

CHAPTER 3

Chapter 3

3.1 Introduction

Microorganisms (bacteria, phytoplankton and flagellates) produce high molecular weight, hydrated polymeric compounds called exopolysaccharides (EPS) during their life-cycle (Ducklow and Mitchell 1979). EPS may exist as capsules, sheaths, slimes (loosely attached to the cell wall), apical pads or mesh-like fibrils in the natural environment (Beveridge and Graham 1991, Costerton et al 1992, Herndl 1993, Hoagland et al 1993, Takeda et al 1998). Capsules are tightly bound to the cell wall by non-covalent linkages where as sheaths are linear EPS-containing structures surrounding chain of cells. Slime layer is a less organized form of capsule or sheath that diffuses into the surrounding environment (Wingender et al 1999). Microorganisms produce different forms of EPS to perform diverse functions.

Although not an essential feature, EPS does provide structural and functional stability to microbial assemblages in the natural environment (Decho 2000). Most microorganisms produce EPS either for attachment to substratum (adhesion), formation of micro-consortium/biofilms or binding to other particulate matter (cohesion or aggregation). EPS produced for attachment by microorganisms may influence biofouling by conditioning the substratum (Characklis and Escher 1988). Other functions like gliding motility, protection against osmotic shock, predation, desiccation and detoxification of toxic compounds, nutrient sequestering, chelation of metals, horizontal transfer of

genetic material etc. have also been attributed to microbial EPS (Decho 1990, Hoagland et al 1993).

EPS in the natural environment is generally heteropolymeric (made of different monomeric units) (Decho 1990, Wingender et al 1999). EPS contain non-sugar components like uronic acid, methyl esters, sulphates, pyruvates, proteins, nucleic acids and lipids (Corpe 1980, Sutherland 1980, Cadieux et al 1983, Hoagland et al 1993, Wingender et al 1999, Sutherland 2001). EPS also contain divalent metal cations that act as ionic bridges linking adjacent polysaccharide chains (Rees 1969 & 1972, Fletcher 1980). The presence of side-linkages and organic molecules influence the overall charge, stability, binding capacity, rheology and solubility of the polymer (Hoagland et al 1993).

Many bacterial cultures produce different types of EPS during its life-cycle. For example, most bacteria produce capsular form of EPS during the exponential growth phase and slime type EPS during the stationary growth phase (Decho 1990). Similarly, the chemical characteristics of EPS changes with the age of the culture (Gloaguen et al 1995), nutrient levels (Williams and Wimpenny 1978, De Philippis et al 1991) and growth conditions (Smith and Underwood 1998, Jahn et al 1999).

In the marine environment, the bacterial numbers are in the range of 10^5 to 10^8 ml⁻¹ (Cho 1991, Ramaiah et al 1999, Bode et al 2001). Moreover, up to 45% of organic carbon like glucose incorporated by bacteria is converted into EPS, which is then released into the surrounding waters (Stoderegger and

Herndl 1998). Heissenberger and Herndl (1994) have shown that metabolically active bacteria produce EPS throughout their living period and may contribute to the EPS concentrations in marine waters, especially in oligotrophic growth conditions. Based upon the information available on the ubiquitous distribution of bacterial EPS in the oceans and its contribution to the organic carbon pool (Stoderegger and Herndl 1998), it is clear that bacterial EPS might play an important role in regulating various marine processes and fuelling the marine food-web (Decho 1990). However, most of the laboratory and *in situ* studies on the factors regulating important marine processes like aggregation, flux and trophic web have focused on the role of phytoplankton and its extracellular polymers (Decho 1990, Sell and Overbeck 1992, Passow et al 1994, Niven et al 1995, Passow and Alldredge 1995, Passow et al 2001, Prieto et al 2001). Thus, very little is known about the behaviour of bacterial EPS in the marine environment despite its wide distribution and high abundance.

A seasonal variation in the distribution of EPS as discrete particles (TEP) or in association with other particulate matter has been observed in near-shore waters (see Chapter 2A). Some forms of EPS showed good correlation with bacterial production suggesting its utility as a possible organic carbon source. On the other hand, EPS in the form of TEP was inert to bacterial activity and appeared to be a major sink of organic carbon in these waters (Chapter 2B). Keeping in mind the ecological significance of the EPS in general and the limited information available on the role of bacterial EPS in marine processes, a study was carried out to characterize the bulk and molecular chemistry of

EPS isolated from a marine bacterium. Furthermore, experiments were also carried out to assess the effect of bacterial growth conditions on EPS production.

3.2 Material and Methods

3.2.1 Isolation and screening of bacterial cultures for EPS production

Surface seawater samples from Dona Paula Bay were collected in 500 ml sterile flasks. Similarly, sediment samples were collected from Dona Paula and mangrove forests along the Zuari river. Macroalgal samples were collected from the intertidal regions of Dona Paula Bay. In order to isolate bacterial cultures, sediments and macroalgal samples were suspended in sterile filtered seawater, vortex mixed, serially diluted and appropriate dilutions were plated on Zobell Marine Agar (ZMA) plates. Similarly, seawater samples were serially diluted and plated on ZMA plates. The plates were incubated at room temperature ($30 \pm 2^\circ\text{C}$) for 48 h. Bacterial colonies with mucoidal appearance or colonies that had a sticky surface were picked up and purified by repeated streaking on ZMA. On an average 5 to 7 colonies were randomly selected from each plate during every sampling for further purification. The purified cultures were then transferred to ZMA slants and stored at 4°C . Bacterial cultures obtained from the Materials & Material Research Division (MCMRD) culture collection were also screened for their potential to produce EPS.

Loopful of cultures from the slants were transferred into test-tubes having 5 ml of BSS medium (Appendix IA) supplemented with 1% glucose as the sole source of carbon and grown at room temperature ($28 \pm 2^\circ\text{C}$) for 24-48 h. The cultures were sub-cultured twice in the same medium before measuring their EPS production. Cells were centrifuged at 10,000 rpm (16750 g) for 30 mins, supernatant was filtered through 0.22 μm filters and dialysed against glass-distilled water at 4°C using 8000 daltons (8 kDa) MW cut-off dialysis bags (Decho 1990). After dialysis, the dialysate was made to a known volume and analysed for total carbohydrates using the phenol-sulphuric acid method (Dubois et al 1956). Bacterial isolate that produced the highest amount of EPS was chosen for further studies.

3.2.2 Identification of the selected culture

The selected EPS producing bacterium was identified using both conventional physiological and biochemical tests followed by the molecular identification method based on 16S-rRNA sequencing.

a) *Conventional identification methods*- The bacterial isolate was studied for morphological, physiological and biochemical characteristics following standard methods described by Gerhardt et al (1981). The culture characteristics and biochemical characteristics were compared with those given in Bergey's Manual of Systematic Microbiology (1984). Using these methods, the culture could be identified up to its family. For further identification, molecular methods were used.

b) *Molecular identification method*- The 16S-rRNA sequencing procedure can be divided into 3 stages: i) chromosomal DNA extraction, ii) gene amplification by polymerase chain reaction (PCR) and iii) DNA sequencing and analyses.

i) *Chromosomal DNA extraction*

The chromosomal DNA was extracted by repeated freezing (-80°C) and heating (at 95°C) of the bacterial cells for 30 min each in Tris buffer at pH 8. The cells were centrifuged at 10,000 x g for 10 min at 4°C to separate the cells. To the supernatant, few microlitres of proteinase K, DNAase free RNAase and SDS were added and incubated at 37°C for half an hour. Further extraction and precipitation was done following Sambrook et al (1989).

ii) *Gene amplification*

An approximately 1500 bp-segment of the 16S-rRNA gene was amplified by PCR with a pair of primers targeting the 5' end (sequence-AGAGTTTGATC(AC)TGGC) and the 3' end (sequence-TACCTTGTTACGACTT; MWG, Germany), respectively. The PCR cycle involved denaturing of the strand at 95°C for 5 min followed by 30 cycles of annealing (for 1 min at 40°C), extension (for 3 min at 72°C) and denaturing at 95°C (for 1 min). A final extension for 10 min at 72°C was carried out before the amplified sequences were loaded on 0.8% agarose gel and separated by gel electrophoresis. The gel was then immersed in ethidium bromide for 2 h

and the products were detected using a transilluminator. The PCR products were then purified using QIAquick Purification Kit (Qiagen, USA).

iii) *DNA sequencing and analyses*

The DNA sequencing was carried out using DYEnamic Direct cycle sequencing kit US79535 (Amersham Life Science Inc.) and a Li-Cor Long 4200 automated DNA sequencer (MWG, Germany). Sequencing primers used were GM 3F (5'-AGAGTTTGATC(AC)TGGC-3') and GM 8R (5'-TGGGTATCTAATCCT-3', MWG, Germany) and were labelled with IRDyeTM800. The sequencing protocol consist of an initial denaturing step at 95°C for 5 min, followed by 30 cycles of denaturing 95°C for 0.5 min, annealing at 40°C for 0.5 min and 1 min extension at 70°C. The reaction was immediately cooled to 4°C and maintained till loading in the sequence gel. Electrophoresis was carried out at 45°C for 16h (Biocym, Germany). The sequences thus obtained were aligned and compared with those available in the Genebank of the National Centre for Biotechnology Information (NCBI), USA (Altschul et al 1990).

3.2.3 Optimization of growth conditions of Marinobacter sp.

In order to optimize growth conditions, *Marinobacter* culture (1% v/v) was first grown in test-tubes having 5 ml BSS medium (Appendix-Ia) supplemented with 1% glucose and low MW fractions of yeast extract (0.05%) and peptone (0.1%) at $28 \pm 2^\circ\text{C}$ (pH 7.6) for 48 h. Before use, 100 ml 10x stock solutions of

yeast extract and peptone were passed through 10 kDa MW cut-off membrane filters using Amicon filtration system (Amicon, USA) to remove high molecular weight contaminants.

- 1) *Effect of glucose concentration-* *Marinobacter* sp. was grown in BSS medium containing varying glucose concentrations (0%, 0.5%, 1%, 2%, 3% and 5%) to study the effect of glucose concentration on the growth and EPS production.

- 2) *Effect of nitrogen source and concentration-* The effect of nitrogen source was assessed by growing the culture in glucose optimized BSS media supplemented with 0.1% of different nitrogen sources like NH_4Cl , NaNO_3 , urea, $(\text{NH}_4)_2\text{SO}_4$ and KNO_3 . The nitrogen source that enhanced EPS production was selected. The concentration of this N-source was then varied to assess the influence of increasing nitrogen concentration (0%, 0.0026%, 0.0052%, 0.013% and 0.026%) on EPS production.

- 3) *Effect of phosphate concentration-* In order to assess the effect of phosphate concentrations on the growth and EPS production of *Marinobacter*, the culture was grown in BSS medium (optimized for glucose and nitrogen) having increasing phosphate concentrations (0%, 0.017%, 0.034%, 0.056% and 0.085%).

- 4) *Effect of magnesium concentration-* The culture was grown in the presence of increasing magnesium concentrations (0%, 0.0048%,

0.0096%, 0.0168% and 0.024%) to assess the role of magnesium concentrations in EPS production and bacterial growth.

During each experiment, the bacterial growth was monitored by measuring the turbidity at 540 nm. The amount of EPS produced by the culture was estimated by the phenol-sulphuric acid method (Dubois et al 1956).

3.2.4 Growth and EPS production of Marinobacter sp.

The bacterial culture was grown in 1 l capacity conical flask containing 500 ml optimized BSS medium (Appendix Ib) at pH 7.6. The culture was grown for 170 h during which sub-samples (5 ml) were withdrawn at regular intervals for both EPS production and growth. Bacterial growth was estimated by turbidity measurements at 540nm. In order to extract EPS, the cells were first centrifuged at 10,000 rpm (16750 g) for 30 mins and the supernatant (dissolved fraction) was decanted. The dissolved fraction was filtered through 0.22 µm filters. The cell pellet was resuspended in 10mM EDTA solution, vortexed for 15 mins and centrifuged at 10,000 rpm (16750 g) for 10 mins. The supernatant thus obtained was mixed with the dissolved fraction and then dialysed against distilled water using 8kDa MW cut-off dialysis bag for 48 h. During the dialysis period, water was changed every 10 h. After the dialysis, 1 ml of the dialysate was used to estimate EPS as carbohydrates following Dubois et al (1956).

