*

EPS Protein and polysaccharide at different sizes of filtrates (Activated sludge B3 set)

Before digestion (VS = 7.97g/L)

	Protein (mg/L)			Polysaccharide (mg/L)		
Size	Base	CER	Sulfide	Base	CER	Sulfide
1.5 µm	378.4	586.4	124.1	90.7	208.2	29.7
0.45 µm	330.1	421.6	95.1	65.4	134.3	24.4
500 kDa	90.9	76.1	41.6	15.3	35.4	5.2
100 kDa	92.2	75.4	39.6	14.2	33.8	5.2
30 kDa	84.4	68.7	38.4	12.0	31.6	4.5
3 kDa	71.6	63.7	28.0	9.3	30.5	3.3

After anaerobic digestion (VS = 4.83 g/L)

	Protein (mg/L)			Polysaccharide (mg/L)		
Size	Base	CER	Sulfide	Base	CER	Sulfide
1.5 µm	190.1	403.7	73.2	44.7	104.8	29.4
0.45 µm	107.2	210.3	31.2	21.4	57.7	12.2
100 kDa	13.2	28.3	1.1	1.8	8.0	2.1
30 kDa	10.5	25.6	0.9	1.3	6.7	1.3
3 kDa	7.3	21.5	0.0	0.0	3.5	0.1

After anaerobic digestion (VS = 5.48 g/L)

	Protein (mg/L)			Polysaccharide (mg/L)		
Size	Base	CER	Sulfide	Base	CER	Sulfide
1.5 µm	218.2	262.7	91.7	69.5	96.6	26.8
0.45 µm	168.8	154.9	64.0	53.0	67.6	16.0
100 kDa	39.7	36.2	19.9	5.5	15.3	3.2
30 kDa	32.0	29.7	17.4	3.8	15.1	2.3
3 kDa	26.4	26.0	11.3	1.2	9.4	0.6

Appendix B (Chapter 4)

Recovery of proteins and polysaccharides during sequential ammonium sulfate precipitation (Sludge A)

Protein	Ammonium Sulfate fraction	Protein (mg/L)	Volume (mL)	Mass of proteins (mg)	Fractions of proteins (%)	Qumulative fractions (%)
CER	Crude	998	30	29.9	100	
	0-20%	1181	7.8	9.2	30.8	30.8
	20-40%	1415	4.5	6.4	21.3	52.1
	40-60%	1647	3.7	6.1	20.4	72.4
	60-90%	816	2.9	2.4	7.9	80.3
Base	Crude	927	30	27.8	100	
	0-20%	1020	6.3	6.4	23.1	23.1
	20-40%	1223	4	4.9	17.6	40.7
	40-60%	1335	2.9	3.9	13.9	54.6
	60-90%	647	2.7	1.7	6.3	60.9
Sulfide	Crude	299	60	17.9	100	
	0-20%	733	6	4.4	24.5	24.5
	20-40%	773	4.8	3.7	20.7	45.2
	40-60%	641	4.5	2.9	16.1	61.3
	60-90%	462	3.5	1.6	9.0	70.3

Poly-Saccharides	Ammonium Sulfate fraction	poly-saccharide (mg/L)	Volume (mL)	Mass of poly-saccharide (mg)	Fractions of poly-saccharide (%)	Qumulative fractions (%)
CER	Crude	453	30	13.6	100	
	0-20%	515	7.8	4.0	29.5	29.5
	20-40%	332	4.5	1.5	11.0	40.5
	40-60%	320	3.7	1.2	8.7	49.2
	60-90%	106	2.9	0.3	2.3	51.5
Base	Crude	300	30	9.0	100	
	0-20%	480	6.3	3.0	33.6	33.6
	20-40%	289	4	1.2	12.8	46.5
	40-60%	316	2.9	0.9	10.2	56.6
	60-90%	63	2.7	0.2	1.9	58.5
Sulfide	Crude	142	60	8.5	100	
	0-20%	420	6	2.5	29.6	29.6
	20-40%	153	4.8	0.7	8.6	38.3
	40-60%	225	4.5	1.0	11.9	50.2
	60-90%	64	3.5	0.2	2.6	52.8

Recovery of proteins and polysaccharides during sequential ammonium sulfate precipitation (Sludge B)

