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ISOLATION, SEPARATION AND IDENTIFICATION OF THE EXTRACELLULAR POLYMERIC SUBSTANCE (EPS) PROTEIN FRACTION FROM THE ACTIVATED SLUDGE FLOC

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ABSTRACT

Isolation, Separation and Identification of the Extracellular Polymeric Substance (EPS) Protein Fraction from the Activated Sludge Floc

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Environmental Applied Science and Management
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The purpose of this study was to expand the current knowledge of the composition of extracellular polymeric substances (EPS), principally EPS proteins, and their influence on structure, stability and surface chemistry of microbial flocs in activated sludge. It was proposed that a substantial portion of EPS proteins contains glycoproteins or proteins that are strongly bound noncovalently to carbohydrates. Various buffer additives, purification and precipitation methods were employed for protein purification and several glycoprotein detection methods were utilized for glycoprotein detection in the EPS. The proteins were separated with success, with a substantial portion suggesting either a possible glycosylation or strong noncovalent interactions with carbohydrate moiety. An enzyme, oligoendopeptidase F from M3B family was tentatively identified as a major protein present. These results suggest that proteins in the activated sludge EPS may exist in a very intricate arrangement. Furthermore, the EPS peptides may get degraded by naturally present enzymes in the EPS after the protein is digested prior to mass spectrum (MS), making the identification challenging.

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ABBREVIATIONS

AcN	Acetonitrile
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CER	Cation exchange resin
DNA	Deoxyribonucleic acid
DOC	Deoxycholate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
EPS	Extracellular polymeric substances
ESI-LC-MS/MS	Electron spray ionization-liquid chromatography-tandem mass spectrum
GHCl	Guanidine hydrochloride
HCl	Hydrogen chloride
IPA	Isopropanol
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometer
MLSS	Mixed liquor suspended solids
MS	Mass spectrometry
NaOH	Sodium hydroxide
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
P-E	Phenol-ether
RPLC	Reverse phase liquid chromatography
SBR	Sequencing batch reactor
SDS	Sodium dodecyl sulphate
1-D SDS-PAGE	One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
WWTP	Wastewater treatment plant

CHAPTER 1. INTRODUCTION

1.1 Background

The activated sludge process is one of the chief biological wastewater treatment technologies (Hoa *et.al.*, 2003) and is frequently used in wastewater treatment for the removal of organic compounds (Lim and Bai, 2003). Activated sludge consists of biological flocs, which are intricate aggregates comprised of an assortment of microorganisms, including bacteria (Wilén *et.al.*, 2003), organic fibres, and inorganic particles embedded in a highly charged polymeric network, known as extracellular polymeric substances (EPS) (Frølund *et.al.*, 1996; Jorand *et al.*, 1998; Wilén *et.al.*, 2003). Microbial flocculation is a key factor in the solid-liquid separation that takes place in a clarifier in the activated sludge process and its settling performance must be adequate to attain efficient solid-liquid separation.

EPS are the main components of the activated sludge floc matrix (Hoa *et.al.*, 2003) and are composed of polysaccharides, proteins, nucleic acids, lipids, and other polymeric compounds in the intracellular space of microbial aggregates at or outside the cell surface (Neyens *et.al.*, 2004). EPS are essential in wastewater treatment as a result of their significance in pollutant removal from wastewater (Dignac *et al.*, 1998), bioflocculation (floc formation), bacterial attachment, biofilm structure and function (Wolfaardt *et al.*, 1999; Lim and Bai, 2003), and floc properties, such as settling and dewatering (Bura *et al.*, 1998). EPS are involved in cellular associations, bacterial nutrition, interaction of bacteria with their bio-physicochemical environment, and they have an effect on the macroenvironment (Wolfaardt *et al.*, 1999; Lim and Bai, 2003; Geesey and Van Ommen Kloeke, 2005).

Previous studies indicate that protein is the major constituent in EPS (Frølund *et al.*, 1996; Liao *et al.*, 2001) and is the most important EPS component for flocculation (Higgins and

Novak, 1997; Jorand *et al.*, 1998). EPS contains several functional groups (*e.g.*, hydroxyl and negatively charged carboxyl groups) that may possibly bind through specific protein-polysaccharide interactions, hydrophobic interactions, hydrogen bonding, and ionic interactions (Higgins and Novak, 1997; Jorand *et al.*, 1998; Gorner, 2003). The predominant constituents of EPS comprise of proteins and polysaccharides (Jahn and Nielsen, 1995; Frølund *et al.*, 1996; Dignac *et al.*, 1998; Liu and Fang, 2002; Harshey, 2003), which suggests a possible protein-polysaccharide interaction within the EPS (Higgins and Novak, 1997). Furthermore, many proteins in the EPS involved in the protein-polysaccharide interactions may be prokaryotically glycosylated.

The interest in prokaryotic glycoproteins in the EPS is due to the fact that most of the characterization of glycosylated proteins was done on eukaryotic organisms (Sandercock, 1994; Suzuki *et al.*, 1995); however, now it is established that glycosylation occurs in all domains of life (Moens and Vanderleyden, 1997; Schäffer *et al.*, 2001). The significance of prokaryotic glycoproteins in the EPS arises from the various biological functions which prokaryotic glycoproteins execute, including enzymatic catalysis, lubrication, surface protection, structural support, and cell adhesion (Wagh and Bahl, 1981). Some of the prokaryotic glycoproteins include surface-associated S-layer proteins, flagellins, pillins, and fimbriaes (Moens and Vanderleyden, 1997; Schäffer *et al.*, 2001), which may be present in the microbial floc EPS. Therefore, glycoproteins in the EPS may explain microbial floc structure.

EPS play an important role in floc and biofilm structure, activity and performance in wastewater treatment plants (WWTPs); nevertheless, various bacterial EPS exoproteins and exopolysaccharides have been given insufficient attention to the identity of structure and

function of these EPS constituents. Consequently, the specific physicochemical characteristics of EPS are still not entirely clear (Robinson *et al.*, 1984; Liss *et al.*, 1996).

1.2 Research Objectives

The aim of this research was to gain a better understanding regarding the nature of EPS proteins, and reveal if the majority of the protein-polysaccharide interactions in the EPS are through glycosylation. This may assist in controlling the bioflocculation and improve solid-liquid separation in biological wastewater treatment. The ultimate goal of the floc analysis was to be able to manipulate flocs in order to achieve flocs that settle well, have good dewatering characteristics and are highly active, in order to improve the solid-liquid separation and dewatering in biological wastewater treatment (Wilén *et.al.*, 2003).

The underlying hypothesis of this study is that proteins in the EPS are strongly associated with polysaccharides through non-covalent associations and possibly through glycosylation. The motivation for this research is that these EPS proteins are not easily extracted or resolved employing standard purification procedures due to many interactions within the EPS matrix. Therefore, these interactions must be broken and the EPS protein must be purified and extracted from the activated sludge EPS.

The goal of this research was to resolve the protein fraction in the activated sludge EPS by describing a novel purification, isolation and extraction method. Furthermore, this study attempts to demonstrate that a considerable portion of the EPS proteins consists of glycoproteins. The eventual goal was to characterize the EPS proteinaceous material by either chemical, physical or enzymatic methods and thus approximate the EPS protein molecular weight and

composition. Determination of proteins in the EPS may assist in elucidating the structure of the EPS.

The four specific objectives established to reach the overall objective were to:

1. Investigate the ability to isolate, separate and purify the EPS proteins from microbial activated sludge floc EPS.
2. To estimate the extent of glycosylation in the microbial EPS matrix.
3. Identify the microbial activated sludge EPS proteins.
4. Gain a better understanding of the function of EPS proteins on the floc structure and formation in the activated sludge by assessing the nature of the protein and its function.

1.3 Thesis Outline

This report comprises of seven chapters and five appendices. Chapter 1 provides an introduction and the thesis outline. Chapter 2 consists of a literature review, containing the background on previous research on factors affecting microbial EPS flocculation and surface characteristics of EPS, as well as experimental techniques applied in this study. Chapter 3 outlines the experimental approach used and Chapter 4 presents the results acquired in this study and a detailed discussion of the results, whereas Chapter 5 includes conclusions and recommendations for prospective future studies. The list of references is included in Chapter 6 and the details of experimental data and results are incorporated in Chapter 7 appendices.

CHAPTER 2. LITERATURE REVIEW

2.1 Microbial Sludge Flocs

Naturally occurring microbial flocs are important in influencing the efficiency of the solid-liquid phase process streams in the engineered biological systems. (Seka and Verstraete, 2003; Chu and Lee, 2004; Daims, 2005; Geesey and Kloeke, 2005; Jarvis *et al.*, 2005). Poor floc stability causes a rise in turbidity of the wastewater effluent (Liao *et al.*, 2002) and thus the floc structure is alleged to control flocculation and dewatering behaviours of the biomass (Jin *et al.*, 2003).

2.1.1 Sludge Floc Significance

Flocculation is fundamental for mediating the physical, chemical, and biological properties of suspended flocs as well as the aquatic and engineered system as a whole (Droppo *et al.*, 2005). In wastewater and drinking water research, it is crucial to understand the function-structure relationships of flocs, in order to resolve such problems as poor settling sludge (Liss *et al.*, 2005), which occurs in the secondary clarifier of wastewater treatment plant (Schmid *et al.*, 2003). Poor settling sludge stems from two chief problems, which include filamentous or non-filamentous sludge bulking and poor sludge flocculation (i.e. dispersed growth, pinpoint flocs, and foaming or scum formation) (Rittmann and McCarty, 2000; Jin *et al.*, 2003).

Filamentous bulking arises from excessive growth of filamentous bacteria in activated sludge, diminishing settling properties and effluent quality of the sludge (Slijkhuis, 1983; Andreasen and Nielsen, 1997; Jin *et al.*, 2003; Martins *et al.*, 2004). Non-filamentous bulking and pinpoint flocs arise from excess EPS in activated sludge flocs (Urbain *et al.*, 1993). Foaming

or scum formation arises from the presence of *Nocardia* sp. and/or *Microthrix parvicella* or by non-degradable surfactants (Martins *et al.*, 2004).

2.1.2 Sludge Floc Structure

Typical microbial floc is 25–1000 mm in diameter and floc constituents, such as bacteria, are approximately 0.5–5 μm in diameter (Li and Ganczarczyk, 1990; Snidaro *et al.*, 1997). The sludge flocs have a loose structure (Wingender *et al.*, 1999), which is composed of bacteria, fungi, protozoa, organic fibres, inorganic particles, and extracellular polymeric substances (EPS) (Figure 2.1) (Li and Ganczarczyk, 1990; Jorand *et al.*, 1995; Frølund *et al.*, 1996; Wingender et al., 1999).

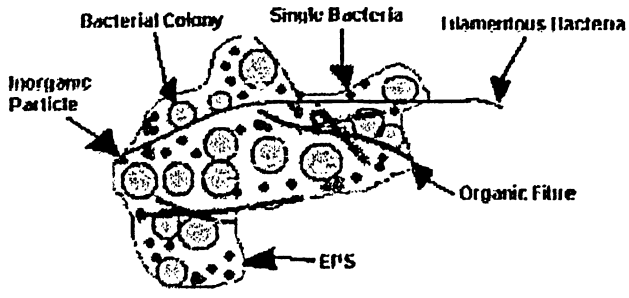


Figure 2.1 Microbial floc composition (Adapted from Keiding and Nielsen, 1997).

The majority of activated sludge floc is comprised of heterotrophic bacteria, such as *Pseudomonas, Achromobacter, Flavoracterium, Alcaligenes, Arthrobacter, Citromonas* and *Zooglea*, which may be accountable for the floc formation (Dias and Bhat, 1964).

The microbial floc's structure is highly irregular with microbial communities varying considerably, depending on the operating conditions of the activated sludge process (Li and Ganczarczyk, 1990; Sheng *et al.*, 2006). There are several proposed models on the floc structure

(Parker *et al.*, 1972; Keiding and Nielsen, 1997; Liao *et al.*, 2002; Sheng *et al.*, 2006). One of these models is a polymer-bridging (filament backbone) model (Parker *et al.*, 1972; Sheng *et al.*, 2006), which proposes that microbial sludge floc is a multi-layer structure with two distinct regions: outer and inner (Figure 2.2). The outer fraction of sludge floc is loose and layered, entangled in EPS that may be readily extracted. The inner fraction sludge floc is stable and firmly attached, which may only dissociate under highly adverse environment (Sheng *et al.*, 2006). The bacteria attach to each other (microstructure) and the filament backbone (macrostructure) by polymer bridge bonds (Parker *et al.*, 1972; Parker, 2005; Sheng *et al.*, 2006). Liao *et al.* (2002) proposed that the floc structure is affected by settling retention time (SRT). Therefore, at lower growth rate and hence higher SRT, the floc's inner EPS region is more hydrophobic and thus is more compact than at lower SRT at which the floc's inner EPS region is more diffuse (Figure 2.2).

2.1.3 Microbial Floc Physicochemical Properties

For good settling and dewatering, large and dense flocs are desirable (Liao *et al.*, 2006). The most significant parameters that affect size, structure and settleability of the microbial flocs are solid retention time (SRT), dissolved oxygen concentration (DO), mixed liquor suspended solids concentration (MLSS), and sludge volume index (SVI) (Eriksson and Hardin, 1984; Wilén and Balmér, 1999; Liao *et al.*, 2006).

SRT determines the characteristics of the sludge produced. Liao *et al.* (2001) studied the influence of SRT on the EPS and physicochemical properties of sludge, including hydrophobicity and surface charge using laboratory-scale sequencing batch reactors (SBRs). The study demonstrated that EPS concentration was not dependent on SRT. The sludge surface was

more hydrophobic and less negatively charged at higher SRTs (16 and 20 days) than at lower SRTs (4-9 days). Liao *et al.* (2006) studied the effect of SRT (4-20 days) on sludge floc structure, size distribution and morphology in SBRs. The results showed no clear relationship between SRT and floc size. However, sludge flocs at lower SRTs (4-9 days) were more irregular and variable than at higher SRTs (16 and 20 days).

High dissolved oxygen (DO) concentration is important for improvement of EPS production by microorganisms and thus improvement in bioflocculation. Unlike high DO, low DO levels (< 2.0 mg/l) in the mixed liquor were found to lead to poor settling properties and turbulent effluent (Sürücü and Dilek, 1990; Wilén and Balmér, 1999).

The mixed liquor suspended solids (MLSS) concentration measures the amount of suspended solids in the effluent. Even though EPS concentration is key in obtaining successful floc-particle aggregation, its levels do not need to be at maximum in order to obtain the best effluent quality. Some authors reported that the effluent suspended solids discharged from full-scale plant are negatively affected by increases in the MLSS levels (Chapman, 1983; Wahlberg *et al.*, 1994).

The sludge volume index (SVI) measures the morphology and physical characteristics of activated sludge (Schmid *et al.*, 2003; Liao *et al.*, 2006) and establishes the required sludge recirculation rate for calculation of the MLSS concentration needed in the aeration tank. The SVI is defined as the volume (ml) occupied by 1 gram of activated sludge following the settling the aerated liquor after 30 minutes. The efficiency of the settling will depend upon initial settling rate and the activated sludge characteristics (Dick and Vesilind, 1969). Therefore, the results of SVI cannot be used to predict settling behaviour in full-scale plants with complete confidence (Dick and Vesilind, 1969).

2.1.4 Extracellular Polymeric Substances (EPS)

Microbial extracellular polymeric substances (EPS) are high molecular-weight metabolic products accumulating on the bacterial cell surface (Frølund *et al.*, 1996) and the construction materials for microbial aggregates such as biofilms, flocs and sludge (Flemming and Wingender, 2001; Sheng *e t al.*, 2006). These biosynthetic polymers vary in quantity and composition of its constituents (range from 10-90%) depending on the growth conditions and environmental stresses (Christensen and Characklis, 1990). The EPS may be actively excreted from the floc or biofilm organisms, absorbed from the medium or they are a product of cellular lysis (Jahn and Nielsen, 1995).

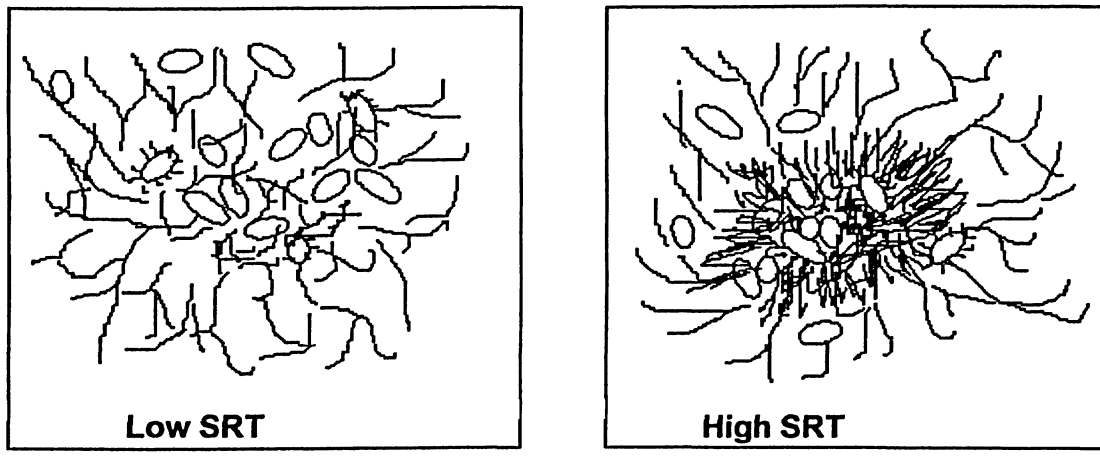


Figure 2.2 Two-layer microbial sludge floc structural model in biological wastewater treatment at low and high SRT (Adapted from Liao *et al.*, 2002).

2.1.4.1 Microbial Sludge EPS Significance

EPS in flocs, biofilms and biological sludges can be both harmful and valuable. Accumulation of EPS may cause biofouling, biocorrosion of metals, and bioweathering. EPS have been implicated as an important cause of membrane fouling in membrane bioreactors. EPS in the water phase is crucial for the filterability of activated sludge (Evenblij and van der Graaf, 2004).

Sorption properties of EPS are valuable for water purification (Brown and Lester, 1980). Furthermore, EPS hold a great biotechnological potential (Flemming and Wingender, 2001) and have an important commercial use in the industry such as production of Dextran derived Sephadex products for the use in gel filters and chromatography media (Sutherland, 1998). EPS is also essential to the flocculation, settleability and dewatering of activated sludge (Jia *et al.*, 1996; Keiding and Nielsen, 1997; Liao *et al.*, 2001; Mikkelsen and Keiding, 2001; Shin *et.al.*, 2001; Liu and Fang, 2002; Wilén *et.al.*, 2003).

2.1.4.2 Microbial Sludge EPS Structure and Ecological Aspects

EPS can be classified into two types: tight capsules (bound) that closely bind cells and loosely attached (soluble) slime secretions (Zhang *et al.*, 1999; Liss *et al.*, 2005). Unlike slime EPS, capsular EPS are involved in attachment (Liss *et al.*, 2005).

Researchers have overlooked the importance of EPS until recently, studying mainly subcultured individual bacterial strains in pure cultures using artificial growth media. Under these laboratory culture conditions the bacterial isolates may not produce EPS given that EPS are not essential for bacteria when the EPS loss does not impair cell growth or viability (Wingender *et al.*, 1999; Liu and Fang, 2002).

Bacteria spend a considerable amount of its carbon and energy (more than 70%) in manufacture of EPS (Liss *et al.*, 2005). This suggests that EPS plays a significant role in the performance of microbial communities and their arrangement (Liss *et al.*, 2005). It was found that in natural environments EPS production functions as carbon and energy reserve during starvation (Wingender *et al.*, 1999; Liu and Fang, 2002).

The activated sludge EPS have two different origins: one from the activated sludge bacterial cells (due to metabolism and cell autolysis) and another from compounds in the incoming wastewater (Frølund *et al.*, 1996). Activated sludge EPS is a main sludge floc constituent keeping the floc together in a three-dimensional matrix (Sheng *et al.*, 2006).

2.1.4.3 EPS Constituents

EPS are a blend of different polymers such as proteins, polysaccharides, uronic acids and nucleic acids (Table 2.1) (Frølund *et al.*, 1996; Neu, 1996; Neu and Lawrence, 1999; Liu and Fang, 2002). These constituents contain functional groups that may participate in the various interactions that keep the floc structure together (Liu and Fang, 2002).

Polysaccharide is the predominant constituent in the EPS of many pure cultures (Liu and Fang, 2002), comprised of both neutral and acidic sugars (Sutherland, 1990). EPS polysaccharide plays an important role for the structure in biofilms (Jahn and Nielsen, 1995). Protein is found in substantial quantities in the sludges of many wastewater treatment reactors (Liu and Fang, 2002) and it may be significant in aggregate structure stabilization (Laspidou and Rittmann, 2002). Less is known about other constituents found in the EPS, which include lipids (Goodwin and Forster, 1985; Bura *et al.*, 1998), nucleic acids (Urbain *et al.*, 1993; Bura *et al.*, 1998) and humic compounds (Frølund *et al.*. 1995).

Table 2.1 Common constituents of bacterial EPS (Adapted from Wingender *et al.*, 1999).

EPS Class of Constituents	Principal Components	Examples of Substituents
Carbohydrates	Monosaccharides	Organic: O-acetyl, N-acetyl,
	Uronic acids	succinyl, pyruvyl,
	Amino sugars	Inorganic: sulfate, phosphate
Proteins	Amino acids	Oligosaccharides: glycoproteins
		Fatty acids: lipoproteins
Nucleic acids	Nucleotides	
(Phospho)lipids	Fatty acids Glycerol Phosphate Ethanolamine Serine Choline Sugars	
Humic substances	Phenolic compounds Simple sugars Amino acids	

Knowing the composition of EPS is key in order to determine several significant properties of floc and biofilm including density, porosity, diffusivity, strength, elasticity, frictional resistance, thermal conductivity, and metabolic activity. Additional information about their constituents will contribute to a better understanding of the physical and physiological behaviour of flocs and biofilms in environmental systems (Zhang *et al.*, 1999) in order to be able to improve their manipulation to attain more favourable flocs (*e.g.*, flocs which settle well, have good dewatering characteristics and have high functionality in terms of activity) (Wilén, 2003).

2.2 EPS Proteins

EPS proteins contain such carboxyl containing groups as aspartic and glutamic acids (Higgins and Novak, 1997), which contribute to a high level of negative charge in the EPS (Dignac *et al.*, 1998; Harshey, 2003) and thus may play a key role in floc structure stability (Higgins and Novak, 1997; Jorand *et al.*, 1998; Laspidou and Rittmann, 2002).

The protein involvement in floc formation is explained by hydrophobic interactions (Jorand *et al.*, 1998) and by cation bridging (Gorner, 2003). Previous studies indicate that since the EPS is ionically linked to divalent cations such as calcium, protein may be involved in sludge settleability (Urbain *et al.*, 1993). Therefore, knowing more about the proteins in the EPS, which constitute a large portion of EPS in activated sludge flocs (Frølund *et al.*, 1996; Liao *et al.*, 2001), may lead to resolution of numerous problems caused by EPS in activated sludge systems. For instance, characterization and isolation of EPS proteins extracted from activated sludge systems can assist to better understand the role of protein in bioflocculation (Higgins and Novak, 1997).

2.3 EPS Protein Interactions

A number of classes of proteins are present in the exocellular environment of bacteria (*e.g.*, extracellular enzymes, proteinaceous S-layers, lectins, intracellular protein from cell lysis or cell wall turnover, or polypeptide capsular material). The exocellular protein extracted from activated sludge samples could be from a mixture of these sources (Higgins and Novak, 1997). EPS contains several functional groups (*e.g.*, hydroxyl and negatively charged carboxyl groups) which may possibly bind through van der Waals forces (Nielsen *et al.*, 1996), electrostatic binding with bivalent cations (*e.g.*, Ca^{2+} and Mg^{2+}) (Frølund *et al.*, 1996), hydrogen bonds

(Frølund *et al.*, 1996), hydrophobic interactions (Frølund *et al.*, 1996; Higgins and Novak, 1997), and in some cases covalent bonds (*e.g.*, disulfide bonds in proteins) (Emerson and Ghiorse, 1993; Higgins and Novak, 1997; Jorand *et al.*, 1998; Gorner, 2003). Because the predominant constituents of EPS comprise of proteins and polysaccharides (Jahn and Nielsen, 1995; Frølund *et al.*, 1996; Dignac *et al.*, 1998; Liu and Fang, 2002; Harshey, 2003), the majority of proteins in EPS may possibly bind through specific protein-polysaccharide interactions (Higgins and Novak, 1997). Furthermore, a substantial portion of these proteins in the EPS that are involved in the protein-polysaccharide interactions may be prokaryotically glycosylated (Goodwin and Forster, 1985; King and Forster, 1990).

2.4 EPS Glycoproteins

Glycoproteins, as a class of biomolecules, present a number of differences in structure and function from non-glycosylated proteins (Wagh and Bahl, 1981; Robertson and Kennedy, 1996). The transformation of a protein into a glycoprotein involves glycosylation of the peptide chain during biosynthesis. Thus, glycoprotein is a protein in which one or more saccharides are covalently attached to the peptide chain. The nature of covalent bond that links the carbohydrate to the peptide chain is a major aspect of the structural uniqueness of glycoproteins (Wagh and Bahl, 1981).

Glycoproteins are ever-present in nature (Maley *et al.*, 1989) (*e.g.*, animals, plants, and microorganisms). Glycoproteins have also been established in the extra- and intracellular fluids, connective tissue, and cellular membranes (Wagh and Bahl, 1981). Of all biologically occurring macromolecules, glycoproteins, which consist of carbohydrate moieties covalently linked to a polypeptide backbone, represent the most varied assembly, ranging from substances in which the

carbohydrate component represents less than 1% of the total weight to those in which it corresponds to over 80% of the total molecule. The three types of sugars that commonly occur in glycoproteins include neutral sugars (*e.g.*, galactose, mannose, glucose, and fucose), amino sugars (*e.g.*, N-acetylglucosamine and N-acetylgalactosamine) and acidic sugars (*e.g.*, sialic acids) (Kornfeld and Kornfeld, 1976; Wagh and Bahl, 1981).

2.4.1 Protein Glycosylation

The glycosylation of proteins is a complex ordered biological pathway which is more abundant and structurally varied than all other types of post-translational modifications combined (Hart, 1992). Furthermore, protein glycosylation is a highly specific process (Meynial-Salles and Combes, 1996) dependent upon protein sequence, cellular phenotype, and the physiological environment (Cumming, 1992). Glycosylation can affect signal transduction and cell-cell communication properties and hence influence critical cell decisions (*e.g.*, regulation of differentiation and apoptosis) (Murrell, 2004).

Modification of oligosaccharide part of a glycoprotein (glycan) can lead to a modification of the activity of the glycoprotein (Meynial-Salles and Combes, 1996). The biosynthesis of glycans is not template-driven and there is no mechanism for proofreading and correcting differently glycosylated biomolecules. The structure of glycans depends on the competition of glycosylating enzymes for the same substrate, substrate specificity of these enzymes and substrate availability. Consequently, glycoproteins are created in a number of forms (glycoforms) that have the same peptide backbone, but vary in nature and site of glycosylation (Khmelnitsky, 2004).

Consistent with the nature of glycosidic bonds, glycosylated proteins encompass N-linked (amide group of asparagines) and O-linked (hydroxyl groups of amino acids such as serine, threonine, hydroxyproline, and hydroxylysine) residues (Ploegh and Neefjes, 1990; Suzuki *et al.*, 1995).

2.4.1.1 N-Glycosylation

N-linked glycosylation is imperative for correct protein folding and for glycoprotein function (Shi and Elliott, 2004), and is the most abundant naturally occurring glycan attachment to proteins (Spiro, 2002). The reaction is extremely specific and occurs only at asparagine (Asn) embedded in the consensus sequence Asn–Xxx–Ser/Thr, where Xxx is any amino acid except proline (Pro), Ser is serine and Thr is threonine (Figure 2.3) (Khmelnitsky, 2004).

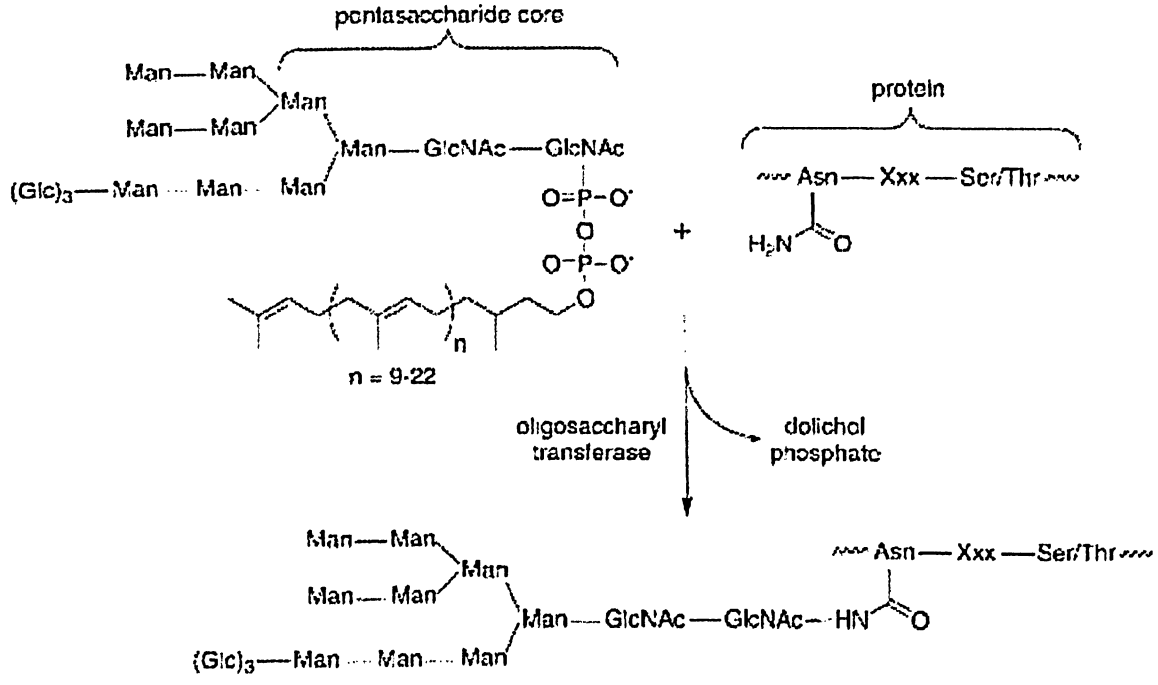


Figure 2.3 *N*-Glycosylation of proteins catalyzed by oligosaccharyltransferase (Khmelnitsky, 2004).

The entire *N*-glycosylation process occurs co-translationally, during protein synthesis, but prior to the final protein molecule formation and adoption of its conformation. Oligosaccharyltransferase (OST) is a highly complex membrane-bound multi-enzyme complex consisting of at least nine subunits. OST concentration in tissues is very low, and it is difficult to isolate (Meynial-Salles and Combes, 1996). In combination with the virtual unavailability of the enzyme and its sophisticated glycosyl donor, this makes OST of little practical value for in vitro glycoprotein synthesis (Khmelnitsky, 2004).

2.4.1.2 O-Glycosylation

O-Glycosylation of proteins is normally a less complicated process than *N*-glycosylation, given that there is no need for a complex lipid-linked oligosaccharide precursor for transfer to protein. The initiating event in *O*-glycosylation is the transfer of *N*-acetylgalactosamine (GalNAc) from UDP–GalNAc to Ser or Thr in the polypeptide chain. However, unlike *N*-glycosylation, a consensus sequence of amino acids in the polypeptide chain has not been found for GalNAc addition, although several prognostic algorithms have been created (Elhammer *et al.*, 1999). The reaction is catalyzed by a polypeptide *N*-acetylgalactosaminyl transferase, the enzyme that catalyzes the addition of the initiating monosaccharide to the protein in the process of *O*-glycosylation, and occurs post-translationally (Meynial-Salles and Combes, 1996; Elhammer *et al.*, 1999). *N*-acetylgalactosaminyl transferase takes place in an assortment of isozyme forms, has a high tolerance to variations in amino acid sequences flanking Ser or Thr in the polypeptide chain and demonstrates a good stability in purified form (Elhammer *et al.*, 1999; Wang *et al.*, 1992). The enzyme is challenging to isolate and it is not commercially available (Khmelnitsky, 2004).

2.4.1.3 EPS Prokaryotic Glycoproteins

Glycoproteins were found to be the main components of eukaryotic cells (Suzuki *et al.*, 1995), but in the last decades, many prokaryotic glycoproteins have also been identified (Sandercock, 1994; Benz and Schmidt, 2002). In addition to the incidence of glycosylated enzymes, antigens and other cell envelope components, surface layer (S-layer) glycoproteins represent the best-studied examples of glycosylated prokaryotic proteins. The prokaryotic glycoprotein studied in greatest detail is the cell surface glycoprotein of the archaebacterium *Halobacterium halobium* (Wieland, 1988).

Significance of the prokaryotic glycoproteins is mainly due to the assorted biological functions that they execute. These incorporate, along with others, enzymatic catalysis, lubrication, surface protection, structural support, and cell adhesion (Wagh and Bahl, 1981). The glycoproteins in the EPS may explain the rigidity of the EPS and its role in floc formation and floc structure.

2.5 Methods Employed in the Analysis and Characterization of EPS Proteins

This section includes a literature review on the potential methods that may be employed in EPS extraction, EPS protein purification and EPS protein characterization. The protein purification methods mentioned have been used on complex protein samples, such as plants and blood serum and thus offer a reasonable comparison.

2.5.1 EPS Extraction Procedure

Quantification of EPS, such as determining the composition and the amount of different EPS compounds present in a sample, depends a great deal on the extraction procedure used

(Frølund *et al.*, 1996; Liu and Fang, 2002). There is no universal method for quantitative EPS extraction process recognized thus far; hence, making it challenging to significantly compare and interpret published results (Liu and Fang, 2002). Consequently, a suitable approach must be chosen after thorough evaluation of requirements and available procedures.

Table 2.2 lists several EPS extraction methods from activated sludge that may be employed and compares the extraction efficiency of the methods. Physical extraction techniques that have been used previously for their low generation of cell lysis products include high-speed centrifugation, ultrasonication and heating (Horan and Eccles, 1986). The general chemical EPS extraction techniques include uses of alkaline (ammonium hydroxide and sodium hydroxide), sulfuric acid, EDTA, boiling benzene and formaldehyde (Liu and Fang, 2002; Liss *et al.*, 2005). It is imperative to generate negligible cell lysis, maintain cell membrane structure undamaged and release most of the EPS biopolymers for successful EPS extraction (Wingender *et al.*, 1999).

From Table 2.2, it is observed that sonication, heating, steaming, and NaOH treatments give the lowest amount of extraction efficiency (Brown and Lester, 1980; Urbain *et al.*, 1993; Frølund *et al.*, 1996), and sonication and heating possibly cause cell lysis (hence the high amount of protein and polysaccharide). The NaOH treatment gives relatively high amount of protein but with low extraction efficiency. The alkaline treatments cause many charged groups to be ionized (i.e. proteins and polysaccharides) as a result of low isoelectric points (pH 4-6) and thus causing strong repulsion between EPS within the matrix and higher water solubility of the compounds. However, this method causes denaturation of proteins by pH and vast amount of cellular disruption, as concluded by Brown and Lester (1980).

NaOH-formaldehyde treatment gives the best extraction efficiency and relatively high amount of protein compared to formaldehyde-ultrasound treatment (Table 2.2) (Liu and Fang,

2002). However, the NaOH treatment as stated previously ionizes charged groups in the microbial floc EPS and denatures the EPS proteins. Formaldehyde used in the EPS extraction treatment interferes with protein analysis. Therefore, to remove the formaldehyde from the treated samples, dialysis must be employed. Dialysis utilizes a semipermeable membrane that permits the passage of smaller molecules (*e.g.*, formaldehyde) and excludes the passage of larger molecules (*e.g.*, protein) (Pierce Biotechnology, 2004a). The disadvantages of using dialysis are that it is designed for large protein volumes with minimum samples and the procedure takes days to accomplish. Dilute protein samples, such as EPS protein samples, will produce very low recovery (Bonomo and Swaney, 1988).

EDTA demonstrates a high EPS extraction efficiency (Table 2.2) (Liu and Fang, 2002) by removing divalent cations from floc. However the use of EDTA as EPS extraction treatment removes such constituents as lipopolysaccharides from the cell wall and as a result leads to the cell wall instability (Johnson and Perry, 1976).

Cation exchange resin (CER) procedure gives a high amount of protein and carbohydrate with high extraction efficiency (Table 2.2) (Frølund *et al.*, 1996). However, according to Liu and Fang (2002), CER procedure gives a low amount of protein and carbohydrate with moderate extraction efficiency. The discrepancy in EPS composition may be due to varied activated sludge sources studied (Urbain *et al.*, 1993), different extraction techniques utilized (Brown and Lester, 1980) and the different analytical tools employed (Urbain *et al.*, 1993). CER procedure employs both a physical and chemical means of extraction, has been widely acknowledged and utilized for EPS extraction from activated sludge because it has been reported to be twice as high in yield, providing high extraction efficiency with little or no cell lysis and exopolymer disruption than other frequently used procedures (Jahn and Nielsen, 1995; Frølund *et al.*, 1996; Nielsen *et*

al., 1996; Bura *et al.*, 1998; Zhang et al., 1999; Liss *et al.*, 2005). The CER extraction technique detaches divalent cations (*e.g.*, Ca^{2+} and Mg^{2+}) from the EPS matrix and substitutes them with monovalent cations, thereby reducing EPS stability and separating it from the cellular material (Jahn and Nielsen, 1995; Frølund *et al.*, 1996; Wingender *et al.*, 1999; Liss *et al.*, 2005).

Table 2.2 EPS composition and extraction methods from activated sludge.

Method	Protein (mg/g)	Polysaccharide (mg/g)	DNA (mg/g)	Uronic Acid (mg/g)	Humic Acid (mg/g)	Extraction Efficiency (%org C extracted from total org C)	Reference
Sonication	279.9	60.54	67.34	-----	-----	10	Urbain *et al.*, 1993
Heating (80°C)	121	8	-----	-----	-----	9	Frølund *et al.*, 1996
Steaming (10 min)	77.1	15.8	3.7	-----	-----	10	Brown and Lester, 1980
NaOH (pH 11)	96	22	-----	3.1	-----	8	Frølund *et al.*, 1996
Formaldehyde-NaOH	54.6	40.5	0.35	4.2	50.4	164.9	Liu and Fang, 2002
Formaldehyde-Ultrasound	20.4	28.9	0.13	1.8	18.9	77.9	Liu and Fang, 2002
EDTA	22.9	12.4	0.47	2.1	59.2	146.8	Liu and Fang, 2002
CER	243	48	-----	6.1	126	27	Frølund *et al.*, 1996
CER	17.6	12.7	0.14	1.2	16.4	57.8	Liu and Fang, 2002

2.5.2 EPS Protein Identification and Characterization

2.5.2.1 EPS Protein Extraction from Activated Sludge

One of the difficulties in proteomics is the complex matrices (Romijin *et al.*, 2003) including floc EPS. To study such proteins, the sample must be purified by protein extraction and separation methods. (Michalski and Shiell, 1999).

2.5.2.1.1 Buffer Additives

Proteins are extremely heterogeneous molecules that require stable pH environment (Ahmed, 2005). The first choice to make for an extraction buffer is determination of the nature and pH of the desired buffer. It is favourable to avoid time and protein loss caused by additional buffer exchange step. The most common buffers used are listed in Table 2.3.

Insoluble proteins will cause artifacts and sample loss. Therefore, protein solubilization must be employed in order to break interactions that aggregate or precipitate the protein out of solution (Rabilloud 1996; Berkelman *et al.*, 2004). In order to remove the strongly bound proteins from the EPS matrix, improve stability of the EPS proteins and keep them in solution, it is necessary to employ chemical additives in the extraction buffer to facilitate dissociation (Pierce Biotechnology, 2004a). The chemical additives employed for sample solubilization include such compounds as acids, bases, salts, chelating agents, detergents, chaeotropic agents, organic agents, and reducing agents (Berkelman *et al.*, 2004). Table 2.4 lists various chemical additives, their purpose and their optimum concentration.

The pH of a solution will have a strong effect on protein solubility. Typically proteins are optimally soluble at high pH; nonetheless, various proteins have different optimum pH values and a range of proteins will be extracted at different pH values (Berkelman *et al.*, 2004).

