CHAPTER 4

Production and biochemistry of extracellular polymeric substances by thraustochytrid protists
4.1. Introduction

Extracellular polymeric substances (EPS) are organic macromolecules that are formed by polymerization of similar building blocks, which may be arranged as repeating units within the polymer molecule (Wingender et al., 1999). Microbial EPS are studied for several reasons (Neu and Lawrence, 1999).

1. EPS confer several ecological advantages to the cells producing them and also play a major role in biogeochemical cycles in the ecosystem.
2. EPS often represent a structural feature of microbial cell and is, therefore, investigated for pure and basic research.
3. EPS are recognized as antigen determinants of the microbial cell surface. The knowledge of their structure is of great significance in medical microbiology.
4. EPS are polymers with unique properties and so are important in biotechnological applications.

Most marine microorganisms produce EPS in their ecosystem (Decho, 1990).

The amount of EPS produced by various organisms may range from low levels, such as 0.24 g L\(^{-1}\), as in a *Pseudomonas* species, to very high amounts of 33 g L\(^{-1}\) as in a *Xanthomonas* species after optimization (Christensen et al., 1985; Sutherland, 1998).

EPS is mostly composed of polymer chains with high molecular weight ranging from 70 – 2800 kDa (Philippis and Vincenzini, 1998). The composition and production of EPS is very variable and is dependent on (Decho, 1990):
a) The physiological state or growth stage of the cell. It has been observed that production of EPS starts in the exponential phase of growth but maximum production occurs during the stationary phase in most organisms (Whitefield, 1988). Some strains are known to produce different polymers at different growth stages. (Sutherland, 1997).

b) The composition of the nutrient media in which the culture is grown. Many microorganisms in laboratory culture can be induced to yield large quantities of EPS when grown in a high-carbon: low-nitrogen growth media (Williams and Wimpenny, 1978). Although excess carbon in media increases EPS production, it is not a necessity for its production. EPS production has also been observed when the cultures were grown only on amino acids but the production was very low (Sutherland, 1979; Sutherland, 1982; Wicken, 1985).

c) The ionic and physical condition of the media. Temperature and pH are also known to affect the composition of the polymer.

The ability of microorganisms to produce exopolymers under varying conditions is thought to reflect the important function of these secretions under fluctuating nutrient and environmental conditions.

4.1.1. Structure and Composition of EPS

Backbone polymer: The backbone polymer in EPS is usually a polysaccharide, although some organisms are known to secrete a proteinaceous polymer also (Platt et al., 1985; Frølund et al., 1996). Polysaccharides form a large portion of
EPS. Heteropolysaccharides are more common in the marine ecosystem, being made up of repeating units of 6 or fewer monosaccharides (Anton et al., 1998). The commonly occurring hexoses are glucose, galactose and mannose, while xylose, arabinose and ribose are common among the pentoses. Deoxyhexoses are mostly represented by fucose and rhamnose (Decho, 1990; Philippis and Vincenzini, 1998).

A variety of organic and inorganic substituents are found associated with EPS. These non-carbohydrate components make up a relatively smaller portion of the EPS on a per weight basis but are extremely important to the tertiary structure, as well as physicochemical and biological properties of the EPS. These components are mostly in the form of residues and side groups on the polysaccharide chain (Philippis and Vincenzini, 1998; Majumdar et al., 1999).

**Organic substituents:** These include amino acids, amino sugars, proteins, uronic acids, acyl, ketals and pyruvates. Proteins can also be glycosylated with oligosaccharides to form glycoproteins or can be substituted with fatty acids to form lipoproteins (Sutherland, 1980; Orr et al., 1982). Proteinaceous material, in the form of exoenzymes is also found to be associated with EPS. However, the concentration of nitrogenous material is generally less than 10 % in purified polymer. Removal of the proteinaceous moiety drastically reduces the viscosity of the aqueous solution of the polysaccharides (Arad et al., 1993).

Uronic acids are carboxylated forms of sugars and the commonly found uronic acids are glucuronic and galacturonic acids. Their presence confers an
overall negative charge and acidic property to the polymer. The absolute amounts of uronic acids increase in the EPS with increase in age and metabolic stress. It has been postulated that the presence of acyl groups such as O-acetyl and pyruvate groups are essential to protect uronic acids from epimerisation (Decho, 1990).

Presence of acetyl groups and the deoxy sugars, fucose and rhamnose confer a lipophilic character to EPS (Shepherd et al., 1995). Presence of acetyl groups hinders cation binding (Geddie and Sutherland, 1994). Moreover acetyl groups may contribute to the stabilization of the ordered form of the polymer, as has been reported in xanthan structure (Sutherland, 1994).

Pyruvates are often ketal linked and contribute to the water binding properties of the EPS (Sutherland, 1979).

Inorganic substitutents: These include phosphate and sulfate groups, and are very common in the marine ecosystem. These groups confer a negative charge to the polymer (Decho, 2000). Polysaccharides having concentrations of charged components usually form stable gels in the presence of metallic ions. However, mere determination of the quantity of the charged groups is not enough for predicting the metal binding capacity of the polymer, as the accessibility of charged groups to ions depends on the conformational structure of the molecule (Philippis and Vincenzini, 1998).

The objectives of the experiments carried out under this chapter are:

1. Microscopic confirmation of the presence of EPS;
2. Kinetics of EPS production and the effect of media ingredients on EPS production;

3. Gross biochemical characterization of the EPS;

4. Preliminary structural elucidation of the EPS.
4.2. Materials and Methods

4.2.1. Cultures and Growth conditions

Five isolates of thraustochytrids - NIOS-1, NIOS-2, NIOS-4, NIOS-7, and NIOS-12 were used to study production of EPS. The protocol given in Chapter 2 was followed for inoculum preparation and growing the cultures to study the EPS production. M4 medium was used for all the studies. The experiments were set up in triplicates and the cultures were grown for 7 day.

4.2.2. Light and scanning electron microscopy

Microscopic mounts of cells from seven-day old cultures grown in M4 broth were examined by phase contrast microscopy for the presence of EPS. The following methods were used for further microscopic examination using light and scanning electron microscopy. Sterile cut pieces of microscope slides, measuring approximately 25 mm\(^2\) (5 mm X 5 mm) were placed in sterile 5 cm Petri dishes and were covered with M4 medium (see Chapter 2). A loopful of culture grown in M4 broth was inoculated into these plates. Following growth for 10 days, some of the glass pieces were stained with 1.0 % alcian blue in 3.0 % acetic acid, pH 2.5 to test for acidic polysaccharides (Passow and Alldredge, 1994). Standard procedures were followed for scanning electron microscopy (Heywood, 1971). Cells grown on glass pieces as above were dehydrated in 10, 20, 50, 80, 90, and 100 % acetone for 15 min each, followed by dehydration in 100 % acetone for 2 rounds of 15 min each. The cells growing on the glass pieces were immediately critical point dried using a SPI supplies, US, make
instrument. This was followed by sputter-coating with gold-palladium for 45 s. Following gold palladium sputter coating, the pieces were examined under a Jeol JSM-5800LV scanning electron microscope.