3.2.5 Bulk EPS production for chemical characterization

Pure culture of *Marinobacter* sp. was grown in 2 l flasks containing 1 l of the optimized BSS medium adjusted to pH 7.6. The culture was grown at 28°C ± 2°C over a period of 14 days. At the end of the incubation period, the cells were harvested by centrifugation at 10,000 rpm (16750 g) for 30 mins and the supernatant was collected. The cell pellet was treated with 10mM EDTA as described earlier to extract cell-bound EPS. The EDTA-extracted EPS was pooled with the supernatant EPS solution and filtered through 0.22 µm filters, concentrated at 35°C to less than 50 ml using a rotary evaporator.

The concentrated EPS was then precipitated using ice-cold absolute ethanol (Bhosle et al 1995) while continuously stirring and kept at 4°C overnight. The supernatant was decanted; the precipitate was redissolved in minimum volume of distilled water and dialysed for 48 h using 8 kDa MW cut-off dialysis bags against distilled water to remove salts. During dialysis, the water was changed every 10 h. The high molecular weight EPS retained in the dialysis bag was then reprecipitated, lyophilised and stored at -20°C until further analyses.

3.2.6 Chemical characterization of EPS

The EPS of *Marinobacter* sp. was analysed for its bulk chemical characteristics like total carbohydrates (TCHO), proteins, uronic acid (UA),

sulphates, total carbon and nitrogen. The EPS sample was also analysed for its monosaccharide and amino acid constituents.

3.2.6a Reagent preparation

Protein assay

Bicinchoninic acid (BCA)- BCA reagent for protein analyses was freshly prepared by adding 1 ml of 4% CuSO₄ to 50 ml of BCA solution (Sigma Chemicals USA). The reagent was prepared for immediate use.

Uronic acid assay

Sulfamic acid- Four molar sulfamic acid solution was prepared by adding sulfamic acid in half volume of deionized water under continuous mixing and dissolved by drop-wise addition of saturated KOH. Once completely dissolved, the final volume was made with distilled water. This reagent was used to reduce the caramelization of sugars during uronic acid estimation.

Metahydroxy biphenyl reagent- Biphenyl reagent for uronic acid estimation was prepared by dissolving 150 mg of metahydroxy biphenyl in 100 ml of 0.5% (w/v) NaOH solution. This solution is stored under refrigeration.

Borax reagent- The borax reagent is prepared by adding 2.86 g of sodium tetraborate in 100 ml of concentrated sulfuric acid.

Sulphate assay

BaCl₂-gelatin reagent- The BaCl₂-gelatin reagent for sulphate estimation was prepared by dissolving 0.5% gelatin in warm water (60-70°C). The solution was stored overnight at 4°C. The following day, 0.5% of BaCl₂ was added and the resultant cloudy solution was allowed to stabilize for 2-3 h at room temperature before using for sulphate estimation.

3.2.6b Analytical methods

1. Bulk characterization

Carbohydrates (TCHO)- TCHO concentrations were estimated following the phenol-sulphuric method (Dubois et al 1956) with a slight modification. A sub-sample of the lyophilised EPS was weighed and dissolved in an appropriate volume of deionized water. If the samples were in the form of clear solution, EPS sample was directly used for total carbohydrate estimation. One ml of the EPS solution was mixed with 1 ml of cold 5% phenol and 5 ml of concentrated sulfuric acid was added to develop colour. The intensity of the colour was measured spectrophotometrically at 490 nm. Glucose was used as a standard for calibration of the method.

Proteins- The protein content of the EPS was estimated following Smith et al. (1993). A known amount of the EPS was weighed, dissolved in appropriate volume of distilled water and used directly for protein estimation. In order to analyze the protein content of the EPS, 100 µl of the EPS sample was

transferred into clean test-tubes and 2 ml of bicinchoninic acid (BCA) reagent was added to it. The samples were then incubated at 37°C for 30 min to complete the reaction and develop color. The intensity of the color formed was measured spectrophotometrically at 562 nm. Bovine serum albumin was used as a standard for the calibration of the method.

Uronic acid (UA)- UA content in the EPS was measured following the modified meta-hydroxy biphenyl method of Filisetti-cozzi and Nicholas (1993). The modified method remains the same as the old meta-hydroxy biphenyl method (Blumenkrantz and Asboe 1973) except for the pretreatment with sulfamic acid. A known amount of EPS was dissolved in deionised water and 0.4 ml was taken in a clean test-tube. To this, 40 µl of 4 M sulfamic acid was added and mixed thoroughly. After mixing properly, 2.4 ml of sulfuric acid-tetraborate solution was added and vortex mixed. The test-tube was then placed in boiling water bath (100°C) for 20 min and then immediately placed in crushed ice. Once cooled, 80 µl of metahydroxy biphenyl was added to the mixture, vortex mixed and the colour developed was measured at 525 nm. Galacturonic acid was used as a standard for the calibration of the method.

Sulphate estimation- Sulphates in the EPS were estimated following BaCl₂-gelatin method (Dodgson and Price, 1963). A known amount of EPS was dissolved in deionized water and 1-2 ml of the sample was transferred into glass ampoule and dried under flow of nitrogen gas. To this ampoule, 1 ml of 1 N HCl was added and the sample was flushed with nitrogen. Thereafter, the ampoule was sealed and the sample was hydrolysed at 105°C for 17 h. After

hydrolyses, the sample was cooled and the content was transferred to test-tube containing 3.8 ml of 3% trichloro acetic acid (TCA) solution. To this mixture, 1 ml of BaCl₂-gelatin reagent was added and mixed thoroughly. The solution was kept at room temperature for 15-20 min and the turbidity formed was measured at 360 nm. Potassium sulphate dissolved in 1 N HCl was used as a standard for calibration.

In order to estimate blanks in all the above estimations, deionized water was used in place of the samples and analysed.

Carbon and Nitrogen- Total carbon and nitrogen content of the bacterial EPS was analysed using a ThermoFinnigen CNS analyzer. A known amount of dry EPS sample was weighed accurately in tin foils and combusted in the CNS analyzer. Methionine was used as a standard for the calibration of the method. Empty tin cups were used as blanks.

FTIR analyses- Fourier Transformed Infra-Red absorption spectrum of the EPS sample was obtained following KBr technique. Small amount of lyophilised EPS sample was mixed with KBr crystals using mortar and pestle, placed in cup and compressed to form a thin pellet. The spectrum of the pellet was then obtained using a Shimadzu FTIR spectrophotometer.

2. Molecular characterization

Monosaccharide composition- Freeze-dried EPS sample was weighed and dissolved in a defined volume of deionised water. One ml of the EPS was

transferred into 10-ml ampoule and mixed with 10 μ l of 10M HCl (final concentration 100 mM HCl), flushed with nitrogen, sealed and hydrolysed at 100°C for 20 h. After hydrolysis, the samples were dried at 60°C over a continuous flow of nitrogen gas. The dried samples were suspended in 10 ml of deionized water and passed through ion-exchange Dowex 50 W-X 8 columns (H^+ form, 100 mesh, Sigma USA). Aldoses were then detected amperometrically with a HPLC system equipped with a CarboPac PA 10 column (Dionex, USA) using 20 mM NaOH as an eluent (HPLC-PAD; Borch and Kirchman 1997). The solvents (MilliQ water and 200 mM NaOH) were pumped at the rate of 1 ml/min in a ratio of 0.1 ml NaOH and 0.9 ml MilliQ water. A standard sugar solution containing 100mM concentration of glucose, galactose, rhamnose, mannose/xylose, fucose, fructose and arabinose (Sigma Chemicals, USA) was prepared and used for the calibration of the method.

Amino acid composition- The total hydrolysable amino acid (THAA) composition and concentrations in the EPS were analysed by HPLC with a fluorescence detector (Lindroth and Mopper 1979, Bhosle and Wagh 1997). Suitable amount of EPS was weighed and dissolved in an appropriate amount of deionised water. Equal volumes of EPS sample and 12N HCl, 20 μ l of ascorbic acid and 20 μ l of 100 μ M α -aminobutyric acid (internal standard) were taken in clean glass ampoules, flushed with nitrogen, sealed and hydrolysed at 110°C for 20h. After hydrolysis, the samples were dried completely over a stream of nitrogen at 60°C and reconstituted in 1ml of deionised water.

Fluorescent amino acid derivatives were prepared by mixing 50 µl of the hydrolysate with 50 µl of o-phthalaldehyde (OPA) and 2-mercaptoethanol at pH 9.5 for 2 min. The samples were then separated using reverse-phase HPLC using a binary solvent system i.e. methanol and sodium acetate buffer (40 mM sodium acetate having 3% tetrahydrofuran (THF) at pH 6.0). The retention time of the standards were matched with those of the samples to identify the different amino acids and the concentrations were calculated by integrating the peaks relative to those of standard component peaks. The method was calibrated by running standards having all the 16 amino acids with a concentration of 20 nM each.

For blanks, deionised water instead of samples was used, processed and analysed as above.

3.3 Results

3.3.1 Screening and identification of EPS producing bacterial isolate

About 150 bacterial isolates obtained from various natural samples and departmental culture collection were screened for EPS production. The morphology and the gram characteristics of some of the isolates and their EPS production estimated after 48 h of incubation in 1% glucose supplemented BSS medium is shown in **Table 3.1**. From the table, its clear that isolates P3R and Ti-21d(3) produced the maximum amount of EPS. Based upon its ability for EPS production, a bacterial isolate (P3R) obtained from the sediments of mangrove forest along the Zuari river was chosen for

Table 3.1: Some of the bacterial isolates examined for their EPS production after 48 h of incubation at $28 \pm 2^\circ\text{C}$. The cultures were grown in 1% glucose supplemented BSS medium.

Bacterial isolates	Growth in 1% glucose medium in 48 h	EPS production ($\mu\text{g ml}^{-1}$)	Cell-morphology and Gram characteristics isolated on ZMA plates
Seawater			
SW 1	++	9.5	Gram negative cocco-bacilli
SW 2	+	3.6	Gram negative short thick rods
SW 4	+	2.6	Gram negative long rods
SW 6	+	8.9	Gram negative, short thick rods
SW 6b	++	12.6	Gram negative coccobacilli
SW 7	++	9.1	Gram negative coccobacilli
SW 10	⊥	3.3	Gram negative short rods
SW 12	⊥	2.1	Gram negative long slender rods
SW 14	+⊥	1.7	Gram negative coccobacilli
SW 15	+⊥	2.6	Gram negative short rods
SW 17	+⊥	2.6	Gram negative coccobacilli
SW 21	+⊥	0.9	Gram negative coccobacilli
Sediment			
Sed. I	++	2.6	Gram negative short fat rods
Sed. II	+	5.2	Gram negative medium sized curved bacteria
Sed. II(a)	++	2.9	Gram negative short rods
P3R	+++	20.2	Gram negative short medium sized rods
Macroalgae			
Algal(I)	-	-	Gram negative medium sized thin rods
Departmental collection			
Ti-21d(3)	+++	20.2	Gram negative short rods
Ti-21d(5)	++	13.5	Gram negative medium sized rods
Ti-21d(16)	++	11.0	Gram negative short thick rods

- = no growth, ⊥ = poor; + = moderate; ++ = good; +++ = excellent

further studies. The cells were gram negative short-rods, non-sporulating and motile that produced creamy white, circular colonies with smooth shiny surface when grown on Zobell-Marine agar. Other tests carried out indicated that the organism was facultative aerobic, both catalase and oxidase positive and reduced nitrate (**Table 3.2**). The culture was found to utilize many sugars as sole carbon source and was non-fermentative (**Table 3.3**). Based upon these characteristics of the isolate, the culture was identified as a member of

Table 3.2: Typical culture characteristics of *Marinobacter* sp.

