MARINE BIOTECHNOLOGY

Extracellular Polysaccharide Production by Thraustochytrid Protists

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Abstract

Four strains of marine stramenopilan protists, the thraustochytrids, were studied for their ability to produce extracellular polysaccharides (EPSs). Observations by light and scanning electron microscopy revealed the production of a matrix of EPS around groups of cells in stationary cultures. EPS in shake culture filtrates ranged from 0.3 to 1.1 g/L. EPS production, which was studied in greater detail in 2 isolates, SC-1 and CW1, increased with age of cultures, reaching a peak in the stationary phase. Anion exchange chromatography yielded a single fraction of the EPS of both species. The EPS contained 39% to 53% sugars, besides proteins, lipids, uronic acids, and sulfates. Molecular weight of the EPS produced by SC-1 was approximately 94 kDa, and that of CW1, 320 kDa. Glucose formed the major component in the EPS of both isolates—galactose, mannose, and arabinose being the other components. Cultures of both isolates survived air-drying up to a period of 96 hours, suggesting a role for EPS in preventing desiccation of cells.

Key words: exopolysaccharides — thraustochytrids — marine — protist

Introduction

Extracellular polysaccharides (EPSs) are widely secreted by various marine organisms, including plants, animals, diatoms, microalgae, and bacteria (Decho, 1990; Gutierrez et al., 1996; Philippis et al., 1998; Philippis and Vincenzini, 1998). The EPSs produced by these organisms have been explored for various biotechnological applications, such as antitumor agents, anticoagulants (heparin analogues), and wound dressings for eye and joint surgery. Apart from medical applications, EPSs are also important as emulsion stabilizers (in food and thixotrophic paints), flocculants (in water clarification and ore extraction), foam stabilizers (in beer and fire-fighting fluids), gelling agents (in cell and enzyme technology and foods), hydrating agents (in cosmetics and pharmaceuticals), and as inhibitors of crystal formation in frozen foods and sugar syrups (Colwell et al., 1986; Sogawa, 1998; Sutherland, 1998). It is likely that hitherto unexplored groups of marine microorganisms produce novel and useful EPSs.

In heterotrophic bacteria and cyanobacteria, EPS can exist as tight capsules closely surrounding the cell or as a dispersed slime, in no apparent association with any one cell. EPS confers several benefits to the cells that produce it (Decho, 1990; Phillips and Vincenzini, 1998).

EPSs produced by marine organisms also have a role in ecological processes (Decho, 1990). In the pelagic water column, EPSs help in sedimenting phytoplankton blooms and other types of marine snow and play a major role in aggregate formation. They also influence the transfer of heavy metals and other toxic compounds from water column to sediments through the food web. In the benthic system they help in sediment binding and stabilization and regulate the attachment of certain invertebrate larvae to surfaces. Extracellular polysaccharides play a major role in biofilm formation and biofouling and in the localization of microbiogeochemical processes within aggregates and sediments. They also serve as food for meiofauna, such as harpacticoid copepods, nematodes, turbellarians, and nauplii of various animals (Bhosle et al., 1995; Decho, 1990). Therefore it is apparent that EPS-producing organisms, when present in substantial numbers, will have important ecological roles.

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One group of organisms that have been identified as an extremely common component of microbial consortia in the sea, often reaching biomass comparable to that of bacteria, are the thraustochytrids (Naganuma et al., 1998; Kimura et al., 1999; Raghukumar et al., 2000, 2001). Thraustochytrids are heterotrophic, obligate marine protists, belonging to the kingdom Straminipila, which also includes several heterotrophic flagellates, oomycetous fungi, diatoms, and brown algae. Organisms belonging to this kingdom have tubular cristae in the mitochondria and produce heterokont zoospores with tripartite hairs (Porter, 1990; Raghukumar, 2002). They are widespread and have been isolated from numerous habitats in coastal and oceanic regions. Although usually saprophytic, some are parasitic, causing diseases in a few marine animals. They have been shown to be present in microbial films that form on submerged surfaces in the sea (Raghukumar et al., 2000; Raghukumar, 2002). In view of their abundant presence in the sea, it is of intrinsic interest to examine the production of EPS by thraustochytrids, from the perspective of their biotechnological importance, as well as their ecological role. This study addresses the production of EPS by thraustochytrids.