Protein	Ammonium Sulfate fraction	Protein (mg/L)	Volume (mL)	Mass of proteins (mg)	Fractions of proteins (%)	Qumulative fractions (%)
CER	Crude	1190	27	32.1	100	0
	0-20%	2006	4	8.0	25.0	25
	20-40%	2476	3.5	8.7	27.0	52
	40-60%	2866	2.8	8.0	25.0	77
	60-90%	1529	2.1	3.2	10.0	87
Base	Crude	720	27	19.5	100	0
	0-20%	1333	3	4.0	20.6	21
	20-40%	1811	2.1	3.8	19.5	40
	40-60%	1138	2.4	2.7	14.0	54
	60-90%	696	1.4	1.0	5.0	59
Sulfide	Crude	561	54	30.3	100	0
	0-20%	2142	2.9	6.2	20.5	21
	20-40%	2188	2.7	5.9	19.5	40
	40-60%	1790	2.2	3.9	13.0	53
	60-90%	551	2.2	1.2	4.0	57

Poly-saccharides	Ammonium Sulfate fraction	poly-saccharide (mg/L)	Volume (mL)	Mass of poly-saccharide (mg)	Fractions of poly-saccharide (%)	Qumulative fractions (%)
CER	Crude	626	27	16.9	100	0
	0-20%	1394	4	5.6	33.0	33.0
	20-40%	483	3.5	1.7	10.0	43.0
	40-60%	628	2.8	1.8	10.4	53.4
	60-90%	241	2.1	0.5	3.0	56.4
Base	Crude	218	27	5.9	100	0
	0-20%	610	3	1.8	31.0	31.0
	20-40%	309	2.1	0.6	11.0	42.0
	40-60%	221	2.4	0.5	9.0	51.1
	60-90%	84	1.4	0.1	2.0	53.1
Sulfide	Crude	165	54	8.9	100	0
	0-20%	890	2.9	2.6	29.0	29.0
	20-40%	320	2.7	0.9	9.7	38.7
	40-60%	425	2.2	0.9	10.5	49.1
	60-90%	117	2.2	0.3	2.9	52.0

Recovery of proteins and polysaccharides during sequential ammonium sulfate precipitation (Sludge C)

Protein	Ammonium Sulfate fraction	Protein (mg/L)	Volume (mL)	Mass of proteins (mg)	Fractions of proteins (%)	Qumulative fractions (%)
CER	Crude	871	27.5	24.0	100	0
	0-30%	1615	6.2	10.0	41.8	42
	30-60%	1797	3.4	6.0	25.1	67
	60-90%	1134	3.7	4.1	17.3	84
Base	Crude	603	27.5	16.6	100	0
	0-30%	1344	3.8	5.1	30.8	31
	30-60%	965	4.0	3.9	23.3	54
	60-90%	749	2.6	1.9	11.7	66
Sulfide	Crude	162	27.5	4.5	100	0
	0-30%	535	3.1	1.7	37.2	37
	30-60%	368	3.0	1.1	24.7	62
	60-90%	182	2.1	0.4	8.6	70

Poly-saccharide	Ammonium Sulfate fraction	poly-saccharide (mg/L)	Volume (mL)	Mass of poly-saccharide (mg)	Fractions of poly-saccharide (%)	Qumulative fractions (%)
CER	Crude	333	27.5	9.2	100	0
	0-30%	408	6.2	2.5	27.6	28
	30-60%	442	3.4	1.5	16.1	44
	60-90%	290	3.7	1.1	11.5	55
Base	Crude	176	27.5	4.8	100	0
	0-30%	402	3.8	1.5	31.6	32
	30-60%	203	4.0	0.8	16.7	48
	60-90%	137	2.6	0.4	7.4	56
Sulfide	Crude	42	27.5	1.2	100	0
	0-30%	130	3.1	0.4	35.1	35
	30-60%	71	3.0	0.2	18.4	54
	60-90%	65	2.1	0.1	11.9	65

Recovery of proteins and polysaccharides during sequential ammonium sulfate precipitation (Sludge D2)

Protein	Ammonium Sulfate fraction	Protein (mg/L)	Volume (mL)	Mass of proteins (mg)	Fractions of proteins (%)	Qumulative fractions (%)
CER	Crude	1330	27.5	36.6	100	0
	0-30%	2783	6.8	18.9	51.7	52
	30-60%	1750	3.6	6.3	17.2	69
	60-90%	1074	3.3	3.5	9.7	79
Base	Crude	589	27.5	16.2	100	0
	0-30%	1347	4.4	5.9	36.2	36
	30-60%	1048	3.6	3.7	23.0	59
	60-90%	358	3.2	1.1	7.0	66
Sulfide	Crude	247	27.5	6.8	100	0
	0-30%	469	4.0	1.9	27.6	28
	30-60%	723	2.9	2.1	30.3	58
	60-90%	302	2.1	0.6	9.1	67