Ionic strength also has a strong effect on protein solubility and thus various salts (*e.g.* 150 mM NaCl) are typically added to sample preparation solutions for protein extraction (Berkelman *et al.*, 2004). The major cations found in activated sludge include sodium, ammonium, calcium, magnesium, and iron. Urbain *et al.* (1993) have found that protein in the floc and EPS have a strong affinity for calcium and magnesium cations. Murthy and Novak (2001) have demonstrated that ferric and sodium cations coagulated protein and carbohydrate through potential adsorptive interactions.

Detergents disrupt hydrophobic interactions between and within proteins and bring insoluble proteins into solution. The usual concentrations of detergents used are 1-4% and they are typically categorized according to their charge in solution. There are three types of detergents, including anionic (i.e. sodium dodecyl sulfate (SDS)), cationic (ethyl trimethyl ammonium bromide (ETAB)), neutral (i.e. TRITON® X-100), or zwitterionic (i.e. 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)). SDS detergent is frequently used for its ability to quickly and effectively solubilize proteins (Berkelman *et al.*, 2004).

Chaeotropic agents disrupt hydrogen bonds and hydrophobic interactions between and within proteins. Typically, Chaeotropes are used in high concentration (neutral urea: 5-9 M; charged guanidine hydrochloride (GHCl): 5-8 M) in order to disrupt secondary protein structure and bring insoluble proteins into solution (Berkelman *et al.*, 2004).

Reducing agents are frequently used to cleave disulfide bond crosslinks within proteins and between protein subunits. Common reducing agents include sulfhydryl (dithiothreitol (DTT); dithioerythritol (DTE); β-mercaptoethanol) and phosphine (tributylphosphine (TBP); tris-carboxyethylphosphine (TCEP)) reducing agents (Berkelman *et al.*, 2004).

Table 2.3 Common buffers utilized with a wide range of buffering capacity (Adapted from Ahmed, 2005).

Buffer	PH RANGE	Reagents
Glycinate-HCl	2.6-3.6	Glycine, HCl
Citrate	3.0-6.2	Citric acid, sodium citrate
Acetate	3.6-5.6	Acetic acid, sodium acetate
Citrate-Phosphate	2.7-7.0	Citric acid, dibasic sodium phosphate
Succinate	3.8-6.0	Succinic acid, NaOH
Phosphate	5.7-8.0	Monobasic sodium phosphate, dibasic sodium phosphate
Tris	7.2-9.0	Tris (hydroxymethyl) aminomethane
Glycine-NaOH	8.6-10.6	Glycine, NaOH
Carbonate-bicarbonate	9.2-10.7	Sodium carbonate, sodium bicarbonate

Table 2.4 Various classes of additives used in a protein extraction buffer (Adapted from Berkelman *et al.*, 2004).

Class of additive	Example	Concentration	Purpose
Salts	NaCl	50mM-1M	Maintain ionic strength of medium
Detergents	Deoxycholate, SDS	0.1-1.0%	Solubilize poorly soluble proteins
Chaeotropes	GHCl, urea	5-9M	Solubilize poorly soluble proteins
Glycerol		5-10%	Stabilizes proteins
Glucose or sucrose		1-40%	Stabilize lysosomal membranes and reduce protease release
Borate		0.2M	Disrupts interactions due to glycoproteins and other saccharides
Metal chelators*	EDTA, EGTA	1mM-50mM	Reduce oxidation damage and chelate metal ions
Reducing agents	DTT	1-10mM	Reduce oxidation damage
Ligands, metal ions	Mg^{2+}, Ca^{2+}	1-100mM	Stabilizes proteins
Protease inhibitors	Benzamidine, PMSF, ABESF, EDTA, EGTA	1-50mM	Reduce protease release

*NOTE: The use of EDTA and EGTA is not compatible with the presence of Ca^{2+} or Mg^{2+} ions.

2.5.2.1.2 Reverse Phase Liquid Chromatography

Reverse phase liquid chromatography (RPLC) is a powerful technique used for protein and peptide separation (Tarr and Crabb, 1983). RPLC separates proteins and peptides with various hydrophobicity based on their reversible interaction and binding with the hydrophobic surface of a chromatographic medium (i.e. silica beads) (Mant and Hodges, 1991). The downside to using RPLC is the denaturing of proteins due to the presence of strong organic solvents (Mant and Hodges, 1991).

2.5.2.1.3 Protein Precipitation

The goal of EPS protein extraction procedure is to prepare a clarified sample of EPS protein from the source material, as well as removal of particulate matter or other contaminants, which are not compatible with chromatography or Tricine-PAGE. The EPS sample contains impurities that may reduce protein solubility. Therefore, it may be beneficial to remove the nonprotein impurities by protein precipitation methods. Protein precipitation methods are used to concentrate proteins or eliminate interferences before electrophoresis or protein determination. It must be noted however that in almost all the cases the proteins will be denatured and will lose their biological activity (Strop and Brunger, 2005). There are several protein precipitation protocols available in the literature. However, the three protocols commonly used are the Chloroform-Methanol, Trichloroacetic acid (TCA) and Phenol-Ether (PE) protocols (Sauve *et al.*, 1995; Ziegler *et al.*, 1997; Aguilar *et al.*, 1999). These methods employ chemicals in order to effectively precipitate and purify proteins in a given sample.

2.5.2.2 Polyacrylamide Gel Electrophoresis

For purification and separation of small amounts of proteins in a sample, polyacrylamide gel electrophoresis (PAGE) offers best results and excellent resolution (Michalski and Shiell, 1999). PAGE alters the pore size consistent with the nature of the proteins separated (Gersten, 1996). PAGE is also used for its compatibility with mass spectrometry (MS) for subsequent protein characterization (Chevalier *et al.*, 2004). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is frequently employed for separation of proteins based on their molecular mass (Romijin *et al.*, 2003). However, the SDS-PAGE suffers from poor resolution and streaking of proteins below 20 kDa (Schagger and von Jagow, 1987). Tricine-PAGE gives a superior resolution of proteins in the range of 5 and 20 kDa (Schagger and von Jagow, 1987).

To detect proteins in polyacrylamide electrophoresis gels, Coomassie Brilliant Blue and silver nitrate are the most frequent techniques of protein staining, with sensitivity ranges of about 1 mg for Coomassie Brilliant Blue and 1 ng for silver nitrate stain (Wray *et al.*, 1981; Gradilone *et al.*, 1998; Blackstock and Weir, 1999).

2.5.3 EPS Glycoprotein Detection

A vast number of approaches exist for the detection, isolation and characterization of glycoproteins aiming at the identification of the covalent linkage between the carbohydrate and the polypeptide fraction (Schäffer *et al.*, 2001). Relatively few techniques that sensitively and reliably detect carbohydrate residues on proteins in polyacrylamide gels (Liu and Jiang, 1993; Steinberg *et al.*, 2001) are listed below.

2.5.3.1 FTSC Fluorescent Labeling

Gao *et al.* (1997) have improved their method of detecting glycoproteins on SDS-PAGE by using a fluorescent probe, fluorescein 5-thiosemicarbazide (FTSC) (Molecular Probe), in solution before SDS-PAGE. The fluorescent bands of the labeled glycoproteins may then be examined and analyzed in wet or dry gels under a UV lamp with a detection limit of 1-2 ng (Gao *et al.*, 1997). Nonetheless, this method is time consuming (staining takes approximately six hours) compared to periodic acid-Alcian blue stain which takes less than three hours to stain.

2.5.3.2 Periodic Acid—Alcian Blue Stain

The conventional silver staining protocols only weakly stain heavily glycosylated proteins due to steric interferences. To overcome this, Moller and Poulsen (1995) proposed a method for glycoprotein detection in SDS-PAGE by initial periodic acid oxidation/Alcian blue staining and subsequent staining with silver nitrate. Periodate is used to oxidize the carbohydrate groups and generate free aldehyde groups, making them detectable using Alcian blue staining. The Alcian blue stain is often used to stain acidic glycoproteins (Steinberg *et al.*, 2001) and detect up to 1.6 ng of glycoprotein (Moller and Poulsen, 1995). The results obtained are reproducible and obtained within three hours. Conversely, this procedure requires the use of potassium metabisulfite as a washing step during the prestaining of the gels.

2.5.3.3 Periodic Acid—Schiff Stain

Doerner and White (1990) have developed a procedure for staining glycoproteins in a nondenaturing polyacrylamide gel electrophoresis by use of periodic acid-Schiff (PAS) staining which became one of the most commonly practiced methods (Steinberg *et al.*, 2001). The glycols

present in the glycoproteins are first oxidized by periodic acid and then the chromogenic substrate, acid fuchsin sulfite, reacts with the aldehydes on the glycoproteins to create a fuchsia colour product. The PAS stain may detect 25–100 ng of glycoproteins depending on the nature and degree of glycosylation of the protein (Doerner and White, 1990) and the stain is specific for *N*- and *O*-linked oligosaccharides.

2.5.3.4 Deglycosylation of EPS Glycoproteins

Glycosylation often presents problems in protein analysis procedures because glycopeptides usually do not readily ionize during MS analysis, leading to inadequate spectral data. Moreover, proteolytic digestion of the native glycoprotein is frequently unfinished due to steric hindrance by oligosaccharides. Removal of the carbohydrate groups from a glycoprotein prior to protein identification is preferred. For instance, removing N-linked glycans from glycoproteins is very valuable to remove or diminish heterogeneity for MALDI-TOF mass spectrometric analysis (Gates *et al.*, 2004). For entirely sequenced genomes, this alone may be adequate to identify the proteins in the complex.

Deglycosylation of glycoproteins is important in elucidation of structure, function, and biosynthesis of biologically significant glycoproteins. For such studies, it is imperative that the integrity of the polypeptide chain is maintained during deglycosylation (Thotakura and Bahl, 1987). Both chemical and enzymatic methods are available for removing oligosaccharides from glycoproteins.

Several authors have employed hydrazinolysis for removal of both *N*- and *O*-linked sugars (Mizuochi, 1993; Patel *et al.*, 1993; Byers, *et al.*, 1999). The disadvantage of

hydrazinolysis treatment is that it causes complete destruction of the protein component and is as a result not suitable if the recovery of the protein is desired (Harvey, 2001).

A number of papers mention *O*-linked glycans release from glycoproteins by β-elimination with alkali and a reducing agent such as sodium borohydride (Slomiany *et al.*, 1984; Chiba *et al.*, 1993; Hong and Kim, 2000). This method also suffers from disadvantages, including the use of borohydrate, which releases some *N*-linked glycans, and removal of *O*-acetyl groups (Harvey, 2001).

Milder chemical method, which has been extensively used, is trifluoromethanosulfonic acid (TFMS) treatment (Sojar and Bahl, 1987; Edge, 2003). This method has been successfully used on *O*-linked (Edge *et al.*, 1981; Florman and Wassarman, 1985; Herzberg *et al.*, 1985) and *N*-linked glycans (Karp *et al.*, 1982; Harrison, 1983; Paxton *et al.*, 1987; Naim *et al.*, 1988). However, this method even when optimized, results in incomplete sugar removal and partial protein destruction (Thotakura and Bahl, 1987).

However, when using the enzymatic method, complete sugar removal is achieved without any protein degradation (EMD Biosciences, 2005). A wide variety of specific glycosidases which cleave carbohydrates and oligosaccharide chains have been found to be potentially useful. Enzymatic deglycosylation can be brought about by exoglycosidases either used sequentially or in a mixture (Thotakura and Bahl, 1987; Benz and Schmidt, 2002). The most common enzyme used for release of *N*-glycans is *N*-glycosidase F (PNGase-F) (Harvey, 2001). Enzymatic deglycosylation of glycoproteins may be accomplished in-solution or in-gel digestion of glycoprotein samples followed by MS.

Glycosylation sites and oligosaccharide heterogeneity in glycoproteins have been characterized based on a combination of nonspecific proteolysis using a pronase enzyme,

enzymatic deglycosylation using PNGase F enzyme, and matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FT MS) (An *et al.*, 1993). It has been identified that the peptide moiety of the glycopeptides to be *Xenopus laevis* egg cortical granule lectin. Thus, the enzymatic deglycosylation method was found to be useful for determining protein site heterogeneity and structural heterogeneities of the oligosaccharide moiety of glycoproteins (An *et al.*, 1993).

The native glycoproteins and the material obtained after enzymatic deglycosylation may be analyzed on polyacrylamide electrophoresis gels (Schäffer *et al.*, 2001; Kilz *et al.*, 2002). The separation is based on protein mass and a considerable reduction in molecular weight, and thus a rise in electrophoretic mobility, is a good indication that Asn-linked oligosaccharides have been freed enzymatically (Tarentino *et al.*, 1989). The polyacrylamide electrophoresis gel may obtain the molecular weight of the glycoprotein, the glycoprotein's purity and homogeneity, and the degree of glycosylation (Schäffer *et al.*, 2001).

2.5.4 EPS Protein Identification Using Mass Spectrometry (MS)

Due to numerous possible structural isomers in glycoproteins, a complete structural analysis is not possible. Nuclear magnetic resonance (NMR) is a good technique for protein and glycoprotein structural analysis; however, the method is very insensitive when compared to mass spectrometry (MS) and chromatography (Harvey, 2001). MS is the best technique for structural analysis of proteins (Gygi and Aebersold, 2000) and especially *N*-linked glycans (Harvey, 2001). The fragment-ion masses obtained on MS are unique identifiers for that peptide (Gygi and Aebersold, 2000). The molecular weight of a protein is insufficient for protein identification; thus, most approaches to protein identification rely on proteolysis of the separated protein with

trypsin and analysis of the resulting peptides, often without any further separation (Blackstock and Weir, 1999).

Database searching uses uninterpreted fragment-ion MS and a cross-correlation algorithm to compare the sequence obtained with the predicted spectra (Blackstock and Weir, 1999). These databases may be accessed via available internet software tools (Hillenkamp *et al.*, 1991; Kellner, 2000). These tools include such molecular biology servers as EXPASY (www.expasy.ch; SIB Swiss Institute for Bioinformatics, Geneva, Switzerland), which is dedicated to analysis of protein sequences and structures as well as 2-D PAGE. Search engine MASCOT (www.matrixscience.com; Matrix Sciences Ltd., London, UK) uses MS data to identify proteins from primary sequence databases.

2.5.4.1 Tandem Electrospray Ionization Mass Spectrometry (LS/ESI-MS/MS)

MS is one of the most sensitive methods for protein and in particular glycoprotein analyses but it is unable to separate isomers. As a result, MS is used in combination with high performance liquid chromatography (HPLC) utilizing an electrospray (ESI) interface for total glycoprotein profiles (Harvey, 2001). The main feature of these alternative separation methods integrated with MS is the capability of a tandem mass spectrometer to collect sequence information from a specific peptide, even if other peptides are present in the sample. When the mixtures are very complex, on-line reverse-phase liquid chromatography (RPLC) is utilized to concentrate and separate the peptides before sequencing in the MS.

Crude protein digestion and subsequent LC-ESI-ION TRAP approach has been a successfully utilized for yeast, rice, and wheat analysis (Washburn et al., 2001; Koller et al., 2002; Andon et al., 2002) and LC/ES-MS has been used in order to selectively detect

glycopeptides in proteolytic digests of glycoproteins (Carr et al., 1991). Therefore, this approach may be able to successfully analyze EPS proteins in activated sludge flocs.

2.5.4.2 Matrix-Assisted-Laser-Desorption-Ionization Mass Spectrometry (MALDI MS)

Mass spectrometers continue to develop, due to the requirement for improved sensitivity, higher mass accuracy and resolving power, enhanced duty cycle and more effective fragmentation of peptides in tandem mass spectra (MS/MS) (Jensen, 2004). Fast atom bombardment (FAB) used to be the preferred choice for analysis of intact glycans but this method is not sensitive enough due to a high matrix background. Therefore, matrix-assisted-laser-desorption-ionization (MALDI) and ESI are favoured for proteomic study with a sensitivity of low pmole to high fmole range (Harvey, 2001). MALDI MS/MS methods are currently used for sequencing of post-translationally modified peptides (*e.g.*, phosphopeptides (Lee *et al.*, 2001; Bennett *et al.*, 2002) due to MALDI robustness and high sensitivity (Larsen *et al.*, 2002) and usefulness in identifying the carbohydrate moiety (Stimson *et al.*, 1995).

Currently there is a growing interest in combining MALDI MS/MS techniques with miniaturized peptide separation techniques. *In situ* liquid-liquid extraction is an easy and quick approach for the separation of hydrophobic and hydrophilic peptides before MALDI MS and MS/MS. This technique may permit more efficient detection and sequencing of modified, hydrophobic peptides, especially for acylated peptides. Graphite columns allow capture of otherwise elusive hydrophilic peptides, such as phosphopeptides and glycopeptides, for their analysis by MS (Larsen *et al.*, 2002). Moreover, capillary chromatography and electrophoresis have been interfaced to MALDI MS/MS in various ways (Mo and Karger, 2002) to facilitate in-depth analysis of crude peptide mixtures (Larsen *et al.*, 2002).

2.5.4.3 Matrix-Assisted-Laser-Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Matrix-assisted-laser-desorption-ionization time-of-flight (MALDI-TOF) MS is a widely used technique for sensitive and rapid identification of mixtures of peptides, crude extracts or large proteins separated by gel electrophoresis (Hillenkamp *et al.*, 1991; Blackstock and Weir, 1999; Gates *et al.*, 2004). This tool may be utilized in order to determine the molecular weights of EPS glycoprotein released oligosaccharides. The drawback of this procedure is a possible poor mass resolution of glycoproteins.

Based on the aforementioned review of various methods that may be utilized for EPS protein analysis and characterization, the next section will describe the methods chosen for this study in purification and identification of EPS proteins in activated sludge flocs.

CHAPTER 3. EXPERIMENTAL MATERIALS AND METHODS

3.1 Overview

This study encompassed an extraction of extracellular polymeric substances (EPS) and EPS proteins obtained from microbial flocs (Figure 3.1). Furthermore, the glycoprotein contribution to EPS structure was examined (Figure 3.1). This chapter describes the materials and methods employed during the present study, including experimental approach, extraction and chemical analysis of EPS, extraction and identification of EPS proteins, glycoprotein detection, and statistical analysis.

3.2 Experimental Approach

Biomass samples were collected for this study from four parallel 2L glass laboratory-scale sequencing batch reactors (SBRs) and full-scale Ashbridges Bay Wastewater Treatment Plant (WWTP) (formerly known as Toronto Main WWTP; Toronto, ON, Canada).

The SBRs were run for a two-year period to obtain true stable conditions (Liu *et al.*, 2006). All of the stages in the activated sludge process occurred in the same vessel in SBRs. A single cycle consists of four steps, which include fill, aeration, settling, and withdrawal. Initially, the reactor was filled with wastewater. Subsequently, the biological reaction stage took place for 5 hours under mechanical aeration, where the nutrients in the wastewater are broken down by the activated sludge. Following the reaction stage, mixing and aeration stopped, and the solid-liquid separation stage occurred for 6 hours during which the solids in the sludge settled. Lastly, the top liquid effluent was drawn off and solids remained in the reactor.

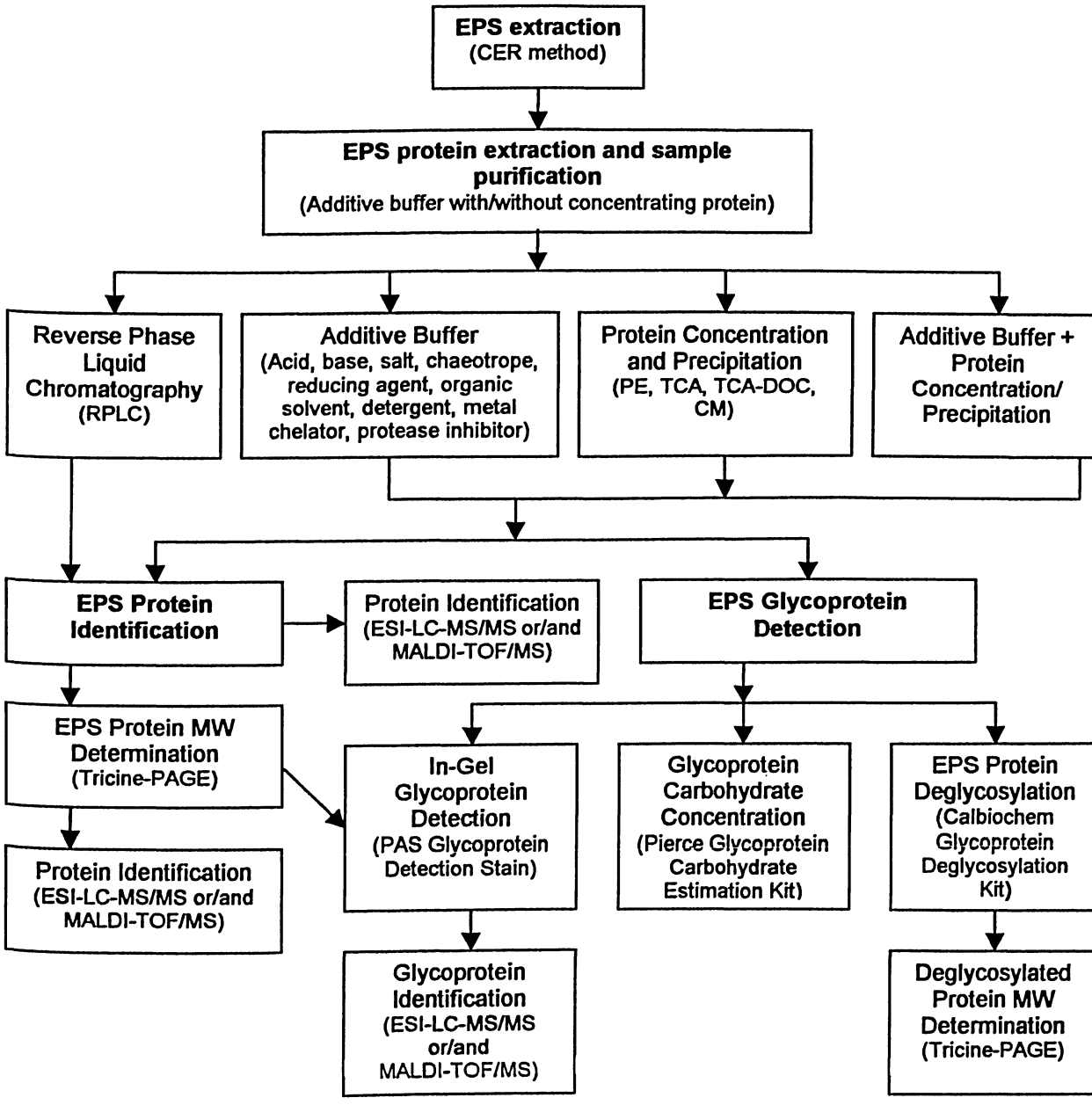


Figure 3.1 Experimental procedures for EPS protein extraction, purification and identification.

The pH was controlled (7.0 +/-0.2) by pH controllers (pH/ORP controller Model 5656-00, Cole Parmer, Canada) and by addition of sodium hydroxide (NaOH) solution (0.02N). Air was introduced through a stone air diffuser as an oxygen supply and a magnetic stir bar was used for mixing the contents inside the reactor. Electronic timers controlled the four stages of the activated sludge process cycle in the SBRs. The SBRs were fed daily with 4°C synthetic water containing glucose (Sigma Chemical Co., St. Louis, MO) and inorganic salts (Fisher Scientific, Fair Lawn, NJ) at different COD:N:P ratios. Reactors 1 and 2 contained COD:N:P ratios of 100:5:1 and reactors 3 and 4 contained COD:N:P ratios of 100:5:0.1. The sludge retention time (SRT) of the reactors was 6-8 days.

When the reactors were suspended, an aerobic sludge was obtained from the Ashbridges Bay Treatment Plant (formerly known as Main Treatment Plant, Toronto, Ontario) and immediately utilized for EPS extraction to retain sludge properties (Nielsen *et al.*, 1996; Bura *et al.*, 1998).

3.3 Microbial Floc Physicochemical Properties

Mixed liquor suspended solids (MLSS) concentration was measured in accordance with Standard Methods (APHA, 1995). MLSS is comprised of active biomass, non-active biomass, nonbiodegradable organic mass, and inorganic mass. An aliquot of well-mixed mixed liquor sample (5 mL) was filtered through a weighed standard glass-fibre filter (Whatman, 45 μm) and the retained solids on the filter were dried at 103-105°C for one-two hours. The MLSS presented the weight of the retained solids of the filter.

Sludge volume index (SVI) was used to determine the separation and settling ability of the sludge prior to extraction of the EPS from activated sludge flocs. The measurement was

taken in a 1,000 ml glass cylinder following sedimentation of the sludge after 30 minutes according to the Standard Methods (APHA, 1995). Typically, the sludge with good stability contains SVI values of less than 100 ml/g and poor sedimenting sludge has SVI values of above 100 ml/g (Martinez et al., 2004).

3.4 Extracellular Polymeric Substances (EPS)

3.4.1 Cation Exchange Resin (CER)

The cation ion exchange resin (DOWEX® C-211, Na^+ form, 16–50 mesh) (Sigma Chemical Company, St. Louis, MO, USA) method was employed for the extraction of EPS (Frølund *et al.*, 1996; Bura *et al.*, 1998). The total EPS concentration was measured as the total carbohydrate, protein and DNA given that these are the major constituents that represent the extracted EPS fraction (Frølund *et al.*, 1996; Bura *et al.*, 1998; Liao *et al.*, 2001). An activated sludge was diluted to one fifth of the original mixed liquor suspended solids (MLSS) concentration (determined by standard methods (APHA, 1995)) and washed at least three times with the EPS extraction buffer [Na3PO4 (2 mN w/v), NaH_2PO_4 (4 mN w/v), NaCl (9 mN w/v), KCl (1 mN w/v) in distilled deionized water (1 L, pH 7.0)] and centrifuged at 2000 *g* for 5 minutes at 4°C (IEC Model HN-S Centrifuge; Needham Heights, MA, USA). The exchange resin (after determination of the amount of MLSS in the washed sludge) was added to a washed sludge sample (45 mL) and mixed with a single blade paddle at 600 rpm for 2 hours at 4°C to minimize cell lysis (Frølund *et al.*, 1996). The mixture was then centrifuged at 12,000 *g* for 15 min at 4°C (Hermle Labnet Z 323 K Centrifuge) to remove solids and filtrated through 0.45-um acetate cellulose membrane. The EPS fraction was stored at –20°C for future chemical analysis of carbohydrates, proteins, DNA, and protein extraction.

3.4.2 Chemical Analyses of EPS

The CER-extracted EPS was analyzed for total carbohydrates (Masuko *et al.*, 2005), proteins (Pierce Biotechnology, 2002), and DNA (Brunk *et al.*, 1979) and EPS composition was determined by employing colorimetric methods. ELISA Plate Reader and SP6-500 UV spectrophotometer (Pye Unicam/Thermo Electron Corporation, Waltham, MA, USA) were utilized for protein and carbohydrate analyses. Fluorometer (Model 110, G.K. Turner Associates, USA) was used for DNA analysis. Ultrapure water was used as a blank solution. All chemicals were of analytical grade. A standard curve was produced for each sample tested and concentrations were determined from the linear regression equation of the standard curve.

3.4.2.1 EPS Protein Concentration

The protein concentration in microbial EPS was initially measured calorimetrically by the methods of Lowry *et al.* (1951), bicinchoninic acid (BCA™) (Pierce Biotechnology, 2002) and modified Bradford (1976) (Ghosh *et al.*, 1988), using bovine serum albumin (BSA) solution as a standard. However, BCA protein assay was found to be ideal for this study.

3.4.2.1.1 Bicinchoninic Acid (BCA) Procedure

Bicinchoninic acid (BCA) (Smith et al., 1985; Tylianakis *et al.*, 1994; Pierce Biotechnology, 2002) protein assay, similar to Lowry *et al.* (1951) procedure, is based on its ability to reduce of Cu^{2+} to Cu^{+} by amides. The BCA™ Protein Assay (Pierce Biotechnology, 2002) microplate procedure was employed based on bicinchoninic acid (BCA) for the colorimetric detection and measurement of the total protein. Bovine serum albumin (BSA) was

used to prepare a standard calibration curve (absorbance at 560 nm vs. protein concentration) for each unknown protein sample.

An aliquot (25 µl) was pipetted into a microplate well (working range 20-2,000 µg/ml). Working reagent [50:1 ratio of BCA™ Reagent A to BCA™ Reagent B] was added to each well and the microplate was mixed thoroughly. The microplate was covered and incubated at 37°C for 30 minutes. The microplate was cooled to room temperature and the absorbance was measured at 560 nm on a plate reader. The BCA was determined to be the method of choice for total protein content in the EPS.

3.4.2.2 EPS Polysaccharide Concentration

Both the anthrone method (Gaudy, 1962) and phenol-sulfuric acid procedure (Dubois *et al.*, 1956) are often employed in order to measure the concentration of total polysaccharides in EPS (Frølund *et al.*, 1996), with glucose as the standard and the amount of exocellular polysaccharide expressed as µg of glucose/ml of sample. The total polysaccharide concentration in microbial EPS was quantified calorimetrically by the Anthrone method (Gaudy, 1962) and phenol-sulfuric acid method (Masuko *et al.*, 2005) modified from Dubois *et al.* (1956), using glucose as a standard.

3.4.2.2.1 Phenol-Sulfuric Acid Microplate Procedure

The phenol-sulfuric acid approach employs EPS combined with 5% phenol in water and concentrated sulfuric acid. In contrast to the Anthrone procedure, the phenol-sulfuric acid approach is very practical for the quantitative colorimetric microdetermination of methylated sugars, oligosaccharides and polysaccharides. In addition, the phenol-sulfuric acid method is

simple, economical, rapid and sensitive with reproducible results (Dubois *et al.*, 1956). The phenol-sulfuric acid method was employed in a 96-well microplate format, which is specifically optimized for a smaller sample analysis. D-glucose was used to prepare a standard calibration curve and the procedure was carried out as described by Masuko *et al.* (2005) with minor modifications.

Fifty microliters of aliquot solution was added to a 96-well microplate. 50 ul of 5% (v/v) phenol (Fisher Scientific, Fairlawn, NJ, USA) was added to the samples and mixed. 250 ul of concentrated sulfuric acid (Fisher Scientific, Fairlawn, NJ, USA) was immediately added following phenol addition. The samples were incubated for 10 minutes at 37°C. The samples were measured spectrophotometrically by the use of an ELISA plate reader at 490 nm.

3.4.2.3 EPS DNA Concentration

Deoxyribonucleic acid (DNA) was measured by the use of a standard fluorescent DNA quantification kit (Bio-Rad Laboratories, Hercules, CA, USA) and a Turner-Model 111 Fluorometer (Coleman Inc., Lorton, VA, USA). Calf thymus DNA was used to prepare a standard calibration curve. Hoechst dye was added to each sample in triplicate and the absorbance was measured employing 360 nm excitation filter and 460 nm emission filter.

3.5 EPS Protein Extraction and Purification

In order to satisfy the objectives in this study, a flow chart of protein identification and characterization methodology was established (Figure 3.2). In order to remove EPS proteins from the complex and rigid EPS that surrounds the bacteria in activated sludge flocs, some preliminary extraction steps must be conducted. Once the appropriate EPS extraction method is

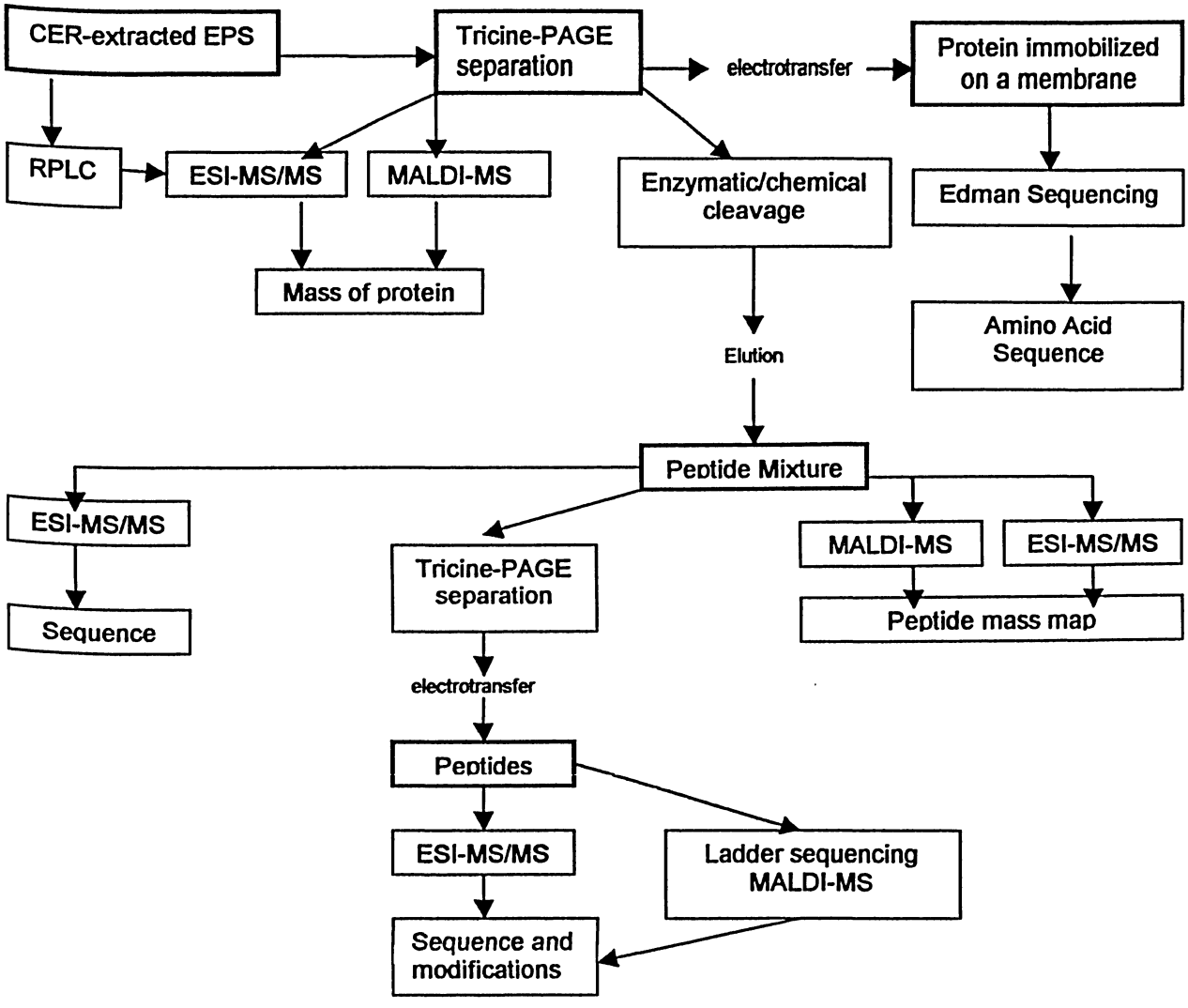


Figure 3.2 A flow chart for obtaining a sequence of electrophoretically separated EPS proteins (Adapted from Michalski and Shiell, 1999).

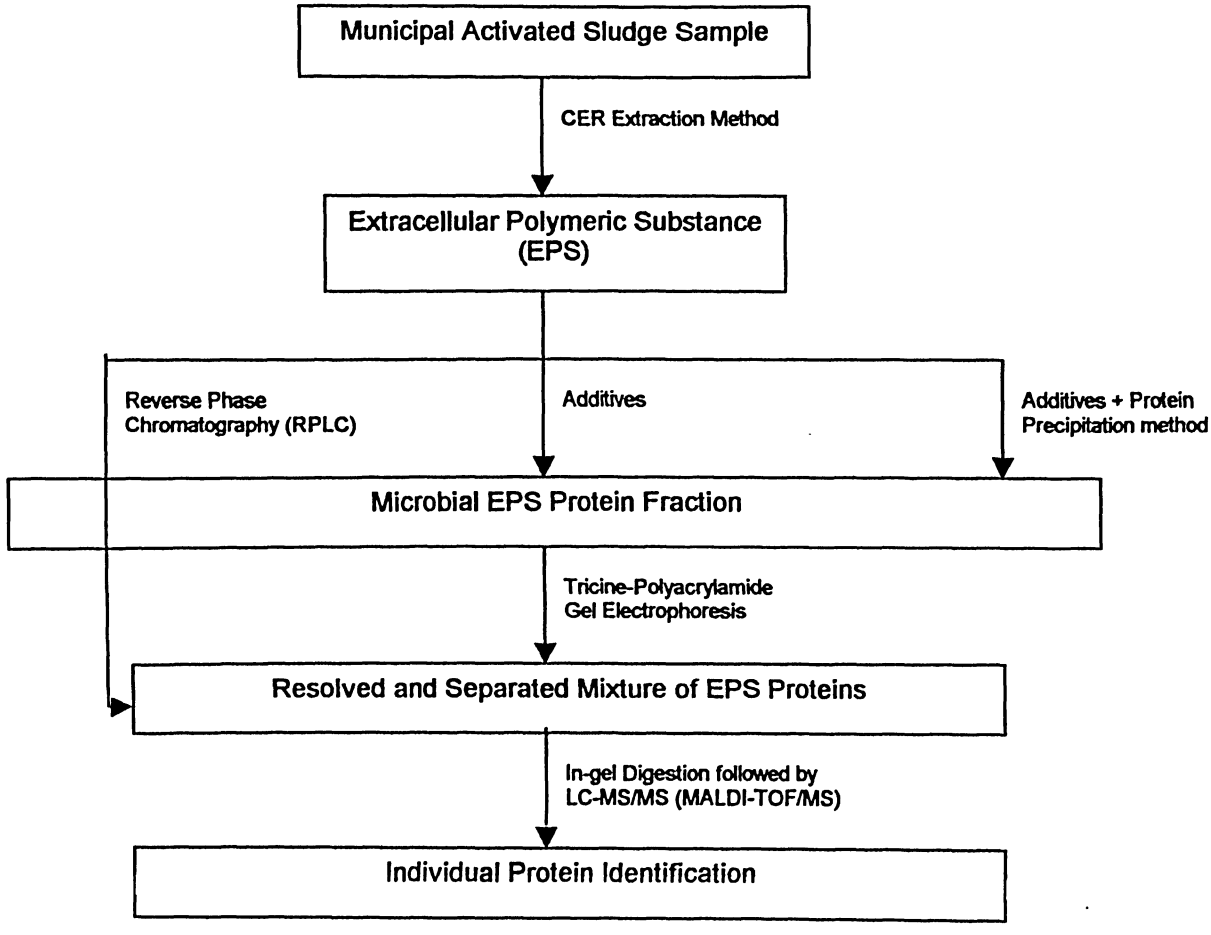


Figure 3.3 Flow chart of methodology for extracting, purifying, separating, and identifying EPS proteins.

selected, the microbial EPS protein fraction should be extracted, separated, purified, and identified. The microbial EPS protein fraction would then be identified by the use of mass spectrometry. Initially, the experimental flow chart in Figure 3.2 was devised and later on it was translated into flow chart methodology in Figure 3.3. The ultimate goal of the separation and identification of individual proteins in the EPS matrix is to obtain a primary amino acid sequence in order to identify and characterize the unknown proteins and protein families in the microbial EPS.

From the flow chart it may be observed that there are two steps for general protein identification: electrophoresis and mass spectrometry. These two methods can provide microscale analyses of peptides and proteins (Cohen and Chait, 1997). Initially, electrophoresis is employed for separation of complex protein mixtures and obtaining estimates of protein molecular masses (Cohen and Chait, 1997). Mass spectrometry provides a rapid, sensitive, and accurate determination of proteins and peptides (Michalski and Shiell, 1999).

All experiments involving EPS protein extraction and purification were conducted in an ice-bath to ensure a temperature of approximately 4°C. A 1:100 dilution inhibitor cocktail P8340 (Sigma Chemical Company, St. Louis, MO, USA) mix (4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin) was added prior to protein extraction process to prevent protein degradation.

3.5.1 Sources of Error

The CER-extracted EPS from WWTP was assessed for potential induced lysis by rupturing the cells present in the activated sludge portion or examining the cells under Zeiss inverted microscope (Axiovert 200, Carl Zeiss) at a magnification of 400x, and comparing the

released proteins in the activated sludge to the possible EPS proteins from activated sludge floc. The EPS was tested for protein interferences originating from dust. The activated sludge and EPS were sonicated, heated with SDS (1-2% w/v), lysed with TRIZOL® Reagent, and violently agitated to test for induced cell lysis proteins interferences.