4.2.3. Extraction and estimation of exopolysaccharides

The isolates NIOS-1, NIOS-4, NIOS-7 and NIOS-12 were grown in M4 medium as described above. The EPS was extracted as follows. Cultures were transferred to 50 ml centrifuge tubes and centrifuged at 7500 rpm (5200 g) for 15 min. The pellet was discarded and the supernatant was first filtered through Whatman GF/F filter and then through 0.45 μm filters. The filtrate was then concentrated 10 X by ultrafiltration using an Amicon make ultrafilter having a molecular cut off of 10 kDa and dialyzed against distilled water. Three volumes of cold absolute ethanol was added to the retained solution and the mixture was left overnight at 4 °C. The precipitated EPS was centrifuged as above and lyophilized using a Thermo Savant Micro Modulyo freeze drier prior to storage at -20 °C. Total EPS produced was estimated gravimetrically. This EPS was used for all further analyses.

4.2.4. Production of EPS

4.2.4.1. Kinetics of EPS production with respect to growth in isolates NIOS-1 and NIOS-4

The growth and EPS production by isolates NIOS-1 and NIOS-4 in M4 medium were monitored. Three-milliliter aliquots were removed for turbidity measurements (OD 660 nm) and EPS production at regular intervals. In order to
estimate EPS, samples were centrifuged (5200 g, for 15 min) and serially filtered through a Whatman GF/F glass fiber filter and 0.45 µm membrane filters. The filtrate was collected and dialyzed. EPS was extracted as above. EPS was estimated as total carbohydrates using the phenol-sulfuric acid method (Dubois et al., 1956). The experiment was carried out until the culture reached the stationary phase.

Assimilation of organic carbon into EPS in NIOS-4 was studied following the basic protocols provided by Deming (1993). Twenty-five milliliters of M4 medium in a 100 ml Erlenmeyer flask was inoculated with a 2-day culture as above. Radiolabelled glucose (¹⁴C glucose, 0.05 mCi ml⁻¹), obtained from Bhabha Atomic Research Centre, Trombay, India, was used for the experiments. An end concentration of 20 µCi of ¹⁴C glucose was added to the 25 ml culture. A sterile Eppendorf tube containing a filter paper soaked in phenylethylamine was suspended in the flask using a string to measure the amount of CO₂ respired from the glucose. The flasks were stoppered tightly using rubber stoppers. The culture was incubated for 24 h at room temperature on a shaker at 150 rpm. The cells were then harvested by vacuum filtration through 0.22 µm filter paper. Labeled EPS in the culture filtrate was precipitated by ethanol (70 % final concentration) and its radioactivity was recorded by using Perkin Elmer Wallac 1409DSA liquid scintillation counter. The radioactivity in the respired CO₂, in the cells and unutilized glucose (culture filtrate after EPS precipitation and removal) was also measured.
4.2.4.2. Effect of media ingredients on the production of EPS

The same set of cultures used for optimizing DHA production by the isolate NIOS-1 using the Response Surface Methodology (RSM), as given in Chapter 2, was also used to study the relationship between media ingredients, DHA and EPS production. After harvesting the cells (RSM experiment) the remaining culture filtrate was used for EPS estimations.

4.2.5. Biochemical composition of the EPS

4.2.5.1. Total Carbohydrates

The total carbohydrates were estimated according to the protocol of Dubois et al. (1956). To 0.5 ml sample, 0.3 ml 5 % phenol and 1.8 ml concentrated sulfuric acid (sp.gr. 1.84) were added and mixed thoroughly. After 20 min incubation, the OD was read at 480 nm. Sugar content was estimated by referring to a standard graph prepared by using D-galactose (5-50 μg / 0.5 ml).

4.2.5.2. Protein estimation

Composition of the various reagents is given in Appendix 2. A modified Lowry’s method was followed to estimate the total protein (Peterson, 1977). EPS at a concentration of 1 mg ml\(^{-1}\) in distilled water was taken, to which 0.1 ml of 0.15 % deoxycholate was added. The solution was incubated for 10 min at room temperature, to which 0.1 ml 72 % TCA was added. The mixture was centrifuged for 7500 rpm for 15 min. The pellet was dissolved in 1 ml of distilled water to which 1 ml Reagent A was added (Appendix 2), mixed and the mixture was
incubated at room temperature for 10 min. This was followed by an addition of 0.5 ml of Reagent B (Appendix 2) and the OD was read at 750 nm after 30 min. The concentration of protein was estimated by referring to a standard curve prepared using bovine serum albumin (0 – 100 µg ml⁻¹).

4.2.5.3. Uronic acids

Knutson and Jeanes' (1968) method was followed to estimate the total uronic acid content of the EPS. To 0.5 ml sample, at ice-cold temperature, 3 ml of concentrated sulfuric acid was added drop wise and mixed thoroughly. This solution was boiled in a boiling water bath for 20 min, cooled and 0.1 ml carbazole solution was added (0.1 ml, 0.1 % prepared by dissolving recrystallized carbazole in alcohol. Carbazole was thrice recrystallized by using benzene in order to remove contaminants in the commercially available sample). The tubes were incubated in dark for 2 h and the OD was read at 530 nm. Uronic acids content was determined by referring to the standard graph prepared by using D-galacturonic acid (10-50 µg 0.5 ml⁻¹).

4.2.5.4. Sulfate estimation

Total sulfates were estimated according to the procedure of Dodgson (1961). Twenty milligrams of sample was hydrolyzed with 3 ml of 60 % formic acid at 100 °C for 8 h. The hydrolysate was evaporated to dryness and reconstituted with 2 ml distilled water. To 0.5 ml hydrolysate, 3.8 ml of 4 % TCA and 1 ml of 5 % gelatin in 50 % barium chloride were added and incubated at
room temperature for 10 min. The OD was read at 500 nm and the sulfate content was determined by referring to a standard curve prepared by using potassium sulfate (0 – 50 µg).

4.2.5.5. Reducing Sugars

The method of Imoto and Yagishita (1971) was followed to estimate the total reducing sugars. To 1 ml of sample, 2 ml of potassium ferricyanide reagent (0.05 % potassium ferricyanide in 0.5 M sodium carbonate) was added and boiled for 15 min. The samples were cooled and OD was read at 420 nm. Reducing sugar content was estimated by referring to a standard curve prepared by using D-glucose (0-100 µg).