Examination	Observation
Gram characteristics	Gram negative
Cell morphology	Short rods
Spores	Non-sporulating
Motility	Motile
Pigmentation	Creamy white
Colony shape	Small round
Elevation	Low convex
Consistency	Butyrous
Oxidase	Positive
Catalase	Positive
Indole production	Negative
Citrate	Negative
Methyl red	Negative
Voges-Proskauer	Negative
Nitrate reduction	Positive
Urea	Positive
Hugh-Liefson's medium	Facultative aerobic
TCBS agar	No growth
MaConkey agar	No growth

the family Alteromonadeaceae following Bergey's Manual of Systematic Bacteriology (1984). The culture was identified as *Marinobacter* sp. following the 16S-rRNA base sequences (**Fig. 3.1**) and showed 99% similarity with a strain *Marinobacter* sp. *Trimyema-2*.

Table 3.3: Fermentative properties of *Marinobacter* sp.

Fermentation Tests	Acid	Gas	Alkaline
Glucose	+	-	-
Sucrose	+	-	-
Galactose	+	-	-
Lactose	+	-	-
Maltose	+	-	-
Xylose	+	-	-
Arabinose	-	-	-
Mannitol	-	-	-
Sorbitol	-	-	-
Mannose	+	-	-
Starch	+	-	-
Inositol	-	-	-
TSI slant	Butt	-	Slant

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GTTTgATCATGRaTCAGATTGAACGCTGRCGRcAgGCTTAAcAcATgCAAgTCGAGCGGT
aACAGGGGGAGCTTGCTCCCTGcTGACGAGCGGGCGACGGGTGAGTAATGCATAGGA
ATCTGCCAGTAGTGGGGGATAGCCCCGGGGAAACCCGGATTAATACCGCATAACGCc
TACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTATTGGATGAGCCTATGTCGGA
TTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGGCAGACGATCCGTAGCTGGTCTGAGA
GGATGATCAGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGCAAgCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAACCTGATGGTTAATACCC
ATTGGGCTTGACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGC
GGTAATACGGAGGGTGCAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGG
CGTTTTGGTAAGCGAGATGTGAAATCCCCGGGCTTAACCTGGGAAGTGCATTTGAA
CTGCCAGGCTAGAGTATGGTAGAGGGTAGTGGAATTTCTGTGTAGCGGTGAAATGC
GTAGATATAGGAAGGAACACCAGTGGCGAAGGCGGCTACCTGGACCAATACTGACGC
TGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGTCAACTAGCCGTTGGGACTCTTGAAGTCTTAGTGGCGCA-
CTAACGCACTAAgTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACCTCAAATGAA
TTgACGGGGGCCCGCACAAAGCGTGGAGcaTGTGGTTAATTCC.

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Fig 3.1. 16S-rRNA sequence of *Marinobacter sp.* obtained after amplifying 1500 bp-segment of the 16S rRNA gene with a pair of primers targeting at the leading (5'-AGAGTTTGATC(AC)TGGC-3') and terminating end of the 16S rRNA gene (5'-TACCTT GTT ACG ACT T-3'). Sequencing primers used were GM 3F (5'-AGAGTTTGATC(AC)TGGC-3') and GM 8R (5'-TGGGTATCTAATCCT-3'), both labeled with IRDyeTM800.

3.3.2 Optimization of growth medium for EPS production

1) *Effect of glucose concentration-* *Marinobacter sp.* showed maximum growth when the medium was supplemented with 3% glucose (**Fig. 3.2**). However, EPS concentration was maximal when the culture was grown in 1% glucose. Thereafter, EPS concentrations did not increase with the increase in glucose concentrations, although cell growth increased up to 3% glucose (**Fig. 3.2**).

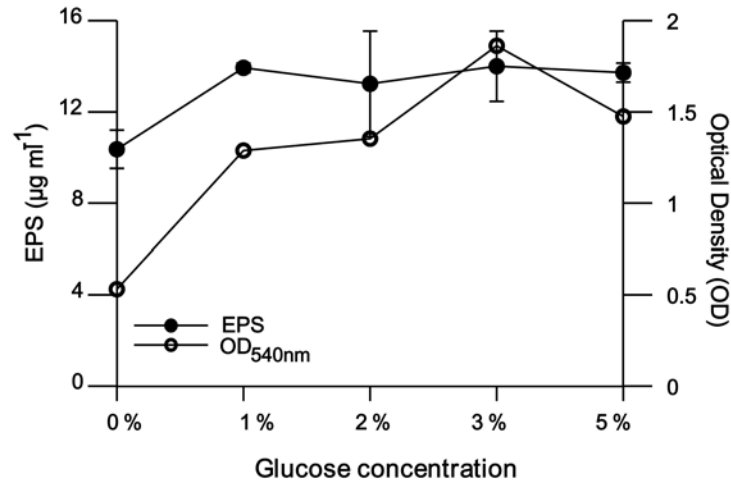


Fig. 3.2 Effect of glucose concentrations on the growth and EPS production of *Marinobacter* sp.

2) *Effect of different nitrogen sources and concentration-* Ammonium chloride was most suitable source of nitrogen amongst the five nitrogen sources examined for both bacterial growth and EPS production (**Fig. 3.3a**). Thus, ammonium chloride was used to assess the effect of nitrogen concentrations on EPS production. It is clear from the graph (**Fig 3.3b**) that there was an almost 1.5-fold increase in EPS production by the culture when the medium was supplemented with 0.0026% N. Thereafter, the EPS concentration decreased with increasing N-concentration whereas cell density showed a small initial increase followed by a decrease in cell density with increase in N-concentrations.

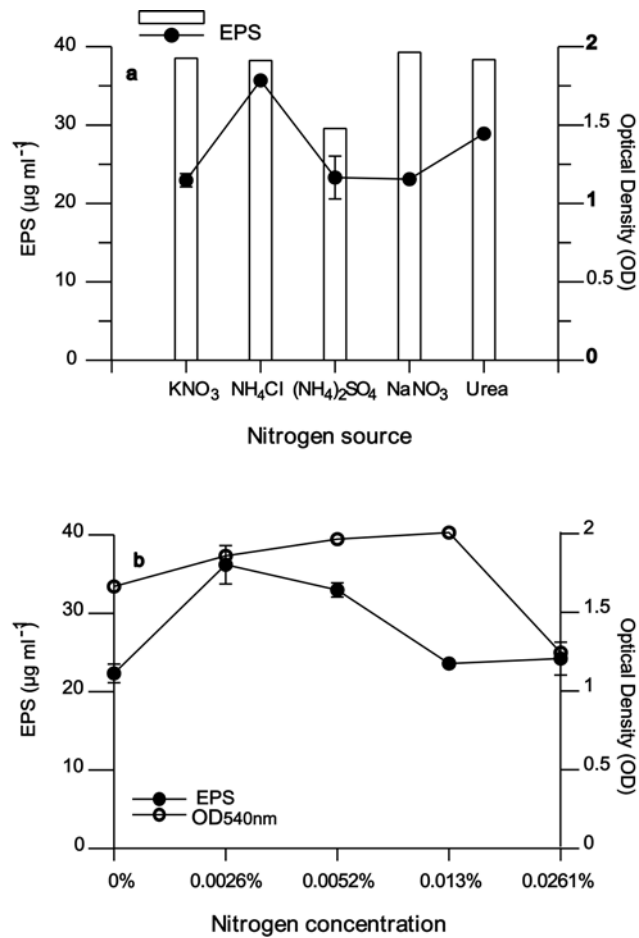


Fig. 3.3 Effect of various nitrogen sources (a) and different nitrogen concentrations (b) on the growth and production of EPS by *Marinobacter* sp.

3) *Effect of phosphate concentration-* EPS production by the culture was maximal when the medium was supplemented with 0.017% phosphate (**Fig. 3.4**). On the other hand, cell density increased with increase in phosphate concentrations followed by a decrease when the medium was supplemented with 0.085% phosphate.

4) *Effect of magnesium concentration*- Increasing concentrations of magnesium appeared to support EPS production more than cell growth. In contrast, there was not much difference in the cell density except for a sharp increase in the medium supplemented with 0.024% magnesium concentrations (**Fig. 3.5**).

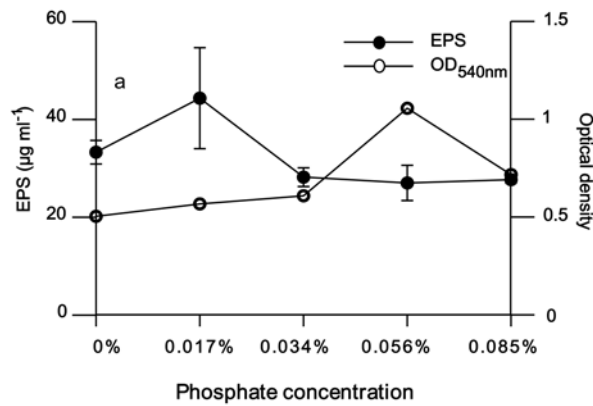


Fig. 3.4 Effect of different phosphate concentrations on the growth and EPS production by *Marinobacter* sp.

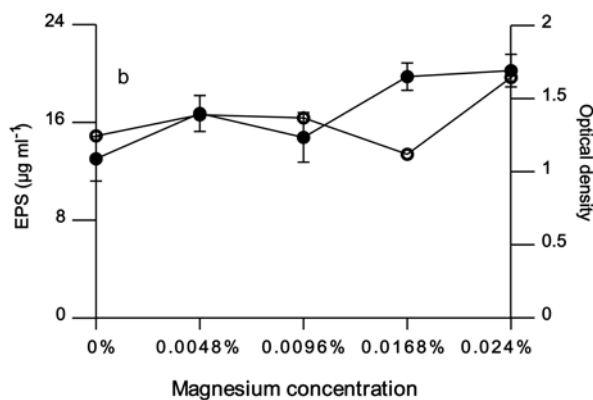


Fig. 3.5 Effect of different magnesium concentrations on the growth and EPS production by *Marinobacter* sp.

3.3.3 Growth and EPS production

The growth curve of the bacterium is shown in **Figure 3.6**. The culture growth increased gradually from an optical density of 0.04 at 0 h to 0.819 after 120 h. Thereafter, the growth of the culture did not vary much until the end of the incubation. The production of EPS was however different. In the beginning, EPS concentration rose from 9 $\mu\text{g ml}^{-1}$ at 6 h to 18.8 $\mu\text{g ml}^{-1}$ after 20 h of incubation and did not vary much until 61 h. Thereafter, the EPS concentration increased to 26.9 $\mu\text{g ml}^{-1}$ between 61 h and 100 h followed by a decrease at the end of the experiment.

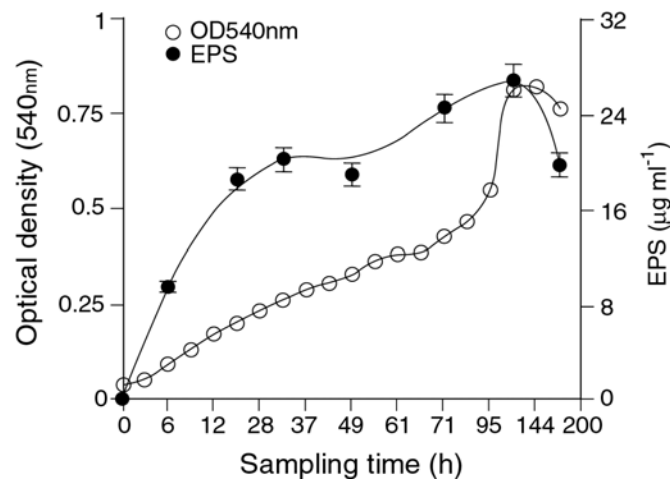


Fig. 3.6 Growth curve and EPS production of *Marinobacter* sp. grown in BSS medium supplemented with 3% glucose, 0.0026% N, 0.05% yeast extract and 0.25% peptone.