3.5.1.1 Error from Dust

A dust sample (30 mg), from five different locations in the laboratory room where the experiments were carried out, was boiled in 1%-2% SDS for 10-15 minutes to solubilize and denature all proteins in the dust sample and these proteins were compared to the proteins in the EPS fraction and to the proteins in the activated sludge supernatant (sludge water). The sludge supernatant was found to contain a negligible amount of protein, carbohydrate and DNA. This finding is supported by Nielsen *et al.* (1996), and thus it was required to concentrate the sludge supernatant in order to visualize the proteins on Tricine-PAGE. It was observed that the overall protein pattern found in the dust sample does not match the possible activated sludge derived EPS protein pattern. However, the bands in the regions 67-70 kDa of the dust protein pattern coincided with the possible microbial EPS protein pattern (Appendix F). Due to the variable nature of sludge, it is foreseeable that these keratin bands may be interferences from numerous potential sources (*e.g.*, from laboratory or from WWTP effluent). To limit the interference from potential ambient dust sources within the laboratory environment, samples were handled under a flame or in the fumehood.

3.5.1.2 Error from Induced Cell Lysis

To test for potential induced cell lysis, the biomass sample and CER-extracted EPS were sonicated with Dawe Soniprobe (Dawe Instruments Ltd., London, UK) at 60 W with a frequency of 20 Hz. The samples were sonicated with bursts lasting no longer than several seconds, with cooling on ice between bursts for a total of three minutes. The activated sludge and EPS were boiled in SDS (1-2% w/v) for 10-15 minutes. The activated sludge and EPS were lysed with TRIZOL® Reagent (Molecular Probes/Invitrogen, Eugene, OR, USA) and/or violently agitated at 3000 *g* with Mini-Bead-Beater-8™ (BioSpec Products, Inc., Bartlesville, OK, USA). In this method the samples were agitated with bursts lasting no longer than thirty seconds, with cooling on ice between bursts for a total of three minutes. The bead size of 0.1 mm was utilized for bacterial cell lysis. TRIZOL®, Reagent (1 part TRIZOL® to 10 parts sample) was found to be incompatible with the Tricine-PAGE system.

3.5.2 Separation of Intracellular and Exocellular Proteins in the EPS

To separate possible intracellular proteins in the EPS and in the sludge supernatant from the exocellular protein fraction, the dried fractions were washed with ice-cold ethanol (80% w/v) or ice-cold SDS (0.1% w/v) three to four times. The EPS tested was untreated (control), treated with only protease inhibitors, or treated with protease inhibitors and 2mM DTT (concentrated by phenol-ether method).

3.5.3 Buffer Additives

The protein buffer consisted of ultrapure water (Milli-Q water system, water quality 17-18 MΩ·cm at room temperature) containing protease inhibitors at neutral pH. If necessary the pH

was adjusted to neutral by 50mM Tris-HCl pH 8.8. The initial preparation of protein extraction involved placing 0.5-1.5ml of CER-extracted EPS into a microcentrifuge tube (1.5 ml) and speed drying the EPS sample in SpeedVac vacuum concentrator (Model: DNA110-120; Savant Instruments, Farmingdale, NY, USA). The EPS samples were resuspended in extraction buffer with additives and a protease inhibitor cocktail to the original volume and the mixture was incubated for up to 96 hours (Tables 3.1-3.3).

After the addition of chemical additives buffer in the EPS sample mixture, it was observed that the additives caused incompatibility with Tricine-PAGE. When the sample was speed dried under vacuum, many additives remained in the sample and did not dissolve in the 2x sample dye buffer (Laemmli, 1970) (Data not shown). Therefore the samples containing salts (NaCl, NH_4Cl, $CaCl_2$, $MgCl_2$, $Fe(NO_3)_3$) or a chelating agent (EDTA) were desalted employing a group separation utilizing Sephadex (a beaded gel filtration medium prepared by cross-linking Dextran with epichlorohydrin) G-25 in superfine size beads (Pharmacia Biotech, Piscataway, NJ, USA). Microspin column (3,000-5,000 *g* for 3-5 minutes) desalting procedure was employed for high efficiency at high flow rates. Molecules larger than 1-5 kDa were excluded while smaller molecules entered the beads. The beads were preswollen in ethanol (20% v/v) to prevent microbial contamination and stored in 4°C. Resin slurry (1 ml) was pipetted into a microspin column and rinsed three times with ultrapure water. The EPS sample (300 µl) was added to the resin and spun in a centrifuge at 3,000 *g* for 3 minutes. The flow through eluate was collected and the samples were dried in the SpeedVac and resuspended in sample dye buffer. The resuspended samples were loaded on Tricine-PAGE gel (8-12%) and the protein bands were visualized by employing Coomassie blue, colloidal Coomassie silver, SYPRO Ruby, and silver nitrate stains. The best Tricine-PAGE gel concentration was found to be 10% (data not shown).

Table 3.1 A summary of various chemical additives (salts and metal chelator) that may be used to extract proteins from activated sludge EPS.

Class of additive	Specific additive used	Temperature of incubation (°C)	Additive concentration	Time of incubation (hours)	Manufacturer
Monovalent Salt	NaCl	23, 4	0.1-1M	3	US Biological, Swampcott, MA, USA
	NH_4Cl	4	0.1M	3*, 24, 48, 96	Sigma Chemical Company, St. Louis, MO, USA
Divalent Salt	$CaCl_2$	23, 4	0.1M	3*, 24, 48, 96	J.T. Baker, Philipsburg, NJ, USA
	$MgCl_2$	23, 4	0.1M	3, 24, 48, 96	J.T. Baker, Philipsburg, NJ, USA
Trivalent Salt	$Fe(NO_3)_3$	4	0.1M	3, 24, 48, 96	J.T. Baker, Philipsburg, NJ, USA
Metal chelator	EDTA	23, 4	10mM, 50mM pH 6.5	3*, 24, 48, 96	Sigma Chemical Company, St. Louis, MO, USA
	EGTA	23, 4	10mM pH 6.5	3, 24, 48, 96	BioShop Canada, Burlington, ON

Table 3.2 A summary of various chemical additives (detergents, chaeotropic agents, organic solvents) that may be used to extract proteins from activated sludge EPS.

Class of additive	Specific additive used	Temperature of incubation (°C)	Additive concentration	Time of incubation (hours)	Manufacturer
Detergent	DOC	4	0.05%	1	Sigma-Aldrich, Oakville, ON
	SDS	23, 4	0.1%	1-30minutes, 1, 3	BioShop Canada, Burlington, ON
	TRITON® X-100	4	0.1%	3	BioShop Canada, Burlington, ON
Chaeotropic agent	GHCl	23	6M	24	ICN Biomedicals, Costa Mesa, CA, USA
	GHCl + 0.1% TFA†	23	6M	24	Sigma-Aldrich, Oakville, ON
	Urea	23, 4	8M	3*, 24	Sigma Chemical Company, St. Louis, MO, USA
Organic solvent	50% acetonitrile	23, 4		3*, 24	Caledon Laboratories, Georgetown, ON
	50% acetonitrile + 0.1%TFA†	23, 4		3*, 24	Sigma-Aldrich, Oakville, ON
	Ethanol	4	70%	48	Caledon Laboratories, Georgetown, ON
	Formic acid	23	0.2%	24	Sigma Chemical Company, St. Louis, MO, USA

†NOTE: TFA was used in order to provide conditions for complete protein denaturation and unfolding.

Table 3.3 A summary of various chemical additives (acid, base, reducing agent, borate) that may be used to extract proteins from activated sludge EPS.

Class of additive	Specific additive used	Temperature of incubation (°C)	Additive concentration	Time of incubation (hours)	Manufacturer
Acid*	HCl	23, 4	0.1-0.2%	3, 24	Sigma Chemical Company, St. Louis, MO, USA
Base*	NaOH	23, 4	0.1%	3, 24	EMD Biosciences, Darmstadt, Germany
Reducing agent*	DTT	4	2mM	3	EMD Biosciences, Darmstadt, Germany
Borate*		4	0.2M pH 7.6	3	Sigma Chemical Company, St. Louis, MO, USA

3.5.4 Reverse Phase Liquid Chromatography

Reverse phase liquid chromatography (RPLC) was utilized in a batch mode to separate proteins and peptides within the EPS with various hydrophobicity, and to obtain samples with limited number of carbohydrates. Aqueous activated sludge EPS proteins and peptides were bounded to C_{18} reversed phase silica beads resin (Fluka Biochemica/Sigma-Aldrich, Oakville, ON, Canada) through hydrophobic interactions, allowing small molecules—including salts, buffers and chaeotropes—to be removed. The EPS proteins and peptides were subsequently eluted with acetonitrile (AcN) (50% v/v) (Caledon Laboratories LTD, Georgetown, ON, Canada) in water with trifluoroacetic acid (TFA) (0.1% v/v) and formic acid (5% v/v) (Naldrett *et al.*, 2005).

Resin (1 ml) was presoaked in 5ml of 100% AcN for 20-30 minutes in a 15-ml tube (1:5 ratio). Suspended resin (250 µl) was transferred to a microcentrifuge tube (tube 1) and centrifuged at 11,000 *g* for 30 seconds. The supernatant was decanted and AcN (400 µl of 50% v/v) + formic acid (0.1% v/v) was added to tube 1, resuspended and centrifuged at 11,000 *g* for 30 seconds. The supernatant was decanted once again and 400ul of AcN (5% v/v) + formic acid (0.1% v/v) was added to tube 1, resuspended and centrifuged at 11,000 *g* for 30 seconds. The addition of AcN (400 µl of 5% v/v) + formic acid (0.1% v/v) to tube 1 was repeated two more times. The dried EPS sample was resuspended in second microcentrifuge tube (tube 2) with acetic acid (200 µl of 0.1% v/v) + TFA (10 µl) (Fluka Biochemica/Sigma-Aldrich, Oakville, ON, Canada). Accounting for the binding capacity of the resin an appropriate amount of sample from tube 2 was transferred to tube 1, resuspended and centrifuged at 11,000 *g* for 30 seconds. The EPS sample in tube 1 was then resuspended in of formic acid (400 µl of 0.1% v/v) and this was repeated two more times. For protein elution, AcN (400 µl of 70% v/v) was added to tube 1,

resuspended and centrifuged at 11,000 *g* for 30 seconds. The supernatant collected was dried in a SpeedVac and either resuspended in 2x sample buffer or digested with trypsin in 1x digestion buffer and ran on ESI/LC-MS/MS.

3.5.5 EPS Protein Concentration and Precipitation

In this study, the Chloroform-Methanol (C-M), Trichloroacetic acid (TCA) and Phenol-Ether (P-E) protocols (Sauve *et al.*, 1995; Ziegler *et al.*, 1997; Aguilar *et al.*, 1999) were employed for protein precipitation and purification.

3.5.5.1 Chloroform-Methanol (C-M) Method

Four volumes of methanol were added to 1 volume of EPS and vortexed well. Chloroform (100 µl) (Caledon Laboratories LTD, Georgetown, ON, Canada) was added and mixed. Ultrapure water (300 µl) was added and vortexed. The mixture was centrifuged at 15,000 *g* for 5 minutes at 4°C and the proteins are located at the interphase. The aqueous top layer was gently removed and 4 volumes of methanol were added and vortexed. The samples were centrifuged at 14,000 *g* for 5 minutes at 4°C. The supernatant was removed and the lower aqueous phase was dried by centrifugation under vacuum. Finally, the dry samples were mixed with 2x sample buffer for Tricine-PAGE.

3.5.5.2 Trichloroacetic Acid (TCA) Method

3.5.5.2.1 TCA Method

TCA (20% w/v) (Fisher Scientific, Fairlawn, NJ, USA) was added to the EPS sample and the sample was left on ice for 1 hour before centrifugation at 12,000 *g* for 30 minutes at 4°C. The residual liquid was removed and dried before mixing with 2x sample buffer for Tricine-PAGE.

3.5.5.2.2 TCA-Acetone Method

TCA (20% w/v) was added to the EPS sample and the sample was left on ice for 1 hour before centrifugation at 12,000 *g* for 30 minutes at 4°C. The residual liquid was removed and the remaining pellet was rinsed twice with cold acetone (200 µl) and dried before mixing with 2x sample buffer for Tricine-PAGE.

3.5.5.2.3 TCA-Sodium Deoxycholate (DOC) Method

TCA (20% w/v) was added to the EPS sample and 0.05% sodium deoxycholate (DOC) (Sigma-Aldrich, Oakville, ON, Canada). The sample was left on ice for 1 hour before centrifugation at 12,000 *g* for 30 minutes at 4°C. The residual liquid was removed and dried before mixing with 2x sample buffer for Tricine-PAGE.

3.5.5.2.4 TCA-DOC-Ethanol Method

TCA (20% w/v) and deoxycholate (DOC) (0.05% w/v) were added to the EPS sample. The sample was left on ice for 1 hour before centrifugation at 12,000 *g* for 30 minutes at 4°C. The residual liquid was removed and the remaining pellet was rinsed twice with pure cold ethanol (400 µl) and dried before mixing with 2x sample buffer for Tricine-PAGE.

3.5.5.3 Phenol-Ether (P-E) Method

Phenol (Fluka Biochemica/Sigma-Aldrich, Oakville, ON, Canada) was added to EPS in a 1:1 ratio and the sample mixture was vortexed for 20 seconds, followed by centrifugation at 12,000 *g* for 5 minutes at 4°C. After removal of the top aqueous phase, two volumes of ether (Sigma Chemical Company, St. Louis, MO, USA) (1ml maximum) were added to the phenol phase. The mixture was vortexed for 20 seconds and centrifuged at 12,000 *g* for 5 minutes at 4°C (proteins should be at the interphase). Two volumes of ether were added once again to the EPS samples, vortexed for 20 seconds, and centrifuged at 12,000 *g* for 5 minutes at 4°C to remove residual phenol. The lower aqueous phase was dried by centrifugation under vacuum and the dry samples were mixed with 2x sample buffer for Tricine-PAGE.

3.5.6 Additive Buffer Combined with EPS Protein Concentration/Precipitation

After successfully precipitating the EPS proteins, the next step was to extract the protein by using additives and then effectively concentrate the dilute protein solution prior to Tricine-PAGE.

3.5.7 Tricine Polyacrylamide Gel Electrophoresis

For purification and separation of small amounts of protein sample, polyacrylamide gel electrophoresis (PAGE) offers best results and excellent resolution (Michalski and Shiell, 1999) by altering the pore size consistent with the nature of the proteins separated (Gersten, 1996). PAGE is also used for its compatibility with mass spectrometry (MS) for subsequent protein characterization (Chevalier *et al.*, 2004). Sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) is frequently employed for separation of proteins based on their molecular mass (Romijin *et al.*, 2003). However, the SDS-PAGE suffers from poor resolution and streaking of proteins below 20 kDa (Schagger and von Jagow, 1987). Tricine-PAGE gives a superior resolution of proteins in the range of 5 and 20 kDa and thus was employed instead of SDS-PAGE in this study (Schagger and von Jagow, 1987). This method separates negatively charged proteins in polyacryamide gel with a built-in pH gradient using Tris-tricine (Michalski and Shiell, 1999) discontinuous buffer system of Laemmli (Laemmli, 1970).

Approximately 10 ml of 10% separating gel mix [5.0 ml of 30% Acrylamide-Bis mix (29:1) (BioShop Canada, Burlington, ON), 4.5 ml of 3.0 M Tris-HCl in 0.3% (w/v) SDS, pH 8.0 (Fisher Scientific, Fairlawn, NJ, USA), 4.0 ml of ultrapure water, 1.5 ml glycerol (Fisher Scientific, Fairlawn, NJ, USA), 50 µl of 20% (w/v) ammonium persulfate (APS) (Bio-Rad Laboratories, Hercules, CA, USA), and 20 µl of TEMED (BioShop Canada, Burlington, ON, Canada)] was poured between the glass plates. The separating gel was overlaid with water and after 20 minutes this overlay was removed and replaced with stacking gel mix [Acrylamide-Bis mix (1.62 ml of 30% v/v, 29:1), Tris-HCl (3.10 ml of 30% w/v, pH 8.8), ultrapure water (7.78 ml), ammonium persulfate (APS) (50 µl of 20% w/v), and TEMED (20 µl)].

After purification, the dried proteins were solubilized in 20-60 µl of denaturing 2x Tricine sample buffer [4x Tris-HCl/SDS (2 ml, pH 6.8), glycerol (2.4 ml), sodium dodecyl sulfate (SDS) (0.8 g) (BioShop Canada Inc., Burlington, ON, Canada), DTT (0.31 g) (EM Science, Darmstadt, Germany), and Coomassie blue G-250 (2 mg)] and loaded into the wells. Bio-Rad Precision Plus Protein™ molecular weight markers with and without dual colour (Bio-Rad Laboratories, Hercules, CA, USA) were employed for determination of the molecular weight of protein samples.

10x SDS running buffer [Tris (121 g) (Sigma Chemical Company, St. Louis, MO, USA), Tricine (179 g) (BioShop Canada Inc., Burlington, ON) and SDS (10 g) were dissolved in ultrapure water (1000 ml) and autoclaved before use] was poured into the upper and lower reservoirs. The gels were electrophoresed at 30 V for 30 minutes and then 125 V until the dye front reached the bottom of the gel.

To detect proteins in Tricine polyacrylamide gels, Coomassie Brilliant Blue and silver nitrate are the most frequent techniques of protein staining, with sensitivity ranges of about 1 mg for Coomassie Brilliant Blue and 1 ng for silver nitrate stain (Wray *et al.*, 1981; Gradilone *et al.*, 1998; Blackstock and Weir, 1999).

Coomassie dyes stain the protein molecules according to dye-dye ionic or hydrophobic associations with the Coomassie dyes and basic amino acids of proteins (Candiano *et al.*, 2004). Coomassie Brilliant Blue stain for detection of proteins suffers from low sensitivity including the improved colloidal blue stain (sensitivity of 30 ng) (Neuhoff et al., 1990).

The silver nitrate staining procedure includes soaking the polyacrylamide gel in solution containing soluble Ag^+ and subsequently reducing the gel to Ag^0 with formaldehyde, a silver reductant (Yan *et al.*, 2000). Silver nitrate stain is much more sensitive than Coomassie stain, but suffers from a possible interference with MS protein analysis (Chevalier *et al.*, 2004). To overcome the classical stain limitations, several fluorescent dyes were introduced to overcome the sensitivity and MS compatibility issues. Such stains include SYPRO Ruby protein gel stain (Molecular Probes, Eugene, OR, USA), which is as sensitive as silver stain but does not require glutaraldehyde, which can create false positive responses on the polyacrylamide gel.

SYPRO Ruby dye contains ruthenium, which interacts noncovalently with proteins and the detection sensitivity is in the range of 0.5–1 ng. The stained proteins may then be visualized

on a UV light box of 302 nm and the stain is fully compatible with subsequent protein characterization using MS (Candiano *et al.*, 2004). Nevertheless, even this improved fluorescent stain has its drawbacks, such as high cost and technical difficulties (Chevalier *et al.*, 2004).

For the following study several stains were employed, including Coomassie Brilliant Blue, colloidal blue, silver nitrate, and SYPRO Ruby dye stains.

The gel was stained with either Coomassie Brilliant Blue stain solution [Coomassie blue R-250 (0.025% w/v) (Bio-Rad Laboratories, Hercules, CA, USA), methanol (40% v/v) (Caledon Laboratories LTD, Georgetown, ON, Canada), acetic acid (7% v/v) (Caledon Laboratories LTD, Georgetown, ON, Canada)], colloidal Coomassie stain solution (designated "blue silver" owing to its significantly higher sensitivity than conventional Coomassie blue) [Coomassie blue G-250 (100 mg) (Sigma Chemical Company, St. Louis, MO, USA), phosphoric acid (30 ml of 83% v/v) (Sigma Chemical Company, St. Louis, MO, USA), ammonium sulfate (170 g), and distilled water (460 ml)] (Gersten, 1996; Candiano *et al.*, 2004), silver nitrate staining [wash I (acetic acid (7% v/v)), wash II (methanol (50% v/v) with aqueous glutaraldehyde (0.03% v/v)), wash III (ultrapure water), staining solution (NaOH (0.36% w/v) (EMD Biosciences, Darmstadt, Germany) in water (21 ml) and ammonium hydroxide (1.4 ml of 14.8 M w/v) (Sigma Chemical Company, St. Louis, MO, USA)), developing solution (citric acid (1ml of 1% w/v)) (Sigma Chemical Company, St. Louis, MO, USA), formaldehyde (100 µl of 37% v/v) (Sigma Chemical Company, St. Louis, MO, USA) and ultrapure water (200 ml)] (Wray *et al.*, 1981; Gersten, 1996), or SYPRO Ruby dye (Molecular Probes, Eugene, OR, USA), according to instructions of the manufacturer.

The Coomassie- and colloidal Coomassie-stained gels were destained in methanol (40% v/v) and 10% acetic acid (v/v), while the SYPRO Ruby stain was destained in ultrapure water.

All the stains were visualized colorimetrically except SYPRO Ruby stain, which was visualized fluorimetrically with a standard UV light box. However, data acquisition and fluorescence intensity could not be integrated and quantitative information could not be obtained using this system. The sizes of the EPS proteins were determined by generating a standard curve from molecular mass standards on each Tricine-PAGE gel.

3.6 EPS Glycoprotein Detection

A vast number of approaches exist for the detection, isolation and characterization of glycoproteins aiming at the identification of the covalent linkage between the carbohydrate and the polypeptide fraction (Schäffer *et al.*, 2001). However, relatively few techniques sensitively and reliably detect carbohydrate residues on proteins in polyacrylamide gels or on electroblot membranes (Steinberg *et al.*, 2001). The glycoproteins were detected on Tricine-PAGE gel versus on a nitrocellulose, since nitrocellulose interferes with glycoprotein analysis. The unglycosylated proteins bind strongly to nitrocellulose, although highly-glycosylated proteins bind weakly or not at all (Walker, 1994). Therefore, to detect glycoprotein in the crude EPS and the EPS protein samples on Tricine-PAGE gels, Periodic acid-Schiff (PAS) stain was utilized. For glycoprotein carbohydrate estimation, a Glycoprotein Carbohydrate Estimation Kit (23260) (Pierce Biotechnology, Rockford, IL, USA) was employed. Figure 3.4 demonstrates the various methods used in determining the glycosylation extent in EPS.

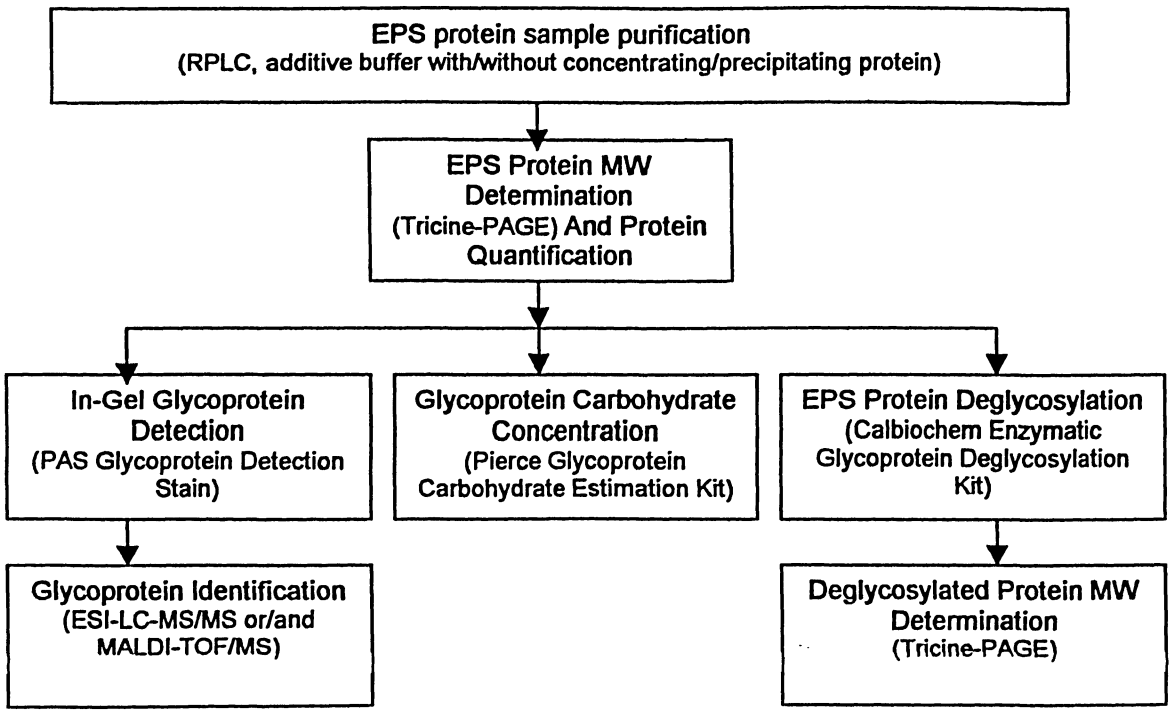


Figure 3.4 Flow chart of methodology for detecting and deglycosylating EPS glycoproteins.

3.6.1 Periodic Acid—Schiff Stain

The periodic acid-Schiff (PAS) reagent is commonly employed for general glycoprotein detection due to its ability to stain icinal diol groups that are mainly found on peripheral sugars and sialic acids in glycoproteins (Walker, 1994). The PAS staining using a chromogenic substrate, acid fuchsin, was employed for glycoprotein detection, which may detect 25–100 ng of glycoprotein (Doerner and White, 1990). The procedure for PAS staining was modified from Doerner and White (1990) and from Pro-Q Fuchsia Glycoprotein Detection kit (Molecular Probes, Eugene, OR, USA).

Following the EPS protein separation on Tricine-polyacrylamide electrophoresis, the gel was fixed in the fume hood by immersing it in a Fix solution [methanol (50% v/v)] for 1 hour with gentle agitation. The gel was then washed twice in the Wash solution [acetic acid (3% v/v)] for 20 minutes each time. The gel was incubated in Oxidizing solution [periodic acid (2.5 g) in acetic acid (250 ml of 3% v/v)] with gentle agitation for 30 minutes-1 hour. The gel was washed in Wash solution three times for 10 minutes each time. The gel was then incubated in the dark in Fuchsia reagent [fuchsin acid stain (2.5 g) in ultrapure water (500 ml)] with gentle agitation for 30 minutes-1 hour. Following the staining with Fuchsia reagent, the gel was incubated in the dark in Reducing solution [sodium metabisulfite (1.25 g) in ultrapure water (250 ml)] for 10-20 minutes. The gel was then washed repeatedly with the Wash solution following a wash in ultrapure water. After the washes, the glycoproteins are visible as fuchsia-coloured bands.

3.6.2 Glycoprotein Carbohydrate Estimation

Glycoprotein Carbohydrate Estimation Kit (Pierce Biotechnology, Rockford, IL, USA) was used to simply and rapidly detect glycoproteins and estimate carbohydrate content. Glycoprotein is first oxidized with sodium *meta*-periodate to form aldehydes that react with the proprietary Glycoprotein Detection Reagent. The resulting purple reaction has an absorption maximum at 550 nm that is proportional to the carbohydrate content in the glycoprotein. Unknowns are compared with protein standards of known glycoprotein content. Non-glycosylated proteins, such as lysozyme and bovine serum albumin, produce a low absorbance at 550 nm. This kit was utilized for glycoprotein estimation in crude and treated EPS samples.

Standards (50 µl each) and the sample (50 µl) were placed in the 96-well plates (use 50 µl of Glycoprotein Assay Buffer as a blank). Each sample and standard were tested in triplicate.

Sodium *meta*-periodate solution (25 µl) was added to each well and the plate was mixed for 30 seconds in a microplate shaker. Subsequently, the plate was covered and incubated at room temperature for 10 minutes. Glycoprotein Detection Reagent (150 µl) was added to each well and the plate was mixed for 30 seconds in a microplate shaker. The plate was covered and incubated at room temperature for 1 hour. The absorbance was measured at 550 nm in a microplate reader and a standard curve was plotted.

3.6.3 Enzymatic Deglycosylation of EPS Glycoproteins

The Calbiochem® glycoprotein deglycosylation kit (EMD Biosciences, Darmstadt, Germany) was employed for the removal of N-linked and O-linked oligosaccharides. The kit components include 5 different enzymes for deglycosylation of approximately 200µg of an average glycoprotein in the given time and can be employed for EPS glycoproteins from activated sludge:

1. **N-Glycosidase F**—removes virtually all N-linked oligosaccharides from glycoproteins.
2. **Endo-α-N-acetylgalactosaminidase**—removes the Galβ1,3GalNAc core structure, attached to serine and threonine, intact.
3. **α2-3,6,8,9-Neuraminidase**—removes trisialyl structure by cleaving the Neu5Acα 2,8-Neu5Ac bond.
4. **β1,4-Galactosidase**—hydrolyzes glycoprotein with the sequence GlcNAcβ1,3-Galβ1,4GlcNAc at the β–1,4 linkage; required for complete removal of O-linked structure or its derivatives.

5. **β-N-Acetylglucosaminidase**—hydrolyzes Galβ1,3GalNAc core disaccharide from Ser/Thr residues in glycoprotein; required for complete removal of O-linked structure or its derivatives.

Steric hindrance inhibits or slows the action of the deglycosylating enzymes on certain residues of glycoproteins (GlycoProfile™I Kit PP0200). Various denaturants have been employed to expose otherwise inaccessible oligosaccharide cores, including ionic, nonionic, and zwitterionic detergents as well as chaeotropic salts and thiols. Of these, heating in sodium dodecyl sulfate (SDS) appears to be the simplest and most effective method for unfolding glycoproteins. TRITON® X-100 is used in the kit to enhance the ability of oligosaccharide-cleaving enzymes to deglycosylate certain glycoproteins in metabolically labeled cell extracts (Tarentino *et al.*, 1989).

The denaturing protocol was used in order to deglycosylate the glycoproteins in the EPS completely. The EPS protein sample was dissolved in ultrapure water (30 µl). 5X reaction buffer (10 µl) and denaturation solution (2.5 µl) was added to the mixture, mixed gently and heated for 5 minutes at 100°C. After the sample cooled to room temperature, TRITON® X-100 (2.5 µl) was added to the sample and gently mixed. Subsequently, five enzymes (1 µl each) in the kit were added to the sample. The mixture was incubated overnight instead of the recommended three hours at 37°C for complete deglycosylation and extent of deglycosylation was analyzed by Tricine-PAGE. The position of the native protein was then compared with the deglycosylated protein to judge the extent of glycosylation. Bovine fetuin, which contains both N- and O-linked oligosaccharides, was used as a control.

3.7 EPS Protein Identification

In order to identify the proteins in the EPS, the EPS samples were either purified by the RPLC method or combining chemical buffer additive with precipitating the protein. The combined method of addition of chemical buffer additive with protein precipitation was followed by 10% Tricine-PAGE and staining with Coomassie blue, colloidal Coomassie, silver nitrate, or SYPRO Ruby stain. After throughoutly destaining, the gel bands were dissected and analyzed by electrospray ionization mass spectrometry (LC/ESI-MS/MS).

3.7.1 Tandem Electrospray Ionization Mass Spectrometry (LC/ESI-MS/MS)

After RPLC procedure, the EPS sample was digested with 0.5 g of sequencing grade trypsin (Promega, Madison, WI, USA) in 5% acetonitrile, 200 mM urea and 100 mM Tris-HCl, pH 8.8 at 37°C overnight. After the last incubation with trypsin (overnight), the pH was adjusted to 4 using formic acid. The enzymatic peptides obtained from the EPS sample were desalted using C_{18} Zip-Tips (Millipore Laboratories, Bedford, MA, USA) and separated on a C_{18} column (150 mm x 0.3 mm, 5 um particle size) (Grace Vydac, Hesperia, CA, USA) with an Agilent 1100 HPLC pump (Agilent, Palo Alto, CA, USA). Following the HPLC protein separation, the EPS peptide samples were loaded unto ESI-LC-MS/MS which employs ion trap. The sample was analyzed over a 90-minute gradient from 5% to 65% acetonitrile at a flow rate of 2 *i*L per minute. The obtained MS/MS data files from the Bruker Esquire 6000 Mass Spectrometer (Bruker Daltonics, Bremen, Germany) and the mass spectra obtained was searched against a nonredundant library of proteins such as BLAST (http://www.ncbi.nlm.nih.gov/BLAST/; BLAST, Bethesda, MD, USA) and Matrix Science Mascot Algorithm

(http://www.Matrixscience.com; Matrix Science, Boston, MA, USA). The MALDI-TOF-MS was not utilized due to insufficiency of equipment parts.

After optimization of the chemical additive method with protein precipitation, the gel band obtained from the Tricine-PAGE was excised and digested with 0.5 g of sequencing grade trypsin overnight in 1x digestion buffer containing, ZipTipped, and ran on ESI-LC-MS/MS. The destaining and removal of the protein extract was performed on individual gel slices containing a single protein band as depicted in Figure 3.5.

The protein bands were excised from the silver-stained gel with a clean, sharp straight-edge razor as close to the boundaries of the protein as possible. The band was further cut into 1mm x 1mm sections in minimal ultrapure water and transferred to a 1.5 microcentrifuge tube with tweezers. The destaining process was performed by adding 1.0 ml of 0.1M NH_4HCO_3 in 50% AcN for 30 minutes at 30°C. This process was repeated once. The gel piece was dried completely in SpeedVac and rehydrated in 10 µl of 1x digestion buffer containing 0.5 µg of trypsin. Additional 200 µl of 1x digestion buffer containing 0.5 µg of trypsin was added to the gel band and the sample was incubated overnight at 37°C. The digestion buffer (extract) was carefully removed and placed into a clean 1.5 ml microcentrifuge tube. 200 µl of 0.1% TFA in 60% AcN and 50 µl of 5% acetic acid was added to the gel pieces. The tube was then incubated for 30 minutes at room temperature. The extract was carefully removed from tube 1 with the gel

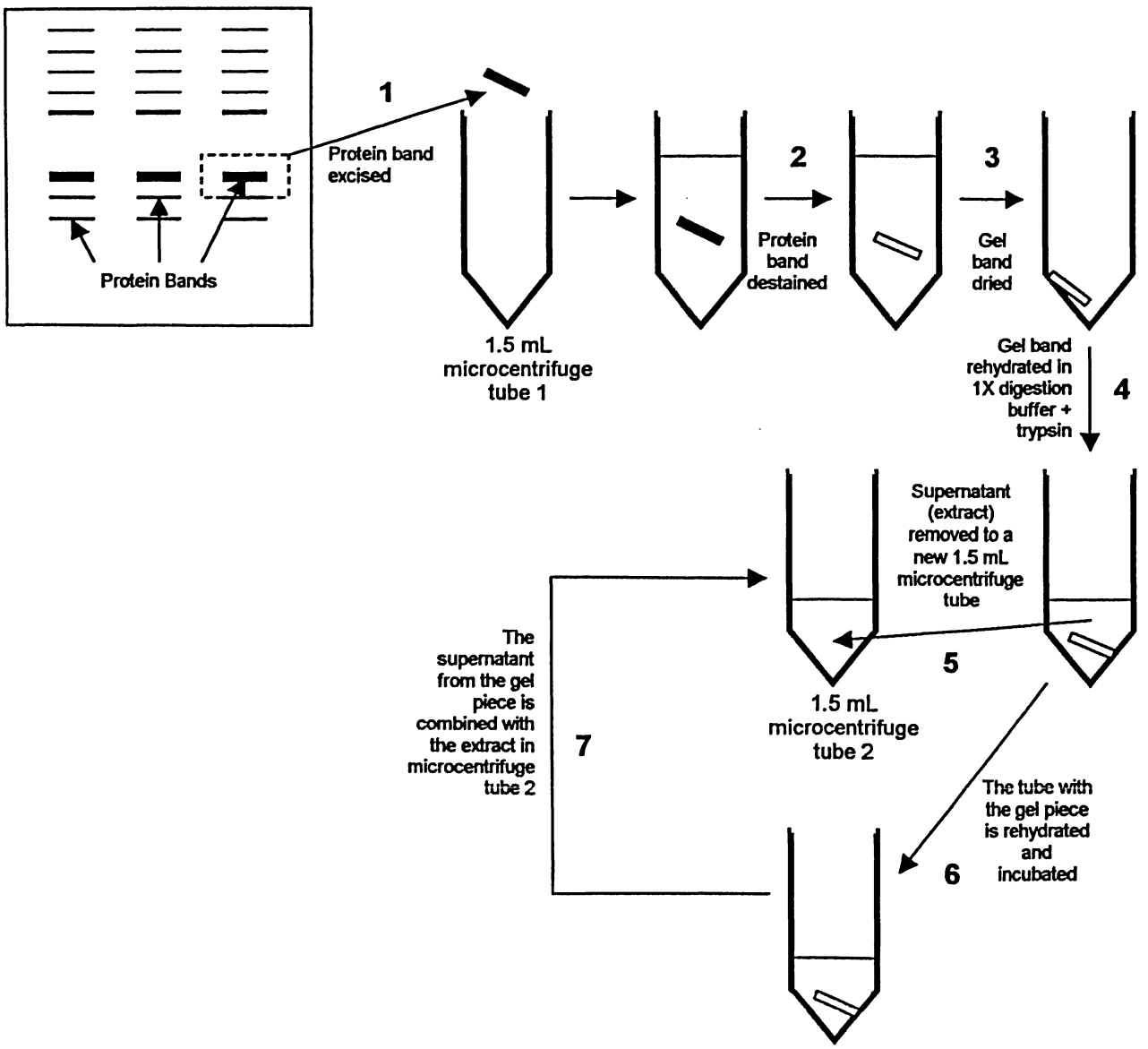


Figure 3.5 In-gel digestion of protein bands after Tricine-PAGE (Adapted from Cohen and Chait, 1997).

pieces into tube 2 with the extract from the digestion buffer (Figure 3.5) and the volume in the tube 2 was reduced by SpeedVac.

3.8 Statistical Analysis

The variability in measurements was determined using standard deviation of the average (mean +/- standard deviation), employing Microsoft Excel (Windows 2000). The t-test was performed for determination of significance between sample means. The null hypothesis that there is no significant difference between various treatments for protein isolation and purification was tested using a two-tailed t-test. If the probability for the calculated t-statistic was equal or less than 0.05, then the null hypothesis was rejected and the two or more samples were concluded to be different and statistically significant. The ANOVA was performed to determine if the observations have come from the same population and the $F_{calculated}$ was compared to $F_{significant}$ from the F table (Mendenhall and Sincich, 1992).

CHAPTER 4. RESULTS AND DISCUSSION

The purpose of this study was to resolve the protein fraction in the activated sludge extracellular polymeric substances (EPS) by developing a novel purification, isolation and extraction method and endeavor to reveal that a considerable portion of the EPS proteins consist of glycoproteins. The objectives in this study were four-fold; 1) to investigate the ability to isolate, separate and purify the EPS proteins from microbial activated sludge floc EPS; 2) to estimate the extent of glycosylation in the microbial EPS matrix; 3) to identify the microbial activated sludge EPS proteins; and 4) to gain a better understanding of the function of EPS proteins on the floc structure and formation in the activated sludge by assessing the nature of the protein and its function. To accomplish the first objective, the untreated cation exchange resin (CER)-extracted EPS samples and microbial EPS samples treated with various buffers (acids, bases, salts, chelating agents, detergents, chaeotropes, organic agents, and reducing agents), reverse-phase liquid chromatography (RPLC), and precipitation methods, were run on one-dimensional Tricine-polyacrylamide gel electrophoresis (Tricine-PAGE) and visualized under a variety of stains (Coomassie Brilliant Blue, colloidal Coomassie, silver nitrate, and SYPRO Ruby). To accomplish the second objective, following Tricine-polyacrylamide electrophoresis, the resulting gel was stained with periodic acid Schiff (PAS) stain and the glycoprotein carbohydrate content was estimated in the untreated and treated EPS samples. To accomplish the third objective, the untreated and treated CER-extracted EPS samples (in-solution or in-gel) were run on tandem electrospray ionization mass spectrometry (ESI-MS/MS) and various protein database searches. To accomplish the fourth objective, the nature of the EPS proteins was theorized from the conducted study.

4.1 Microbial Floc Physicochemical Properties

Sludge volume index (SVI) for activated sludge used in this study demonstrates relatively good settling ability, with SVI values of less than 100 ml/g, as can be observed in Table 4.1.

Table 4.1 The sludge volume indexes for SBR- and WWTP-derived activated sludge samples prior to CER extraction of EPS.

Source of the activated sludge sample	SVI
SBR 1	52.2
SBR 2	69.0
SBR 3	65.9
SBR 4	72.0
1st sample from WWTP (April)	-----
2nd sample from WWTP (May)	75.98
3rd sample from WWTP (June)	67.5
4th sample from WWTP (July)	57.17

4.2 Extracellular polymeric substances (EPS)

4.2.1 Cation Exchange Resin (CER)

Various EPS extraction methods have been endeavored with the intention of finding an appropriate technique that would yield high amount of protein with minimal lysis. The cation exchange resin (CER) method was used for its high extraction efficiency with minimum cell lysis and no interference during the protein analysis.