4.2.6.6. Solubility and Viscosity measurements

Solubility of the polymers was studied in 0.1 M EDTA, 0.5 M EDTA, 0.1 N NaOH, 1 N NaOH, 10 % SDS, 0.1 N H₂SO₄, 1 N H₂SO₄, 1 N HCl, DMSO, hexane and distilled water. EPS was dispersed at a concentration of 1 mg ml⁻¹ in each of above for 4 h. The insolubles were removed by centrifugation at 7500 rpm for 15 min. The solubility was determined by estimating the concentration of total sugar by phenol-sulfuric acid method in the supernatant.

The polysaccharide (50 mg) was suspended in 10 ml distilled water and stirred for 4 h at room temperature. This solution was centrifuged and the pellet was air dried at room temperature for 5 d, thereby evaluating the solubility of the polymer. For viscosity measurements, 0.6 % EPS in distilled water was analyzed.
using a Brookfield Model DV-III programmable rheometer. The values are expressed as centipoises. The standard used was distilled water, with a viscosity of 0.89 cp.

The polymer had maximum solubility in distilled water. Therefore, the water-soluble fractions of both the polymers were lyophilized and stored at -20°C for further use. The sugar, protein, lipid, uronic acid and surface contents were estimated for the total, native polymer as well as the soluble fraction.

4.2.6. Molecular weight determination

4.2.6.1. Ion exchange chromatography

The purity of the lyophilized EPS was confirmed by ion exchange chromatography, using DEAE-cellulose (HiMedia, India) (Khandeparkar and Bhosle, 2001; Jayaraman, 2001). The lyophilized EPS was suspended in 200 mM phosphate buffer containing 0.1 M NaCl (pH 7.5), and applied to a column (12.5 cm X 1 cm) of DEAE-cellulose. Samples were eluted with a linear gradient of 0.1 M to 1.0 M NaCl (pH 7.5) in phosphate buffer at a flow rate of 8 ml h\(^{-1}\). One-milliliter fractions were collected and analyzed for total sugars by phenol - sulphuric acid method.

4.2.6.2. Gel permeation chromatography

Molecular weight of the EPS was determined by gel permeation chromatography (GPC) according to the procedure of Brown and Volence (1989). GPC was performed on Sepharose CL – 2B (1.6 cm X 92 cm). One
milliliter of sample (5 mg ml\(^{-1}\)) was loaded to the column and was eluted with
degassed triple distilled water at a constant flow rate of 1.6 ml min\(^{-1}\). Fractions
(1.6 ml) were collected and analyzed for the presence of total sugars. The
column was calibrated with T-series dextran standards (T-70, T-150, T-500, T-
2000, Pharmacia). A calibration curve was prepared by plotting elution volume
(Ve)/ Void volume (Vo) versus log molecular weight and the molecular weight of
the unknown sample was determined (Plummer, 1994).

4.2.6.3. High performance size exclusion chromatography (HPSEC)

The molecular weight of the EPS was confirmed by HPSEC. HPSEC was
carried out using Shimadzu HPLC system (LC-8A model), equipped with
refractive index (RI) detector and CR 4A recorder. E-Linear (7.8 mm X 30 cm)
and E-1000 (3.9 mm X 30 cm) columns connected in series, were used for
analysis. The EPS sample (5 mg ml\(^{-1}\)) were dissolved in triple distilled water. 10
µl was loaded and eluted with triple distilled water at a flow rate of 0.6 ml min\(^{-1}\).
Similarly standards (T-70, T-150, T-500 and T-2000) were loaded individually.
The elution was monitored by using RI detector set at 8 X10\(^{-6}\) RIU (Kobayashi \textit{et al}, 1985).

4.2.7. Structural elucidation of EPS

4.2.7.1. Determination of neutral sugar composition by GLC

The neutral sugar composition was determined according to the procedure
of Sawardekar \textit{et al.} (1967). The EPS (10 mg) was suspended in water and was
completely hydrolyzed by prior solubilization with 72 % sulfuric acid at ice-cold temperature followed by diluting to 8 % acid and heating at 100 °C for 10-12 h. The above mixture was neutralized with solid barium carbonate, filtered, deionized with Ambrelite IR-120-H+ resin and concentrated. Ten milligram of inositol was added as an internal standard. The monosaccharides were reduced by adding 20 mg of sodium borohydride at room temperature for overnight. Excess borohydride was decomposed by adding 2 N acetic acid, dropwise till effervescence of hydrogen stopped. The boric acid formed was removed by co-distilling with methanol (2 ml X 3). The dry glycitols were acetylated with acetic anhydride and pyridine (1 ml each). The mixture was kept at 100 °C for 2 h. Excess reagent was removed by co-distilling with water and toluene. The alditol acetates were extracted with chloroform, filtered through glass wool and dried under a jet of nitrogen. The derivatives were reconstituted in a known volume of chloroform and injected into GLC for quantitative and qualitative analyses.

Analysis of alditol acetates was carried out using Shimadzu GLC system (GC-15A), fitted with flame ionization detector and CR4-A monitor. OV-225 (3 %) stainless steel column (8 feet X 1/8 inch internal diameter) was used for analysis with column, injector and detector port temperatures maintained at 200, 250 and 250 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 35 ml min⁻¹. Fucose, rhamnose, xylose, mannose, galactose and glucose were used as standards.
4.2.8. Linkage studies

4.2.8.1. Periodate Oxidation

Periodate oxidation helps in the determination of the type of linkages and the substituent group arrangements. When α, β and γ-triols are treated with periodate, one molecule proportion of formic acid is produced. Therefore, (1→6) linked hexopyranose units yield formic acid, whereas (1→2), (1→3) and (1→4) linked units do not (Fred, 1966).