3.3.4 Chemical characterization

Bulk characterization

The chemical composition of the *Marinobacter* EPS is shown in **Table 3.4**.

The EPS was enriched in proteins ($438.9 \mu\text{g mg}^{-1}$) relative to carbohydrates ($168.9 \mu\text{g mg}^{-1}$). The polymer contained $175.8 \mu\text{g mg}^{-1}$ uronic acid and $26.9 \mu\text{g mg}^{-1}$ sulphate. The C:N ratio of the polymer was 2.81.

Table 3.4: Bulk chemical composition of EPS ($\mu\text{g/mg}$) isolated from *Marinobacter* sp. in comparison with EPS composition of other bacterial isolates.

Parameters	TCHO	Proteins	Uronic acid	Sulfates	C:N	Reference
<i>Marinobacter sp</i>	168.9	438.9	175.8	26.9	2.81	Present study
<i>Vibrio sp.</i>	510.9	17.93	148.89	--	--	Majumdar et al (1999)
<i>Halomonas eurihalina</i>	363	102	12	--	--	Bejar et al (1998)
<i>A. infernus</i>	570	40	420	88	--	Guezennec et al (1998)
<i>A. macleodii</i>	420	40	380	50	--	Rougauex et al (1996)

FTIR spectra

The FTIR-spectra of bacterial EPS showed bands of carbohydrates at 1029.9 and 1107.1 cm^{-1} . On the other hand, carboxylate and sulphate peaks

were observed at 1612.4 cm^{-1} and 613.3 cm^{-1} , respectively in the EPS of *Marinobacter* (Fig. 3.7).

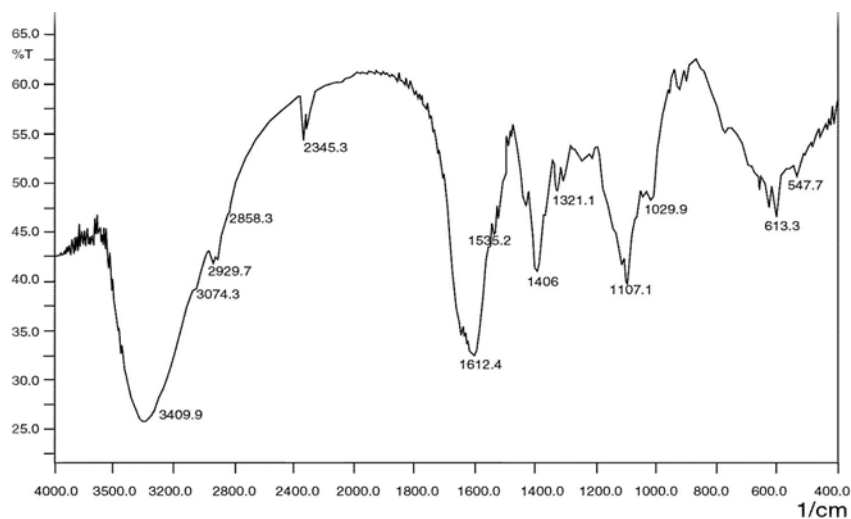


Fig. 3.7. FTIR-spectrum of bacterial EPS

Monosaccharide concentrations

Glucose was the most abundant (79.9%) monosaccharides in the bacterial EPS followed by galactose (17.6%) (Table 3.5). Mannose/xylose and fructose were present in smaller amounts (< 3%). Pentoses like ribose and arabinose and deoxy sugars like rhamnose and fucose were not detected in the EPS (Fig. 3.8).

Monosaccharides	Mole%
Fuc	---
Rib	---
Rha	---
Ara	---
Gal	17.6
Glu	79.8
Man+Xyl	1.5
Fru	1.1

Table 3.5: Monosaccharide (Aldose) composition (mole%) of EPS isolated from *Marinobacter* sp.

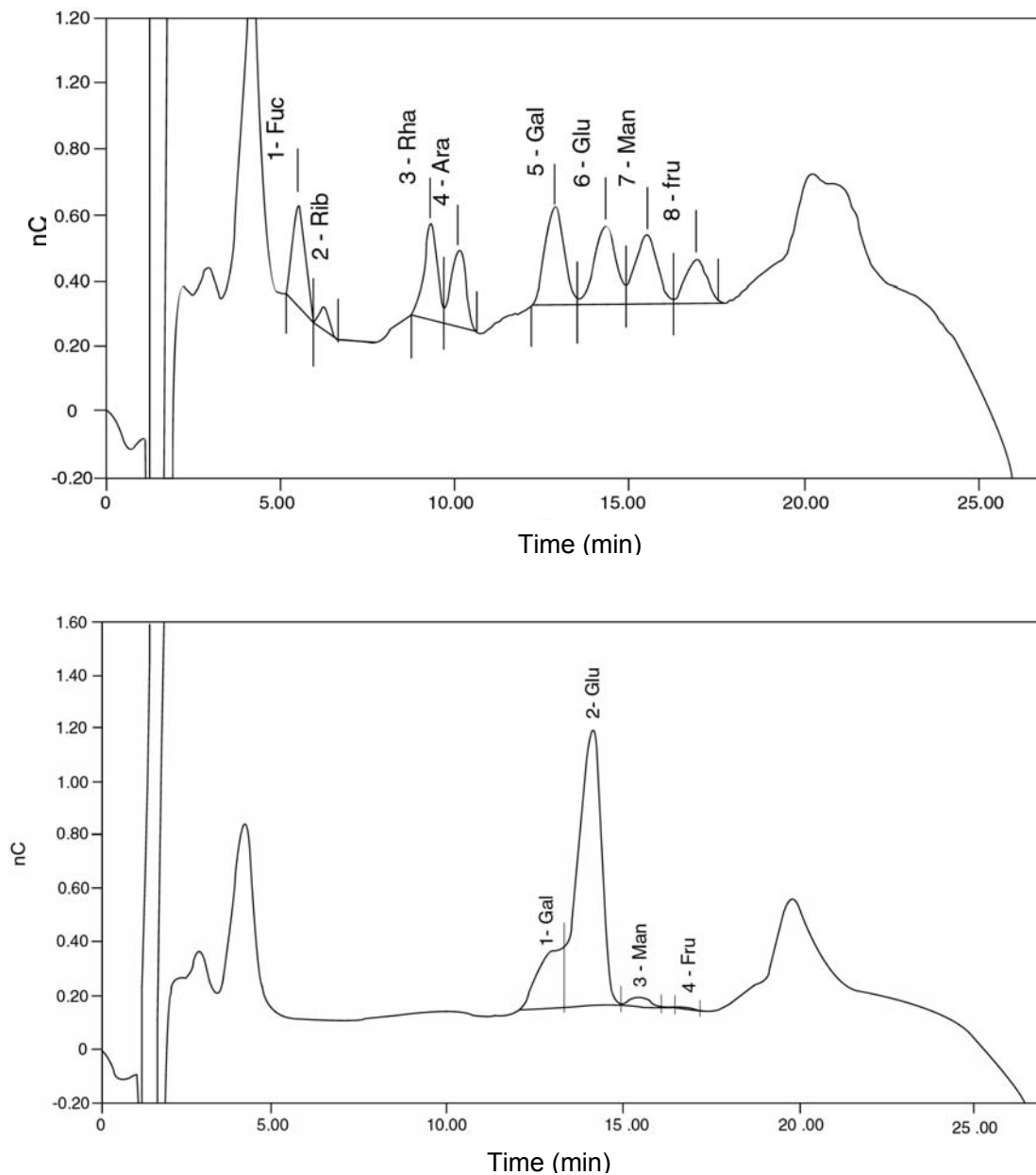


Fig. 3.8 HPLC-PAD chromatographs of a) aldo standard and b) bacterial EPS. A mixture of 8 aldo sugars at 20 nM each was used as a standard for the calibration. The sugars estimated are: i) Fuc- Fucose, ii) Rib- Ribose, iii) Rha- Rhamnose, iv) Ara- Arabinose, v) Gal-Galactose, vi) Glu- Glucose, vii) Man- Mannose/Xylose and viii) Fru- Fructose

Total hydrolysable amino acid (THAA)

The individual amino acid concentration was expressed as mole% and is shown in the **Table 3.6**. Aspartic acid was the major amino acid in the EPS derived from *Marinobacter* and contributed 79.2 mole% to the THAA. This was followed by serine (7.4 mole%) and glutamate (6.9 mole%) (**Fig. 3.9**). Amino acids like glycine/threonine, valine, alanine, phenylalanine and leucine

THAA	Mole %	THAA	Mole%
Aspartate	79.2	β -Alanine	0.07
Glutamate	3.5	Alanine	2.5
Asparagine	0.2	Tyrosine	0.68
Serine	3.7	γ -ABA	0.18
Glutamine	1.6	Methionine	0.02
Histidine	0.5	Valine	1.7
Citrulline	0.2	Phenylalanine	0.68
Glycine/Threonine	2.8	Isoleucine	1.3
Arginine	0.43	Leucine	1.3

Table 3.6: Total hydrolysable amino acid (THAA) composition (mole %) of EPS isolated from *Marinobacter* sp.

contributed around 0.6 to 2.8 mole%. Histidine, citrulline, β -alanine, tyrosine, methionine, isoleucine and γ -ABA were the minor amino acids.

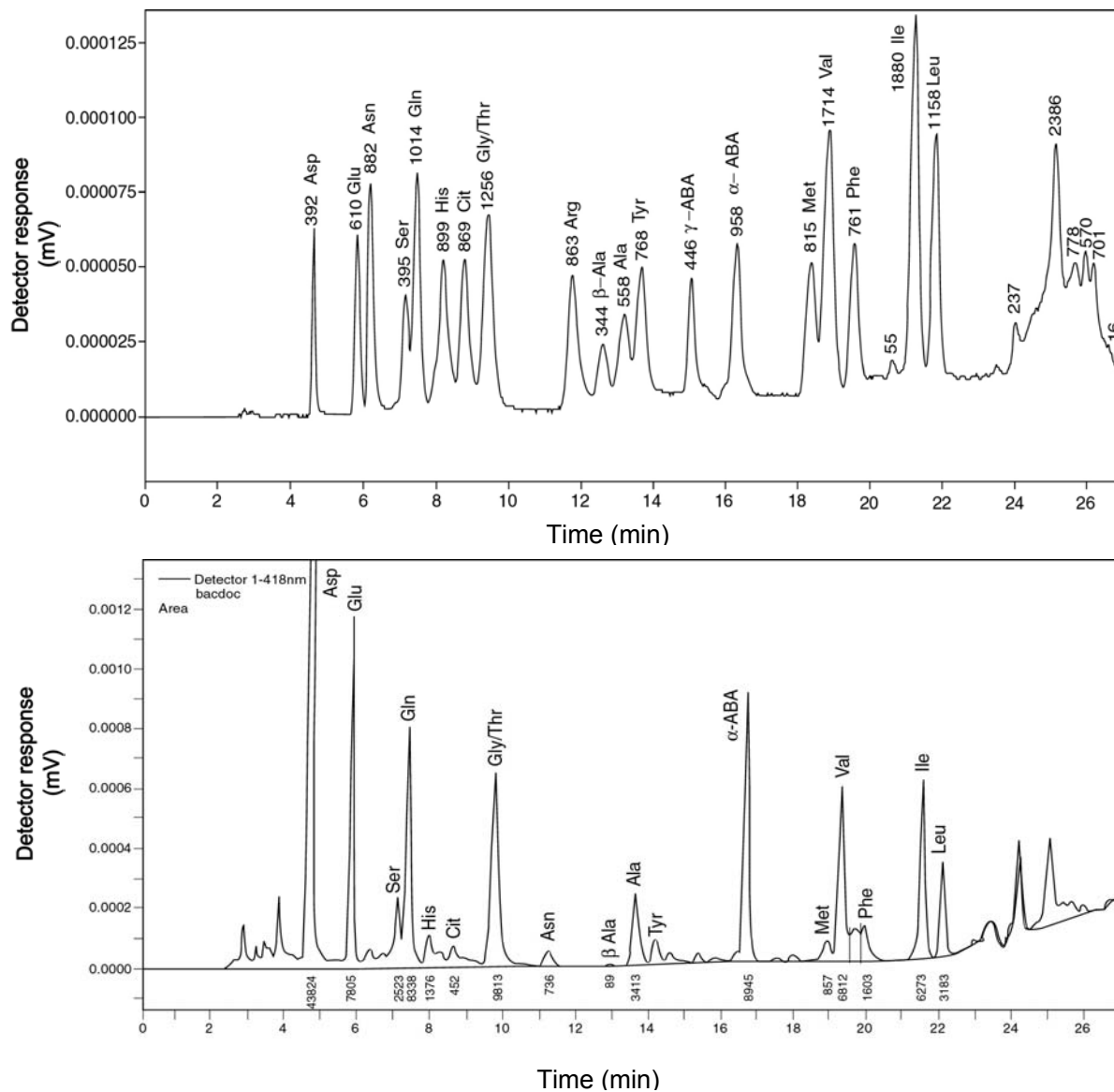


Fig. 3.9 HPLC chromatographs of total hydrolysable amino acids (THAA) in the a) standard and b) bacterial EPS. The standard contained 18 amino acids and α -ABA was used as internal standard. For the list of entire 18 amino acids, please refer to **Table 3.6**.