4.2.1.1 Chemical Analyses of EPS

The concentrations of protein, carbohydrate and DNA in the EPS from activated sludge obtained from four laboratory sequencing batch reactors (SBRs) and Ashbridges Bay wastewater treatment plant (WWTP) were assessed (Figures 4.1-4.2), using BCA protein assay (Pierce Biotechnology, Rockford, IL, USA), phenol-sulfuric acid method (Masuko *et al.*, 2005) modified from Dubois *et al.* (1956), and standard fluorescent DNA quantification kit (Bio-Rad Laboratories, Hercules, CA, USA), respectively (Appendix A contains results in a table format for the concentrations in μg/ml and mg/g MLSS).

From Figures 4.1 and 4.2 it may be observed that the laboratory-scale SBR EPS samples contain less total protein, total carbohydrate and total DNA compared to WWTP EPS samples. This may be because these are different systems (*e.g.*, varying effluent age, SRT, and organic load).

Figures 4.1 and 4.2 demonstrate the major quantities of EPS constituents. The major EPS constituents comprise of proteins, carbohydrates and DNA originated within the activated sludge floc matrix derived from laboratory-scale SBRs or WWTP. In this study the protein: carbohydrate ratio varied from 1.16-3.42 (w/w) (Figure 4.3), which situates within the established range of 0.2-5.0 (w/w) (Frølund *et al.*, 1995).

It was determined that protein was the dominant constituent in the EPS, followed by carbohydrate and a smaller portion of DNA in both SBRs and WWTP samples, as exhibited in previous studies (Urbain *et al.*, 1993; Frølund *et al.*, 1996; Bura *et al.*, 1998; Liao *et al.*, 2001).

The protein and carbohydrate content (μg/ml and mg/g MLSS) for SBRs 1 and 2 (t-test, $p>0.05$), and 3 and 4 (t-test, $p>0.05$), were not significantly different. The protein and content in all four reactors (ANOVA, $p>0.05$) was not significantly different (Figures 4.1a and 4.2a).

However, the carbohydrate content in all four reactors (ANOVA, $p<0.05$) was significantly different (Appendix C). The carbohydrate content may have differed in all four reactors but was similar in reactors 1 and 2 and reactors 3 and 4, because reactors 1 and 2 were fed with high phosphorous levels and reactors 3 and 4 were fed with limited phosphorous levels. Phosphorus limitation causes morphological changes in the floc structure and the composition of EPS (Liu *et al.*, 2006).

Samples from WWTP were further divided into subsamples (S), from which the EPS was extracted individually.

CER-extracted EPS subsample from WWTP biomass collected in May ($S2_1$) contained significantly greater protein content (μg/ml and mg/g MLSS) (ANOVA, $p<0.05$) than EPS subsamples $S2_2$ and $S2_3$ (Figures 4.1b and 4.2b). EPS subsample $S2_2$ contained significantly greater carbohydrate content (μg/ml and mg/g MLSS) (ANOVA, $p<0.05$) than EPS subsamples $S2_1$ and $S2_3$ (Figures 4.1b and 4.2b) (Appendix C).

There was not significant difference in protein content (μg/ml and mg/g MLSS) of CER-extracted EPS subsamples from WWTP biomass collected in June ($S3_1$, $S3_2$, $S3_3$, $S3_4$) (ANOVA, $p>0.05$) (Figures 4.1b and 4.2b). EPS subsamples $S3_1$ and $S3_2$ contained significantly lower carbohydrate content (μg/ml and mg/g MLSS) (ANOVA, $p<0.05$) when compared to EPS subsamples $S3_3$ and $S3_4$ (Figures 4.1b and 4.2b) (Appendix C).

CER-extracted EPS subsample from WWTP biomass collected in July ($S4_1$) contained significantly greater protein content (μg/ml and mg/g MLSS) (ANOVA, $p<0.05$) than EPS subsample $S4_2$ (Figures 4.1b and 4.2b). There was no significant difference in carbohydrate content (μg/ml and mg/g MLSS) between EPS subsamples $S4_1$ and $S4_2$ (ANOVA, $p>0.05$) (Figures 4.1b and 4.2b) (Appendix C).

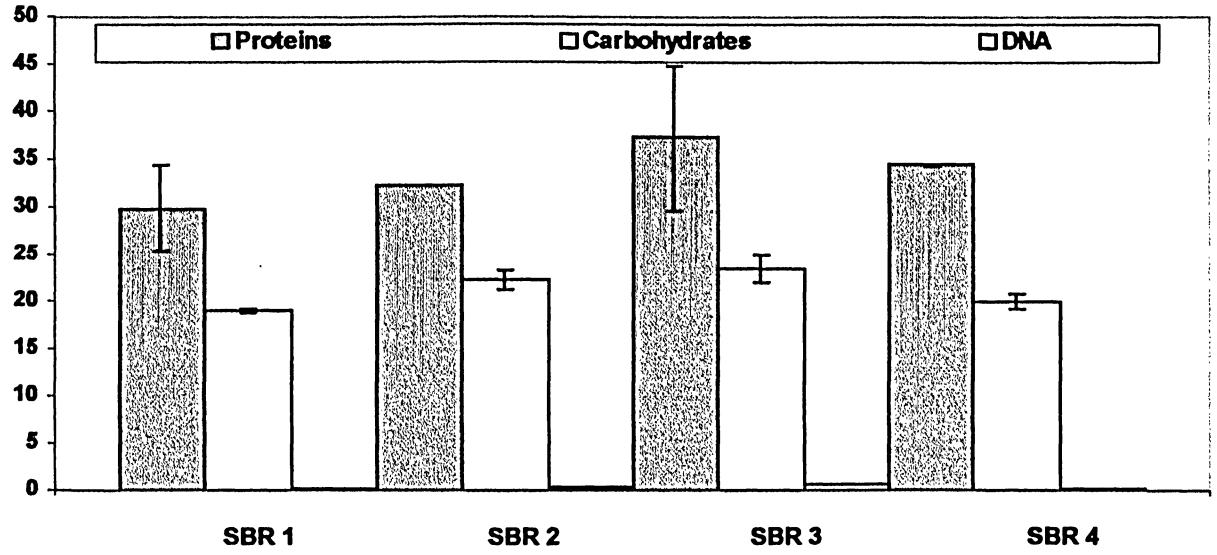
This demonstrates that the samples derived from SBRs contain protein and carbohydrate concentrations that level off over time and are relatively constant unlike samples derived from WWTP because these are different systems. The difference in DNA concentrations between SBR- and WWTP-derived EPS samples could not be determined using ANOVA analysis because the standard deviations of DNA content were at zero.

The ratio of proteins to carbohydrates was constant for all four laboratory SBRs and no significant difference was found in samples from WWTP (ANOVA, $p>0.05$) (Appendix B). A slight change that was seen within the samples in the WWTP may be due to growth rate and microbial community of sludge.

The DNA content in the EPS of the activated sludge samples in WWTP was found to be highly variable compared to the total EPS, ranging from 0.4-7.4%. The extracellular DNA is likely to be dependent on physicochemical factors, rather than the biomass, as was verified by Urbain *et al.* (1993). The nucleic acid and protein content found in the CER-extracted EPS samples have been shown previously to be naturally occurring in the EPS from cell lysis or cell excretion (Urbain *et al.*, 1993; Frølund *et al.*, 1996; Nielsen and Jahn, 1999; Liao *et al.*, 2001). The presence of DNA and protein in the EPS has been also linked to structural stability of the floc (Urbain *et al.*, 1993; Liao *et al.*, 2001). From microscopic examinations it has been observed that before and after CER extraction the cells were not ruptured (results not shown).

From the study conducted, no correlation was found between EPS constituents and SVI, which coincides with Liao *et al.* (2001) and (2006) findings.

A



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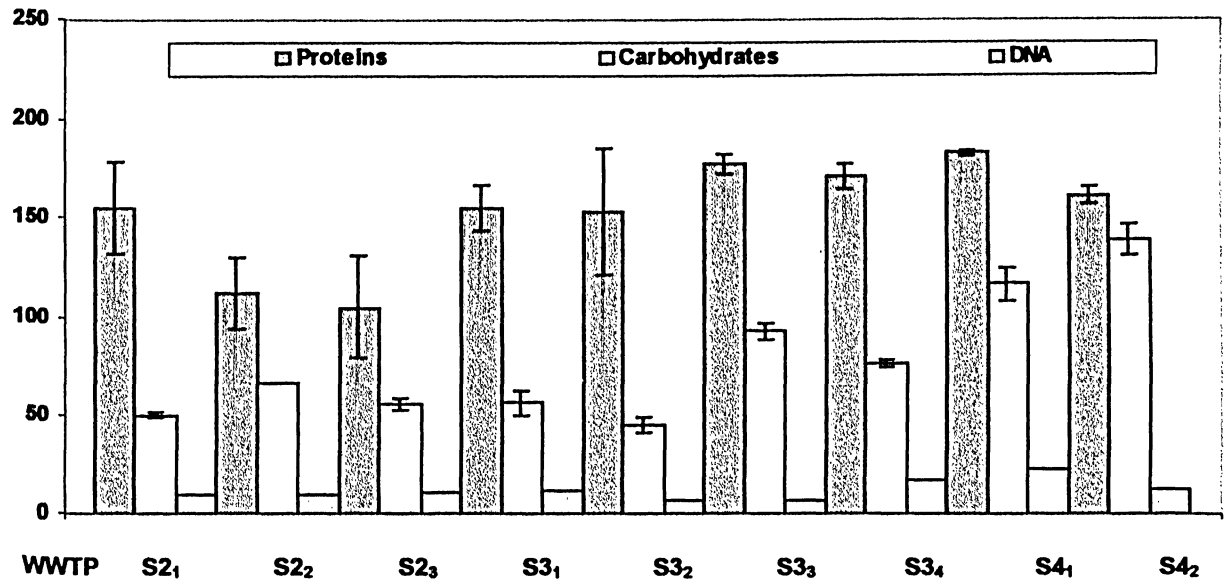
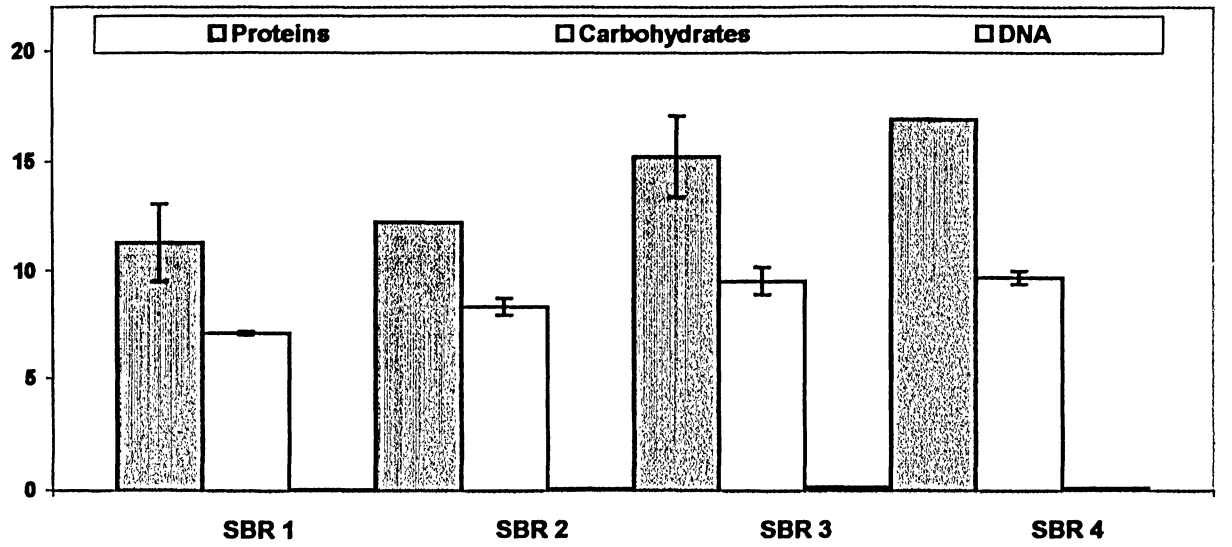


Figure 4.1 SBR-derived (A) and WWTP-derived activated sludge (B) CER-extracted EPS components (μg/ml). Results are expressed as the average of ± one standard deviation. Note: EPS subsamples from WWTP-derived activated sludge obtained in April ($S1_1$ and $S1_2$) are not shown since the sludge was stored anaerobically at 4°C for 5 days and then reaerated for approximately 3-4 hours prior to sludge analysis. The data can be found in Appendix A.

A



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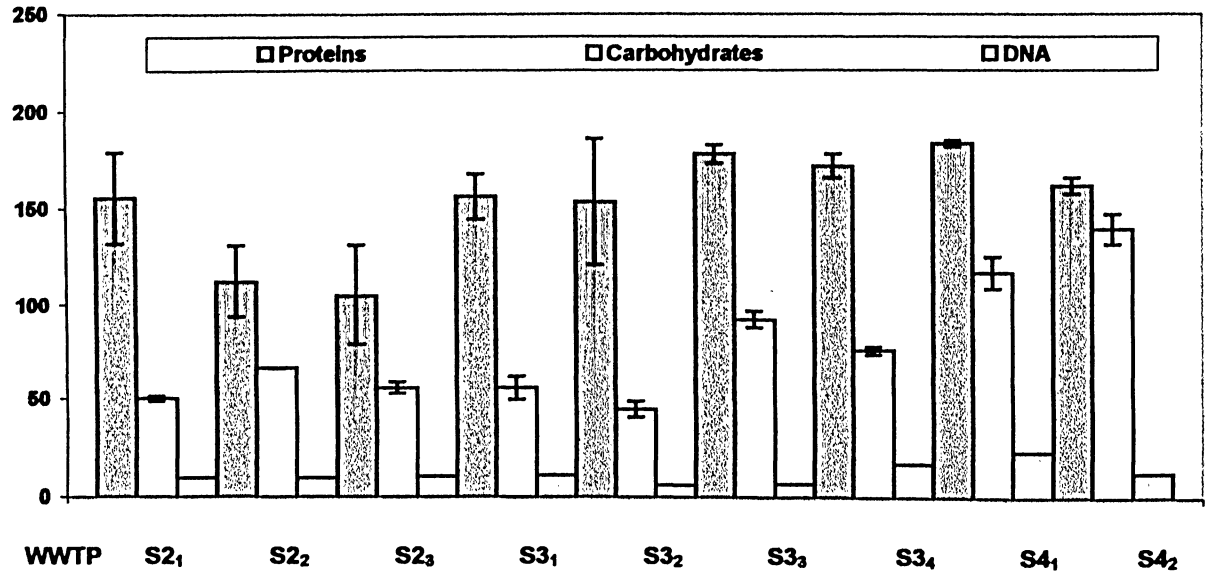


Figure 4.2 SBR-derived (A) and WWTP-derived activated sludge (B) CER-extracted EPS components (mg/g MLSS). Results are expressed as the average of ± one standard deviation. Note: EPS subsamples from WWTP-derived activated sludge obtained in April ($S1_1$ and $S1_2$) are not shown since the sludge was stored anaerobically at 4°C for 5 days and then reaerated for approximately 3-4 hours prior to sludge analysis. The data can be found in Appendix A.

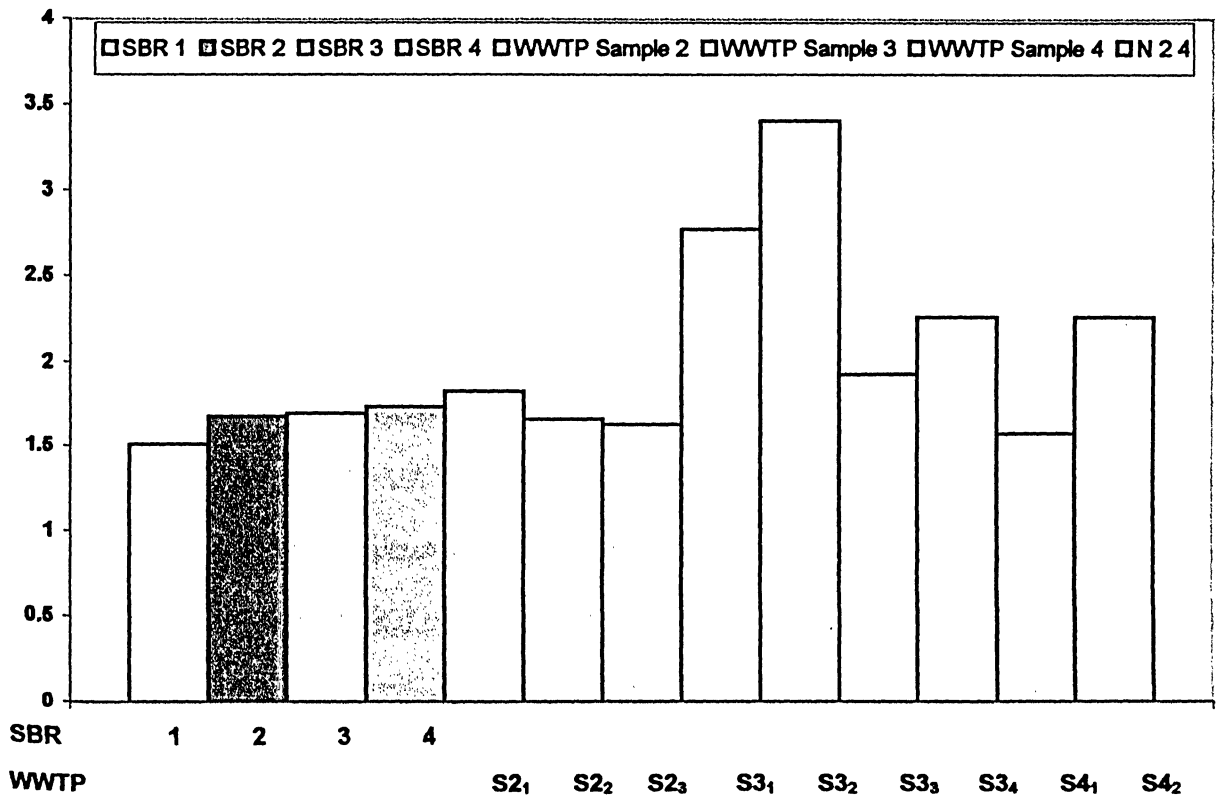


Figure 4.3 The protein to carbohydrate ratio of SBR- and WWTP-derived activated sludge CER-extracted EPS. The data can be found in Appendix A, Table A.4.

4.3 EPS Protein Extraction and Purification

From previous studies done in our lab (Williams, 2005; Lang, 2005) it was found that the resolution of proteins from biomass and crude CER-extracted EPS was very poor utilizing conventional polyacrylamide electrophoresis methods for protein separation and purification. The results observed from Figure 4.4a correspond to the prevous findings. The dense regions that possibly contain protein fraction lie within the 100-250, 50-80, 35-55, and 25 killodaltons. The poor resolution of crude microbial EPS sample on Tricine-PAGE may occur due to interactions within EPS, which is a very complicated matrix of various constituents, majority of which consisting of proteins and carbohydrates. Further it is hypothesized that some of these protein-polysaccharide interactions may contain glycoproteins.

The results of the WWTP-derived microbial EPS (Figure 4.4B) demonstrate resolution of the crude untreated EPS. Eleven distinct bands (shown by arrows) were identified as approximately: 170, 129, 114, 97, 71, 62, 51, 28, 21, 13, and 8 kDa.

From the results in Figure 4.4B, the proteins that are visualized may come from cell lysis that may occur as a result of lower microbial growth rates and a higher level of endogenous metabolism. The accumulation of protein and DNA in the crude EPS extract cannot be used as indicators for cell lysis given that it has been established that the EPS naturally contains large amounts of protein and DNA (Urbain *et al.*, 1993; Frølund *et al.*, 1996; Nielsen and Jahn, 1999), which are linked to structural stability of the floc (Urbain *et al.*, 1993; Liao *et al.*, 2001). Furthermore, from microscopic observations, the cell lysis was observed to be minimal (Results not shown).

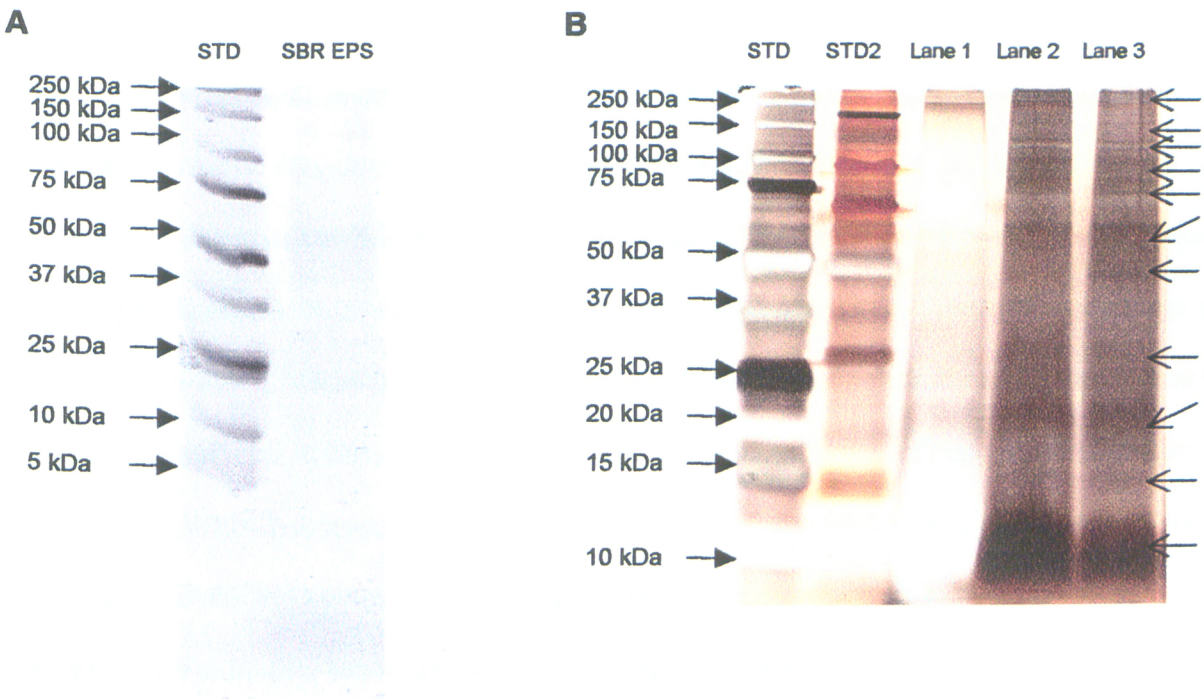


Figure 4.4 Coomassie Brilliant Blue (A) and silver nitrate stained Tricine-PAGE (9 and 10% respectively) showing crude activated sludge floc EPS from SBR (A) and WWTP (B). Lane 1, untreated EPS; lanes 2 and 3, EPS with protease inhibitors. Arrows (←) indicate the regions of density of the proteins in the EPS fraction.

The extracted EPS from WWTP was also examined for possible induced cell lysis by rupturing the cells present in the activated sludge portion or observing the cells before and after CER extraction under inverted microscope, and comparing the released proteins in the activated sludge to the possible EPS proteins from activated sludge floc. As stated previously, the cell lysis was observed to be minimal (Results not shown) under the microscope.

In order to evaluate the quantity of proteins visualized in the possible EPS protein fraction that may be cellular, activated sludge was sonicated, boiled in 1-2% SDS, lysed with TRIZOL®, and/or violently agitated with Mini-Bead-Beater-8™ system. TRIZOL® was found to be incompatible with the Tricine-PAGE system. From Figure 4.5A it may be observed that the protein pattern obtained from activated sludge sample by using Mini-Bead-Beater-8™ overall is very similar to the crude EPS pattern from WWTP. This may be due to the fact that the proteins extracted from activated sludge, even after cell lysis, contain proteins that are both of exocellular and intracellular origin. There are, however, at least seven protein bands in the activated sludge (arrows on the left in Figure 4.5A) that do not match with the possible proteins from the EPS. The molecular weights of these proteins are approximately: 153, 116, ~61, 47, 35, 20, and 12 kDa. There is also a band in the possible EPS protein fraction that is not present in the activated sludge fraction at ~170 kDa.

When WWTP samples were treated with hot SDS (1% w/v), there is an increased quantity of proteins that become more distinct as observed on Tricine-PAGE of Figure 4.5B in the microbial EPS fraction (1103.83 ± 18.1 μg/ml) (Appendix A). There are approximately thirteen distinct protein bands that are visible in Figure 4.5B. These proteins are roughly: 147, 138, 131, 119, 107, 95, 92, 72, 54, 40, 35, 30, and 23 kDa. These results may possibly suggest

that the EPS fraction contains insoluble proteins that become more solubilized in SDS. This may also suggest that their hydrophobic interaction with the EPS matrix was destabilized by hot SDS.

The proteins solubilized by SDS and visualized on polyacrylamide electrophoresis gel may be glycoproteins, which are possibly still covalently linked to a polysaccharide portion because SDS cannot destabilize covalent interactions since they require a large quantity of energy to break. The prominent protein bands from the SDS treatment do not generate cell lysis products since the sonication treatment (172.96 ± 11.7 μg/ml) demonstrates similar protein quantity on the Tricine-PAGE (Figure 4.5B) and merely a 10% protein increase when compared to the untreated EPS fraction (155.62 ± 12.5 μg/ml) (Appendix B).

The prominent bands from the sludge supernatant proteins are of 114 and 10 kDa (Figure 4.6C). These proteins appear to be distinct from the possible EPS proteins in activated sludge floc but the intracellular proteins are probably still present from natural cell lysis or cell wall turnover (Higgins and Novak, 1997). Therefore, it was attempted to remove these intracellular proteins from the EPS sample in order to isolate the exocellular protein fraction in the EPS. It was assumed that the structure might be configured in a honeycomb-type of arrangement, with intracellular proteins positioned on the outer shell of the structure.

To separate possible intracellular proteins in the EPS and in the sludge supernatant from the exocellular protein fraction, the dried fractions were washed with ice-cold ethanol (80% w/v) or ice-cold SDS (0.1% w/v). It may be observed in Figure 4.6 that the majority of the proteins were found in the wash fraction when control (Figure 4.6C), EPS treated with protease inhibitors and DTT (2mM w/v) (concentrated by phenol-ether method) (Figure 4.6A), and sludge supernatant (Figure 4.6A) were washed with ice-cold SDS. Nonetheless, when the ice-cold SDS

was used on EPS treated with protease inhibitors, the proteins did not wash away completely (Figure 4.6B).

When ice-cold ethanol was utilized instead of ice-cold SDS on EPS fraction, the proteins corresponding with the sludge supernatant proteins appeared to be washing away (Figure 4.6A). However, by the third washing the protein fraction became soluble in the ethanol and displayed a similar protein fraction in the washings as Figure 4.6B.

From results obtained, it is possible that the CER-extracted EPS proteins may be associated with the water-soluble fraction of activated sludge floc. Furthermore, EPS treated with protease inhibitors followed by washings with ice-cold SDS or ice-cold ethanol resembled the same pattern as the control. Consequently, perhaps these proteins in the possible EPS protein fraction may play an important role in the EPS and thus may not be easily removed from the EPS matrix.

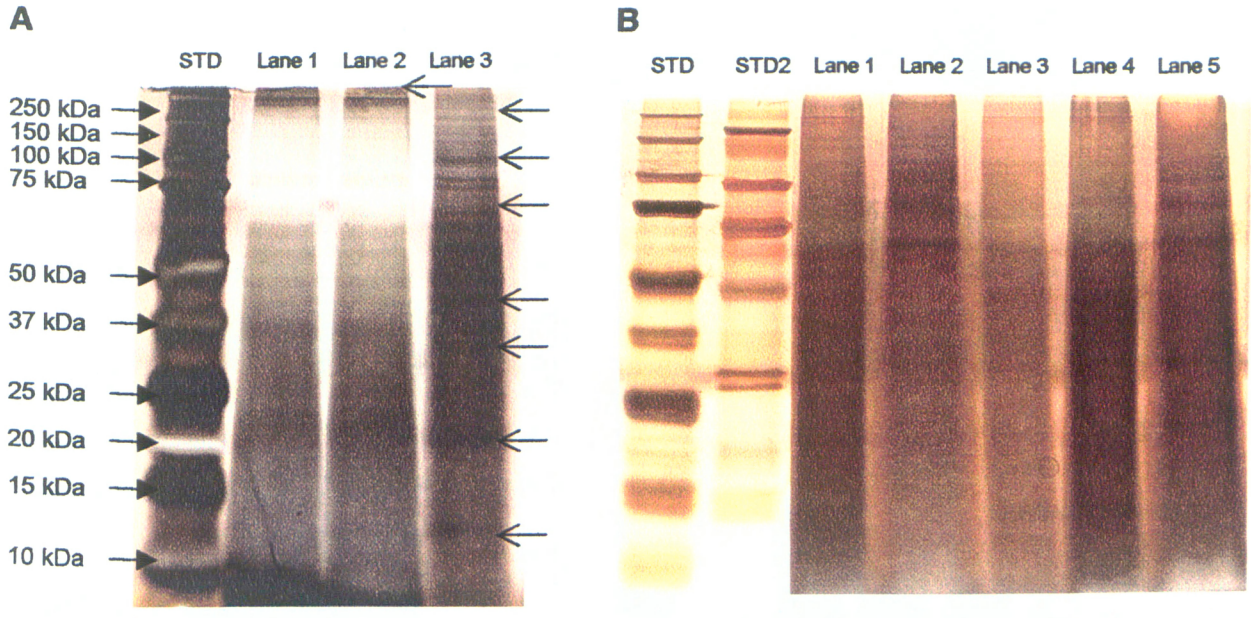


Figure 4.5 Silver nitrate stained Tricine-PAGE (10%) showing a comparison between crude activated sludge EPS and EPS/biomass that is lysed. (A). Lanes 1 and 2, WWTP-derived crude EPS; lane 3, activated sludge (400 µl) violently agitated in Mini-Bead-Beater-8™. (B). Lane 1, WWTP-derived EPS with protease inhibitors; lanes 2 and 5, EPS treated with boiled SDS (1% w/v) for 10 minutes; lane 3, EPS sonicated for 2 minutes at 60W (20 Hz); lane 4, Crude EPS. Arrows (←) indicate the regions of density of the proteins in the activated sludge floc and EPS fraction.

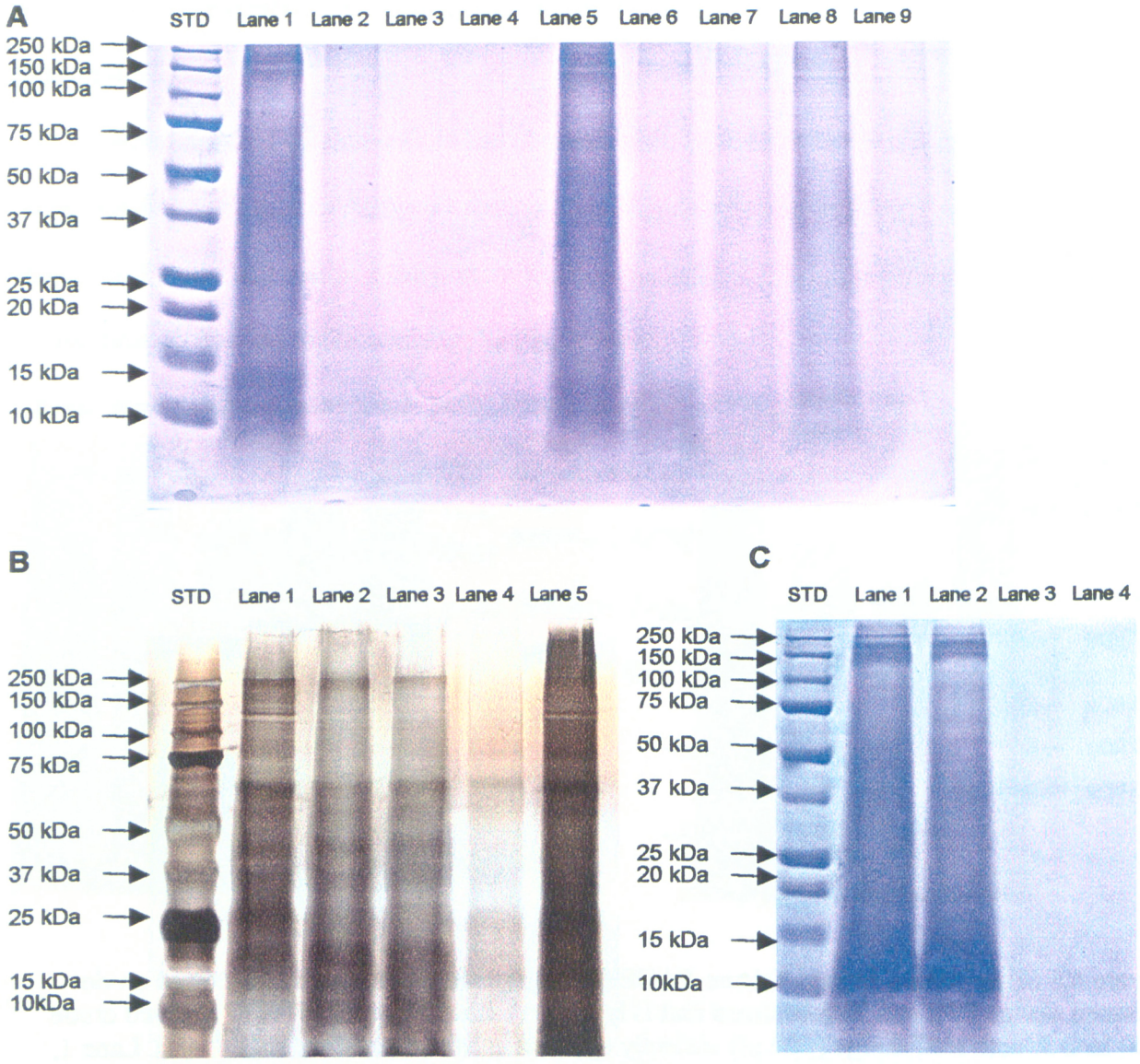


Figure 4.6 Coomassie Brilliant Blue (A and C) and silver nitrate (B) stained Tricine-PAGE (10%) of EPS washed in SDS and ethanol. (A). Lane 1, WWTP-derived EPS with protease inhibitors treated with DTT (2mM w/v) and concentrated by P-E method (treated) SDS (0.1% w/v) first wash; lane 2, treated EPS SDS (0.1% w/v) second wash; lane 3, treated EPS (0.1% w/v) third wash; lane 4, 2x sample buffer; lane 5, treated EPS washed in ethanol (80% v/v); lane 6, treated EPS ethanol (80% v/v) first wash; lane 7, treated EPS ethanol (80% v/v) second wash; lane 8, treated EPS ethanol (80% w/v) third wash; lane 9, treated EPS washed in SDS (0.1% w/v). (B). Lane 1, WWTP-derived EPS with protease inhibitors; lane 2, EPS with protease inhibitors treated with SDS (0.1% w/v) first wash; lane 3, EPS with protease inhibitors treated with SDS (0.1% w/v) second wash; lane 4, EPS with protease inhibitors treated with SDS (0.1% w/v) third wash; lane 5, EPS with protease inhibitors washed in SDS (0.1% w/v). (C) Lane 1, WWTP-derived untreated EPS; lane 2, EPS treated with SDS (0.1% w/v) first wash; lane 3, EPS treated with SDS (0.1% w/v) second wash; lane 4, EPS treated with SDS (0.1% w/v) third wash.

After staining the untreated WWTP-derived microbial EPS with PAS glycoprotein detection stain, the results display a possible glycosylation or a strong association with carbohydrate in the EPS protein fraction (Figure 4.7). This demonstrates that a substantial portion of the CER-extracted EPS is either glycosylated or the EPS proteins are strongly bound with carbohydrates.

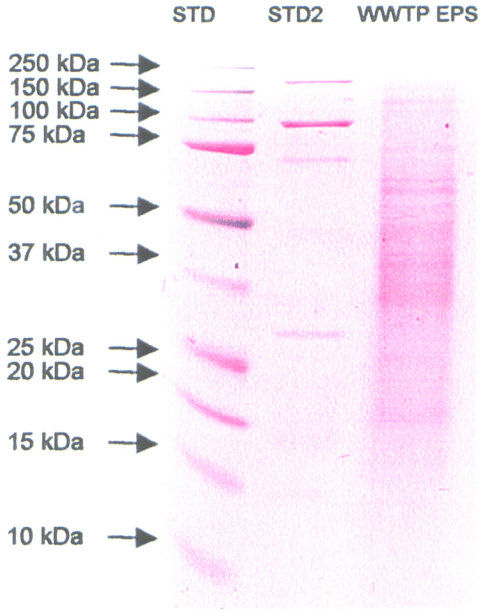


Figure 4.7 PAS acid fuchsin stained Tricine-PAGE (10%) of WWTP-derived activated sludge floc EPS with protease inhibitors.

Microorganisms have evolved a variety of adhesins. Proteinaceous adhesins include flagella (Jarrel *et al.*, 1996; DeFlaun *et al.*, 1990), fimbriae (Larkin and Nelson, 1987), pili (Ong *et al.*, 1990), and various surface proteins (Heilmann *et al.*, 1996; Timmerman *et al.*, 1990; Wilcox *et al.*, 1989; Staat *et al.*, 1980). These adhesins are known to contain glycosylation occurring outside the cytoplasmic membrane and thus act as lectins; therefore, it is probable that the glycosylated proteins visualized under PAS stain are lectins coming from these sources.

Bacterial lectins were described in the range of 15-20 kDa previously (Higgins and Novak, 1997). More recent data demonstrates that these lectins may range from 10-240 kDa (Jin and Zhao, 2000; Van den Broeck *et al.*, 2000; Brimer and Montie, 1998; Jarrel *et al.*, 1996).

Flagella are intricate organelles that may propel bacteria through liquids, extremely viscous environments or along surfaces (McCarter, 2006). Archaeal flagellin contains glycosylation proteins in the range of 10-53 kDa (Brimer and Montie, 1998; Jarrel *et al.*, 1996).

Pili are hair-like adhesins that project from the bacterial surface. Fimbriae are pili that attach bacteria to a surface. These organelles are present in both Gram-negative and Gram-positive organisms (Pizarro-Cerda and Cossart, 2006). Fimbrial glycosylated proteins are in the range of 16-240 kDa (Jin and Zhao, 2000; Van den Broeck *et al.*, 2000).

Surface proteins include S-layer proteins, which were isolated from *Campylobacter fetus* by Dworkin *et al.* (1995) (97-149 kDa). These S-layer proteins also function as adhesins.

The glycosylated moieties on these exposed organelles are probably a protective feature from proteases and other enzymes (Varki, 1993).

4.3.1 Buffer Additives

To obtain a better resolution of proteins in SBR-derived and WWTP-derived activated sludge floc EPS, four methods were employed. The first method involved buffer additives, which comprised of acids, bases, salts, chelating agents, detergents, chaeotropes, organic agents, and reducing agents, in order to resolve EPS protein bands based on ionic interactions and increased protein stability and solubilization. The second method involved the use of reverse-phase liquid chromatography in order to get a better resolution of EPS proteins from activated sludge flocs based on protein hydrophobicity. The third method employed the use of precipitation methods to

effectively concentrate the proteins and remove interfering substances. The fourth method was a combination of precipitation and buffer additives to improve protein resolution, stabilize the EPS proteins and remove interferences within the sample. The effectiveness of each method was judged by running the samples on 10% Tricine-PAGE.

To stabilize activated sludge EPS proteins, a range of several concentrations of acid and base were added to the CER-extracted EPS fraction in order to determine the optimum pH of the EPS proteins (Figure 4.8). The optimum pH for the total proteins in the activated sludge EPS will possibly bring these proteins closer to their isoelectric point. The appropriate pH of the total EPS proteins was found to be more than 3.5 and less than 11.0, where the usual pKa for sludge flocs is 2.9-5.0 (Liao et al., 2002). With a slight NaOH concentration increase (0.05% (w/v) NaOH), the pH has risen from pH of 7.5 to pH of 11.0 (Figure 4.8) and the Tricine-PAGE displayed protein degradation (Results not shown), which became more prominent with increased pH. The pH lower than 3.5 has demonstrated a similar degradation pattern.

The effect of highly alkaline pH (i.e. >11) or highly acidic pH (i.e. <3.5) on activated sludge EPS may effect the functional groups, and in particular carboxyl and amino groups from proteins. A high pH (>11) causes ionization of several charged functional groups, including carboxylic groups in proteins and carbohydrates, in the activated sludge EPS (Wingender *et al.*, 1999). At pH of greater than 11, the disulfide binding in proteins tends to break (Nielsen and Jahn, 1999) and the inner structure of the EPS could be damaged, with particles inside the EPS dispersed into solution. Sheng *et al.* (2006) have found that stability of flocs did not change from pH 5.0 to 9.0; however, as pH increased to 11.2, the dispersed mass dramatically increased from 0.082 g SS/L to 0.20 g SS/L. This dispersal of particles into solution may be due to protein destruction at pH of more than 11.