To a 5 ml aqueous solution of 0.1 % polysaccharide 5 ml of 20 mM sodium meta periodate solution was added, mixed thoroughly and kept at 4 °C in dark. Aliquots (0.5 ml) of sample were withdrawn at regular intervals of 4 h, mixed with a solution containing 8 ml distilled water, 4 ml 20 % potassium iodide and 0.5 N, 0.6 ml sulfuric acid. The liberated iodine was immediately titrated with 0.1 N sodium thiosulfate using starch as an indicator. The consumption of periodate was calculated using the formula;

\[
\text{Periodate consumption} = \frac{E - (V_1 - V_2) \times C \times M}{1000G}
\]

Where;
- \(E\) = Actual moles of periodate taken
- \(V_1\) = Titre value of blank
- \(V_2\) = Tire value of sample
- \(C\) = Concentration of sodium thiosulfate solution
- \(M\) = Molecular weight of sugar
- \(G\) = Weight of sample taken in grams
4.2.8.2. Formic acid liberation

Formic acid liberation was studied according to the procedure of Fred (1966). To 0.5 ml of above reaction mixture, after periodate consumption became constant, 2 ml of 50 % ethylene glycol and 2 - 3 drops of 0.02 % methylene red were add as an indicator. This mixture was titrated against 0.01 N NaOH. A reagent blank was prepared in the same way and the difference in the acidity between the blank and the sample represented the formic acid liberated from the polysaccharide and it was calculated by the formula

\[
\text{Formic acid liberated} = \frac{V_1 - V_2 \times N_{NaOH} \times MW}{0.5}
\]

- \( V_1 \) = sample titer value
- \( V_2 \) = Blank titer value
- \( N_{NaOH} \) = Normality of NaOH
- \( MW \) = Molecular weight of the sugar
- \( 0.5 \) = Volume of sample taken for titration

4.2.8.3. Optical Rotation

Aqueous solution of the polysaccharide (1.0 %) was used to measure the optical rotation in a Perkin Elmer (model 243) polarimeter. Optical rotation is calculated using the formula

\[
\text{Optical Rotation} \ (\alpha)_D = \frac{100 \ \theta}{1C}
\]

Where, \( \theta \) = Angle of rotation of plane polarized light
- \( 1 = \) Path length (1 cm)
4.2.8.4. Infra-Red Spectroscopy

Polysaccharide (~2.5 mg) was blended thoroughly with solid KBr. IR spectra were obtained with Perkin Elmer Spectrophotometer (2000 system GC-IR) operating at 4 cm⁻¹ resolution. Spectra were recorded between 400 – 4000 cm⁻¹ (Lijour et al., 1994).

4.2.9. Enzymatic method to deduce the structure of EPS

4.2.9.1. Screening enzymes, hydrolyzing the polysaccharide

Fifty microlitres of 1 mg ml⁻¹ enzyme, pectinase (Rhizopus sp.), cellulase, α-galactosidase, α-amylase, amyloglucosidase, pullulanase and isoamylase (all from Sigma) were added to 0.5 ml of 5 mg ml⁻¹ polysaccharide and incubated for 1 h at room temperature. Two volume of absolute ethanol was added to precipitate the polysaccharide and the mixture was kept at 4 °C for 2 h. This solution was centrifuged at 7500 rpm for 15 min and the supernatant was flash-evaporated to dryness. The resultant oligomers were reconstituted in 0.5 ml distilled water and the reducing sugars were estimated according to the method of Imoto and Yagishita (1971).
4.2.9.2. Separation and molecular weight determination of oligomers by Gel permeation chromatography

Gel permeation chromatography was performed on Biogel P2 (0.9 cm X 91 cm) according to the procedure of Marry et al. (2003). One milliliter of sample (enzyme hydrolysed polysaccharide) was loaded onto it and was eluted with degassed triple distilled water at a constant flow rate of 1.6 ml min\(^{-1}\). Fractions (1.6 ml) were collected and analyzed for the presence of reducing sugars. The void volume was determined using blue dextran and the column was calibrated with maltose, raffinose, maltotetraose and maltopentaose.

4.2.9.3. Identification of hydrolytic products by HPLC

The enzyme hydrolytic oligomers were identified using a Shimadzu HPLC system (LC-8A model), equipped with RI detector and CR 4A recorder. An aminopropyl column was used for analysis and 10 μl sample was loaded and eluted with acetonitrile : water (80 : 20) at a flow rate of 1.0 ml min\(^{-1}\). Similarly standards (galactose, maltose, raffinose, maltotetraose and maltopentaose) were loaded individually. The elution was monitored by using RI detector set at 8 X10\(^{-6}\) RIU (Suzuki and Honda, 2001).
4.3. Results

4.3.1. Light and scanning electron microscopy

Phase contrast microscopic examination of all the cultures grown for 10 days on M4 medium showed the production of particulate EPS. The EPS was produced as amorphous particles or fibrillar material surrounding the cells (Fig. 4.3.1 a and b). The EPS was present as extensive sheaths, surrounding and embedding the cells of thraustochytrids. Scanning electron microscopy of thraustochytrids grown on glass pieces confirmed the production of EPS sheaths (Fig. 4.3.1 c and d). Extensive sheaths were particularly noticeable in culture NIOS-4. Further, EPS, which was secreted over the entire surface of the glass piece, stained positively with alcian blue (Fig. 4.3.2).

4.3.2. Estimation of EPS production

Quantitative estimation of EPS in culture filtrates confirmed that all four thraustochytrid isolates produced them (Fig. 4.3.3). The isolate NIOS-4 produced the maximum EPS, amounting to $1.1 \text{ g L}^{-1}$ (dry weight). Isolate NIOS-12 produced the least amount, corresponding to $0.3 \text{ g L}^{-1}$ dry weight. Since NIOS-1 and NIOS-4 produced the maximum quantity of EPS, these cultures were used for further studies. The production of EPS during different phases of growth was studied for the two isolates, under batch culture conditions (Fig. 4.3.4 a and b). The cultures showed characteristic sigmoidal growth curves with a lag phase of about 18 h for NIOS-4 and 9 h for NIOS-1 and reached the stationary phase after 46 h and 34 h, respectively. EPS production was observed at all stages, but
lagged behind growth. The concentration increased with age, reaching the highest values during the stationary phase. The concentration of the polymer did not show any decline during 73 h of growth.

Carbon assimilation studies during the late exponential growth phase showed that most of the carbon was assimilated into the cell biomass. About 7.0% of assimilated carbon was converted to EPS (Fig. 4.3.5).

4.3.3. EPS with relation to media and DHA production

EPS production by the isolate NIOS-1 was studied simultaneously during the Response Surface Methodology (RSM) experiment designed to optimize the media for biomass, total lipids and DHA production (Chapter 2). EPS was estimated for the 20 trials used for this RSM experiments, the results of which are shown in Table 4.3.1. The highest EPS production was seen in Trial 10 containing 3.62% glucose, 0.3% peptone and 0.25% yeast extract. The minimum EPS production was seen in Trial 1 containing 1.0% glucose, 0.15% peptone and 0.1% yeast extract (Table 4.3.1). Correlation coefficient values (r) were estimated to examine the relationship between EPS on one hand and biomass, lipids and DHA on the other (Table 4.3.2) It was observed that DHA production was positively related to biomass and EPS production (Table 4.3.1). In general EPS production was positively influenced by glucose.
4.3.4. Biochemical characterization of EPS

The solubility of EPS produced by NIOS-1 and NIOS-4 in various solvents is shown in Table 4.3.3. The EPS produced by both the isolates dissolved best in distilled water. The EPS of isolate NIOS-1 was 99.4 % soluble whereas that of NIOS-4 was 72.4 % soluble in distilled water.

