OPTIMIZED PROCEDURES FOR EXTRACTION, PURIFICATION AND CHARACTERIZATION OF EXOPOLYMERIC SUBSTANCES (EPS) FROM TWO BACTERIA (*Sagittula stellata* and *Pseudomonas fluorescens* Biovar II) WITH RELEVANCE TO THE STUDY OF ACTINIDE BINDING IN AQUATIC ENVIRONMENTS

A Thesis

by

CHEN XU

Submitted to the Office of Graduate Students of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2007

Major Subject: Oceanography

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Approved by:

Chair of Committee, Peter H. Santschi
Committee Members, Luis A. Cifuentes
Rainer Amon
Head of Department, Piers Chapman

December 2007

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ABSTRACT

Optimized Procedures for Extraction, Purification and Characterization of Exopolymeric Substances (EPS) from Two Bacteria (*Sagittula stellata* and *Pseudomonas fluorescens* Biovar II) with Relevance to the Study of Actinide Binding in Aquatic Environments.

(December 2007)

Chen Xu, B.S., Xiamen University

Chair of Advisory Committee: Dr. Peter H. Santschi

The extracellular polymeric substances (EPS) of marine bacterium *Sagittula stellata* and soil bacterium *Pseudomonas fluorescens* Biovar II, were extracted by six methods referred to the bibliography, efficacies of which were compared based on the EPS yield, composition as well as cell disturbance. Purification methods on these EPS were also improved, which proved to be more cost-effective and involve less interference from broth, compared to previous methods. Size exclusion chromatography (SEC) proved to be a useful tool, providing the "fingerprints" of the EPS extracted by different methods or after each purification step. Studies of the EPS production and composition at different growth stages provided abundant information and a basis for further in-depth studies. Results from SEC demonstrated that bacterial EPS had a constant molecular weight distribution all through the life but with various polymers in different proportions. Three fractions were successfully isolated by a combination of SEC and anion exchange chromatography for "non-attched" EPS produced by *Pseudomonas flurorescens* Biovar II. Protein turned out to be a major component of EPS in their native states, which was

mixed with the broth material and couldn't be recognized previously. The EPS harvested at the optimal time of the bacterial life was purified according to the improved method and was more enriched in polysaccharides, with small amounts of proteins, giving the molecules amphiphilic properties. In addition, simultaneous determination of neutral sugars and uronic acids by GC-EI-MS provided more information on the monosaccharide composition of the exopolysaccharides. Isoelectric focusing (IEF) spectra of the bacterial EPS spiked with Pu/Th, and Pu-enriched Rocky Flats Environmental Technology Site (RFETS) soil organic colloid spiked with Th showed similar activity distributions of both actinides along the pH gradient, with the activities of both actinides focusing on the low pH region. Characterizations of this Pu-enriched IEF extract from RFETS soil by spectrophotometric methods and ATR-FTIR indicated the co-presence of lipids, proteins and polysaccharides, in contrast to the bacterial EPS, which showed a simpler composition. This suggests that Th/Pu binding to organic macromolecules is more determined by the availability of binding functional groups rather than the exact specific compounds.

DEDICATION

To my husband and my parents

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I. INTRODUCTION

I.1.Extracellular Polymeric Substances (EPS): Their Formation, Importance to Microorganisms and Implication to Natural Environment and Industry

The vast majority of microorganisms, including both prokaryotes (bacteria and archaea) and eukaryotes (phytoplankton and fungi), mostly live and grow in aggregated forms such as biofilms, which are attached to natural or man-made surfaces, flocs and in a free planktonic state (Bhaskar and Bhosle, 2005). Microorganisms are ubiquitously distributed in natural soil and aquatic environments, on tissues of plants, animals and humans (Prouty and Gunn, 2003) as well as in medical and industrial systems, such as filters, sewage, treated effluent, pulp mill, fermented products, etc. Modern microscopy techniques have demonstrated that these microorganisms are actually embedded in a complex matrix of extracellular polymeric substances (EPS). Individual EPS macromolecules are fibrillar, a few nm thick and a few µm long (Decho, 1990; Leppard, 1995, 1997; Santschi et al., 1998; Passow, 2002), thus are included in the dissolved organic matter fraction (DOC, <0.7 µm) (McConville, 1985), the colloidal fraction (1-1000 nm) or particulate fraction (>0.4 µm) (Decho, 1990; Alldredge and Crocker, 1995; Chin et al., 1998; Krembs et al, 2002; Bhaskar et al., 2005). These polymers have been characterized as a heterogeneous mixture of polysaccharides, proteins, with minor amounts of lipids, nucleic acids, and other polymers, such as flagella, phages, debris from lysed cells, outer membrane vesicles, pili, etc. (Kumar et al., 2004; Hunter and

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This thesis follows the style of Marine Chemistry.

Beveridge, 2005 and references therein). After they're secreted (e.g., Quesadaa et al., 2006), they might remain tightly bound to the cell surface either by linkage between the carboxyl groups of EPS and hydroxyl groups of lipopolysaccharides (LPS) or by a covalent bond through phospholipids and glycoprotein (Bhaskar et al., 2005). Thus these densely packed and less diffusible capsules with a more organized polymeric structure are called "capsular" EPS, or "attached" EPS. Those polymers that more loosely adhere in the form of slime and can more easily be shed into the extracellular medium, as well as those which are already free in the medium, are called "non-attached" EPS (Figure 1). Improved high-pressure freeze-substitution and transmission electron microscopy (TEM) have further confirmed this formation mechanism (Figure 2).

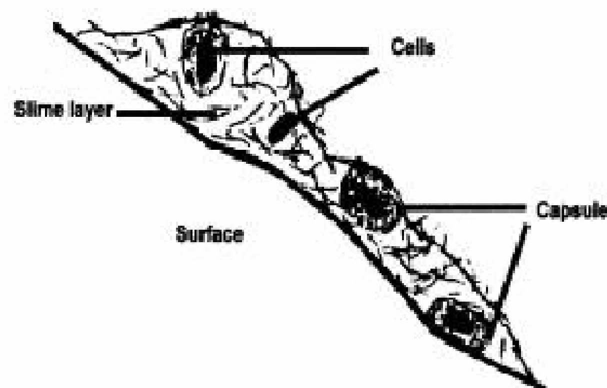


Figure 1 Schematic diagram showing bacterial cells and secreted exopolymers on the surface of a sediment or detrital particles (reproduced from Decho, 1990)

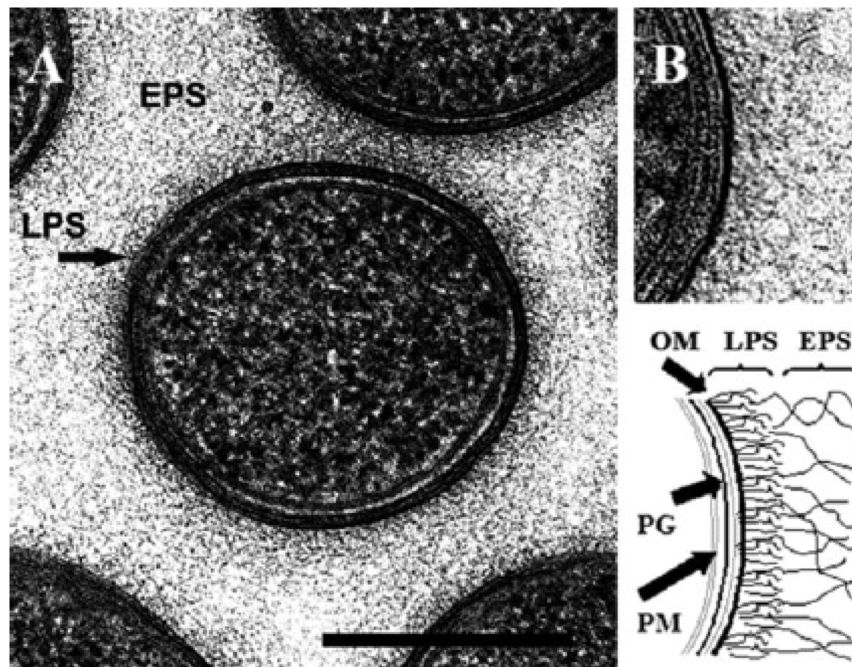


Figure 2 TEM micrograph of freeze-substituted biofilm cells. (PM, stained plasma membrane; PG, Periplasmic gel; OM, a taut outer membrane; LPS, lipopolysaccharides; Bar=0.5μm, reproduced from Hunter and Beveridge, 2005)

At the molecular level, EPS provide a protective mechanism for the microorganisms: (1) the buffering zone against micro-environmental changes, since extracellular mucilaginous substances are secreted especially under the stress of low nutrient concentrations, high concentrations of toxins, metals or of extreme salinity, pH and temperature. Recently, high concentrations of exopolymeric substances in Arctic winter sea ice were reported and correlated well with bacterial abundance and diatoms activity alternately (Krembs et al., 2002), which further confirmed that EPS are produced in response to very different kinds of growth stress and could be an important self-protection mechanism of microorganisms, e.g., against harsh winter conditions of high salinity and potential ice-crystal damage in this case. (2) Exoenzymes attached to

the cell surface can effectively hydrolyze large foreign molecules into more readily utilizable molecules, e.g., amino acids, monosaccharides, etc., to be utilized by the bacteria itself. This is a very efficient mechanism that utilizes the limited food sources in the waters, since in natural environments, rarely are all the compounds necessary to support microbes present in sufficient quantities. (3) Microbial EPS are highly hydrated (~97% of EPS mass exists as water) that provide protection to the cells against desiccation (Wingender et al., 1999; Hunter et al., 2005; Bhaskar and Bhosle, 2006). Also this hydrated structure makes EPS to work as a sorptive sponge that enable them to sequester essential nutrients, DOM and dissolved metals, etc. (Decho et al., 1990).

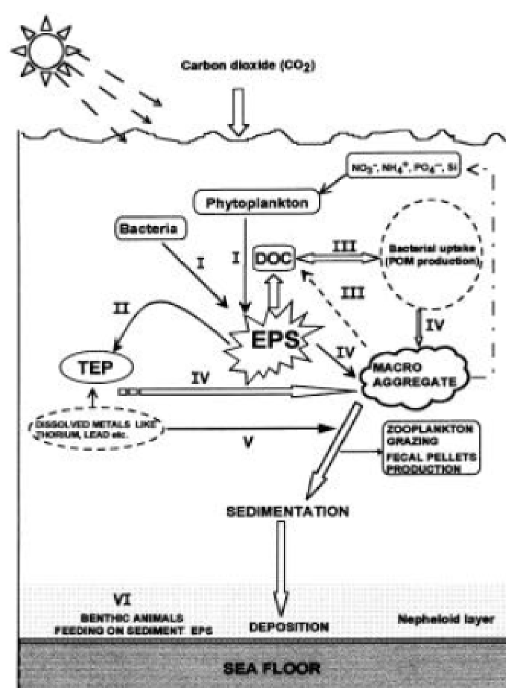


Figure 3 Schematic graph indicating various processes in which EPS are involved in aquatic systems. I, production of EPS by bacteria and algae; II, Production of TEP from EPS; III, Microbial loop; IV, Form ation of particles; V, Chelation of dissolved metals and VI, EPS as a carbon source for benthic community (reproduced from Bhaskar et al., 2005)

Recent microscopic (transmission electron and atomic force microscopy) and NMR techniques revealed that fibrillar polysaccharides formed an important fraction of marine colloidal organic matter and oceanic HMW DOM composition was physically and chemically quite similar to the EPS shed by certain phytoplankton species (Santschi et al., 1998; Aluwihare and Repeta, 1999). Generally EPS, released by algae or bacteria, may constitute <3% to more than 90% of the primary production in the aquatic environment. Thus they are an important fraction (up to 50%) of the semi-labile DOM pool in the oceanic waters (Sell and Overbeck 1992; Bhaskar et al., 2005). EPS serve as biological glue that modify the surface features or the sticking coefficients (α) of colliding particles and are usually regarded as the precursor of transparent exopolymer particles (TEP). Formation of TEP from EPS involves either biotic or abiotic aggregation processes, and the collectively formed larger particles from all these processes are called "marine snow" (Passow et al., 1994; Stoderegger and Herndl, 1998; Bhaskar and Bhosle, 2005; Bhaskar et al., 2005). Furthermore, through the continuous and sophisticated aggregation and dis-aggregation of EPS/TEP matrix throughout the water column (Chester, 2003), other suspended particles or dissolved trace metals (e.g., Pb, Co, Th, Cd, Sr, Cr, etc.) would be either directly scavenged or trapped in this mesh-like structure and may thus be transported out of the euphotic zones and eventually be sequestered in the sediments (Ford et al., 1987; Roane and Pepper, 2000; Quigley et al., 2002; Salehizadeh and Shojaosadati, 2003; Bhaskar et al., 2005). Therefore, EPS play a significant role in the global carbon flux as well as in metal biogeochemical cycling (Figure 3).

The strong binding capacity of EPS has led to their wide application in the removal

of heavy metals and radionuclides from wastewater and natural waters (Philippis et al., 2001). Choppin (1992) has predicted, based on binding studies with humic material, that organic material plays an important role in the mobilization and immobilization of Pu and other actinides in natural waters. In addition, it has been demonstrated that bacteria species, e.g., *pseudomonas* sp., can catalyze the transformation of uranium, neptunium and plutonium into less soluble, i.e., reduced, forms (REFS), thus acting as "living" backfill to immobilize actinides, either through surface binding or metabolic uptake.

I.2.Current Methodology of Extraction and Purification of EPS

Vander Waal forces, electrostatic interactions, hydrogen bonds, hydrophobic interactions and covalent bonds such as disulfide bonds in glycoprotein, etc., are usually the main forces between EPS and the cell surface (Christensen and Characklis, 1990, Wingender et al., 1999). There are many methods proposed for "stripping" (i.e., extraction) capsular polymeric substances (or called "attached" exopolymeric substances) from the cell, relying on applying a shear to the EPS matrix to break the physical and chemical forces. However, the dominant forces might be very different from one EPS matrix to another. Furthermore, studies with different chemically indentifiable target components, e.g., extracellular proteins vs. extracellular polysaccharides, the extraction method could be quite different even for the same EPS matrix. Therefore, there are no universal extraction methods and comparative experiments of various methods are needed. Current extraction methods can be subdivided into chemical and physical

extraction. Physical extraction includes high-speed centrifugation, ultrasonication, stirring, heating, autoclaving, cation exchange chromatorgraphy, etc., while chemical extraction involves addition of mild extractants such as tap water, sodium chloride and strong extractants such as alkaline reagents (e.g., sodium hydroxide, ammonium hydroxide), EDTA, acidic reagents (e.g., sulfuric acid, hydrochloric acid) and aldehydic solution, etc. In most cases, physical and chemical extraction methods are combined together to give the most efficient yield. However, caution must be taken to avoid any cell disruption or cell lysis, which would cause intracellular material to leak out and be collected also by the extraction procedure. Actually it's possible that cell disruption or cell lysis would always happen, no matter what kind of extraction method is used, but it should be minimized to the greatest extent. There are several ways of determining whether cell disruption or cell lysis has occurred by microscopic observation or measurement of intracellular material content, e.g., nucleic acid, ATP, glucose-6-phosphate dehydrogenase (G6PDH) (Platt et al., 1985; Grotenhuis et al., 1991a and b; Wingender et al., 1999).

Purification of extracellular polysaccharides will remove various interfering "contaminants", such as cell debris, protein, nucleic acid, ions, etc, for further composition and structure analysis. In general, the complexity of isolation and purification methods is mainly determined by the culture medium used for microorganism growth. Usually more than one method is used in order to achieve the highest recovery of EPS, elimination of the impurities to the greatest extent and the least modification of EPS structure. Table 1 lists the common methods used for isolation and

purification of EPS.

Table 1
Isolation and purification methods for extracellular polymeric substances

	"Contaminants" removed	References
Dialysis or dialfiltration against distilled water	Any contaminants with molecular weight less than membrane cutoff	Cerning et al., 1988; Marshall et al., 1995; van Kranenburg et al., 1997; Gehrke et al., 1998; Beech et al., 1999; Bergmaier et al., 2001; Vaningelgem et al., 2004; Corradi da Silva et al., 2005; Hung et al., 2005; Alvarado Quiroz et al., 2006
Acohol (e.g., ethanol, methanol, acetone, etc.) precipitation	Small molecules like ions could be eliminated, but proteins and other macromolecules could also be co-precipitated.	Van Geel-Schutten et al., 1999; Petry et al., 2000; Torino et al., 2000b; Dal Bello et al., 2001; Degeest et al., 2001; Rimada and Abraham, 2001; Ricciardi et al., 2002; Goh et al., 2005; Hung et al. 2005
Trichloroacetic acid precipitation (final concentration ranging from 4 to 14%)	Amino acids, peptides, proteins. Some polysaccharides might also be co-precipitated.	Garcı´a-Garibay and Marshall, 1991; Cerning et al., 1994; Grobben et al., 1995; van Marle and Zoon, 1995; Dupont et al., 2000; Frengova et al., 2000; Knoshaug et al., 2000; Marshall et al., 2001a,b; Ruas-Madiedo et al., 2002; Van Calsteren et al., 2002; Harding et al., 2003; Ruas-Mediedo and Reyes-Gavilan, 2005;
Enzyme digestion	Proteins and nucleic acids by protease and nuclease, respectively	Cerning et al., 1986, 1988, 1992; Abbad-Andaloussi et al., 1995; Mozzi et al., 1995, 1996; Bouzar et al., 1996, 1997; Torino et al., 2000a, 2001; Hung et al., 2005; Ruas-Mediedo and Reyes-Gavilan, 2005
Size exclusion chromatography	Any contaminants with molecular weights different from target compounds	Chen et al., 1999; Kim et al., 2003; Hwang et al., 2003; Corradi da Silva et al., 2005; Yang et al., 2005; Denkhaus et al., 2007;
Anion exchange chromatorgraphy	Any macromolecules with surface net charges different from target compounds	Wu et al., 2005; Yang et al, 2005; Ray et al., 2006;
Single dimension gel electrophoresis (i.e., isoelectric focusing)	Any macromolecules with isoelectric point different from target compounds	Quigley et al., 2002; Santschi et al., 2003;Alvarado Quiroz et al., 2006
Two-dimension SDS-PAGE	Any macromolecules with surface net charges and molecular masses different from target compounds	Quigley et al., 2002; Santschi et al., 2003; Alvarado Quiroz et al., 2006

I.3.Current Analytical Methods for Characterization of EPS

Table 2
Some spectrophotometric methods for measuring EPS composition

Composition	Methods	References
Total carbohydrates (TCHO)	Phenol-sulfuric acid	Dubois et al., 1956
	Anthrone-sulfuric acid	Morris, 1948; Vanden Berg et al., 1995; Levander et al., 2001; Rimada and Abraham, 2001
	TPTZ (2,4,6-tripyridyl-s-triazine)	Myklestad et al., 1997; Witter and Luther, 2002; Hung et al., 2001.
	MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride)	Burney and Sieburth, 1977; Johnson and Sieburth, 1977; Johnson et al., 1981
Acid polysaccharides (APS)	Ruthenium red adsorption	Figueroa and Silverstein, 1989
	Alcian blue stain	Passow and Alldredge, 1995
Uronic acids (URA)	meta-hydroxyphenyl method	Filisetti-Cozzi and Carpita 1991; Hung and Santschi, 2001
Protein	Lowry	Lowry et al., 1951
	BCA	Smith, 1985.
	Bradford	Bradford, 1976

Spectrophotometric methods, including colorimetric and dye adsorption methods, are the most common and simplest ways of quantifying the contents of various components (total carbohydrates, acid polysaccharides, uronic acid, protein, nucleic acids contents, etc.) of EPS. Table 2 lists some common spectrophotometric methods for measuring EPS composition. There are several drawbacks of these methods in that: (1) Dye, e.g., alcian blue, on one hand, usually only stains acidic polysaccharides on the particle surface rather than reacting with all acidic polysaccharides within the particle

itself; on the other hand, it can also stain non-acid polysaccharides, which would result in an overestimate (Leppard, 1995, 1997); (2) All colorimetric methods are semi-quantitative and use a single standard (e.g., glucose for total carbohydrates, xanthan for acid polysaccharides, glucuronic acid for uronic acid and BSA for protein analysis, respectively), which hardly represents the real composition of natural samples, since different compounds have different responses to the colorimetric reagents, sometime the accumulated differences could greatly bias the result. Moreover, usually all of these methods have relatively high detection limits for field samples. Thus, a pre-concentration of the samples is usually needed (e.g., Hung et al., 2001).

I.4.Relevance of This Study

Surficial soils of the Rocky Flats Envrionmental Technology Site (RFETS), Colorado, USA, contain elevated ^{240}Pu due to dispersal of soil particles by wind and waters, contaminated in the 1960's by leaking drums stored on the 903 Pad. A wide range of work at RFETS has demonstrated that "particulate" forms (≥0.45 μm, 40-90%) and colloidal forms (~2 nm or 3 kDa to 0.45 μm; 10-60%) of Pu make up the major fraction of actinides in surface waters and that particle transport is greatest during rainfall events (Santschi et al., 2002a, b). Furthermore, controlled laboratory experiments confirmed that most of the Pu in the 0.45 μm filter passing phase was in the colloidal phase (~80%) and the colloidal Pu is in the four-valent state and was mostly associated with a negatively charged ion-containing organic macromolecule with pH_{IEF} of 3.1 and a molecular weight of 10-15 KDa, rather than with the more abundant inorganic (e.g.

aluminosilicates) colloids. However, further characterization at the molecular level on the composition and structures of this Pu-enriched colloidal fraction isolated by isoelectric focusing method hasn't been carried out yet. Moreover, it has also been hypothesized that microbially produced EPS can act as a colloidal trap by trapping mineral particles that result in an enhancement of the K_d value for Pu onto mineral particles. Thus, controlled laboratory experiments on the production of EPS by microbial strain (i.e., *Pseudomonas fluorescens* Biovar II) isolated from the surface soil in this environmental site as well as the characterization of these EPS at both semi-quantitative and molecular levels would be of great help to elucidate the interaction between plutonium and bacterial EPS, the possible ligands of functional groups within the EPS macromolecules for plutonium binding. In a broader sense, the results could help to better understand microorganisms' role in environmental transport behavior of plutonium and the potential of bacterial remediation.

In the ocean, where ^{238}U is abundant, and produces ^{234}Th by radioactive decay, $^{234}Th/^{238}U$ disequilibria and ^{234}Th/POC ratios can be used to calculate carbon flux (Bruland and Coale, 1986; Cochran et al., 1992; Buesseler et al., 1992, 1998; Murray et al., 1996; Santschi et al., 1999; Guo et al., 2002; Hung et al., 2004). This is possible because Th is a highly particle reactive element and has strong affinities to particles and colloids, thus most (i.e., >90%) of the dissolved thorium is complexed with surface-active exopolysaccharide excreted either by marine algae or bacteria (Quigley et al., 2001, 2002; Guo et al., 2002; Santschi et al., 2003; Hung et al., 2004). However, there is often a discrepancy in the estimate of carbon flux, since production and

physicochemical characteristics of exopolymers vary considerably with hydrographic regimes, nutrients concentration and status, age of phytoplankton blooms, particle sizes, phytoplankton and bacteria species composition, temperature, salinity, etc., and this variability can make the Th/OC ratios greatly variable (Quigley et al., 2002; Hung et al., 2004). Therefore, controlled laboratory experiments on the production of exopolymeric substances by marine bacteria (i.e., *Sagittula stelleta* in this study) and characterization of these EPS would enhance the understanding of the nature of ^{234}Th (IV) binding with different fractions of marine organic matter.

I.5.Objectives

The primary objective of this research is to optimize the extraction, isolation and purification of EPS produced by soil bacteria, i.e., *Pseudomonas fluorescens* Biovar II and marine bacteria, i.e., *Sagittula stelleta*, respectively, thus, to establish the optimal routine methodology to yield enough and purified EPS suitable for further binding experiments or molecular-level analysis, e.g., NMR. Secondly, the growth of these two species in commercial broths, without any other additives and used in previously published research, are to be monitored as well as the EPS production and composition, which would guide further research on the manipulation of the growth conditions (e.g., carbon and nitrogen sources) and selection of the best growth condition, under which the two species could produce the most “sticky” EPS having the highest binding capacity for ^{240}Pu/^{234}Th and potential application in bacterial remediation in contaminated soils. Thirdly, a more quantitative methodology for the simultaneous determination of neutral

sugars and uronic acid by GC-EI-MS are to be developed. Fourthly, these bacterial EPS and Pu-enriched IEF extracts from RFETS soil colloids will be characterized, using both spectrophotometric methods and qualitative and/or quantitative techniques at the molecular level, i.e., GC-EI-MS, HPLC and ATR-FTIR. Finally, laboratory harvested EPS will be used as "model" substances to help interpret the binding mechanism of $^{240}Pu/^{234}Th$ with natural colloidal organic matter.

The hypotheses for the second objective, i.e., the study on the kinetics of the bacterial EPS production and composition, are stipulated as follows:

I. The null hypothesis (H_{01}) was set for the analysis of a correlation between the EPS composition (carbohydrates, proteins and/or uronic acids) and bacterial growth.

II. The null hypothesis (H_{02}) was set for the analysis of a corrrelation of the EPS varieties (carbohydrates, proteins and/or uronic acids) between "non-attached" and "attached" EPS.

II. MATERIALS AND METHODS

II.1.Bacterial Species

II.1.1.*Pseudomonas fluorescens* Biovar II (ATCC 55241)

Pseudomonas fluorescens Biovar II (ATCC 55241) was isolated from surface soil in Rocky Flats Envrionmental Technology Site (RFETS) and kindly offered by American Type Culture Collection (ATCC). This species is gram-negative, rod-shaped, 0.5-0.8 μm×1-3μm; strickly aerobic, mobile by polar flagella and can grow very well in a complex synthetic soy broth (BD company), which is composed of pancreatic digest of casein (17.0 g), papaic digest of soybean meal (3.0 g), sodium chloride (5.0 g), dipotassium phosphate (2.5 g) and dextrose (2.5 g). Usually 30 g of soy broth was dissolved in 1 L of double distilled water (18.0 Ω) and the solution was autoclaved in 121°C for 15 min. No other growth factors were needed. The species has preferable growth temperature within 28-30°C (Todar, 2004).

II.1.2.*Sagittula stellata* (Strain ATCC 700073)

Sagittula stellata (Strain ATCC 700073) was obtained from the ATCC. Cell of this marine species are rod-shaped (approximately 2.3μm long, and 0.9μm in diameter), gram-negative, strictly aerobic, and oxidase and catalase positive. Cell envelope has numerous surface vesicles derived from the outer membrane. They were inoculated in 2L Erlenmeyer flask with complex synthetic Marine Broth 2216 (Difco Laboratories), which has been demonstrated as one of the best growth media for this species (Gonzalez et al., 1997). It's composed of peptone (5.00g), yeast extract (1.00 g), Fe(III) citrate

(0.10 g), NaCl (19.45 g), $MgCl_2$ (5.90 g), $NaSO_4$ (3.24 g), $CaCl_2$ (1.80 g), KCl (0.55 g), Na_2CO_3 (0.16 g), KBr (0.08 g), $SrCl_2$ (34.00 mg), H_3BO_3 (22.00 mg), Na-silicate (4.00 mg), NaF (2.40 mg), $(NH_4)NO_3$ (1.60 mg), and Na_2HPO_4 (8.00 mg). Usually 37.4 g of marine broth was dissolved in 1 L of double distilled water (18.0 Ω) and the solution was autoclaved in 121°C for 15 min. The optimal growth temperature is 30°C and the best pH is 7.5 (Gonzalez et al., 1997).

The two species were pre-inoculated in their respective growth medium and incubated until both reached their late exponential phase, which was monitored by reading the optical density in a Turner SP-890 UV-VIS spectrophotomer at 600 nm. After more than ten transfers on the same medium, only one type of colony was observed on plate for each species (nutrient agar for *Pseudomonas fluorescens* Biovar II and marine argar 2216 for *Sagittula stellata*), which means both cultures were pure. For the latter experiments below, axenity of all cultures were always examined at the end, by agar plating.

II.2.Capsular EPS Extraction

For both bacteria species, four chemical (NaCl, EDTA, HCl and NaOH solutions), and two physical methods (ultrasonication and heating) were compared, in order to choose the best extraction method to give the highest yield at the minimal cell lysis. Centrifugation and blending (i.e., stirring) were always used, thus they were not considered here as experimental variables.

Specifically, for *Sagittula stelleta*, there were six groups of treatment: 3% NaCl, 2%

EDTA, 0.5 N HCl, 0.5 N NaOH, 3% NaCl with ultrasonication and 3% NaCl with heating. Extraction with 3% NaCl solution was used as the control method since it's the usual ionic strength in seawater and previously used by Hung et al. (2005) for this bacterium. 0.5 N acidic or alkaline reagents were chosen to give a similar ionic strength as the control method. The dosage of EDTA was chosen based on previous work by Sheng et al., (2005). Heating temperature as 75 ºC was chosen according to Gehr and Henry (1983), Sheng et al. (2005). Two physical extraction methods were combined with the control method (i.e., 3% NaCl solution), respectively. 1.8 liter of *Sagittula stelleta* culture was harvested when it was about to enter the stationary phase, judged from OD 600 nm. The solution was homogenously split into six groups and each group has two duplicates, thus each sample contained 150 ml of bacterial suspension and was then centrifuged at 1900×g for 30 min. The supernatant was carefully discarded without disrupting the pellet. The pellet was then extracted with the above solutions, respectively for three hours (Sheng et al, 2005). For comparison, pellets were also extracted in 3% NaCl solution combined with ultrasonication or heating at 75 ºC for one hour. Ultrasonication was carried out in a Bransonic Ultrasonic bath (continuous output: 40 W). While the extraction was going on, all solutions, except the solution using ultrasonication method, were stirred vigorously on a stir plate. After extraction, all solutions were centrifuged at 1900×g for 30 min and filtered through a 0.22 µm polycarbonate filter (GTTP, Millipore Corporation, USA) to get rid of any bacteria cell, and all the filtrates were dialfiltered against double distilled water through 10 KDa regenerated cellulose membrane (PLGC, Millipore Corporation, USA) by use of an

Amicon stirred cell series 8400 (Millipore Corporation, USA) and concentrated to a final volume of 15 ml. This step was required in order to remove any chemicals (e.g., EDTA) which might interfere with the later chemical characterization.

For *Pseudomonas fluorescens* Biovar II, there were seven groups of treatments: 0.005 N NaCl, 3% NaCl, 2% EDTA, 0.01 N HCl, 0.01 N NaOH, 0.005N NaCl with ultrasonication and 0.005N NaCl with heating. 0.005 N NaCl was chosen as the control group since it's the average ionic strength in soil solution (Uehara and Gillman, 1981; Black and Campbell, 1982, Kookana and Naidu, 1998). Accordingly, acid or alkaline solutions having ionic strengths of the same magnitude were used. 3% NaCl was also chosen, as referred to Hung et al. (2005). The same processing method for *Sagittula stelleta* described above was used.

II.3.Bacterial Growth Status and Sample Collection

To study the bacterial growth and EPS production and composition at different growth phases, 7.5 ml of each strain from pre-inoculated culture was inoculated in a 2 L Erlenmeyer flask, containing 1.5 L of respective medium for both species (section II1.1 and 1.2.). In order to reduce and estimate the error from sampling, extraction, purification and analysis, duplicates were harvested at certain time intervals for each species. Agitation was performed in a rotary shaker at 60 rev min^{-1} to keep the medium homogeneous. Temperature was kept constant at 30ºC. Optical density at 600 nm was read at regular time intervals. 200 ml of sample collected at various intervals from each flask were immediately centrifuged at 1900×g for 30 min, and the resulting supernatant,

which the "non-attached" EPS were extracted from, was filtered first through Whatman glass fiber filter (GF/F, Whatman International Ltd, Maidstone, UK) and later a 0.22 μm polycarbonate filter. The filtrate was collected and dialfiltered thoroughly against double distilled water through a 10 KDa regenerated cellulose membrane in a stirred cell. The retentate was concentrated to around 30 ml and pooled for later analysis. Both the GF/F and polycarbonate filter, as well as the pellet from previous centrifugation step were combined and extracted in the optimal extractant for each species, which was determined in previous experiment (section II.2 and section III.1). After extraction for a certain time (three hours for both species), which was also determined by the previous comparative experiment, the solution was centrifuged at 1900×g and the supernatant was collected, filtered through a 0.22μm polycarbonate filter and the filtrate was diafiltered against double distilled water through 10 KDa regenerated cellulose membrane. The retentate was also concentrated to around 30 ml for further analysis. This was defined as crude "attached EPS".

II.4.Purification of EPS by Alcohol Precipitation

The methodology of purifying bacterial EPS from the broth medium was modified and improved, based on the methods of Hung et al.(2005) and Alvarado Quiroz et al.(2006), in order to establish a more efficient, economic and routine way of EPS production of in large scale in the lab.

Three parallel experiments were designed in order to resolve three questions: (1) what's the best solvent, which could yield the highest extracellular polysaccharides

under the same condition? Three kinds of common alcohols were chosen to compare here: methanol, ethanol and isopropanol. (2) What's the best ratio of volume of alcohol to that of sample, 1:1, 2:1, 3:1 or 4:1, etc.? (3) How many times of alcohol precipitation and TCA precipitation are needed? For every parallel experiment, bacterial suspension from the same batch was used to eliminate the error caused by batch differences. Duplicates for each treatment were also taken.

II.5.Size Exclusion Chromatography and Anion Exchange Chromatography

II.5.1.Size Exclusion Chromatography

The molecular size distributions of bacterial EPS were measured by a Waters High Performance Liquid Chromatographic (HPLC) system. Millenium 4.00 software was used to operate the HPLC system and to acquire and integrate the chromatograms. The Waters System components used in this analysis were a 600S gradient controller, a 626 non-metallic pump, a 200 µL sample loop, a 717-plus autosampler, and a 2417 refractive index (RI) detector. The RI detector was set at a temperature of 30°C and a sensitivity of 4. The mobile phase was a solution of 0.078 M $NaNO_3$ with 10 mM phosphate buffer, pH 6.8, ionic strength 100 mM, maintained at a flow rate of 0.5 ml min^{-1} through Tosoh Biosciences guard and analytical columns G4000 PWxl, 6 mm x 4 cm and 7.8 mm x 30 cm, respectively, particle size 10 µm. The Tosoh Biosciences G4000 PWxl analytical column is rated to quantify dextran molecular weights (MW) from 1 to 700 kDa and globular proteins ranging in size from 10 to 1500 kDa. The temperature of the autosampler was set to match ambient conditions, at 22°C, which also was the

temperature of the columns (Schwehr et al., 2007, manuscript in preparation).

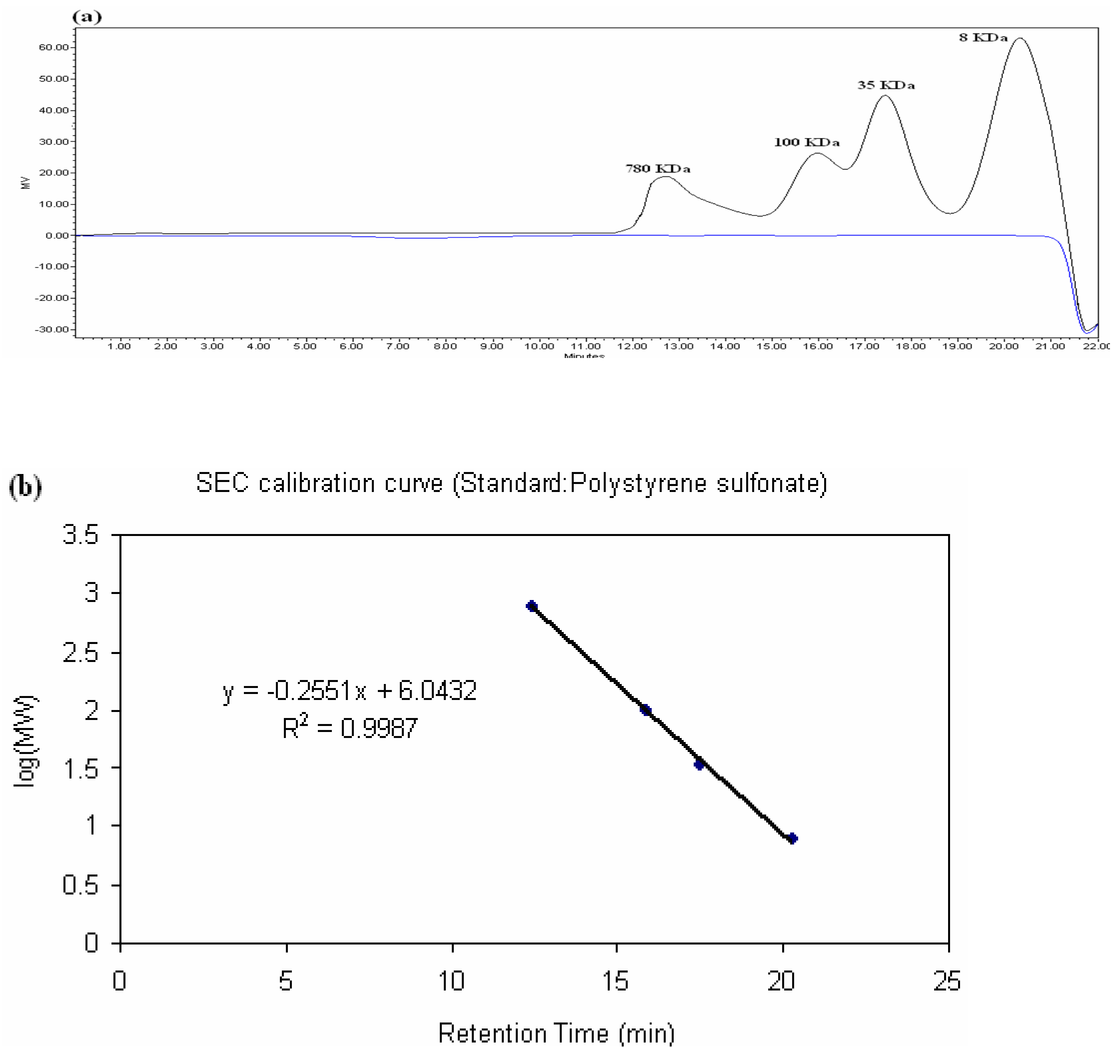


Figure 4 Calibration of size exclusion chromatogram (SEC). (a) Size exclusion chromatogram of different polystyrene sulfonate standards including molecular weights of 8, 35, 100 and 780 KDa; (b) calibration curve for size exclusion chromatography

Solutions of EPS were prepared at concentrations of 1mg/ml in Micro and

acid-cleaned, combusted vials at least 12 hours before analysis to allow uncoiling and solubilization. During this period, the EPS solutions were stored in the dark under refrigeration apart from any volatile organics, and then filtered through 0.2 μm sterile syring filter (cellulose acetate, VWR international) before injection into the HPLC to remove non-rehydrated aggregates and bacteria (Wilkinson et al., 1997). The injection volume was 150 μL, which is close to 1% of the column bed volume, 14.3 ml.