The pH of EPS protein samples' buffer was chosen to be neutral or slightly acidic to stabilize these proteins. At pH of 7.0, the proteins in the EPS will have an overall negative charge with some positively charged groups, although less so than carbohydrates in the EPS, which are considered to be negatively throughout (King and Forster, 1990). Nonetheless, the change in pH alone did not assist with the resolution of EPS proteins (Results not shown) and thus other additives were utilized.

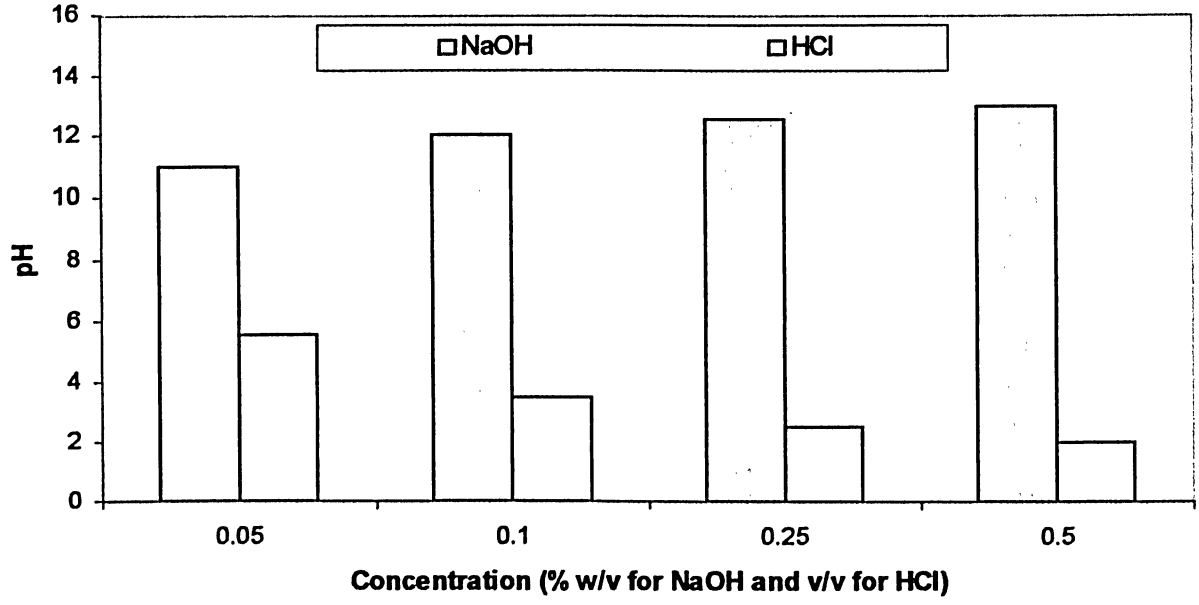


Figure 4.8 Effect of pH on the CER-extracted activated sludge EPS with increasing NaOH and HCl concentrations.

The floc and EPS stability has been reported in the literature to be affected by the ionic strength; therefore, the effect of ionic strength was assessed on EPS protein stability by addition

of monovalent (NaCl and NH_4Cl), divalent ($CaCl_2$, $MgCl_2$) and trivalent ($Fe(NO_3)_3$) cation salts and a chelating agent (EDTA) to the activated sludge EPS.

From the Table 4.2 it may be observed that CER extracted EPS treated with monovalent cation salts, NaCl and NH_4Cl, demonstrates a greater stability of the proteins in the EPS compared to crude EPS that has been desalted on a Sephadex G-25 column. From the literature, it has been reported that monovalent cations, such as sodium and ammonium displace divalent cations within the cation bridged floc structure by ion exchange, making flocs weak and sensitive to any physiochemical changes on the floc structure (Keiding and Nielsen, 1997). Evidently from this study it was found that the monovalent cations seem to strengthen the proteins in the microbial CER-extracted EPS fraction after treatment. The CER-extracted EPS treated with monovalent cation salts, NaCl and NH_4Cl, does indeed exhibit a greater amount of protein than other treatments. The results obtained from Tricine-polyacrylamide gel stained with silver nitrate and PAS stain also support that indeed 10 mM of NaCl and NH_4Cl (Figures 4.9-4.11) contain a considerable quantity of protein after the EPS sample was desalted on the Sephadex G-25 column. These findings validate the concentration results obtained from BCA protein concentration assay (Table 4.2) (Appendix B).

The EPS treated with divalent cation salts, $CaCl_2$ and $MgCl_2$, were found to contain less protein than the desalted EPS (Table 4.2) after treatment. In the literature, it has been reported that divalent cations, primarily calcium and magnesium, contribute to the ion bridging between the EPS matrix and cells (Kakii et al., 1985; Higgins and Novak, 1997; Keiding and Nielsen, 1997; Sheng *et al.*, 2006). The results found in this study demonstrate that $MgCl_2$ had low binding to the CER extracted EPS proteins. However, the EPS proteins were observed to form a solid complex with calcium ion and thus precipitate out of the solution. Keiding and Nielsen

(1997) observed an almost linear increase in proteins and carbohydrates with decrease of dissolved calcium ions.

The EPS sample treated with chelating agent EDTA was found to contain less protein than crude EPS desalted on G-25 Sephadex spun column (Table 4.2). EDTA is known for its ability to remove divalent cations from the activated sludge, causing the EPS matrix to fall apart and causing breakdown of the floc configuration (Sheng *et al.*, 2006). The EDTA did not demonstrate improvement in the EPS protein stability. The results obtained from Tricine-polyacrylamide gel stained with silver nitrate and PAS stain also demonstrate that 5mM of EDTA (Figure 4.9-4.11) contains small quantity of protein after the EPS sample was desalted on the Sephadex G-25 spun column.

The EPS sample treated with trivalent cation salt $Fe(NO_3)_3$ displays the lowest protein yield after desalting on the Sephadex G-25 column (Table 4.2). A decreased microbial EPS resolution is also observed on 10% Tricine-PAGE stained with silver nitrate and PAS stain (Figure 4.10a and Figure 4.11). It has been proposed in the past that trivalent cations, mainly iron and aluminum play a role in EPS stability and ferric iron has a high affinity for protein (Murthy and Novak, 2001). This finding was not supported by our results.

Table 4.2 Various chemical additives (salts and chelator) employed for extraction of EPS proteins From WWTP- and SBR-derived activated sludge EPS*.

Class of additive	Specific additive used	Additive concentration	Time of incubation (hours)	Sludge Sample Origin	EPS Protein Concentration (μg/ml) after treatment	EPS Carbohydrate Concentration (μg/ml) after treatment
Control	----	----	----	WWTP $S2_1$	155.31 ± 23.6	50.04 ± 1.33
				SBR 1	29.88 ± 4.64	18.83 ± 0.236
EPS desalted on Sephadex G-25 Column	----	----	----	WWTP $S2_1$	41.20 ± 3.54	28.26 ± 7.50
				SBR 1	----	----
Monovalent Salt†	NaCl	10mM-1M	3	WWTP $S2_1$	57.71 ± 6.08	46.60 ± 3.97
				SBR 1	----	----
	NH_4Cl	0.1M, 10mM	3, 24, 48, 96	WWTP $S2_1$	47.51 ± 0.919	35.97 ± 4.33
				SBR 1	----	----
Divalent Salt†	$CaCl_2$	0.1M, 10mM	3, 24, 48, 96	WWTP $S2_1$	6.33 ± 0.262	29.11
				SBR 1	----	----
	$MgCl_2$	0.1M, 10mM	3, 24, 48, 96	WWTP $S2_1$	29.19 ± 1.44	46.07 ± 9.67
				SBR 1	----	----
Trivalent Salt†	$Fe(NO_3)_3$	0.1M, 10mM	3, 24, 48, 96	WWTP $S2_1$	0.00 ± 1.88	31.32 ± 12.8
				SBR 1	----	----
Metal chelator	EDTA	10mM, 50mM pH 6.5, 5mM	3, 24, 48, 96	WWTP $S2_1$	39.34 ± 6.43	45.21 ± 12.5
				SBR 1	----	----

*NOTE: All the additives were incubated at 4°C on ice.
†NOTE: The salts were desalted on a Sephadex G-25 chromatography media employing a spun column technique.

Table 4.3 Various chemical additives (detergents and chaeotropic agents) employed for extraction of EPS proteins From WWTP- and SBR-derived activated sludge EPS*.

Class of additive	Specific additive used	Additive concentration	Time of incubation (hours)	Sludge Sample Origin	EPS Protein Concentration (µg/ml) after treatment	EPS Carbohydrate Concentration (µg/ml) after treatment
Control	----	----	----	WWTP $S2_1$	155.31 ± 23.6	50.04 ± 1.33
				SBR 1	29.88 ± 4.64	18.83 ± 0.236
Detergent	SDS	0.01-1%	1-30 minutes, 1, 3	WWTP $S2_1$	----	----
				SBR 2	882.22 ± 11.35	58.75
	TRITON® X-100	0.1%	3	SBR 2	324.67 ± 61.8	35.75 ± 20.2
				SBR 1	----	----
Chaeotropic agent	GHCl	6M	24	WWTP $S2_1$	----	----
				SBR 2	817.57 ± 16.3‡	0.00
				SBR 3	453.15	----
	GHCl + 0.1% TFA†	6M	24	WWTP $S2_1$	----	----
				SBR 3	155.69	----
	Urea	8M	3, 24	WWTP $S2_1$	----	----
				SBR 1	----	----

*NOTE: All the additives were incubated at 4°C on ice.
†NOTE: TFA was used in order to provide conditions for complete protein denaturation and unfolding.

Table 4.4 Various chemical additives (organic solvents, acid, base, reducing agent) employed for extraction of EPS proteins from WWTP- and SBR-derived activated sludge EPS*

Class of additive	Specific additive used	Additive concentration	Time of incubation (hours)	Sludge Sample Origin	EPS Protein Concentration (µg/ml) after treatment	EPS Carbohydrate Concentration (µg/ml) after treatment
Control	----	----	----	WWTP $S2_1$	155.31 ± 23.6	50.04 ± 1.33
				SBR 1	29.88 ± 4.64	18.83 ± 0.236
Organic solvent	Acetonitrile With/without TFA†	50%	3, 24	WWTP $S2_1$	----	----
				SBR 1	----	----
	Ethanol	70%	48	WWTP $S2_1$	----	----
				SBR 1	----	----
Acid	HCl	0.1-0.2%	3, 24	WWTP $S2_1$	102.14 ± 11.8	----
				SBR 1	----	0.00
Base	NaOH	0.05%, 0.10%, 0.25%, 0.50%	3, 24	WWTP $S2_1$	126.11 ± 5.93	35.30 ± 0.338
				SBR 1	----	0.00
Reducing agent	DTT‡	2mM	3	WWTP $S2_1$	----	----
				SBR 2	1527.28 ± 20.2	68.83 ± 3.78

*NOTE: All the additives were incubated at 4°C on ice.
†NOTE: TFA was used in order to provide conditions for complete protein denaturation and unfolding.
‡NOTE: Concentration was measured after the sample was concentrated with Phenol-Ether.

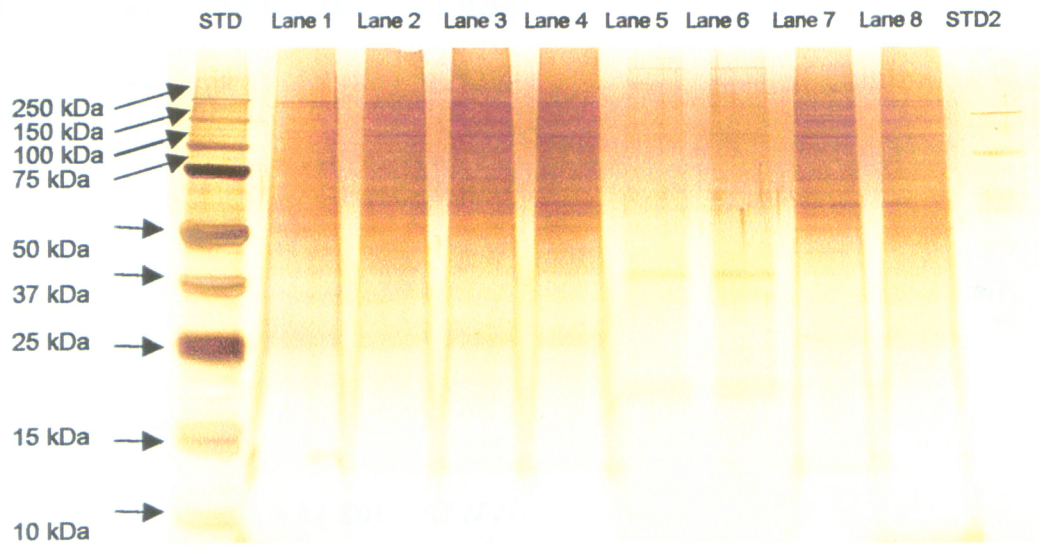


Figure 4.9 Silver stained Tricine-PAGE (10%) of WWTP-derived activated sludge EPS protein extraction and detection by use of salts and chelating agent following desalting on a Sephadex G-25 column. Lane 1, EPS with protease inhibitors; lane 2, EPS fraction collected after desalting on Sephadex G-25; lane 3, EPS treated with NH_4Cl (10mM w/v); lane 4, EPS treated with NaCl (10mM w/v); lane 5, EPS treated with $CaCl_2$ (10mM w/v); lane 6, EPS treated with $CaCl_2$ (10mM w/v) in the presence of DTT (2mM w/v); lane 7, EPS treated with $MgCl_2$ (10mM w/v); lane 8, EPS treated with EDTA (5mM w/v).

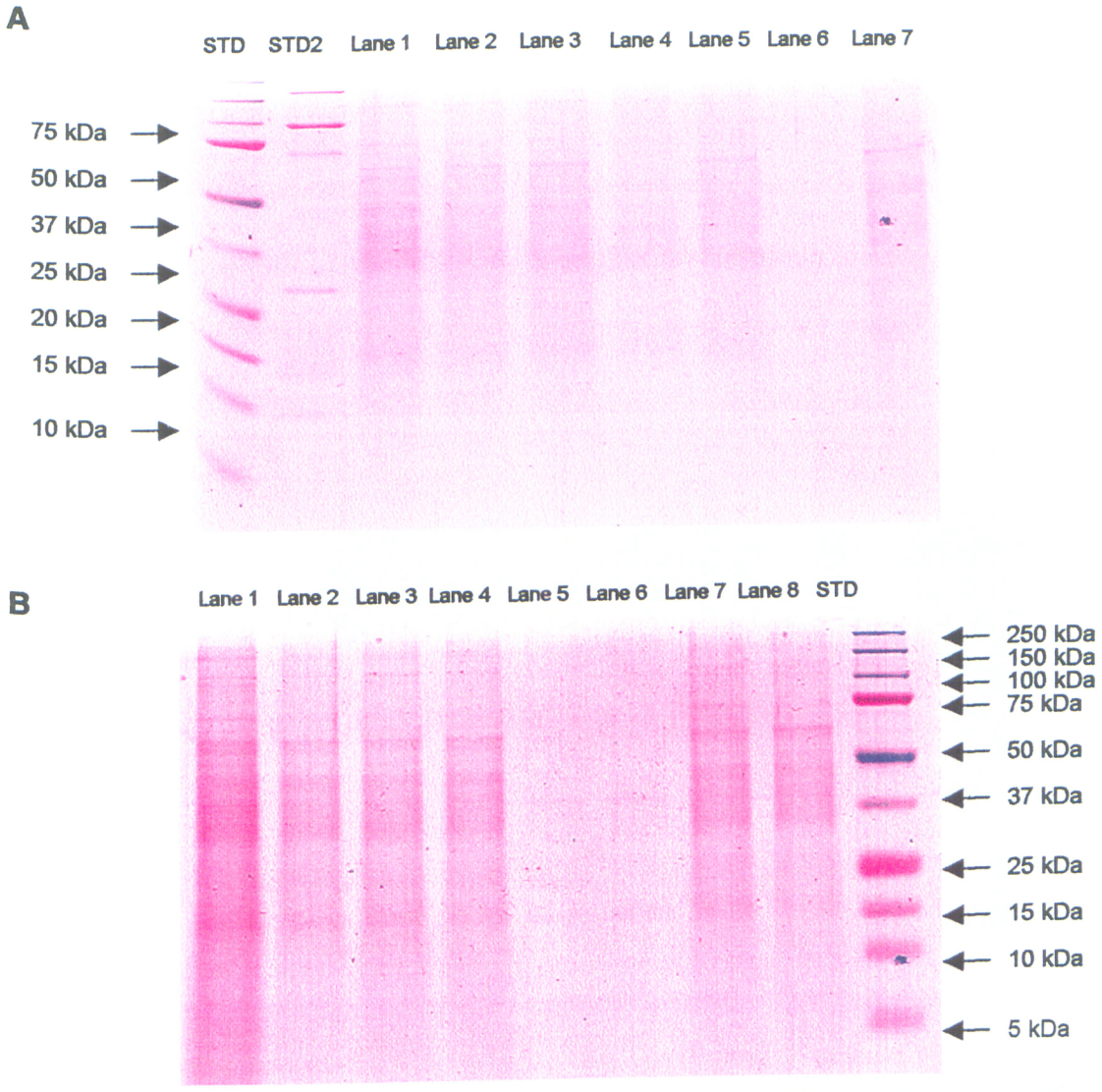


Figure 4.10 PAS acid fuchsin stained Tricine-PAGE (10%) of WWTP-derived activated sludge EPS protein and glycoprotein extraction and detection by use of salts and chelating agent following desalting on a Sephadex G-25 column. (A). Lane 1, EPS with protease inhibitors prior to desalting; lane 2, EPS treated with 10mM NH_4Cl; lane 3, EPS treated with NaCl (10mM w/v); lane 4, EPS treated with $CaCl_2$ (10mM w/v); lane 5, EPS treated with $MgCl_2$ (10mM w/v); lane 6, EPS treated with $Fe(NO_3)_3$ (10mM w/v); lane 7, EPS treated with EDTA (5mM w/v). (B). Lane 1, EPS with protease inhibitors; lane 2, EPS fraction collected after desalting on Sephadex G-25 column; lane 3, EPS treated with NH_4Cl (10mM w/v); lane 4, EPS treated with NaCl (10mM w/v); lane 5, EPS treated with $CaCl_2$ (10mM w/v); lane 6, EPS treated with $CaCl_2$ (10mM w/v) in the presence of DTT (2mM w/v); lane 7, EPS treated with $MgCl_2$ (10mM w/v); lane 8, EPS treated with EDTA (5mM w/v).

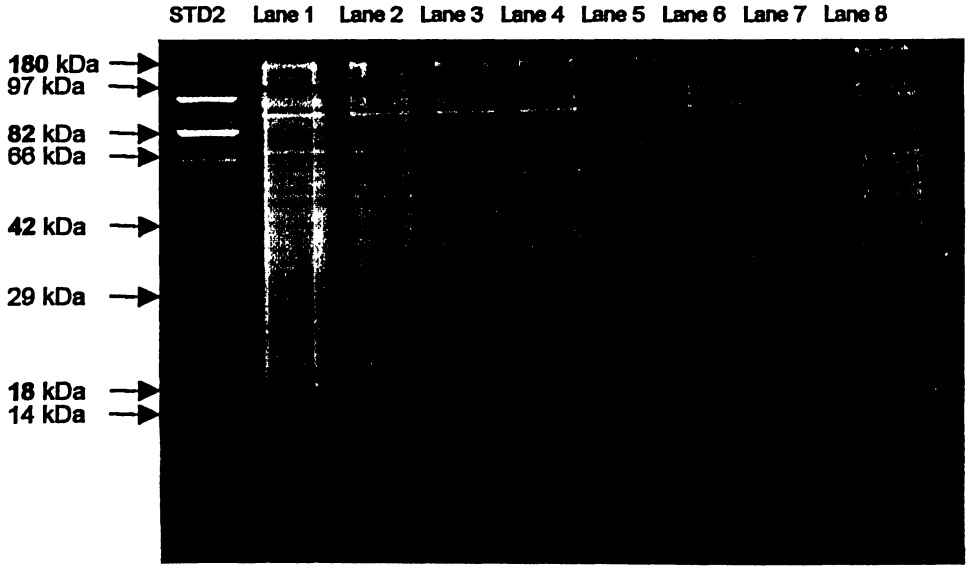


Figure 4.11 SYPRO Ruby stained Tricine-PAGE gel (10%) of WWTP-derived activated sludge EPS protein and glycoprotein extraction and detection by use of salts and chelating agent following desalting on a Sephadex G-25 column. Lanes 1 and 2, EPS with protease inhibitors prior to desalting; lane 3, EPS treated with NH_4Cl (10mM w/v); lane 4, EPS treated with NaCl (10mM w/v); lane 5, EPS treated with $CaCl_2$ (10mM w/v); lane 6, EPS treated with $MgCl_2$ (10mM w/v) lane 7, EPS treated with $Fe(NO_3)_3$ (10mM w/v) lane 7, EPS treated with EDTA (5mM w/v).

The overall advantage of desalting procedure is the visualization of distinct protein bands after electrophoresis (Figures 4.9 and 4.11). The disadvantage of the desalting procedure is the large loss of proteins, which were lost in the process. The EPS prior to desalting procedure contained 155.31 ± 23.6 μg/ml of protein and 50.04 ± 1.33 μg/ml of carbohydrate (Table 4.2). After desalting the same sample on G-25 Sephadex column, the EPS contained 41.20 ± 3.54 μg/ml of protein (control) and 28.26 ± 7.50 μg/ml of carbohydrate (control) (Table 4.2). This demonstrates an almost 4-fold decrease in total amount of proteins and a 1.2-fold decrease in total amount of carbohydrates in microbial EPS sample compared to control sample. Therefore, other additives were considered for protein stabilization.

Guanidine hydrochloride (GHCl) stabilized and solubilized the EPS proteins the best compared to crude EPS sample (approximately 4 fold increase) (Table 4.3). However, GHCl is not compatible with the 2x sample buffer and requires pure denatured ethanol to remove the residual GHCl. This method may have washed a portion of proteins off. Ethanol did not improve protein resolution (results not shown). A nonionic detergent TRITON® X-100 exhibited a higher protein concentration when compared to the crude EPS (approximately 2 fold increase), although less than SDS (Table 4.3). The ionic detergent sodium dodecyl sulfate (SDS) improved protein and glycoprotein stability (Figure 4.12) and exhibited a high protein extraction yield (SBR: 882.22 ±11.3; WWTP: 1103.83 ± 18.1 μg/ml) compared to control (SBR: 32.39 ±12.5 μg/ml; WWTP: 155.62 ±12.5 μg/ml) (approximately 27 fold increase in SBR- and 7-fold increase in WWTP-derived activated sludge EPS sample). This suggests that SDS may have solubilized the insoluble glycoprotein fraction (Table 4.3). The EPS proteins were denatured by boiling in SDS prior to electrophoresis in order to eliminate the majority of secondary and tertiary structures. Reducing agent dithiothreitol (DTT) demonstrated an increase in EPS proteins (SBR: 1527.28

+/-20.2 μg/ml (following P-E precipitation); WWTP: 6013.31 ± 126 μg/ml) (approximately 47-fold increase in SBR- and 39-fold increase in WWTP-derived activated sludge EPS sample) and thus was also considered to be an excellent additive (Table 4.4) (Appendix B).

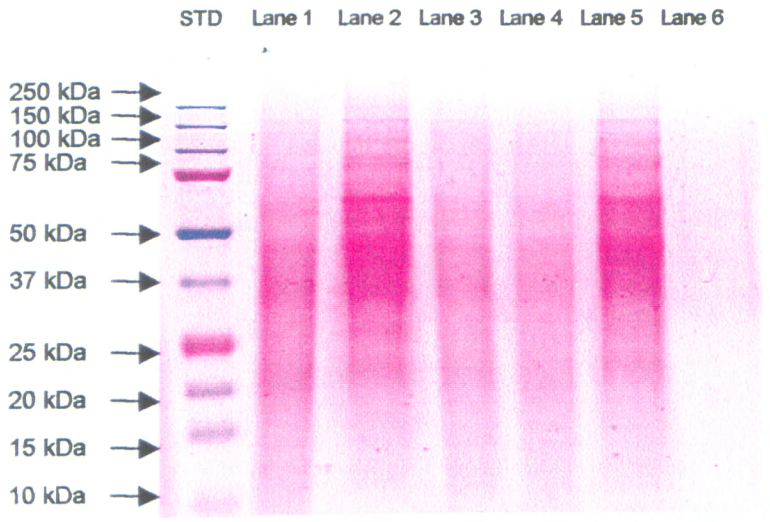


Figure 4.12 PAS stained Tricine-PAGE (10%) of WWTP-derived activated sludge EPS protein and glycoprotein extraction and detection by use of SDS detergent and sonication. Lane 1, EPS with protease inhibitors; lanes 2 and 5, EPS treated with boiled SDS (1% w/v) for 10 minutes; lane 3, EPS sonicated for 3 minutes; lane 4, untreated EPS; lane 6, 2x sample buffer.

4.3.2 Reverse Phase Liquid Chromatography

Reverse phase liquid chromatography (RPLC) employing a C_{18} column in the batch mode was utilized to clean up the complex microbial EPS sample, to separate proteins and peptides within the EPS with various hydrophobicity, and to obtain samples with limited number of carbohydrates. It has been demonstrated in previous studies that the hydrophobic fraction of the EPS is comprised of proteins (amino acids with hydrophobic side groups) and the hydrophilic portion is comprised of mainly neutral carbohydrates (Jorand *et al.*, 1998; Liao *et al.*, 2000).

After employing the RPLC procedure on SBR-derived activated sludge EPS sample, a thick band was visualized by Tricine-PAGE (Figure 4.13) in the region of 40-60 killodaltons. From Figure 4.13, it is apparent that the RPLC method employing C_{18} column achieved a better protein and peptide separation than the buffer additive method.

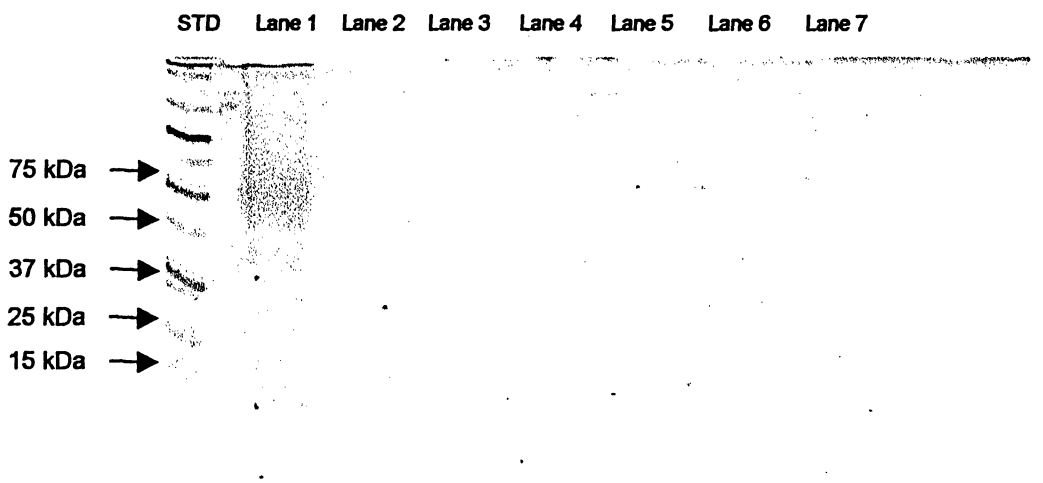


Figure 4.13 Colloidal Coomassie blue stained Tricine-PAGE (10%) of SBR-derived activated sludge EPS protein extraction by reverse-phase liquid chromatography (RPLC). Lane 1, EPS proteins extracted in 70% (v/v) acetonitrile elution buffer; lane 2, first wash in acetonitrile (5% v/v); lane 3, second wash in acetonitrile (5% v/v); lane 4, third wash in acetonitrile (5% v/v); lane 5, supernatant; lane 6, EPS proteins extracted in 90% (v/v) acetonitrile elution buffer; lane 7, EPS proteins extracted in 100% (v/v) acetonitrile elution buffer.

Nevertheless, the WWTP-derived microbial EPS sample was not concentrated but in fact diluted when the RPLC procedure was employed (Tricine-PAGE results not shown). The RPLC method using C_{18} column demonstrates that the bead cutoff in 70% acetonitrile elution buffer was approximately at 40 μg/ml of protein and 20 μg/ml of carbohydrate, whether the crude sample contained more protein or less protein than this. Accordingly, the crude samples containing a lower EPS protein and carbohydrate concentration were concentrated (SBR-derived) and crude

Table 4.5 Protein and carbohydrate concentration in WWTP- and SBR-derived activated sludge EPS during RPLC protein extraction. Results are expressed as the average of ± one standard deviation.

Elution Buffer	Sludge Sample Origin	Protein Concentration (µg/ml)	Carbohydrate Concentration (µg/ml)
Crude EPS	SBR 4	34.59 +/-0.0600	19.89 +/-0.839
	WWTP $S3_1$	155.62 +/-11.7	56.17 +/-6.32
Supernatant	SBR 4	82.90 +/-19.4	------
	WWTP $S3_1$	------	33.43 +/-4.69
Wash 1	SBR 4	0.00	0.00
	WWTP $S3_1$	0.00	0.00
Wash 2	SBR 4	0.00	0.00
	WWTP $S3_1$	0.00	0.00
Wash 3	SBR 4	0.00	0.00
	WWTP $S3_1$	0.00	0.00
Elution in 70% Acetonitrile	SBR 4	41.81 +/-1.58	21.35 +/-2.83
	WWTP $S3_1$	42.58 +/-0.499	23.24+/-6.88

samples containing a much higher EPS protein concentration were diluted (WWTP-derived).

It was hypothesized that further protein concentration of dilute activated sludge EPS samples may increase the resolution of proteins on Tricine-PAGE and perhaps resolve them into clear, distinct bands.

4.3.3 EPS Protein Concentration and Precipitation

Protein precipitation methods are used to concentrate proteins or eliminate interferences before electrophoresis or protein determination. Out of the numerous protein precipitation protocols available in the literature, the three that are frequently utilized are the Chloroform-Methanol (C-M), Trichloroacetic acid (TCA) and Phenol-Ether (P-E) protocols (Sauve *et al.*, 1995; Ziegler *et al.*, 1997; Aguilar *et al.*, 1999).

After the SBR-derived crude activated sludge EPS was treated with various protein precipitation techniques, the treated EPS sample was ran on 10% Tricine-polyacrylamide electrophoresis in order to assess the method of choice. From Figure 4.14, it is evident that the P-E precipitation protocol is the method of choice. Both the P-E and TCA-DOC methods effectively precipitated proteins in the EPS sample fraction. However, only P-E gave distinct protein bands.

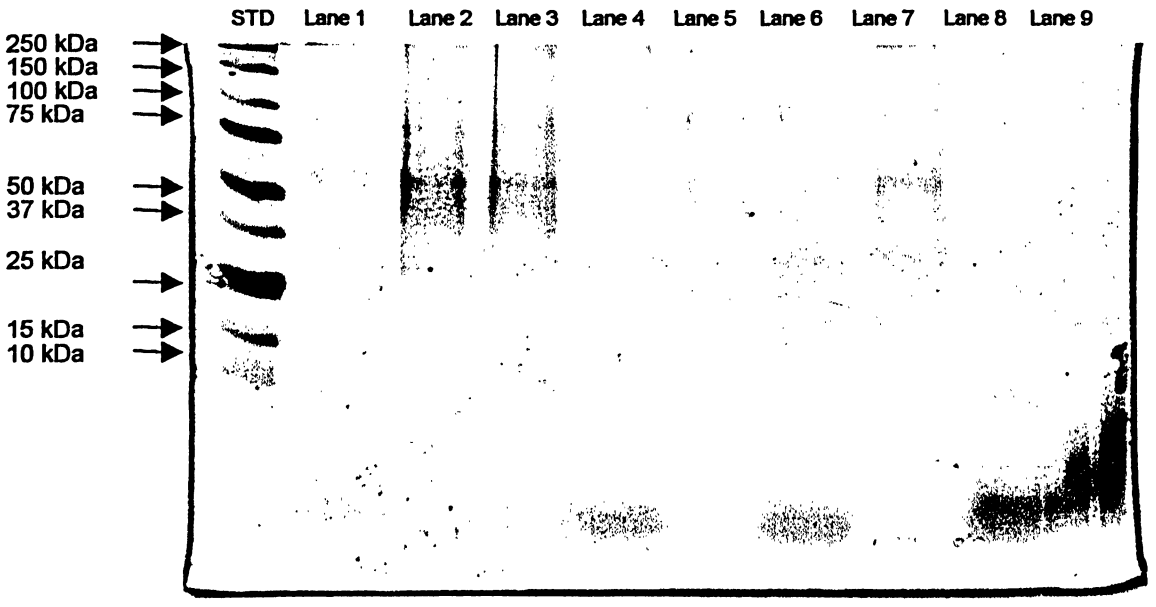


Figure 4.14 Colloidal Coomassie blue stained Tricine-PAGE (10%) of SBR-derived activated sludge EPS protein extraction by precipitation method. Lane 1, untreated EPS (control); lanes 2 and 3, P-E precipitation method; lane 4, TCA precipitation method; lane 5, TCA-acetone precipitation method; lane 6, TCA-DOC precipitation method; lane 7, TCA-DOC-ethanol precipitation method; lanes 8 and 9, C-M precipitation method.

4.3.4 Additive Buffer Combined with EPS Protein Concentration/Precipitation

After successfully precipitating the activated sludge EPS proteins using the P-E precipitation protocol, the EPS proteins were subsequently extracted by using several buffer additives (i.e. SDS, acetonitrile with and without trifluoroacetic acid (TFA), dithiothreitol (DTT), and borate) to achieve a higher protein yield in conjunction with the protein precipitation (P-E) method prior to Tricine-PAGE.

SDS was used within its critical micelle concentration (CMC) (0.1% w/v) to resolve EPS proteins. Urea chaeotrope was employed because it does not interfere with 2x sample buffer. Acetonitrile (50% v/v) with and without TFA (0.1% v/v) was utilized as an organic solvent to achieve a higher EPS protein separation with or without reducing conditions. Borate (0.2 M w/v) was utilized in order to remove glycosylated moiety that might cause a poorer extraction of EPS proteins.

The results in Figure 4.15 demonstrate that DTT had the highest protein yield when it was added to the crude EPS sample and thus it was employed in combination with the P-E precipitation protocol.

The results of this experiment demonstrate that including DTT (2 mM w/v) before EPS protein precipitation increases the separation and quantity of proteins in the SBR-derived activated sludge EPS fraction (Figure 4.15). There are thirteen protein bands that are demonstrated in Figure 4.15. These possibly exocellular EPS proteins are approximately: 123, 91, 84, 67, 66, 56, 51, 38, 31, 28, 23, 18, and 13 kDa.

The same method of adding DTT following P-E precipitation was employed on WWTP-derived microbial EPS fraction in order to isolate the exocellular proteins from the EPS matrix. It was determined that employing DTT following P-E treatment did eliminate some interactions in

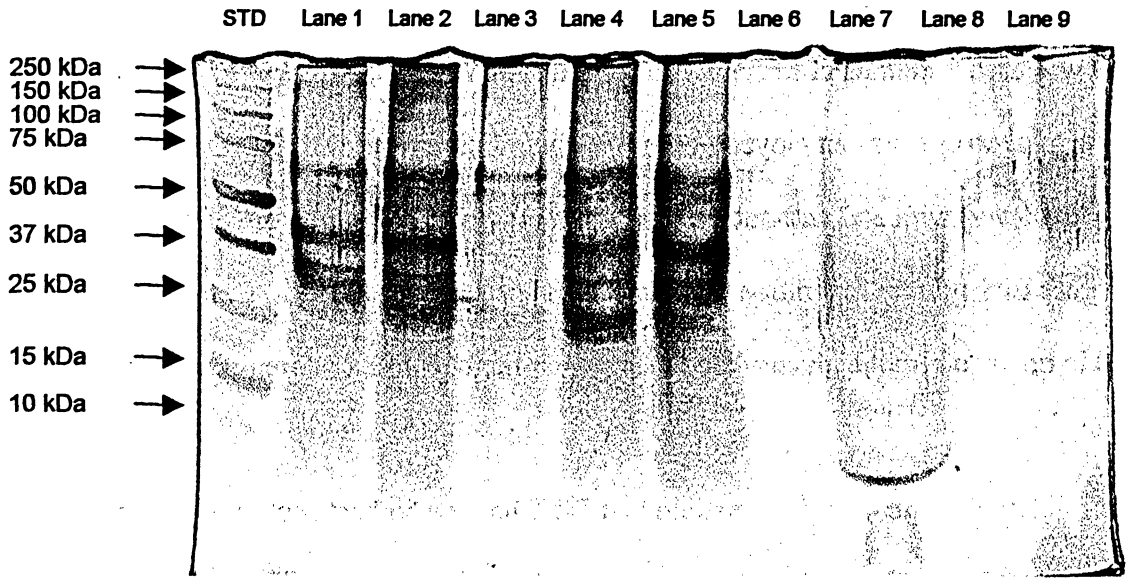


Figure 4.15 Silver nitrate stained Tricine-PAGE (10%) of SBR-derived activated sludge EPS protein extraction by use of additives following P-E precipitation method. Lane 1, P-E precipitation; lane 2, SDS (0.1% w/v) following P-E precipitation method; lane 3, acetonitrile (50% v/v) following P-E precipitation method; lane 4, acetonitrile (50% v/v) + trifluoroacetic acid (0.1% v/v) following P-E precipitation method; lane 5, DTT (2mM w/v) following P-E precipitation method; lane 7, borate (0.2M w/v) following P-E precipitation method; lanes 8 and 9, urea (6M w/v) following P-E precipitation method.

the EPS sample; however, the proteins observed in the treated fraction match the proteins in the EPS fraction containing only protease inhibitors. Addition of calcium to the EPS sample along with DTT followed by P-E precipitation did not improve protein resolution but in fact hindered it. Employing DTT alone also did not improve protein visualization on Tricine-PAGE (Figure 4.16).

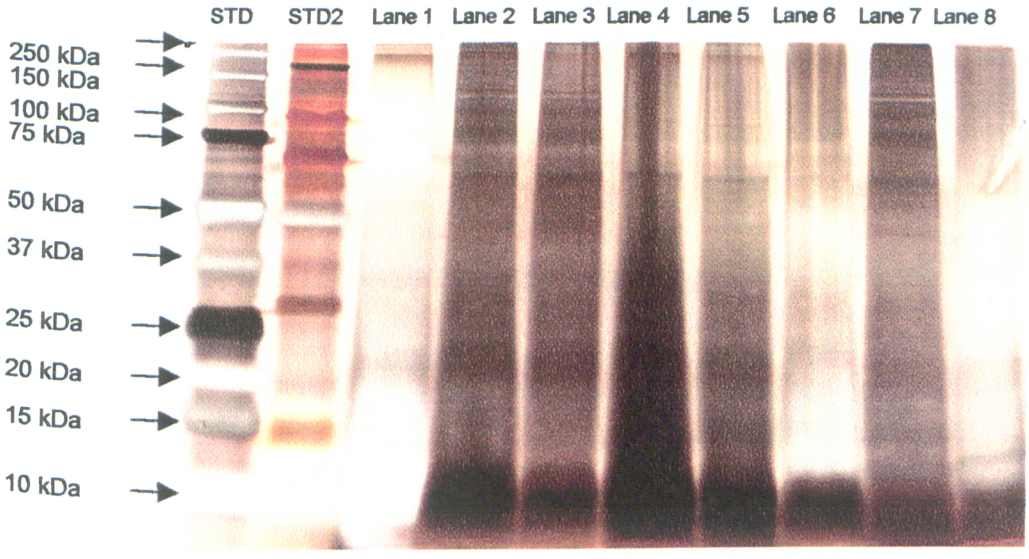


Figure 4.16 Silver nitrate stained Tricine-PAGE (10%) of EPS containing proteins from WWTP-derived samples. Lane 1, untreated EPS (control); lanes 2 and 3, EPS with inhibitor cocktail added; lanes 4, 5 and 6, EPS treated with 2mM DTT; lane 7, EPS treated with 2mM DTT and concentrated by P-E precipitation method; lane 8, EPS treated with DTT (2mM w/v) and $CaCl_2$ (10mM w/v) and concentrated by P-E precipitation method.

From the BCA protein assay (Figure 4.17), it is evident that P-E method gives a 10-fold increase (291.91 +/-5.91 µg/ml) in protein compared to crude EPS sample (32.39 +/-0.00 µg/ml). The 2mM DTT following P-E method gives a 5-fold increase (1527.28 +/-20.2 µg/ml) compared

with the P-E method alone. RPLC method gives an increase of only 1-fold (41.67 +/-1.55 μg/ml) (Appendix B).