Poly-saccharide	Ammonium Sulfate fraction	poly-saccharide (mg/L)	Volume (mL)	Mass of poly-saccharide (mg)	Fractions of poly-saccharide (%)	Qumulative fractions (%)
CER	Crude	416	27.5	11.4	100	0
	0-30%	623	6.8	4.2	37.1	37
	30-60%	307	3.6	1.1	9.7	47
	60-90%	177	3.3	0.6	5.1	52
Base	Crude	159	27.5	4.4	100	0
	0-30%	324	4.4	1.4	32.2	32
	30-60%	187	3.6	0.7	15.1	47
	60-90%	64	3.2	0.2	4.6	52
Sulfide	Crude	67	27.5	1.8	100	0
	0-30%	153	4.0	0.6	33.3	33
	30-60%	150	2.9	0.4	23.4	57
	60-90%	81	2.1	0.2	9.0	66

Appendix C (Chapter 5)

Recovery of proteins during sequential ammonium sulfate precipitation and protein reduction by sludge digestion

Before digestion (VS = 13.486 g/L; two time concentrate from raw sludge)

	Ammonium Sulfate fraction	Protein (mg/L)	Volume (mL)	Mass of proteins (mg)	Proteins (mg/g VS)
CER	Crude	998	30	29.9	74.0
	0-20%	1181	7.8	9.2	22.8
	20-40%	1415	4.5	6.4	15.7
	40-60%	1647	3.7	6.1	15.1
	60-90%	816	2.9	2.4	5.9
Base	Crude	927	30	27.8	68.8
	0-20%	1020	6.3	6.4	15.9
	20-40%	1223	4	4.9	12.1
	40-60%	1335	2.9	3.9	9.6
	60-90%	647	2.7	1.7	4.3
Sulfide	Crude	299	60	17.9	22.2
	0-20%	733	6	4.4	5.4
	20-40%	773	4.8	3.7	4.6
	40-60%	641	4.5	2.9	3.6
	60-90%	462	3.5	1.6	2.0

After anaerobic digestion (VS = 8.188 g/L; two time concentrate from raw sludge)

	Ammonium Sulfate fraction	Protein (mg/L)	Volume (mL)	Mass of proteins (mg)	Proteins (mg/g VS)	Reduction of proteins (%)
CER	Crude	490	30.0	14.7	59.8	19.2
	0-20%	737	5.0	3.7	15.0	34.1
	20-40%	867	3.4	2.9	12.0	23.8
	40-60%	833	2.8	2.3	9.5	36.9
	60-90%	609	2.5	1.5	6.2	-6.0
Base	Crude	423	30	12.7	51.6	24.9
	0-20%	929	6.0	5.5	22.5	-41.6
	20-40%	561	4.2	2.4	9.6	20.6
	40-60%	612	2.5	1.5	6.1	36.2
	60-90%	402	2.2	0.9	3.6	16.6
Sulfide	Crude	170	52	8.9	20.8	6.2
	0-20%	534	5.5	2.9	6.9	-27.0
	20-40%	435	4.4	1.9	4.5	1.9
	40-60%	414	1.9	0.8	1.8	49.5
	60-90%	148	2.3	0.3	0.8	59.9

After aerobic digestion (VS = 6.980 g/L; two time concentrate from raw sludge)

	Ammonium Sulfate fraction	Protein (mg/L)	Volume (mL)	Mass of proteins (mg)	Proteins (mg/g VS)	Reduction of proteins (%)
CER	Crude	202	30.0	6.1	29.0	60.8
	0-20%	394	4.2	1.7	7.9	65.3
	20-40%	476	3.3	1.6	7.5	52.4
	40-60%	360	2.5	0.9	4.3	71.5
	60-90%	131	2.4	0.3	1.5	74.4
Base	Crude	382	30	11.5	54.7	20.4
	0-20%	344	8.9	3.1	14.6	8.1
	20-40%	583	4.2	2.4	11.7	3.3
	40-60%	543	3.2	1.7	8.3	13.3
	60-90%	404	2.9	1.2	5.6	-29.7
Sulfide	Crude	131	54	7.1	18.8	15.2
	0-20%	239	6	1.4	3.8	30.1
	20-40%	437	4.4	1.9	5.1	-11.2
	40-60%	327	3.8	1.2	3.3	7.4
	60-90%	173	3.7	0.6	1.7	14.9

Appendix D
Hemaaglutination and Hemaaglutination Inhibition Assay On Sludges used in Chapter 5

Two-fold serial dilution assay in 96 well
2% of trypsinized B-type human red blood cells were used to determine hemagglutinating activity of activated sludge EPS

Before digestion (VS = 13.486 g/L; two time concentrate from raw sludge)