Polystyrene sulfonate molecular weight standards of 8, 35, 100, and 780 kDa, from Polysciences, Inc., were used to calibrate the samples (Figure 4 a-b). These standard solutions were prepared in the same manner as and were run in parallel to the samples.

II.5.2.Anion Exchange Chromatography

EPS purified by alcohol and TCA precipitation step was re-dissolved in D.I. (~1mg/ml) and filtered through a 0.22 μm polycarbonate membrane and subjected to a preparative BioSuite™ anion exchange column (21.5×150 mm) connected to a Waters HPLC 600 pump. The injection volume was 5 ml. The sample was eluting at a constant flow rate of 4 ml/min successively with 20mM Tris-HCl buffer (pH=8.1, Sigma-Aldrich) for 15 min and then a gradient 0-0.75 N NaCl in 70 min. The eluate was detected by a Waters 474 scanning fluorescence detector (excitation wavelength: 285 nm; emission wavelength: 580 nm; gain: 100; attenuation: 256) and 2487 Dual λ-UV absorbance detector (λ_1=200nm, λ_2=285 nm). The same Millenium platform was used to operate the HPLC system and to acquire and integrate the chromatograms.

II.6.Radiolabelling EPS with ^{234}Th(IV) and ^{240}Pu

5 mg of dried purified EPS was re-dissolved in 1 ml of double distilled water. Addition of both isotopes was followed by an adjustment of pH to near neutral (6.5-7.0) with reagent-grade NaOH (Quigley et al., 2001). The neutralized Th-EPS or Pu-EPS mixture was stirred in a Teflon beaker with a Teflon coated stir bar at 60 rpm for about 30 min. In order to get rid of those unbound or "free" ^{234}Th (IV) or ^{240}Pu, the solution was dialfiltered thoroughly against double distilled water by use of a disposable Centriplus Centrifugal Filter Device (MW cutoff: 1 KDa, Millipore) until the activity of ^{234}Th in the permeate, measured by a liquid scintillation counter, kept constant or was close to the background. This step was also required for further isoelectric focusing experiment since radiolabeling and neutralization steps would make EPS-Th or EPS-Pu solution with a high ionic strength, which would severely affect gel pH stability. The final retentate was concentrated to around 100-150 μl and ready for the isoelectric focusing electrophoresis experiment.

II.7.Isoelectric Focusing Electrophoresis (IEF)

IEF is an electrophoretic method that separates molecules based on their isoelectric point (pI). In a pH gradient under the influence of an electric field, amphoteric macromolecules possessing a positive, negative or zero charge, depending on their surrounding pH, migrate either towards the cathode or the anode until they reach a zero net charge. This specific point in the pH gradient, where the net charge of the macromolecules is zero, is called isoelectric point (pI). Under the electric field,

molecules with a negative charge migrate towards anode and concentrate there, thus having a low pI; molecules with positive charge migrate towards cathode and concentrate there, thus having a high pI. Specifically, the net charge of a macromolecule is the sum of all the negatively (e.g., carboxyl, phosphate, sulphato) and positively charged (amino) functional groups. Therefore, IEF is a useful method to not only characterize the net surface charge of macromolecules, but also separate macromolecules with the highest resolution and the cleanest results, based on very small charge differences (Trubetskoj et al., 1992; Berkelman and Stenstedt, 1998).

IEF is performed under denaturing conditions by mixing the sample with the rehydrated solution, which includes urea and detergent (see Appendix I), to make sure that each polymer is present only in one configuration and aggregation and molecular interactions are minimal. Dry colloid (0.22μm-1KDa) powder (~11 mg) extracted from Rocky Flats Environmental Site (RFETS) soil by a series of centrifugation and ultrafiltration steps, and kindly provided by Kim Roberts was dissolved in 1.1 ml of double distilled water and mixed very well with 1.54 ml of rehydrated solution. Radiolabelled bacterial EPS with ^{234}Th(IV) or ^{240}Pu (see section II.7) didn't need to be diluted in D.I.. 240 μl of this solution (100μl sample +140μl rehydrated solution) was loaded in each IPG strip (Amersham Biosciences, Immobiline Dry Strip, pH 3-10, 11 cm, Cat. No: 18-1016-61). One strip, as the blank, was loaded with 100 μl of D.I. without sample and 140 μl of rehydrated solution. For each IEF running, there were 11 sample strips and one blank strip. Thus the sample load was 1 mg colloid /strip. All strips were placed into the reswelling tray for at least 12 hours and then placed in the electrophoresis

apparatus. (Amersham Biosciences, Multiphor II Electrophoresis System) and ran for 17.5 hours with a programmed current (See Appendix II).

After the program was finished, pH values of each strip at every centimeter were measured immediately, by a bench pH/mv/temperature meter (pH/Ion 510 series, OAKLON Instrument, USA). Strips were carefully cut evenly into 11 fractions and the same fraction in every strip was pooled and extracted in 1% sodium dodecyl sulfate (SDS) for 24 hours. The fractions were further processed respectively for either ^{234}Th(IV) or ^{240}Pu activity analysis.

II.8. ^{234}Th (IV) and ^{240}Pu Activity Analysis

Liquid scintillation cocktail (Ecolume, ICN) was added to the previous SDS solution and the vials were capped and vigorously shaken and loaded into a Beckman Model 8100 Liquid Scintillation Counter (LSC) and counted for 10 min. The conventional energy window for ^{234}Th (IV) was 0.199 MeV and detection limit was 63-65 dpm (a personal communication with Kim Roberts). Analysis yielded ±10% or 2-sigma error (Quigley et al., 2001; Alvarado Quiroz et al., 2006).

Pu analysis was referred to Santschi et al., 2002a and b. Briefly, previous SDS solution was transferred in a 250 ml beaker and the vial was thoroughly triply rinsed with HCl and then HNO_3. All the washings were combined. Pu-242 (2.21 dpm) was added as tracer at this moment. Acid were evaporated to dryness on a hot plate. 5ml of concentrated HCl and 5 ml of concentrated HNO_3 were added to rinse the glass wall of the beaker and then evaporated to dryness. This was repeated twice. 5 ml of concentrated

HCl was added and evaporated to dryness. The residue was dissolved in 75 ml of 9 N HCl and 1 ml of freshly made $NaNO_2$ (0.85 g/ml) was added. The mixture was then run through a well-packed and pre-conditioned cation exchange column (AG×8, Biorad econo-pac, Cat. No.732-1010). 40 ml of 9 N HCl was used to rinse the column and discarded. This was repeated one more time. Pu was eluted by rinsing the column with a mixture composed of 20 ml of 9 N HCl and 1 ml of freshly made NH_4I (0.14494g/ml). This fraction was collected and 1 ml of concentrated HNO_3 was added. The whole solution was evaporated to dryness. The Pu was then microprecipiteated as (LaF_3) on a Pall Gelman Metrice 0.1 um filer, mounted on a stainless steel planchet, and α-counted.

II.9.Chemical Characterization by Spectrophotometric Methods

Turner SP-890 UV-VIS spectrophotomer was used for all spectrophotometric analysis. Total carbohydrates were measured by a modified TPTZ method (Hung and Santschi, 2001). Uronic acids were measured by meta-hydroxyphenyl method (Filisetti-Cozzi and Carpita 1991), modified by Hung and Santschi (2001). The protein assay followed BCA method (Smith, 1985). Nucleic acid content was quantitated by measuring the absorbance at 260 nm with a quartz cuvette having a 1 cm light pathlength and multiplied the absorbance by the extinction coefficient 50 µg/ml. This is based on the principle that an absorbance of 1 unit at 260 nm corresponds to 50 µg plasmid DNA per ml (Manchester, 1996).

For total phosphate content, Pu enriched colloidal fraction extracted by isoelectric focusing was digested following Solórzano and Sharp (1980) and then measured by a

colorimetric method (Murphy and Riley, 1962). Total Organic Carbon (TOC) was measured by use of a Shimadzu TOC 5000 carbon analyzer (Guo et al. 1994).

II.10.Simultaneously Determination of Neutral Sugars and Uronic Acids by GC-EI-MS

II.10.1.Hydrolytic Conditions

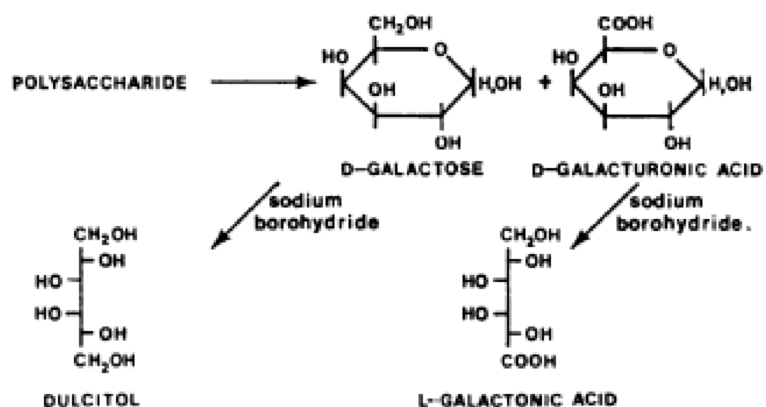
Experiments (acid type, temperature and time) on hydrolytic conditions were carried out. Two volatile acids were chosen according to the literature (Section III.3.1), trifluoroacetic acid and hydrochloric acid. There were three parallel experiments conducted to answer three questions: (1) What are the best concentrations for both acids, which could yield the best recovery for both bacterial EPS? (2) What are the best hydrolysis temperatures? (3) At the optimal temperature and acid concentration, how long does the hydrolysis take in order to give the best yield? After hydrolysis, the total carhydrates contents were measured by the TPTZ method (Myklestad et al., 1997; Hung and Santschi, 2001) and expressed as % of EPS dry weight.

II.10.2.Derivatization of Neutral Sugars and Uronic Acids

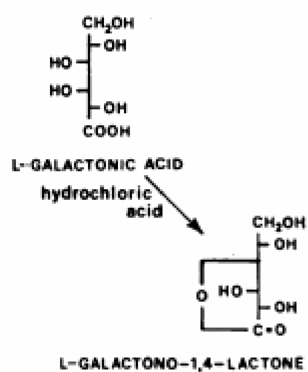
Derivatization of neutral sugars and uronic acids were performed according to Walters and Hedges (1988), with a small modification. Briefly, after hydrolysis, samples were spiked with 20 µl of 1000 µM of allose and ribonolactone, which were used as internal standards for neutral sugars and uronic acids, respectively. Samples were then blown to dryness under a nitrogen stream. To split any lactones present, 0.5ml of a 0.1 M sodium carbonate solution was added to the residue in each tube and incubated in a

low-temperature oven at 30 ºC for one hour. The carbonyl carbons of aldoses and uronic acid salts were reduced to the corresponding alditols and aldonic acid, respectively (Reaction 1), when 0.5 ml of 6% freshly-made sodium borohydride solution was added and tubes were all closely capped. After 1.5 hours, 25% acetic acid solution was added drop by drop to the solution in order to decompose the excessive sodium borohyride. Cations were removed by pouring the solution onto a column containing 2 ml of cation-exchange resin (Dowex 50W-X8 400 mesh) and washed with 7 ml of distilled water. Both eluate and washing were combined and a stream of N_2 was used to dry the solution. Borate was removed as the volatile trimethyl borate by three times evaporating the residue with 2 ml of methanol. 1 ml of 2 N HCl was added to each tube and then blown to dryness under a N_2 stream. The residue was then heated at 85 ºC for 2 h under a N_2 environment to completely convert the aldonic acids into aldonolactones (Reaction 2). Lower temperature and /or shorter heating time might result in only partial conversion and isolation of some free aldonic acid. Complete conversion was necessary since free aldonic acids wouldn't form the N-hexylaldonamides. To produce the N-hexylaldonamides from the aldonalactones, 0.5 ml of pyridine and 0.5 ml of N-hexylamine were added to each tube and the capped samples were heated in the block at 55 ºC for 30 min (Reaction 3). After the tubes were removed and cooled to below 45 ºC, N_2 is blown over the solutions until dry residues remain. To produce peracetylated alditols and N-hexylaldonamides, 0.5 ml of pyridine and 0.5 ml of acetic anhydride were added and heated at 95 ºC for 1 h (Reaction 4). Basically the original aldoses were converted into alditol acetates and the original uronic acids converted into corresponding

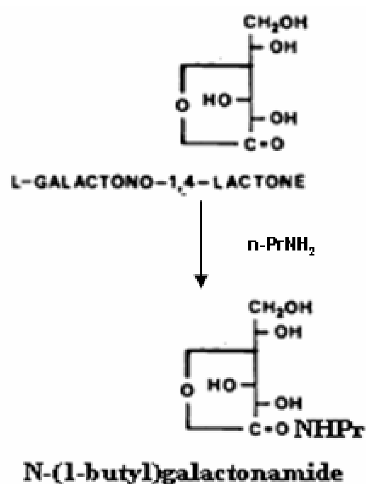
peracetylated N-hexylaldonamide. A stream of nitrogen was used to remove the solvents and 1 ml of distilled water was added to dissolve the residue. Finally, 1 ml of ethyl acetate was added to extract the sample, and this process was repeated twice and the extracts were combined. A stream of N_2 was blown over the final residue until dryness and 200ul of ethyl acetate was used to re-dissolve the sample to the concentrated volume and this was ready for GC-EI-MS analysis.

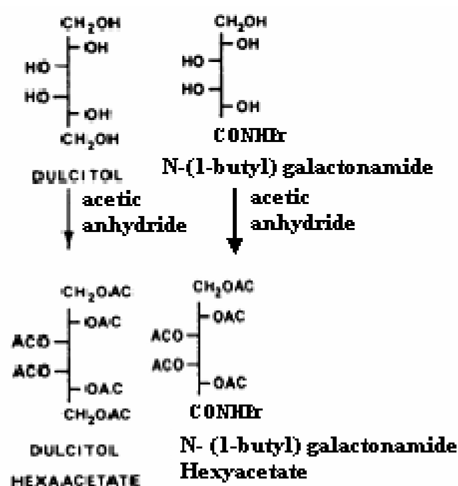


Reaction 1



Reaction 2





Reaction 3 Reaction 4

II.10.3.GC-EI-MS

The sample was injected to a GC (Thermo Finnigan) coupled with an AS3000 autosampler and a Finnigan Polaris Q external ion source mass spectrometer. A splitless type injector was used having a glass injection liner filled with pre-combustered glass wool. The injection port was kept at 250 ºC and ion source was kept at 280 ºC. A full scan mode was used and the mass range was 50-650. The injection volume was 1 µl and helium, as the carrier gas, was kept at a constant flow rate of 1.2 ml/min. Temperature of the GC program was set up as follows: the initial temperature was set to 80 ºC and held for one minute; then the column was heated up to 220 ºC at a rate of 10 ºC /min, and held for another two minutes; finally the column was heated up to 260 ºC at a rate of 1 ºC /min and held for 5 minutes. Xcalibur™ Data System was used to acquire and integrate the real-time plot of Total Ion Current (TIC) and mass spectrum.

II.11.ATR-FTIR

Attenuated Total Reflection-Fourier Transform-Infrared Spectroscopy, so called ATR-FTIR is a non-destructive on line technique, allowing users to not only identify the functional groups but also study *in situ* adhesion and biofilm development of bacteria as well as adsorption of isolated polysaccharides. In this study, a Variant 3100 FTIR was connected with a single reflection horizontal ATR accessory from PIKE Technologies, Inc. (Madison, WI). It uses a diamond plate as the internal reflection element. Dry EPS samples or colloids were mounted at the surface of the diamond. Since the ATR effect only took place very close to the surface of the diamond, an intimate contact and highest sampling sensitivity were achieved by using the pressure clamps. The spectrum of absorbance from 4000 to 650 cm^{-1} was collected and integrated by the Varian Resolution Pro 4.0 software. A sensitivity of 3 and a resolution of 4 cm^{-1} were used. All ATR spectra used the spectrum of a clean diamond as the background, which was auto-subtracted from the spectra of the sample.

II.12.Statistical Analysis

Statistical analysis was conducted using SPSS 11.0 (SPSS Inc. Chicago, IL 60606). Data were first tested for normality using Kolmogorov-Smirnov test (α=0.05). If normally distributed, they would be further tested for correlation using 2-tailed, bivariate Pearson analysis (α=0.05). If data were not normally distributed, non-parametric Spearman analysis would be performed, which was more conservative than the Pearson test against Type I error, i.e, rejecting a true null hypothesis (Dytham, 1999). With a

critical level for rejection (α) of a hypothesis set at 0.05, the null hypotheses is rejected when *p*-value is much smaller than 0.05, i.e., accepting the alternative hypotheses that a significant correlation relationship exists between different variables.

III. RESULTS

III.1.Capsular EPS Extraction

The contents of capsular EPS varieties extracted by different treatments are shown in Figures 5 and 7 for *Sagittula stellata* and *Pseudomonas fluorescens* Biovar II, respectively. Total carbohydrates content is expressed as glucose equivalents in mg/L-bacterial suspension. Protein is expressed as BSA equivalents in mg/L-bacterial suspension and nuclecic acid as plasmid DNA equivalents in mg/L-bacterial suspension. EPS varieties in the latter text, if measured by the same spectrophotometric methods and not specified anywhere else, are all following this way. Ratio of carbon in total carbohydrates (40%) to that in protein (33%) is summarized in Figures 6 and 8. It varied from 0.10 to 0.92 for *Sagittula stellata* and from 0.05 to 0.34 for *Pseudomonas fluorescens* Biovar II, dependent on different treatments.

In order to assess to what extent different chemical or physical extraction methods would hydrolyze or break down macromolecules, thus change their molecular weight, EPS extracted by different methods were further analyzed in a size exclusion column connected with Waters HPLC.

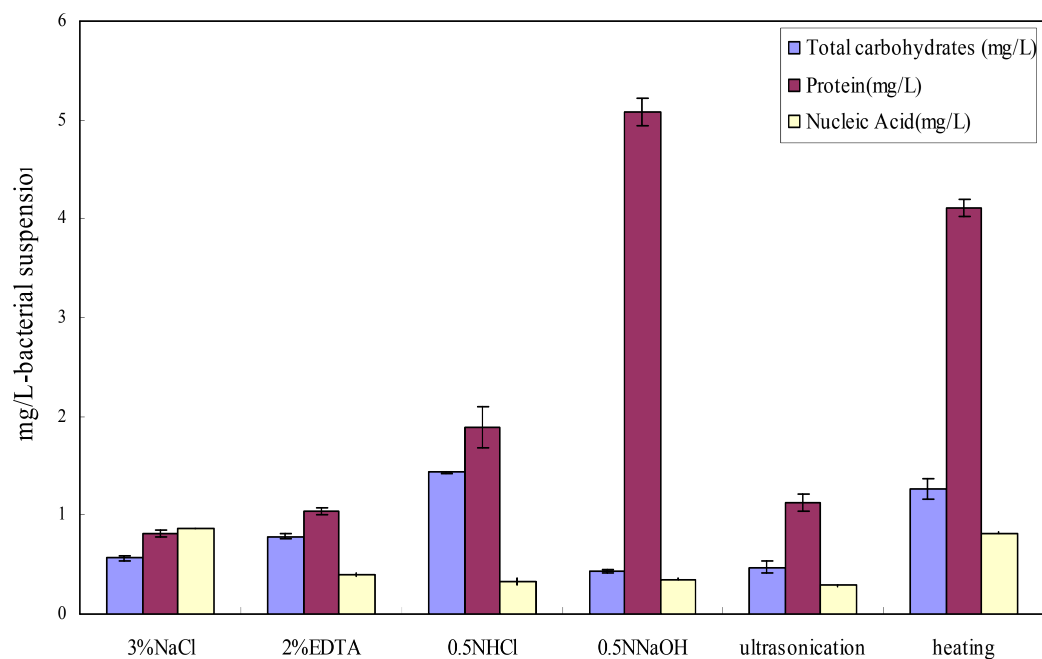


Figure 5 “Attached” EPS varieties produced by *Sagittula stellata* and extracted by different methods

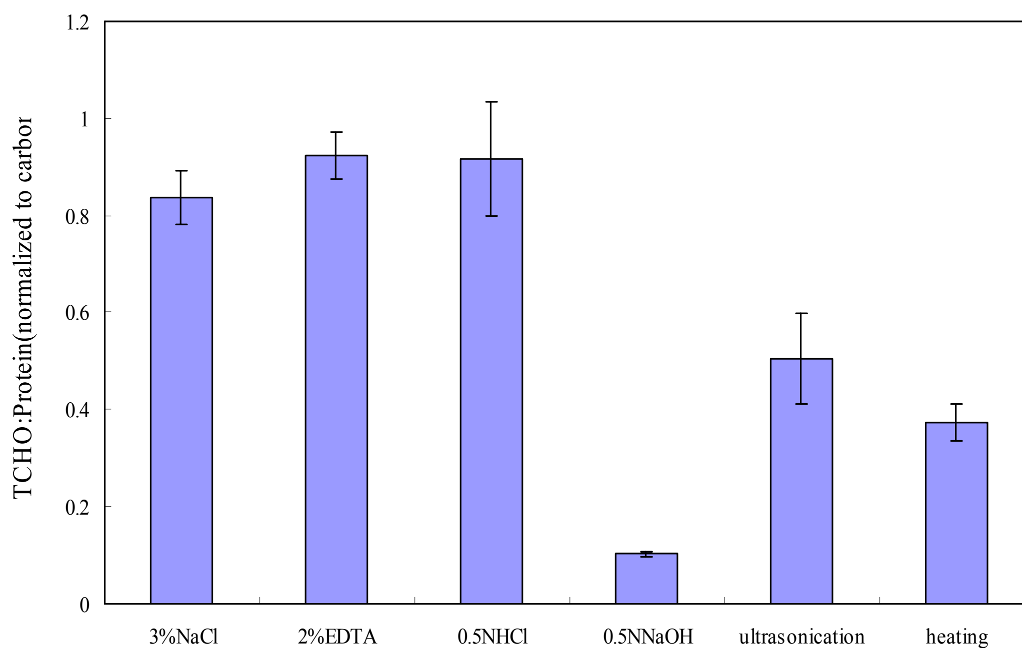


Figure 6 Carbohydrates-C to protein-C ratio by different extraction methods on “attached” EPS produced by *Sagittula stellata*

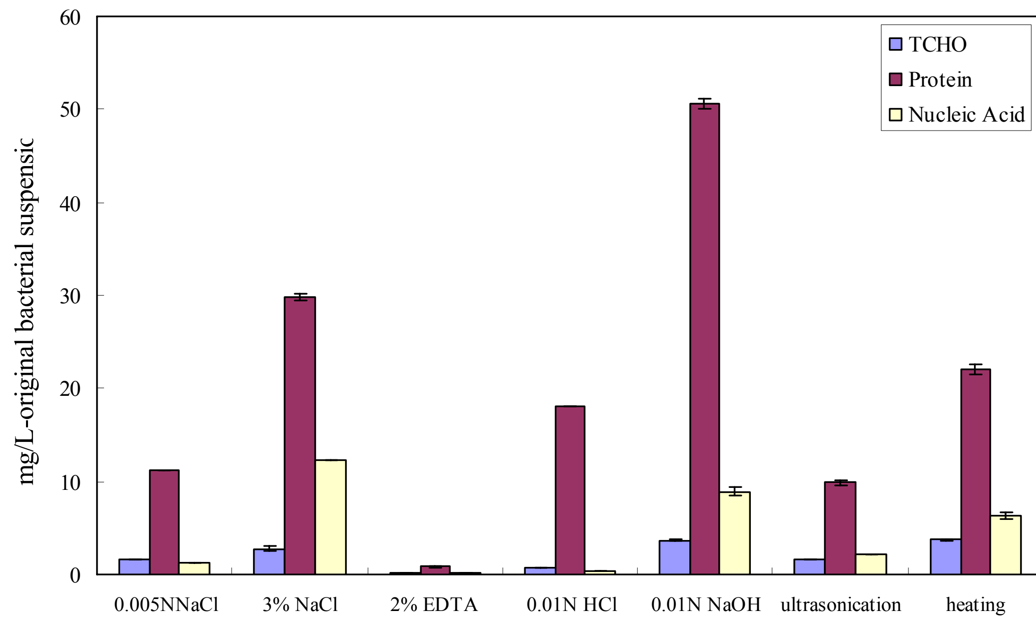


Figure 7 “Attached” EPS varieties produced by *Pseudomonas fluorescens* Biovar II and extracted by different methods

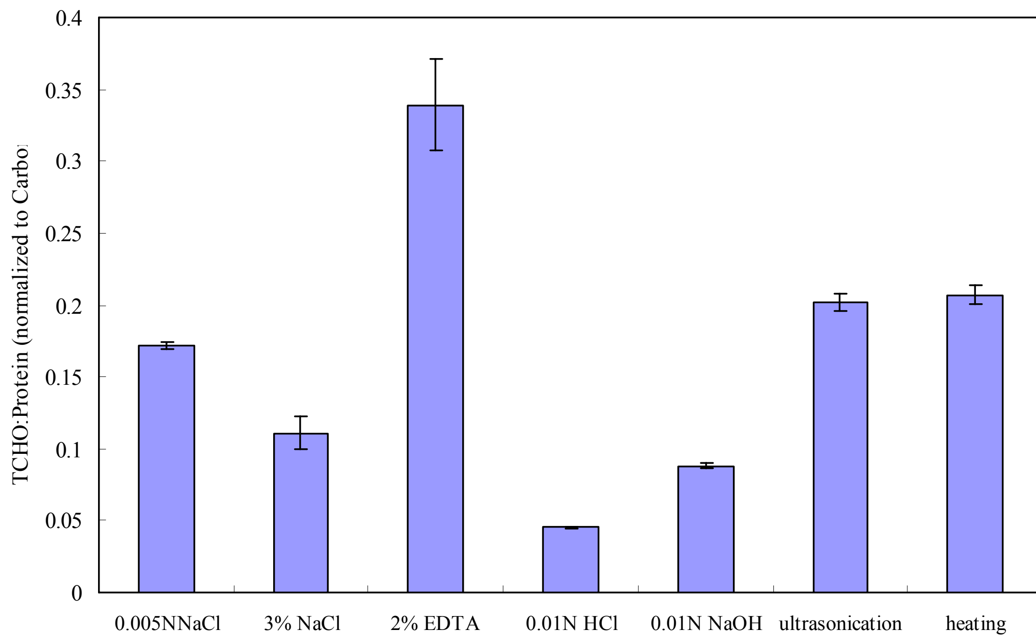


Figure 8 Carbohydrates-C to protein-C ratio by different extraction methods on “attached” EPS produced by *Pseudomonas fluorescens* Biovar II

Results for *Sagittula stellata* EPS showed that extraction with 3% NaCl, 2% EDTA, 0.5 N HCl, 0.5 N NaOH or ultrasonication won't change the molecular distribution of the EPS variety (single peak at very similar retention time, molecular weight: (28.1±1.4) KDa) while EPS extracted with a combination of 3% NaCl solution and heating at 75 °C for one hour have double peaks and very different retention times, indicating molecular weights of 31.9 KDa and 12.8 KDa respectively (Figure 9.(a)-(f)). This means that either intracellular material was leaked into the solution, or EPS had been chemically modified under high temperature. The content of nucleic acid for the six groups was in the order: 3% NaCl ≥3% NaCl with heating (1 hour)> 2% EDTA>0.5 N NaOH>0.5 HCl>3% NaCl with ultrasonication (1 hour). Results from HPLC-SEC and nucleic acid contents were complementary to demonstrate that all these treatments wouldn't cause severe cell lysis, while only extraction by 3% NaCl with heating would probably cause modification of the macromolecules. Ultrasonication under 3% NaCl solution for one hour didn't significantly increase the extraction yield, including both extracellular polysaccharide and protein content. 0.5 N HCl turned out to be the most efficient method to extract capsular polysaccharides with the highest yield (1.43 mg total carbohydrates /L-bacterial culture), while having the most similar ratio of carbohydrates to protein to that of the control group. 0.5 N NaOH is the most efficient method to extract capsular protein with the highest yield (5.07±0.14) mg protein/L-bacterial culture. Thus 0.5 N HCl would be considered as the best extractant for capsular extracellular polysaccharides of *Sagittula stellata*.

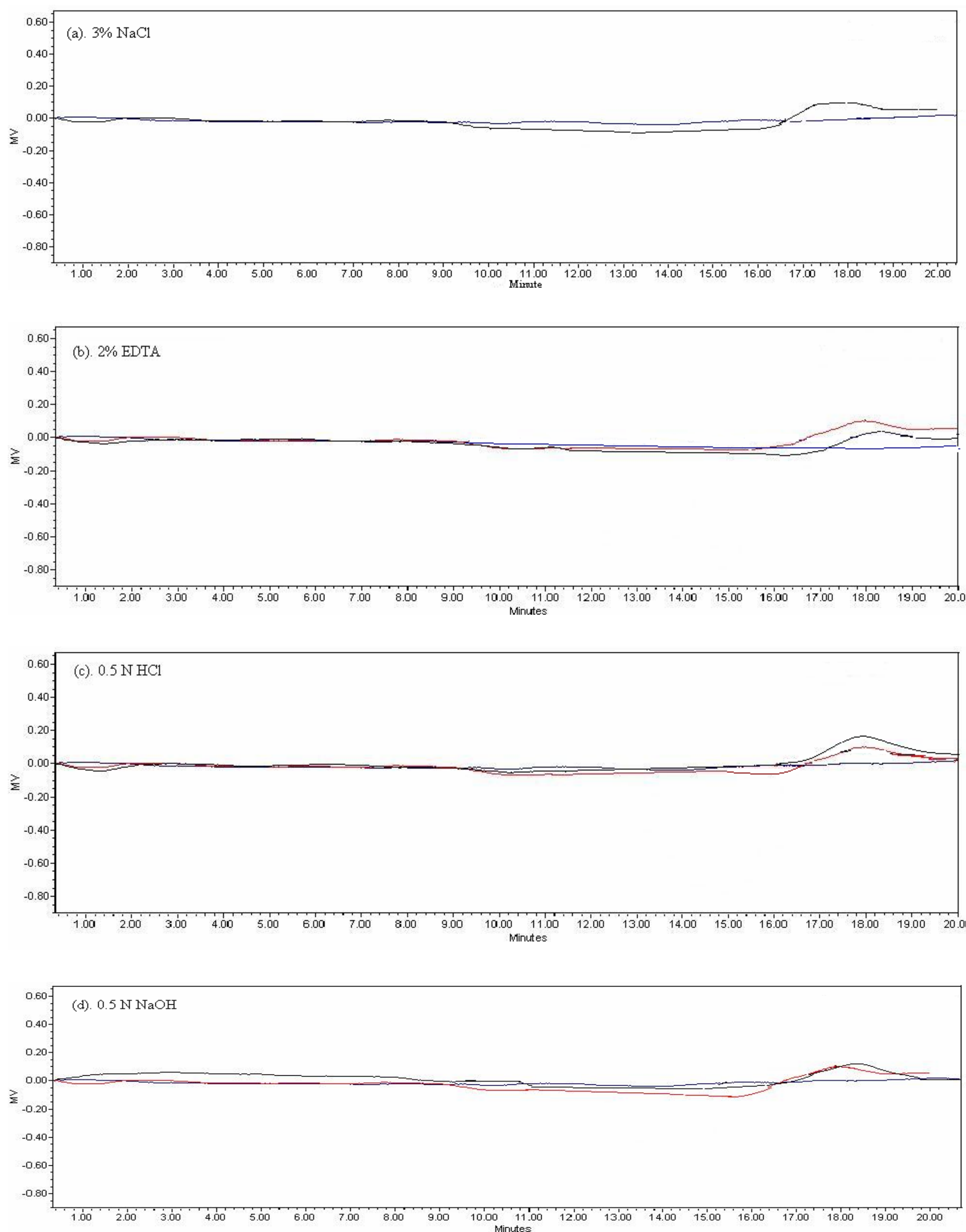


Figure 9 Size exclusion chromatograms of capsular EPS of *Sagittula stellata* extracted by different methods (blue line represents "blank"; red line represents the control group, i.e., 3% NaCl; black line represents current method)

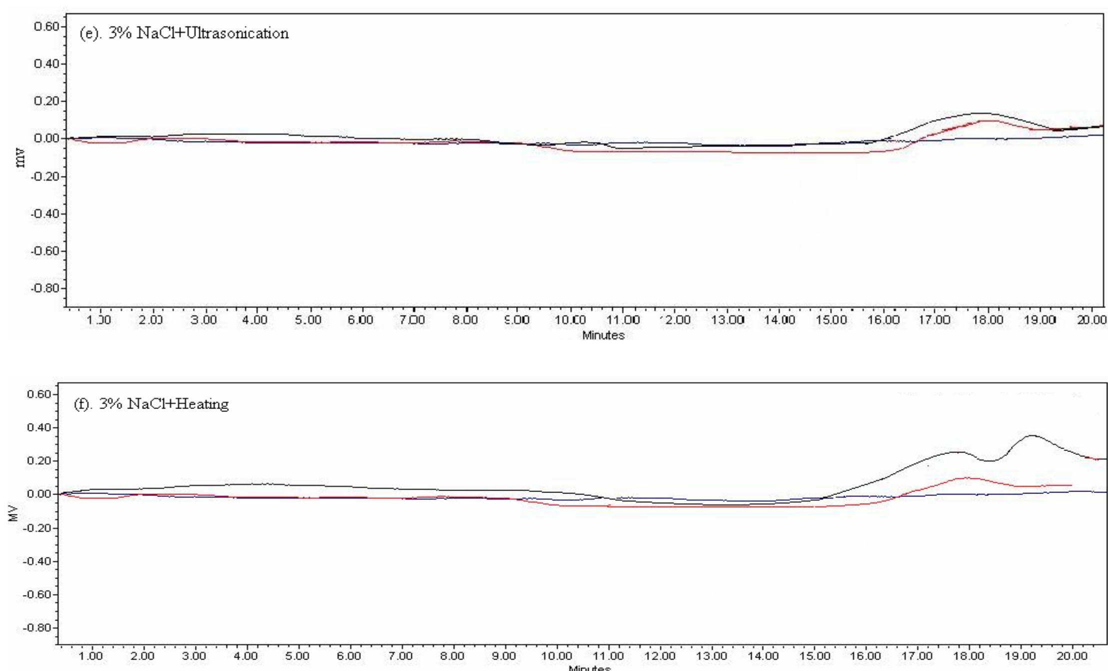


Figure 9 continued

Results for *Pseudomonas fluorescens* Biovar II showed that previous extractions with 3% NaCl had probably caused cell lysis, with double peaks in the size exclusion chromatogram. One is 18 KDa and the other is 167 KDa, indicating either cell membrane material or intracellular molecules probably leaked into solution at high ionic strength. Heating also caused double peaks in the SEC spectra. EPS extracted with 0.01 N NaOH showed a very broad peak, which indicated a possible cell lysis. All the other methods showed similar patterns in their chromatograms to that of the control group, with an overall average molecular weight of (25.5±3.6) KDa (Figure 10. (a)-(g)). The content of nucleic acid in EPS extracted by the seven methods was in the order: 3% NaCl>0.01NaOH>0.005N NaCl with heating> 0.005N NaCl with

ultrasonication>0.005N NaCl (control)>2% EDTA>0.01N HCl. When 3% NaCl, 0.01 N NaOH and 0.005 N NaCl with heating were used to extract EPS, the nucleic acid content was 10.3, 7.4 and 5.2 times higher than that of the control (0.005N NaCl), indicating that cell lysis was severe and a large amount of intracellular material leaked out. Ultrasonication would not significantly increase the polysaccharide yield. Both 2% EDTA and 0.01 N HCl caused less cell lysis (0.11 and 0.34 mg/L, respectively, vs.1.2 mg/L). However, the extracellular polysaccharides yield was also very low (0.23 and 0.67 mg/L, respectively, vs. 1.59 mg/L). Therefore, 0.005 N NaCl would be the best out of the seven groups.