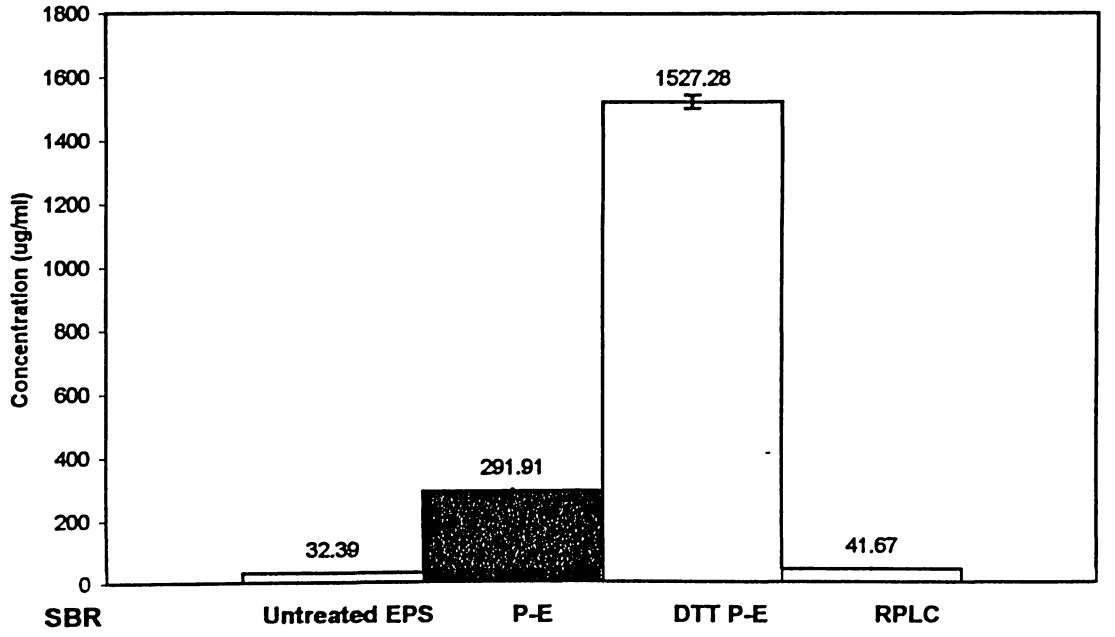


Figure 4.17 Protein concentration (μg/ml) comparison between SBR-derived untreated EPS (control), EPS concentrated by P-E precipitation method, EPS treated with 2mM DTT and concentrated by P-E precipitation method, and EPS treated with RPLC. Results are expressed as the average of ± one standard deviation.

Glycoprotein Carbohydrate Estimation Kit (23260) was utilized to simply and rapidly detect glycoproteins and estimate carbohydrate content. From the results obtained the WWTP-derived EPS samples contained a larger portion of glycosylated proteins in the untreated EPS portion. The glycosylation in WWTP-derived EPS varied from 24-100% (Figure 4.18). The glycosylated carbohydrate content was compared to the total carbohydrates derived from WWTP EPS samples (Figure 4.18-4.19). It was found that within samples 2, 3 and 4 there was no

apparent association between quantities of total carbohydrates to glycoprotein carbohydrate estimate. Furthermore, when protease inhibitor cocktail is added to the untreated EPS, the glycosylated carbohydrate content reaches 100% (results not shown) (Appendix B).

When the untreated fraction was precipitated with P-E, a 100% glycoprotein carbohydrate content was also reached (Figure 4.20). This demonstrates that the P-E precipitation method precipitates the glycoproteins present in the activated sludge EPS sample. However, DTT by itself or with $CaCl_2$ followed by P-E precipitation appears to diminish glycosylation present in the EPS sample 13- and 9-fold, respectively. Even the 2mM DTT treatment of EPS followed by P-E precipitation diminished glycosylation compared to the EPS only treated with P-E precipitation (1.3-fold). Conceivably, the removal of disulfide bridge links by DTT deglycosylates a portion of the EPS proteins and thus an alkylation must be conducted to stabilize the DDT-reduced protein fraction. It may also be possible that the DTT interferes with the assay kit utilized; however, there were no possible interferences listed in the kit.

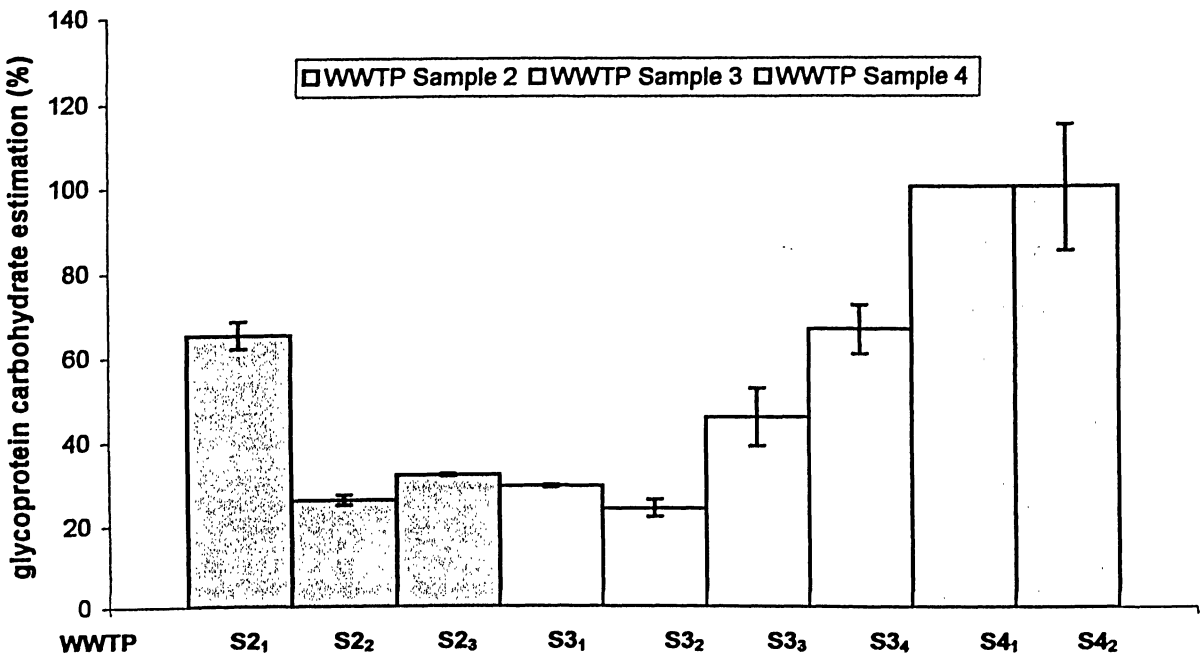
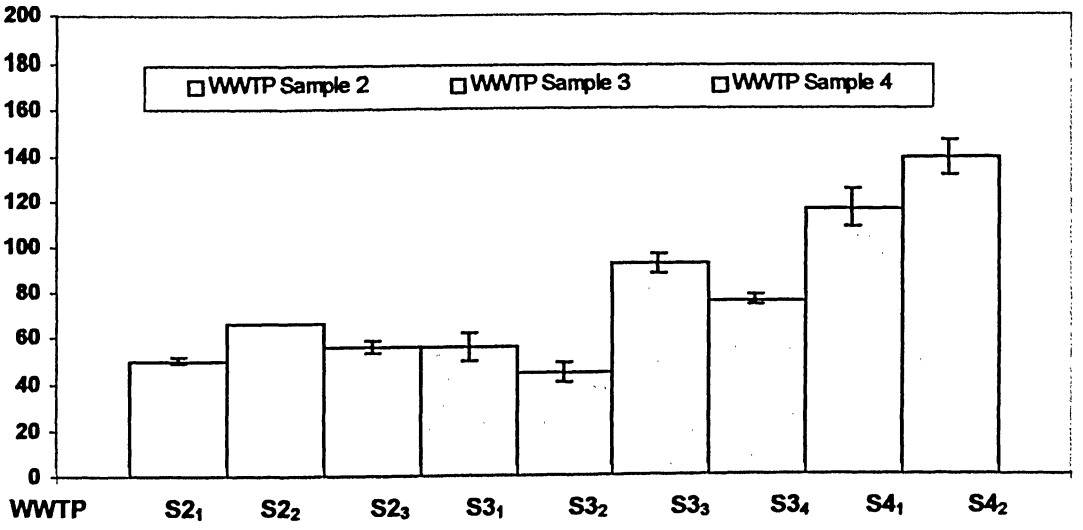


Figure 4.18 Glycoprotein carbohydrate content estimate (%) comparison between the untreated WWTP-derived EPS. Results are expressed as the average of ± one standard deviation.

A



B

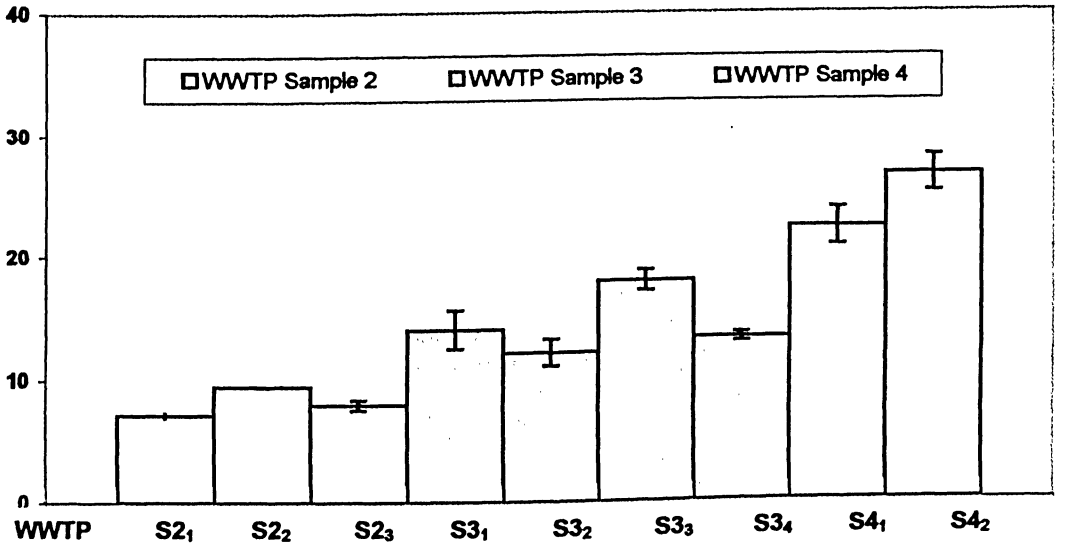


Figure 4.19 Carbohydrate content in (A) μg/ml and mg/g MLSS (B) comparison of WWTP-derived EPS. Results are expressed as the average of ± one standard deviation.

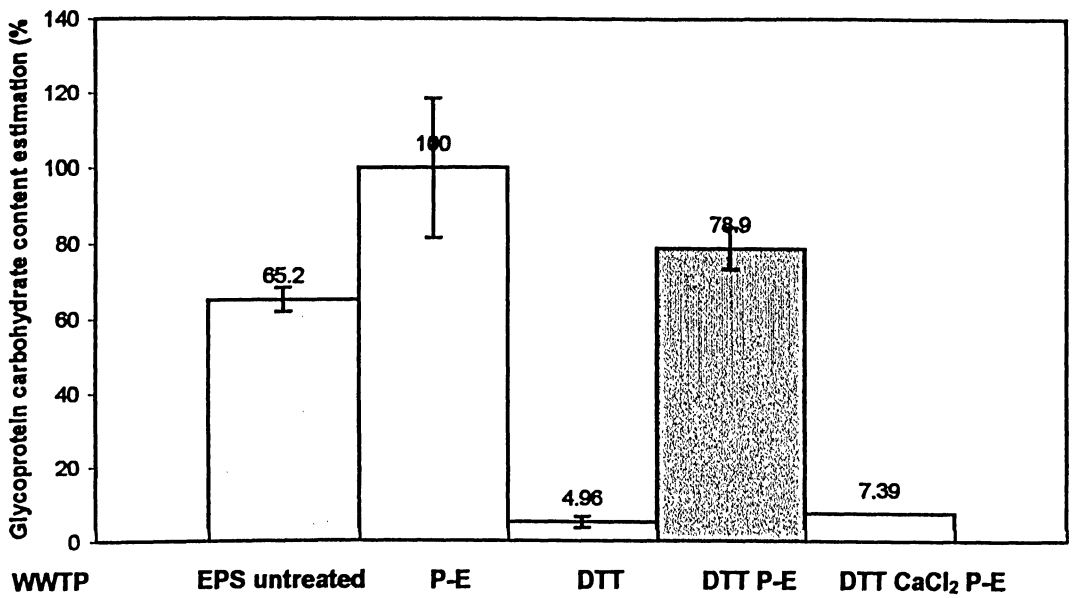


Figure 4.20 Glycoprotein carbohydrate content estimate comparison between untreated EPS (control), EPS concentrated by P-E precipitation method, EPS treated with DTT (2mM w/v), EPS treated with DTT (2mM w/v) and concentrated by P-E precipitation method, and EPS treated with DTT (2mM w/v) and $CaCl_2$ (10mM w/v) and concentrated by P-E precipitation method. Results are expressed as the average of ± one standard deviation.

4.3.4 Enzymatic Deglycosylation of EPS Glycoproteins

In order to determine how much of the molecular mass discrepancy for EPS proteins could be accounted for by the addition of glycosylated groups, the EPS was enzymatically deglycosylated by the use of enzymatic glycoprotein deglycosylation kit (EMD Biosciences/Calbiochem, Darmstadt, Germany). The kit employed completely removes N- and O-linked oligosaccharides from the glycosylated protein without any protein degradation (Thotakura and Bahl, 1987).

From the results obtained in Figure 4.21a and b, it may be observed that the molecular masses of the EPS proteins did not change significantly after treating the fraction with various deglycosylating enzymes. The presence of O-linked or N-linked carbohydrates in CER-extracted activated sludge EPS could not be determined by this method. The bovine fetuin used as a control does show a deglycosylation and a major protein drop from 70 killodaltons to 50 killodaltons. The overall band pattern is shifted for the bovine fetuin control, demonstrating that the denaturing deglycosylation does work for the 200 μg of glycoprotein. The WWTP- and SBR-derived samples were not able to be deglycosylated possibly due to EPS carbohydrates remaining associated with exocellular proteins in the EPS following enzymatic deglycosylation. Another possibility is that the EPS glycoproteins are not attached through classical N- or O-linkages.

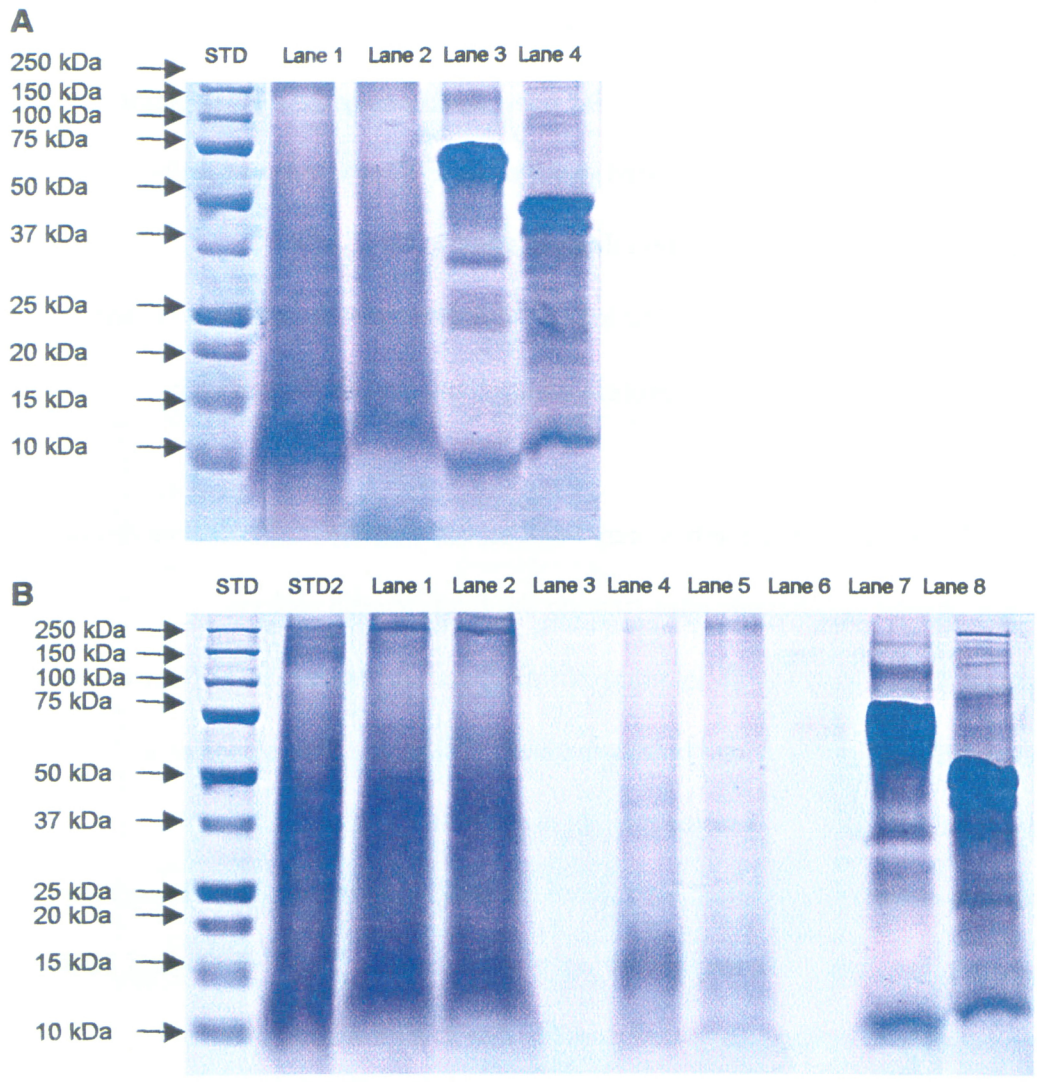


Figure 4.21 Coomassie Brilliant Blue stained Tricine-PAGE (10%) of SBR- and WWTP-derived activated sludge EPS proteins treated with deglycosylating enzymes. (A). Lane 1, untreated WWTP-derived EPS (control); lane 2, WWTP-derived EPS enzymatically deglycosylated and denatured; lane 3, bovine fetuin (200 μg) (glycosylated control); lane 4, bovine fetuin (200 μg) enzymatically deglycosylated and denatured (deglycosylated control). (B). Lane 1, untreated WWTP-derived EPS (control); lanes 2 and 3, WWTP-derived EPS enzymatically deglycosylated and denatured; lane 4, 2x sample buffer; lane 5, SBR-derived EPS treated with DTT (2mM w/v) and concentrated by P-E method; lane 6, SBR-derived EPS treated with 2mM DTT and concentrated by P-E method followed by enzymatic deglycosylation and denaturation; lane 7, 2x sample buffer; lane 8, bovine fetuin (200 μg) (glycosylated control); lane 9, bovine fetuin (200 μg) enzymatically deglycosylated and denatured (deglycosylated control).

4.4 EPS Protein Identification

In order to identify the proteins in the EPS, the EPS samples were either purified by the RPLC method or combining chemical buffer additive with precipitating the protein. The combined method of addition of chemical buffer additive with protein precipitation was followed by 10% Tricine-PAGE and staining with Coomassie blue, colloidal Coomassie, or silver nitrate. After throughoutly destaining, the gel bands were excised and analyzed by ESI-LC-MS/MS.

4.4.1 Tandem Electrospray Ionization Mass Spectrometry (ESI-LC-MS/MS)

After RPLC, the EPS sample was digested with 0.5 μg of serine protease (i.e. trypsin) in 1x digestion buffer. After incubation with trypsin (overnight), the enzymatic peptides obtained from the EPS sample were ZipTipped through C_{18} column and loaded unto ESI-LC-MS/MS which employs ion trap. The MALDI-TOF-MS was not utilized due to insufficiency of equipment parts. After running the obtained sequence on data searches such as BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and then running the obtained spectrum on Mascot (http://www.Matrixscience.com), the parent protein producing significant alignments was identified to be a 3+ fragment of a peptidase (BLAST locus: ZP_00907499 http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=protein&val=82744985; Mascot locus gi|82744985) (Table 4.6). (Appendix E contains the sequences producing significant alignments from BLAST data search).

Peptidase M3B is a proteolytic enzyme that catalyzes the splitting of proteins into smaller peptide fractions and amino acids by proteolysis. This result was expected due to the naturally present extracellular enzymes in the activated sludge which hydrolyze organic matter in order to degrade the polymers into smaller assimilable molecules that may be taken up by bacteria for

Table 4.6 The highest peptide matches of RPLC-treated EPS sample from BLAST and Mascot search engines producing considerable alignments.

Name of Protein	Score	Expect	Identities	Positives	Length	Organism	Search Engine
Peptidase M3B, oligoendo -peptidase F	74	6e-13	22/22 (100%)	22/22 (100%)	594 aa	Clostridium beijerincki	BLAST
Peptidase M3B, oligoendo -peptidase F	51	0.52	43/210	---	2289.2004 bases	Clostridium beijerincki	Mascot

intracellular metabolism (Ubukata, 1998; Sanders *et al.*, 2000; Gessesse *et al.*, 2003). The origin of the enzymes in the activated sludge may be from being attached to cell surface (ecto-enzymes) or released (exo-enzymes) into the medium in the free form prior to developing complexes with humic substances or other polymers (Cadoret *et al.*, 2002). The oligoendopeptidase F found in this study is an exoenzyme because of its association with the EPS (Frølund *et al.*, 1995). This exoenzyme is thought to be approximately 22 kDa in length, which matches as one of the proteins bands obtained from the EPS sample (21-23 kDa region).

After optimization of the chemical additive method with protein precipitation, the gel band obtained from the Tricine-PAGE was excised, digested, ZipTipped, and ran on ESI-LC-MS/MS. The destaining and removal of the protein extract was performed on individual gel slices containing a single protein band. The results of this digestion did not yield meaningful results, suggesting that the protein band within the Tricine-PAGE gel still contains substances that interfere with the protein analysis of the activated sludge EPS sample. Furthermore, the EPS proteins may not have been characterized and thus may not contain entries in Mascot and BLAST databases.

Overall, obtaining an exoenzyme in a digested RPLC-treated EPS sample demonstrates that EPS indeed contains a large quantity of this naturally present enzyme that degrades simple wastewater polymers into monomers in order for these substances to be taken up by the cells. This would explain the importance of high molecular weight compounds, such as glycoproteins, in the EPS that are most likely strategically formed by the cells in order to protect these compounds from enzyme hydrolysis. It has been found by Ubukata (1998) that high molecular weight compounds are hydrolyzed slowly by hydrolases. This would explain the glycoprotein formation and potential structural role in the EPS.

CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS

The purpose of this study was to resolve the protein fraction in the activated sludge EPS by describing a novel purification, isolation and extraction method and endeavor to reveal that a considerable portion of the EPS proteins consist of glycosylated proteins. With the obtained data, a better understanding of the function of EPS proteins on the floc structure and formation in the activated sludge has been acquired. Specific conclusions that can be drawn from the results presented are summarized below:

1. SBR-derived EPS samples contained less total EPS than WWTP-derived EPS samples because these are different systems. The laboratory- and full-scale systems both contained protein as the predominant constituent of the EPS, followed by carbohydrate and a smaller portion of DNA. No correlation was found between EPS content and SVI values in either sample.
2. A crude EPS samples could not be adequately resolved on Tricine-PAGE. In order to adequately resolve these EPS samples, the samples should be treated with 2mM DTT followed by P-E precipitation to cleave disulfide bond crosslinks within proteins and between protein subunits in the EPS. The EPS sample might also be treated with 1% (w/v) SDS to dissociate hydrophobic interactions from the EPS, which contains both soluble and insoluble proteins.
3. Monovalent cations, especially NaCl, were found to stabilize CER-extracted EPS proteins the best followed by Sephadex G-25 desalting column treatment.
4. Possible glycosylation or strongly noncovalent interactions between EPS protein and carbohydrate were observed using PAS stain and glycoprotein carbohydrate estimation kit, especially after removal of hydrophobic and ionic interactions as well as disulfide

linkages. These glycoproteins or noncovalent proteins strongly bound with carbohydrates may potentially be adhesins, including flagella, fimbriae, and/or pili, and may act as protection against hydrolases that are naturally present in the EPS matrix. Nevertheless, these proteins were not effectively deglycosylated using enzymatic deglycosylation possibly due to their resistance to deglycosylation or not enough enzymes added to fully deglycosylate the glycoconjugates found in the EPS glycoprotein portion.

5. A 22-kDa oligoendopeptidase F from M3B family was tentatively identified as the major protein in RPLC-treated SBR-derived in-solution EPS sample. This exoenzyme is naturally present in the EPS and hydrolyzes organic matter in order for the bacteria to uptake the smaller molecules for intracellular metabolism. The in-gel digested sample did not yield meaningful results due to potentially interferences still present after various treatments and electrophoresis.

This study examined the function of proteins in activated sludge floc EPS. The proteins were found to be the largest constituent of CER-extracted EPS and may possibly play a significant role in EPS structure and function. EPS arrangement and allocation is of great importance seeing as it effects floc formation and other sludge properties. Consequently, a deeper knowledge of proteins in the EPS may aid in manipulation of these natural biopolymers in activated sludge flocs. The EPS proteins may assist in the research of their effects on sludge properties.

Furthermore, Higgins and Novak (1997) have identified a single 15-killodalton lectin by sonication extraction and NaOH digestion. Flemming and Wingender (2001) found proteins in activated sludge ranging from 5-150 kDa with no mention of the quantity of protein bands observed. Martinez *et al.* (2004) were the only group to list molecular weights of various

exopolymeric proteins obtained by EDTA EPS extraction following dialysis. However, not a single author in previous studies has published their protein bands as attained on SDS-PAGE and only Higgins and Novak (1997) mention the nature of these proteins. This study provided the electrophoresis gels obtained and listed various proteins that were found. This study also attempted to identify the nature of these proteins by testing for possible interferences (i.e. from keratin, sludge supernatant and cellular proteins) and treating the EPS with various additives and a variety of purification techniques. The large quantity of extracellular proteins found in this study from WWTP may originate from wastewater products, bacterial products from cell lysis or cell secretion (exoenzymes and structural proteins).

Due to the novelty of the approach in this particular area and small amount of literature published on exocellular proteins in the EPS, it is difficult to judge and compare the previously published results to the results obtained in this study. The variation of methods used in each study, such as sample handling (Bura *et al.*, 1998), and relying on sludge samples from different full-scale activated sludge systems makes the comparison more tedious. To validate the results obtained in this study it would be necessary to replicate the results by using samples from various WWTPs and compare if there are common proteins among the samples that may be significant for EPS structure and function. These samples should also be extracted by the same method, preferably CER extraction, since this method is well established. CER is preferred to such chemical compounds as EDTA (Johnson and Perry, 1976; Brown and Lester, 1980) and NaOH due to limited risk of contamination of the samples by organic compounds and easy removal of resin from cells by settling. This method also preserves integrity of bacterial cells (Brown and Lester, 1980; Urbain *et al.*, 1993; Frølund *et al.*, 1996).

The identified peptidase exoenzyme in the digested protein fraction may be significant for understanding and optimizing organic matter removal in WWTPs. The information that may be significant is the spatial and temporal variation of enzymes in EPS, the organisms that may be generating these enzymes and factors that may influence enzyme activity (Amann *et al.*, 1995; Gessesse *et al.*, 2003). The identification of a peptidase may also reveal that this enzyme cleaves the peptides produced from protein digestion and thus makes it arduous to identify these proteins on MS. To protect the CER-extracted EPS proteins from degradation by exoenzymes, BSA should be added prior to protein digestion for MS. Owing to inadequate quantity of parts for MALDI-Q-TOF-MS/MS, it could not be utilized in this study. Therefore, for future work it is advisable to employ MALDI-Q-TOF-MS/MS for EPS protein identification.

The accuracy of glycoprotein detection in the EPS should be tested by employing other glycoprotein detection devices, such as periodic acid Pro-Q Emerald 300 dye glycoprotein detection kit (Molecular Probes, Eugene, OR, USA). The Pro-Q Emerald 300 dye kit is specific for glycoproteins that are susceptible to precise deglycosylating enzymes and offers high sensitivity (~300 pg of glycoprotein per band).

When the proteins and the glycoproteins isolated from the EPS matrix and visualized on polyacrylamide electrophoresis gel will be established, it may be feasible to sequence these proteins using Edman Degradation Sequencing technique. Urbain *et al.* (1993) have found that alanine, leucine and glycine are important amino acids in EPS proteins, and are involved in hydrophobic bonds. Edman Degradation Sequencing of treated EPS samples may reveal similar results.

Following disulfide reduction, alkylation, and proteolysis of all proteins in EPS sample, for future work glycopeptide fragments may be separated by an immobilized lectin column, such

as convalin A (Con A). Con A is a good choice due to its broad selectivity and high affinity for N-type glycoproteins. If glycopeptides will be identified on MS, after further resolving the samples on RPLC, with identified structure and mass, then it may be useful to use an immobilized lectin column of narrow selectivity, such as lectin from Bandeiraea simplicifolia (BS-II). BS-II demonstrates high selectivity for N-acetylglucosamine (GlcNAc) derivatized oligosaccharides (Geng *et al.*, 2001).

If, in fact, you could identify structural proteins in the EPS, it should be realized that the use of antibodies on EPS proteins would be very difficult to attain. The reason for this may be that the EPS protein fraction must be in a very pure form to guarantee high specificity and little cross-reactivity (Neu and Lawrence, 1999).

CHAPTER 6. REFERENCES

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APPENDICES

Appendix A: Extracellular Polymeric Substances

Table A.1 Data for CER-extracted activated sludge EPS protein concentrations (mg/L) derived from laboratory-scale SBRs and WWTP using BCA protein assay.

Absorbance @ 560 nm	Standard Curve y=mx+b	Correlation Coefficient r^2	Sample	Absorbance @ 560 nm	Conc. (mg/L)	Average (mg/L)	Standard Deviation
0.988	y=0.000x+0.084	0.960	SBR 1	0.096	24.52	29.88	4.64
				0.100	32.69		
				0.099	32.43		
0.767			SBR 2	0.099	32.39	32.39	0.00
				0.099	32.39		
0.613			SBR 3	0.129	39.19	37.25	7.48
				0.119	28.99		
				0.133	43.49		
0.334			SBR 4	0.124	34.59	34.59	0.0600
				0.124	34.59		
0.309			WWTP1_1	0.135	45.40	52.16	10.94
				0.149	58.92		
				0.154	63.73		
				0.130	40.58		
0.221			WWTP2_1	0.157	67.31	66.27	1.47
				0.155	65.23		
0.150			WWTP1_2	0.272	182.52	155.31	23.6
				0.230	140.18		
				0.233	143.24		
0.090			WWTP2_2	0.195	105.26	112.00	18.35
				0.229	139.36		
				0.190	100.28		
				0.193	103.11		
			WWTP3_2	0.224	134.30	104.98	25.7
				0.176	86.21		
				0.184	94.44		
			WWTP1_3	0.210	141.01	155.62	11.7
				0.202	131.48		
			WWTP2_3	0.266	175.83	152.98	32.3
				0.220	130.13		
			WWTP3_3	0.271	180.83	177.48	4.74
				0.264	174.13		
			WWTP4_	0.266	175.77	171.40	6.17

	3	0.257	167.04		
	WWTP1_	0.274	184.26	183.13	1.60
	4	0.272	182.00		
	WWTP2_	0.256	166.26	161.46	4.16
	4	0.249	158.86		
		0.249	159.25		

Table A.2 Data for CER-extracted activated sludge EPS carbohydrate concentrations (mg/L) derived from laboratory-scale SBRs and WWTP using phenol-sulfuric acid carbohydrate assay.

Absorbance @ 490 nm	Standard Curve y=mx +b	Correlation Coefficient r^2	Sample	Absorbance @ 490 nm	Conc. (mg/L)	Average (mg/L)	Standard Deviation
0.367	y=0.003x -0.002	0.907	SBR 1	0.055	18.99	18.83	0.236
				0.054	18.66		
0.241			SBR 2	0.075	22.96	22.18	1.09
				0.070	21.41		
0.187			SBR 3	0.072	24.61	23.41	1.52
				0.071	21.74		
				0.078	23.89		
0.107			SBR 4	0.055	19.02	19.89	0.839
				0.058	19.95		
				0.060	20.70		
0.064			WWTP1	0.102	31.12	32.08	1.35
			_1	0.109	33.03		
0.060			WWTP2	0.110	37.22	37.22	0.00
			_1	0.110	37.22		
			WWTP1	0.151	50.98	50.04	1.33
			_2	0.145	49.10		
			WWTP2	0.198	66.64	66.46	0.177
			_2	0.197	66.44		
				0.197	66.28		
			WWTP3	0.173	58.48	56.09	2.96
			_2	0.169	57.00		
				0.156	52.77		
			WWTP1	0.171	51.70	56.17	6.32
			_3	0.201	60.63		
0.282	y=0.002x +0.052	0.910	WWTP1 _3	0.177	54.42	-----	-----
0.257			WWTP2	0.163	48.40	44.76	4.01
			_3	0.145	40.45		
				0.156	45.43		
0.165			WWTP3	0.271	95.53	92.41	4.41
			_3	0.257	89.29		
			WWTP4	0.223	74.76	76.24	2.10
			_3	0.230	77.73		
0.131			WWTP1	0.274	96.67	116.75	8.13
			_4	0.297	122.5		
0.094			WWTP2	0.319	133.5	139.00	7.78
			_4	0.341	144.5		

0.093

Table A.3 Data for CER-extracted activated sludge EPS DNA concentrations (mg/L) derived from laboratory-scale SBRs and WWTP using Biorad DNA assay. (N=4)

Excitation @ 360 nm and Emission @ 460 nm	Standard Curve y=mx +b	Correlation Coefficient r^2	Sample	Fluorescence (ng)	Volume (µl)	Total DNA (ng)	Conc. (mg/L)
193	y=0.207x	0.9647	SBR 1	1	20.00	4.81	0.241
124			SBR 2	2	20.00	6.42	0.321
57			SBR 3	3	20.00	10.4	0.522
32			SBR 4	2	20.00	5.62	0.281
19			WWTP1_1	3	40.00	14.4	0.361
9			WWTP2_1	3	40.00	31.28	0.782
			WWTP1_2	41	20.00	197.3	9.86
			WWTP2_2	41	20.00	197.3	9.86
			WWTP3_2	49	20.00	216.0	10.8
			WWTP1_3	48	20.00	231.0	11.5
			WWTP2_3	27	20.00	129.9	6.50
			WWTP3_3	30	20.00	144.4	7.22
			WWTP4_3	74	20.00	356.1	17.8
			WWTP1_4	99	20.00	476.4	23.8
			WWTP2_4	54	20.00	259.9	13.0

Table A.4 Concentration (μg/ml) of CER-extracted activated sludge EPS samples and its constituents derived from SBR and WWTP.

Source of the activated sludge EPS sample	Replicate #	Protein Conc. (μg/ml)	Carbohydrate Conc. (μg/ml)	DNA Conc. (μg/ml)	Carbohydrate: Protein: DNA Concentration Ratio	Total EPS Conc. (μg/ml)
EPS from SBR	1	29.88 ± 4.64	18.83 ± 0.236	0.241	1: 1.51: 0.0128	48.95
	2	32.39 ± 0.00	22.18 ± 1.09	0.321	1: 1.46: 0.0145	54.89
	3	37.25 ± 7.48	23.41 ± 1.52	0.522	1: 1.63:0.0229	60.59
	4	34.59 ± 0.0600	19.89 ± 0.839	0.281	1: 1.74: 0.0141	54.76
1st sample from WWTP (April)*	1	52.16 ± 10.94	32.08 ± 1.35	0.361	1: 1.63: 0.0112	84.60
	2	66.27± 1.47	37.22 ± 0.00	0.782	1: 1.78: 0.0210	104.27
2nd sample from WWTP (May)	1	155.31 ± 23.6	50.04 ± 1.33	9.86	1: 3.10: 0.197	215.2
	2	112.00 ± 18.35	66.46 ± 0.177	9.86	1: 1.68: 0.148	188.3
	3	104.98 ± 25.7	56.09 ± 2.96	10.8	1: 1.87: 0.192	171.9
3rd sample from WWTP (June)	1	155.62 ± 11.7	56.17 ± 6.32	11.5	1: 2.77: 0.205	223.3
	2	152.99± 32.3	44.76 ± 4.01	6.50	1: 3.42: 0.145	203.9
	3	177.48 ± 4.74	92.41 ± 4.41	7.22	1: 1.91: 0.0781	277.1

	4	171.40 ± 6.17	76.24 ± 2.10	17.8	1: 2.25: 0.233	265.4
4th sample from WWTP (July)	1	183.13 ± 1.60	116.75 ± 8.13	23.8	1: 1.57: 0.204	323.7
	2	161.46 ± 4.16	139.00 ± 7.78	13.0	1: 1.16: 0.0935	313.46

*NOTE: The sample was stored anaerobically in at 4°C for 5 days and then reaerated for approximately 3-4 hours prior to sludge analysis

Table A.5 Concentration (mg/g MLSS) of CER-extracted activated sludge EPS samples and its constituents derived from SBR and WWTP.

Source of the activated sludge EPS sample	Replicate #	MLSS (g/L)	Protein Conc. (mg/g MLSS)	Carbohydrate Conc. (mg/g MLSS)	DNA Conc. (mg/g MLSS)	Total EPS Conc. (mg/g MLSS)
EPS from SBR	1	2.65	11.3 +/- 1.75	7.11 +/- 0.0891	0.0909	18.5
	2	2.65	12.2 +/- 0.00	8.37 +/-0.410	0.121	20.7
	3	2.45	15.2 +/- 3.05	9.55 +/-0.620	0.213	24.7
	4	2.05	16.9 +/- 0.0293	9.70 +/-0.293	0.137	26.7
1st sample from WWTP (April)*	1	4.67	11.2 +/- 2.34	6.87 +/-0.289	0.0773	18.1
	2	10.5	6.31 +/- 0.140	3.550 +/- 0.00	0.0745	9.93
2nd sample from WWTP (May)	1	7.00	22.2 +/- 3.37	7.15 +/-0.190	1.41	30.76
	2	7.03	15.9 +/- 2.61	9.45 +/-0.0252	1.40	26.75
	3	7.03	14.9 +/- 3.66	7.98 +/-0.421	1.54	24.42
3rd sample from WWTP (June)	1	4.03	38.6 +/- 2.90	13.9 +/-1.57	2.85	55.35
	2	3.73	41.0 +/- 8.66	12.0 +/-1.07	1.74	53.29
	3	5.20	34.1 +/- 0.910	17.8 +/-0.848	1.39	45.67
	4	5.80	29.5 +/- 0.360	13.1 +/-0.362	3.07	53.29
4th sample from WWTP (July)	1	5.23	35.0 +/- 0.310	22.3 +/-1.55	4.55	61.85
	2	5.20	31.1 +/- 0.800	26.7 +/-1.50	2.50	60.3

*NOTE: The sample was stored anaerobically in at 4°C for 5 days and then reaerated for approximately 3-4 hours prior to sludge analysis

Appendix B: EPS Protein Purification Methods

Table B.1 Data for treated CER-extracted activated sludge EPS protein concentrations (mg/L) derived from laboratory-scale SBRs and WWTP using BCA protein assay.