The total as well as the soluble fraction of EPS produced by both the isolates contained sugars, proteins, lipids and uronic acids (Table 4.3.4 and 4.3.5). Sugars were the most abundant constituent of the EPS in both the isolates, comprising 53 and 39 % in NIOS-1 and NIOS-4, respectively. However, the soluble fraction of EPS in NIOS-1 contained less sugars, while that of NIOS-4 contained much higher levels. Sulfate contents were higher in the soluble fractions of both EPS. Proteins were much less in the soluble fractions of NIOS-1 and NIOS-4, compared to the total EPS. Protein and uronic acid contents were almost the same. EPS of both isolates were viscous in nature, that of NIOS-1 being more so (Table 4.3.4.). The IR spectra of the EPS produced by both NIOS-1 and NIOS-4 were similar. The broad peaks around 3500 cm⁻¹ and 1050 cm⁻¹ for OH⁻¹ and C-O(H) respectively showed the presence of sugars. The broad peak at around 1650 cm⁻¹ confirmed the presence of uronic acids in the EPS. The peak at 890 cm⁻¹ and 1240 cm⁻¹ indicated the presence of SO₄²⁻ (Fig. 4.3.6 a and b).

The EPS of both NIOS-1 and NIOS-4 were retained by 10 kDa ultrafilters. The extracted EPS from both gave a single peak when subjected to ion exchange chromatography and gel permeation chromatography (Figs. 4.3.7 and 4.3.8 a and b) showing that they produced only one type of EPS. Gel permeation
chromatography, as well as HPSEC indicated that the molecular weights of the EPS produced by NIOS-1 and NIOS-4 were more than 2000 kDa (Fig. 4.3.8 a and b). HPSEC also showed that polysaccharide extracted from both NIOS-1 and NIOS-4 was more than 95 % pure in both the cases (Fig. 4.3.9 a and b).

Gas chromatography analysis showed the presence of galactose, mannose, arabinose and fucose or rhamnose in the polymers produced by both NIOS-1 and NIOS-4 (Fig 4.3.10). Galactose formed the major component of the polysaccharide (65 % in NIOS-1 and 89 % in NIOS-4). Mannose and arabinose were present as minor constituents (Table 4.3.6).

Periodate consumption by the EPS of NIOS-1 and NIOS-4 was 0.071 moles and 0.084 moles respectively. No formic acid was liberated from EPS of NIOS-4, while 72 moles were liberated from that of NIOS-1. The optical rotation was −0.140 and +0.060 for NIOS-1 and NIOS-4 polymers (Table 4.3.7).

The elucidation of the structure of EPS was also carried out enzymatically. The various enzymes tested for hydrolysis are listed in Table. 4.3.8. The activity of various enzymes was similar on the EPS produced by both NIOS-1 and NIOS-4. The maximum hydrolysis was by pectinase whereas α-galactosidase, pullulanase, isoamylase and amyloglucosidase had very little or no activity on the polymer. Cellulase and amylase had some activity on the polymer.

Pectinase cleaved the EPS produced by NIOS-4 into 2 types of oligomers (Fig. 4.3.11a) and that of NIOS-1 was cleaved into one (Fig. 4.3.11b) as shown by GPC and HPLC. The smaller oligomer was a monosaccharide, identified as
galactose in NIOS-4 by HPLC and the larger oligomer was a disaccharide from both NIOS-1 and NIOS-4 (Fig. 4.3.12 a and b).

Fig. 4.3.1: a. Phase contrast photomicrograph of EPS matrix produced by the isolate NIOS-2. b. Phase contrast photomicrograph of EPS sheath produced by the isolate NIOS-1. Bar represents 10 μm. c. SEM photograph of EPS sheath produced by the isolate NIOS-1. d. SEM photograph of EPS matrix produced by the isolate NIOS-4. Arrow represents the cells and the diamond arrow, the EPS.
Fig. 4.3.2: Positive staining of EPS by alcian blue (arrow) in isolate NIOS-1. Bar represents 10 μm.

Fig. 4.3.3: Production of EPS by 4 isolates of thraustochytrids. The error bars indicate SD.
**Fig. 4.3.4 a:** Kinetics of EPS production with respect to growth in isolates NIOS-1. The error bars indicate SD.

**Fig. 4.3.4 b:** Kinetics of EPS production with respect to growth in isolates NIOS-4. The error bars indicate SD.
Fig. 4.3.5: Assimilation of $^{14}$C carbon into EPS in isolate NIOS-4. The error bars indicate SD.
<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Percent</th>
<th>glucose</th>
<th>Peptone</th>
<th>Yeast extract</th>
<th>Biomass</th>
<th>Lipids</th>
<th>DHA</th>
<th>DHA</th>
<th>EPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.15</td>
<td>0.1</td>
<td>3.81</td>
<td>35.97</td>
<td>27.81</td>
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<td>43.20</td>
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<td>36.08</td>
<td>0.978</td>
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<tr>
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<td>0.25</td>
<td>7.58</td>
<td>42.00</td>
<td>33.91</td>
<td>1.080</td>
<td>0.379</td>
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</tr>
<tr>
<td>20</td>
<td>2</td>
<td>0.3</td>
<td>0.25</td>
<td>6.56</td>
<td>34.80</td>
<td>36.17</td>
<td>0.825</td>
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</table>

Table 4.3.1: Biomass, total lipids and DHA for the 20 trials carried out under RSM