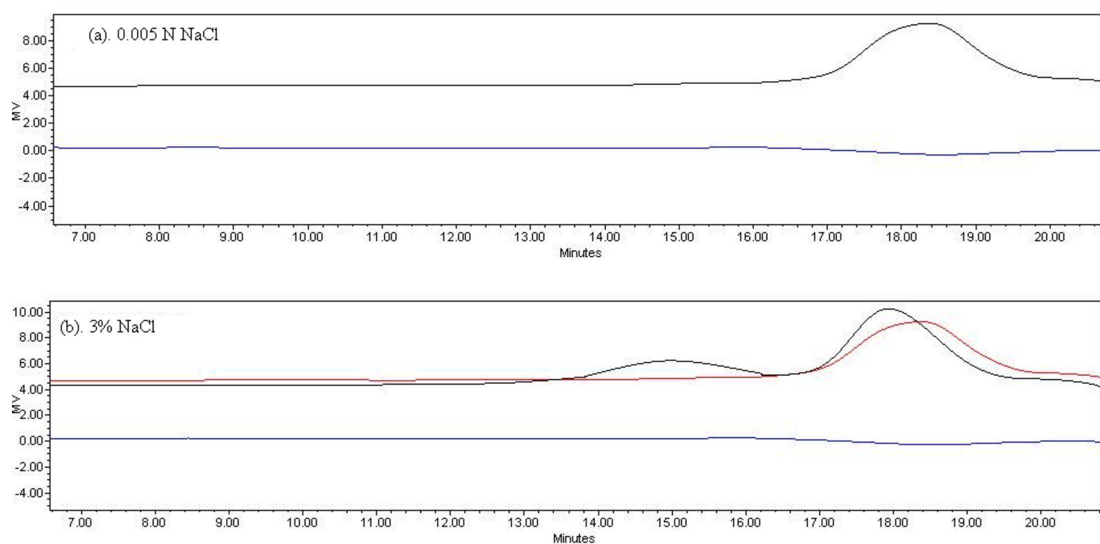


Figure 10 (a)-(g) Size exclusion chromatograms of capsular EPS of *Pseudomonas fluorescens* Biovar II extracted by different methods. (blue line represents “blank”; red line represents the control group, i.e., 0.005 N NaCl; black line represents the current method)

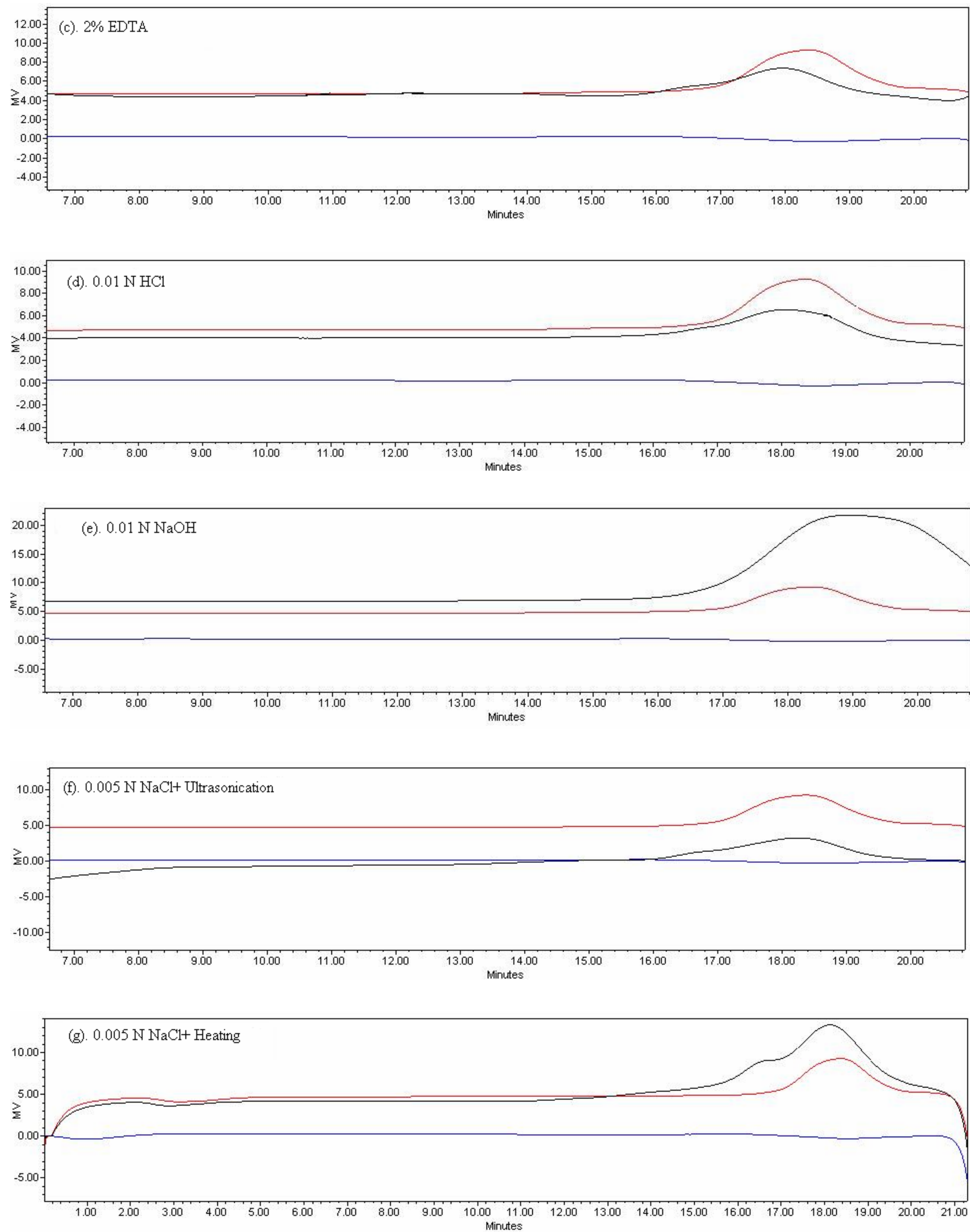


Figure 10 continued

In subsequent experiments, both concentrations of the optimal extractants and extraction time were tested, for both bacteria species, respectively. It was found out that the higher the HCl concentration, the higher the yield of extracellular polysaccharides from *Sagittula stellata*. However, the nucleic content also increased by 1.6 and 2.6 times when extracted with 1 and 2 N HCl, compared to that of the control concentration (0.5 N HCl) (Figure 11). Extraction time seemed to have little influence on the contents of polysaccharides, protein and nucleic acid in the EPS of *Sagittula stellata* (Figure 12). For *Pseudomonas fluorescens* Biovar II, when NaCl concentration increased from 0.005 N (control) to 0.05 N, there was slight increase in contents of polysaccharides, protein and nucleic acid in capsular EPS (Figure 13). When the concentration of NaCl increased to 0.1 N NaCl, the nucleic acid content was 2.1 times higher than that of the control concentration. Therefore the lower concentration 0.05 N NaCl is regarded as a more safe concentration with no significant elevated nucleic acid content compared to that of the control group. Different from *Sagittula stellata*, polysaccharides, protein and nucleic acid contents were dependent on extraction time for *Pseudomonas fluorescens* Biovar II (Figure 14). From three hours to five hours, extraction time had only a slight influence on the polysaccharides content while after 5 hours, both protein and nucleic acid content increased significantly. Therefore, extracted with 0.05 N NaCl for 3-5 hours would be the optimal condition for extraction of capsular EPS of *Pseudomonas fluorescens* Biovar II, while for *Sagittula stellata*, extraction with 0.5 N HCl for three hours would still be the optimal condition, considering the best extracellular polysaccharides yield, the least cell lysis or distruption and the minimal modification of the extracellular

macromolecules.

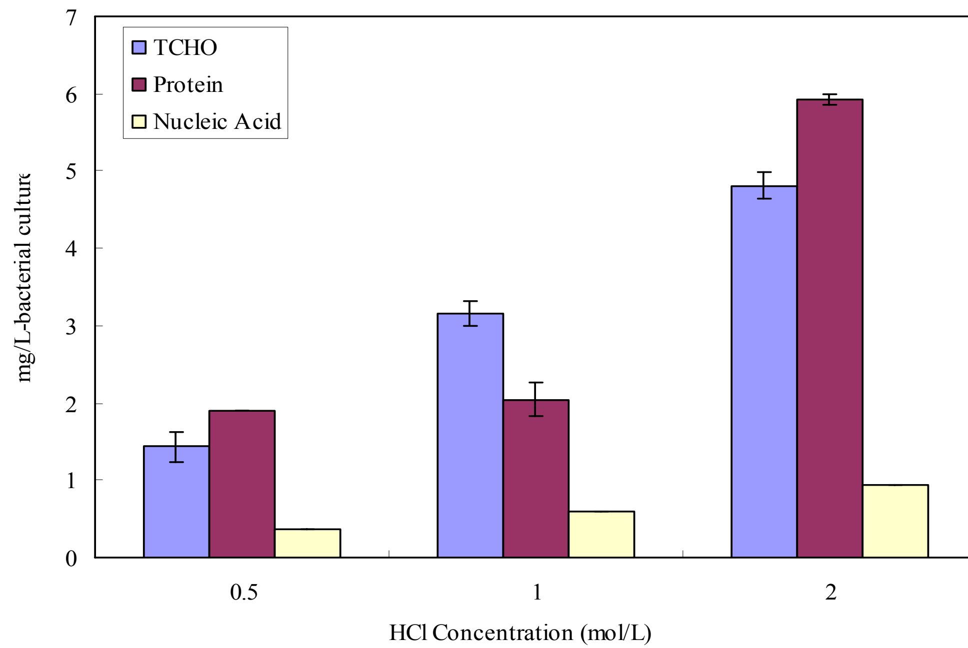


Figure 11 “Attached” EPS composition of *Sagittula stellata* extracted by HCl of different concentrations

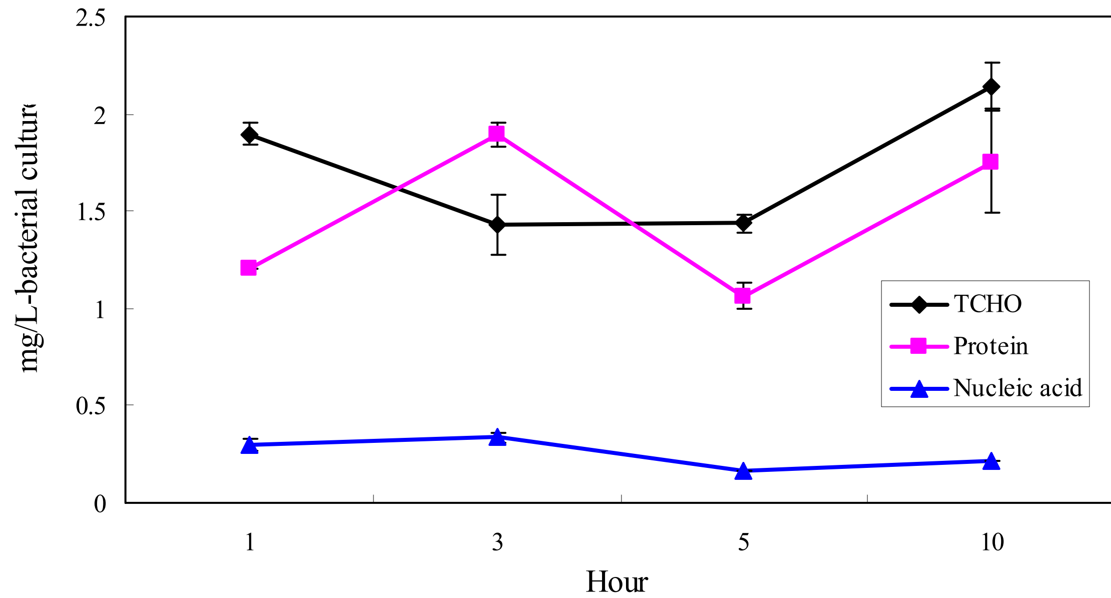


Figure 12 “Attached” EPS composition of *Sagittula stellata* extracted by 0.5 N HCl at different time

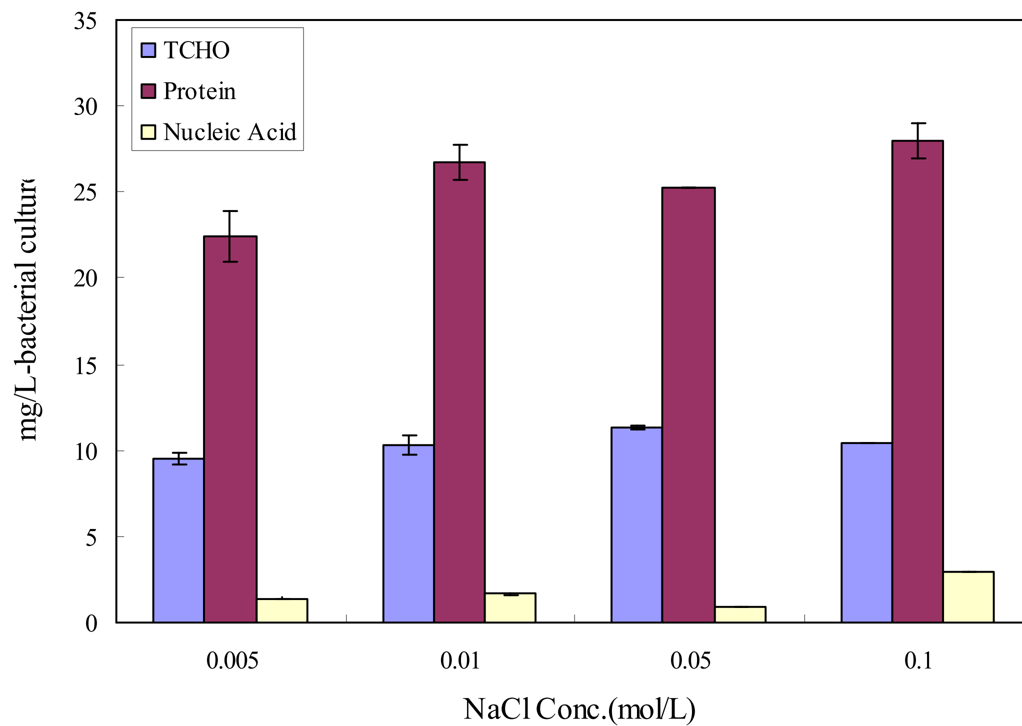


Figure 13 “Attached” EPS composition of *Pseudomonas fluorescens* Biovar II extracted by NaCl solution of different concentrations

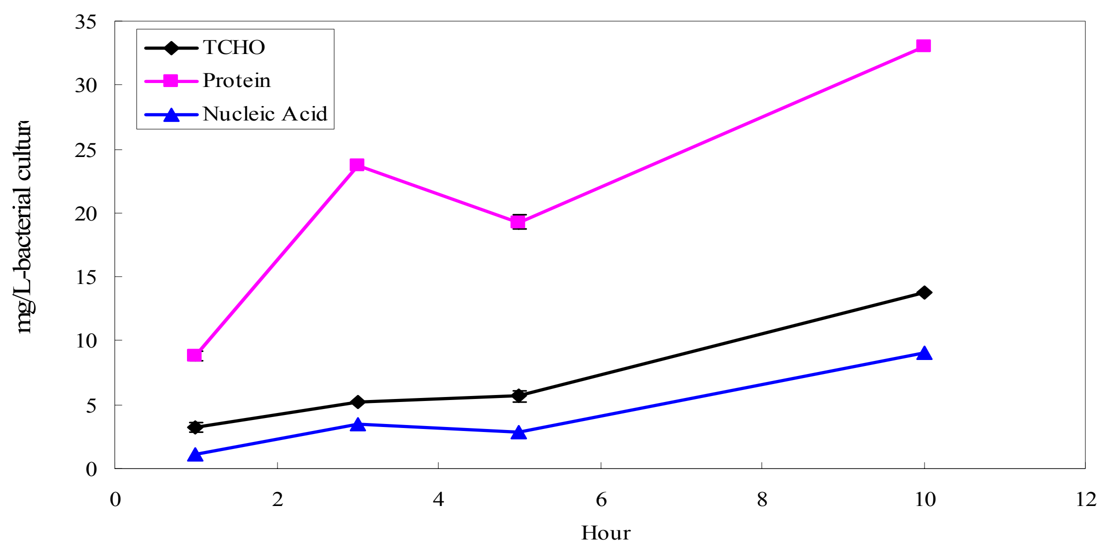


Figure 14 “Attached” EPS composition of *Pseudomonas fluorescens* Biovar II extracted by 0.05 N NaCl at different time

III.2.Purification of EPS

Filtered marine broth 2216 (<0.22μm) for *Sagittula stellata* in size exclusion chromatogram showed one peak at (5.28±0.70) KDa, while filtered soy broth for *Pseudomonas fluorescens* Biovar II showed two peaks: (7.21±0.16) KDa and (4.81±0.13) KDa (spectra not shown here for brevity). Therefore a 10 KDa regenerated cellulose dialfiltration membrane was chosen to effectively “wash out” all these broth materials. A size fractionation step, i.e., pre-filteration and diafilteration, collecting material from the bacterial culture, with size from 10 KDa to 0.22μm, was added before alcohol precipitation. This step also concentrated the culture suspension from a volume of 1-2 L to around 100-150 ml. There was, however, some brown precipitate formed after diafiltration. The solution was filtered through 0.22 μm polycarbonate membrane again,

and the protein and total carbohydrate content in the precipitate and the filtrate was measured respectively (Table 3). The aggregate was mostly protein with trace amounts of carbohydrates. Adding either HCl or $NaHCO_3$ dropwise could make this brown aggregate re-dissolved. Thus a filtration after dialfiltration is needed to remove this high-protein-low-carbohydrate aggregate.

Table 3
Carbohydrate and protein content retained on the 0.22 um polycarbonate filter (%, of total amount) after dialfiltration and concentration

	carbohydrates (%, of total carbohydrates in EPS)		Protein (%, of total protein in EPS)	
	Retentate	Filtrate	Retentate	Filtrate
Sagittula stellata	98-98.3	1.7-2.0	45.31-48.63	51.37-54.69
Pseudomonas fluorescens Biovar II	92.73	7.27	22.79	77.21

Retentate carbohydrates%= carbohydrates in retentate/ Total carbohydrates in both retentate and filtrate*100%; Retentate protein%=protein in retentate/Total protein in both retentate and filtrate*100%; Filtrate carbohydrates%= carbohydrates in filtrate/Total carbohydrates in both retentate and filtrate*100%; Filtrate protein%=protein in filtrate/Total protein in both retentate and filtrate*100%

Ethanol was demonstrated to preferentially precipitate polysaccharides while isopropanol preferentially precipitate protein (Figures 15 and 16). The higher the percentage of ethanol volume to total volume (ethanol+EPS solution), the higher yield of both carbohydrates and protein were obtained, though the carbohydrates-C to protein-C ratio decrease a little bit (Figure 17).

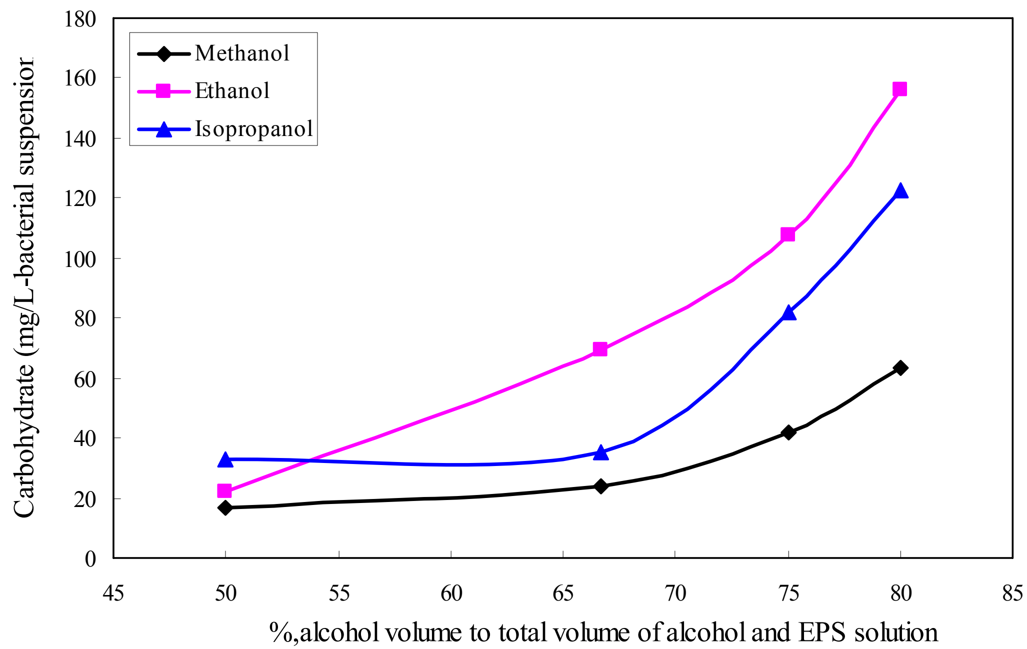


Figure 15 Total carbohydrates (mg/L-bacterial suspension) extracted by different alcohol from EPS of *Pseudomonas fluorescens* Biovar II

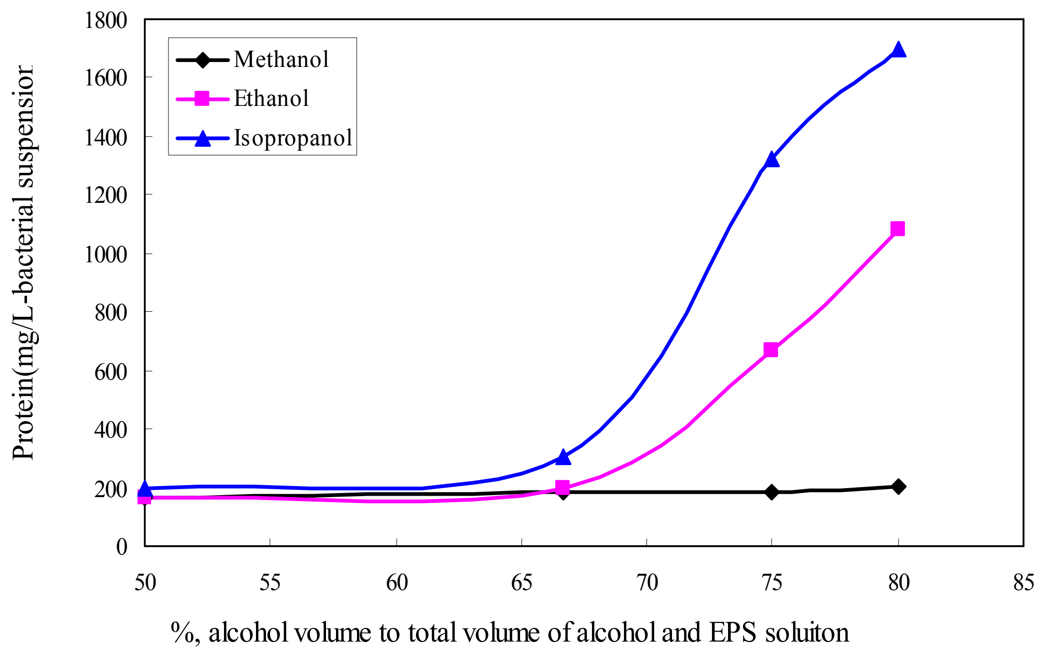


Figure 16 Total protein (mg/L-bacterial suspension) extracted by different alcohol from EPS of *Pseudomonas fluorescens* Biovar II

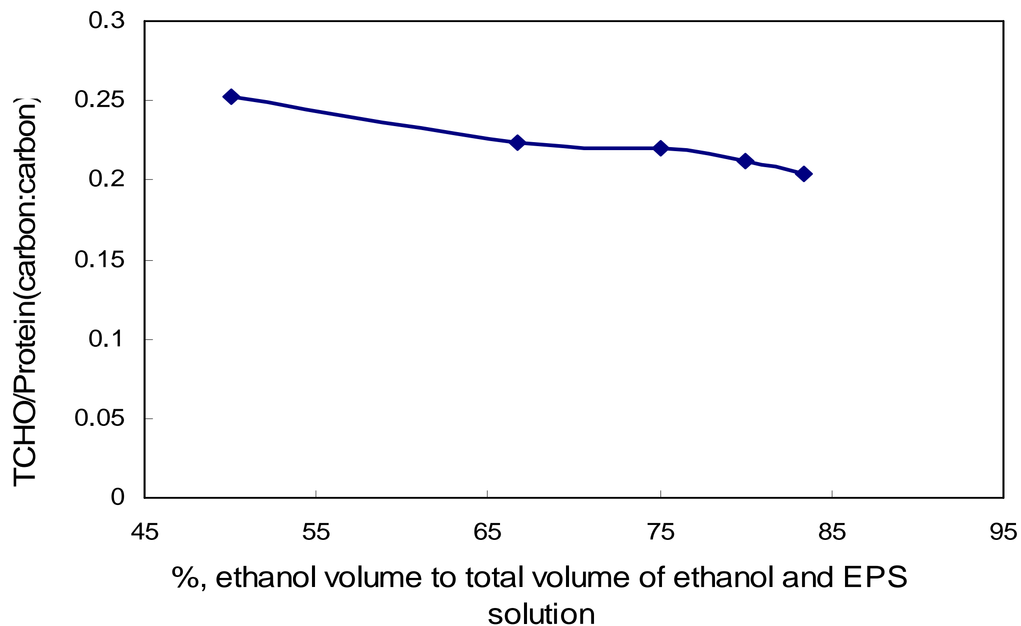


Figure 17 Carbohydrate-C to protein-C ratios as a function of the percentage of ethanol volume to total volume

Figures 18 and 19 show the changes of contents of total carbohydrates, proteins and uronic acids, normalized to the percentage of EPS dry weight, with the progression of alcohol and TCA precipitation, for *Sagittula stellata* and *Pseudomonas fluorescens* Biovar II, respectively. Final ethanol concentration was 80%, while final TCA concentration was ~6%. For *Sagittula stellata*, carbohydrates increased a little bit after the first time ethanol precipitation, i.e., by ~4%, compared to a sample without any ethanol precipitation, and then remained almost constant within the three times of ethanol precipitation and finally increased by ~14% with TCA precipitation. Uronic acid content had the same trend as total carbohydrates, though the magnitude was different (less than 1%). Protein became more enriched with the progression of ethanol

precipitation but decreased dramatically by 39% after TCA precipitation. For *Pseudomonas fluorescens* Biovar II, total carbohydrates increased by 4% after the first time ethanol precipitation and remained almost constant for the latter twice ethanol precipitation and one time of TCA precipitation. Protein content decreased by ~13% after twice ethanol precipitation, and by another ~5% after the TCA precipitation. Actually, TCA precipitation was conducted twice for both species but for the second time, there was no visible precipitate formed after centrifugation or filtration through a 0.22μm polycarbonate membrane.

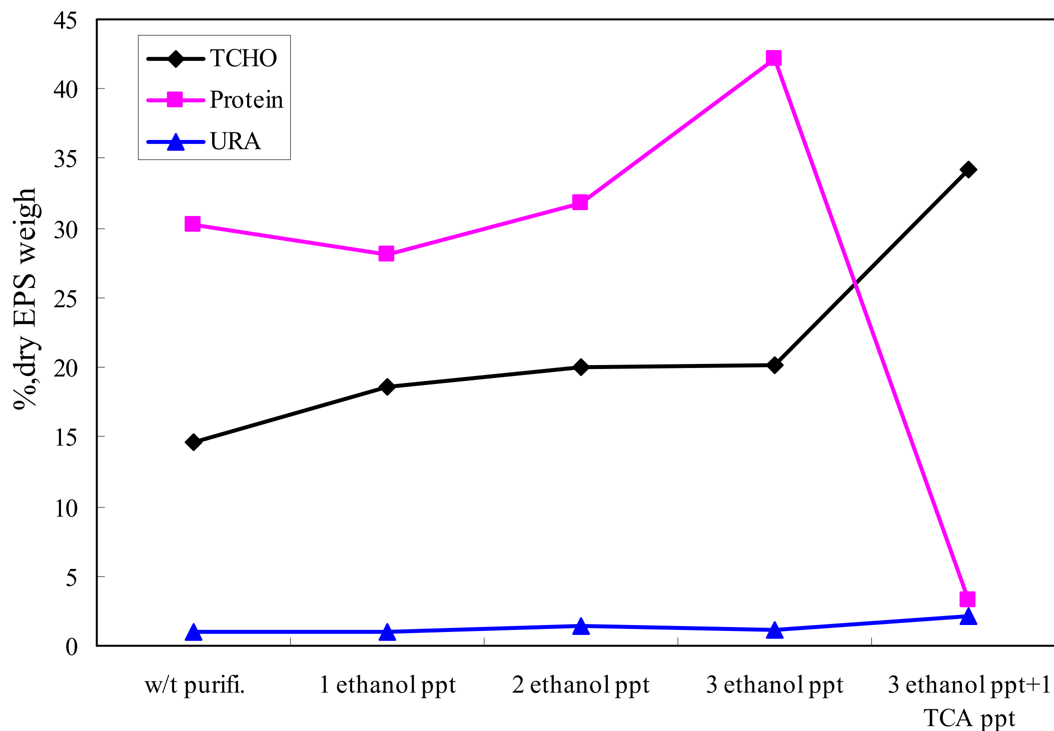


Figure 18 TCHO (total carbohydrates), Protein and URA (uronic acids) changes with purification of *Sagittula stellata* EPS

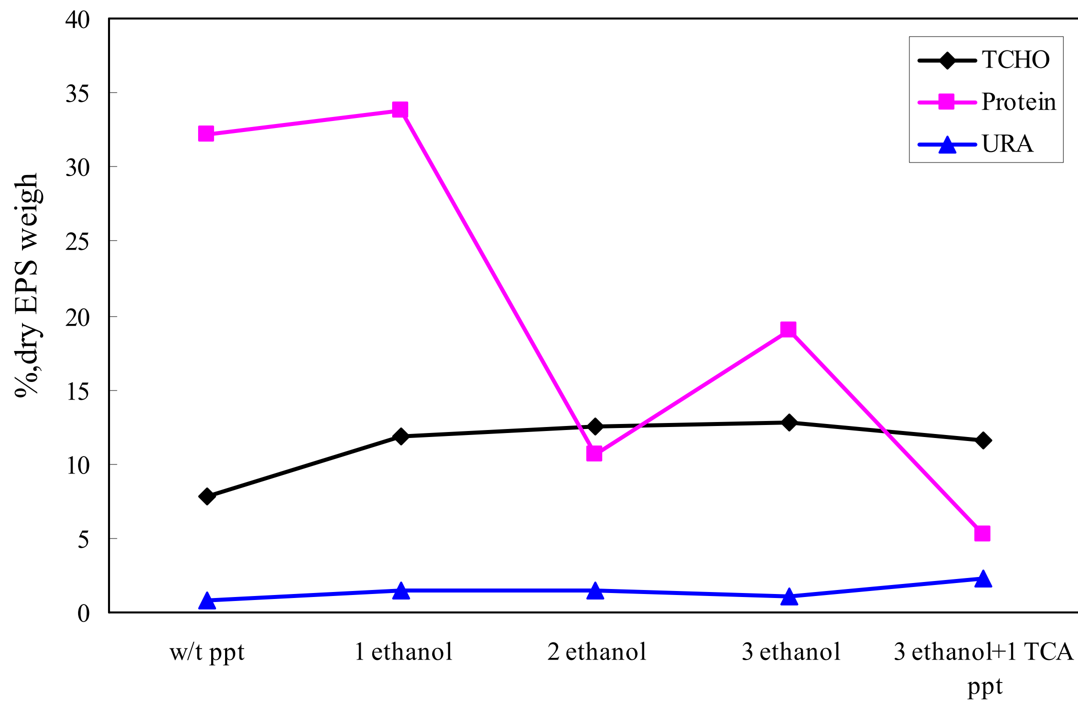


Figure 19 TCHO (total carbohydrates), Protein and URA (uronic acids) changes with purification of *Pseudomonas fluorescens* Biovar II EPS

Therefore a finalized optimal procedure for extraction and purification of both bacterial EPS was shown in Figure 20. For *Sagittula stellata* EPS, once ethanol precipitation and once TCA precipitation would achieve a high yield of polysaccharides while removing a significant fraction of protein. For *Pseudomonas fluorescens* Biovar II EPS, twice ethanol precipitation and once TCA precipitation would be required to achieve the purification purpose.

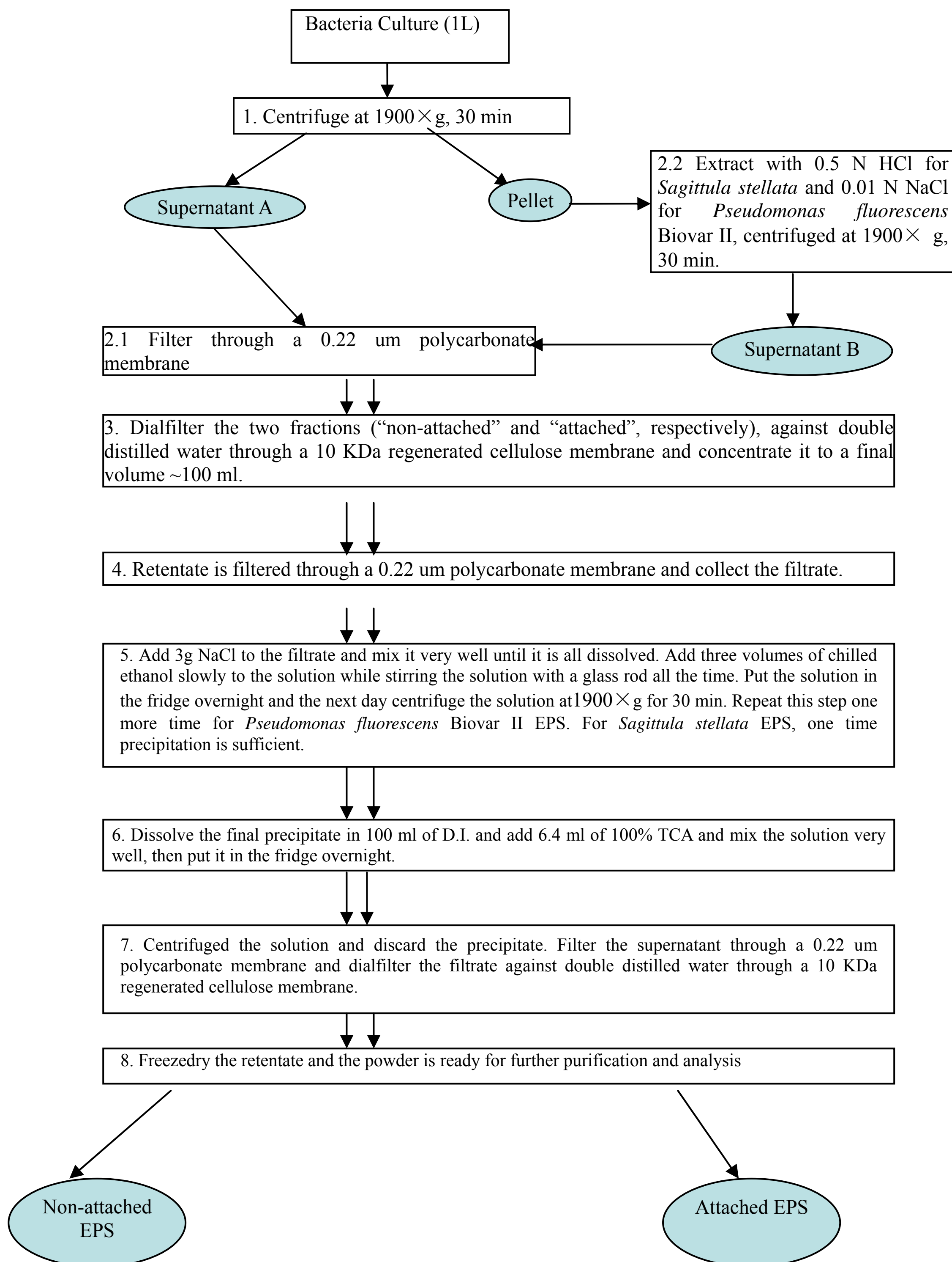


Figure 20 A sketch for initial extraction and purification of bacterial EPS

III.3.Simultaneous Determination of Neutral Sugar and Uronic Acids by GC-EI-MS

III.3.1.Hydrolysis Conditions

As HCl and TFA are both volatile and can be easily evaporated, they are often used to hydrolyze polysaccharides before the derivatization step in the GC-MS analysis (Jones and Albersheim, 1972, Walters and Hedges, 1988, Myklestad et al., 1997, Aluwihare et al., 1999, 2002, Witter and Lurther III, 2002). However, hydrolysis efficiencies of different exopolymers could vary a lot due to their different composition and linkage. There are no universal ideal hydrolysis conditions for all polysaccharides, with respect to (1) acid type and concentration; (2) temperature; (3) time. Jones and Albersheim (1972) recommended 0.2 N TFA for hydrolysis of cell wall polysaccharides; Walters and Hedges (1988) recommended an acid strength of 0.5 N TFA and a temperature of 135 ^{o}C for the best balance between hydrolysis time and high, stable uronic acid and aldose recoveries from sediment samples; Myklestad et al. (1997) and Witter (2002) both recommended a final concentration of 0.09 N HCl for hydrolysis of polysaccharides in seawater. Aluwihare et al. (1999, 2002) used an acid strength of 2 N TFA for hydrolysis of HMW DOM. Thus, HCl and TFA of different concentrations, which are usually used in the literature, were tried out on both bacterial EPS. A tentative temperature of 150 oC and hydrolysis time of 1 hour for these two bacterial EPS was chosen also based on the previous libterature (Hung et al., 2005; Alvarado Quiroz et al., 2006). Basically, EPS were dissolved in D.I. water and the above acids were added to make different final concentrations (HCl: 0.05, 0.1, 0.5, 1N; TFA: 0.3, 0.5, 1, 2 N) and sealed in ampoules and heated in an oven at 150 ^{o}C for 1 hour. After hydrolysis, the pH

of the solution was adjusted to neutral by dropwise addition of NaOH solution. All the volumes had already been corrected when comparing different treatments. Total carbohydrates were measured using the TPTZ method. The hydrolysis efficiency was expressed as percentage of total carbohydrates as glucose equilvalents in dry EPS weight. For *Sagittulla stellata* EPS, 0.05 N HCl was chosen as it gives the best efficiency, while for *Pseudomonas fluorescens* Biovar II EPS, either 0.3 N TFA or 0.5 N HCl was chosen (Figure 21).

For both EPS, the optimal hydrolysis temperature was 120 ^{o}C, when using the optimal acid and concentration (0.05 N HCl for EPS of *Sagittula stellata* and 0.5 N HCl for EPS of *Pseudomonas fluorescens* Biovar II, respectively) (Figure 22). For *Pseudomonas fluorescens* Biovar II EPS, the best hydrolysis time was three hours while for *Sagittula stellata* EPS, hydrolysis efficiency seemed to increase "endlessly" with time, when the optimal acid and temperature were applied. Hydrolysis efficiency over 10 hours wasn't tested since hydrolysis time seemed to have less effect on the efficiency than temperature (Figure 23). After 3 hours, the efficiency increased much more slowly. Thus for effective and efficient hydrolysis of both kinds of EPS, 0.05 N HCl at 120 ^{o}C for 3-5 hours was chosen for *Sagittula stellata* EPS and 0.5 N HCl at 120 ^{o}C for 3 hours was chosen for *Pseudomonas fluorescens* Biovar II EPS.