Absorbance @ 560 nm	Standard Curve y=mx+b	Correlation Coefficient r^2	Sample	Absorbance @ 560 nm	Conc. (mg/L)	Average (mg/L)	Standard Deviation
0.988	y=0.000x+0.084	0.960	SBR 1 RPLC 70% AcN	0.104 0.103	42.77 40.57	41.67	1.55
0.767			SBR 2 PE	0.215 0.219	287.73 296.09	291.91	5.91
0.613			SBR 2 0.05% SDS	0.165 0.163	178.00 173.38	175.69	3.26
0.334			SBR 2 0.1% SDS	0.198 0.199	249.47 252.33	250.90	2.02
0.309			SBR 2 0.5% SDS	0.448 0.470	798.99 848.90	823.94	35.3
0.221			SBR 2 1% SDS	0.482 0.489	874.19 890.24	882.22	11.3
0.150			SBR 2 2mM DTT PE	0.785 0.772	1541.57 1512.98	1527.28	20.2
0.090			SBR 2 4mM DTT PE	0.338 0.349	557.54 581.51	569.53	16.9
			SBR 2 6mM DTT PE	0.510 0.506	935.98 927.62	931.80	5.91
			SBR 2 6M GHCl	0.461 0.451	829.11 806.02	817.57	16.3
			SBR 2 TRITON® X-100	0.252 0.212	368.43 280.91	324.67	61.8
			SBR 2 0.1% HCl	0.127 0.135	93.78 110.49	102.14	11.8
			SBR 2 0.1% NaOH	1.912 2.009	Max Max	Max	-----
1.048	y=0.000x+0.114	0.992	WWTP 1_1 PE	0.141 0.142	124.57 126.77	125.67	1.55
0.851			SBR 1 1mM DTT PE	0.478 0.372	760.71 537.99	649.35	157
0.582			SBR 1	0.622	1060.31	1060.3	-----

			2mM DTT PE			1	
0.494			SBR 1	0.373	540.08	478.24	87.4
			4mM DTT PE	0.314	416.40		
0.383			SBR 1	0.395	587.30	583.01	6.06
			6mM DTT PE	0.391	578.73		
0.238			SBR 1	0.197	174.79	168.82	5.61
			PE	0.193	164.85		
0.164			SBR 1	0.169	1147.06	1188.8	59.1
			0.1% NaOH		(1:10)	4	
				0.173	1230.63		
					(1:10)		
0.098							
3.340	y=0.002	0.987	WWTP 1_2	0.249	43.70	41.20	3.54
	x+0.179		G-25	0.241	38.69		
			Sephadex				
2.638			WWTP 1_2	0.257	48.16	47.51	0.919
			10mM NH_4Cl	0.255	46.86		
			G-25				
			Sephadex				
1.886			WWTP 1_2	0.265	53.41	57.71	6.08
			10mM NaCl	0.279	62.01		
			G-25				
			Sephadex				
1.288			WWTP 1_2	0.189	6.52	6.33	0.262
			10mM $CaCl_2$	0.189	6.15		
			G-25				
			Sephadex				
1.140			WWTP 1_2	0.228	30.21	29.19	1.44
			10mM $MgCl_2$	0.224	28.17		
			G-25				
			Sephadex				
0.612			WWTP 1_2	0.235	34.79	39.34	6.43
			5mM EDTA	0.250	43.89		
			G-25				
			Sephadex				
0.347			WWTP 1_2	0.122	0.00	0.00	1.88
			10mM	0.127	0.00		
			$Fe(NO_3)_3$				
			G-25				
			Sephadex				
0.121			WWTP1_2	1.136	5924.22	6013.3	126

			2mM DTT		(1:10)	1	
				1.165	6102.40		
					(1:10)		
			WWTP 1_2	1.285	6841.74	6872.0	42.9
			10mM $CaCl_2$		(1:10)	6	
			2mM DTT	1.294	6902.38		
					(1:10)		
			WWTP 1_1	0.359	111.20	111.66	0.656
			PE	0.360	112.13		
			WWTP 2_1	0.374	120.61	122.21	2.27
			PE	0.379	123.82		
			WWTP 1_1	1.769	984.06	980.87	4.51
			2mM DTT PE	1.759	977.68		
			WWTP 1_1	0.221	26.38	21.09	7.48
			0.2M borate	0.204	15.80		
			2mM DTT PE				
1.915	y=0.001	0.965	SBR 4	0.125	40.70	41.81	1.58
	x+0.090		RPLC 70%	0.127	42.93		
			AcN				
1.198			WWTP 1_3	0.127	42.93	42.58	0.499
			RPLC 70%	0.126	42.23		
			AcN				
0.924			WWTP 1_2	0192	119.37	126.11	5.93
			0.25% NaOH	0.200	128.42		
				0.201	130.54		
0.733			WWTP 1_2	0.168	91.15	90.79	0.502
			PE	0.167	90.44		
0.529			WWTP 1_2	0.761	789.07	793.48	6.24
			2mM DTT PE	0.769	797.89		
			SBR 3	0.509	491.67	492.26	0.832
			2mM DTT PE	0.510	492.85		
0.332			WWTP 1_2	3.766	Max	Max	-----
			10mM $CaCl_2$	4.016	Max		
			2mM DTT				
0.210			WWTP 1_3	0.167	181.11	172.96	12.5
			Sonication		(1:2)		
				0.167	179.23		
					(1:2)		
				0.158	158.54		
					(1:2)		
0.111							

2.893	y=0.001 x+0.031	0.928	WWTP 1_3 2mM DTT PE	0.522 0.523 0.531	382.97 383.52 389.67	385.39	3.72
1.647			WWTP 1_3 10mM $CaCl_2$ 2mM DTT PE	1.552 1.646 1.631	1185.60 1258.70 1246.85	1230.38	39.2
1.168			WWTP 3_3 Sonication	0.218 0.204	291.37 (1:2) 270.96 (1:2)	281.16	14.4
0.979			WWTP 1_3 400 µl Trizol (1:10) and Mini-Bead-Beater-8	1.676 1.678 1.693	1282.54 1283.87 1295.24	1287.22	6.98
0.689			WWTP 1_3 1 ml Trizol (1:10) and Mini-Bead-Beater-8	2.599 2.711 2.721	2001.48 2089.07 2096.47	2062.34	52.8
0.396			WWTP 4 sludge 400 µl Trizol (1:10) and Mini-Bead-Beater-8	0.145 0.142 0.147	88.80 86.54 90.98	88.77	2.22
0.243			WWTP 4 sludge 1 ml Trizol (1:10) and Mini-Bead-Beater-8	1.278 1.318 1.323	971.70 1003.34 1006.84	993.96	19.3
0.123			WWTP2_4				
1.984	y=0.001 x+0.182	0.985	SBR 3 100 µl 2mM DTT PE	0.334 0.336 0.348	173.57 165.30 177.76	172.21	6.34
1.631			SBR 3 250 µl 2mM DTT PE	0.570 0.542 0.517	416.22 386.68 359.50	387.47	28.4
1.145			SBR 3 500 µl 2mM DTT PE	0.722 0.719 0.701	579.16 576.37 556.93	570.82	12.1
0.667			WWTP 1_1 100 µl	0.422 0.409	256.92 242.96	262.94	23.6

			2mM DTT PE	0.451	288.93		
0.402			WWTP 1_1	0.834	700.01	727.58	28.7
			250 µl	0.858	725.36		
			2mM DTT PE	0.888	757.36		
0.370			WWTP 1_1	1.056	938.68	967.15	43.1
			500 µl	1.063	945.98		
			2mM DTT PE	1.129	1016.77		
0.107			WWTP 2_1	0.286	111.48	108.33	7.50
			100 µl	0.275	99.77		
			2mM DTT PE	0.288	113.74		
			WWTP 2_1	0.716	573.58	595.56	19.2
			250 µl	0.745	604.30		
			2mM DTT PE	0.749	608.81		
			WWTP 2_1	0.906	776.81	764.70	16.7
			500 µl	0.901	771.65		
			2mM DTT PE	0.877	745.66		
0.988	y=0.001 x+0.085	0.980	SBR 2	0.215	287.73	291.91	5.91
			PE	0.219	296.09		
0.767			SBR 2	0.104	42.77	41.67	1.55
0.613			RPLC 70% AcN	0.103	40.57		
0.334			N 1 2	0.645	1121.74	1103.8	18.1
			1% SDS	0.627	1085.45	3	
				0.637	1104.31		
0.309							
0.221							
0.150							
0.009							
1.999	y=0.001 x+0.129	0.990	SBR 3 Sludge Supernatant	0.111	0.00	-----	-----
1.655			SBR 3 Sludge 50mM Tris buffer pH 6.5 wash 1	0.114	0.00	-----	-----
1.099			SBR 3 Sludge 50mM Tris buffer pH 6.5 wash 2	0.107	0.00	-----	-----

0.887	SBR 3 Sludge 50mM Tris buffer pH 6.5 wash 3	0.110	0.00	-----	-----
0.656	SBR 3 Sludge 1% SDS 1 min	0.132	31.44 (1:10)	31.44	-----
0.373	SBR 3 Sludge 1% SDS 15 min	0.141	123.12 (1:10)	123.12	-----
0.229	SBR 3 Sludge 1% SDS 30 min	0.171	434.18 (1:10)	434.18	-----
0.104	SBR 3 Sludge 1% SDS 1 hr	0.181	538.21 (1:10)	538.21	-----
	SBR 3 Sludge 0.1% SDS wash 1	0.126	0.00 (1:10)	0.00	-----
	SBR 3 Sludge 0.1% SDS wash 2	0.118	0.00 (1:10)	0.00	-----
	SBR 3 Sludge 0.1% SDS wash 3	0.124	0.00 (1:10)	0.00	-----
	SBR 3 Sludge 95% ETOH wash 1	0.600	4850.94 (1:10)	4850.94	-----
	SBR 3 Sludge 95% ETOH wash 2	0.590	4750.94 (1:10)	4750.94	-----
	SBR 3 Sludge 95% ETOH wash 3	0.598	4834.38 (1:10)	4834.38 (1:10)	-----
	SBR 3 Sludge 6M GHCl 3 hr	0.245	1194.32 (1:10)	1194.32 (1:10)	-----
	SBR 3 Sludge After 1 min SDS and GHCl: 6M GHCl and 5mM DTT and 0.1M $CaCl_2$	0.396	2746.55 (1:10)	2746.55	-----
	SBR 3 Sludge After 15 min SDS and GHCl:	0.460	3408.84 (1:10)	3408.84	-----

			6M GHCl and 5mM DTT and 0.1M $CaCl_2$				
			SBR 3 Sludge After 30 min SDS and GHCl: 6M GHCl and 5mM DTT and 0.1M $CaCl_2$	0.443	3230.65 (1:10)	3230.65	-----
			SBR 3 Sludge After 1 hr SDS and GHCl: 6M GHCl and 5mM DTT and 0.1M $CaCl_2$	0.338	2151.20 (1:10)	2151.20	-----
			SBR 3 1% SDS 1 min	0.231	105.63	105.63	-----
			SBR 3 1% SDS 5 min	0.223	96.88	96.88	-----
			SBR 3 1% SDS 10 min	0.178	50.73	50.73	-----
			SBR 3 1% SDS 15 min	0.200	73.29	73.29	-----
			SBR 3 1% SDS 30 min	0.181	54.13	54.13	-----
			SBR 3 1% SDS 1 hr	0.166	38.16	38.16	-----
			SBR 3 6M GHCl with 50mM Tris	0.280	155.69	155.69	-----
			SBR 3 6M GHCl with 5mM DTT and 0.1M $CaCl_2$	0.569	453.155	453.155	-----
2.904	y=0.001 x - 0.037	0.921	SBR 3 Sludge Supernatant	0.102	0.00	0.00	-----
1.606			SBR 3 Sludge 1.0 M Tris buffer pH 8.8	0.151	140.07	140.07	-----

			wash 1				
1.362			SBR 3 Sludge 1.0 M Tris buffer pH 8.8 wash 2	0.143	133.80	133.80	-----
1.043			SBR 3 Sludge 0.1% SDS wash 1	0.392	319.96	319.96	-----
0.464			SBR 3 Sludge 0.1% SDS wash 2	0.213	186.33	186.33	-----
0.267			SBR 3 Sludge 0.1% SDS wash 3	0.242	207.59	207.59	-----
0.188			SBR 3 Sludge 95% ETOH wash 1	0.573	4546.32	4546.32	-----
0.115			SBR 3 Sludge 95% ETOH wash 2	0.490	3928.52	3928.52	-----
			SBR 3 Sludge 95% ETOH wash 3	0.484	3886.00	3886.00	-----
2.938	y=0.001 x +0.216	0.983	SBR 1	0.232 0.215	11.72 Min	-----	-----
2.430			SBR 2	0.216 0.223	0.03 5.00	-----	-----
1.707			SBR 3	0.247 0.246	21.81 21.11	21.46	0.495
1.360			SBR 4	0.214 0.224	Min 6.19	-----	-----
1.021			SBR 3 1% SDS 1 min	0.504	201.82		
0.583			SBR 3 1% SDS 5 min	0.493	194.19		
0.342			SBR 3 1% SDS 10 min	0.400	129.05		
			SBR 3 1% SDS 15 min	0.433	152.16		
			SBR 3 1% SDS 30	0.365	104.53		

	min		
	SBR 3	0.362	102.29
	1% SDS 1 hr		

Table B.2 Data for treated CER-extracted activated sludge EPS carbohydrate concentrations (mg/L) derived from laboratory-scale SBRs and WWTP using phenol-sulfuric acid carbohydrate assay.

Absorbance @ 490 nm	Standard Curve y=mx+b	Correlation Coefficient r^2	Sample	Absorbance @ 490 nm	Conc. (mg/L)	Average (mg/L)	Standard Deviation
0.367	y=0.003x - 0.002	0.907	WWTP 1_1 RPLC supernatant	0.078 0.103	23.89 31.18	27.53	5.15
0.241			WWTP 1_1 RPLC wash 1	0.059 0.072	Min 21.98	21.98	-----
0.187			WWTP 1_1 RPLC wash 2	0.061 0.055	18.90 Min	18.90	-----
0.107			WWTP 1_1 RPLC 70% AcN	0.062 0.064	18.96 19.80	19.38	0.591
0.064			SBR 4 RPLC 70% AcN	0.076 0.063	23.35 19.35	21.35	2.83
0.060			WWTP 1_1 RPLC 100% AcN	0.092 0.070	27.92 21.44	24.68	4.58
			WWTP 1_1 RPLC 100% IPA	0.049 0.054	0.00 0.00	0.00	-----
			WWTP 1_1 0.2M borate PE	0.071 0.075	21.71 22.81	22.26	0.782
			WWTP 1_1 0.2M borate 2mM DTT PE	1.621	Max	Max	-----
			WWTP 1_2 2mM DTT	0.220 0.387	66.16 Max	-----	-----
			WWTP 2_1 2mM DTT PE	0.063 0.066	19.41 20.36	19.89	0.676
			SBR 3 2mM DTT PE	0.107 0.116 0.097	32.46 35.15 29.39	32.33	2.88
			WWTP 1_2 10mM $CaCl_2$ 2mM DTT PE	0.209 0.301	62.93 90.60	76.77	19.6
			WWTP 1_2	0.116	35.06	35.30	0.338

			0.25% NaOH	0.117	35.54		
			WWTP 1_3	0.093	56.62	58.79	3.92
			Sonication	0.093	56.44		
				0.104	63.31		
0.282	y=0.002	0.910	WWTP 1_3	0.136	36.74	33.43	4.691
	x		RPLC	0.121	30.11		
	+0.052		supernatant				
0.257			WWTP 1_3	0.078	0.00	0.00	-----
			RPLC wash 1	0.087	0.00		
0.165			WWTP 1_3	0.074	0.00	0.00	-----
			RPLC wash 2	0.086	0.00		
0.131			WWTP 1_3	0.083	0.00	0.00	-----
			RPLC wash 3	0.070	0.00		
0.094			WWTP 1_3	0.116	28.10	23.24	6.88
			RPLC 70%	0.094	18.37		
			AcN				
0.093			WWTP 1_3	0.081	0.00	0.00	-----
			RPLC 100%	0.115	27.32		
			AcN				
			WWTP 1_3	0.082	0.00	0.00	-----
			RPLC 100%	0.065	0.00		
			IPA				
			WWTP 1_3	0.145	40.50	38.53	2.78
			PE	0.136	36.57		
			WWTP 1_3	0.154	44.47	38.80	8.02
			2mM DTT PE	0.128	33.12		
			WWTP 1_3	0.142	39.14	42.61	4.91
			10mM $CaCl_2$	0.158	46.08		
			2mM DTT PE				
			WWTP 3_3	0.164	97.93	87.19	15.2
			Sonication		(1:2)		
				0.140	76.46		
					(1:2)		
			WWTP 1_3	0.263	91.91	80.82	15.7
			400 µl	0.212	69.74		
			Trizol and				
			Mini-Bead-				
			Beater-8				
			WWTP 1_3 1	0.309	Max	Max	-----
			ml	0.287	Max		
			Trizol and	0.295	Max		
			Mini-Bead-				
			Beater-8				
			WWTP 4	0.140	38.53	31.91	5.84

			sludge 400 µl Trizol (1:10) and Mini-Bead-Beater-8	0.120 0.115	29.72 27.49		
			WWTP 4 sludge 1 ml Trizol (1:10) and Mini-Bead-Beater-8	0.249 0.219	85.93 72.71	79.32	9.35
			SBR 1 RPLC 70% AcN	1.152 1.320	Max Max	Max	-----
			SBR 1 RPLC 100% IPA	0.072 0.093	23.16 61.78	42.47	27.3
0.114	y=0.001 x +0.060	0.833	WWTP 1_1 PE	0.090 0.111	56.48 97.19	76.83	28.8
0.107			WWTP 2_1 PE	0.153 0.094	Max 64.81	64.81	-----
0.083			SBR 1 1mM DTT PE	0.094	63.67	63.67	-----
0.080			SBR 1 2mM DTT PE	0.095 0.098	65.57 71.82	68.69	4.42
0.067			SBR 1 4mM DTT PE	0.095 0.112	66.70 99.27	82.99	23.0
0.072			SBR 1 6mM DTT PE	0.089 0.094	54.59 64.24	59.41	6.83
			SBR 2 0.05% SDS	0.071 0.063	21.45 Min	21.45	-----
			SBR 2 0.1% SDS	0.107 0.082	88.86 40.95	64.90	33.8
			SBR 2 0.5% SDS	0.089 0.078	55.34 33.19	44.27	15.7
			SBR 2 1% SDS	0.086 0.096	49.85 67.65	58.75	12.6
			SBR 2 6M GHCl	0.051 0.052	0.00 0.00	0.00	-----
			SBR 2 0.1% TRITON® X-100	0.071 0.086	21.45 50.04	35.75	20.2
			SBR 2	0.048	0.00	0.00	-----

			0.1% HCl	0.049	0.00		
			SBR 2	0.113	99.46	-----	-----
			0.1% NaOH	0.077	32.62		
				0.047	Min		
				0.048	Min		
0.407	y=0.001 x +0.060	0.868	WWTP 1_2 G-25 Sephadex	0.185 0.144	33.56 22.95	28.26	7.50
0.401			WWTP 1_2 10mM NH_4Cl G-25 Sephadex	0.183 0.207	32.90 39.03	35.97	4.33
0.342			WWTP 1_2 10mM NaCl G-25 Sephadex	0.226 0.248	43.79 49.41	46.60	3.97
0.178			WWTP 1_2 10mM $CaCl_2$ G-25 Sephadex	0.096 0.166	Min 29.11	-----	-----
0.113			WWTP 1_2 10mM $MgCl_2$ G-25 Sephadex	0.208 0.262	39.23 52.90	46.07	9.67
0.101			WWTP 1_2 5mM EDTA G-25 Sephadex	0.196 0.266	36.35 54.07	45.21	12.5
			WWTP 1_2 10mM $Fe(NO_3)_3$ G-25 Sephadex	0.212 0.141	40.37 22.27	31.32	12.8
			WWTP1_2 2mM DTT	0.503 0.335	Max 71.51	-----	-----
			WWTP 1_2 10mM $CaCl_2$ 2mM DTT	0.258 0.236	52.04 46.30	49.17	4.06
			WWTP 1_1 PE	0.168	29.13	29.13	-----
			WWTP 2_1 PE	0.354	76.19	76.19	-----
			WWTP 1_1	0.173	30.30	30.30	-----

	2mM DTT PE				
	WWTP 1_1	0.148	24.02	25.81	2.54
	0.2M borate	0.162	27.61		
	2mM DTT PE				

Table B.3 Data for CER-extracted activated sludge EPS glycoprotein carbohydrate content (%) estimate derived from laboratory-scale SBRs and WWTP using glycoprotein carbohydrate estimation kit.

Absorbance @ 550 nm	Standard Curve y=mx +b	Correlation Coefficient r^2	Sample	Absorbance @ 550 nm	Conc. (mg/L)	Average (mg/L)	Standard Deviation
0.889	y=0.0174x +0.1504	0.9751	SBR 3 2mM DTT PE	0.751	220.18 (2:1)	100	0.386
				0.753	(100) 220.73 (2:1) (100)		
0.502			WWTP 1_1 PE	0.635	93.43	93.6	0.264
				0.638	(4:1) 93.80 (4:1)		
0.335			WWTP 1_1 2mM DTT PE	0.416	247.72 (100)	100	2.40
				0.410	244.33 (100)		
0.178			WWTP 1_1 2mM DTT 0.2M borate PE	0.598	88.08	88.6	0.711
				0.605	(4:1) 89.09 (4:1)		
0.151			WWTP 2_1 2mM DTT PE	0.214	131.57 (100)	100	0.488
				0.215	132.26 (100)		
0.134							
1.64	y=0.0487x	0.7209	SBR 3 2mM DTT PE	0.388	7.97	7.97	-----
1.60			WWTP 2_1 2mM DTT PE	0.267	5.48	5.48	-----
0.60			WWTP 1_2	3.352	68.83	65.2	3.21
				3.056	62.75		
				3.116	63.98		
0.429			WWTP 1_2 2mM DTT	0.293	6.02	4.96	1.49
				0.190	3.90		
0.246			WWTP 1_2 10mM $CaCl_2$	0.360	7.39	7.39	-----

	2mM DTT PE				
0.162	WWTP 1_2	1.180	24.29	24.3	-----
	0.25% NaOH				
	WWTP 2_2	1.323	27.17	25.8	1.21
		1.240	25.46		
		1.212	24.89		
	WWTP 3_2	1.557	31.97	31.8	0.276
		1.538	31.58		
	WWTP 1_3	1.427	29.30	28.9	0.380
		1.390	28.54		
		1.407	28.89		
	WWTP 1_3	6.000	123.20	100	0.00
	Protease		(100)		
	inhibitors	6.000	123.20		
			(100)		
	WWTP 1_3	0.558	11.46	11.3	0.189
	Sonication	0.545	11.19		
	WWTP 1_3	4.732	97.17	100	18.4
	PE	6.000	123.20		
			(100)		
	WWTP 1_3	3.637	74.68	78.9	5.53
	2mM DTT PE	3.738	76.76		
		4.146	85.13		
	WWTP 1_3	6.000	123.20	100	-----
	Trizol 400 µl		(100)		
	WWTP 1_3	6.000	123.20	100	0.00
	Trizol 1 ml		(100)		
		6.000	123.20		
			(100)		
	WWTP 2_3	1.266	26.00	23.7	2.04
		1.077	22.11		
		1.118	22.96		
	WWTP 3_3	2.591	53.20	45.3	6.91
		1.980	40.66		
		2.042	41.93		
	WWTP 3_3	0.509	10.45	10.5	-----
	Sonication				
	WWTP 4_3	3.516	72.20	65.5	5.78
		3.038	62.38		
		3.020	62.01		
	WWTP 4	5.276	108.34	100	12.4
	Sludge		(100)		
	Mini-Bead-	6.000	123.20		
	Beater-8 1ml		(100)		

	4.799	98.54		
WWTP 1_4	6.000	123.20 (100)	100	0.00
	6.000	123.20 (100)		
	6.000	123.20 (100)		
WWTP 4_4	4.732	114.52 (100)	100	15.0
	6.000	123.20 (100)		
	6.000	123.20 (100)		

Appendix C: Statistical Analysis

T-Test

The t-test employed for independent samples of unequal variance was based on Pagano (1993). If $p<0.05$, then the null hypothesis (Ho), which states that the two populations are not significantly different, would be rejected. T-test was calculated using Excel Software and results are presented in Table C.1.

Table C.1 Statistical results of t-test to determine if there is any difference between CER-extracted activated sludge EPS protein content (μg/ml) derived from laboratory-scale SBRs.

Samples	Average Protein Concentration (μg/ml)
SBR 1	29.88
SBR 2	32.39
SBR 3	37.59
SBR 4	34.59
Observations	5
P(T<=t) one-tail	0.224
Between SBR1 and 2	
t critical 1-tail	6.31
P(T<=t) two-tail	0.448
Between SBR1 and 2	
t critical 2-tail	12.7
P(T<=t) 1-tail	0.301
Between SBR 3 and 4	
P(T<=t) two-tail	0.603
Between SBR 3 and 4	

Since $p>0.05$, Ho is accepted and the SBR1 and 2 and SBR 3 and 4 are not significantly different.

Table C.2 Statistical results of t-test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrate content (μg/ml) derived from laboratory-scale SBRs.

Samples	Average Carbohydrate Concentration (ug/ml)
SBR 1	18.83
SBR 2	22.18
SBR 3	23.41
SBR 4	19.89
Observations	SBR 1/2 (4) 3/4 (6)
P(T<=t) one-tail	0.0656
Between SBR1 and 2	
t critical 1-tail	4.30
P(T<=t) two-tail	0.132
Between SBR1 and 2	
t critical 2-tail	12.7
P(T<=t) 1-tail	0.0174
Between SBR 3 and 4	
P(T<=t) two-tail	0.0345
Between SBR 3 and 4	

Table C.3 Statistical results of t-test to determine if there is any difference between CER-extracted activated sludge EPS protein content (mg/g MLSS) derived from laboratory-scale SBRs.

Samples	Average Protein Concentration (mg/g MLSS)
SBR 1	11.3
SBR 2	12.2
SBR 3	15.2
SBR 4	16.9
Observations	5
P(T<=t) one-tail	0.224
Between SBR1 and 2	
t critical 1-tail	6.31
P(T<=t) two-tail	0.448
Between SBR1 and 2	
t critical 2-tail	12.7
P(T<=t) 1-tail	0.220
Between SBR 3 and 4	
P(T<=t) two-tail	0.439
Between SBR 3 and 4	

Table C.4 Statistical results of t-test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrate content (mg/g MLSS) derived from laboratory-scale SBRs.

Samples	**Average Carbohydrate Concentration (mg/g MLSS)**
SBR 1	7.11
SBR 2	8.37
SBR 3	9.31
SBR 4	9.70
Observations	SBR 1/2 (4) 3/4 (6)
P(T<=t) one-tail	0.0658
Between SBR1 and 2	
t critical 1-tail	4.30
P(T<=t) two-tail	0.132
Between SBR1 and 2	
t critical 2-tail	12.7
P(T<=t) 1-tail	0.375
Between SBR 3 and 4	
P(T<=t) two-tail	0.750
Between SBR 3 and 4	

ANOVA

The ANOVA test was based on Pagano (1993). The means were calculated in each group (μ_1, μ_2...etc) and overall mean was found (u). The variance was calculated in each group (S_1^2, S_2^2...etc). The S_S^2 was calculated to find out what group the measurement is in (k represented the number of groups, n represented the sample size, and N represented the population size) and S_W^2 was calculated to find out the variance within each group. F-test was conducted and H_0 tested if all observations came from the same population ($\mu_1 = \mu_2$). ANOVA was calculated using Excel Software and results are presented in Tables C.2-C.16.

Table C.5 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS proteins derived from laboratory-scale SBRs.

Protein Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Protein content (μg/ml)**	**N**	**df_1**	**df_2**
29.88	3	4.64	33.61	10	3	9
32.39	2	0.00				
37.59	3	7.48				
34.59	2	0.06				
Calculations:	**F calculated**	**F critical**	**Fcalc.<Fcritic.**			
94.1562125	1.22	3.86	Accept Ho			
31.38540417			Significantly similar at α=0.05			
154.9636						
6						
25.82726667						

Table C.6 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates derived from laboratory-scale SBRs.

Carbohydrate Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Carbohydrate content (μg/ml)**	**N**	**df_1**	**df_2**
18.83	2	0.236	21.08	10	3	9
22.18	2	1.09				
23.41	3	1.52				
19.89	3	0.839				
Calculations:	**F calculated**	**F critical**	**Fcalc.>>Fcritic.**			
33.0856625	9.10	3.86	Reject Ho and accept Ha Significantly different at α=0.05			
11.02855417						
7.272438						
6						
1.212073						

Table C.7 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS protein: carbohydrate derived from laboratory-scale SBRs.

Protein: Carbohydrate Ratio	**n**	**Standard Deviation**	**Sum of Total Protein: Carbohydrate Ratio**	**N**	**df_1**	**df_2**
1.51	3	19.7	1.66	10	3	9
1.68	2	0.00				
1.70	3	4.92				
1.74	2	0.0715				
Calculations:	**F calculated**	**F critical**	**Fcalc.<<Fcritic.**			
0.0853125	0.000208	3.86	Accept Ho Significantly similar at α=0.05			
0.0284375						
821.5500543						
6						
136.9250091						

Table C.8 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS proteins (μg/ml) derived from WWTP sample 2.

Protein Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Protein content (μg/ml)**	**N**	**df_1**	**df_2**
155.31	3	23.6	124.10	10	2	9
112.00	4	18.35				
104.98	3	25.35				
Calculations:	**F calculated**	**F critical**	**Fcalc.>Fcritic.**			
4604.474744	4.727	4.256	Reject Ho and accept Ha			
2302.237372			Significantly different at α=0.05			
3409.3325						
7						
487.0475						

Table C.9 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS proteins (mg/g MLSS) derived from WWTP sample 2.

Protein Content (mg/g MLSS)	**n**	**Standard Deviation (mg/g MLSS)**	**Sum of Total Protein content (mg/g MLSS)**	**N**	**df_1**	**df_2**
22.2	3	3.37	17.7	10	2	9
15.9	4	2.61				
14.9	3	3.66				
Calculations:	**F calculated**	**F critical**	**Fcalc.>Fcritic.**			
97.1011111	4.859	4.256	Reject Ho and accept Ha			
48.5505556			Significantly different at α=0.05			
69.9413						
7						
9.99161429						

Table C.10 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates (μg/ml) derived from WWTP sample 2.

Carbohydrate Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Carbohydrate content (μg/ml)**	**N**	**df_1**	**df_2**
50.04	2	1.33	57.53	8	2	7
66.46	3	0.18				
56.09	3	2.96				
Calculations:	**F calculated**	**F critical**	**Fcalc.>>Fcritic.**			
357.6557	46.20	4.737	Reject Ho and accept Ha			
178.82785			Significantly different at α=0.05			
19.354758						
5						
3.8709516						

Table C.11 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates (μg/ml) derived from WWTP sample 2 replicates 1 and 3.

Carbohydrate Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Carbohydrate content (μg/ml)**	**N**	**df_1**	**df_2**
50.04	2	1.33	53.07	5	1	4
56.09	3	2.96				
Calculations:	**F calculated**	**F critical**	**Fcalc<Fcritic.**			
45.753125	7.115	7.709	Accept Ho			
45.753125			Significantly similar at α=0.05			
19.2921						
3						
6.4307						

Table C.12 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates (mg/g MLSS) derived from WWTP sample 2.

Carbohydrate Content (mg/g MLSS)	**n**	**Standard Deviation (mg/g MLSS)**	**Sum of Total Carbohydrate content (mg/g MLSS)**	**N**	**df_1**	**df_2**
7.15	2	0.19	8.19	8	2	7
9.45	3	0.0252				
7.98	3	0.421				
Calculations:	**F calculated**	**F critical**	**Fcalc.>>Fcritic.**			
7.05125556	44.99	4.737	Reject Ho and accept Ha Significantly different at α=0.05			
3.52562778						
0.39185208						
5						
0.07837042						

Table C.13 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates (mg/g MLSS) derived from WWTP sample 2 replicates 1 and 3.

Carbohydrate Content (mg/g MLSS)	**n**	**Standard Deviation (mg/g MLSS)**	**Sum of Total Carbohydrate content (mg/g MLSS)**	**N**	**df_1**	**df_2**
7.15	2	0.190	7.57	5	1	4
7.98	3	0.421				
Calculations:	**F calculated**	**F critical**	**Fcalc<Fcritic.**			
0.861125	6.614	7.709	Accept Ho			
0.861125			Significantly similar at α=0.05			
0.390582						
3						
0.130194						

Table C.14 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS protein: carbohydrate derived from WWTP sample 2.

Protein: Carbohydrate Ratio	**n**	**Standard Deviation**	**Sum of Total Protein: Carbohydrate Ratio**	**N**	**df_1**	**df_2**
1.83	2	17.7	1.71	8	2	7
1.66	3	103				
1.63	3	8.68				
Calculations:	**F calculated**	**F critical**	**Fcalc<<Fcritic.**			
0.0545889	6.214E-06	4.737	Accept Ho			
0.0272944			Significantly similar at α=0.05			
21961.53						
5						
4392.306						

Table C.15 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS proteins (μg/ml) derived from WWTP sample 3.

Protein Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Protein content (μg/ml)**	**N**	**df_1**	**df_2**
155.62	2	11.7	164.37	8	3	7
152.98	2	32.3				
177.48	2	4.74				
171.4	2	6.17				
Calculations:	**F calculated**	**F critical**	**Fcalc.<<Fcritic.**			
855.1752	0.9190	4.347	Accept Ho			
285.0584			Significantly similar at α=0.05			
1240.7165						
4						
310.179125						

Table C.16 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS proteins (μg/ml) derived from WWTP sample 3 replicates 1 and 2.

Protein Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Protein content (μg/ml)**	**N**	**df_1**	**df_2**
155.62	2	11.7	154.30	4	1	3
152.98	2	32.3				
Calculations:	**F calculated**	**F critical**	**Fcalc<<Fcritic.**			
6.9696	0.01181	10.13	Accept Ho			
6.9696			Significantly similar at α=0.05			
1180.18						
2						
590.09						

Table C.17 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS proteins (μg/ml) derived from WWTP sample 3 replicates 3 and 4.

Protein Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Protein content (μg/ml)**	**N**	**df_1**	**df_2**
177.48	2	4.74	174.44	4	1	3
171.40	2	6.17				
Calculations:	**F calculated**	**F critical**	**Fcalc.<<Fcritic.**			
36.9664	1.221	10.13	Accept Ho			
36.9664			Significantly similar at α=0.05			
60.5365						
2						
30.26825						

Table C.18 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates (μg/ml) derived from WWTP sample 3.

Carbohydrate Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Carbohydrate content (μg/ml)**	**N**	**df_1**	**df_2**
56.17	2	6.32	67.395	9	3	8
44.76	3	4.01				
92.41	2	4.41				
76.24	2	2.1				
Calculations:	**F calculated**	**F critical**	**Fcalc.>>Fcritic.**			
3196.999425	55.53	4.066	Reject Ho and accept Ha			
1065.666475			Significantly different at α=0.05			
95.9607						
5						
19.19214						

Table C.19 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates (μg/ml) derived from WWTP sample 3 replicates 1 and 2.

Carbohydrate Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Carbohydrate content (μg/ml)**	**N**	**df_1**	**df_2**
56.17	2	6.32	50.47	5	1	4
44.76	3	4.01				
Calculations:	**F calculated**	**F critical**	**Fcalc.<<Fcritic.**			
162.735125	6.771	7.709	Accept Ho			
162.735125			Significantly similar at α=0.05			
72.1026						
3						
24.0342						

Table C.20 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates (μg/ml) derived from WWTP sample 3 replicates 3 and 4.

Carbohydrate Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Carbohydrate content (μg/ml)**	**N**	**df_1**	**df_2**
92.41	2	4.41	84.33	4	1	3
76.24	2	2.10				
Calculations:	**F calculated**	**F critical**	**Fcalc. >Fcritic.**			
261.4689	21.92	10.13	Reject Ho and accept Ha Significantly different at α=0.05			
261.4689						
23.8581						
2						
11.92905						

Table C.21 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates (mg/g MLSS) derived from WWTP sample 3.

Carbohydrate Content (mg/g MLSS)	**n**	**Standard Deviation (mg/g MLSS)**	**Sum of Total Carbohydrate content (mg/g MLSS)**	**N**	**df_1**	**df_2**
13.9	2	1.57	14.2	9	3	8
12.0	3	1.07				
17.8	2	0.848				
13.1	2	0.362				
Calculations:	**F calculated**	**F critical**	**Fcalc. >Fcritic.**			
43.04	12.80	4.066	Reject Ho and accept Ha Significantly different at α=0.05			
14.3466667						
5.604848						
5						
1.1209696						

Table C.22 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS protein: carbohydrate derived from WWTP sample 3.

Protein: Carbohydrate Ratio	**n**	**Standard Deviation**	**Sum of Total Protein: Carbohydrate Ratio**	**N**	**df_1**	**df_2**
2.77	2	1.85	2.59	9	3	8
3.40	3	8.05				
1.92	2	1.07				
2.25	2	2.94				
Calculations:	**F calculated**	**F critical**	**Fcalc<<Fcritic.**			
3.170025	0.03695	4.066	Accept Ho			
1.056675			Significantly similar at α=0.05			
142.976479 5						
28.5952958						

Table C.23 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS proteins (μg/ml) derived from WWTP sample 4.

Protein Content (μg/ml)	**n**	**Standard Deviation (μg/ml)**	**Sum of Total Protein content (μg/ml)**	**N**	**df_1**	**df_2**
183.13	2	1.6	172.30	4	1	3
161.46	2	4.16				
Calculations:	**F calculated**	**F critical**	**Fcalc.>Fcritic.**			
469.5889	47.28	10.13	Reject Ho and accept Ha			
469.5889			Significantly different at α=0.05			
19.8656 2						
9.9328						

Table C.24 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS proteins (mg/g MLSS) derived from WWTP sample 4.

Protein Content (mg/g MLSS)	**n**	**Standard Deviation (mg/g MLSS)**	**Sum of Total Protein content (mg/g MLSS)**	**N**	**df_1**	**df_2**
35.0	2	0.31	33.05	4	1	3
31.1	2	0.80				
Calculations:	**F calculated**	**F critical**	**Fcalc.>Fcritic.**			
15.21	41.33	10.13	Reject Ho and accept Ha			
15.21			Significantly different at α=0.05			
0.7361						
2						
0.36805						

Table C.25 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates (µg/ml) derived from WWTP sample 4.

Carbohydrate Content (µg/ml)	**n**	**Standard Deviation (µg/ml)**	**Sum of Total Carbohydrate content (µg/ml)**	**N**	**df_1**	**df_2**
116.75	2	8.13	127.88	4	1	3
139.00	2	7.78				
Calculations:	**F calculated**	**F critical**	**Fcalc.<Fcritic.**			
495.0625	7.819	10.13	Accept Ho			
495.0625			Significantly similar at α=0.05			
126.6253						
2						
63.31265						

Table C.26 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS carbohydrates (mg/g MLSS) derived from WWTP sample 4.

Carbohydrate Content (mg/g MLSS)	n	Standard Deviation (mg/g MLSS)	Sum of Total Carbohydrate content (mg/g MLSS)	N	df_1	df_2
22.3	2	1.55	24.5	4	1	3
26.7	2	1.50				
Calculations:	**F calculated**	**F critical**	**Fcalc.<Fcritic.**			
19.36	8.322	10.13	Accept Ho Significantly similar at α=0.05			
19.36						
4.6525						
2						
2.32625						

Table C.27 Statistical results of ANOVA-single factor test to determine if there is any difference between CER-extracted activated sludge EPS protein: carbohydrate derived from WWTP sample 4.

Protein: Carbohydrate Ratio	n	Standard Deviation	Sum of Total Protein: Carbohydrate Ratio	N	df_1	df_2
1.56	2	0.197	1.35	4	1	3
1.14	2	0.535				
Calculations:	**F calculated**	**F critical**	**Fcalc.<Fcritic.**			
0.1764	1.087	10.13	Accept Ho Significantly similar at α=0.05			
0.1764						
0.32464						
2						
0.16232						

Appendix D: Tricine-PAGE Molecular Weight Estimation

Table D.1. Tricine-PAGE molecular weight estimation in Figure 4.3 of WWTP-derived EPS sample with protease inhibitors.

Molecular Weight (kDa)	**log_{10} (Molecular Weight)**	**Distance traveled down the gel (cm)**	**Rf value (Distance/Dye front)**	
250	2.397940009	0.21	0.02692308	y = -1.5241x +2.2735
150	2.176091259	0.55	0.07051282	
100	2	1.22	0.15641026	
75	1.875061263	1.47	0.18846154	
50	1.698970004	2.75	0.3525641	
37	1.568201724	3.35	0.42948718	
25	1.397940009	4.25	0.54487179	
20	1.301029996	5.19	0.66538462	
15	1.176091259	5.65	0.72435897	
10	1	6.8	0.87179487	
WWTP 1 2 with protease inhibitors				
Molecular Weight (kDa)	*log_{10} (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*	
170.025	2.230512564	0.22	0.02820513	
129.217	2.111320128	0.83	0.10641026	
114.436	2.058562821	1.1	0.14102564	
96.887	1.986265769	1.47	0.18846154	
71.350	1.853395513	2.15	0.27564103	
62.341	1.794776282	2.45	0.31410256	
51.375	1.710755385	2.88	0.36923077	
28.241	1.450876795	4.21	0.53974359	
20.611	1.31409859	4.91	0.62948718	
12.909	1.110885256	5.95	0.76282051	
8.419	0.925257692	6.9	0.88461538	

Table D.2. Tricine-PAGE molecular weight estimation in Figure 4.4a of violently agitated WWTP-derived activated sludge sample.