<table>
<thead>
<tr>
<th>Variables</th>
<th>Biomass (g L(^{-1}))</th>
<th>Lipids %</th>
<th>DHA %</th>
<th>DHA (g L(^{-1}))</th>
<th>EPS (g L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass (g L(^{-1}))</td>
<td>1.00</td>
<td>-0.05</td>
<td>0.61</td>
<td>0.90</td>
<td>0.52</td>
</tr>
<tr>
<td>Lipids %</td>
<td>-0.05</td>
<td>1.00</td>
<td>-0.47</td>
<td>-0.12</td>
<td>-0.12</td>
</tr>
<tr>
<td>DHA %</td>
<td>0.61</td>
<td>-0.47</td>
<td>1.00</td>
<td>0.85</td>
<td>0.44</td>
</tr>
<tr>
<td>DHA (g L(^{-1}))</td>
<td>0.90</td>
<td>-0.12</td>
<td>0.85</td>
<td>1.00</td>
<td>0.53</td>
</tr>
<tr>
<td>EPS (g L(^{-1}))</td>
<td>0.52</td>
<td>-0.12</td>
<td>0.44</td>
<td>0.53</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 4.3.2: A correlation matrix of biomass, total lipids, percent DHA and absolute values of DHA. Values in bold are significant at p < .05000, N=20.
<table>
<thead>
<tr>
<th>No.</th>
<th>Solvent</th>
<th>OD 480 NIOS-4</th>
<th>OD 480 NIOS-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1 M EDTA</td>
<td>0.256</td>
<td>0.239</td>
</tr>
<tr>
<td>2</td>
<td>0.5 M EDTA</td>
<td>0.278</td>
<td>0.224</td>
</tr>
<tr>
<td>3</td>
<td>0.1 N NaOH</td>
<td>0.105</td>
<td>0.113</td>
</tr>
<tr>
<td>4</td>
<td>1 N NaOH</td>
<td>0.125</td>
<td>0.165</td>
</tr>
<tr>
<td>5</td>
<td>10 % SDS</td>
<td>0.098</td>
<td>0.106</td>
</tr>
<tr>
<td>6</td>
<td>0.1 N H₂SO₄</td>
<td>0.253</td>
<td>0.236</td>
</tr>
<tr>
<td>7</td>
<td>1 N H₂SO₄</td>
<td>0.165</td>
<td>0.169</td>
</tr>
<tr>
<td>8</td>
<td>1 N HCl</td>
<td>0.085</td>
<td>0.063</td>
</tr>
<tr>
<td>9</td>
<td>DMSO</td>
<td>0.035</td>
<td>0.048</td>
</tr>
<tr>
<td>10</td>
<td>Hexane</td>
<td>0.046</td>
<td>0.026</td>
</tr>
<tr>
<td>11</td>
<td>Chloroform</td>
<td>0.071</td>
<td>0.052</td>
</tr>
<tr>
<td>12</td>
<td>Distilled water</td>
<td>0.265</td>
<td>0.248</td>
</tr>
</tbody>
</table>

**Table 4.3.3:** Solubility of EPS produced by NIOS-1 and NIOS-4 in various solvents.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIOS-1</td>
</tr>
<tr>
<td>Sugars</td>
<td>53.27 %</td>
</tr>
<tr>
<td>Proteins</td>
<td>23.73 %</td>
</tr>
<tr>
<td>Lipids</td>
<td>14.17 %</td>
</tr>
<tr>
<td>Sulfates</td>
<td>10.95 %</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>4.07 %</td>
</tr>
<tr>
<td>Viscosity (6 mg in 1 ml)</td>
<td>2.47 cp</td>
</tr>
</tbody>
</table>

**Table 4.3.4:** Physical and chemical characteristics of the total EPS produced by the two isolates.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIOS-1</td>
</tr>
<tr>
<td>Sugars</td>
<td>48 %</td>
</tr>
<tr>
<td>Proteins</td>
<td>2.56 %</td>
</tr>
<tr>
<td>Sulfates</td>
<td>12.7 %</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>4.8 %</td>
</tr>
</tbody>
</table>

**Table 4.3.5:** Chemical characteristics of the soluble fraction of EPS produced by the two isolates.
Fig. 4.3.6 a: IR spectrum of the soluble portion of the EPS produced by NIOS-1

Fig. 4.3.6 b: IR spectrum of the soluble portion of the EPS produced by NIOS-4
4.3.7: Elution profile of the EPS produced by NIOS-1 and NIOS-4 in an ion exchange column.
4.3.8 a: Elution profile of the EPS produced by NIOS-1 through Sepharose CL-2B column.

4.3.8 b: Elution profile of the EPS produced by NIOS-4 through Sepharose CL-2B column.
4.3.9 a: Elution profile of the EPS produced by NIOS-1 through E-linear E-1000 columns.

4.3.9 b: Elution profile of the EPS produced by NIOS-4 through E-linear E-1000 columns.
Fig. 4.3.10: GC profiles of component sugars in EPS of NIOS-1 (B) and NIOS-4 (C). 'A' represents standards. Numbered peaks are: 1 = rhamnose / fucose, 2 = xylose, 3 = arabinose, 4 = mannose, 5 = galactose, 6 = glucose, 7 = inositol (internal standard).

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Isolate</th>
<th>NIOS-1</th>
<th>NIOS-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose/Rhamnose</td>
<td></td>
<td>16.29</td>
<td>3.63</td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td></td>
<td>1.43</td>
<td>1.10</td>
</tr>
<tr>
<td>Mannose</td>
<td></td>
<td>16.89</td>
<td>5.67</td>
</tr>
<tr>
<td>Galactose</td>
<td></td>
<td>65.37</td>
<td>89.58</td>
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<tr>
<td>Glucose</td>
<td></td>
<td>-</td>
<td>-</td>
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</table>

Table 4.3.6: Monosaccharide composition (mole %) of EPS produced by the isolate NIOS-1 and NIOS-4
4.3.7: Linkage analysis of EPS produced by isolate NIOS-1 & NIOS-4

<table>
<thead>
<tr>
<th>Linkage analysis</th>
<th>Isolate</th>
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<tr>
<td></td>
<td>NIOS-1</td>
<td>NIOS-4</td>
<td></td>
</tr>
<tr>
<td>Periodate</td>
<td>0.071 moles</td>
<td>0.084 moles</td>
<td></td>
</tr>
<tr>
<td>consumption</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Formic acid</td>
<td>72 moles</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>liberation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optical rotation</td>
<td>-0.140</td>
<td>+0.060</td>
<td></td>
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4.3.8: Enzyme hydrolysis of EPS produced by isolate NIOS-1 & NIOS-4. The amount of reducing sugar released was estimated by potassium ferricyanide method.
Fig. 4.3.11 a: Elution profile of the Pectinase digest of the EPS produced by NIOS-1 through Biogel P-2 column.

Fig. 4.3.11 b: Elution profile of the Pectinase digest of the EPS produced by NIOS-4 through Biogel P-2 column.
4.3.12 a: Elution profile of the Pectinase digest of the EPS produced by NIOS-1 through aminopropyl column using HPLC.

4.3.12 b: Elution profile of the Pectinase digest of the EPS produced by NIOS-4 through aminopropyl column using HPLC.
4.4. Discussion

This study presents the first conclusive evidence for the production of extracellular polymeric substances (EPS) in thraustochytrids (Jain et al. 2005). All 4 species of thraustochytrids examined in this study produced noticeable amounts of EPS (Fig. 4.3.1, 4.3.2 and 4.3.3). An earlier scanning electron microscopic study by Bremer (1976) indicated the presence of adhesive pad like material in *Thraustochytrium kinnei* growing on the surface of the larvae of the brine shrimp, *Artemia*. Raghukumar et al. (2000) did not notice any adhesive EPS by thraustochytrids cells growing in biofilms on solid substrata immersed in seawater. It is likely that growth on high nutrient substrates promotes the production of EPS. Several earlier studies have shown that high concentrations of glucose in the culture medium promote EPS production (Sutherland, 1994). EPS was always produced in the 4 thraustochytrids cultures when grown in M4 medium containing 2 % glucose, 0.15 % peptone, 0.1 % yeast extract, 0.025 % KH$_2$PO$_4$ in sea water.