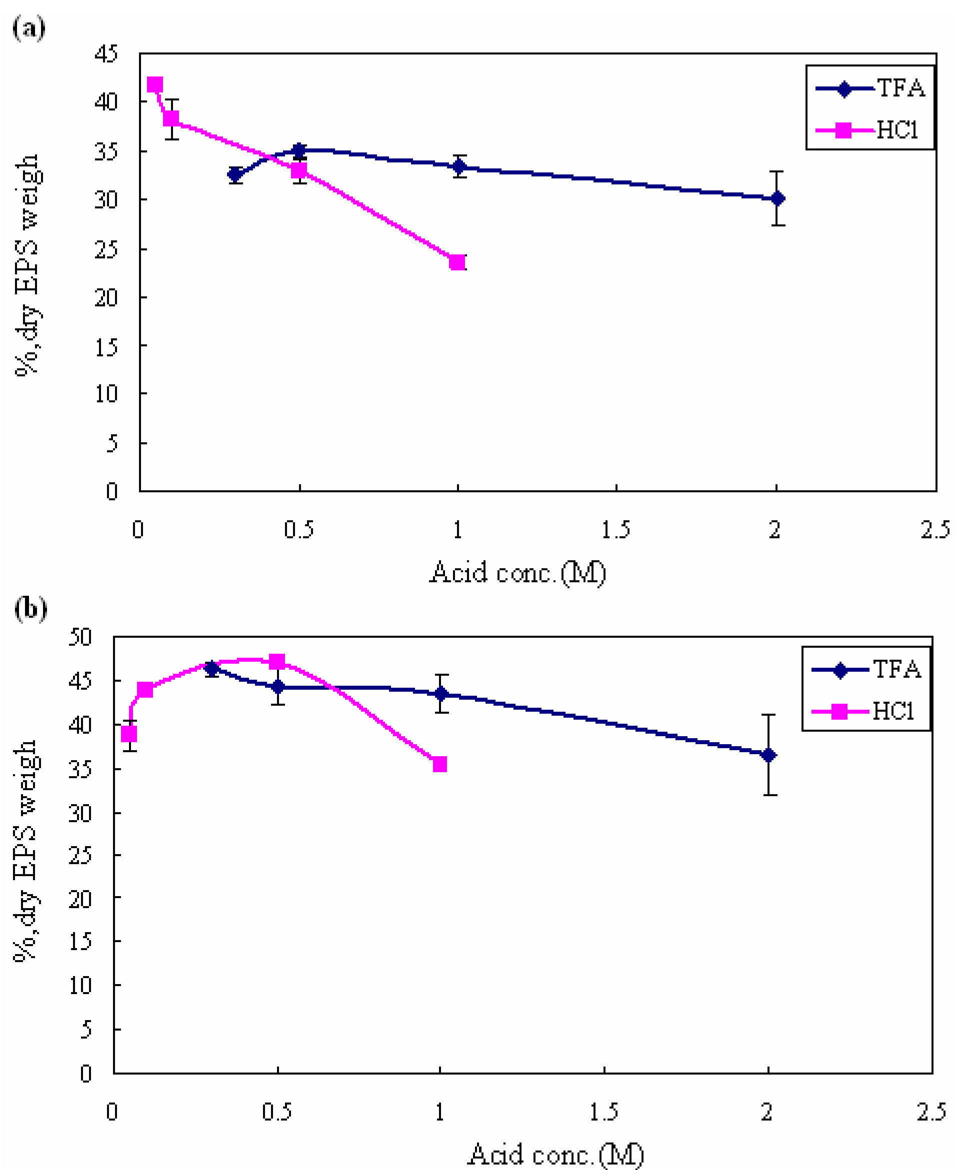


Figure 21 Effects of acid types and concentrations on the hydrolysis efficiency of the two bacterial "non-attached" EPS. a: *Sagittula stellata*; b: *Pseudomonas fluorescens* Biovar II

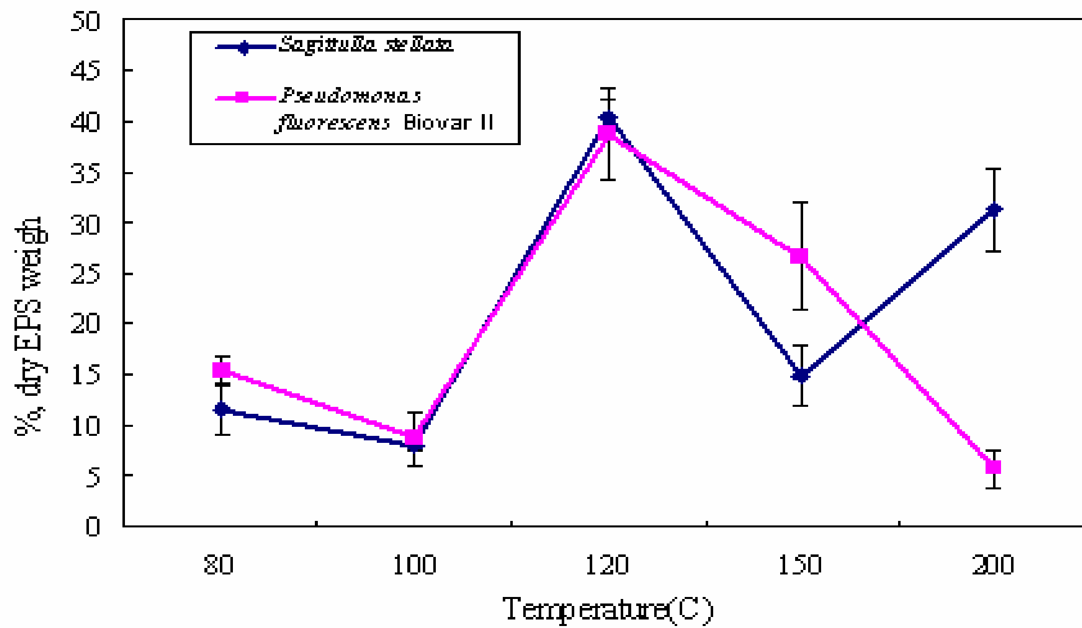


Figure 22 Temperature effects on hydrolysis efficiency of the two bacterial EPS

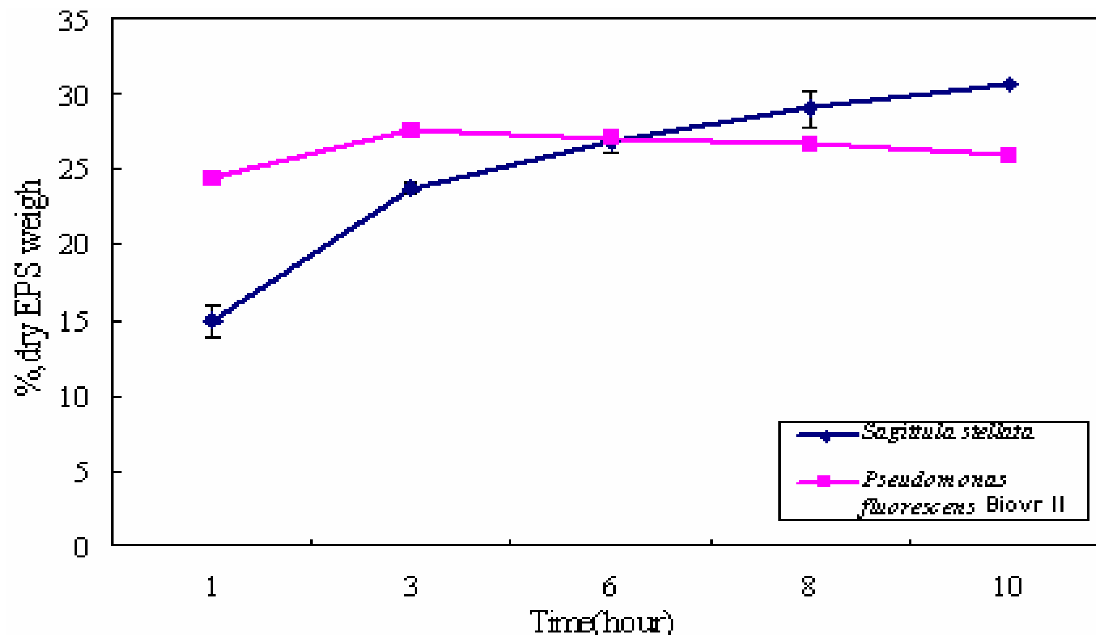


Figure 23 Time effects on hydrolysis efficiency of the two bacterial EPS

III.3.2.Simultaneous Derivatization of Neutral Sugars and Uronic Acids

Results from gas chromatographic analysis of monosaccharide standards, including seven neutral sugars, three uronic acids and two internal standards, i.e. allose and ribonolactone, which were used as the internal standard for neutral sugars and uronic acids respectively, are shown in Figure 24. Derivatives of all monosaccharides were well separated by the chromatography program within 45 minutes described before. The retention time and relative response factors are shown in Table 4. Relative response factor was calculated as follows,

$$RRF = \frac{C_i \times A_{\text{int}}}{C_{\text{int}} \times A_i}$$

where RRF represents the relative response factor of individual monosaccharide; C_i, C_{int} represent the concentrations (μmol/L) of individual monosaccharide and internal standard (allose for neutral sugars and ribonolactone for uronic acids), respectively; A_i and A_{int} represent the integrated peak area of individual monosaccharide and internal standard, respectively.

Good relative response factor linearity (r^2 values ≥ 0.99) and reproducibility (% mean deviation ≤ 8%) were obtained for an injection volume of 1μl and concentrations of standards between 10 μM to 200μM. Sample blanks (Table 5), i.e., double distilled water, which went through the whole procedure, including acid hydrolysis and derivatization, were injected in the same volume as the sample. Practical detection limit for each monosaccharide under the normal integration settings was calculated as the sum of average blank and three-fold mean deviation of blanks, and it ranged from 0.59 to

4.14 ng for different monosaccharides.

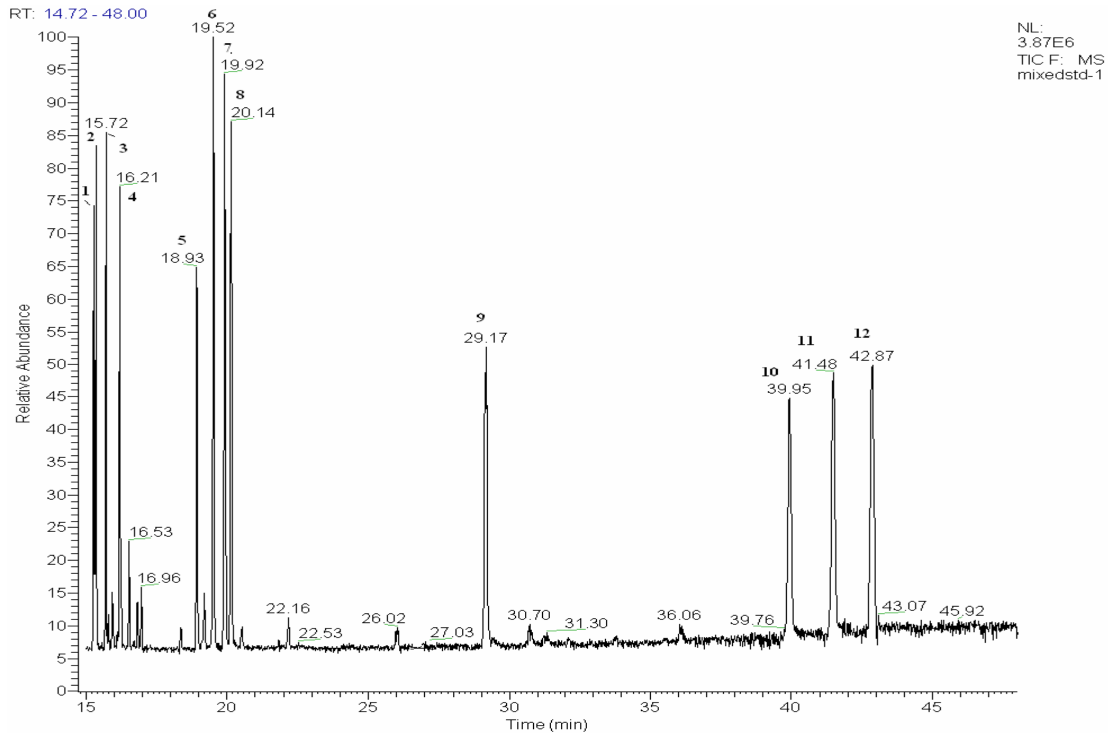


Figure 24 Gas chromatographic traces of peracetate derivatives of seven neutral sugar standards, three uronic acid and two internal standards. Peak identification numbers (in terms of original compounds) are as follows: 1. Rhamnose (Rha); 2. Fucose (Fuc); 3. Arabinose (Ara); 4. Xylose (Xyl); 5. Allose (All); 6. Mannose (Man); 7. Galactose (Gal); 8. Glucose (Glu); 9. Ribonolactone (RibAc); 10. Mannuronic acid (ManAc); 11. Glucuronic acid (GluAc); 12. Galacturonic acid (GalAc). The abbreviations are the same in other figures below

Table 4
Retention time and relative response factors of different monosaccharides

	Retention Time (min)	Relative Response Factor	%mean deviation of RRF
Neutral sugar	Allose: 18.93±0.007	Internal standard: Allose	
Rhamnose	15.26±0.02	0.88±0.07	7.6
Fucose	15.35±0.02	1.26±0.08	6.4
Arabinose	15.72±0.02	0.83±0.04	4.3
Xylose	16.21±0.02	0.76±0.04	5.2
Manose	19.52±0.03	1.42±0.07	4.6
Glucose	19.92±0.03	1.38±0.05	3.5
Galactose	20.14±0.02	1.31±0.03	2.6
Uronic Acid	Ribonolactone:29.17±0.03	Internal standard: Ribonolactone	
Mannuronic Acid	39.95±0.04	1.15±0.03	2.8
Glucuronic Acid	41.48±0.05	1.31±0.02	1.5
Galacturonic Acid	42.87±0.04	1.24±0.04	3.6

Table 5
Blanks for individual monosaccharides and practical detection limit of GC-EI-MS

	Rha	Fuc	Ara	Xyl	Man	Gal	Glu	ManAc	GluAc	GalAc
Average blank (ng)	1.74	1.68	0.79	2.13	0.44	0.42	0.31	0.49	0.81	0.41
mean deviation (ng)	0.58	0.56	0.04	0.67	0.05	0.07	0.13	0.06	0.14	0.19
detection limit (ng)	3.48	3.37	0.91	4.14	0.59	0.61	0.69	0.68	1.22	0.99

III.4.EPS Production as a Function of Growth Status

III.4.1.*Sagittula stellata*

For *Sagittula stellata*, the lag phase could last as long as 11 hours under the inoculation and growth conditions described before. The exponential phase was around 34 hours and the stationary phase was 43 hours (Figure 25). For “non-attached” EPS, protein increased almost linearly with time at the exponential phase and during the first 15 hours after the species entered the stationary phase. After that, protein content kept almost constant. Total carbohydrates almost increased all the way even at the death phase. Nucleic acid detected in “non-attached” EPS could be regarded as an indicator of cell lysis during growth and it had a similar trend with protein content. For “attached” EPS, total carbohydrates kept increasing slowly while protein content dropped abruptly in the middle of the stationary phase. Nucleic acid could be regarded as an indicator of cell lysis during extraction process. As it is seen from the graph, little lysis occurred during the extraction operation. “Non-attached EPS” were more enriched in polysaccharides, with carbohydrates-C to protein-C ratio ranging from 0.84-1.93, while “attached” EPS contained more protein with this ratio from 0.13-1.49. It’s interesting to note that if not considering the bacterial death phase, the carbohydrate-C to protein-C ratio remained relatively constant in “non-attached” EPS, with an average value of 1.04 and a coefficient of variations as low as 13%, while in “attached” EPS, this ratio increased relatively slowly from 0.16 to 0.30 during the lag and log phases and then increased dramatically to 1.21 at the stationary phase (Figure 26). Little uronic acid could be significantly detected in either “non-attached” EPS or “attached” EPS, by

spectrophotometric methods. But it could also be due to the high detection limit of the method itself (Hung et al., 2001).

Statistical analysis revealed that the contents of “non-attached” EPS varieties, i.e., polysaccharides and proteins, were significantly and positively correlated with bacterial growth, i.e., OD_{600}, (p <<0.05); while in “attached” EPS, they were weakly positively correlated with bacterial growth with both p values close to the critical level, i.e., 0.05. “Non-attached” polysaccharide content was significantly positively correlated with that in “attached” polysaccharide, so did the protein contents in both EPS (both $p<0.05$).

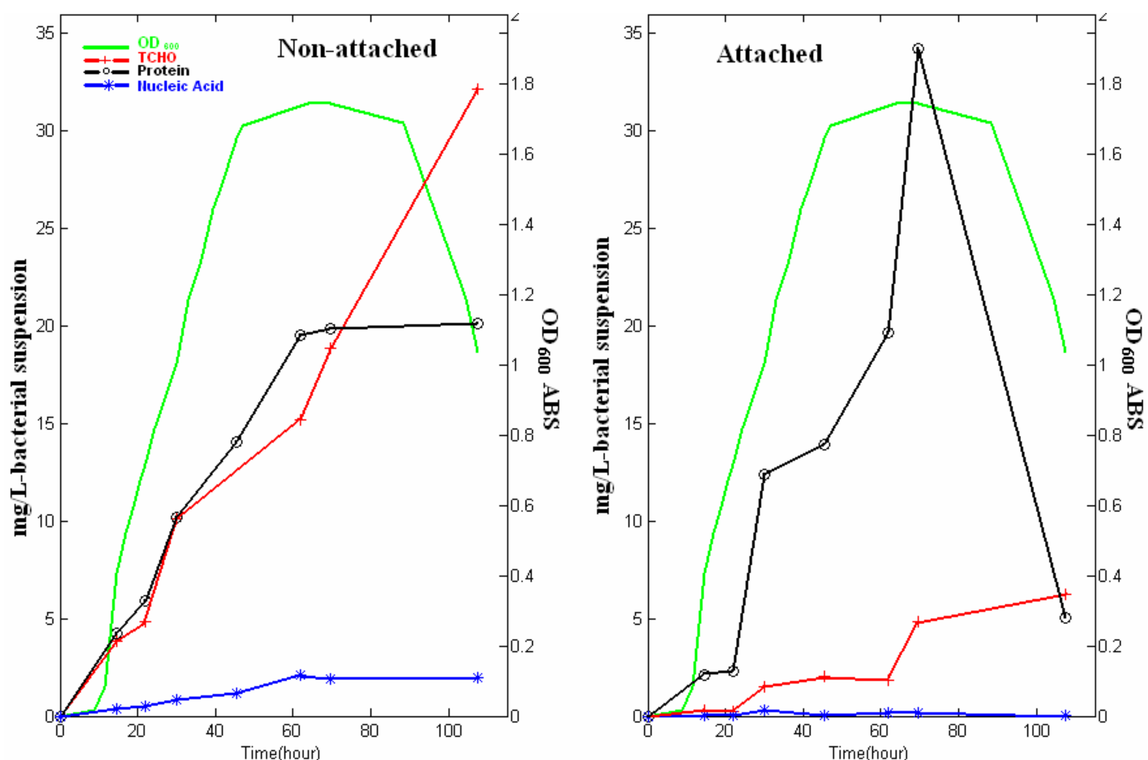


Figure 25 *Sagittula stellata* growth curve and change of EPS varieties (left: “non-attached” EPS; right: “attached” EPS)

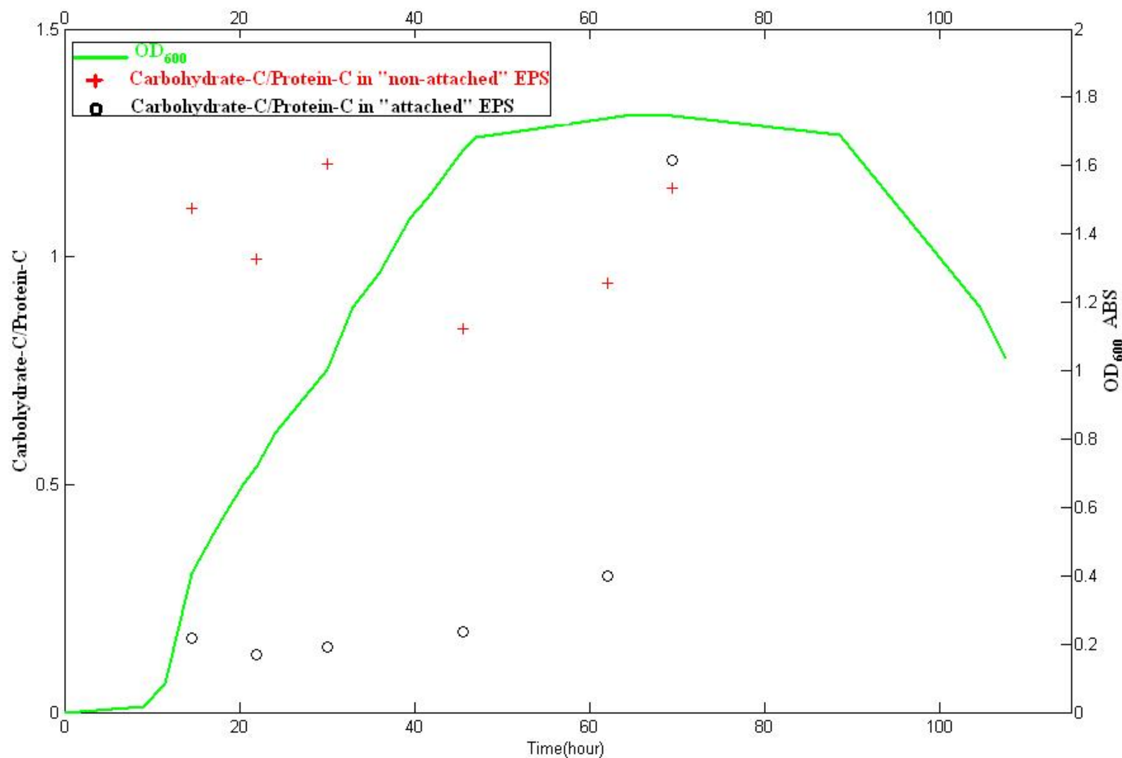


Figure 26 Carbohydrate-C to protein-C ratios during *Sagittula stellata*'s growth life

"Non-attached" EPS were produced at more or less similar rates during the bacterial growth phase, which was estimated as the mass of EPS or polysaccharides or proteins per OD_{600} (initial blank subtracted) (Figure 27), assuming that one unit of OD_{600} corresponds to a certain amount of dry cell mass (Vaningelgem, et al., 2004; Torino et al., 2005). Nevertheless, the maximal specific productivity, which was actually only slightly higher than others, occurred at the stationary phase. Although there were variations of specific productivity for "attached" EPS, the general trend was increasing, especially when the bacteria entered the stationary phase.

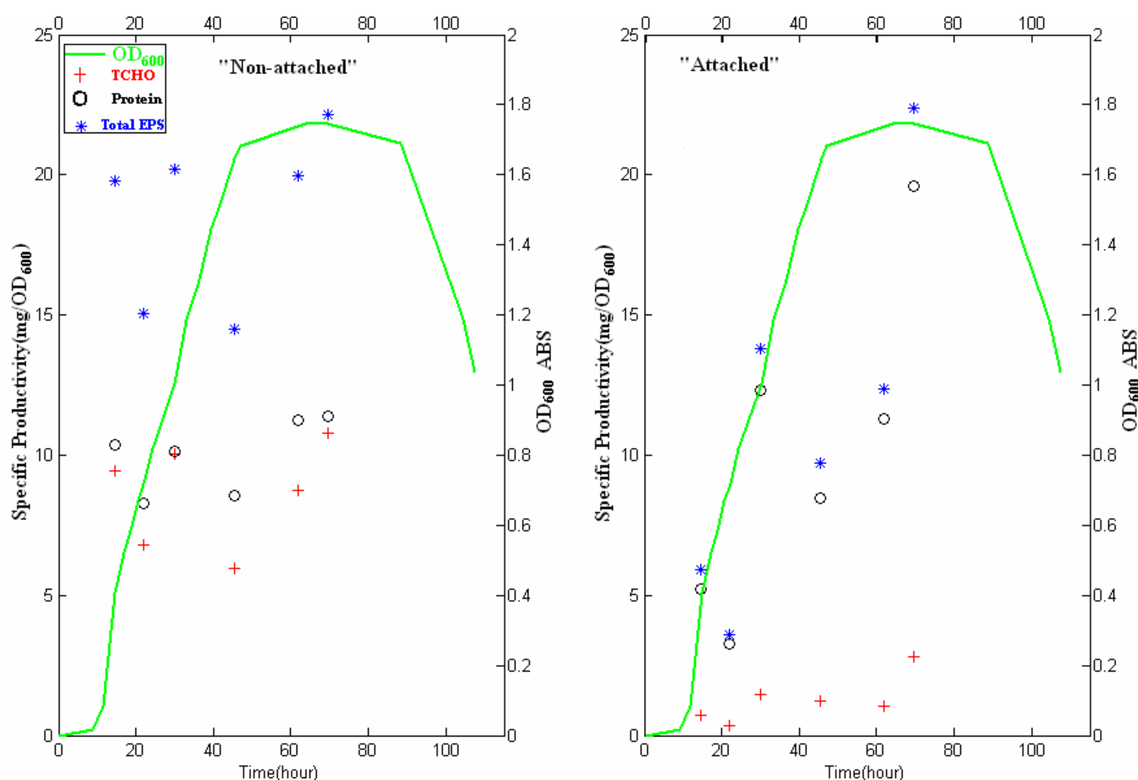


Figure 27 Specific productivity of total EPS (sum of polysaccharides and proteins), polysaccharides and proteins in "non-attached" (left) and "attached" EPS (right) of *Sagittula stellata*

Figure 28 showed the monosaccharide composition of both "non-attached" (up) and "attached" (down) exopolysaccharides. In the case of "non-attached" EPS, galactose and mannose were the two predominant carbohydrates, with minor quantities of other sugars. Mannose remained as the biggest fraction until bacterial growth entered the stationary phase. Then galactose increased more rapidly than mannose and became the main fraction. For "attached" EPS, galactose, glucose and mannose were the three main carbohydrates. Higher concentrations of glucose and mannose were detected than that of galactose, when culture was in the exponential phase. After it entered the stationary

phase, galactose increased more rapidly than the other two and became the dominant monosaccharide. Both graphs indicate that various polysaccharide strains were present in both the EPS mixtures and their proportions had changed during the bacterial life period. Interestingly, glucose, which was found in small amount in “non-attached” EPS, was abundant in “attached” EPS and even in higher concentration than both mannose and galactose before culture entered its growth stationary phase. When comparing the two major monosaccharides (galactose and mannose, respectively), statistical analysis showed that each of them significantly correlated in “non-attached” EPS, with its corresponding sugar in “attached” EPS ($p<0.05$). This is consistent with the conclusion that total polysaccharide contents in both types of EPS were significantly correlated. Both EPS were depleted of uronic acids, with only trace amount of glucuronic acid being detected, indicating that EPS excreted by *Sagittula stellata* were mainly neutral polysaccharides.

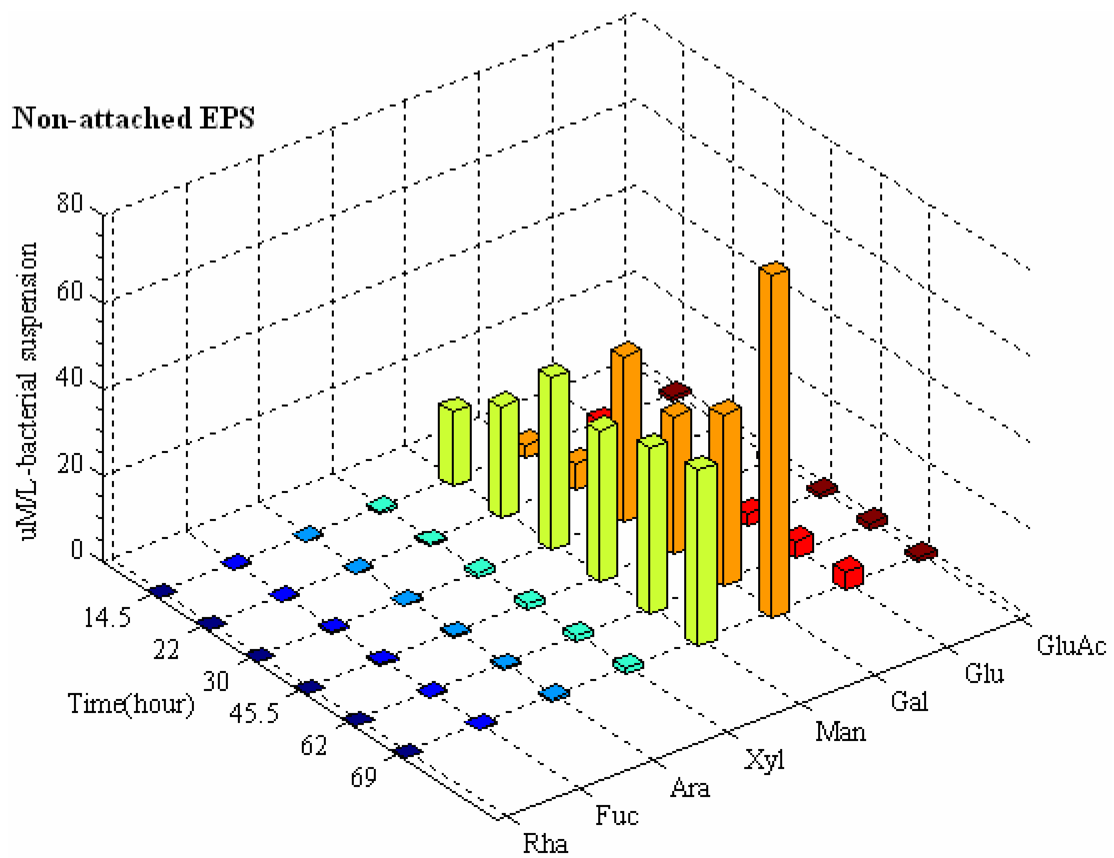


Figure 28 Individual monosaccharides in EPS of *Sagittula stellata* at different growth status (up: “non-attached” EPS; down: “attached” EPS)

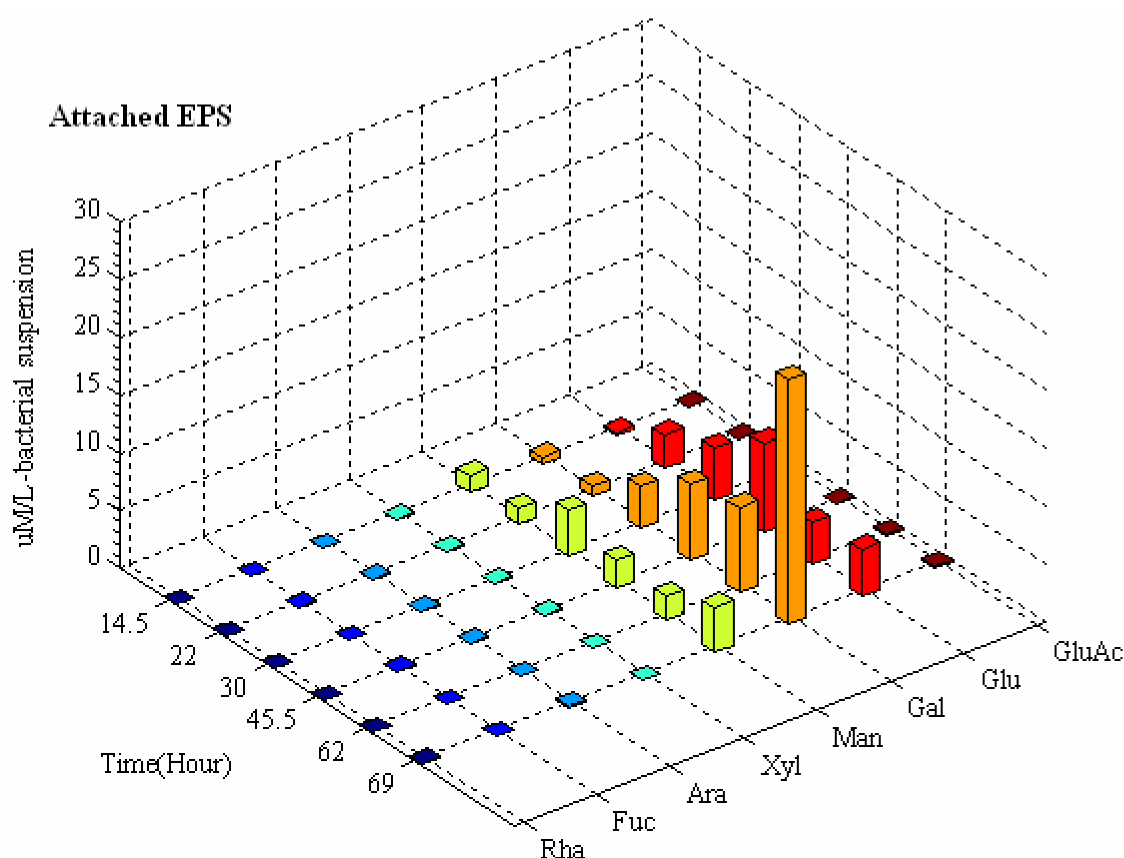


Figure 28 continued

Results from HPLC-SEC (Figure 29c) showed that molecular weight distribution of both “non-attached” EPS and “attached” EPS in different growth phases didn’t change too much all through the bacterial life period. Only the chromatograms of “non-attached” and “attached” EPS harvested at the end of stationary phase were shown for brevity.There was only one peak, with molecular weight as (24.17±1.96) KDa for “attached” EPS (Figure 29a). Two peaks were found for “non-attached” EPS, with molecular weight as (64.49±0.78) KDa and (24.74±2.37) KDa (Figure 29b).

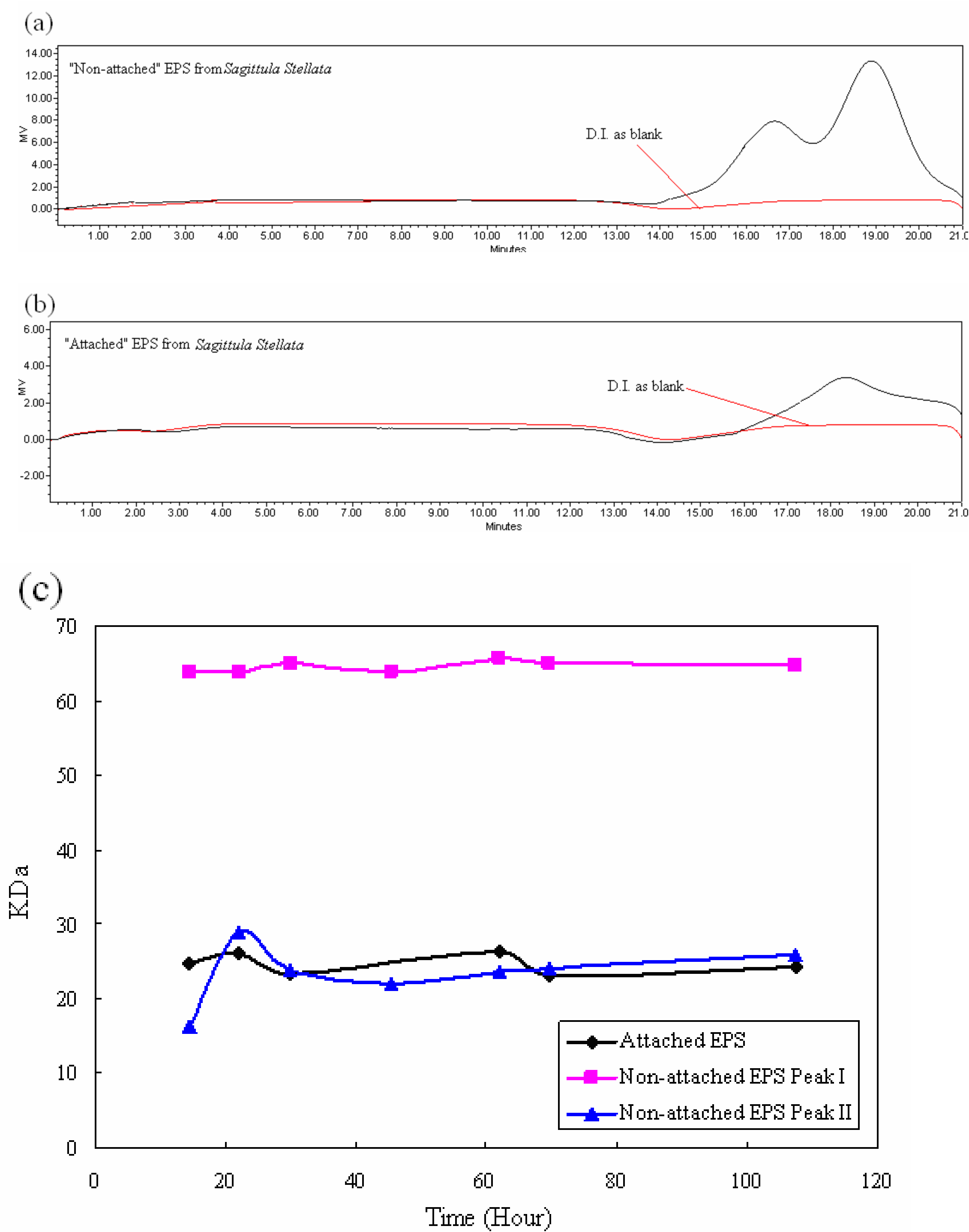


Figure 29 Molecular weight distribution of *Sagittula stellata* EPS. (a) and (b) "non-attached" and "attached" EPS harvested at the end of stationary phase, respectively. (c) Molecular weight distribution of both "non-attached" and "attached" EPS throughout the life of *Sagittula stellata*

III.4.2.*Pseudomonas fluorescens* Biovar II

The lag phase for *Pseudomonas fluorescens* Biovar II lasted around nine hours, and the exponential phase was around 15 hours, under the incubation conditions described before. Different from *Sagittula stellata*, it showed a little incline at its stationary phase and it lasted as long as around 62 hours before the growth declined. For “non-attached” EPS, both protein and total carbohydrates increased rapidly during the exponential growth phase, then more modestly after the culture reached the stationary phase (Figure 30). At the middle of the stationary phase, total carbohydrates declined abruptly, while the protein content increased steadily. Uronic acid could be detected in “non-attached” EPS, when the colorimetric method was used. It increased three-fold during the culture’s exponential growth phase and stayed constant during the first half of the stationary growth phase of the culture, after which it slightly decreased. Nucleic acid, as an indicator of natural cell lysis, increased by four- to five-fold during the culture’s exponential growth phase and then stayed constant during the whole stationary phase. In the case of “attached” EPS, total carbohydrates reached the “stationary phase” earlier than the culture’s stationary growth phase. After that, it continued to increase, though more modestly, and started to decrease abruptly just when the culture entered the stationary growth phase. Protein content showed a distinct lag phase and started to grow rapidly until the culture reached the stationary phase. No significant “stationary phase” was observed for protein, since it decreased sharply at the late exponential growth phase. Uronic acid, though in lower concentration compared with that in “non-attached” EPS, still could be detected. Nucleic acid content, as an indicator of operational cell lysis,

remained as low as 0.14-1.21 mg per liter of bacterial suspension.