Molecular Weight (kDa)	**$\log_{10}$ (Molecular Weight)**	**Distance traveled down the gel (cm)**	**Rf value (Distance/Dye front)**	
250	2.397940009	0.18	0.02337662	y = -1.4031x +2.2553
150	2.176091259	0.58	0.07532468	
100	2	1.05	0.13636364	
75	1.875061263	1.58	0.20519481	
50	1.698970004	2.72	0.35324675	
37	1.568201724	3.49	0.45324675	
25	1.397940009	4.61	0.5987013	
20	1.301029996	5.33	0.69220779	
15	1.176091259	5.88	0.76363636	
10	1	7.3	0.94805195	
Sludge violently agitated by Mini-Bead-Beater-8				
Molecular Weight (kDa)	*$\log_{10}$ (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*	
153.482	2.186056104	0.38	0.04935065	
115.869	2.063968182	1.05	0.13636364	
81.452	1.910902727	1.89	0.24545455	
47.407	1.675837922	3.18	0.41298701	
34.899	1.542816753	3.91	0.50779221	
19.890	1.298640909	5.25	0.68181818	
11.674	1.067220519	6.52	0.84675325	
		N 1 2		
166.388	2.220678052	0.19	0.02467532	

Table D.3. Tricine-PAGE molecular weight estimation in Figure 4.4b of SDS-boiled WWTP-derived EPS sample.

Molecular Weight (kDa)	**$\log_{10}$ (Molecular Weight)**	**Distance traveled down the gel (cm)**	**Rf value (Distance/Dye front)**	
				y = -1.3179x
250	2.397940009	0.25	0.03246753	+2.2537
150	2.176091259	0.65	0.08441558	
100	2	1.2	0.15584416	
75	1.875061263	1.65	0.21428571	
50	1.698970004	2.75	0.35714286	
37	1.568201724	3.59	0.46623377	
25	1.397940009	4.62	0.6	
20	1.301029996	5.26	0.78051948	
15	1.176091259	6.01	0.91038961	
10	1	7.01	0.91038961	

SDS-boiled WWTP sample

Molecular Weight (kDa)	*$\log_{10}$ (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*	
147.273	2.168122078	0.5	0.06493506	147
138.273	2.140737143	0.66	0.08571429	138
130.851	2.116775325	0.8	0.10389610	131
119.041	2.075697922	1.04	0.13506494	119
106.604	2.027774286	1.32	0.17142857	107
95.466	1.979850649	1.6	0.20779221	95
91.777	1.962735065	1.7	0.22077922	92
72.451	1.860041558	2.3	0.29870130	72
53.868	1.731332364	3.052	0.39636364	54
40.115	1.603307792	3.8	0.49350649	40
34.946	1.543403247	4.15	0.53896104	35
30.444	1.483498701	4.5	0.58441558	30
23.104	1.36368961	5.2	0.67532468	23

Table D.4. Tricine-PAGE molecular weight estimation in Figure 4.5a of WWTP-derived EPS sample and SDS-boiled dust.

Molecular Weight (kDa)	**log_{10} (Molecular Weight)**	**Distance traveled down the gel (cm)**	**Rf value (Distance/Dye front)**	
				y = -1.5247x
250	2.397940009	0.17	0.02207792	+2.2316
150	2.176091259	0.5	0.06493506	
100	2	0.95	0.12337662	
75	1.875061263	1.33	0.17272727	
50	1.698970004	2.2	0.28571429	
37	1.568201724	2.9	0.37662338	
25	1.397940009	3.8	0.49350649	
20	1.301029996	4.33	0.66233766	
15	1.176091259	5.1	0.77662338	
10	1	5.98	0.77662338	
WWTP 1 2	Coomassie			
Molecular Weight (kDa)	*log_{10} (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*	
153.482	2.186057013	0.23	0.02987013	153
103.225	2.013785714	1.1	0.14285714	103
78.520	1.894977922	1.7	0.22077922	79
68.482	1.835574026	2	0.25974026	68
58.381	1.766269481	2.35	0.30519481	58
38.205	1.582117403	3.28	0.42597403	38
13.821	1.140548442	5.51	0.71558442	14
Dust	Coomassie			
Molecular Weight (kDa)	*log_{10} (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*	
78.520	1.894977922	1.7	0.22077922	79
58.381	1.766269481	2.35	0.30519481	58
40.538	1.607859091	3.15	0.40909091	41
28.797	1.459349351	3.9	0.50649351	29
13.884	1.142528571	5.5	0.71428571	14
11.054	1.043522078	6	0.77922078	11

Table D.5. Tricine-PAGE molecular weight estimation in Figure 4.5c of sludge supernatant and WWTP-derived EPS sample.

Molecular Weight (kDa)	**log_{10} (Molecular Weight)**	**Distance traveled down the gel (cm)**	**Rf value (Distance/Dye front)**	
				y = -1.5897x
180	2.255272505	0.38	0.04935065	+2.166
97	1.986771734	0.65	0.08441558	
82	1.913813852	0.975	0.12662338	
66	1.819543936	1.52	0.1974026	
42	1.62324929	2.13	0.27662338	
29	1.462397998	3.5	0.45454545	
18	1.255272505	4.5	0.58441558	
14	1.146128036	5.07	0.65844156	

Sludge supernatant

Molecular Weight (kDa)	*log_{10} (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*
114.457	2.058643636	0.52	0.06753247
9.755	0.989209091	5.7	0.74025974

WWTP 1 2

Molecular Weight (kDa)	*log_{10} (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*
145.860	2.163935455	0.01	0.00129870
143.794	2.157741818	0.04	0.00519481
11.250	1.051145455	5.4	0.70129870

Table D.6. Tricine-PAGE molecular weight estimation in Figure 4.11 of WWTP-derived EPS sample.

Molecular Weight (kDa)	**log_{10} (Molecular Weight)**	**Distance traveled down the gel (cm)**	**Rf value (Distance/Dye front)**	
				y = -1.6179x
250	2.397940009	0.75	0.09615385	+2.3568
150	2.176091259	1.06	0.13589744	
100	2	1.47	0.18846154	
75	1.875061263	1.82	0.23333333	
50	1.698970004	2.77	0.35512821	
37	1.568201724	3.51	0.45	
25	1.397940009	4.485	0.575	
15	1.176091259	5.69	0.72948718	
10	1	6.99	0.89615385	
WWTP desalted on G-25 Sephadex (Silver)				
Molecular Weight (kDa)	*log_{10} (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*	
153.860	2.187127923	0.818	0.10487179	154
150.086	2.176341923	0.87	0.11153846	150
140.915	2.148962077	1.002	0.12846154	141
140.311	2.147095269	1.011	0.12961538	140
119.795	2.078438231	1.342	0.17205128	120
113.718	2.055829115	1.451	0.18602564	114
87.072	1.939879615	2.01	0.25769231	87
79.671	1.901298923	2.196	0.28153846	80
73.845	1.868318654	2.355	0.30192308	74
68.904	1.838242308	2.5	0.32051282	69
60.279	1.780163846	2.78	0.35641026	60
53.239	1.726233846	3.04	0.38974359	53
45.044	1.653635769	3.39	0.43461538	45
27.806	1.444138462	4.4	0.56410256	28
19.434	1.288571154	5.15	0.66025641	19
10.904	1.037589231	6.36	0.81538462	11

Table D.7. Tricine-PAGE molecular weight estimation in Figure 4.12c of WWTP-derived EPS sample.

Molecular Weight (kDa)	**log_{10} (Molecular Weight)**	**Distance traveled down the gel (cm)**	**Rf value (Distance/Dye front)**	
				y = -1.5396x
180	2.255272505	0.835	0.10705128	+2.2436
97	1.986771734	1.2	0.15384615	
82	1.913813852	1.4	0.17948718	
66	1.819543936	1.8	0.23076923	
42	1.62324929	2.71	0.3474359	
29	1.462397998	3.87	0.49615385	
18	1.255272505	5.185	0.66474359	
14	1.146128036	5.73	0.73461538	

WWTP desalted on G-25 Sephadex (SYPRO)

Molecular Weight (kDa)	*log_{10} (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*	
148.104	2.170567692	0.37	0.04743590	148
119.076	2.075823077	0.85	0.10897436	119
116.930	2.067927692	0.89	0.11410256	117
105.324	2.022529231	1.12	0.14358974	105
81.324	1.910217385	1.689	0.21653846	81
73.887	1.868569231	1.9	0.24358974	74
70.604	1.848830769	2	0.25641026	71
67.467	1.829092308	2.1	0.26923077	67
64.469	1.809353846	2.2	0.28205128	64
60.221	1.779746154	2.35	0.30128205	60
55.743	1.746190769	2.52	0.32307692	56
34.905	1.542884615	3.55	0.45512821	35
26.107	1.416755846	4.189	0.53705128	26
19.598	1.292206154	4.82	0.61794872	20
7.580	0.879672308	6.91	0.88589744	8

Table D.8. Tricine-PAGE molecular weight estimation in Figure 4.17 of SBR-derived EPS sample.

Molecular Weight (kDa)	**$\log_{10}$ (Molecular Weight)**	**Distance traveled down the gel (cm)**	**Rf value (Distance/Dye front)**	
				y = -1.7076x
250	2.397940009	0.19	0.02435897	+2.2419
150	2.176091259	0.55	0.07051282	
100	2	0.9	0.11538462	
75	1.875061263	1.25	0.16025641	
50	1.698970004	2.05	0.26282051	
37	1.568201724	2.65	0.33974359	
25	1.397940009	3.56	0.45641026	
15	1.176091259	4.62	0.70512821	
10	1	5.5	0	

SBR 4

Molecular Weight (kDa)	*$\log_{10}$ (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*	
122.646	2.088653846	0.7	0.08974359	123
90.636	1.9573	1.3	0.16666667	91
84.035	1.924461538	1.45	0.18589744	84
67.319	1.828135385	1.89	0.24230769	67
65.643	1.817189231	1.94	0.24871795	66
56.147	1.749323077	2.25	0.28846154	56
50.762	1.705538462	2.45	0.31410256	51
38.471	1.585130769	3	0.38461538	38
31.446	1.497561538	3.4	0.43589744	31
27.722	1.442830769	3.65	0.46794872	28
23.238	1.366207692	4	0.51282051	23
18.061	1.256746154	4.5	0.57692308	18
12.691	1.1035	5.2	0.66666667	13

Table D.9. Tricine-PAGE molecular weight estimation in Figure 4.18 of WWTP-derived EPS sample.

Molecular Weight (kDa)	**log_{10} (Molecular Weight)**	**Distance traveled down the gel (cm)**	**Rf value (Distance/Dye front)**	
180	2.255272505	0.165	0.02115385	
				y = -1.8927x
97	1.986771734	0.475	0.06089744	+2.1778
82	1.913813852	0.98	0.12564103	
66	1.819543936	1.4	0.17948718	
42	1.62324929	2.5	0.32051282	
29	1.462397998	3.32	0.42564103	
18	1.255272505	4.185	0.53653846	
14	1.146128036	5	0.64102564	

WWTP

Molecular Weight (kDa)	*log_{10} (Molecular Weight)*	*Distance traveled down the gel (cm)*	*Rf value (Distance/Dye front)*	
101.846	2.007942308	0.7	0.08974359	102
72.837	1.86235	1.3	0.16666667	73
66.981	1.825951923	1.45	0.18589744	67
52.382	1.719184231	1.89	0.24230769	52
50.939	1.707051538	1.94	0.24871795	51
42.838	1.631828846	2.25	0.28846154	43
38.309	1.583298077	2.45	0.31410256	38
28.173	1.449838462	3	0.38461538	28
22.531	1.352776923	3.4	0.43589744	23
19.594	1.292113462	3.65	0.46794872	20
16.113	1.207184615	4	0.51282051	16
12.186	1.085857692	4.5	0.57692308	12
8.241	0.916	5.2	0.66666667	8

Appendix E: Matched Sequences Obtained From ESI-LC-MS/MS and BLAST

Peptide View

MS/MS Fragmentation of YNNALEASLKPNNIPLEVYK
Found in gi|82744985, Peptidase M3B, oligoendopeptidase F [Clostridium beijerincki NCIMB 8052]

Match to Query 168: 2288.858172 from(763.960000,3+) intensity(45846.0000)

Cmpd 82, +MSn(764.1) 31.8 min

From data file D:\elena b\EPS_70percAcN_mar2320000k.d.mgf

Monoisotopic mass of neutral peptide Mr(calc): 2289.2004

Ions Score: 51 **Expect:** 0.52

Matches (Bold Red): 43/210 fragment ions using 42 most intense peaks

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}
1	164.0706	82.5389				
2	278.1135	139.5604	261.087	131.0471		
3	392.1565	196.5819	375.1299	188.0686		
4	463.1936	232.1004	446.167	223.5872		
5	576.2776	288.6425	559.2511	280.1292		
6	705.3202	353.1638	688.2937	344.6505	687.3097	344.1585
7	776.3573	388.6823	759.3308	380.169	758.3468	379.677
8	863.3894	432.1983	846.3628	423.685	845.3788	423.193
9	976.4734	488.7404	959.4469	480.2271	958.4629	479.7351
10	1104.5684	552.7878	1087.5418	544.2746	1086.5578	543.7825
11	1201.6211	601.3142	1184.5946	592.8009	1183.6106	592.3089
12	1315.6641	658.3357	1298.6375	649.8224	1297.6535	649.3304
13	1429.707	715.3571	1412.6805	706.8439	1411.6964	706.3519
14	1542.7911	771.8992	1525.7645	763.3859	1524.7805	762.8939
15	1639.8438	820.4256	1622.8173	811.9123	1621.8333	811.4203
16	1752.9279	876.9676	1735.9013	868.4543	1734.9173	867.9623
17	1881.9705	941.4889	1864.9439	932.9756	1863.9599	932.4836
18	1981.0389	991.0231	1964.0123	982.5098	1963.0283	982.0178
19	2144.1022	1072.5547	2127.0757	1064.0415	2126.0917	1063.5495

Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
Y							20
N	2127.1444	1064.0758	2110.1179	1055.5626	2109.1338	1055.0706	19
N	2013.1015	1007.0544	1996.0749	998.5411	1995.0909	998.0491	18
A	1899.0586	950.0329	1882.032	941.5196	1881.048	941.0276	17
L	1828.0214	914.5144	1810.9949	906.0011	1810.0109	905.5091	16
E	1714.9374	857.9723	1697.9108	849.4591	1696.9268	848.967	15
A	1585.8948	793.451	1568.8682	784.9378	1567.8842	784.4458	14
S	1514.8577	757.9325	1497.8311	749.4192	1496.8471	748.9272	13
L	1427.8257	714.4165	1410.7991	705.9032	1409.8151	705.4112	12
K	1314.7416	657.8744	1297.715	649.3612	1296.731	648.8692	11
P	1186.6466	593.827	1169.6201	585.3137	1168.6361	584.8217	10
N	1089.5939	545.3006	1072.5673	536.7873	1071.5833	536.2953	9
N	975.5509	488.2791	958.5244	479.7658	957.5404	479.2738	8
I	861.508	431.2576	844.4815	422.7444	843.4974	422.2524	7
P	748.424	374.7156	731.3974	366.2023	730.4134	365.7103	6
L	651.3712	326.1892	634.3446	317.676	633.3606	317.184	5
E	538.2871	269.6472	521.2606	261.1339	520.2766	260.6419	4
V	409.2445	205.1259	392.218	196.6126			3
Y	310.1761	155.5917	293.1496	147.0784			2
K	147.1128	74.06	130.0863	65.5468			1

Table E.1 Sequences from RPLC-treated EPS and LC/ESI-MS/MS producing significant alignments.

Sequence	Name	Score	Expect
gi|82744985|ref|ZP_00907499.1|	Peptidase M3B, oligoendopeptid...	74.0	6e-13
gi|18145115|dbj|BAB81159.1|	oligopeptidase [*Clostridium perfr...*	57.9	4e-08
gi|28204024|gb|AAO36464.1|	oligoendopeptidase F [*Clostridium* ...	55.8	2e-07
gi|76260026|ref|ZP_00767668.1|	Peptidase M3B, oligoendopeptid...	43.9	7e-04
gi|76259709|ref|ZP_00767355.1|	Peptidase M3B, oligoendopeptid...	42.2	0.002
gi|89209725|ref|ZP_01188120.1|	Peptidase M3B, oligoendopeptid...	39.2	0.018
gi|67916420|ref|ZP_00510131.1|	Peptidase M3B, oligoendopeptid...	38.8	0.024
gi|76797206|ref|ZP_00779541.1|	Peptidase M3B, oligoendopeptid...	37.1	0.077
gi|71492373|gb|EAO24679.1|	Peptidase M3B, oligoendopeptidase ...	36.3	0.14
gi|85683739|ref|ZP_01030375.1| COG1164:	Oligoendopeptidase F [*Cl*	36.3	0.14
gi|33322731|gb|AAQ07100.1|	oligoendopeptidase f [*Lactobacillus d*	34.1	0.61
gi|20516018|gb|AAM24265.1|	Oligoendopeptidase F [*Thermoanaero...*	34.1	0.61
gi|62516282|ref|ZP_00387639.1|	COG1164: Oligoendopeptidase F ...	34.1	0.61
gi|60545050|gb|AAQ08885.2|	oligopeptidase F [*Bacillus amylolique*	32.5	2.0
gi|2633508|emb|CAB13011.1|	yjbG [Bacillus subtilis subsp. sub...	32.5	2.0
gi|1651216|dbj|BAA13561.1|	Pz-peptidase [*Bacillus licheniformis*]	32.5	2.0
gi|82500204|ref|ZP_00885628.1|	Peptidase M3B, oligoendopeptid...	32.5	2.0
gi|23497235|gb|AAN36782.1|	hypothetical protein [*Plasmodium f...*	32.0	2.6
gi|32446842|emb|CAD78748.1|	peptidase [*Rhodopirellula baltica...*	32.0	2.6
gi|66810784|ref|XP_639099.1|	hypothetical protein DDB0218506 ...	32.0	2.6
gi|89331546|dbj|BAE81139.1|	oligopeptidase F/B [*Chlamydophila fe*	31.6	3.5
gi|82539509|ref|XP_724137.1|	hypothetical protein PY00416 [Pl...	31.2	4.8
gi|68073545|ref|XP_678687.1|	hypothetical protein [*Plasmodium...*	31.2	4.8
gi|44983477|gb|AAS52613.1|	AEL072Wp [Ashbya gossypii ATCC 108...	30.8	6.4
gi|52002867|gb|AAU22809.1|	oligoendopeptidase F,Pz peptidase ...	30.3	8.6

gi|67009983|dbj|BAD99434.1|	oligopeptidase [*Geobacillus sp.* MO-1	29.9	11
gi|67468871|ref|XP_650429.1|	receptor protein kinase [*Entamoe...*	29.9	11
gi|45655974|ref|YP_000060.1|	hypothetical protein LIC10060 [*L...*	29.9	11
gi|24193410|gb|AAN47266.1|	conserved hypothetical protein [*Le...*	29.9	11
gi|88945331|ref|ZP_01148508.1|	Peptidase M3B, oligoendopeptid...	29.9	11
gi|62554016|emb|CAB05816.2|	Hypothetical protein T25C8.1 [*Cae...*	29.5	15
gi|6459395|gb|AAF11188.1|	oligoendopeptidase [*Deinococcus rad...*	29.5	15
gi|34451899|gb|AAQ72430.1|	endopeptidase F [*Lactobacillus helvet*	29.1	21
gi|33632977|emb|CAE07788.1|	possible-TPR Domain containing pr...	29.1	21
gi|3183372|sp|Q58823|	Y1428_METJA Hypothetical protein MJ1428 ...	29.1	21
gi|62148292|emb|CAH64059.1|	putative peptidase [*Chlamydophila...*	29.1	21
gi|2651|emb|CAA39501.1|	alpha-glucosidase [*Pseudozyma tsukuba...*	29.1	21
gi|23002983|ref|ZP_00046654.1|	COG1164: Oligoendopeptidase F [*La*	29.1	21
gi|82180758|sp|Q63ZM9|	CCD16_XENLA Coiled-coil domain-containi...	29.1	21
gi|85684143|ref|ZP_01030766.1|	COG1455: Phosphotransferase sy...	29.1	21
gi|50306319|ref|XP_453133.1|	unnamed protein product [*Kluyver...*	28.6	28
gi|10581433|gb|AAG20172.1|	oligopeptidase; YjbG [*Halobacteriu...*	28.6	28
gi|83859151|ref|ZP_00952672.1|	ribosomal large subunit pseudo...	28.6	28
gi|56754847|gb|AAW25606.1|	SJCHGC01836 protein [*Schistosoma japo*	28.2	37
gi|29841109|gb|AAP06122.1|	hypothetical protein [*Schistosoma jap*	28.2	37
gi|49331565|gb|AAT62211.1|	oligoendopeptidase F [*Bacillus thu...*	28.2	37
gi|65304212|emb|CAI76591.1|	methylase-like protein, putative ...	28.2	37
gi|42780388|ref|NP_977635.1|	oligoendopeptidase F [*Bacillus c...*	28.2	37
gi|790694|gb|AAA87313.1|	mannuronan C-5-epimerase [*Azotobacte...*	28.2	37
gi|67005319|gb|AAY62245.1|	TPR [*Rickettsia felis* URRWXCal2] >...	28.2	37

gi|51977606|gb|AAU19156.1|	oligoendopeptidase F [*Bacillus cer...*	28.2	37
gi|29894890|gb|AAP08179.1|	Oligoendopeptidase F [*Bacillus cer...*	28.2	37
gi|9655999|gb|AAF94651.1|	tail-specific protease [*Vibrio chol...*	28.2	37
gi|67158322|ref|ZP_00419313.1|	Hemolysin-type calcium-binding...	28.2	37
gi|47501617|gb|AAT30293.1|	oligoendopeptidase F [*Bacillus ant...*	28.2	37
gi|82705206|ref|XP_726874.1|	cysteine repeat modular protein ...	28.2	37
gi|47568400|ref|ZP_00239101.1|	oligoendopeptidase F [*Bacillus...*	28.2	37
gi|89305398|gb|EAS03386.1|	hypothetical protein TTHERM_007303...	28.2	37
gi|89204581|ref|ZP_01183158.1|	Peptidase M3B, oligoendopeptid...	28.2	37
gi|75826862|ref|ZP_00756297.1|	COG0793: Periplasmic protease ...	28.2	37
gi|75823391|ref|ZP_00752896.1|	COG0793: Periplasmic protease [*Vi*	28.2	37
gi|75817802|ref|ZP_00748086.1|	COG0793: Periplasmic protease [*Vi*	28.2	37
gi|75761255|ref|ZP_00741238.1|	Oligoendopeptidase F [*Bacillus...*	28.2	37
gi|56122499|gb|AAV74373.1|	RpoC2 [*Acorus gramineus*]	27.8	50
gi|22759003|gb|AAN05636.1|	acetylcholinesterase 1 [*Necator ameri*	27.8	50
gi|16945695|emb|CAD11604.1|	cystathionine beta-synthase [*Osterta*	27.8	50
gi|46361219|emb|CAG25080.1|	conserved hypothetical protein; h...	27.8	50
gi|39648184|emb|CAE26704.1|	FAD linked oxidase, C-terminal:FA...	27.8	50
gi|56379199|dbj|BAD75107.1|	thimet oligoendopeptidase [*Geobac...*	27.8	50
gi|19918004|gb|AAM07269.1|	ammonium transporter [*Methanosarci...*	27.8	50
gi|66476080|ref|XP_627856.1|	hypothetical protein cgd6_5380 [...	27.8	50
gi|28204357|gb|AAO36795.1|	serine/threonine kinase, putative ...	27.8	50
gi|42519744|ref|NP_965674.1|	oligoendopeptidase F [*Lactobacil...*	27.8	50
gi|13092977|emb|CAC31288.1|	putative conserved membrane prote...	27.8	50
gi|58255337|gb|AAV43574.1|	oligopeptidase [*Lactobacillus acid...*	27.8	50
gi|29834748|gb|AAP05383.1|	oligoendopeptidase F [*Chlamydophil...*	27.8	50
gi|71675014|ref|ZP_00672760.1|	hypothetical protein TeryDRAFT...	27.8	50
gi|68244965|gb|EAN27107.1|	hypothetical protein Mmc1DRAFT_035...	27.8	50

gi|69216853|gb|AAZ03979.1|	RNA polymerase beta" chain [Acoru...	27.8	50
gi|54650760|gb|AAV36959.1|	LP06206p [*Drosophila melanogaster*]	27.4	67
gi|17646194|gb|AAL40927.1|	IKAP [*Oryctolagus cuniculus*]	27.4	67
gi|55242080|gb|EAA07706.2|	ENSANGP00000010808 [*Anopheles gamb...*	27.4	67
gi|5901854|gb|AAD55435.1|	SNF1A [*Drosophila melanogaster*]	27.4	67
gi|60459958|gb|AAX20150.1|	AMPK-alpha subunit [*Aedes aegypti*]	27.4	67
gi|45553923|ref|NP_996327.1|	SNF1A/AMP-activated protein kina...	27.4	67
gi|52217925|dbj|BAD50518.1|	conserved hypothetical protein [*B...*	27.4	67
gi|17741159|gb|AAL43637.1|	conserved hypothetical protein [*Ag...*	27.4	67
gi|15157864|gb|AAK88377.1|	AGR_C_4816p [*Agrobacterium tumefac...*	27.4	67
gi|33237938|gb|AAQ00006.1|	ATP adenylyltransferase [*Prochloro...*	27.4	67
gi|9656099|gb|AAF94743.1|	alpha-acetolactate decarboxylase [*V...*	27.4	67
gi|71080755|ref|XP_779456.1|	hypothetical protein GLP_487_346...	27.4	67
gi|50740602|ref|XP_419507.1|	PREDICTED: similar to zinc finge...	27.4	67
gi|86608313|ref|YP_477075.1|	hypothetical protein CYB_0831 [C...	27.4	67
gi|57167637|ref|ZP_00366777.1|	outer membrane efflux family p...	27.4	67
gi|89093551|ref|ZP_01166499.1|	pseudouridine synthase [*Oceano...*	27.4	67
gi|75818097|ref|ZP_00748315.1|	COG3527: Alpha-acetolactate decar	27.4	67
gi|87121506|ref|ZP_01077395.1|	sensory box sensor/GGDEF/EAL d...	27.4	67
gi|54643763|gb|EAL32506.1|	GA15892-PA [*Drosophila pseudoobscura*]	27.4	67
gi|71042477|pdb|1ZSY|	A Chain A, The Structure Of Human Mitoch...	26.9	90
gi|77747830|ref|NP_637005.2|	hypothetical protein XCC1635 [*Xa...*	26.9	90

>gi|82744985|ref|ZP_00907499.1| **Peptidase M3B, oligoendopeptidase F [*Clostridium beijerincki* CIMB 8052]**
gi|82727170|gb|EAP61905.1| Peptidase M3B, oligoendopeptidase F [Clostridium beijerincki NCIMB 8052]
Length=594

Score = 74.0 bits (167), Expect = 6e-13
Identities = 22/22 (100%), Positives = 22/22 (100%), Gaps = 0/22 (0%)

Query 1 KYNNALEASLKPNNIPLEVYKN 22
KYNNALEASLKPNNIPLEVYKN
Sbjct 249 KYNNALEASLKPNNIPLEVYKN 270

>gi|18145115|dbj|BAB81159.1| **oligopeptidase [*Clostridium perfringens* str. 13]**
gi|18310435|ref|NP_562369.1| oligopeptidase [Clostridium perfringens str. 13]
Length=599

Score = 57.9 bits (129), Expect = 4e-08
Identities = 17/20 (85%), Positives = 20/20 (100%), Gaps = 0/20 (0%)

Query 1 KYNNALEASLKPNNIPLEVY 20
KYN++LEASLKPNNIP+EVY
Sbjct 252 KYNSSLEASLKPNNIPVEVY 271

>gi|28204024|gb|AAO36464.1| **oligoendopeptidase F [*Clostridium tetani* E88]**
gi|28211583|ref|NP_782527.1| oligoendopeptidase F [Clostridium tetani E88]
Length=626

Score = 55.8 bits (124), Expect = 2e-07
Identities = 16/20 (80%), Positives = 20/20 (100%), Gaps = 0/20 (0%)

Query 1 KYNNALEASLKPNNIPLEVY 20
K+N++LE+SLKPNNIPLEVY
Sbjct 278 KFNSSLESSLKPNNIPLEVY 297

>gi|76260026|ref|ZP_00767668.1| **Peptidase M3B, oligoendopeptidase F [*Chloroflexus aurantiacus* J-10-fl]**
gi|76165118|gb|EAO59256.1| Peptidase M3B, oligoendopeptidase F [Chloroflexus aurantiacus J-10-fl]
Length=603

Score = 43.9 bits (96), Expect = 7e-04
Identities = 15/21 (71%), Positives = 18/21 (85%), Gaps = 0/21 (0%)

Query 2 YNNALEASLKPNNIPLEVYKN 22
Y +ALEA+LKPN IPLEV+ N
Sbjct 251 YASALEAALKPNFIPLEVFHN 271

>[gi|76259709|ref|ZP_00767355.1| **Peptidase M3B, oligoendopeptidase F [*Chloroflexus aurantiacus* J-10-fl]**
gi|76165433|gb|EAO59567.1| Peptidase M3B, oligoendopeptidase F [Chloroflexus aurantiacus J-10-fl]
Length=603

Score = 42.2 bits (92), Expect = 0.002
Identities = 14/21 (66%), Positives = 18/21 (85%), Gaps = 0/21 (0%)

Query 2 YNNALEASLKPNNIPLEVYKN 22
Y++ALEA+L PN IP+EVY N
Sbjct 255 YSSALEAALAPNEIPVEVYHN 275

>[gi|89209725|ref|ZP_01188120.1| **Peptidase M3B, oligoendopeptidase F [*Halothermothrix orenii* H 168]**
gi|89160794|gb|EAR80447.1| Peptidase M3B, oligoendopeptidase F [Halothermothrix orenii H 168]
Length=598

Score = 39.2 bits (85), Expect = 0.018
Identities = 12/22 (54%), Positives = 19/22 (86%), Gaps = 0/22 (0%)

Query 1 KYNNALEASLKPNNIPLEVYKN 22
KYN+ALE++L +N+P++VY N
Sbjct 251 KYNSALESALDDDNVPVDVYNN 272

>[gi|67916420|ref|ZP_00510131.1| **Peptidase M3B, oligoendopeptidase F [*Clostridium thermocellum* ATCC 27405]**
gi|67849613|gb|EAM45213.1| Peptidase M3B, oligoendopeptidase F [Clostridium thermocellum ATCC 27405]
Length=602

Score = 38.8 bits (84), Expect = 0.024
Identities = 12/22 (54%), Positives = 18/22 (81%), Gaps = 0/22 (0%)

Query 1 KYNNALEASLKPNNIPLEVYKN 22
KY+++LEASL +NI ++VY N
Sbjct 255 KYDSSLEASLDADNISVDVYDN 276

>[gi|76797206|ref|ZP_00779541.1| **Peptidase M3B, oligoendopeptidase F [*Thermoanaerobacter ethanolicus* ATCC 33223]**
gi|76587415|gb|EAO63852.1| Peptidase M3B, oligoendopeptidase F [Thermoanaerobacter ethanolicus
ATCC 33223]
Length=596

Score = 37.1 bits (80), Expect = 0.077
Identities = 13/22 (59%), Positives = 18/22 (81%), Gaps = 0/22 (0%)

Query 1 KYNNALEASLKPNNIPLEVYKN 22
KYN++LEASL +N+ +EVY N
Sbjct 249 KYNSSLEASLFEDNVSVEVYNN 270

>[gi|71492373|gb|EAO24679.1| **Peptidase M3B, oligoendopeptidase F [*Syntrophomonas wolfei subsp. wolfei* str. Goettingen]**
gi|71541021|ref|ZP_00662838.1| Peptidase M3B, oligoendopeptidase F [Syntrophomonas wolfei str.
Goettingen]
Length=600

Score = 36.3 bits (78), Expect = 0.14
Identities = 13/20 (65%), Positives = 15/20 (75%), Gaps = 0/20 (0%)

Query 1 KYNNALEASLKPNNIPLEVY 20
KY +ALEASL +NI EVY
Sbjct 251 KYPSALEASLDQDNISPEVY 270

>[gi|85683739|ref|ZP_01030375.1| **COG1164: Oligoendopeptidase F [*Clostridium difficile* QCD-32g58]**
Length=597

Score = 36.3 bits (78), Expect = 0.14
Identities = 12/22 (54%), Positives = 18/22 (81%), Gaps = 0/22 (0%)

Query 1 KYNNALEASLKPNNIPLEVYKN 22
KYN+A+EASL +++ L+VY N
Sbjct 250 KYNSAIEASLFSDDVSLDVYNN 271

>⌜ gi|33322731|gb|AAQ07100.1| **oligoendopeptidase f [*Lactobacillus delbrueckii* subsp. lactis]**
Length=112

Score = 34.1 bits (73), Expect = 0.61
Identities = 11/19 (57%), Positives = 15/19 (78%), Gaps = 0/19 (0%)

Query 2 YNNALEASLKPNNIPLEVY 20
Y +AL A+L NNIP++VY
Sbjct 69 YQDALAAALSENNIPVDVY 87

>⌜ gi|20516018|gb|AAM24265.1| **Oligoendopeptidase F [*Thermoanaerobacter tengcongensis* MB4]**
gi|20807490|ref|NP_622661.1| Oligoendopeptidase F [Thermoanaerobacter tengcongensis MB4]
Length=596

Score = 34.1 bits (73), Expect = 0.61
Identities = 12/21 (57%), Positives = 17/21 (80%), Gaps = 0/21 (0%)

Query 2 YNNALEASLKPNNIPLEVYKN 22
YN++LEASL +N+ +EVY N
Sbjct 250 YNSSLEASLFEDNVSVEVYNN 270

>⌜ gi|62516282|ref|ZP_00387639.1| **COG1164: Oligoendopeptidase F [*Lactobacillus delbrueckii* subsp. bulgaricus ATCC BAA-365]**
Length=600

Score = 34.1 bits (73), Expect = 0.61
Identities = 11/19 (57%), Positives = 15/19 (78%), Gaps = 0/19 (0%)

Query 2 YNNALEASLKPNNIPLEVY 20
Y +AL A+L NNIP++VY
Sbjct 250 YQDALAAALSENNIPVDVY 268

>⌜ gi|60545050|gb|AAQ08885.2| **oligopeptidase F [*Bacillus amyloliquefaciens*]**
Length=684

Score = 32.5 bits (69), Expect = 2.0

Identities = 13/22 (59%), Positives = 16/22 (72%), Gaps = 0/22 (0%)

Query 1 KYNNALEASLKPNNIPLEVYKN 22
KY +A EA+L N+IP EVY N
Sbjct 331 KYKSAREAALSNNSIPEEVYDN 352

>[gi|2633508|emb|CAB13011.1| **yjbG [*Bacillus subtilis subsp. subtilis* str. 168]**
gi|6093669|sp|O31605|PEPF_BACSU **Oligoendopeptidase F homolog**
gi|16078219|ref|NP_389036.1| **hypothetical protein BSU11540 [Bacillus subtilis subsp. subtilis str. 168]**
Length=609

Score = 32.5 bits (69), Expect = 2.0
Identities = 13/22 (59%), Positives = 16/22 (72%), Gaps = 0/22 (0%)

Query 1 KYNNALEASLKPNNIPLEVYKN 22
KY +A EA+L N+IP EVY N
Sbjct 256 KYKSAREAALSNNSIPEEVYDN 277

>[gi|1651216|dbj|BAA13561.1| **Pz-peptidase [*Bacillus licheniformis*]**
Length=628

Score = 32.5 bits (69), Expect = 2.0
Identities = 13/22 (59%), Positives = 16/22 (72%), Gaps = 0/22 (0%)

Query 1 KYNNALEASLKPNNIPLEVYKN 22
KY +A EA+L N+IP EVY N
Sbjct 275 KYKSAREAALSNNSIPEEVYDN 296

>[gi|82500204|ref|ZP_00885628.1| **Peptidase M3B, oligoendopeptidase F [*Caldicellulosiruptor saccharolyticus* DSM 8903]**
gi|82401784|gb|EAP42581.1| Peptidase M3B, oligoendopeptidase F [Caldicellulosiruptor saccharolyticus
DSM 8903]
Length=496

Score = 32.5 bits (69), Expect = 2.0
Identities = 14/22 (63%), Positives = 15/22 (68%), Gaps = 0/22 (0%)

Query 1 KYNNALEASLKPNNIPLEVYKN 22
KYN+ALEASL IP VY N
Sbjct 276 KYNSALEASLAQEFIPRSVYDN 297

Appendix F: Potential Source of Error Originating From Keratin

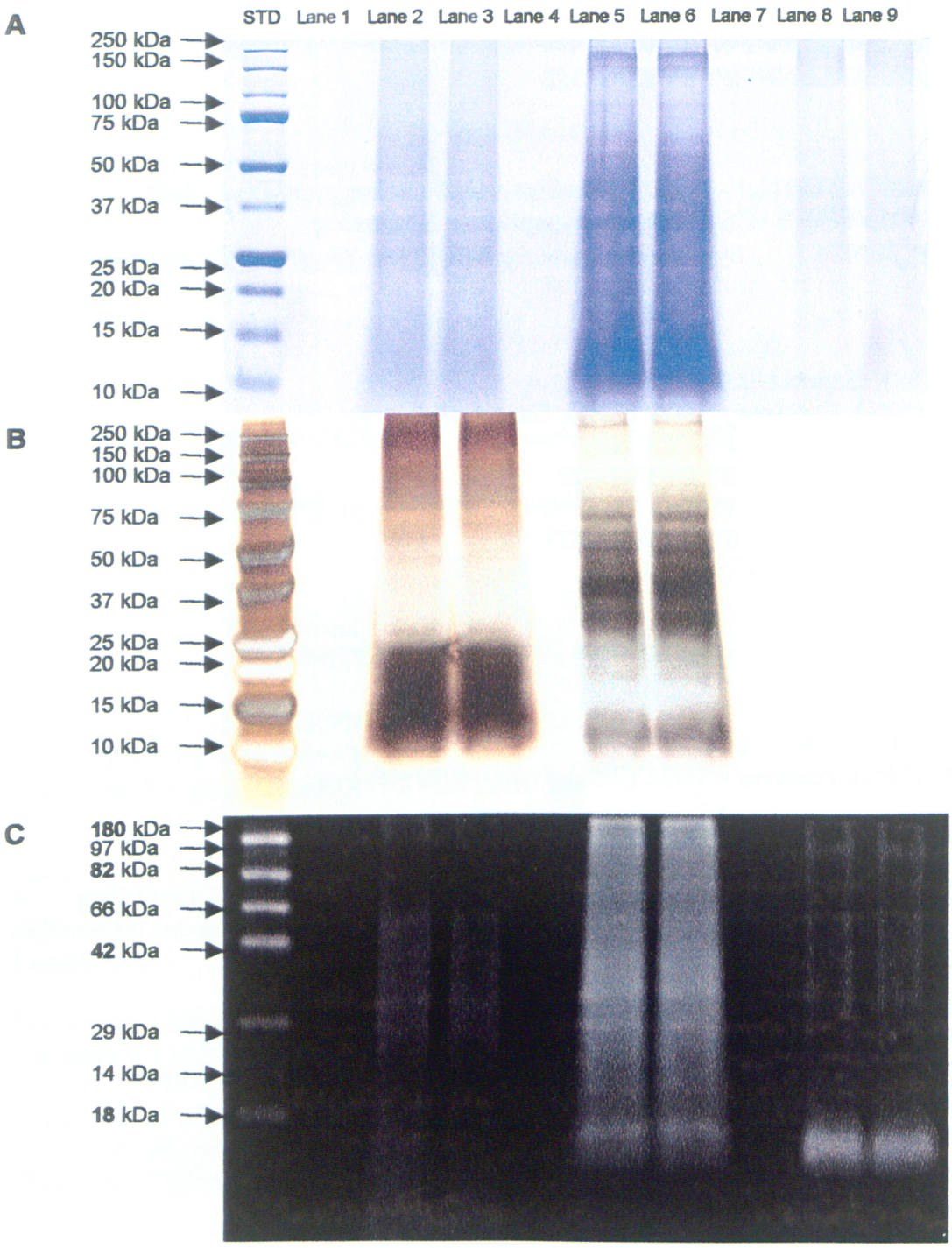

Figure F.1 Coomassie Brilliant blue (A), Silver nitrate (B) and SYPRO Ruby (C) stained Tricine-PAGE (10%) of 2x sample buffer (lanes 1, 4 and 7), dust from laboratory (30 mg) boiled in of 1% (w/v) SDS (500 µl) for 10 minutes (lanes 2 and 3), WWTP-derived EPS with protease inhibitors (lanes 5 and 6), and sludge supernatant concentrated 2x (lanes 8 and 9).