3.4 Discussion

The bacterial isolate was a gram negative, motile, non-sporulating short rods that grew well in most carbohydrate supplemented medium (**Table 3.2b**). Moreover, the bacterial isolate was facultative aerobe. The characteristics of the bacterial isolate were similar to those of *Marinobacter sp.* belonging to family Alteromonadaceae (Gauthier et al 1992, Nguyen et al 1999). Organisms belonging to this genus are gram-negative rods (straight, curved or spiral), non-sporulating having polar flagellum. The members of this genus are strictly aerobes and typically marine in their habitat. *Marinobacter sp.* studied here was isolated from mangrove sediments along the Zuari river, which generally experienced typical marine conditions (higher salinity) during most of the year (Qasim and SenGupta 1977). Like other members of the family Alteromonadaceae, *Marinobacter* culture could ferment most sugars without the production of gas but did not grow in presence of carbon source like sorbitol and mannitol. The culture also had the nitrate reducing properties associated with cultures of the family *Alteromonadaceae*. Moreover, the 16s-rRNA sequences showed 99% similarity with that of *Marinobacter sp. Trimyema-2*. Thus both biochemical and molecular methods of identification suggest that the culture was *Marinobacter*. However, unlike other members of the family Alteromonadaceae like *Alteromonas*, *Pseudoalteromonas* and *Shewanella*, there is no information on the production and characterization of EPS of *Marinobacter sp.*

Various methods including high-speed cold centrifugation (Brown and Lester 1979, Decho 1990), chemical treatments using EDTA (Underwood et al 1995, Bhosle et al 1995), EGTA (Turakhia et al 1983), mild alkali (Bhosle et al 1993, Khandeparker and Bhosle 2001) and sodium chloride (Bhosle et al 1995, Underwood et al 1995) have been reported for extraction of EPS from microbial cultures. Brown and Lester (1979) used only high-speed centrifugation (33000 g) to separate the cells from the EPS. However, it is possible that such high shear may damage the cells and cause contamination of the EPS (Decho 1990). In contrast, treatment of cells with chemicals like EDTA followed by centrifugation has been found to be the most effective extraction method for the isolation of EPS (Decho 1990). We used a combination of both high-speed centrifugation and chemical methods to extract the EPS.

Marinobacter sp. was grown in varying carbon, nitrogen and phosphate concentrations. Microorganisms are known to produce more EPS when grown in carbon enriched medium (high C:N ratios) (Williams and Wimpenny 1977, Sutherland 1983 & 2001, de Souza and Sutherland 1994, Majumdar et al 1999, Nicolaus et al 1999). Limitation of nitrogen is known to trigger increased EPS production by microorganisms (Duguid and Wilkinson 1953, Corpe 1964, Fajon et al 1999). From our data, it was evident that at a constant nitrogen concentration (0.0026% N), the EPS content did not increase much with increasing glucose concentrations. In contrast, bacterial growth increased with glucose concentrations except at 5% glucose concentration (**Fig. 3.1**)

indicating that at higher concentrations, glucose was primarily used to increase the biomass and not for EPS production.

The culture showed a preference for reduced form of nitrogen to support both bacterial growth and maximal EPS production. Moreover, the EPS production was higher when the nitrogen concentrations were low (**Fig. 3.3a & 3.3b**). A similar response in EPS production by *Marinobacter* culture was observed at low phosphate concentrations (**Fig. 3.4**). Increase in EPS production under nutrient limiting growth conditions have been reported in many cultures. For example, optimal EPS production by *Chromobacterium* sp. has been reported when the nitrogen concentrations in the medium were low (Duguid and Wilkinson 1964). Similarly, increase in EPS yield has been reported for *Pseudomonas* PB1 under minimal nitrogen and phosphate concentrations (Williams and Wimpenny 1977). Although not much is known about the mechanism regulating the production of EPS in nutrient depleted conditions, a shift in the biosynthetic pathways might explain the observed changes.

In contrast to the limitation of nitrogen and phosphates, limitations of other essential elements like magnesium has relatively more influence on the composition of the EPS than its production (Davidson 1978). For example, presence of Mg^{2+} caused gelling of bacterial polymer. Similarly, magnesium ions can help in stabilizing the polymer structure (Characklis and Cooksey 1983). In the present study, EPS increased marginally with the increase in magnesium concentrations suggesting that increase in magnesium concentrations did not improve the production of the polymer.

The production of the EPS during the growth of *Marinobacter* had certain distinct features. For example, maximum EPS was produced in the late exponential growth phase of the culture. Moreover EPS production increased rapidly within the first 20 h of culture growth and increased gradually up to 100 h. The EPS production was maximal as the culture approached stationary phase. Bacteria produce EPS during their log-phase depending upon the growth conditions and age of the culture (Decho 1990). *Marinobacter* sp. in the present study appears to produce capsular form of EPS. The decline in EPS concentrations at the end of the incubation was however not explainable by cell density variations (which did not vary much). It is possible that EPS production was less in the stationary phase of the bacterial growth, there by reducing the EPS concentrations in the medium.

EPS produced during different phases of growth have specific properties and functions. For example, capsular form of EPS is generally produced during log phase that forms a tight envelope around the cell, promoting cell attachment to substratum (Carlsson 1967, Costerton 1984). Capsules may also provide better protection against predation and help bacteria survive in low pH (Decho and Lopez 1993). Bacterial capsules also act as a good metal adsorbent (Brown and Lester 1982, Schlekot et al 1998) and may act as a buffer against metal toxicity. Thus the production of EPS in the exponential phase has ecological significance for the organism and may play an important role during the growth of *Marinobacter* sp.

The functions and the properties of the EPS vary due to differences in their chemistry. The bacterial EPS had lesser carbohydrate (TCHO) content compared to proteins (**Table 3.3**). The high protein content is also reflected in the relatively low C:N ratios of the polymer. The TCHO contribution to the total EPS was lower than those reported by Rougaut et al (1996) (42%), Bejar et al (1998), Guezennec et al (1998) (57%) and Majumdar et al (1999) (51%). However, a similar low TCHO content has been reported for the EPS isolated from marine *Pseudomonas* sp. (11.5 to 13.5 $\mu\text{g mg}^{-1}$ EPS) (Beech et al. 1999). The protein content of *Marinobacter* EPS was 22.5% by weight, which was similar to the EPS produced by *Vibrio* sp. (Majumdar et al 1999). Proteins in the microbial EPS play an important role in stabilizing the tertiary polymer structure and to some extent help in adhesion of cells to surfaces (Decho 1990, Hoagland 1993, Wingender et al 1999). It is possible that the proteins present in the bacterial EPS might be important for the stability of the EPS.

Carboxylated sugars like uronic acid formed important fraction of bacterial EPS. The uronic acid content of *Marinobacter* EPS was 17.5% of the polymer by weight (**Table 3.3**). This was relatively greater than EPS of fouling bacteria like *Halomonas* strains (0.6-11.1%, Bejar et al 1998) and *Vibrio* (14.1%) (Majumdar et al 1999) but lower than that of Rougaut et al (1996) (38%). The FTIR-spectrum of the bacterial EPS also showed definite peaks for carboxylates at 1029.9 and 1107.1 cm^{-1} (**Fig. 3.7**). The presence of such functional groups gives a general idea about the nature of the EPS. For example, uronic acid imparts an overall negative charge and reduces the pH of the polymer (Corpe 1970). Similarly, uronic acid may also affect the

solubility of the polymer and may help in metal-complexation (Kaplan et al 1988). The presence of relatively higher uronic acid content in the EPS indicates that the bacterial EPS may be useful in binding of metals and therefore might find industrial application.

On the other hand, the non-sugar component like sulphates was relatively low in the EPS produced by *Marinobacter* (2.7%; **Table 3.3**). This value was less than that of EPS isolated from *Alteromonas infernus* (8.8%; Guezennec et al 1998) and *Alteromonas* EPS (5%; Rougaut et al 1996). The presence of sulphates was also observed in the FTIR-spectrum of the bacterial EPS (**Fig. 3.7**). Sulphate moieties provide flexibility to EPS, impart gel-like consistency, prevent desiccation and help to stabilize the polymer (Hoagland et al 1993). It is possible that sulphates present in bacterial EPS might be useful in flocculation and aggregation of cells. Such aggregation might help the bacterium survive nutrient stress (Hoagland et al 1993).

The bacterial exopolymer was a heteropolysaccharide as observed from the sugar (aldoses) composition. Glucose was the most dominant aldose in the EPS followed by galactose (**Table 3.4**). The glucose contribution in the *Marinobacter* EPS was similar to that of *Vibrio* (75%, Majumdar et al 1999) but was greater than those reported for *Alteromonas macleodii* (11.2 to 14.8%, Rougeaux et al 1996) and *Alteromonas infernus* (1 to 16% glucose, Guezennec et al 1998). The absence of deoxy sugars in the *Marinobacter* EPS was similar to those observed in the EPS isolated from *Alteromonas infernus* (Guezennec et al 1998), a cyanobacterium (*Cyanothece* sp., Philippis

et al 1998) and *Vibrio* sp. (Majumdar et al 1999). *Marinobacter* EPS being enriched in glucose might serve as a carbon source for heterotrophs in the marine environment.

The relatively higher concentrations of proteins in bacterial EPS was also reflected in their amino acid concentrations. Interestingly, aspartic acid was the major amino acid observed in the EPS forming 79.2% of total amino acids. Other major amino acids like serine, glutamate, glycine/threonine, alanine, leucine and valine formed 6.1% to 24.7% of the THAA concentrations (**Table 3.5**). The high aspartate concentration in the EPS was intriguing and could not be explained. It is possible that the amino acids like aspartic acid might belong to high molecular weight proteins involved in the structural organization of the EPS.

3.5 Conclusions

In conclusion, EPS production by *Marinobacter* sp. was influenced by physiological factors and medium composition. Bacterial polymer was nitrogen enriched as observed from the low C:N ratios, which is reflected in its higher protein content. The bacterial EPS was high molecular weight heteropolysaccharide with glucose as the principal component of monosaccharides. *Marinobacter* sp. produced sulphated EPS having a fair amount of uronic acid. Such compounds in the EPS might assist in cell attachment, motility and provide protection against metal toxicity and desiccation.