Both cultures NIOS-1 and NIOS-4 produced EPS throughout their exponential growth phase and this continued even during the stationary phase (Fig 4.3.4 a. and b). Up to 73 % conversion efficiency of glucose into EPS has been observed for some bacteria during the stationary phase (Williams and Wimpenny, 1978). Although only 7 % of organic carbon appeared to be secreted as EPS in the thraustochytrid NIOS-4 (Fig. 4.3.5), the total amount of carbon that goes into the production of EPS over a period of time would be substantial. Moreover, it is known for several bacteria that despite sugar limitation, EPS may
still be produced through utilization of amino acids as carbon sources (Sutherland, 1982). Therefore, it might be expected that EPS will accumulate with increasing age of cultures. Noticeable EPS was evident in 7 d old cultures of thraustochytrids. EPS in microorganisms is generally known to be produced during the stationary phase of growth. Several diatoms such as species of Amphora, Navicula and Melosira, and the brown tide alga, Aureoumbra lagunensis produced EPS throughout the growth phase but more particularly during the stationary growth phase when nutrients were depleted (Bhosle et al., 1995; Liu and Buskey, 2000; Khandeparkar and Bhosle, 2001; Leandro et al., 2003). A similar observation is seen in most marine bacteria (Decho, 1990).

The four isolates studied produced 0.3 to 1.1 g L⁻¹ EPS in M4 medium (Fig. 4.3.3). Bacteria produce EPS ranging from low amounts of about 0.24 g L⁻¹ as in a Pseudomonas species, 0.35 g L⁻¹ Lactobacillus species, to very high amounts of about 33 g L⁻¹ as in a Xanthomonas species after optimization (Christenson et al., 1985; Kimmel et al., 1998; Sutherland et al., 1998). It is likely that under optimal conditions thraustochytrids would produce larger amounts of EPS than those attained in the present experiments.

One of the main aims of this study was to examine the relationship between EPS and DHA production. It is important to understand this since DHA is a commercially important compound and it is necessary to understand how the energy provided to the cell is shunted to the production of other compounds. Likewise, if the EPS of thraustochytrids are found to be biotechnologically useful, it will become relevant if conditions favouring DHA production would suppress
EPS formation. Comparison of EPS production with the results obtained for RSM studies on biomass, lipids and DHA production clearly indicated that EPS production was not detrimental to DHA accumulation (Table 4.3.1.). Indeed, absolute DHA contents in cells and EPS production were positively related (Table 4.3.2). The present study also indicated that EPS production might be positively related to glucose concentration and total biomass. In general, optimal yields of EPS are obtained in the presence of high carbohydrate substrates. Many microorganisms in culture can be induced to yield large quantities of EPS when grown in a high carbon:low-nitrogen growth media (Sutherland, 1994). For many marine bacteria a glucose concentration of 1-2 % yields the highest EPS production, with up to 73 % conversion efficiency of glucose into EPS during the stationary phase (Williams and Wimpenny, 1978). Although EPS is not necessarily induced by high levels of carbon, elevated carbon levels in media promote EPS production. Some marine bacteria can produce EPS merely with ambient levels of nutrients in seawater (Decho, 1990). DHA is a signature compound of thraustochytrids (Ellenbogan et al., 1969). Likewise, EPS also appear to be a signature compound of these protists.

Thraustochytrids did not produce capsular polysaccharides as in the case of Nostoc species (Phillips and Vincenzini, 1998). In stationary cultures the EPS in the thraustochytrids was in the form of a matrix or sheaths around the cells (Fig 4.3.1). This is similar to those produced by many biofilm-forming bacteria, cyanobacteria and diatoms (Bhosle et al., 1995; Phillips and Vincenzini, 1998; Khandeparkar and Bhosle, 2001). However, little such particulate EPS was
observed in shake cultures of two isolates, where most of the EPS was in a soluble form. Both isolates studied produced up to 26% of insoluble EPS. The sheath-like EPS seen around the thraustochytrids cells might constitute insoluble EPS as well as the soluble portion, which had formed sheaths under stationary conditions. Likewise, the diatom *Amphora rostrata* produced both soluble, as well as 'biofilm' EPS (Khandeparkar and Bhosle, 2001).

NIOS-1 and NIOS-4 produced high molecular weight EPS of about 2000 kDa (Figs 4.3.9 a and b). High molecular weight EPS are found in many microorganisms such as the bacteria *Pseudomonas caryopholli* (Sudhamani *et al.*, 2004), the marine periphytic bacterium *Pseudomonas* species (Christensen *et al.*, 1985) and the yeast *Aureobasidium pullulans* (Lee *et al.*, 1999). Many cyanobacteria are also reported to produce high molecular weight EPS in the range of 1400 - 2800 kDa. (Phillips and Vincenzini, 1998). Most water-soluble polymers with high molecular weight readily forms gels in high concentration (Phillips and Vincenzini, 1998).

The EPS of NIOS-1 and NIOS-4 were acidic polysaccharides, staining positively with Alcian blue, indicating the anionic (acidic) nature of the EPS (Fig 4.3.2.) Alcian blue is a copper containing cationic phthalocyanin dye which binds to anionic molecules. Anionic polysaccharides are commonly produced by marine organisms including water column and hydrothermal vent bacteria (Lijour *et al.*, 1994), cyanobacteria, and many phytoplankton species, including diatoms (Christensens *et al.*, 1985; Bhosle *et al.*, 1995; Phillips and Vincenzini, 1998; Liu and Buskey, 2000; Khandeparkar and Bhosle, 2001). Anionic nature is generally
conferred by uronic acids, sulfates and pyruvates (Decho and Lopez, 1993; Leandro et al., 2003). Uronic acids and sulfates were found to be important constituents of the EPS of the two thraustochytrids studied in detail (Table 4.3.4 and Table 4.3.5). In eukaryotic cells, sulfation of EPS occurs in the Golgi apparatus (Ramus and Robins, 1975). Charged groups such as sulfates, pyruvates and uronic acids significantly contribute to the solubility of the polysaccharide in water, thus improving the ability of the EPS to bind water molecules (Sutherland, 1994). Kennedy and Sutherland (1987) showed that bacterial EPS typically contain 20 – 50 % uronic acids. Uronic acids in the EPS increase with increase in age and metabolic stress (Uhlinger and White, 1983). Pyruvates were not estimated in the present study but acyl groups, such as pyruvates and succinates protect uronic acids from epimerisation and thus assure a high uronic acid content in the final polymer.