Materials and Methods

Culture and Growth Conditions. Six isolates of thraustochytrids were used for this study. These were SC-1 (*Schizochytrium* sp.), CW1 (*Schizochytrium* sp.), A6T (*Thraustochytrium aggregatum* Ulken), Mar 3 (*Thraustochytrium aureum* Goldstein), Hof 1 (*Thraustochytrium* sp.), and SR (*Thraustochytrium motivum* Goldstein). All cultures were isolated from mangrove detritus, coastal waters, and sediments using the pine pollen baiting technique (Porter, 1990). The thraustochytrids were brought into axenic cultures and maintained on MV agar (Porter, 1990). For the EPS studies, organisms were grown as batch cultures in M4 medium (2% glucose, 0.025% KH_2PO_4, 0.15% peptone, 0.1% yeast extract, pH 7) at 30°C on a shaker at 150 rpm. Two-day-old cultures were used as inocula. A volume of 100 ml of M4 medium was inoculated with 5% inoculum and incubated as described above for 7 days. The flasks were set up in triplicates.

Light and Scanning Electron Microscopy. Cultures grown in M4 broth were examined by phase-contrast microscopy for the presence of EPS. For alcian blue staining and scanning electron microscopy, cultures were grown on autoclaved, 25-mm^2 glass pieces placed in petri dishes and covered with M4 medium. The plates were then inoculated with the individual cultures. Following growth for 10 days, some of the glass pieces were stained with 1.0% alcian blue in 3% acetic acid, pH 2.5, to test for acidic polysaccharides (Decho, 1993). For scanning electron microscopy, the glass pieces with growth were dehydrated in an acetone series using standard procedures and critical point dried (Raghukumar et al., 2000). Following gold-palladium sputter coating, the pieces were examined under a Jeol JSM-5800LV scanning electron microscope.

Growth and EPS Production by SC-1 and CW1. M4 medium was used to monitor growth and EPS production by CW1 and SC-1. Cultures were incubated at room temperature (approx. 28°C) on a shaker, at 150 rpm. At regular intervals, 3-ml aliquots were removed for turbidity measurements (optical density at 660 nm). Samples were centrifuged (5200 *g*, for 15 minutes) and serially filtered through a Whatman GF/F glass fiber filter and 0.45-μm membrane filters. The filtrate was collected and dialyzed. An aliquot of this was used to estimate total carbohydrates by the phenol–sulfuric acid method (Dubois et al., 1956). The experiment was carried out until the culture reached the stationary phase.

Assimilation of carbon into EPS in CW1 was studied, following the basic protocols provided by Deming (1993). M4 medium (25 ml) was inoculated with a 2-day culture as described above. A total of 20 μCi of ^{14}C glucose was added to the culture. A sterile Eppendorf tube containing a filter paper soaked in phenylethylamine was suspended in the flask to measure the amount of glucose respired. The culture was incubated for 24 hours 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 PerkinElmer Wallac 1409DSA liquid scintillation counter.

Preparation of EPSs. Cultures were grown as described above and then centrifuged at 5200 *g* for 15 minutes. 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 times by ultrafiltration using an ultrafilter having a molecular cutoff of 10 kDa (Amicon) and dialyzed against distilled water. To the retained solution was added 3 volumes of cold absolute ethanol, and the mixture was left overnight. The precipitated EPS was centrifuged as described above and lyophilized prior to storage at −20°C. This material was used for further analyses.

All further analyses were carried out by suspending the EPS in distilled water. EPS of SC-1 grown in shake cultures was totally soluble in distilled water, whereas that of CW1 was only sparingly soluble in distilled water, glycine buffer of pH 4.0, phosphate buffer of pH 7.0, borate buffer of pH 9.0, and carbonate-bicarbonate buffer of pH 10.5. Of these, the phosphate buffer yielded the best solubility. Therefore only the soluble portion of the EPS of CW1 in phosphate buffer was used for viscosity measurements, ion exchange chromatography, and molecular weight determination. However, both the insoluble and soluble fractions were used for the chemical analyses.

Ion Exchange Chromatography. The purity of the lyophilized EPS was confirmed by ion exchange chromatography, using DEAE cellulose (HiMedia, India) (Jayaraman, 2001; Khandeparkar and Bhosle, 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 × 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. Fractions of 1 ml were collected and analyzed for total sugars by the phenol–sulfuric acid method.

Characterization of EPSs. Total sugars were estimated by the phenol–sulfuric acid method (Dubois et al., 1956), and proteins by the modified Lowry method (Peterson, 1977). Lipid content was determined by the method of Parsons et al. (1984). Uronic acids were analyzed according to Filisetti-Cozzi and Carpita (1991), using galacturonic acid as standard. Sulfate contents were determined by the method described by Dodgson (1961). 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 IR spectrum of the exopolymer was recorded with a FTIR Shimadzu spectrophotometer using the KBr technique.