CHAPTER 4

Chapter 4

4.1 Introduction

Macroaggregates are particles that may range from 500 μm to more than 10 cms in length (**Table 4.1**, Simon et al 2002) and occur in varying forms such as amorphous aggregates, stringers, flocs, mats etc (Herndl and Peduzzi 1988, Stachowitsch et al 1990). Macroaggregates form an important constituent of both suspended and sedimenting particles in aquatic environments (Silver and Alldredge 1981, Alldredge and Gotschalk 1989, Eisma et al 1983, Reibesell 1991). Unlike solid particles, macroaggregates are porous, hydrated, and fractal in nature (Logan and Wilkinson 1990, Alldredge and Jackson 1995, Chen and Eisma 1995, Jackson et al 1997). The

Type of organic matter	Size range
Macroaggregates (marine snow)	> 500 μm
Microaggregates	1 to 500 μm
Algae/cyanobacteria	1 to 100 μm
Bacteria	0.1 to 5 μm
Submicron particles	0.05 to 1 μm
Colloids	0.004 to 1 μm
Proteins/Carbohydrates	< 10^4 to 10^6 daltons

Table 4.1: Size spectra of the major organic matter constituents in marine ecosystems.

macroaggregates may contain live cells of phytoplankton, bacteria and protists, cell debris, abandoned larvacean houses and pteropod nets, fecal matter etc entrapped in a matrix of exopolysaccharides (EPS) (Alldredge and Silver 1988, Alldredge and Gotschalk 1989, Herndl 1995, Kiørboe 2001, Simon et al 2002).

The macroaggregates play an important role in biogeochemical cycling of elements, life-cycle of micro-organisms and aquatic trophic-web. For example, phytoplankton cells form macroaggregates under nutrient depleted conditions and sink into deeper waters to escape growth-limiting conditions. These aggregates may act as seed-banks for future blooms and hence plays an important role in the life-cycle of phytoplankton like diatoms (Smetacek 1985). While sedimenting as marine/lake snow, these aggregates act as a vehicle for the vertical transport of organic matter to the benthos (Honjo 1982, Alldredge and Gotschalk 1989, Herndl 1995, Passow et al 2001). Aggregates may also serve as food for grazers like zooplankton (copepods, decapods), krills etc. (Alldredge and Silver 1988, Biddanda and Pomeroy 1988, Herndl 1995, Kiørboe 2001). On the other hand, these particles are also colonized by bacteria and may serve as sites for bacterial mediated mineralisation of organic matter and nutrient regeneration (Azam and Cho 1987, Kirchman and Ducklow 1987, Biddanda 1988, Cho and Azam 1988).

The production of macroaggregates in the natural environments involves collision and sticking together of smaller particles following biotic and abiotic processes. In a biotic process, microorganisms (few microns in length) may act as fine suspended particles that might stick to each other directly i.e. cell-

cell (Engel and Schartau 1999) or aggregate with the help of exopolysaccharides (EPS) produced during its growth to form larger particles (Paerl 1974, Biddanda 1985, Herndl 1995, Decho and Herndl 1995). In contrast to biologically mediated processes, particles might also be produced directly from high molecular weight dissolved organic matter (DOM) by purely abiotic processes including 1) self-coagulation (Kepkay 1994, Mari and Burd 1998, Chin et al 1998, Passow 2000) and 2) bubble adsorption (Mopper et al 1995, Zhou et al 1998, Mari 1999). Similarly, the adsorption of the DOM by charged non-living particles may also lead to the production of aggregates (Gerritsen and Bradley 1987, Eisma 1993).

Microorganisms like bacteria and phytoplankton produce sticky, high molecular weight, surface-reactive EPS that form an important constituent of the DOM in the aquatic environments (Decho 1990). Both field and laboratory observations have shown that EPS released by microorganisms are involved in both biotic and abiotic production of macroaggregates (Kjørboe and Hansen 1993, Passow et al 1994, Schuster and Herndl 1995, Alldredge et al 1995 & 1998, Mopper et al 1995, Mari and Burd 1998, Engel 2000, Passow 2000 & 2002). The presence of microbial EPS influences the aggregation process by altering the surface characteristics and the sticking coefficient of the colliding particles (Kjørboe and Hansen 1993, Engel 2000). Microbial EPS also act as precursors for the production of a class of transparent exopolymer particles or TEP, which is considered important for aggregation (Mopper et al 1995, Stoderegger and Herndl 1998, Passow 2002). Similarly, Coomassie blue stained particles (CSP) are another class of organic particles that are

predominantly rich in proteins and as abundant as TEP in the marine environments (Long and Azam 1996). However unlike TEP, nothing is known about the role of CSP in particle aggregation.

Field and laboratory experiments have reported on the role of EPS in aggregation (Kjørboe and Hansen 1993, Passow et al 1994, Passow and Alldredge 1995, Schuster and Herndl 1996, Passow et al 2001, Prieto et al 2001) and the production of transparent exopolymeric particles (TEP) (Mari and Burd 1998, Stoderegger and Herndl 1998, Passow 2002). Most of the studies on aggregation has been conducted during the growth of microorganisms (Alldredge and Gotschalk 1989, Passow and Alldredge 1995) and there is little information on the production of macroaggregates (visible particles) from dissolved EPS in the absence of growing cells (Stoderegger and Herndl 1998). On the other hand, production of EPS does not necessarily warrant the production of aggregates (Kjørboe and Hansen 1993), suggesting that changes in EPS chemistry can influence the production of macroaggregates in the natural environments. However, there is little information on the influence of the chemical composition of the EPS on the production of macroaggregates.

In this chapter, an attempt is made to study the production of macroaggregates from EPS isolated from two different sources. The study was carried out primarily to assess the variations in macroaggregate size and abundance over time. Apart from abundance of macroaggregates and its size variation, the changes in the concentration of organic particles like TEP and

Coomassie blue stained particles (CSP) and their role in regulating the aggregate abundance and size are also addressed.

4.2 Materials and Methods

4.2.1 Production and isolation of *Marinobacter* EPS

Pure culture of *Marinobacter* was grown in 2 l flasks containing 1 l of BSS medium (pH 7.6) (**Appendix IB**) over 14 days at $30 \pm 2^\circ\text{C}$. The medium was supplemented with 3% glucose, low molecular weight fraction of yeast extract (0.05% final concentration) and peptone (0.1% final concentration). The details of isolation of EPS from the culture are given in Chapter 3.

4.2.2 Production and isolation of EPS from *Skeletonema costatum*

Axenic culture of *Skeletonema costatum* Greve was grown in 6.5 l of Guillard's f/2 medium prepared in aged $0.22 \mu\text{m}$ filtered seawater (**Appendix II**). The medium was distributed in 2 l capacity Schott Duran screw capped bottles, autoclaved and inoculated with axenic cells of *Skeletonema costatum* ($6.3 \times 10^5 \text{ ml}^{-1}$). The axenic cultures were grown under 12 h light: 12 h dark conditions at $15 \pm 0.5^\circ\text{C}$ for 27 days. At the end of the incubation period, the culture was centrifuged at 3000 rpm at 4°C to separate the EPS from the cells. The supernatant was filtered through pre-ashed GF/F filters and then concentrated by passing through a 3 kDa MW spiral membrane filter attached to a tangential flow filtration unit. The low molecular weight fraction of the medium was discarded and the higher molecular weight fraction concentrate

(retentate) was reduced in volume to < 50 ml at 35°C using a rotary evaporator. The diatom EPS was then filtered through 0.22 µm filters and precipitated overnight in absolute ethanol at 4°C. The precipitate was then redissolved in small quantities of deionised water and dialysed against deionised water through 8kDa MW dialysis bags. Once dialyzed, the EPS was reduced in volume, lyophilized and stored at –20°C.

4.2.3 Chemical characterization

The details of the various chemical analyses carried out on *Marinobacter* EPS are given in Chapter 3. EPS isolated from *S. costatum* was also analysed for its bulk chemistry including total carbohydrates, proteins, uronic acid, sulphates and monosaccharide composition following the methods discussed in Chapter 3.

4.2.4 Production of macroaggregates from EPS

In order to produce macroaggregates, four sterile rolling tanks (1.5 l capacity) were filled completely with sterile f/2 medium (Appendix-II). To one tank, nothing was added and was treated as negative control (NC) tank. To the second tank, killed *Marinobacter* cells were added and was treated as killed-bacterial control (KB-C) tank. To the third tank (*Eps*), *Marinobacter* EPS (300 µM-C final concentration) was added to the medium while to the fourth tank (*Eps* + *KB*) killed *Marinobacter* cells were added along with 300 µM-C (final concentration) of *Marinobacter* EPS. The third and fourth tanks were used as experimental tanks. In another experiment, all the experimental conditions

were kept same except that EPS derived from *S. costatum* was used as DOM in place of *Marinobacter* EPS. All rolling tanks were incubated in the dark and rolled at a speed of 2 revolutions per minute on a rolling table (Grossart et al 1998).

Image analyses

Prior to every sampling, images of the samples were captured for 30 s using a Sony HAD CCD handy cam (680K gross pixels) while illuminating the tanks with side-arm focus lamps. In order to analyze the images, a 250x250 pixel window (1 pixel= 60 μm) was chosen and the images were gray-scale transformed to highlight the aggregates within each window. The transformed images were then used to analyze the particle abundance and size using SISview image analyzing software. In each image, aggregates that were clearly focused were selected for abundance and size distribution analyses. The equivalent spherical diameter (ESD) was first calculated using the image analyzing software and used to estimate the size of the aggregates expressed as equivalent spherical area (mm^2). The aggregates were then grouped into different size classes ranging from 0.2 mm^2 to 100 mm^2 . The particle abundance was estimated by summing up the particle numbers in each category to give total abundance. The particle size was the mean size of all the aggregates.

4.2.5 Macroaggregate sampling

A known volume (8 ml) of the sample was removed at regular intervals. Equal volume of sterile medium was added to the tanks so as to maintain the volume of medium in the rolling tanks. Duplicate samples (2 ml each) of macroaggregates were filtered on to polycarbonate membrane filters (5 µm pore size) (Grossart et al 1998) under vacuum (< 10 mbar pressure) to enumerate TEP and CSP. The TEP samples were stained with 0.22 µm prefiltered 0.02% Alcian blue prepared in 0.06% glacial acetic acid (pH 2.5; Alldredge et al 1993). Similarly, CSP samples were stained with 1 ml 0.04% Coomassie brilliant blue G-250 stain (Long and Azam, 1996). The samples were then filtered dry, placed over a drop of oil on a glass slide and enumerated under 100x and 200x total magnification.

4.2.6 Statistical analyses

A simple regression analysis was carried out to assess the relationships between various parameters. One-way ANOVA for unequal data sets was used to assess the differences in abundance of aggregates, TEP, CSP and size of aggregates produced in all the experiments. The statistical analyses were carried out using Statistica 5.0 software installed in the personal computer.

4.3 Results

4.3.1 Chemical composition of EPS derived from *Marinobacter* sp. and *S. costatum*

The bulk chemical and molecular composition of *Marinobacter* EPS have been discussed in Chapter 3 (**Table 3.4 and 3.5**). The bulk chemical composition of the EPS derived from *Skeletonema costatum* is shown in **Table 4.2a**. The diatom EPS contained relatively higher concentrations of sulphates and lesser amounts of protein and uronic acids compared to *Marinobacter* EPS. The monosaccharide composition showed the heterogeneity of the diatom EPS (**Table 4.2b**). Glucose was the predominant monosaccharides in the EPS (53.3 mol%) followed by mannose + xylose (16.8 mol%) and galactose (16.5%) (**Table 4.2b**).

Parameters	Concentration ($\mu\text{g}/\text{mg}$)
TCHO	28.3
Proteins	35.8
Uronic acid	46.2
Sulfates	215.4
C:N (w:w)	8.1

Table 4.2a: Bulk chemical composition of EPS isolated from *S. costatum*.

Monosaccharide composition	Concentration (mole %)
Fuc	2.7
Rib	--
Rha	6.2
Ara	--
Gal	16.5
Glu	53.3
Man+Xyl	16.8
Fru	4.6

Table 4.2b: Monosaccharide (Aldose) composition (mole%) of EPS isolated from *S. costatum*.