Statistical analysis revealed that the “non-attached” EPS varieties, i.e, carbohydrates, proteins, uronic acids and nucleic acids, were significantly positively correctly with bacterial growth ($p<<0.05$). However, this wasn’t observed for “attached” EPS. Moreover, no significant relationships for the individual variety, i.e., carbohydrates, proteins and uronic acids, between “non-attached” EPS and “attached” EPS were observed either.

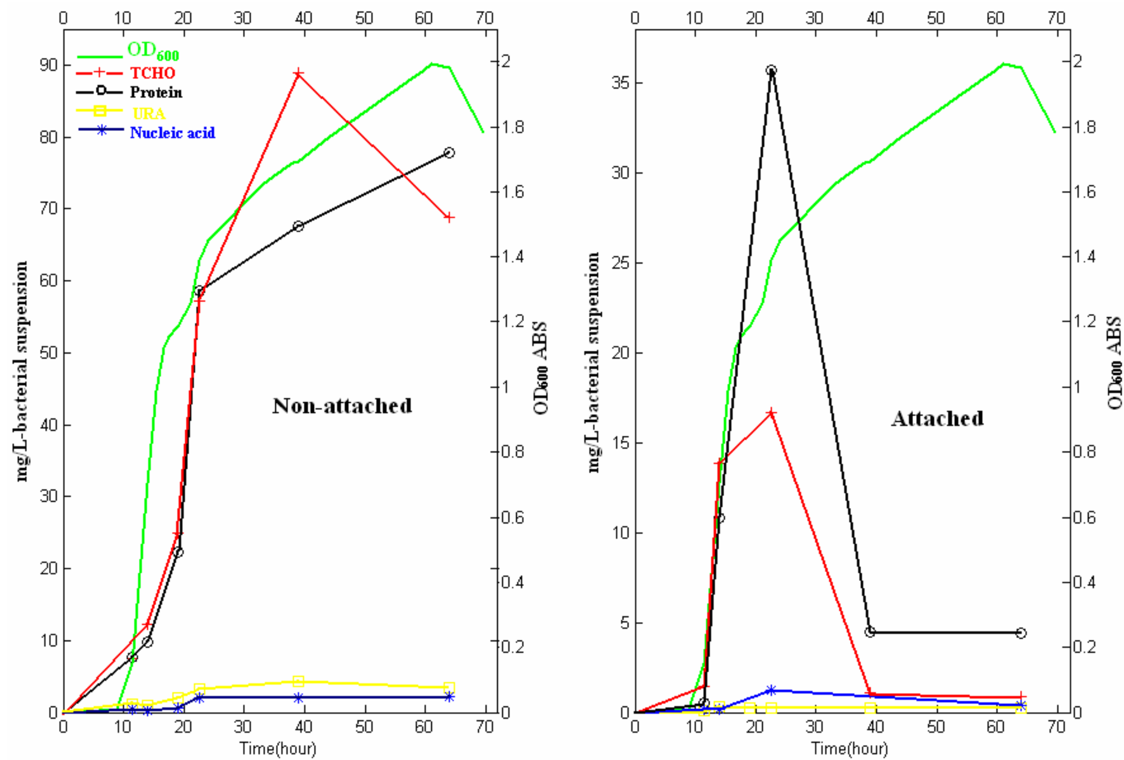


Figure 30 *Pseudomonas fluorescens* Biovar II growth curve and EPS composition change (left: “non-attached” EPS; right: “attached” EPS.)

The carbohydrate-C to protein-C ratio kept relatively constant in “non-attached” EPS, with an average value of 1.32 and the coefficient of variations as 16%, which is similar to “non-attached” EPS in *Sagittula stellata*. In sharp contrast to the “attached” EPS of *Sagittula stellata*, this ratio of “attached” EPS in *Pseudomonas fluorescens* Biovar II decreased dramatically during the bacterial exponentail phase, from 3.07 to 0.57, and then decreased very slightly during the stationary phase (Figure 31).

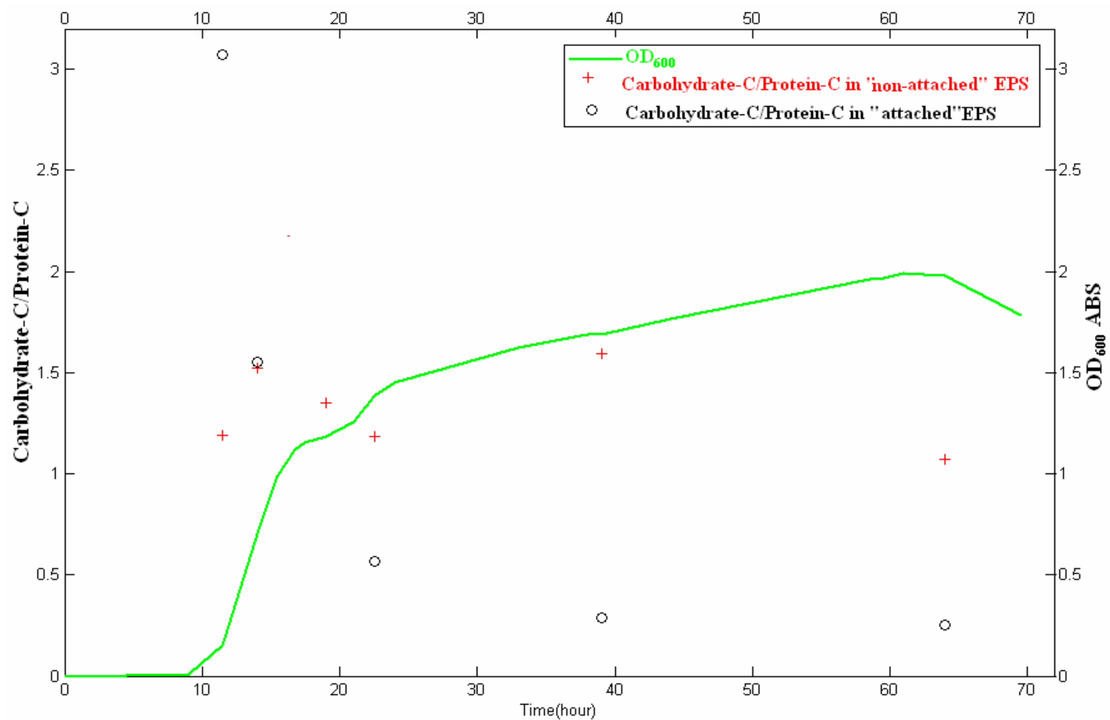


Figure 31 Carbohydrate-C to protein-C ratios during *Pseudomonas fluorescens* Biovar II’s growth life

Specific productivity for “non-attached’ EPS increased dramatically during the

exponential phase and reached the maximum in the middle of the stationary phase, after which it decreased a little (Figure 32). The maximal specific productivity for "non-attached" polysaccharide was in the middle of the stationary phase, while it was at the end of exponental phase (the transitional phase) for "non-attached" proteins. Specific productivity for "attached" EPS and their varities, i.e, carbohydrates and proteins, all have their maximal specifc productivity at the end of the exponential phase.

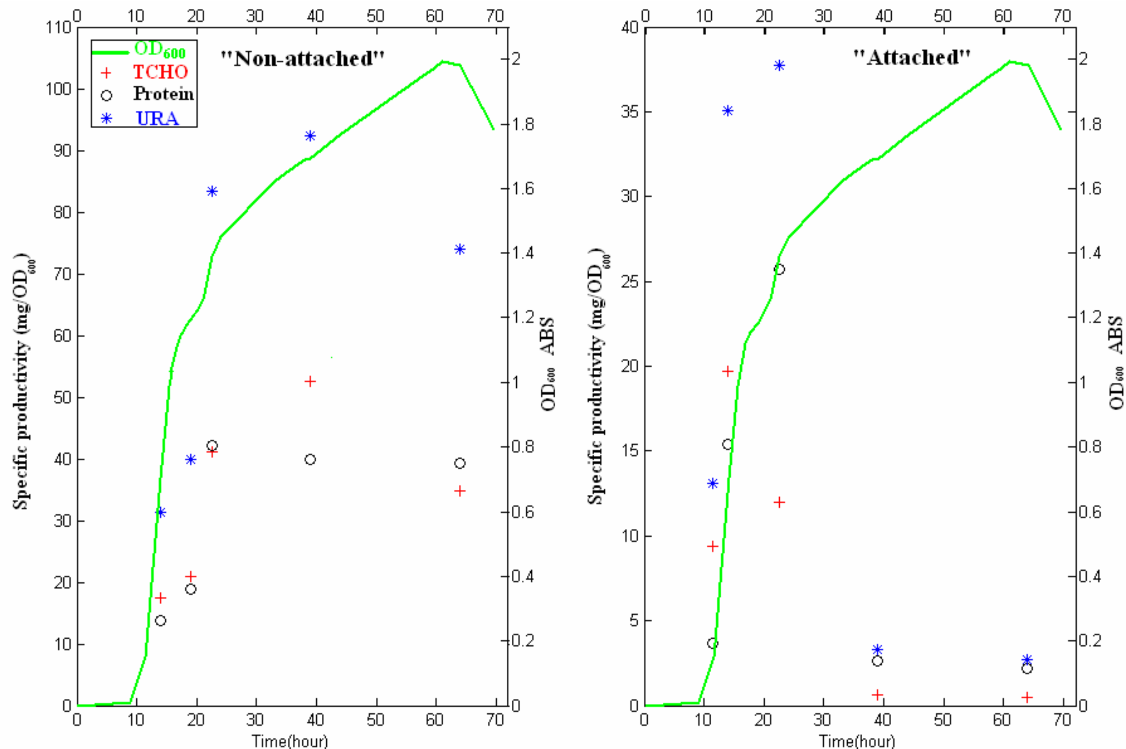


Figure 32 Specific productivity of total EPS (sum of polysaccharides and proteins), polysaccharides and proteins in "non-attached" (left) and "attached" EPS (right) of *Pseudomonas fluorescens* Biovar II

Due to time limitation, only the monosaccharide composition of EPS harvested during the late exponential phase was analyzed. For “non-attached” EPS, galactose, glucose, mannose and glucuronic acid were the main monosaccharides, accounting to 33.44%, 26.64%, 16.44% and 13.27% of total carbohydrates, respectively. “Attached” EPS was predominantly composed of galactose, with minor quantities of other sugars (Figure 33).

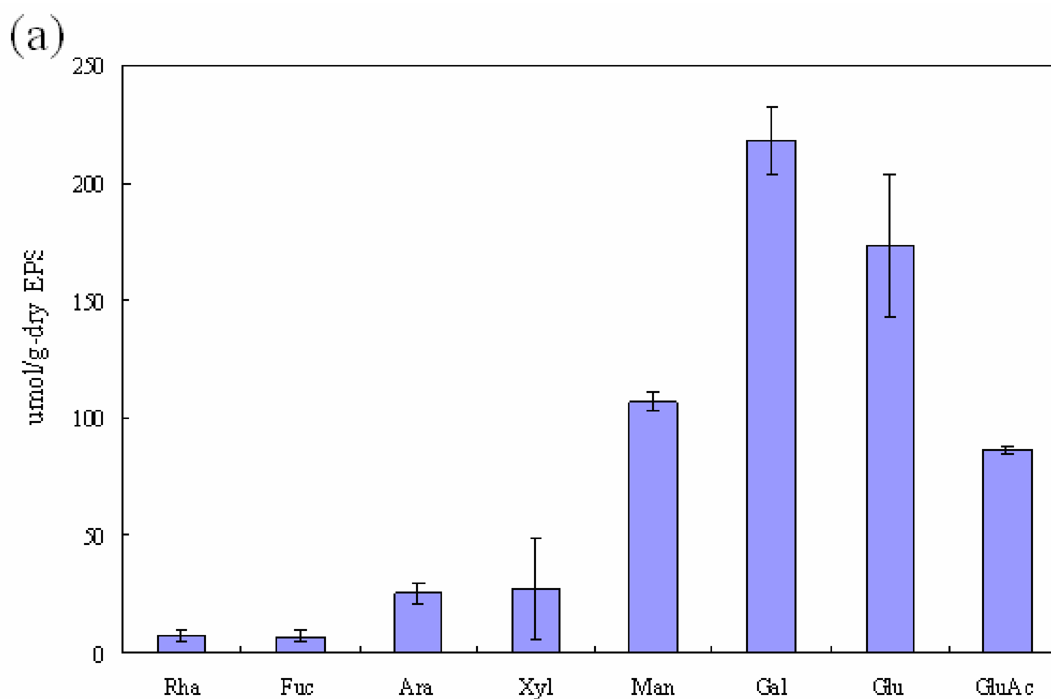


Figure 33 Individual monosaccharide composition of *Pseudomonas fluorescens* Biovar II EPS collected at the late exponential growth phase. (a), “non-attached” EPS; (b), “attached” EPS

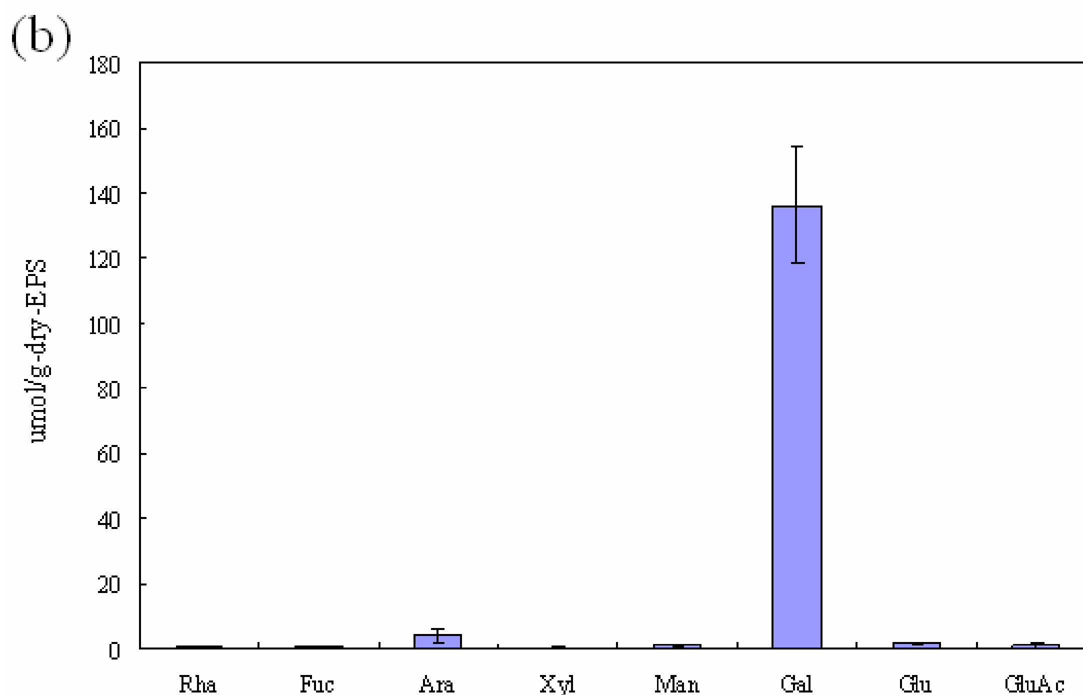


Figure 33 continued

III.5.Three Polysaccharides of "Non-attached" EPS of *Pseudomonas fluorescens* Biovar II Separated by Anion Exchange Chromatography

"Non-attached" EPS of *Pseudomonas fluorescens* Biovar II, collected from its late exponential growth phase, were purified according to the procedure listed in Figure 20. The final product yielded only one single peak in size exclusion chromatogram, indicating the EPS had a homogeneous size with molecular weight (20.3±0.4) KDa (Figure 34). However, in the following anion exchange chromatography, three significant peaks (F1, F2 and F3) were successively eluted in 60 minutes and separately collected (Figure 35). Protein was barely detected in the three fractions. F1 and F3 were

neutral polysaccharides, accounting for 41.9% and 45.2% of total polysaccharides (measured by the TPTZ method and normalized to glucose, the same below). F2 was mainly acidic polysaccharide and accounted for ~12.9% of total polysaccharides. No significant uronic acids were found in F1 and F3, when spectrophotometric methods were used. The uronic acid content was high, i.e., 63.9% in F2. Recovery of anion exchange chromatography was around 70%, which was calculated as percentage of sum of the polysaccharide content of the three fractions to total polysaccharides before injection.

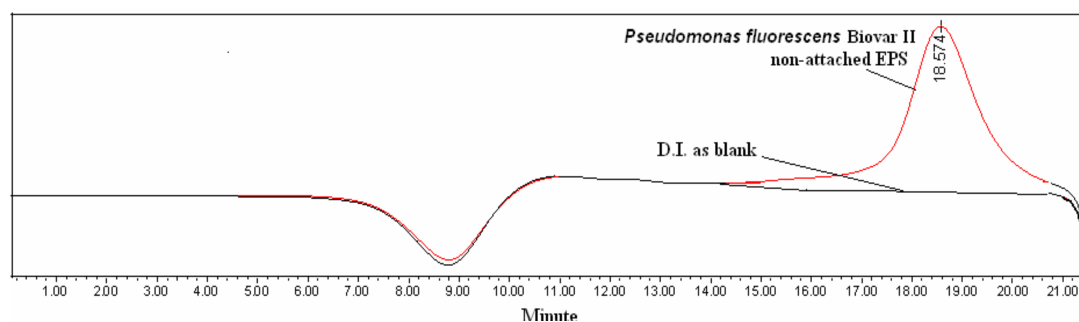


Figure 34 Size exclusion chromatogram of purified “non-attached” *Pseudomonas fluorescens* Biovar II EPS

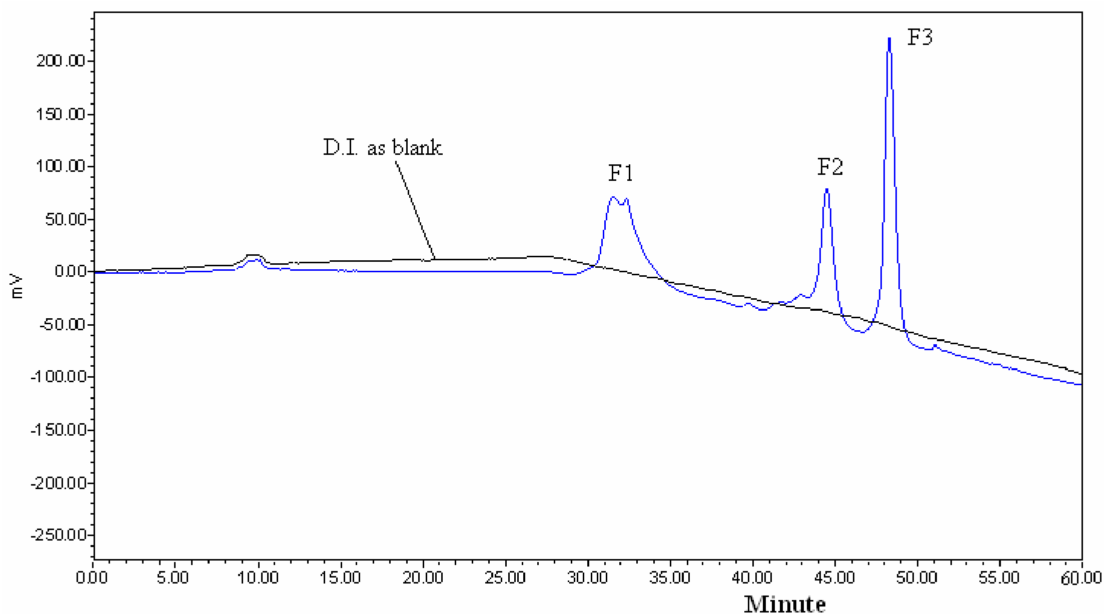


Figure 35 Anion exchange chromatogram of purified "non-attached" *Pseudomonas fluorescens* Biovar II EPS

.III.6.Comparison of IEF Results

While the manufacturer claimed that the pH regions within the gel are immobile and wouldn't migrate during the isoelectric focusing (IEF) step, this was not entirely true, as there was some deviation between measured pH and the pH specified by the manufacturer. Though pH gradient of the IEF gel had a shallower slope of 0.81, the relationship was still linear, especially for the low pH region (less than 7, Figure 36). Therefore, the measured pH scale was used to describe ^{234}Th(IV) or ^{240}Pu association with different organic phases separated by this technique.

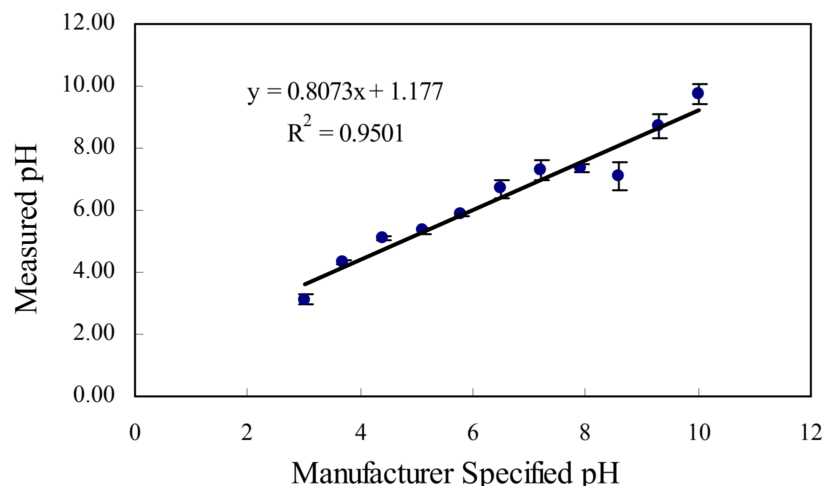


Figure 36 Measured pH values versus manufacturer specified pH values in isoelectric focusing gel after an isoelectric focusing run of RFETS Soil water extract (1 KDa-0.45 µm)

Figure 37 presents the result of an IEF run carried out with Th spiked RFETS Soil water extract (1KDa-0.45µm), which had a specific activity of ^{240}Pu as (297±21) dpm/g. The total activity of ^{240}Pu was calculated from the amount of RFETS soil water extract used in the experiment and its specific activity measured before. The total activity of Th was measured separately on the final retentate of ^{234}Th (IV) radiolabeled RFETS soil water extract using liquid scintillation counting (Section II 6 and II 8). It was found that ^{234}Th (IV) followed the ^{240}Pu activity fairly well, with a significant peak at the low pH end of the gel (0-1 cm), indicating a low isoelectric point of the carrier molecules. Recoveries of both isotopes (^{234}Th (IV) and ^{240}Pu (IV)), calculated as sum of activity within all gel fractions and electrodes to the total activity used in IEF running, were 83(±4) % and 66(±5) %, respectively.

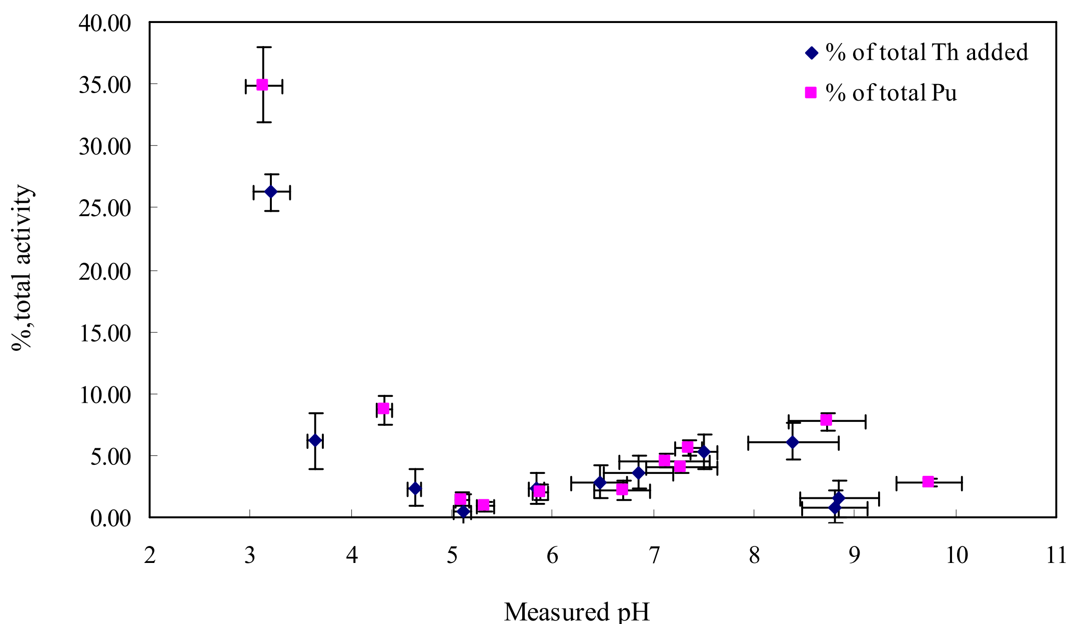


Figure 37 Isoelectric focusing of ^{234}Th (IV) labeled, Pu-enriched RFETS soil water extract

The IEF spectra of Th and Pu spiked *Sagittula stelleta* EPS (Figure 38) exhibited 26% and 16% of total added Th and Pu activity at the low pH end of IEF gel, respectively. IEF spectra of EPS produced by by *Pseudomonas fluorescens* Biovar II exhibited 30% and 21% of total added Th and Pu activity at the same acidic region (Figure 39). Again, Th and Pu had very similar distribution pattern along the pH gradient. Recoveries of Th and Pu for *Sagittula stelleta* EPS were 86(±5) % and 64(±6) %, and for *Pseudomonas fluorescens* Biovar II EPS were 80(±5) % and 60(±8) %, calculated as sum of activity within all gel fractions and both electrodes to total spiked activity. Total added activities of both isotopes were measured separately with the final retentate of radiolabelled bacterial EPS (Section II 6 and II 8).

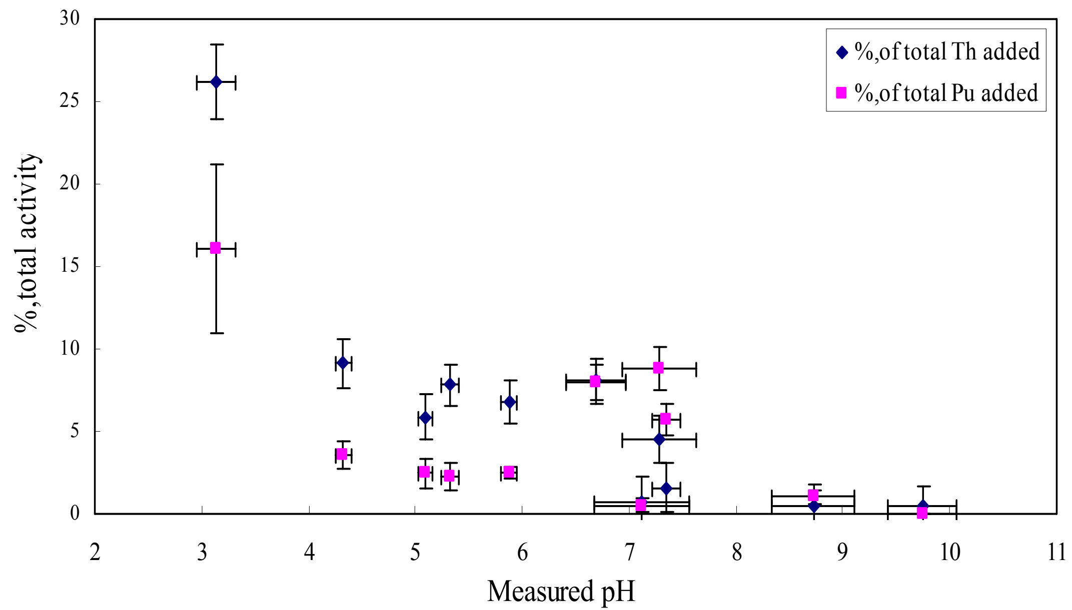


Figure 38 Isoelectric focusing of ^{234}Th(IV) and ^{240}Pu labeled *Sagittula stelleta* “non-attached” EPS

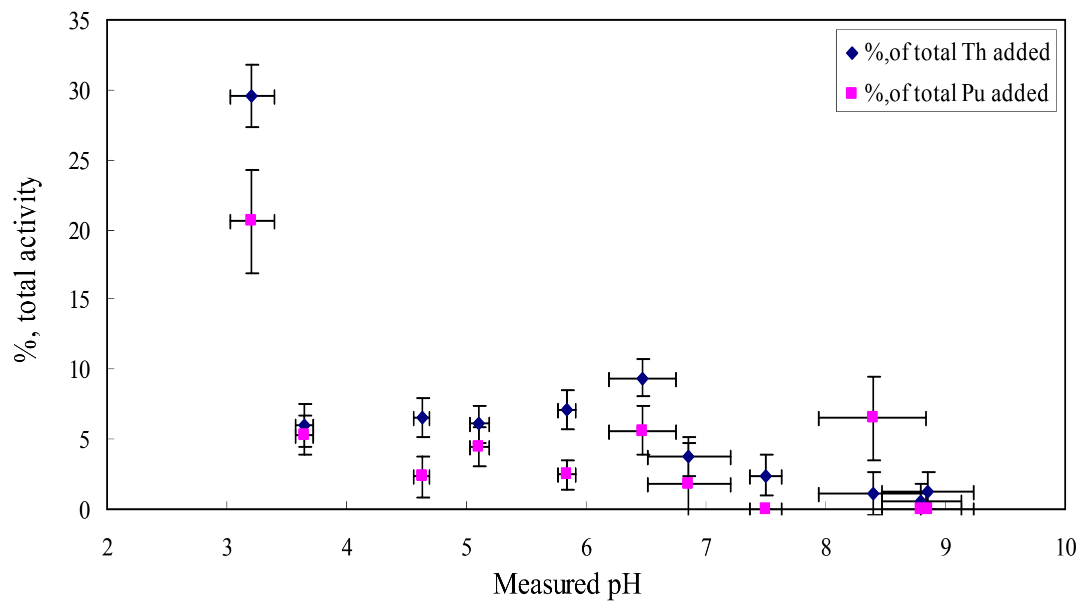


Figure 39 Isoelectric focusing of ^{234}Th(IV) and ^{240}Pu labeled *Pseudomonas fluoresccns* Biovar II “non-attached” EPS

III.7.Chemical Composition of Pu-enriched Colloidal Fraction of RFETS

In order to characterize the mobile, water-soluble colloids from RFETS soil (enriched in Pu), approximately 400mg of colloidal material from 1kg of RFETS soil was gained through water leaching by resuspension, filtration and ultrafiltration, with nominal sizes ranging from 1 KDa to 0.45 μm. About 200 mg of this soil water extract were further separated by IEF, and the Pu enriched fraction in the acidic region, i.e. the low pH end (pH 2-3) was pooled, and underwent further dialfiltration and freezedrying steps to get rid of all the detergent. Around 67 mg of the purified IEF extract was finally collected; therefore the recovery rate was around 30-40% by this separation technique. A comparison of the chemical composition of the original soil, the water extract, and the IEF extract, is given in Table 6.

Table 6
Comparison of RFETS soil, water extract and IEF extract

	% OC	% N	TCHO-C (%,OC)	Protein-C (%,OC)	URA-C (%,OC)	PO_4^{3-} (C/ PO_4^{3-})mol ratio)	Pu (dpm/g)
Original soil ≤2mm	1.9[a]	1.01[a]	n/d	n/d	n/d	58.2	158±3[a]
Water extract	28.6±0.8[a]	1.76±0.06[a]	11.5±1.0[a]	n/d	n/d	2075.6	297±21[a]
IEF-pH2to3 section of water extract	28.4±0.8	n/d	9.5±0.9	5.4±0.15	0.5	5127.5	2175±190

[a], data from DOE report (2007).
n/d, not determined yet.
% OC, percentage of organic carbon in total mass; % N, percentage of nitrogen in total weight; TCHO-C (%, OC), percentage of total carbohydrates-C (40%) in total organic carbon; Protein-C (%, OC), percentage of protein-C (33%) in total organic carbon; URA-C (%,OC), percentage of uronic acid-C (37.11%) in total organic carbon..

GC-EI-MS analysis on the monosaccharide composition exhibited that xylose was the dominant compound, followed by glucose, mannose, galactose, arabinose, rhamnose, fucose, glucuronic acid, galacturonic acid and mannuronic acid (Figure 40).

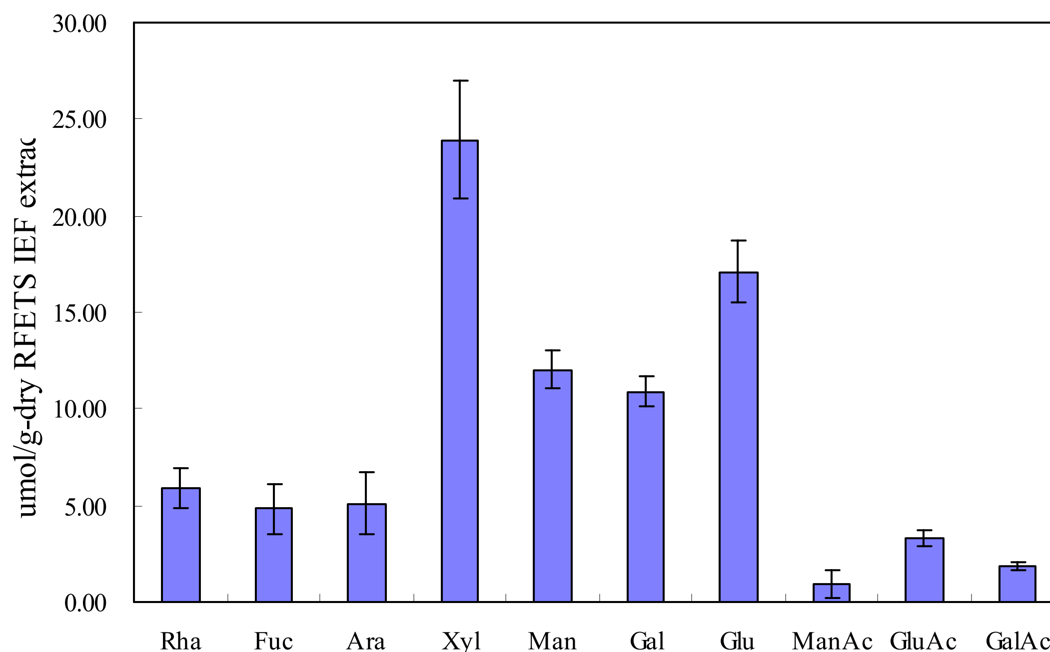


Figure 40 Individual monosaccharides of RFETS soil colloidal IEF extract

III.8.Analysis of ATR-FTIR on the Bacterial EPS and Pu-enriched IEF Extract from RFETS Soil

Spectrum of “non-attached” and “attached” EPS were shown in Figure 41 (a) and (b). Characterization of peaks were compiled based on several references (Casal et al., 1980; Williams and Fleming, 1980; Gendreau et al., 1982; Naumann et al., 1988; Singh and Fuller, 1991; Jackson and Mantsch, 1993; Nivens and Schmitt, 1993; Chen et al.,

1996; Schmitt and Flemming, 1998; Tanaka et al., 2001; Peng et al., 2003; Kim et al., 2004; Lim et al., 2005; Verhoef et al., 2005; Mecozzi and Pietrantonio, 2006; Sheng et al., 2006; Samios et al., 2007). Peaks within 2400-2000 cm^{-1} were caused by the interferences of diamond interface, which has been stated in the manufacturer's instructions. Fortunately, no characteristic peaks of interest in current study occurred in this region. Some authors (e.g. Schmitt and Flemming, 1998; Mecozzi and Pietrantonio, 2006; Sheng et al., 2006) arbitrarily divided the spectrum obtained from FTIR into several characteristic bands representing different compounds: (1) 2956-2850 cm^{-1} (aliphatic CH symmetric and asymmetric stretching in CH_3 and CH_2) as fatty acid region; (2) 1652-1648 cm^{-1} (C=O asymmetric stretching of –NH-CO-R and/or N-H bending of H_2N-CO-R (Amide I)) and 1550-1548 cm^{-1} (N-H bending of –NH- (Amide II)), as protein region; (3) 1300-1245 cm^{-1} (P=O stretching of phosphate PO_4^{3-}), as nucleic acid region; (4)1085-800 cm^{-1} as polysaccharide region. This is not necessarily and completely true, since some compounds, having similar functional groups, are displaying overlapping regions. For example, 2956-2850 cm^{-1} is not specific for lipids, since it could be characteristic of polysaccharides and proteins, which have the CH_3- and -CH_2- functional groups as well, though with different abundance resulting in differences in the intensity of absorbance. Peak in the region of 1720-1750 cm-1 indicates the presence of carboxylic acid, which could be attributed to either uronic acid or fatty acid. Amide I and II bands (peaks within 1650-1540 cm^{-1}) are not necessarily indicative of proteins, since amino sugars or their derivatives, e.g., N-acetyl-glucosamine, would also display peaks within this region. Since C-O stretching of esters (~1245 cm^{-1}) and P=O of phosphate

(1300-1250 cm^{-1}) might probably overlap, it's hard to unambiguously define peaks in this region. Even the presence of phosphate doesn't necessarily indicate the presence of nucleic acids, since some polysaccharides also contain phosphate functional groups. However, the low energy region (1085-800 cm^{-1}) as carbohydrate "fingerprints region" has been widely accepted (Williams and Fleming, 1980; Chen et al., 1996; Schmitt and Flemming, 1998; Kim et al., 2004; Sheng et al., 2006).

Both "non-attached" and "attached" EPS of *Sagittula stellata*, which were extracted and purified according to the modified and improved procedure by this study (Figure 20), displayed peaks within 1300-1240 cm^{-1} (Figure 41 and Table 7), indicating the presence of minor amounts of phosphate groups or ester groups. Though peaks within this region could also result from S=O stretching, the lack of peaks at 850 cm^{-1} and 820 cm^{-1}, which represent C-O-S stretching, makes the presence of sulfate group unlikely. The presence of the carboxylic acid group, as identification of uronic acid, was represented by fairly weak "humps" in both spectra. This is consistent with the colorimetric and GC-MS results, which showed that uronic acid was not the dominant monosaccharide in both "non-attached" and "attached" EPS of *Sagittula stellata*. Though compared to the crude ultra-filtrate (10KDa-0.22µm) (Section III.2), TCA precipitation removed ~39% protein from the EPS, trace amounts of proteins still exist in the polymers. The shift of the characteristic C-O-C group vibrations in the cyclic structures of carbohydrates between "non-attached" EPS and "attached" EPS, i.e., 1034 cm^{-1} vs. 1029 cm^{-1}, indicates a slight difference in the monosaccharide composition of these two types of EPS.