	Ammonium Sulfate fraction	Protein (mg/L)	Volume (mL)	Mass of proteins (mg)	Titer[a]	Total activity (Au)[b]	Specific activity (Au/mg)
CER	Crude	998	30	29.9	128	3840	128
	0-20%	1181	7.8	9.2	512	3994	434
	20-40%	1415	4.5	6.4	128	576	90
	40-60%	1647	3.7	6.1	16	59	10
	60-100%	816	2.9	2.4	0	0	0
	Sum*					4629	534
Base	Crude	927	30	27.8	256	7680	276
	0-20%	1020	6.3	6.4	512	3226	502
	20-40%	1223	4	4.9	512	2048	419
	40-60%	1335	2.9	3.9	512	1485	384
	60-100%	647	2.7	1.7	16	43	25
	Sum*					6802	1329
Sulfide	Crude	299	60	17.9	64	3840	214
	0-20%	733	6	4.4	256	1536	349
	20-40%	773	4.8	3.7	128	614	166
	40-60%	641	4.5	2.9	64	288	100
	60-100%	462	3.5	1.6	16	56	35
	Sum*					2494	649

a) Titer was defined as the reciprocal of final dilution point that showed red blood cell agglutination
b) AU, Hemaaglutination unit (Volume x titer)
*) Sum of ammonium sulfate fractions

After anaerobic digestion (VS = 8.188 g/L; two time concentrate from raw sludge)

	Ammonium Sulfate fraction	Protein (mg/L)	Volume (mL)	Mass of proteins (mg)	Titer	Total activity (Au)	Specific activity (Au/mg)
CER	Crude	490	30.0	14.7	256	7680	523
	0-20%	737	5.0	3.7	512	2560	695
	20-40%	867	3.4	2.9	512	1741	591
	40-60%	833	2.8	2.3	256	717	307
	60-90%	609	2.5	1.5	64	160	105
	sum					5178	1698
Base	Crude	423	30	12.7	256	7680	606
	0-20%	929	6.0	5.5	1024	6093	1102
	20-40%	561	4.2	2.4	512	2150	912
	40-60%	612	2.5	1.5	512	1254	837
	60-90%	402	2.2	0.9	64	141	159
	sum					9638	3011
Sulfide	Crude	170	52	8.9	128	6656	752
	0-20%	534	5.5	2.9	1024	5632	1917
	20-40%	435	4.4	1.9	512	2253	1176
	40-60%	414	1.9	0.8	256	474	618
	60-90%	148	2.3	0.3	32	74	216
	sum					8432	3927

After aerobic digestion (VS = 6.980 g/L; two time concentrate from raw sludge)

	Ammonium Sulfate fraction	Protein (mg/L)	Volume (mL)	Mass of proteins (mg)	Titer	Total activity (Au)	Specific activity (Au/mg)
CER	Crude	202	30.0	6.1	64	1920	316
	0-20%	394	4.2	1.7	256	1075	650
	20-40%	476	3.3	1.6	256	845	538
	40-60%	360	2.5	0.9	128	320	355
	60-90%	131	2.4	0.3	128	307	978
	sum					2547	2521
Base	Crude	382	30	11.5	256	7680	670
	0-20%	344	8.9	3.1	512	4557	1490
	20-40%	583	4.2	2.4	1024	4301	1755
	40-60%	543	3.2	1.7	1024	3277	1885
	60-90%	404	2.9	1.2	256	742	633
	sum					12877	5764
Sulfide	Crude	131	54	7.1	64	3456	488
	0-20%	239	6	1.4	512	3072	2145
	20-40%	437	4.4	1.9	1024	4506	2344
	40-60%	327	3.8	1.2	64	243	196
	60-90%	173	3.7	0.6	32	118	185
	sum					7939	4869

Hemaaglutination inhibition assay on
0~20% ammonium sulfate precipitate from base extract

	Concentration used	Minimum Inhibition Concentration
Saccharides		
Galactose	125 mM	No inhibition
Glucose	125 mM	No inhibition
L-Fucose	125 mM	No inhibition
D-Fucose	125 mM	No inhibition
N-acetylgalactosamine	125 mM	No inhibition
N-acetylglucosamine	125 mM	No inhibition
Mannose	125 mM	No inhibition
Galatouronic acid	125 mM	No inhibition
Arabinose	125 mM	No inhibition
Xylose	125 mM	No inhibition
Lactose	125 mM	No inhibition
Glycoproteins		
Mucin	250 mg/L	125 mg/L
Asialofetuin	251 mg/L	31 mg/L
Fetuin	252 mg/L	250 mg/L
Thyroglobulin	375 mg/L	188 mg/L
Ovalbumin	625 mg/L	156 mg/L

Four units of hemagglutinating activities in 0~20% of base extract was used in this inhibition assay.