4.3.2 Macroaggregate abundance (numbers cm^{-2})

In experiment-I wherein *Marinobacter* EPS was used as dissolved organic carbon, the particle abundance in tank containing only EPS (*Eps*) decreased from 53 cm^{-2} to 25 cm^{-2} in the first 20 h followed by an increase to 61 aggregates cm^{-2} after 70 h (**Fig. 4.1a**). The particle abundance declined thereafter until the end of the experiment. On the other hand, no large-scale temporal variations could be observed in the abundance of particles in tank having both *Marinobacter* EPS and killed *Marinobacter* cells (*Eps + KB*) (**Fig. 4.1a**). The abundance of particles varied from 1.8 to 8.9 aggregates cm^{-2} (**Table 4.3**). In contrast to the experimental tanks, the particle counts in tank having only medium (*NC*) did not vary much up to 99h and declined thereafter

(**Fig. 4.1a**). Similarly, particle abundance in tank containing only killed bacterial cells (*KB-C*) also showed small-scale variation (**Fig. 4.1a**) and ranged from 11 to 27.1 aggregates cm^{-2} (**Table 4.3**).

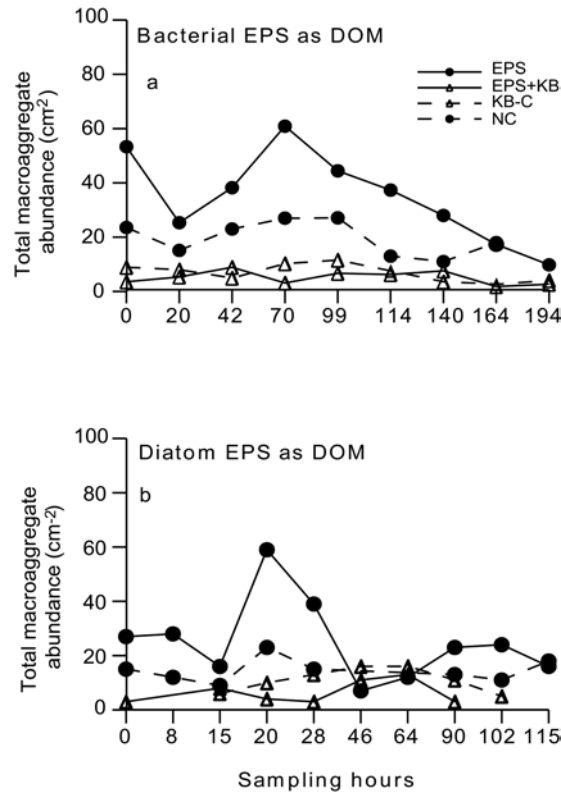


Fig. 4.1 Changes in the abundance of macroaggregates produced in experimental and control tanks having bacterial EPS (a) and diatom EPS (b) as sources of DOM .

In experiment-II where *S. costatum* EPS was used as DOM, the abundance of particles in the tanks having only diatom EPS increased from 28 aggregates cm^{-2} , reached a high (59 aggregates cm^{-2}) after 20 h and declined thereafter (**Fig. 4.1b**). Similarly, the particle abundance in the tank containing both diatom EPS and killed *Marinobacter* cells varied from 3 to 13 aggregates cm^{-2} (**Table 4.3**) and did not show any specific trend over the period of incubation (**Fig. 4.1b**). On the other hand, the total abundance of aggregates in the tank

containing only medium ranged from 9 to 23 aggregates cm^{-2} (**Fig. 4.1b**) and increased till 64 h followed by a decline.

4.3.3 Macroaggregate size (mm^2)

In experiment I, the aggregate size in the tank having *Marinobacter* EPS increased from 1.2 mm^2 in the beginning to 16 mm^2 within 20h of incubation, declined to 7 mm^2 and reached the maximal (22.7 mm^2) by the end of the experiment (**Fig. 4.2a**). On the whole, the aggregate size ranged from 1.1 to 22.7 mm^2 (**Table 4.3**). In contrast, the aggregate size in the tank containing both *Marinobacter* EPS and killed bacterial cells, the aggregate size increased only after 99 h of incubation (**Fig. 4.2a**). The aggregate size in this tank ranged from 0.66 mm^2 to 13.2 mm^2 during the course of the experiment (**Table 4.3**). In contrast, the control tanks (both *NC* and *KB-C*) did not show the formation of macroaggregates except near the end of the experiment (**Fig. 4.2a**). The aggregate size ranged from 0.5 to 9.5 mm^2 and 0.6 to 2.9 mm^2 in the tank having only medium and killed bacterial cells, respectively (**Table 4.3**).

In the second experiment, the macroaggregate size in the experimental tank containing only diatom EPS increased from 1.6 mm^2 to 31.6 mm^2 only after 28 h, increased marginally till 102 h (34.5 mm^2) and then decreased to 19.4 mm^2 (**Fig. 4.2b**). On the other hand, no such increase in the macroaggregate size was observed in the tank with EPS and killed bacterial cells (**Fig. 4.2b**). Similarly, the particle size in the control tanks having only medium and killed

bacterial cells varied from 0.15 mm² to 1.3 mm² and 0.07 mm² to 1.03 mm², respectively and did not show any large-scale variations (**Table 4.3**).

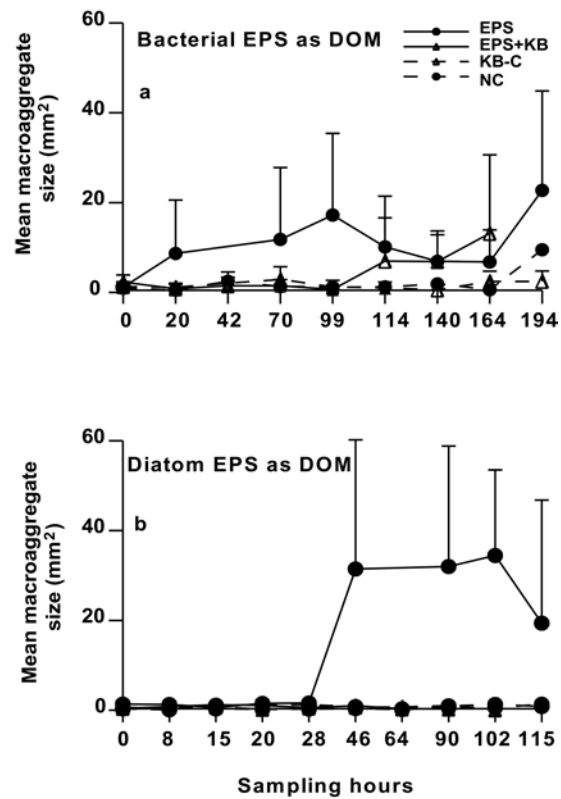


Fig. 4.2 Changes in the mean size of macroaggregates produced in experimental and control tanks having bacterial EPS (a) and diatom EPS (b) as sources of DOM. The error bars indicate the standard deviation.

4.3.4 TEP and CSP abundance (ml⁻¹)

In experiment-I, the abundance of TEP and CSP declined with time in both experimental and control tanks. The abundance of TEP in the tank containing only bacterial EPS increased from 0.6 x 10³ ml⁻¹ to a high of 5.7 x 10³ ml⁻¹

within 20 h followed by a decrease thereafter till the end of the experiment (**Fig. 4.3a**). A similar variation was observed in the abundance of CSP produced in this tank (**Fig. 4.4a**) that varied from 0.6 to $3.7 \times 10^3 \text{ ml}^{-1}$ (**Table 4.3**). The abundance of TEP in tank containing bacterial EPS and killed bacterial cells declined from $1.7 \times 10^3 \text{ ml}^{-1}$ at 0 h to $0.24 \times 10^3 \text{ ml}^{-1}$ after 164 h of incubation (**Table 4.3**). On the other hand, the CSP abundance in the same tank also declined with time (**Fig. 4.4a**) from $2.9 \times 10^3 \text{ ml}^{-1}$ to $0.3 \times 10^3 \text{ ml}^{-1}$ (**Table 4.3**). In contrast, abundance of TEP and CSP in the tank containing only medium decreased from $1.1 \times 10^3 \text{ ml}^{-1}$ on day 0 to almost $0.3 \times 10^3 \text{ ml}^{-1}$ (**Fig. 4.3a**) and $1.1 \times 10^3 \text{ ml}^{-1}$ to $0.1 \times 10^3 \text{ ml}^{-1}$ (**Fig. 4.4a**) by the end of the experiment. On the other hand, TEP counts in tank having killed bacterial cells also decreased from $1.8 \times 10^3 \text{ ml}^{-1}$ on day 0 to $0.1 \times 10^3 \text{ ml}^{-1}$ at the end of the experiment (**Fig. 4.3a**). Moreover, the CSP counts also declined from 2.9 to $0.1 \times 10^3 \text{ ml}^{-1}$ with time (**Fig. 4.4a**).

In experiment-II, the abundance of TEP in tank having only diatom EPS was noticeable only at the end of the experiment (**Fig. 4.3b**) and varied from 0 to $5.7 \times 10^3 \text{ ml}^{-1}$ (**Table 4.3**). In contrast, the abundance of CSP in the tank increased after 8 h of incubation and reached a high of $4.7 \times 10^3 \text{ ml}^{-1}$ after 20 h, declined to $2.6 \times 10^3 \text{ ml}^{-1}$ after 64 h and was maximal at the end of incubation period (**Fig. 4.4b**). The abundance of TEP and CSP in tank having both killed bacterial cells and diatom EPS was negligible ($< 0.05 \times 10^3 \text{ ml}^{-1}$) (**Fig. 4.4b**) with very little variations over time. In contrast, the TEP counts in the tank having only medium did not vary much and remained $< 0.05 \times 10^3 \text{ ml}^{-1}$ except for a small increase at the end of the experiment. However, the CSP

counts in the same tank showed a slight increase with time and ranged from 0 to $3 \times 10^3 \text{ ml}^{-1}$ during the course of the experiment. The abundance of TEP and CSP in tank containing killed bacteria was negligible (**Fig. 4.3b and 4.4b**).

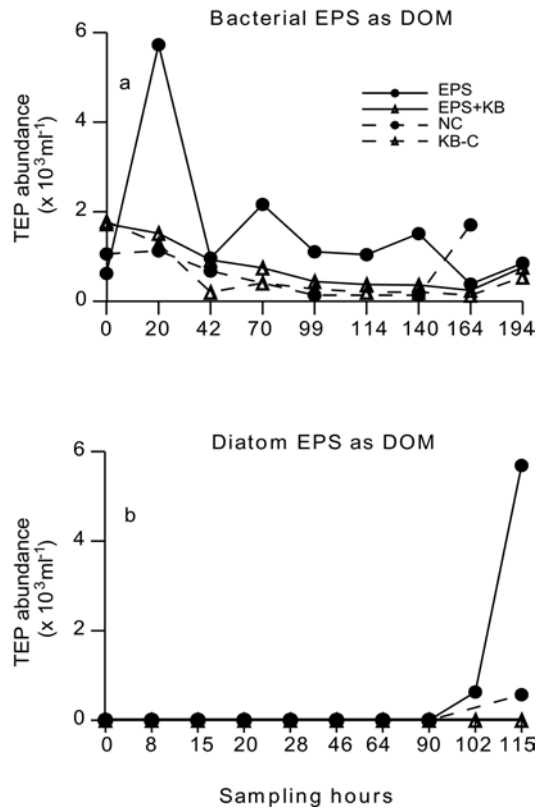


Fig. 4.3 Changes in the abundance of TEP produced in experimental and control tanks having bacterial EPS (a) and diatom EPS (b) as sources of DOM.