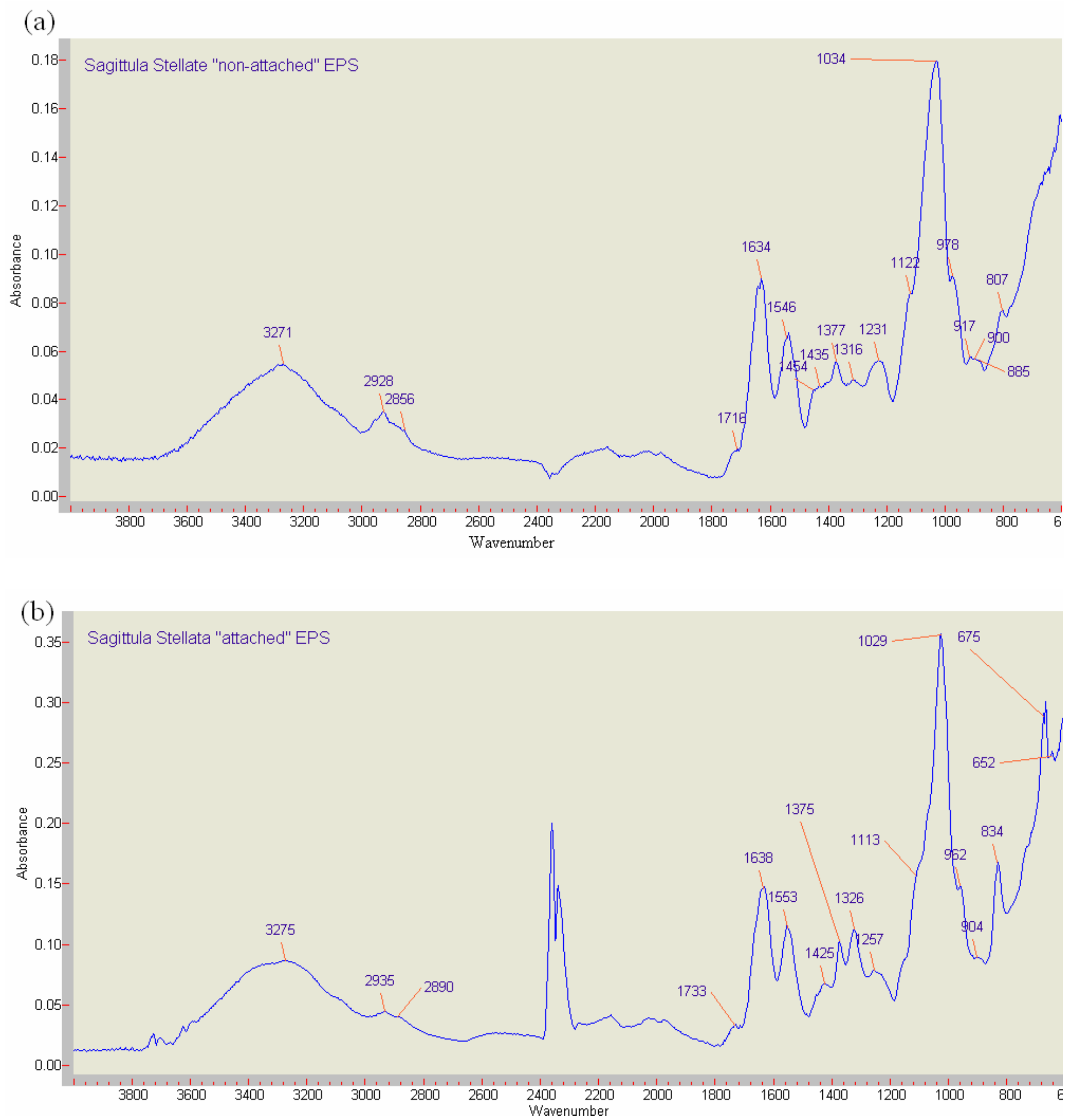


Figure 41 ATR-FTIR spectra of “non-attached” (a) and “attached” (b) EPS of *Sagittula stellata*

Table7

Potential functional groups in “non-attached” and “attached” EPS of *Sagittula stellata*

Potential functional groups	“Non-attached” EPS		“Attached” EPS	
	Wavenumber (cm^{-1})	Characteristics of peak	Wavenumber (cm^{-1})	Characteristics of peak
O-H stretching and hydrogen bonding	3271	Very broad	3275	Very broad
Aliphatic CH stretching (symmetric and asymmetric stretching of CH_3 and CH_2)	2932,2879	Doublet, fairly weak	2935,2890	Doublet, fairly weak
Aliphatic CH bending of CH_3 and CH_2	1435	Fairly weak	1425	weak
C=O stretching of carboxylic acid (COOH)	1716	Fairly weak	1733	Fairly weak
C=O asymmetric stretching of –NH-CO-R and/or N-H bending of H_2N-CO-R (Amide I)	1634	strong	1638	strong
N-H bending of –NH- (Amide II) and/or C=C stretching of aromatic ring	1546	strong	1553	strong
C=O symmetric stretching of carboxylate and/or C-OH stretching of phenolic OH	1377	Weak, a little broad	1375	Sharp, but relatively weak
O-H bending in carboxylic acid	1316	Fairly weak	1326	A little strong
P=O stretching of phosphate PO_4^{3-} and/or C-O stretching of –O-COR	1231	A little broad	1257	Fairly weak
COH bending, C-O-C stretching of alcohols and ethers	1122	Fairly weak	1113	Fairly weak
C-O-C group vibrations in the cyclic structures of carbohydrates	1034	Strong	1054	Strong

Spectra of ATR-FTIR of the three fractions (F1, F2 and F3) of "non-attached" EPS and the whole "attached" EPS from *Pseudomonas fluorescens* Biovar II, are shown in Figure 42 a-d and summarized in Table 8. The peak indicative of phosphate and/or ester in the 1300-1240 cm^{-1} region was almost invisible in F2 and F3, but evident in both F1 and gross "attached" EPS. Weak peaks were found in the region 1720-1740 cm^{-1}, characteristic of carboxylic acid, for F1 and F3, which agrees with the fact that they are mainly neutral polysaccharides. However, no peak was found in this region, for F2, which was found to be acidic polysaccharide. This might be due to a "deprotonation" of the carboxylic oxygen (e.g., metal complexation), which would make a shift of the peak to lower energy as its vibrational mode becomes coupled to that of the other oxygen, giving rise to an asymmetric feature between 1540 and 1650 cm^{-1}. The relatively intensive and sharp peak at 1633 cm^{-1} in F2 over that in the other fractions was observed (Tanaka et al., 2001; Strathmann and Myneni, 2004).

Though ATR-FTIR in this study was not used as a quantitative method, the peaks characteristic of carbohydrates (1085-800 cm^{-1}, carbohydrates "fingerprints region") were relatively intensive and dominant over other peaks (e.g., peaks indicative of the presence of proteins) in all spectra, suggesting these polymers were mainly composed of polysaccharides, after all those purification steps (section III.2).

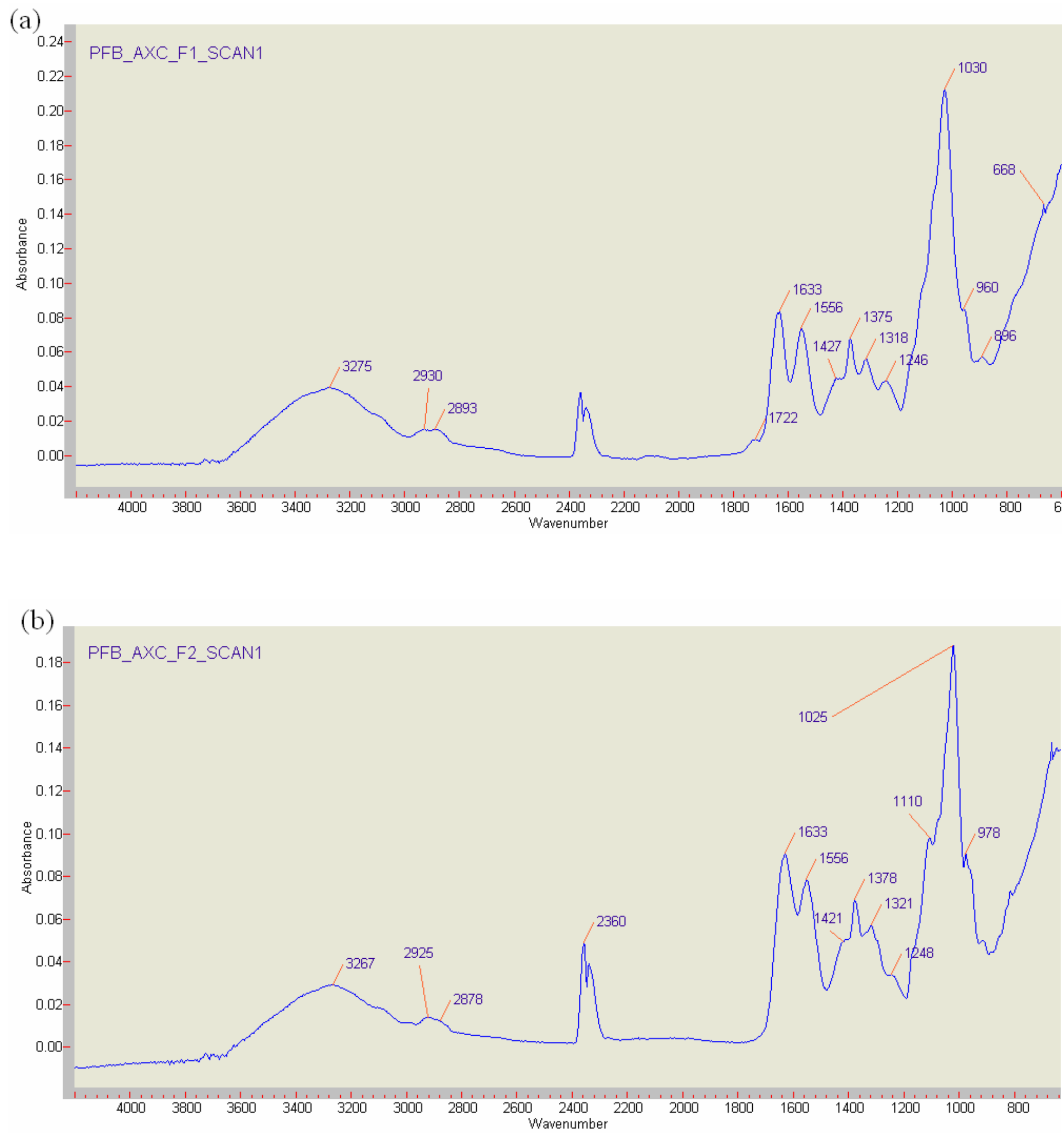
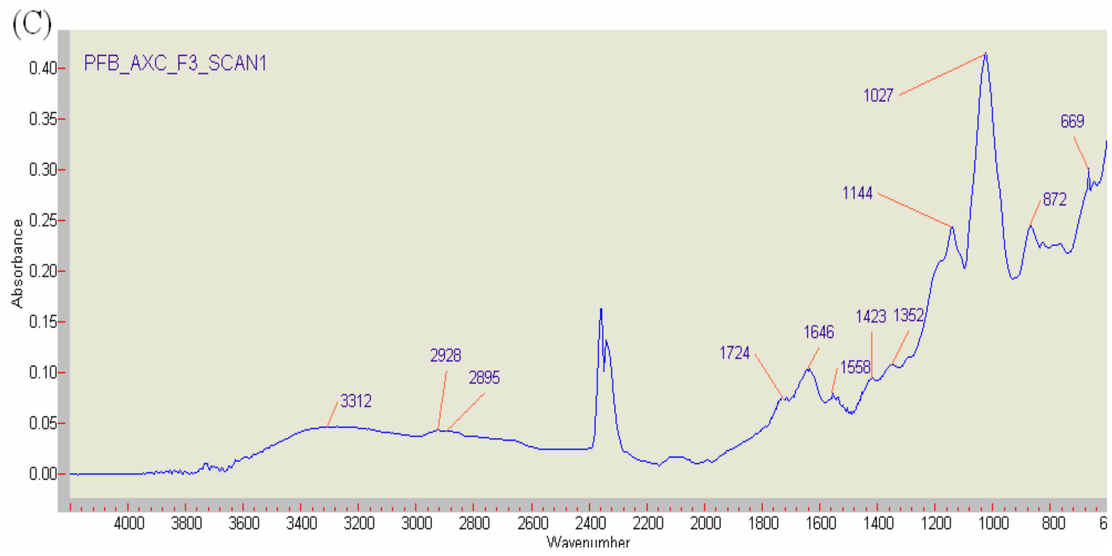


Figure 42 ATR-FTIR spectrum of three fractions (F1, F2 and F3) of “non-attached” EPS of *Pseudomonas fluorescens* Biovar II and “attached” EPS. (a), F1; (b), F2; (c), F3; (d), “attached” EPS



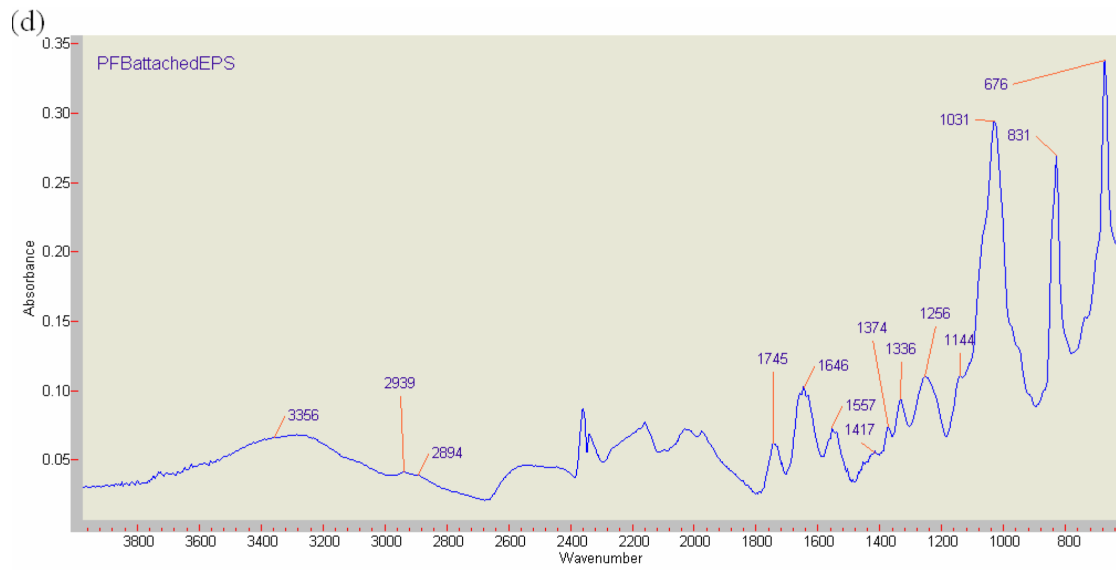


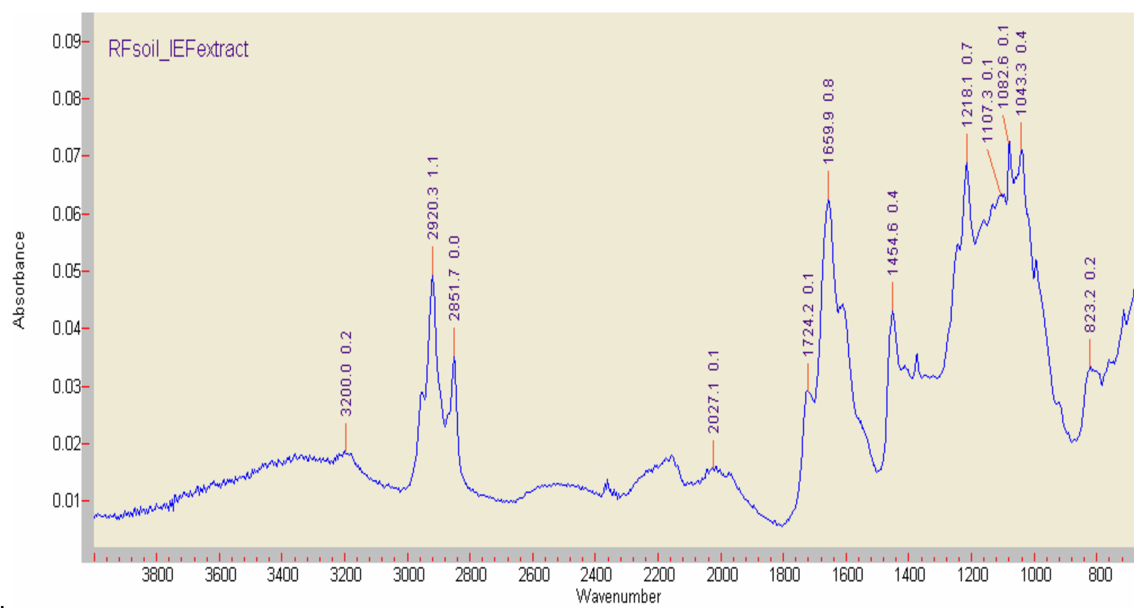
Figure 42 continued

Table 8
Potential functional groups of "non-attached" EPS and "attached" EPS of *Pseudomonas fluorescens* Biovar II

	"Non-attached" EPS			"Attached" EPS
	F1	F2	F3	
O-H stretching and hydrogen bonding	3275, broad	3267, broad	3312, broad and weak	3356, broad
Aliphatic CH stretching (symmetric and asymmetric stretching of CH_3 and CH_2)	2930,2893, doublet, fairly weak	2935, 2878, doublet, fairly weak	2928, 2895, doublet fairly weak	2939, 2894, doublet, farily weak
Aliphatic CH bending of CH_3 and CH_2	1427, fairly weak	1421, fairly weak	1423, fairly weak	1417, fairly weak
C=O stretching of carboxylic acid (COOH)	1722, weak	N/D	1724, weak	1745, strong
C=O asymmetric stretching of –NH-CO-R and/or N-H bending of H_2N-CO-R (Amide I)	1633, sharp and strong	1633, sharp and strong	1646, broad and weak	1646, strong
N-H bending of –NH- (Amide II) and/or C=C stretching of aromatic ring	1556, sharp and strong	1556, sharp and strong	1558, fairly weak	1557, strong
C=O symmetric stretching of carboxylate and/or C-OH stretching of phenolic OH	1375, sharp and strong	1378, sharp and strong	1352, fairly weak	1374, strong
O-H bending in carboxylic acid	1318, sharp and strong	1321, a little weak	N/D	1336, weak
P=O stretching of phosphate PO_4^{3-} and/or C-O stretching of –O-COR	1246, broad and strong	1248, fairly weak	N/D	1256, strong
C-OH bending, C-O-C stretching of aliphatic and aromatic ethers	N/D	1110, weak	1144, sharp	1144, fairly weak
C-O-C group vibrations in the cyclic structures of carbohydrates	1030, intensive and sharp	1025, intensive and sharp	1027, intensive and sharp	1031, intensive and sharp

In the ATR-FTIR spectrum of RFETS soil colloid IEF extract sample, intensive and sharp double peaks were observed at 2920,2852 cm^{-1}(Figure 43 and summarized in Table 9), which are characteristic of aliphatic C-H stretching of CH_3- and -CH_2-. Very strong aliphatic C-H bending was also found at 1455. Though polysaccharide and protein chains can also contain aliphatic C-H, they usually display a very broad and modest "hump" at this region. Thus, such strong absorbance at both regions might indicate the presence of lipids. Since phosphate was relatively low (~0.14 mg/g-IEF extract), the significant peak at 1218 cm^{-1} might be attributed to C-O stretching of esters. The presence of characteristic peak of COOH (1724 cm^{-1}) could be attributed to either fatty acids or uronic acids. Peaks for carboxylate C=O asymmetric (1660 cm^{-1}) and symmetric stretching (1377 cm^{-1}) were very intensive, indicating that carboxylate functional group was highly abundant in this IEF extract. Amide I (H_2N-, 1616 cm^{-1}) was a little bit weak and amide II was fairly weak, appearing as a shoulder due to the strong absorption of the –C=O asymmetric (1660 cm^{-1}) group. Presence of sulfate was suggested by two groups of peaks: 1218 cm^{-1} (S=O stretching) and 823 cm^{-1} (C-O-S stretching). However, several other functional groups also have peaks around 1218 cm^{-1}, making it hard to identify the sulphate groups. Distinct peaks at 1083 and 1043 cm^{-1} were characteristic of C-O-C group vibration in the carbohydrates.

In summary, specific bands of lipids, proteins and polysaccharides were present in the IEF extract of REFTS soil. Presence of other functional group, e.g. sulfate, was suggested with some uncertainty.



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.Figure 43 ATR-FTIR spectrum of RFETS soil IEF extract

Table 9
Potential functional groups in Pu-enriched colloidal IEF extract from RFETS soil

Potential functional groups	Wavenumber (cm^{-1})	Characteristics of peak
O-H stretching and hydrogen bonding	3700-3100	Very broad band
Aliphatic CH stretching (symmetric and asymmetric stretching of CH_3 and CH_2)	2920,2852	Doublet, very strong and sharp
Aliphatic CH bending of CH_3 and CH_2	1455	Very strong and sharp
C=O stretching of carboxylic acid (COOH)	1724	A little weak
C=O asymmetric stretching of –NH-CO-R and/or N-H bending of H_2N-CO-R (Amide I)	1660, 1616	Very strong and sharp
N-H bending of –NH- (Amide II) and/or C=C stretching of aromatic ring	1566	Very weak
C=O symmetric stretching of carboxylate and/or C-OH stretching of phenolic OH	1377	Sharp
P=O stretching of phosphate PO_4^{3-} and/or C-O stretching of –O-COR and/or S=O stretching of SO_4^{2-}	1218	Very sharp and strong
COH bending, C-O-C stretching of aliphatic and aromatic ethers	1164	Fairly weak
C-O-C group vibrations in the cyclic structures of carbohydrates	1083, 1043	Sharp
C-O-S stretching of –O-SO_4-	823	Strong but a little broad

IV. DISCUSSION

IV.1.Extraction Methods for Bacterial Capsular EPS

To select an extraction method which could meet all the three requirements, i.e., a) relatively little cell lysis; b) comparatively high extracellular polymeric substances yield, especially the polysaccharides; c) least chemical modification or denaturing of the extracted polymers, four chemical methods and two physical methods, which were commonly used in previous studies (Brown and Lester 1980; Gehr and Henry, 1983; Liu and Fang, 2002; Sheng et al., 2005; Comte et al., 2006; Comte et al., 2007), were compared with regard to the above aspects as well as being suitable for routine and quantitative operation under current laboratory conditions. The strategy in this research is to (1) compare the cell lysis proxy, i.e, nucleic acid contents, in EPS extracted by each method; (2) compare the absolute yield, especially the polysaccharide contents, as well as relative varieties, i.e. the ratio of carbohydrates-C to protein-C; (3) compare the "fingerprint", i.e. size exclusion chromatograms of different EPS, with those of the control group. The different extraction methods did considerably influence all the three aspects. The influence is also quite different from species to species, i.e. *Sagittula stellata* vs. *Pseudomonas fluorescens* Biovar II. Therefore, no universal applicable method could be found.

IV.1.1.Constituents of Crude Capsular EPS

Samples for different treatments were taken from the same batch and bacterial suspension was mixed very well before splitting, thus batch variation would be

eliminated. In the case of *Sagittula stellata*, the 0.5 N hydrochloric acid extraction turned out to be superior to the other five methods, in terms of polysaccharides yield, which was 2.5 times higher than in the control group (3% NaCl). The cell-destroying degree of this method was far lower, about 38.8% of that of the control group. Ultrasonication couldn't improve the yield at all. However, this is not the same case with *Pseudomonas fluorescens* Biovar II. Surprisingly, the control group, i.e., low concentration of sodium chloride (0.005 N) turned out to be the most suitable method, though it only ranked the fifth highest in polysaccharide yield. Other methods, which yielded much higher amount of polysaccharides than that of the control group, were in the order of heating > 0.01 N NaOH>3% NaCl. However, severe cell lysis occurred since nucleic acid contents were 5.2, 7.4, and 10.3 times higher than that of the control group, respectively. Ultrasonication produced a very slight increase in sugar concentration over the control group, however, the nucleic acid content increased by 82.1%. Therefore, a combination of ultrasonication with the control treatment was not deemed suitable for this case.

As mentioned in Section I.2, there are several kinds of forces that make capsular EPS layer "adhering" to the cell surface. EPS in solution of different ionic strengths, i.e., NaCl solution of different concentrations, would cause the screening of the surface charge of EPS with increasing concentrations of NaCl. The surface charge is the sum of electrostatic repulsion and Van der Waal forces. A low ionic strength needs to be carefully chosen, in order to achieve a net surface repulsion between EPS and the bacterial cell surface. The ionic strength cannot be too low, as this could cause the swelling and breakage of cells, and it cannot be too high, as this could cause cell

shrinkage and breakage. In this study, the ionic strength that prevails for the bacterium in the environment was first considered, e.g., 3% NaCl for marine bacterium *Sagitulla stellata* and 0.005 N NaCl for soil bacterium *Pseudomonas fluorescens* Biovar II. Proper and slow stirring, thus applying moderate shear to the surface, facilitates this process.

Tsai and Manos (1982) found that the composition of the released "attached" EPS was dependent on the pH. They found that the lower the pH, the higher the molecular weight of the desorbed polymer, indicating that EPS of higher molecular weight were more strongly adsorbed. Thus, at low pH, they could be more easily extracted. However, little has been reported about the efficiency of extraction by HCl in the literature. This study has opened a tentative method to extract capsular EPS, for other bacterial species in the future. Great care has to be taken, since under some circumstances, HCl could hydrolyze the exopolymer. However, hydrolysis was not found for EPS from *Sagittula stellata*. For *Pseudomonas fluorescens* Biovar II, this method was not feasible, extracting more protein than polysaccharides and resulted in an overall unsatisfactory yield.

It is possible for some chelating agents, such as EDTA, to chelate the divalent ions "entrapped" in the EPS matrix, e.g., Mg^{2+} and Ca^{2+}, which would reduce "binding" strength between EPS and the cell surface and increase the solubility of EPS in solution, thus facilitating its extraction. Good extraction efficiency has been reported by Sheng et al. (2005). EDTA was the optimal extraction method for a H_2-producing bacterium, *Rhodopseudomonas acidophila*. However, it has been reported by other researchers that EDTA could cause sever cell lysis, due to the removal of cations by EDTA from the cell membrane (Wingender et al., 1999; Liu and Fang, 2002). In addition, it was also

suspected that EDTA could strongly complex with EPS and make it harder for EDTA to be removed by dialysis (Liu and Fang, 2002; Comte et al., 2006; Comte et al., 2007). Furthermore, the presence of EDTA could severely interfere with the analysis of protein by the BCA method (Sapan et al., 1999). In this research, EDTA was used at a similar dosage as in other work (Brown and Lester 1980; Gehr and Henry, 1983; Liu and Fang, 2002; Sheng et al., 2005; Comte et al., 2006; Comte et al., 2007), but it didn't cause severe cell lysis for both bacteria, and neither did it extract significant amounts of EPS from the cell surface.

The addition of NaOH increases the solution pH, resulting in the dissociation of acidic groups in EPS and the repulsion between negatively-charged EPS and bacterial cell surface. The EPS solubility in water also increases and thus allows more EPS to be extracted. Brown and Lester (1980) found that sodium hydroxide caused a vast amount of cellular disruption in all three bacterial cultures (activated sludge, synthetic activated sludge and pure culture) they tested and the extraction efficiency varied a lot. Severe cell lysis was also reported by Sheng et al. (2005), of *Rhodopseudomonas acidophila*. In this study, though no significant cell lysis was found based on nucleic acid content for *Sagittula stellata*, relatively low polysaccharide as well as extremely high protein (72% and 621% of those of the control group, respectively) was extracted, making NaOH an improper candidate for capsular EPS extraction. Polysaccharides might get hydrolyzed under such harsh environment. For *Pseudomonas fluorescens* Biovar II, though a very high amount of protein with a relatively modest increase in the amount of polysaccharides were extracted (452% and 231% of the control group), cell lysis

occurred, based on the observed increase in nucleic acid content and double peaks in SEC spectra. A recent study by Liu and Fang (2002) reported that NaOH combined with formaldehyde could significantly reduce cell lysis while giving a relatively high EPS yield. Still, this could not resolve the problem of potential hydrolyzing EPS.

As for ultrasonication, different devices, i.e., ultrasonic bath or Sonifier cell disruptive tip, different output power, different sonication time and different extractant could all make differences in extraction efficiency. King and Forster (1990) stated that, in general, the sonication method creates disruption of the EPS-bacteria matrix and followed by a period of reflocculation, which decreases the extraction efficiency. This might explain the small increase in EPS yield by sonication in this study. Low extraction efficiency by sonication was also reported in the study of Brown and Lester (1980) and Comte et al. (2006). A detailed investigation by Matias et al. (2003) revealed that a high deflocculation was achieved after 30 seconds of sonication by a phosphate-buffered saline solution with minimal cell lysis while sonication in EDTA gave a larger fraction of damaged cell, shown by transmission electron microscopy. Therefore, sonication in conjunction with another proper extractant for a relatively short period of time may be useful.

Heating creates dispersion in EPS-bacteria matrix facilitating EPS extraction, but the high temperature could easily affect the nature of EPS, e.g., causing protein denaturing or hydrolysis of proteins and polysaccharides (Karapanagiotis et al., 1989). Moreover, heating can also lead to cell lysis, as reported by Sheng et al. (2005).

IV.1.2.Relative Varieties of Crude Capsular EPS

The carbohydrate-C to protein-C ratio has been used recently as an indicator of the composition of extracted extracellular polymeric substances (Liu and Fang, 2002; Sheng et al., 2005; Comte et al., 2006, Hung et al., 2005). For *Sagittula stellata* EPS, this ratio of EPS extracted by 0.5 N HCl was statistically similar to the control group (0.92±0.12 vs. 0.84±0.06), which indicates this method "strips" the similar polymer, e.g., a glycoprotein from the cell surface. Extraction by EDTA also produced a similar ratio; however, the polysaccharides yield was inferior to that of 0.5 N HCl. Other methods, in terms of this ratio, decreased in the order of ultrasonication>heating>0.5 N NaOH, indicating an increase in preferential extraction of protein over polysaccharide.

For *Pseudomonas fluorescens* Biovar II, though EDTA had the highest carbohydrate-C to protein-C ratio, twice as high as the control group (0.34 vs. 0.17), both polysaccharide and protein yields were very low.

Compared to previous studies by Alvarodo Quiroz et al. (2006) and Hung et al. (2005) on EPS of *Sagittula stellata* and *Pseudomonas fluorescens* Biovar II, respectively, relatively lower ratios of carbohydrate to protein (normalized to carbon) were found from this study (0.92 vs. 5.5 for *Sagittula stellata* and 0.17 vs. 3.8 for *Pseudomonas fluorescens* Biovar II). This is not surprising since the EPS they reported both underwent protein digestion, by proteinase, thus removing most of the extracellular protein. In this study, impurities involved (mostly protein and monosaccharides) from both broths (Marine Broth 2216 for *Sagittula stellata* and Trypticase Soy Broth for *Pseudomonas fluorescens* Biovar II) were demonstrated by size exclusion chromatography to have

molecular weights of less than 10 KDa, and were effectively removed by the steps of filtration through a 0.22 μm membrane and following extensive dialfiteration of the filtrate through a 10 KDa regenerated cellulose membrane (see Section III. 2.). Therefore, prefiltration and dialfiltration steps were necessary to distinguish the extracellular protein from broth protein. Thus, the protein content measured afterwards should be the "real" extracellular protein produced by bacteria and their contribution to the EPS matrix shouldn't be ignored.

Table 10
Carbohydrate to protein ratio in EPS of different cultures extracted by different methods

Culture	Optimal extraction method	Carbohydrate-C: Protein-C	References
K. aerogenes	Steaming	2.52	Brown and Lester, 1980
Activated sludge	Steaming	0.42	Brown and Lester, 1980
Synthetic activated sludge	Steaming	0.54	Brown and Lester, 1980
Activated sludge	Cation exchange resin	0.24	Frolund et al., 1996
Activated sludge	Cation exchange resin	0.10	Bura et al.,1998
Rhodovulum sp.	40°C	0.70	Watanabe et al., 1998
Activated sludge	EDTA	0.65	Liu and Fang 2002a
Activated sludge	Cation exchange resin	0.87	Liu and Fang 2002a
Aerobic activated sludge	Formaldehyde-NaOH	1.09	Liu and Fang, 2002b
Acidogenic sludge	Formaldehyde-NaOH	6.02	Liu and Fang, 2002b
Methanogenic sludge	Formaldehyde-NaOH	0.67	Liu and Fang, 2002b
Rhodopseudnomonas acidophila	EDTA	0.13	Liu and Fang, 2002b

* Data modified from the original paper by multiplying the carbohydrate to protein (by weight) by a conversion factor (0.4/0.33), assuming that carbon accounts for 40% and 33% of carbohydrates and protein, respectively (Hung et al., 2005)

For years, polysaccharides were regarded as the dominant component of EPS in pure cultures (Bull, 1972; Sutherland, 1977; Sutherland and Kennedy, 1996). Production of extracellular protein was considered to be very rare, especially for gram negative organisms, due to the complex and impermeable nature of the cell membrane. This is actually not true. Exoenzymes, which are secreted and entrapped in the EPS and become part of the EPS matrix, were usually overlooked, until recent studies found significant amounts of protein (Table 10).

IV.1.3.Implication of Size Exclusion Chromatography

It's already known that releasing of nucleic acids (DNA and RNA) by bacteria into their ambient environment is not necessarily caused by cell lysis or death, since metabolically active bacteria could also "purposely" excrete nuclecic acids concomitantly with cell growth, which form part of organized network of their extracellular milieu. There are some indications that extracellular DNA participates in transformation reactions in situ and catalyzes development of biofilms (Whitchurch, C.B. et al., 2002). Extracellular DNA in deep-sea sediments was found to six- to eight fold higher than that of DNA contained in all benthic prokaryotes inhabiting the top 10 cm of the world marine sediments and have profound implications on deep-sea ecosystem functioning, potentially supplying bacterial P requirement and contributing substantially to the P cycling (Dell'Anno et al., 2004, 2007). Nishimura et al. (2003) and Ando et al. (2006) found that extracellular RNA, excreted by a marine photosynthetic bacterium *Rhodovulum sulfidophilum*, exhibited a similar size and sequence to those of

intracellular RNA and are involved in the bacteria's flocculation. The regulation of extracellular nucleic acid concentration might not be all that well understood, so a certain variability might not necessarily indicate cell lysis. Though applying nucleic acids content as the degree of cell lysis has some limitation, it would be still useful when comparing the relative nucleic acid content to that of the control group in a simple experiment system, i.e., comparing nucleic acid content of the "centrifuged" bacterial cell aggregates under different treatments to the control group. Moreover, the size exclusion chromatography was also used as a supplementary approach to identify cell lysis.

Size exclusion chromatography was successful in showing differences and similarities, i.e., fingerprints, of EPS extracted by different methods. Though in this experiment, size distribution analysis was only carried out on initially purified EPS ultrafiltrate, i.e., 10 KDa-0.22μm, size exclusion chromatography could still be a useful approach to assess the cell disruptive degree as well as to detect any chemical modification. SEC applied in this study has thus opened an innovative approach to monitor the size distribution of EPS extracted by different methods and a useful tool to help assess the effectiveness of these methods as well as the integrity of the cell wall during the extraction steps.

IV.1.4.Dosage Effects and Extracting Time

Once the optimal extractants were found, dosage and extraction time could be tested by a series of experiments. 0.5 N HCl still remained the best concentration for

Sagittula stelleta, compared to the other concentrations. 0.05 N NaCl turned out to be the best concentration to yield EPS with a higher polysaccharides content, relatively similar nucleic acid content, and a carbohydrate-C to protein-C ratio similar to that of the control concentration, i.e., 0.005 N NaCl.

Long extraction times are supposed to allow the complete reaction between extractants and EPS-bacteria matrix. However, cell lysis and degradation of EPS are prone to occur if extraction times are too long. For different methods, the optimal extraction time might vary. For example, an extraction time as short as 30 seconds was found as the optimal time to extract EPS by ultrasonication method from an activated sludge in the study of Matias et al. (2003) with a minimal degree disrupture on the cellular ultrastructure. Progressive lysing effects on the cell walls with time by ultrasonication were observed by TEM. For *Sagittula stellata*, different extraction times seemed to have only small effects; while for *Pseudomonas fluorescens* Biovar II, extraction times longer than five hours appeared to cause significant cell lysis. An improvement in the future could be achieved by carrying out the extraction at relatively low temperature, e.g., 4°C (Liu and Fang, 2002; Matias et al., 2003; Sheng et al., 2005; Comte et al., 2006).

IV.2.Purification of EPS

Alcohol precipitation has often been used to isolate EPS from a culture medium (Staats, 1999; Tuinier et al., 1999; Yang et al., 1999; Hung et al., 2005; Alvarado Quiroz et al., 2006). In this study, bacteria were grown in complex media (Marine broth 2216

for *Sagittula stellata* and Tryticase Soy Broth for *Pseudomonas fluorescens* Biovar II), which would probably interfere with subsequent quantification and characterization of EPS, if the compounds in the media were not effectively eliminated. In the previous studies on purification of the EPS produced by *Sagittula stellata* and *Pseudomonas fluorescens* Biovar II (Hung et al. 2005; Alvarado Quiroz et al., 2006, respectively), direct alcohol precipitation was applied to the crude culture "supernatant" separated by centrifugation. It took around 16 liters of alcohol mixture (mixture composed of 95% ethanol and 5% methanol) to get purified EPS from 1 liter of bacterial culture and the whole processing time was as long as two to three weeks. Moreover, the behavior of broth material with the purification steps has never been explored. Thus, a preliminary experiment was carried out on both Marine broth 2216 and Tryticase Soy Broth without bacterial inoculation. Four volumes of alcohol (mixture composed of 95% ethanol and 5% methanol) was poured directly into axenic broth media with the same concentration as that used for bacterial culture in this study. Significant amount of precipitate formed in the case of marine broth 2216, not Tryticase Soy Broth. The supernatant was discarded and the precipitate of marine broth 2216 was dispersed with 3% NaCl solution and was re-precipitated by alcohol mixture. This procedure was repeated twice more. The final precipitate, getting progressively less, was dissolved in D.I., filtered through 0.22 μm polycarbonate membrane and dialyzed for 5 days using a 1 KDa membrane and the retentate was freezedried. Spectrophotometric methods showed the "residue" was mostly protein, which had a molecular weight of (5.28±0.70) KDa. A fraction of 0.22 μm to 1 KDa retentate of Tryticase Soy Broth was collected by filtration and

dialfiltration steps. It was also mostly protein, having two different molecular weights, i.e., (7.21±0.16) KDa and (4.81±0.13) KDa. This experiment was conducted with “blank” broth media, thus whether broth material and EPS would co-precipitate by direct ethanol precipitation is not certain but likely. Nevertheless, a prefiltration of the supernatant through a 0.22 µm polycarbonate membrane, which was separated from the cell pellet by centrifugation, was applied. The resulting filtrate underwent the dialfiltration in a stirred cell through a 10 KDa regenerated cellulose membrane, based on the results of SEC. Therefore, these steps are necessary to distinguish EPS, especially exoprotein, from the broth material but also to concentrate it to a final sample volume as small as 100-200 ml, instead of one liter, thus largely reducing the usage of alcohol from around 16 liters (Hung et al., 2005; Alvarado Quiroz et al., 2006) to less than one liter (*Sagittula stellata*) or around two liters (*Pseudomonas fluorescens* Biovar II), for the purification of one liter of bacterial suspension.