In order to analyze the component sugar of the polymer, EPS was hydrolyzed with 72% H_2SO_4 for 20 minutes at room temperature, followed by 8.0% H_2SO_4 for 10 hours at 100°C in sealed ampules. The hydrolysate was then neutralized with barium carbonate. The neutral sugars were converted to their alditol acetates and analyzed by gas chromatography as described by Bhat and Tharanathan (1986). The analysis was carried out using a Shimadzu GLC (model CR4A) fitted with a column OV 225 and a flame ionization detector. The column, injector, and detector block were maintained at 200°C, 250°C, and 250°C, respectively. Response factors for the alditol acetates were compared with that of myo-inositol (internal standard) for absolute quantification.

Molecular Weight Determination. Molecular weight of the EPS of isolate SC-1 and CW1 was determined by Sephacryl S-300 gel permeation chromatography, following the instructions given by the manufacturer (Amersham Pharmacia Biotech). Approximately 2 mg/ml of EPS was applied to the column (1.5 cm × 38 cm), which was equilibrated and eluted with 50 mM disodium hydrogen phosphate in 0.15 M sodium chloride, containing 0.02% sodium azide as preservative (pH 7.5), at a flow rate of 25 ml/h. Fractions of 1 ml were collected and analyzed for total sugars by the phenol–sulfuric acid method as described above. The void volume was determined using blue dextran, and the molecular weight was estimated by plotting a standard graph of log molecular weight and K_{av}. The molecular weight standards used were dextran gel filtration standards from Fluka.

Role of EPS in Protection of Thraustochytrids Against Desiccation. Two-day and 7-day cultures of Mar 3, Hof 1, SC-1, and CW1, grown in M4 medium, as described above, at room temperature under shaken conditions, were washed 3 times with 0.22-μm filtered sterile seawater. The cell pellet was resuspended in filtered seawater to form a thick slurry. Single drops were placed in sterile petri dishes and dried for 0, 12, 24, 72, and 96 hours respectively. Viability of the dried cells was checked by adding sterile seawater and pine pollen to the dishes and examining for growth after 3 days.

Results

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 a fibrillar material surrounding the cells (Figure 1, a and b). The EPS was present as an extensive matrix, surrounding and embedding the cells of thraustochytrids. Scanning electron microscopy of glass pieces with thraustochytrid growth confirmed the production of EPS matrix (Figure 1, c and d) particularly noticeable in cultures of CW1. Further, EPS, which was secreted over the entire surface of the glass piece, stained positively with alcian blue (Figure 2).

Quantitative estimation of EPS in culture filtrates of CW1, SC1, A6T, and SR confirmed that all 4 thraustochytrids produced the EPSs. The concentration of EPS in their cultivated medium was 1.1, 0.9, 0.3, and 0.5 g/L, respectively. Because CW1 and