4.3.5 Statistical analyses

In order to assess the relationship between various parameters, Pearson's correlation coefficient was carried out. There was no correlation of

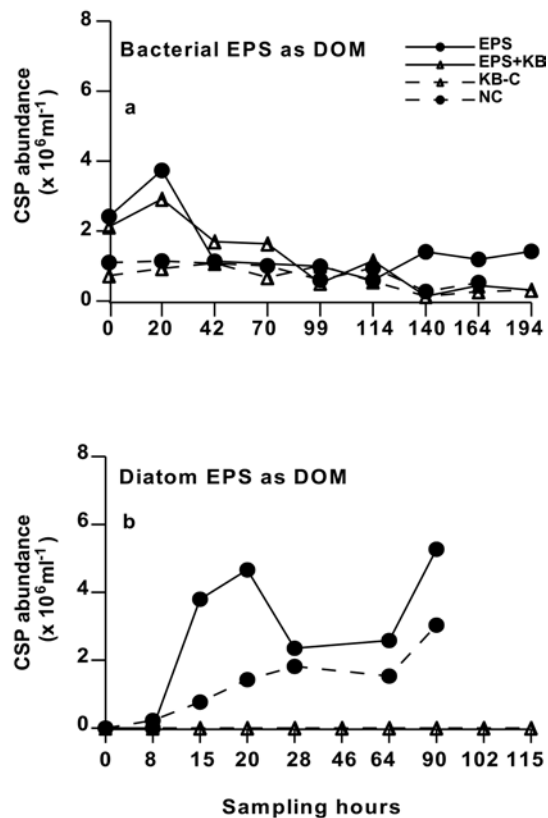


Fig. 4.4 Changes in the abundance of CSP produced in experimental and control tanks having bacterial EPS (a) and EPS of diatom (b) as sources of DOM.

macroaggregate size and abundance with TEP and CSP. Macroaggregate size had no specific trend with particle abundance (Table 4.4). The abundance of TEP had no relation with macroaggregate size except for positive trend in *KB + Eps* tank of experiment-I. On the other hand, TEP abundance had a linear trend with particle abundance. The abundance of CSP did not appear to have any correlation with macroaggregate size and abundance.

In order to assess the effect of EPS derived from different source on the production of macroaggregates, TEP and CSP, a One-way ANOVA for

unequal data set was carried out (**Table 4.5**). Significant differences in the variance of particle abundance and size were observed when aggregates were produced using EPS derived from two different sources. For example, the amount and size of macroaggregates produced in tank having bacterial EPS (experiment I) was significantly different ($p < 0.001$) from that of tank containing diatom EPS (experiment II). On the other hand, significant differences were observed only in the abundance of TEP and not in the abundance of CSP. Similarly, aggregates produced in *Eps + KB* tanks in the two experiments were significantly different in the abundance of macroaggregates, TEP and CSP and the macroaggregate size ($p < 0.001$ and $p < 0.005$). The controls (both *NC* and *KB*) showed significant differences ($p < 0.01$ and $p < 0.001$) in their abundance of macroaggregates, TEP and CSP. However, only moderate differences ($p < 0.05$) were observed in the size of the macroaggregates between the *NC*-tanks.

4.4 Discussion

Various methods including rolling tanks, bubbling and couvette flocculators have been used to study the production of TEP and aggregates. Shanks and Edmondson (1989) were the first to use the rolling tanks to generate marine-snow like aggregates from unfiltered seawater. Since then, rolling tanks have been widely used to produce aggregates from laboratory cultures (Reibesell 1991) and natural limnic and marine waters (Artolozaga et al 1997, Grossart et al 1998, Unanue et al 1998). In this method, shear is generated in the liquid

Table 4.4: Correlation matrix of particle abundance, size and TEP abundance produced using bacterial and diatom EPS in the experimental and control tanks.

Parameters		Experiment I			Experiment II		
Tanks		Size	TEP	CSP	Size	TEP	CSP
Eps	Abundance	-0.08	0.81**	-0.36	-0.32	0.31	0.17
	Size		-0.04	0.26		0.085	0.16
KB+Eps	Abundance	-0.34	-0.06	0.45	0.42	--	--
	Size		0.47	-0.20		--	--
NC	Abundance	0.05	0.09	0.28	0.11	0.29	0.14
	Size		-0.60	-0.51		0.09	0.39
KB-C	Abundance	0.13	0.34	0.65	0.46	--	--
	Size		-0.19	0.04		--	--

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

--: Since the TEP and CSP counts were negligible, correlations were not carried out in this experiment.

thereby causing collisions of aggregates. Upon collision, small aggregates are formed first that produce larger aggregates by differential settling. Although this technique does not completely mimic natural conditions, the method is preferred largely for the convenience of sampling and image analyses (Unanue et al 1998, Simon et al 2002). Moreover, the characteristics of aggregates produced by the rolling tanks have been shown to be similar to those produced in the natural environment and therefore may be considered representative of naturally occurring aggregates.

Variations in aggregate abundance and size was observed in the *Eps* and *NC* tanks of both experiments. The macroaggregate abundance in *Eps* tanks

varied from 9.8 to 60.9 cm⁻² (experiment I) and 7 to 59 cm⁻² (experiment II) (**Table 4.2**). Particles were also observed in the control tanks of both the experiments. It was clear that the autoclaved medium contained particles (mainly salts) at the start of each experiment (**Figs. 4.1a & 4.1b**). It is possible that the initial particle abundance might have influenced the overall aggregation by increasing the chances of particle collisions (Simon et al 2002). However, smaller aggregate size in the controls (*NC* tanks) suggests that initial abundance of inorganic particles alone could not produce macroaggregates. Exopolymers produced by microorganisms are known for their binding and gelling properties, which is essential for particle aggregation (Decho 1990, Herndl 1995, Hoagland et al 1993, Zhou et al 1998). This was evident from the production of larger sized particles in the *Eps* tanks supplemented with EPS derived from both bacteria and diatom.

The observed variations in particle abundance indicate that the production of aggregates was not a unilateral process and varied with time. An increase in particle size with a concomitant decrease in particle abundance suggests that smaller aggregates were coagulating due to shear produced in the rolling tanks to form fewer but larger aggregates. This was evident from the data obtained for experimental tanks supplemented with bacterial and diatom EPS, especially after 20h (experiment I) and 28h (experiment II), respectively (**Fig. 4.2a & 4.2b**). During some samplings, aggregate counts increased with negligible change in the overall size of the aggregates. One reason for the observed variation could be the production of new particles. Similarly, disaggregation of particles might also contribute to the increase in particle

abundance over time. Thus production of new particles combined with aggregation and disaggregation of particles appeared to be occurring during these experiments.

Aggregation experiments in laboratory and in natural environments have showed an increase in abundance of visible aggregates in the presence of polymers released by microorganisms (Reibesell 1991, Kiørboe and Hansen 1993, Passow et al 1994 and 1995, Stoderegger and Herndl 1998). However, all these observations were carried out using growing cultures of microorganisms in the laboratory or during phytoplankton blooms in the natural environments. In the present study, the influence of live cells was minimal. Therefore, an increase in particle abundance in the present study was not always possible and production of new particles was limited.

The presence of organic compounds like EPS help in increasing the chances of colliding particles to stick together and form larger particles (Herndl 1995, Kiørboe and Hansen 1993). In the present study, experimental tanks containing only EPS in both the experiments (I & II) produced larger aggregates in first 20 to 46 h following incubation (**Fig. 4.2a & 4.2b**). Interestingly, similar aggregation rates were not observed when bacterial EPS was mixed with killed bacterial cells. Moreover, killed bacteria did not form any large sized macroaggregates in presence of diatom EPS. A similar variation in macroaggregate size was evident in the killed bacterial control tanks. The presence of killed bacterial cells was expected to serve as nuclei for macroaggregate production. Moreover, the rate of macroaggregate production

is related directly to the stickiness of the colliding particles (Kjørboe et al 1990) and collision rate (which is a function of shear and particle density) (McCave 1984, O'Melia and Tiller 1993). However in the present study, the presence of killed bacterial cells appeared to suppress the aggregation process even in the presence of EPS. It is possible that killed bacterial cells might have released its intra-cellular content in to the medium, which might have had a suppressing effect on the aggregation of killed bacterial cell. Production of organic compounds that lower the particle stickiness has been reported in earlier studies (Decho 1990, Kjørboe and Hansen 1993). However, the exact cause of the suppression of macroaggregates in presence killed bacterial cells could not be ascertained in this study.

Differences in aggregation rates and size were observed in the *Eps* tanks between the two experiments. Single way ANOVA (**Table 4.4**) showed significant differences in the mean size and abundance of aggregates produced by EPS derived from different sources. These EPS differ in their chemistry and binding properties (Kjørboe and Hansen 1993, Passow 2002) and therefore might influence the aggregation rate and macroaggregate size. One reason for the observed differences in aggregation rate, size and abundance could be the difference in the EPS chemistry. For example, *Marinobacter* EPS contained more of protein (44%) and uronic acid (17.6%) and lesser amounts of carbohydrates (17 %) and sulphates (2.7%) (**See Chapter 3**). In contrast, EPS of *S. costatum* had greater amounts of sulphates (21.5%) and relatively lesser amounts of proteins (3.6 %) and uronic acid (4.6 %) (**Table 4.2a**). Although both EPS were heteropolysaccharides, diatom EPS

had greater abundance of different monosaccharides compared to bacterial EPS.

High molecular weight compounds like EPS released by microorganisms generally contain uronic acid, sulphates and proteins. These compounds may impart an overall negative charge to the polymer, thereby help in binding and aggregation processes (Christensen 1989). Moreover, sulphates are known to impart gelling properties and may help in floc formations (Hoagland et al 1993, Zhou et al 1998). For example, Zhou et al (1998) reported the accumulation of sulphates along with deoxy sugars in the foams obtained during bubbling of seawater supplemented with algal polysaccharides, indicating that surface active polymers rich in deoxy sugars and sulphates can form particles abiotically. In the present study, larger sized flocs were produced in presence of diatom EPS that was relatively rich in sulphates whereas aggregates were formed at a slightly faster rate in tanks containing bacterial EPS. The differences in the uronic acid and sulphate content and monosaccharide chemistry of the EPS of *Marinobacter* and *S. costatum* might therefore be responsible for the observed difference in the aggregation rates and size.

Organic particles like TEP are considered important for aggregate production (Kiørboe and Hansen 1993, Passow et al 1995, Stoderegger and Herndl 1998, Simon et al 2002). CSPs are also found abundant in natural environments though its role in particle aggregation is not clear. TEPs were virtually absent or very low in numbers at the start of each experiment (**Fig.**

4.3a & 4.3b) and generally was lower than those reported by Stoderegger and Herndl (1998) and Passow (2002). Moreover, in those experiments the TEP abundance increased with time. In contrast to the observation of TEP, CSP counts in the *Eps* and *NC* tanks varied considerably in the two experiments and were in the range of 10^3 particles ml^{-1} (**Figs. 4.4a & 4.4b**). Only field reports on the distribution of CSP are available (Long and Azam 1996, Berman and Viner-Mozzini 2001) and therefore direct comparison with these reports might not be appropriate due to differences in the experimental conditions. The low amount of TEP in the present study was intriguing since both bacterial and diatom EPS have fair amount of uronic acid, which can serve as a precursors for the production of TEP. However, this was not so as observed from the TEP abundance data (**Table 4.2**). One reason for the observed low numbers of TEP could be the quality of the EPS used. Leppard et al (1977) have reported that precursors of colloids like TEP are few nanometer in size. Moreover these precursors can pass through 8 kDa dialysis bags (Passow 2002). In the present study, the EPS used as a precursor for TEP production had been dialyzed through 8 kDa MW cut-off dialysis bags prior to the aggregation experiments. Therefore, it is possible that fair amount of TEP precursors were lost during dialysis, thereby responsible for the low TEP abundance in these experiments. However, macroaggregates were produced in spite of the low TEP abundance. This is also reflected in the poor correlations between TEP/CSP abundance with macroaggregate size. Thus, it appears that neither TEP nor CSP was necessary for the production of macroaggregates.

4.5 Conclusions

The presence of microbial EPS led to the production of macroaggregates up to the size of 22 to 35 μm^2 . Such abiotic production of macroaggregates carries significance in the DOM-POM transformation at depths unaffected by surface turbulence. The aggregate size and abundance varied with the source of EPS, especially in the absence of killed bacteria. The observed differences in the abundance of macroaggregates, TEP, CSP and macroaggregate size might be related to the differences in polymer chemistry. Moreover, organic particles like TEP and CSP need not always be required for production of macroaggregates.