A systematic tracking of EPS varieties (in terms of carbohydrates, protein and uronic acid contents) during the following purification steps was also carried out to achieve a cost-effective purification design for large scale EPS production. The following criteria were used: (1) the best extraction alcohol (methanol, ethanol vs. isopropanol); (2) the best ratio of alcohol to sample volume; (3) the optimal alcohol precipitation steps. The processing time for purification was shortened from originally 2-3 weeks to 1-2 weeks, since the repetative precipitation was not necessary. If exopolysaccharides would be the final target, an improvement could be made by adding formic acid as a buffer at low pH (pH 3.0), since proteins are then more soluble in

ethanol solution (Tuinier et al., 1999). In addition, it's important to wash the precipitate at least once after TCA precipitation, for complete EPS recovery, since it was proposed that a great proportion of the polysaccharides will co-precipitate in TCA (Cerning, 1995).

The optimization and establishment of suitable methods of extraction and purification for EPS from the specific bacteria in this study would help understand not only the original complex EPS matrix with least disturbance, but also open the perspectives of preferentially isolating specific homogenous fractions with best yield, e.g. exopolysaccharides, which has been hypothesized to carry the binding ligand responsible for strong actinide complexation (Quigley et al., 2001; Guo et al., 2002; Quigley et al., 2002; Santschi et al., 2002; Santschi et al., 2003; Hung et al., 2004; Alvarado Quiroz et al., 2006).

IV.3.Effects of Growth Phases on the EPS Production and Composition

IV.3.1.Effects of Growth Phases on the EPS Production Patterns and Varieties Proportions

The different components of EPS as well as the total yield did vary a lot at different growth stages. The death phase is not discussed here since "contaminants" from the cell interior might be largely involved at this stage, which is hard to quantify and interpret. It is very interesting to find that the "non-attached" EPS produced by both bacterial species were significantly coupled to growth, while the "attached" EPS were either weakly linked to growth (*Sagittula stellata*) or "non-growth-associated" (*Pseudomonas*

fluorescens Biovar II). The positive correlations between "non-attached" EPS and "attached" EPS varieties of *Sagittuala stellata*, i.e., carbohydrates, proteins and major monosaccharides (galactose and mannose), suggest the similarity in the production mechanisms for these two types of EPS, at least to some extent. However, this coupling was missing between the two types of EPS produced by *Pseudomonas fluorescens* Biovar II, which indicates that the secretion of the "non-attached" and "attached" EPS were under different metabolic controls.

By comparing the ratios of carbohydrate-C to protein-C in both types of EPS produced by both species, some common characteristics can be concluded and discussed: 1) "Non-attached" EPS produced by both bacterial species were relatively enriched in polysaccharides, while "attached" EPS were relatively enriched in proteins. This finding demosntrates again that protein is a major fraction of EPS in their native state, especially for "attached" EPS. This is consistent with the results reported by Decho and Lopez (1993). They also found that the capsular EPS were high in protein content, while the "slime" EPS were largely composed of polysaccharides for the marine bacterium, *Pseudomonas atlantica*. 2) In "non-attached" EPS produced by both bacterial species, polysaccharides and proteins were produced almost at a similar rate throughout all bacterial growth stages, while polysaccharides and proteins were produced at very different rates in "attached" EPS, thus displaying a distinct and increaseing trend of carbohydrate-C to protein-C ratio for *Sagittula stellata,* while the opposite trend was observed for *Pseudomonas fluorescens* Biovar II.

It has been suggested by Sutherland (1977) that "non-attached" EPS were secreted

in large amounts resulting from a “metabolic excess response” or an “unbalanced-growth-response”. This usually happens when one kind of nutrient is relatively in excess, usually carbon, because of the depletion of other nutrients, e.g., N and P. The excessive carbon is “discarded” by bacteria into the formation of “non-attached” EPS which is usually high in polysaccharides and low in protein. This explains the significant correlation between the production of “non-attached” EPS and bacterial growth. In contrast, “attached” EPS, or capsualr EPS, were produced in response to very different causes. Further feeding experiments with the ^{14}C radiolabelling and fluorescent dying techniques by these authors demonstrated that these high-protein capsular EPS were less digestible than the “slime” EPS, thus protecting the bacteria against the digestive enzymes, e.g., hydrolases. Though in this current study, no grazers were added to the pure culture, it’s still believable that under most conditions, “attached” EPS probably represent an adaption to protect cells from harsh microenvironments other than to resist enzymatic digestion (Sutherland 1977, Decho and Lopez, 1993). Another explanation for the higher protein content in “attached” EPS was that the relative high protein content could probably enhance the hydrophobic features of the bacterial surface, thus faciliating cell aggregation and TEP formation, which is assumed as a life strategy for microorganisims (Giroldo et al., 2003). Therefore, it’s not suprising that not only were “non-attached” and “attached” EPS produced in different “patterns”, i.e., “growth-associated” versus “non-growth-associated”, but they also displayed different proportions of varieties, i.e, polysaccharides versus proteins.

IV.3.2.Production of Different Polymers in “Non-attached” and “Attached” EPS

Since the monosaccharide composition was determined for the gross mixtures of EPS, the observed variations of individual monosaccharide composition in both “non-attached” EPS and “attached” EPS throughout the bacterial life period, i.e. absolute concentration of individual monosaccharide varying at different growth stages and its relative proportion to the total carbohydrates pool varying as well, could have resulted from different proportions of various polymers produced at different growth stages.

Though data of individual monosaccharide composition at different growth stages for *Pseudomonas fluorescens* Biovar II are lacking, two kinds of successive chromatography (size exclusion chromatography and anion exchange chromatography) on the “non-attached” EPS collected from late exponential phase showed that at least three different polymers with very similar molecular weights were produced at that stage. This result strongly confirms the hypothesis that bacteria did produce different exopolymers.

IV.3.3.Maximal EPS Production and Specific Productivity

For *Sagittula stellata*, both the maximal EPS production (mg-EPS/L-bacterial suspension) and the specific productivity (mg-EPS/OD_{600}) were found to occur at the stationary phase for both “non-attached” and “attached” EPS. For *Pseudomonas fluorescens* Biovar II, the maximal EPS production and the specific productivity of “non-attached” EPS occurred at the stationary phase, while those of the “attached” EPS occurred at the end of exponential phase (the transitional phase).

The synthesis of bacterial EPS involves a large amount of enzymes, which are not unique to EPS formation (Cerning, 1995; Looijesteijn et al., 1999): (1) enzymes responsible for the initial metabolism of a carbohydrate, (2) enzymes involved in sugar nucleotide (EPS precursor) synthesis and interconversion, (3) glycosyltransferases that form the repeating unit attached to the glycosyl carrier lipid, and (4) translocases and polymerases that form the polymer. In the current study, availability of limited nutrients contained in the medium flask to support continuous bacteria growth is the only factor that regulates EPS production and can cause variability. This factor wouldn't affect the enzymes of any group mentioned above, i.e., causing them to be missing or deactivated, therefore only the yields of individual polymers, whose synthesis might involve different kinds of enzymes or utilize different EPS precursors, were influenced. These enzymes might also be needed for the formation of cell wall sugars. Furthermore, the glycosyl carrier lipid involved in EPS formation is undecaprenol phosphate, identical to the carrier lipid also involved in the synthesis of cell wall polymer such as lipopolysaccharides, peptidoglycan and teichoic acids. If cells are growing more slowly, then the cell wall polymer formation rate will also be reduced a lot, thus providing more enzymes and lipid carriers available for EPS synthesis. This explains why the highest specific EPS production occurred in the stationary growth phase or late exponential growth phase rather than during the exponential growth phase.

It has been demonstrated that bacterial EPS production depends on the composition of the medium, e.g. carbon sources, N/P, other elements, and conditions in which the organisms grow, e.g., temperature, pH, incubation time, osmotic stress, etc. (Cerning et

al., 1994; Gandhi et al., 1997; Cerning, 1995; Looijesteijn et al., 1999; Petry et al., 2000; Degeest et al., 2001; Hwang et al., 2003; Vaningelgem, et al., 2004; Aslim et al., 2005; Torino et al., 2005). Therefore, in order to fully understand the specific EPS production mechanisms for these two bacteria species, i.e. *Sagittula stellata* and *Pseudomonas fluorescens* Biovar II, a more complete investigation needs to be carried out on effects of various factors on the EPS production and composition, at a higher level, e.g. isolation of the individual polymers at different growth phases and their characterization at the molecular level.

Though it has been reported that bacteria could excrete certain enzymes to digest exopolymeric substances produced by algae (Giroldo et al., 2003; Radic et al, 2005; Grossart et al., 2006), whether they can self-digest their own exudates under nutrient starvation status, has seldom been investigated. Nevetheless, the results from SEC revealed that the molecular weights of these exopolymers produced by *Sagittula stellata* changed little throughout their life cycle, thus making the self-degradation hypothesis unlikely.

The initial study on the EPS production and composition as a function of growth provides information to help choose the best time to stop bacterial growth and harvest the EPS, in order to gain the highest yield of the target fraction, i.e. exopolysaccharides. For *Sagittula stellata*, the end of stationary phase would be the best time since protein content either grew more slowly (as in the “non-attached” EPS) or dropped abruptly (as in the “attached” EPS), while polysaccharides grew all the way even at the death phase. For *Pseudomonas fluorescens* Biovar II, the end of exponential phase (or called

"transitional phase") would be chosen as the best time for harvesting, considering the yield of polysaccharides in both "non-attached" and "attached" EPS. Moreover, monitoring of the EPS production and composition at different growth stages would provide the base for further study on the effects of various environmental factors on the EPS production and composition. In a broader sense, bacterial EPS obtained under different growth conditions could be used in the studies of ^{234}Th or ^{240}Pu binding experiment to elucidate the contribution of different fractions, e.g. polysaccharides vs. protein, to the complexation of actinide with natural organic matter.

IV.4. Comprehensive Interpretation of the Composition of EPS Produced by the Two Species of Bacteria

Since the composition of EPS would greatly change at different growth stages, it would be more significant to discuss the EPS harvested at the best time i.e., the stage when bacteria yield the highest amount of polysaccharides, purified according to the procedure optimized by this study, and compare the results to those of previous studies. Table 11 and 12 is a summary of characterization of both types of EPS produced by *Sagittula stellata* and *Pseudomonas fluorescens* Biovar II, respectively, in this study as well as comparison to those of previous studies.

Table 11
Summary of characterization of both types of EPS produced by *Sagittula stellata* in this study and a previous study

Sagittula stellata	This study		Alvarado Quiroz et al. (2006)
	"Non-attached"	"Attached"	"Attached"[a]
OC (%, dry weight)	24.2±3.8	31.7	18.5
TCHO-C/OC (%)	39.6±3.6	34.3	14.3
Protein-C/OC (%)	7.7±3.0	5.1	2.6
URA-C/OC (%)	1.3±0.3	1.9	2.2
URA/TCHO (%)	3.3±0.8	5.5	15.4
Molecular weight (KDa)	27.6±0.9	27.5±3.5	3.5
TCHO-C/Protein-C	5.1 (vs. 0.6)[b]	6.7 (vs.0.9) [b]	5.5
Characterized OM (%)	47.3±6.6	39.4	16.9

Note: In current study, results for "non-attached EPS" are the averages of two batches; while results for "attached" EPS are the "average" of combined batches due to the relatively low yield of this type of EPS (the same as the table below). TCHO-C, assuming total carbohydrates contained 40% of carbon, Protein-C, protein with a carbon content of 33%, URA-C, uronic acid with a carbon content of 37.11%.

a. What is called here "attached" EPS, was called by the author "surficial" EPS.

b. Value in the bracket is the carbohydrate-C to protein-C ratio of the initial "supernatant" ultrafiltrate (10KDa-0.22 μm).

Table 12
Summary of characterization of both types of EPS produced by *Pseudomonas fluorescens* Biovar II in this study and a previous study

Pseudomonas fluorescens Biovar II	This study		Hung et al. (2005)*	
	"Non-attached"	"Attached"	"Non-attached" [a]	"Attached" [a]
OC (%, dry weight)	26.3±4.9	20.1	24.5	23.4
TCHO-C/OC (%)	29.7±10.4	20.5	31.2	25.6
Protein-C/OC (%)	9.1±4.3	2.3	8.9	8.7
URA-C/OC (%)	5.9±1.1	0.3	13.9	18.5
URA/TCHO (%)	19.9±7.9	1.5	44.6	72.3
Molecular weight (KDa)	20.7±0.1	20.1±0.0	N/D	N/D
TCHO-C/Protein-C	3.3 (vs.0.6) [b]	8.9 (vs.0.6) [b]	3.5	2.9
Characterized OM (%)	39.8±14.7	22.8	40.1	34.3

Note: a. in Hung et al. (2005), "non-attached" and "attached" EPS were named as "dissolved" and "particulate" EPS, respectively.

b. Value in the bracket is the carbohydrate-C to protein-C ratio of the initial "supernatant" ultrafiltrate (10KDa-0.22 μm).

After purification according to the modified procedure in this study, both bacterial EPS were polysaccharide-rich polymers, with less than 10% protein (protein-C/OC). Carbohydrate-C to protein-C ratios were much enhanced from less than one in the initial "supernatant" ultrafiltrate (10KDa-0.22 μm) to three to nine. These polymers, with molecular weights around 20-28 KDa, were probably mixtures of more than one type of polymer. Three fractions of "non-attached" EPS excreted by *Pseudomonas fluorescens* Biovar II, were successfully isolated by an anion exchange column. However, the above characterized components in this study, if summed, only account for about 29.6% and 32.1 % of the total mass of "non-attached" and "attached" EPS of *Sagittula stellata*, respectively, and about 26.8% and 11.7% of the total mass of "non-attached" and "attached" EPS of *Pseudomonas fluorescens* Biovar II, respectively. The "missing" mass could be partially explained by the presence of hydration water, as EPS are strongly hydroscopic, a typical property which has been suggested by Buffle (1990), Leppard (1995, 1997) and Hung et al. (2005). When comparing the molecular weight distribution between "non-attached" EPS extracted by only centrifugation, prefiltration and dialfiltration and final purified EPS, the fraction with a MW of (64.49±0.78) KDa was missing from the latter, suggesting that this fraction was mainly protein, which had a "growth-associated" pattern and removed with the following purification steps(see the discussion of IV. 3).

Compared to previous studies of EPS from *Sagittula stellata*, there was a significant difference in both composition and molecular weight of EPS. This could be explained by different extraction and purification methods used in the previous study, which

preferentially extracted and isolated substances with a relatively low molecular weight. As stated before, without prefiltration and dialfiltration steps, alcohol precipitation alone would co-precipitate some marine broth material severely, i.e., a substance with a relatively lower molecular weight than that of the real EPS. Besides, whether tap water, which was used in that study, was an effective extractant for capsular EPS of *Sagittula stellata* was never tested. It's therefore possible that the extracted material was mostly from the broth.

For *Pseudomonas fluorescens* Biovar II, results from both studies were more comparable, except for the higher uronic acid content that was found in the previous study by Hung et al (2005), who reported that uronic acids could amount to as much as 44.6% and 72.3% for both types of EPS produced by this bacterium, respectively. The sulfamate/m-hydroxydiphenyl method for uronic acid measurement has been modified by Filisetti-Cozzi and Carpita (1991), by adding 4M sulfamate to suppress the "browning" color interference caused by a 20-fold excess of neutral sugars. Later, Hung et al. (2001) improved the method and found that the addition of 2M sulfamate would give a better response for glucuronic acid standards. However, this was carried out with pure standards without interferences from neutral sugars. Thus, whether the lowered concentration of sulfamate (2M vs. 4M) would still be sufficient to suppress the "browning" was not tested yet. Actually, in this study, a slight "browning" could still be observed after adding Reagent I and II and heating, using 2 M sulfamate to measure the uronic acid content of EPS. This suggests that the uronic acid content in the previous study might have been a little bit overestimated. The uronic acid content in this study

was calculated by subtracting the “browning” absorbance after adding Reagent I, II and heating steps, from the final “pink” absorbance after adding Reagent III. The value calculated by this way agrees very well with the GC-MS results: glucuronic acid, as the only uronic acid detected, was only 13.3% and 0.8% of the sum of all monosaccharides in the chromatogram, for “non-attached” EPS and “attached-EPS” respectively. This is close to the results calculated from colorimetric methods (URA/TCHO (%), Table 12). In addition, it was demonstrated that the 3% NaCl extraction of capsular EPS used previously would probably cause leakage of intracellular material to some extent, thus making the overall composition of “attached” EPS (or “ particulate” EPS) slightly different.

A similar or even lower protein content was obtained by TCA precipitation, compared to the previous study, which applied a proteinase K digestion directly to the bulk bacterial “supernatant”. Involvement of proteinase K, if not removed carefully later, would also become a potential “contaminant”, especially since it has a molecular weight of around 28.5 KDa, according to the datasheet provided by the producer (Sigma-Aldrich), very similar to that of the EPS produced by the two bacteria species. Usually, a TCA precipitation is still needed to precipitate those undigested enzymes, after proteinase incubation (Bernard et al., 1995). Besides, care must be taken to avoid bacterial contamination if using longer incubation times, as was the case in the previous studies by Hung et al., (2005). All in all, more studies on protein removal from these bacterial exopolymers would be necessary, possible with a combination of protein enzymatic digestion and TCA precipitation. Since the molecular weight was not

provided by the previous study (Hung et al., 2005), it's hard to say whether any co-precipitation of soy broth impurities with the real EPS during alcohol precipitation had occurred, though precipitation of soy broth was not observed when using ethanol to precipitate it along.

The monosaccharide content calculated from the sum of individual monosaccharides in *Sagittula stellata* EPS, measured by GC-MS, was similar or a little bit higher than the value obtained from the TPTZ method (Table 13). This is not surprising since the chromatographic analysis, at a molecular level, did take the different responses of the different monosaccharides into account while the TPTZ method, by using glucose as the standard to represent the overall composition of EPS, would certainly be biased. As the major monosaccharides, e.g., galactose, mannose and glucuronic acid, for both EPS (Table 14) all have relatively lower absorbances than 100% compared to that of glucose, results from the TPTZ method would likely be lower than those of GC-MS. However, the lower "recovery" for EPS of *Pseudomonas fluorescens* Biovar II, when comparing the results from GC-MS to those of the TPTZ method could be explained by the potential presence of amino sugars, which were not quantified by GC-MS in this study.

Amino sugars have been found in various bacterial EPS, sometimes in significant amount (Fett et al., 1995; Quintero and Weiner, 1995; Zhang and Amelung, 1996; Benner and Kaiser, 2003; Kumar et al., 2004; Nichols et al., 2005). The simultaneous detection of amino sugars with neutral sugars and uronic acids by GC-EI-MS haven't been resolved in this study, since derivatization of amino sugars to their corresponding

alditol acetates requires a nitrous deamination step (Henry et al., 1983). Without this step, derivatives were thermally unstable, making their quantification unlikely. Actually this was also demonstrated in this study (data not shown). When the "derivatives" of amino sugar standards, i.e., D-glucosamine, D-galactosamine and D-mannosamine, which were obtained by the same way as derivatives of neutral sugars and uronic acids, were injected into the GC-EI-MS, their characteristic "peaks" were eluted out at retention times between those of neutral sugars and uronic acids. However, these derivatives were not stable and their "peaks" would break into a couple of peaks after several repetitive injections. Some unknown peaks, having the similar m/z values to those defined monosaccharides and the same retention times as those amino sugar "derivatives", were occasionally observed in the gas chromatogram of EPS samples. Though amino sugars were not directly measured, spectra from ATR-FTIR did display the presence of relatively strong amide I and II groups, in some purified low-protein EPS, e.g. F1 and F2 of "non-attached" EPS and "attached" EPS produced by *Pseudomonas fluorescens* Biovar II, thus making it likely that they contributed to the overall EPS composition, to some extent.

In all, the evidence for the presence of amino sugars and/or their derivatives in EPS of *Pseudomonas fluorescens* Biovar II come from the following observations: (1) protein was barely detected in the three fractions of "non-attached" EPS separated by anion exchange column; very low protein was present as 2.3% (normalized to OC) in the "attached" EPS; (2) significant amide I and II peaks were detected, which could be attributed to either protein or amino sugars; (3) the significant discrepancy in the results

between the TPTZ method and GC-MS spectra was found, with much lower ratios of results of GC-MS analysis to those of TPTZ method, especially for “attached” EPS.

Table 13
Major monosaccharides of bacterial EPS in this study and “recovery” by GC-MS analysis compared to the TPTZ method

	Sagittula stellata		*Pseudomonas fluorescens* Biovar II	
	Major monosaccharide	%, [GC-MS]/[TPTZ]	Major monosaccharide	%, [GC-MS]/[TPTZ]
“Non-attached”	Gal, Man	101.6-152.9	Gal,Glu,Man,GluAc	60.1
“Attached”	Gal, Glu, Man	109.6-150.6	Gal	28.0

Table 14
Relative absorbance of $Fe(TPTZ)^{2+}$ at 595 nm of some monosaccharides and uronic acids frequently occurring in bacterial EPS, in comparison to glucose

	Relative absorbance,%		Relative absorbance,%
D-glucose [a]	100	D-glyceraldehyde [a]	77
D-mannose [a]	95	D-glucuronic acid [a]	95
D-galactose [a]	96	D-galacturonic acid [a]	71
L-rhamnose [a]	79	D-mannuronic acid [a]	51
L-fucose [a]	74	D-glucosamine [b]	113
D-arabinose [a]	88	D-galactosamine [b]	91
D-xylose [a]	105	D-mannosamine [b]	100

a. Myklestad et al., 1997; b. this study.

IV.5.Comprehensive Interpretation of Pu-enriched Colloidal IEF Extract from REFTS Soil and Its Implication to Pu/Th Binding

The IEF spectra of two bacterial EPS (“non-attached”) and RFETS soil water extract (1KDa-0.45μm) all demonstrated that ^{234}Th and ^{240} Pu were mainly complexed by a negatively charged organic macromolecule, with isoelectric points around 3. ^{234}Th (IV) could track ^{240}Pu (IV) very well, no matter if it was ambient or laboratory spiked. This indicates that both actinides have similar complexation behavior with certain fraction of organic matte. Furthermore, ^{234}Th, due to its simple redox state, could be used as a screening tool to study the potential immobilization of different inorganic and organic phases for ^{240}Pu (IV), which has different other redox states in the environment.

For the Pu-enriched IEF extract from RFETS soil, only 32.3% of total carbohydrates measured by the TPTZ method were identified by GC-MS method. This suggests the possible presence of other types of sugar, e.g., amino sugars, which were not detected by the latter method. The monosaccharide composition of this IEF extract showed a much more diverse and evener distribution than that of EPS harvested from pure cultures, which, on one hand, indicates the different and more variable origins of this natural COM. It’s not surprising since these macromolecules were produced naturally on a massive scale with contribution of many other sources, e.g., cell walls, feces of plant-eaters, detritus of plant tissue, etc. On the other hand, it has been demonstrated by Amon and Benner (2003) that the more degradation the natural organic matter has undergone, the more uniform and heterogeneous composition of the monosaccharides would present. This is very constrast to the freshly produced bacterial

EPS, which displayed a much less even monosaccharide composition with only one or two monosaccharides predominant and trace amounts of others sugars. Besides, it has also been suggested that percentage of polysaccharides in NOM (%, OC) is a good indicator of the diagentic state and lability of DOM, i.e., the fresher the DOM, the higher polysaccharide percentage (%, OC) (Skoog and Benner, 1997; Amon et al., 2001; Benner, 2002 a, b; Amon and Benner, 2003). The relatively low polysaccharide content in the Pu-enriched IEF extract from RFETS soil COM (~10% of OC), compared to the relatively high polysaccharide percentage in fresh bacteria-derived EPS (~30-40%) in this study, as well as the fairly uniform monosaccharide composition, indicates that this natural COM has undergone considerable decomposition.

Moreover, only ~14.9% of the organic carbon of the IEF extract of this RFETS soil colloidal fraction was characterized in this study, as the sum of carbohydrate-C and protein-C. A large unknown fraction, as strongly indicated in the ATR-FTIR spectrum, was attributed to lipids, as fatty acids or fatty esters. 2-D NMR (2DHRMASHSQ) spectra showed numerous alkyl esters and alcohols as components in this IEF extract (Xu et al., 2007 manuscript in preparation), in agreement with the results from ATR-FTIR. Further characterization using these methods is needed. The co-existence of polysaccharides, proteins and lipids in the Pu/Th-enriched-low pH_{IEF} extract suggests that this macromolecule is very complex. In contrast, the two regions which are characteristic of aliphatic C-H bonds in the ATR-FTIR spectrum were relatively low for the two bacterial EPS, suggesting that lipids, even present, might be negligibly low. However, this might be due to different extraction and purification methods applied for

these two kinds of substances since for RFETS soil colloid (water extract), before the application of IEF, only physical centrifugation, filtration and ultrafiltration methods were used; while for the bacterial EPS, both extensive chemical and physical extraction and purification methods were applied, e.g., solvent extraction, alcohol and TCA precipitations, thus most of the lipid fraction might have been already preferentially removed during the purification steps. Anionic hydrophilic functional groups, e.g., carboxylate, phosphate and sulfate have been proposed as the strong binding ligands for A-type metals, e.g., Th and Pu (Quigley et al., 2002; Santschi et al., 2003; 2006; Roberts et al., 2004). Carboxylate as a strong Pu bindng ligand (Neu et al., 2005; Alvarado Quiroz et al., 2006), was found in high abundance in this IEF extract, by the technique of ATR-FTIR. Phosphate was relatively depleted compared to the original colloidal water extract. The presence of sulfate was uncertain and needs to be confirmed. Lipids, polysaccharides, lipopolysaccharides, glycoproteins or proteoglycans are all possible "carriers" for these functional groups. It's also suggested that the hydrophobic "moieties" might be responsible for regulation of bridging flocculation and coagulation (Stenström, 1989; Ahimou et al., 2001; Wilkinson and Reinhardt, 2004; Reinhardt, 2004), which might change the surface activity, i.e., making more hydrophilic ligands avaible for Th/Pu binding. As a matter of fact, the hypothesis that the presence of hydrophobic moieties, by giving the macromolecule an amphiphilic property, would effectively enhance the metal complexation has been tested (Roberts et al., 2007, submitted and in review; Schwehr et al., 2007, manuscript in preparation).

V. SUMMARY

Microbial extracellular polymeric substances (EPS) are ubiquitous in the natural environment and found to be in dissolved or colloidal forms and/or associated with particulate material, e.g., TEP in the marine systems. They are mainly produced by microorganisms, i.e., bacteria and algae, thus their physico-chemical properties and composition might be greatly influenced and regulated by different factors, such as pH, temperature, salinity, light, physical conditions of the media (solid agar vs. liquid broth), carbon sources, nutrients, growth stages, etc. Moreover, the extraction and purification methods applied would drastically influence the final product for later analysis. Generally, the complexity of the methods chosen for extraction and purification of EPS would greatly depend on the composition of the medium and the target of interest, e.g., polysaccharides vs. protein vs. the whole EPS of their native state as source material for experimentation of their complexation ability towards actinides and other trace metals, as well as of their surfactant properties.

The first objective of this thesis was to standardize both the laboratory extraction and purification methods for EPS produced by marine bacterium, *Sagittula stellata* and soil bacterium, *Pseudomonas fluorescens* Biovar II. Cost-effective methods were developed based on the criteria of (1) minimal cell disruption and avoiding inclusion of intracellular material, (2) minimal inclusion of non-EPS components, i.e., broth material, (3) optimal yield of EPS, (4) optimal integrity of biomolecules, i.e., avoiding modification of the EPS structure, e.g., hydrolysis or denaturing. Compared to previous studies, these improved methods resulted in great reduction in the usage of alcohol, in

the presence of any broth "impurities", and in processing time. This was achieved by monitoring the EPS varieties (carbohydrates, proteins, uronic acids, and/or nucleic acids) by spectrophotometric methods, the relative composition (carbohydrate-C to protein-C ratios) and molecular weight distribution ("fingerprint") by SEC under different extraction conditions and at each purification step. It turned out that 0.5 N HCl was the best extractant for *Sagittula stellata* capsular EPS extraction, regardless of extraction time, while extracting with 0.05 N NaCl for three to five hours was the best condition for *Pseudomonas fluorescens* Biovar II capsular EPS. The previous method using 3% NaCl for *Pseudomonas fluorescens* Biovar II capsular EPS might probably have caused some cell lysis and introduced intracellular material into solution, as indicated by both nucleic acid content and molecular weight distribution. Further purification was required by prefiltration and dialfiltration in order to effectively remove the relatively low molecular broth material and pre-concentrate the EPS solution as well. Post-filtration step could effectively remove a large fraction of protein from the EPS. Further once ethanol precipitation followed by once TCA precipitation could effectively yield a "low-protein-polysaccharied-enriched" EPS variety for *Sagittula stellata*, while twice ethanol precipitations followed by once TCA precipitation would be required for *Pseudomonas fluorescens* Biovar II for the same purpose.

The second objective of this study was to monitor the EPS production and composition at different growth stages. Here both "non-attached" and "attached" EPS were extracted and initially purified until the ultrafiltration step, in order to study the whole EPS in their native state. EPS varieties (carbohydrates, proteins, uronic acids and

nucleic acids), composition (carbohydrate-C to protein-C ratio) and individual monosaccharide composition did change greatly as a function of growth state, indicating (1) that bacteria were capable of producing more than one type of exopolymer; (2) the proportion of different exopolymers was varying during the bacterial life cycle; (3) the EPS specific production rate was different at different growth stages. However, the molecular weight distribution remained constant throughout the life cycle. For *Sagittula stellata*, the end of the stationary phase would be the optimal time for harvesting while the end of exponential phase would be the best time for harvesting EPS produced by *Pseudomonas fluorescens* Biovar II, based on the highest polysaccharides yield of both "non-attached" and "attached" EPS. The results of EPS production and composition, as a function of growth phases, would provide the base and model for more systematic studies on influences of different growth factors, e.g., carbon sources, N/P, pH, etc. on the EPS production and composition in the future, in order to select the best conditions favorable for optimal yield of the target of interest.

The third objective of this thesis was to establish the method of derivatization of neutral sugars and uronic acid and detection by GC-EI-MS. A method by Walters and Hedges (1988) was successfully operated with a little modification. High precision and low detection limit for simultaneous determination of both types of sugars were obtained. The sum of individual monosaccharides obtained from GC-EI-MS agree very well with that of the TPTZ method for *Sagittula stellata* EPS, while the "recovery" appeared relatively low for *Pseudomonas fluorescens* Biovar II and Pu-enriched IEF extract from RFETS soil, which could be attributed to the likely presence of amino sugars in these

two types of samples. Uronic acid measured by GC-EI-MS agreed also well with that measured by sulfamate/m-hydroxydiphenyl method if an occasional “browning” caused by excessive neutral sugars was substracted from the final “pink” color. The improved and modified extraction and purification methods were demonstrated to require less broth material and effectively decrease the protein content, without the introduction of any new contaminant.

The fourth objective of this thesis was to use the well extracted and purified bacterial EPS as “model” substances to help understand the colloidal vector responsible for Pu dispersal in the environment. The Isoelectric Focusing (IEF) spectra of these polysaccharide-enriched-low-protein exopolymers, when compared to that of RFETS soil colloid (1KDa-0.45μm), displayed a very similar Th/Pu distribution along the pH gradient. Th/Pu activity was focused in a low pH around 3, which was related to macromolecules with relatively abundant anionic functional groups, thus making their surface negatively charged at neutral pH values. Analysis by the ATR-FTIR technique suggests that this IEF extract at low pH_{IEF} was much more complex than the EPS extracted from pure cultures, due to their more complex origins and more “decomposed” characteristics. A co-existence of lipids, protein and polysaccharides was suggested. Strong peaks characteristic of carboxylate, which is a strong Th/Pu binding ligand, were shown in the ATR-FTIR spectra for the IEF extract. It’s unlikely that the purified EPS have a significant lipid content, as judged from the spectrum of ATR-FTIR. However, peaks characteristic of carboxylate groups were prevalent in both EPS spectra. Phosphate, another strong actinide-binding ligand, was relatively low in this IEF extract

compared to that in the original water extract. Sulfate, as a weakly actinide-binding ligand, are also possible, though with some uncertainty. Therefore, Pu/Th binding might be more determined by the availability of binding sites, i.e., acidic functional groups, such as carboxylate, phosphate and sulfate, rather than the exact specific compounds. The hydrophobic moieties, e.g., proteins and lipids, etc., were shown to give amphiphilic surfactant properties to these macromolecules, enhancing the surface activity and thus their binding capacity to natural particles that also contain hydrophobic moieties due to their natural organic carbon content. Thus, by choosing the proper extraction procedure, it should be possible in the future to obtain EPS with different surfactant properties to carry out experiments to further test the possibility for mobilization and bioremediation of Pu and other actinides in soils, and/or the binding of Th(IV) and particle attachment of the carrier-EPS molecules to marine particles.

VI. PERSPECTIVES

More studies will be needed to study the influence of different factors, e.g., carbon sources, N/P, on the EPS production and composition. Chemically-defined media, composed of only small molecules less than 1 KDa, could be considered to substitute for the complex synthetic commercial soy broth or marine broth. Therefore, it would not only be easier to manipulate the medium constituents to examine the response of EPS production and composition to various growth factors, but also easier to regulate the interference from dissolved molecules of the medium.

Since EPS is most likely composed of more than one type of polysaccharide, an isolation of these polymers by a combination of size exclusion chromatography and anion exchange chromatography is needed to separate them from both types of EPS, i.e., "non-attached" and "attached" EPS produced by the two bacteria species, respectively. In this study, three different individual exopolysaccharides (F1, F2 and F3) have been successfully isolated from the "non-attached" EPS produced by *Pseudomonas fluorescens* Biovar II. Moreover, the analysis of the monosaccharide composition of each polymer by GC-EI-MS that was carried out would be the first step to elucidate their structure.

The hypothesized unknown sugar fraction, i.e., amino sugars, could be analyzed by using high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD), according to Kaiser and Benner (2000).

Total lipid content in both the bacterial EPS and Pu-enriched colloidal IEF extract from RFETS soil could be extracted using a modified Blight/Dyer method (Blight and

Dyer, 1959; Cifuentes and Salata, 2001) and derivatized to their individual fatty acid methyl esters and then measured by GC-EI-MS (Ackman et al., 1964 a, 1964 b, 2006; Bannon et al., 1986, 1987; Craske et al., 1987; Christie, 1993).

For further experimentation in actinide binding and immobilization, an improved method for removing most of the protein from EPS would be needed to yield an “extremely” low protein variety, e.g., by a combination of proteinase and TCA precipitation. The optimal conditions of proteinase digestion for the bacterial EPS, e.g., pH, temperature, cation concentration, stabilizer, etc., are needed to make further progress. Thus, more Pu/Th binding experiment could be carried out on the EPS with high versus low protein content in order to understand the contribution of hydrophobic moieties of EPS molecules to their attachment to particles, and the complexation of actinides with the acidic functional groups in EPS.