Sulfated polysaccharides are of much biotechnological importance and may have applications as blood anticoagulants and antiviral compounds (Sogawa et al., 1998; Alban et al., 2002). The importance of sulphated cell wall polysaccharides in macroalgae is well known (Murano, 1998). In addition, several recent papers describe the importance of extracellular sulfated polysaccharides in organisms such as the cyanobacterium, Aphanocapsa halophytia and the red alga Porphyridium species (Geresh et al., 1991; Matsunaga, 1996). This study demonstrates that thraustochytrids are also characterized by EPS with sulfated polysaccharides, whose biotechnological applications will be worth studying.
EPS with high concentration of charged components usually form stable gels in the presence of metallic ions and are therefore a promising option for the removal of toxic metals from polluted waters (Bender et al., 1994). The accessibility of the ions to the EPS depends upon its conformational status. In the absence of a suitable conformation, some of the charged groups of the EPS will not be accessible for the ions. Therefore, the mere determination of the quantity of charged groups is not enough for anticipating the actual binding capability of the polymer (Sutherland, 1994). Further conformational studies on the EPS of thraustochytrids are necessary to understand their potential in biotechnology.

The enormous structural diversity of the EPS arises from composition of the EPS, broad arrangement of monosaccharides and the additional non-carbohydrate constituents (Decho, 1990). Taxonomic groups of organisms often have certain characteristic sugar composition in their EPS. Most common monomers present in EPS of marine bacteria are glucose, galactose, mannose, rhamnose and fucose. Pentoses are less common (Sutherland, 1977; Powell, 1979) but are a common component in cyanobacteria (Phillips and Vincenzini, 1998). Cyanobacterial EPS are more complex in structure than bacterial EPS as the former usually contain 6 or more monosaccharides, while bacterial and macroalgal EPS usually contain less than 4 (Decho, 1990; Phillips and Vincenzini, 1998). The EPS of the two thraustochytrids studied in detail show that galactose was the predominant sugar in EPS of both the thraustochytrids, in addition to smaller quantities of mannose, arabinose and rhamnose or fucose.
The EPS composition of these indicates some similarities to the general cell wall composition of these protists. The studies of Darley et al. (1973), Ulken et al., (1985), Bahnweg and Jackle (1986) and Chambelain and Moss (1988) have shown that thraustochytrid cell walls are comprised of sulfated galactans and proteins. A minor difference between the EPS and cell wall was the absence of xylose in the latter. Galactose is one of the predominant sugars in diatoms, such as Amphora rostrata (Khandeparkar and Bhosle, 2001). Diatoms are also members of the Kingdom Straminipila to which thraustochytrids belong. Sulfated galactans are characteristic of the polysaccharides of the red alga Gracilaria corticata (Mazumdar et al., 2002).

Monosaccharide composition of the EPS can have a marked effect on the physical properties of the EPS. Bacterial and algal alginates, which differed only in the monosaccharide composition and have similar polyanionic composition of L-guluronic acid and D-mannuronic acid, had different physical characteristics. Rhamnose or fucose, deoxy sugars present in thraustochytrids may impart a lipophilic character to the EPS.

Generally, proteins get adsorbed on the EPS but are also present as an integral component of the EPS. It was shown by Arad et al. (1993), that removal of the proteinaceous moiety of the EPS from Porphyridium species drastically reduces the viscosity of its aqueous solution. Similarly in a strain of Nostoc, 2S9B removal of proteins significantly reduced the adhesive capacity of the polysaccharide to the roots of Triticum vulgar L (Gantar et al., 1995). The
proteins present in the EPS of thraustochytrids might be an integral part of it and might have an important role in its physical characteristics.

The EPS of marine organisms usually possess a polysaccharide backbone, which predominantly contains either 1,3 or 1,4 linkage in either α or more commonly β-configuration (Wingender, 1999). The structural characteristics of the thraustochytrid EPS were determined using periodate oxidation, optical rotation, enzymatic cleavage and IR (Table 4.3.7, Table 4.3.8, Fig 4.3.6 a and b.). IR spectroscopy and $^{13}$C NMR are powerful tools, which have been used to characterize polysaccharides from marine organisms such as the carrageenan producing red algae (Turquois et al., 1996). EPS produced by both the isolates, NIOS-1 and NIOS-4 showed high periodate consumption indicating either a high frequency of adjacent hydroxyl groups or the presence of few substituents on the sugar ring (Table 4.3.7.). However, substantial amounts of substituents such as sulphates and uronic acids were detected in thraustochytrid EPS (Table 4.3.4 and 4.3.5). Besides, the EPS of isolate NIOS-4 showed no formic acid liberation indicating high substitution in form of either uronic acids or sulfates. Therefore, the high periodate consumption might actually indicate the preponderance of 1,4 linkages. Polymers produced by both the isolates were substantially cleaved by α-amylase, suggesting that the linkage was α-1,4. Cellulase also showed a higher activity on the EPS produced by NIOS-1. Therefore the linkage in NIOS-4 is most probably α-1,4.

Marine microorganisms are potential sources of biotechnologically useful EPS. Various groups, including water column bacteria, deep sea hydrothermal
vent bacteria and cyanobacteria have been the objects of research for a variety of applications that include bioleaching, pharmaceuticals, food, cosmetics, microencapsulation, and industries (Rougeaux et al., 1996; Gehre et al., 1998; Murano, 1998; Sutherland, 1998). Thraustochytrids possess sulfated galactans. Sulfated galactans from the red alga Gracilaria corticata are known to be antiviral (Mazumdar et al., 2002). Other interesting applications are the induction of apoptosis of human leukemia cells by the EPS of the dinoflagellate, Gymnodium sp. (Sogawa et al., 1998). In a quest for polysaccharides with new or improved biotechnological applications, it is important to study different groups of organisms. In this context, future studies on thraustochytrid EPS are likely to produce interesting results.
4.5. Conclusion

EPS were produced in all the four thraustochytrids grown in high nutrient media. EPS was produced throughout the growth phase and continued in stationary phase. EPS production was positively related to DHA production, biomass and glucose concentration. Thraustochytrid EPS was produced both as soluble as well as a matrix like insoluble form. The EPS of the two thraustochytrids had a molecular weight in excess of 2000 kDa. The EPS were acidic polysaccharides containing uronic acids and sulfates. Galactose was the predominant sugar of EPS. Sulfates also formed an important component of the EPS. The EPS appeared to possess α-1,4- linkages. The biotechnological applications of the sulfated polysaccharide of thraustochytrids need to be studied further.