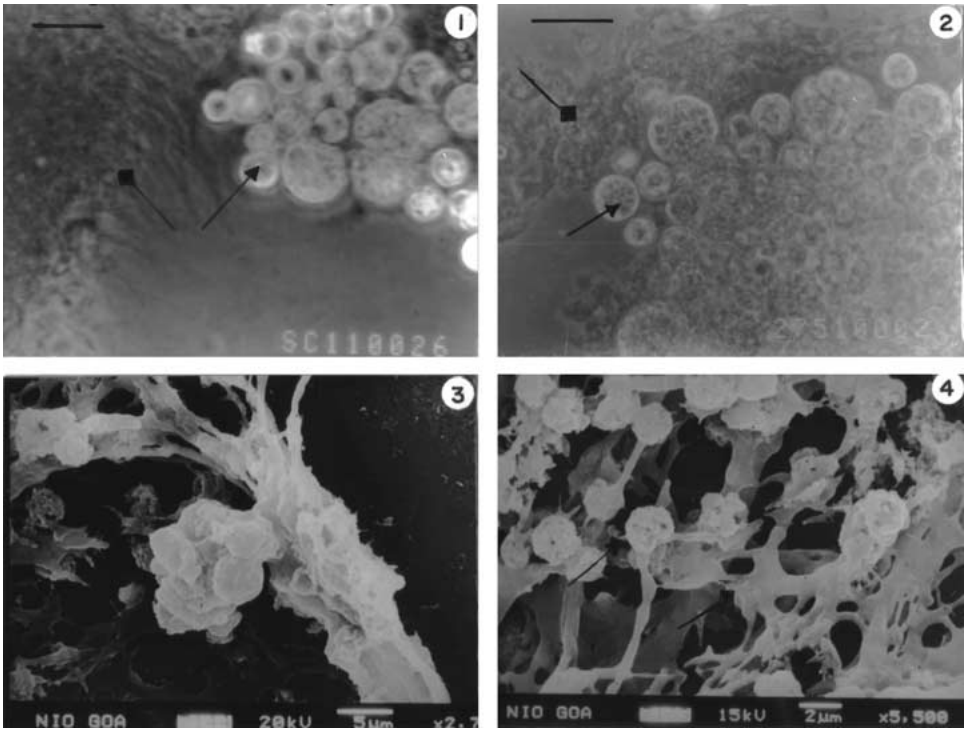

Fig. 1. a: Phase-contrast photomicrograph of EPS matrix produced by the isolate Mar3. Bar represents 10 μm. **b:** EPS sheath produced by the isolate SC-1. Bar represents 10 μm. **c:** SEM of EPS sheath produced by the isolate SC-1. **d:** SEM of EPS matrix produced by the isolate CW1. (Arrows indicate cells; diamond arrows, the EPS.)

SC-1 produced larger amounts of EPS, their cultures were used for further studies. The concentrations of EPS in their media were monitored during cultivation (Figure 3). The cultures showed characteristic sigmoidal growth curves, with a lag phase of about 18 hours for CW1 and 9 hours for SC-1, and reached the stationary phase after 46 hours and 34 hours, respectively. EPS production was observed at all stages of growth, but the concentration increased with age, reaching the highest values during the stationary phase. The concentration of the polymer did not decrease during 73 hours of growth.

The study of glucose metabolism in CW1 using ^{14}C-glucose revealed that about 7% of glucose added in the medium was utilized for EPS and 60% for cells, and the residual glucose (33%) was converted to CO_2. The EPS produced by both the isolates contained sugars, proteins, lipids, and uronic acids (Table 1). The elution profiles of the EPS for SC-1 and CW1, obtained using anion exchange chromatography, yielded a single peak (Figure 4). The sugar content in the EPS of SC-1 and CW1 was approximately 53% and 39%, respectively. The EPS produced by SC-1 had a higher sugar and protein content than that of CW1. However, it had lower lipid and uronic acid contents, EPS of SC-1 and EPS of the soluble portion of CW1 were viscous in nature, that of SC-1 being more so (Table 1).

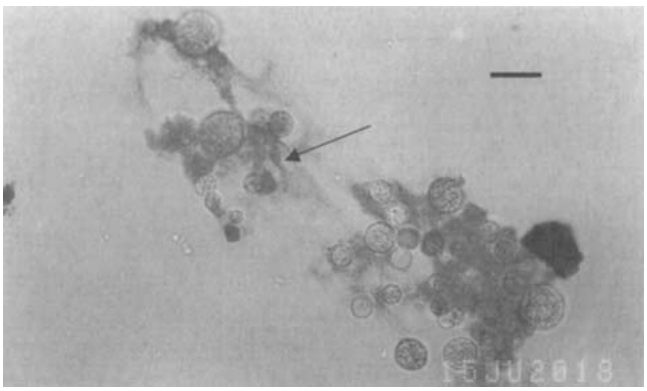

Fig. 2. Positive staining of EPS by alcian blue (arrow) in isolate SC-1. Bar represents 10 μm.

The IR spectra of the EPS produced by both SC-1 and CW1 were similar (Figure 5). The broad peaks around 3500/cm and 1050/cm for OH and C-O(H), respectively, showed the presence of sugars. The broad peak at around 1650/cm confirmed the presence of uronic acids in the EPS. The peak at 890/cm indicated the presence of SO_4^{2-}. The component sugar analysis of both EPSs by gas chromatography showed that the molar ratio of arabinose, mannose, galactose, and glucose in the SC-1 EPS and CW1 EPS were 0.36:1.0:23.6:75.0 and 36:1.8:19.3:78.5, respectively (Figure 6).

The relative molecular mass of the EPS produced by SC-1 and the soluble EPS of CW1 were estimated by gel filtration chromatography to be approximately 94 and 320 kDa, respectively (Figure 7).