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APPENDIX I

REHYDRATION SOLUTION FOR IEF EXPERIMENT

Table 15
Composition of rehydration solution (Immobiline DryStrip Kit for 2-D Electrophoresis Instruction manual, Pharmacia Biotech/Amersham Biosciences)

Compound	Final Conc.	Amount
Urea	8M	12g
CHAPS	2%	0.5 g
IPG buffer	2.0%	500 ul
Bromophenol blue	trace	Trace
Double-distilled water		16 ml
DTT (dithiothreitol)		7mg/2.5 ml stock solution. Add before use

APPENDIX II

ISOELECTRIC FOCUSING ELECTROPHORESIS

Table 16
Current gradient profile for IEF

Phase	Voltage	mA	W	Time (hr)	V_h
1	300	1	5	0.01	1
2	300	1	5	6.5	1,950
3	2000	1	5	5	5,750
4	2000	1	5	6	12,000
final	2000	1	5	17.5	19,700

APPENDIX III

MASS SPECTRUM OF INDIVIDUAL MONOSACCHARIDE

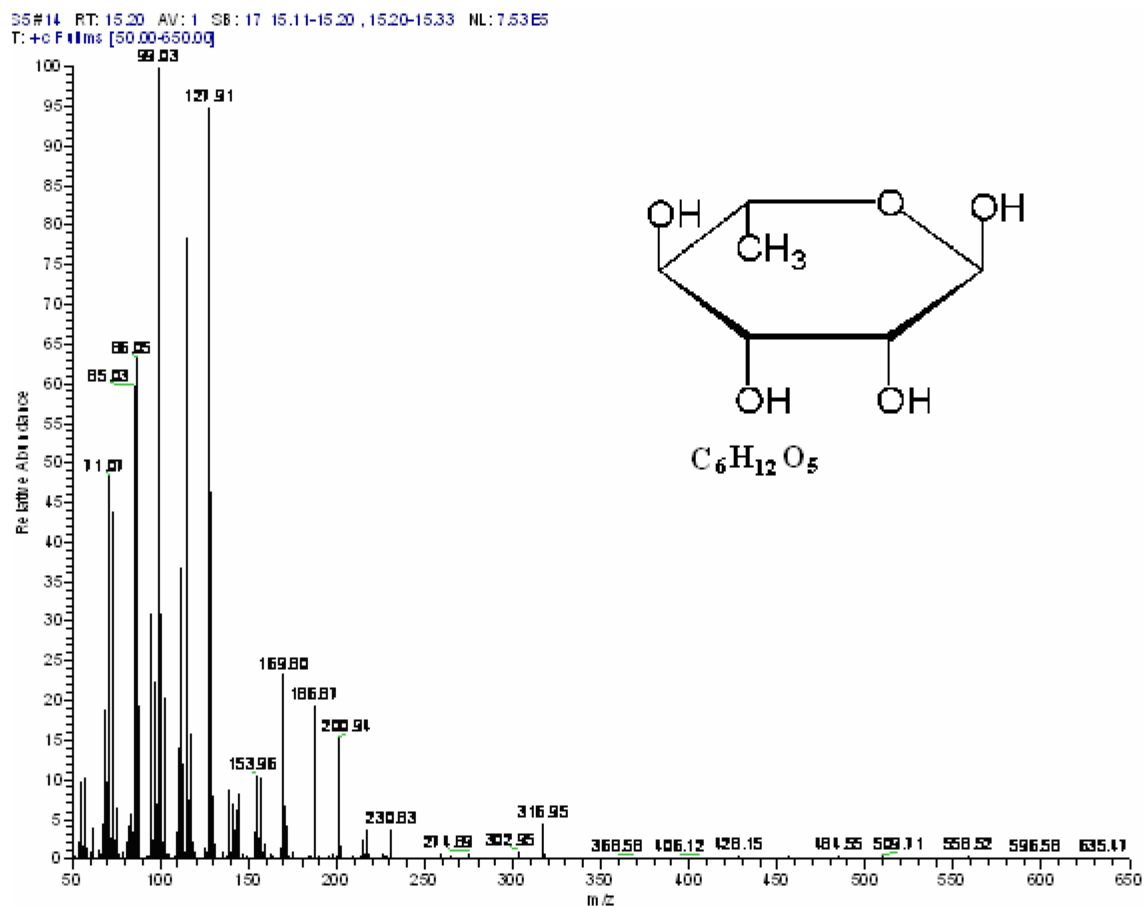

Figure 44 Mass spectra of derivative of Rhamonose

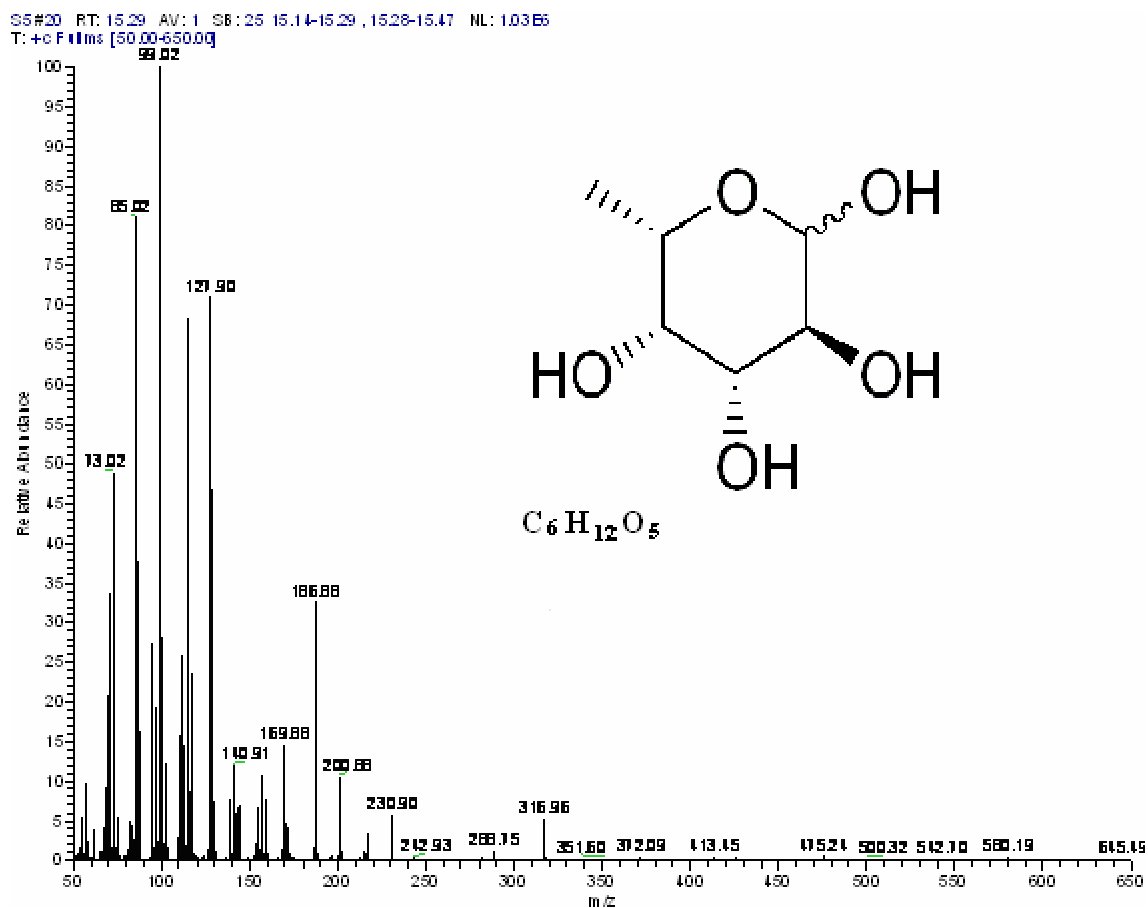

Figure 45 Mass spectra of derivative of Fucose

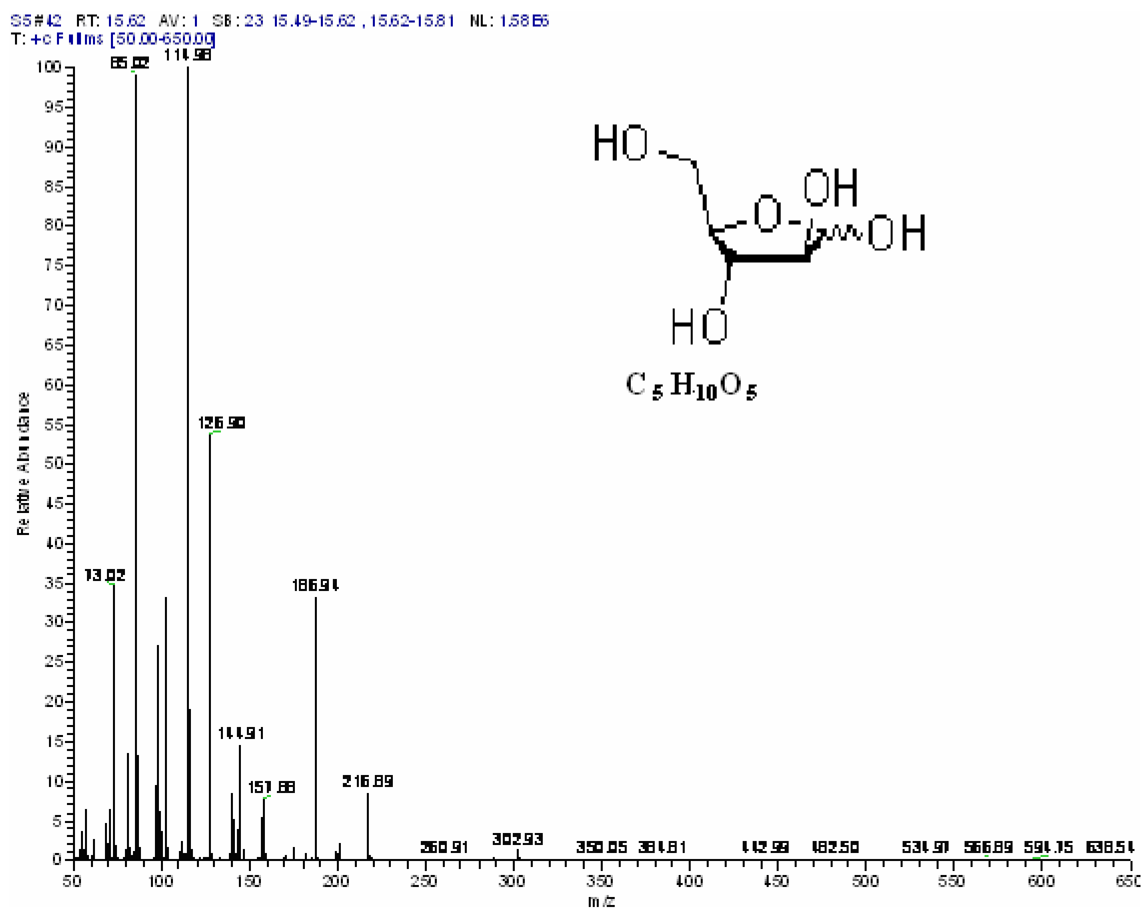

Figure 46 Mass spectra of derivative of Arabinose

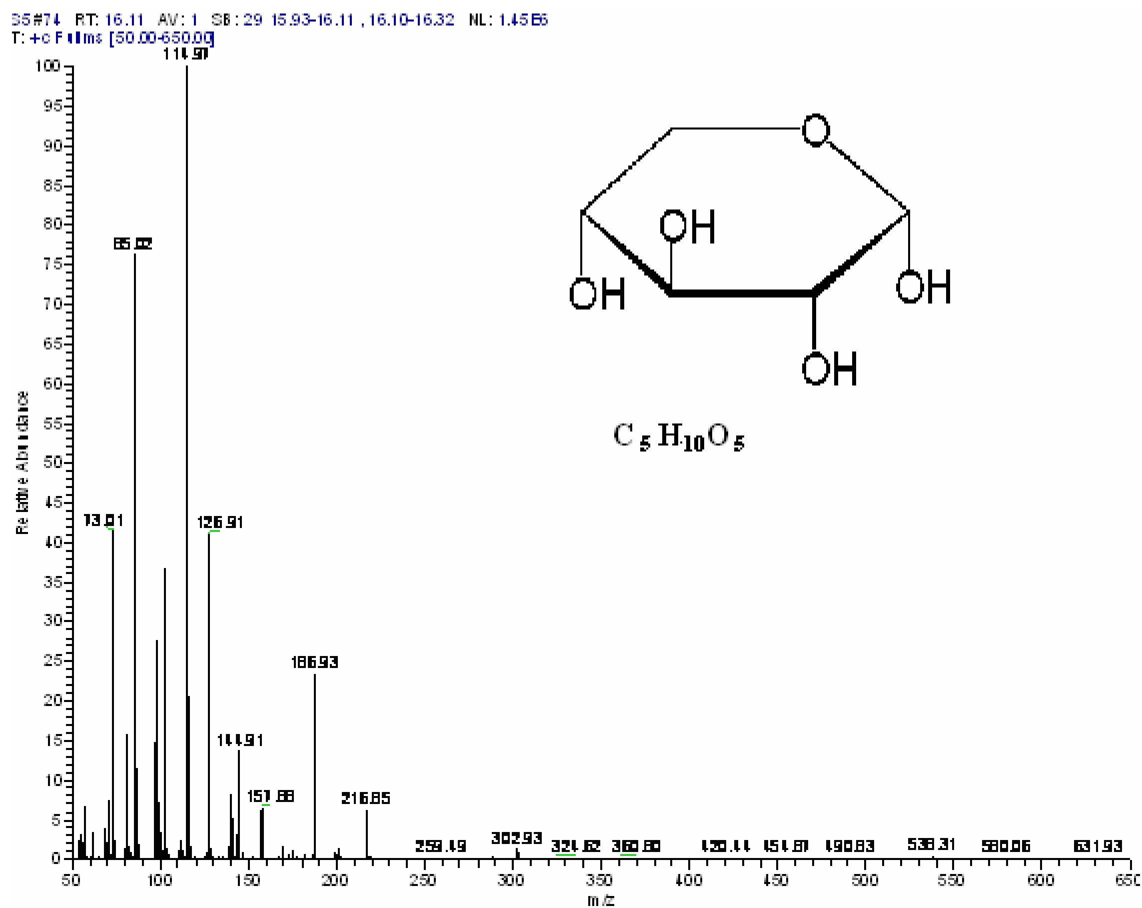

Figure 47 Mass spectra of derivative of Xylose

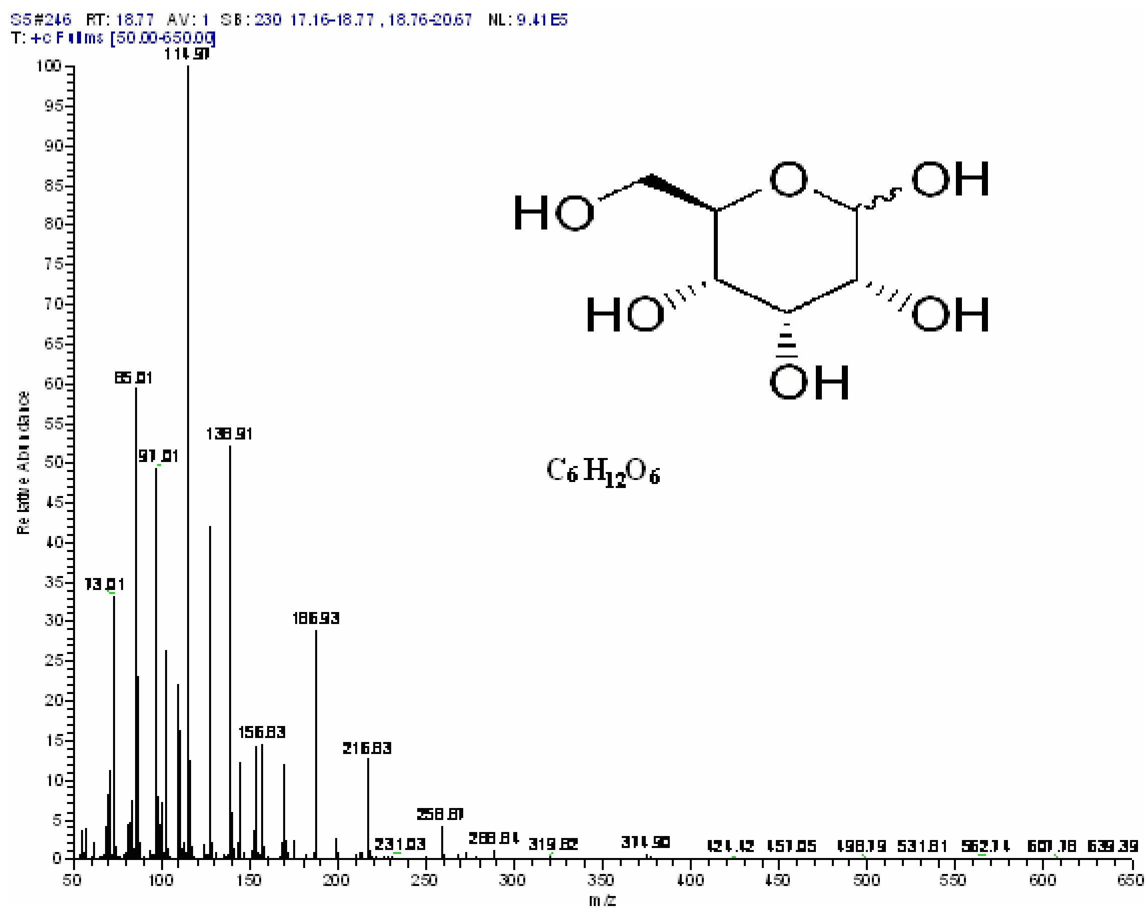

Figure 48 Mass spectra of derivative of Allose

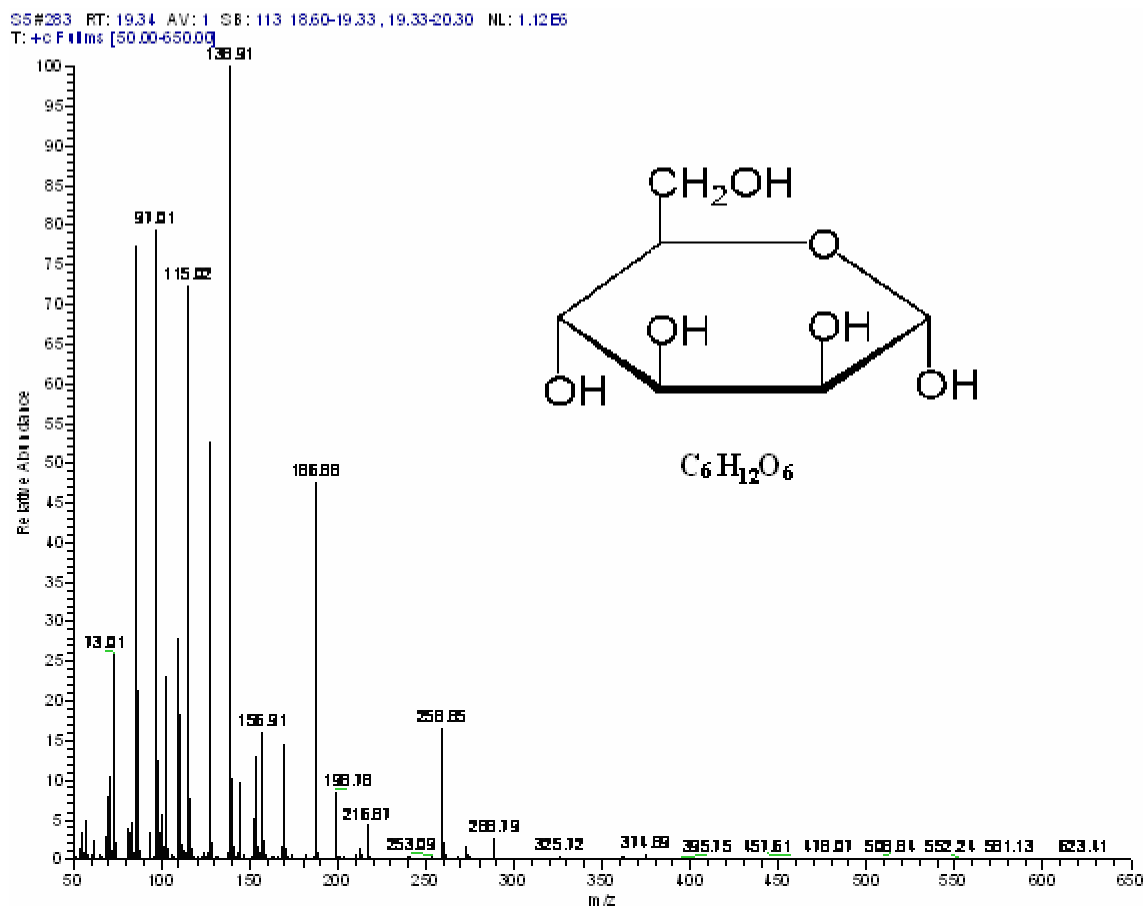

Figure 49 Mass spectra of derivative of Mannose

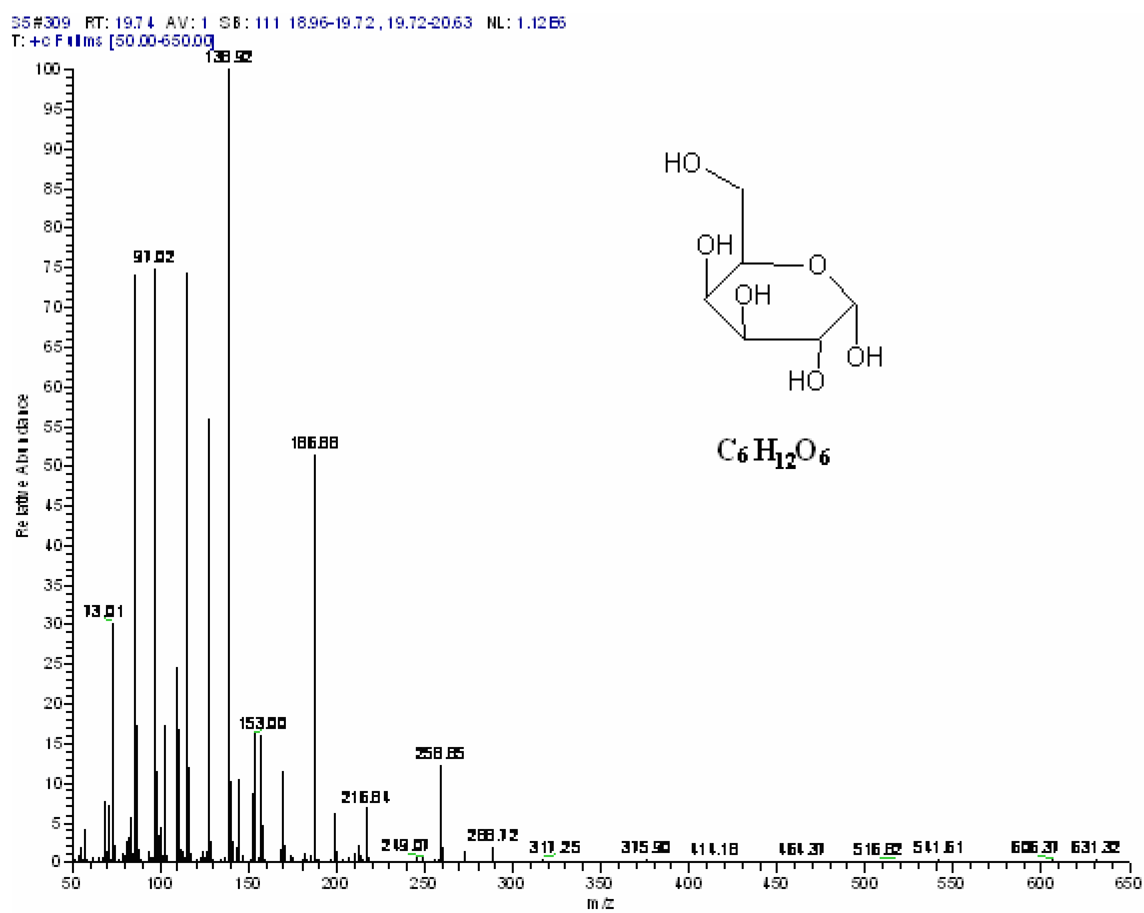

Figure 50 Mass spectra of derivative of Galactose

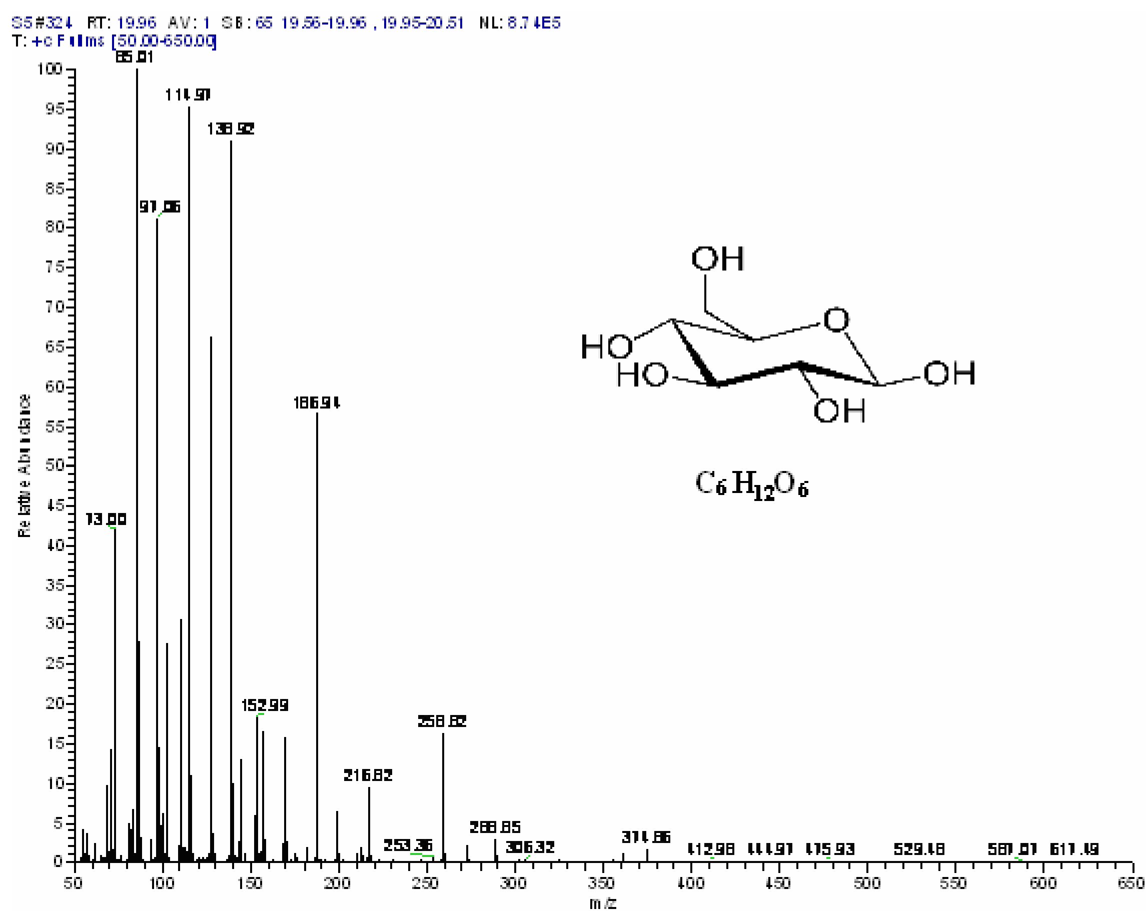

Figure 51 Mass spectra of derivative of Glucose

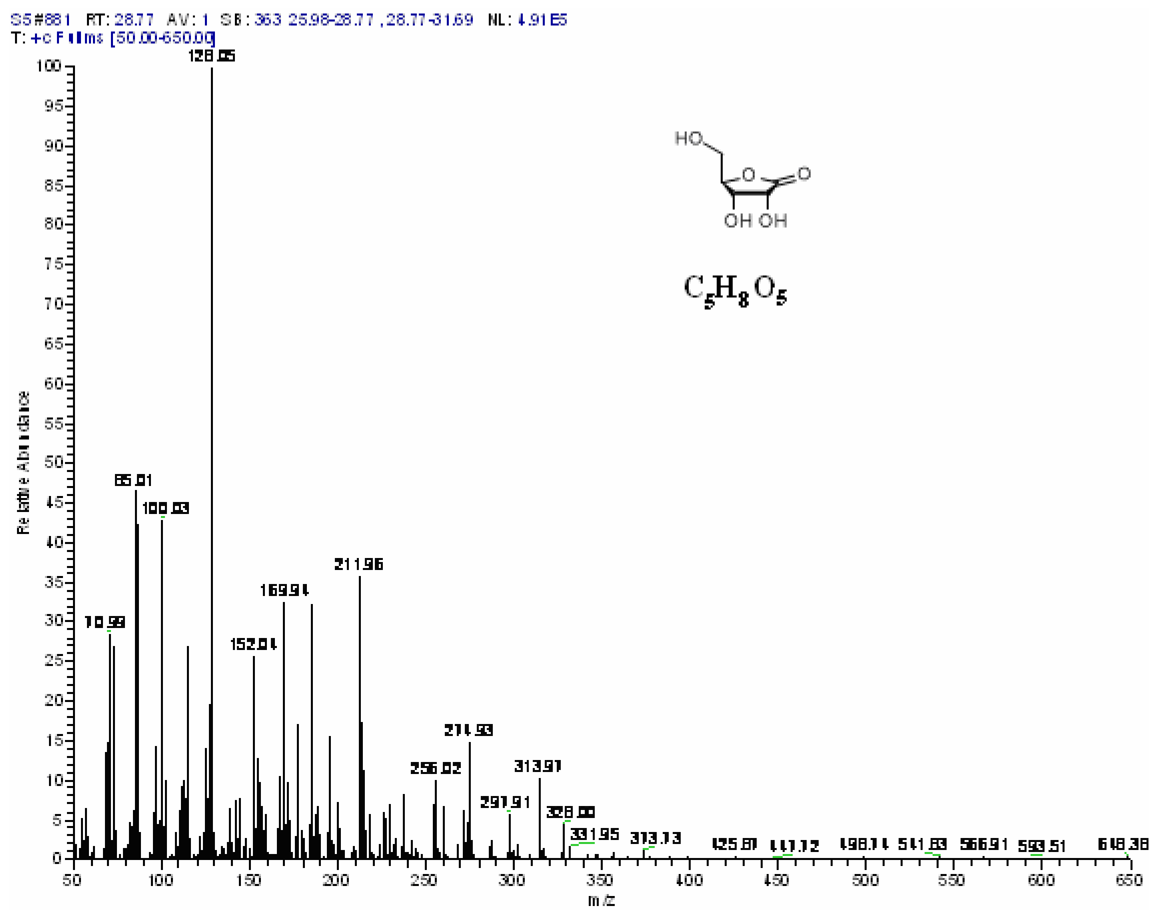

Figure 52 Mass spectra of derivative of Ribonolactone

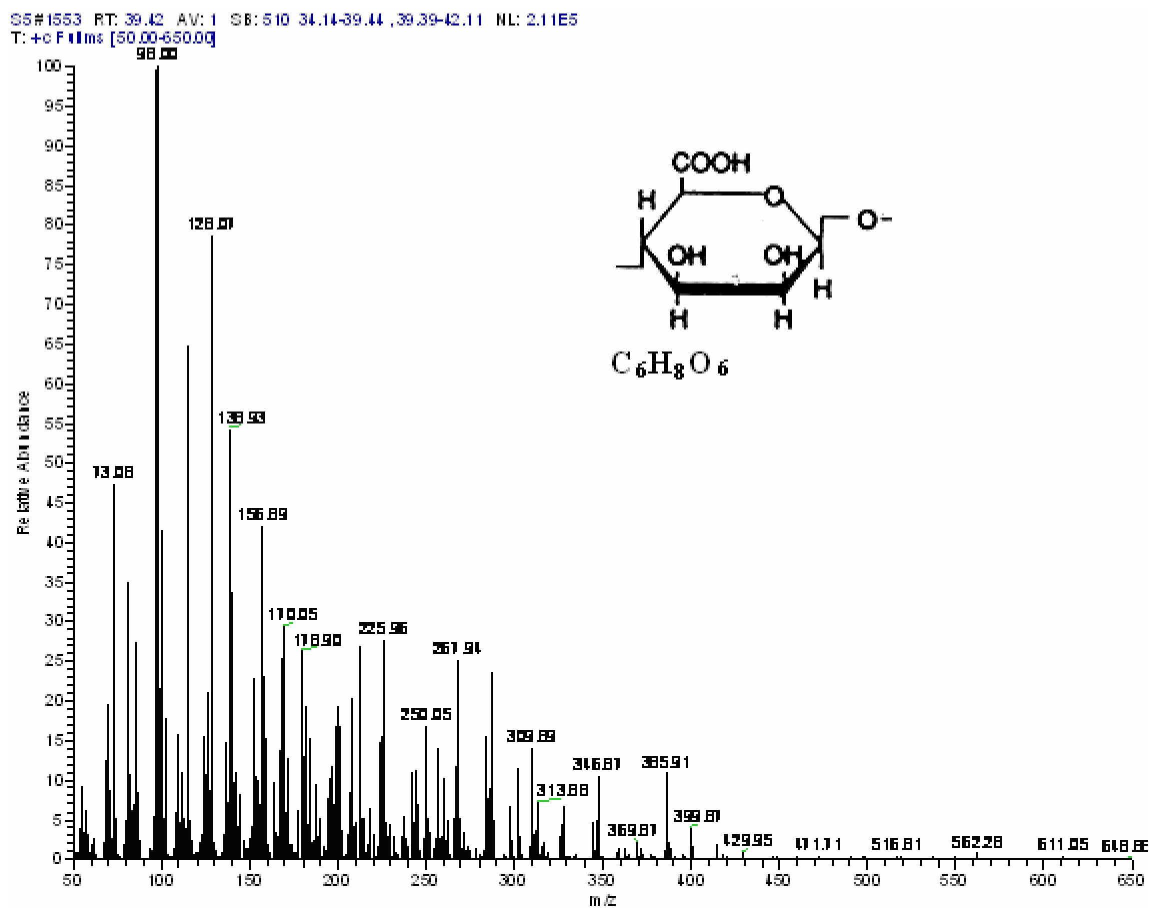

Figure 53 Mass spectra of derivative of D-mannuronic acid lactone

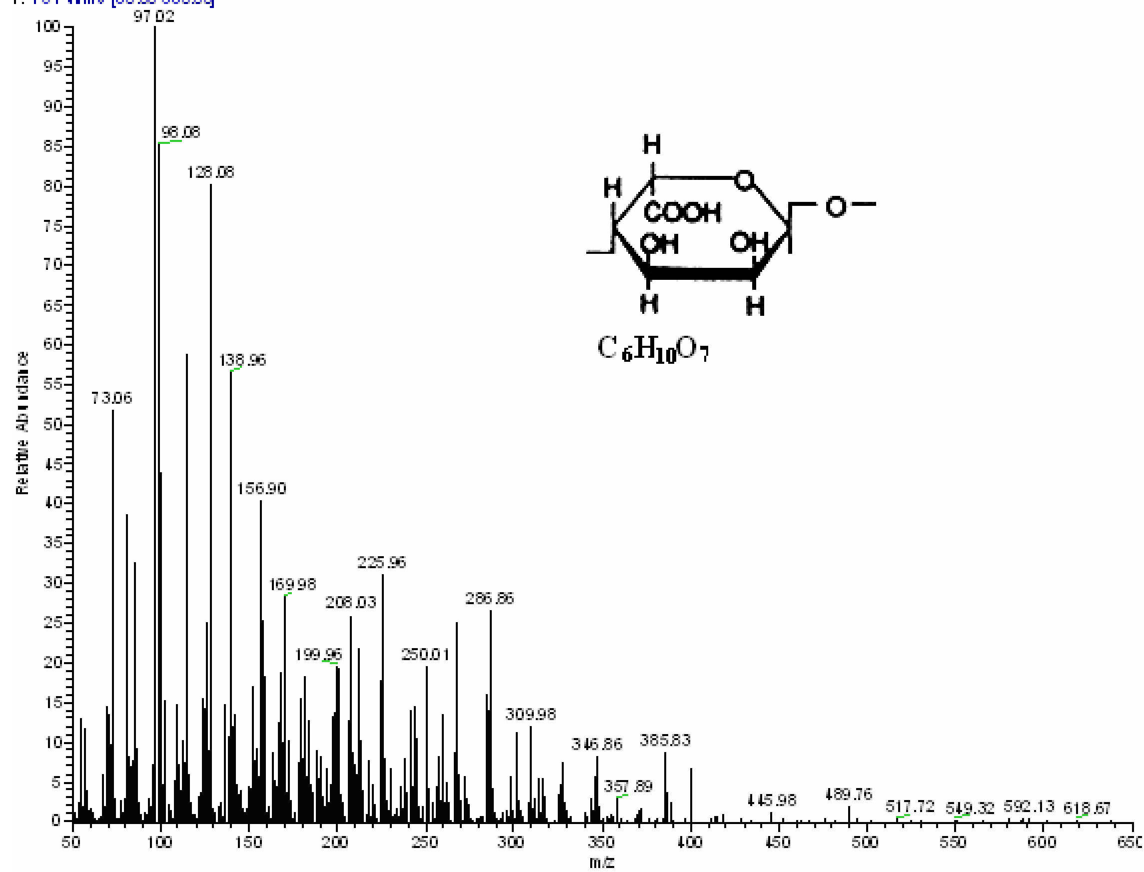

Figure 54 Mass spectra of derivative of Glucuronic acid

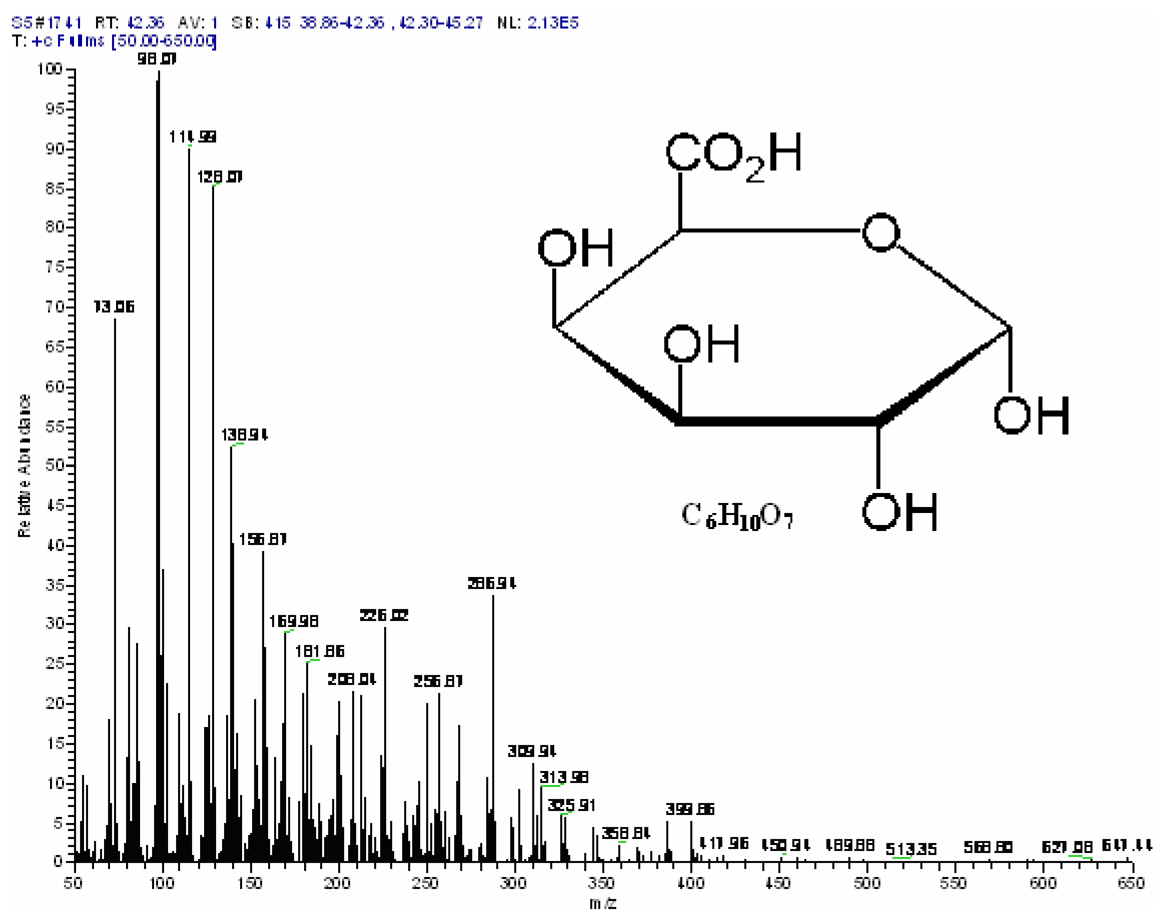

Figure 55 Mass spectra of derivative of Galacturonic acid

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