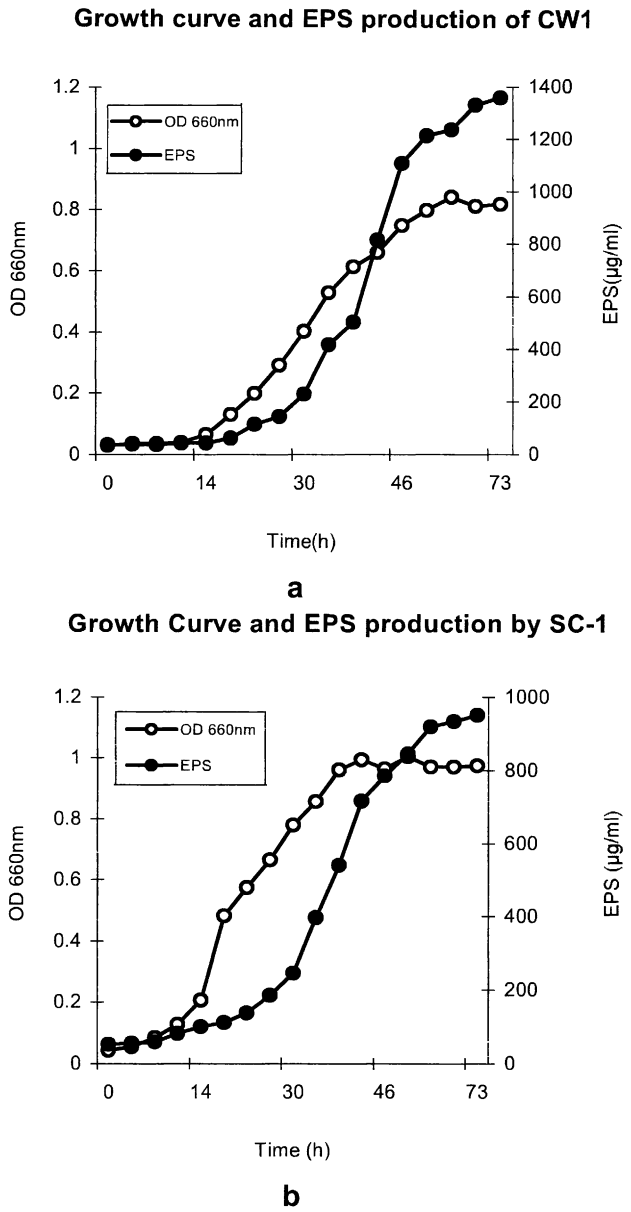

Fig. 3. Growth and EPS production by the thraustochytrids SC-1 (**a**) and CW1 (**b**).

Desiccation experiments showed that cells of all 4 isolates studied remained viable for at least 2 days (Figure 8). Those from 7-day cultures survived longer than those from 2-day cultures. Cells from SC-1 and CW1 survived longer periods of drying, compared with the others. Cells of these isolates obtained from 7-day-old cultures survived even after 96 hours of drying.

Discussion

Thraustochytrids are already considered to be important in biotechnology for production of polyunsaturated fatty acids (Lewis et al., 1999). This study demonstrates for the first time the production of EPS by thraustochytrids, opening up a new avenue of biotechnology research for these protists. Raghukumar et al. (2000), while studying the growth of thraustochytrids in biofilms developing on solid substrata immersed in seawater without addition of extra nutrients, did not observe the production of adhesive EPS by the cells. However, Bremer (1976) noticed the formation of adhesive pad-like material in *Thraustochytrium kinnei* growing on the surface of the larvae of the brine shrimp *Artemia*. It is possible that such adhesive EPS was produced on the high nutrient surface of *Artemia* used by Bremer, but not on the inert material in plain seawater used by Raghukumar et al. (2000). It is well known that high levels of nutrients, particularly carbohydrates, induce production of elevated quantities of EPS in microorganisms (Sutherland, 1985).

Table 1. Physical and Chemical Characteristics of EPS Produced by the Two Isolates

Characteristic	*SC-1*	*CW1*
Sugars	53.27%	39.09%
Proteins	31.73%	18.15%
Lipids	14.17%	23.55%
Sulfates	4.95%	13.08%
Uronic acid	4.07%	1.07%
Viscosity (6 mg/1 ml)	2.47 cp	1.32 cp

The production of EPS by the 4 thraustochytrids varied from 0.3 to 1.1 g/L. The amount of EPS produced by various bacteria may range from low levels, such as 0.24 g/L in a *Pseudomonas* species, to 33 g/L as in a *Xanthomonas* species after optimization (Christensen et al., 1985; Sutherland, 1998). We have not attempted to optimize the production of EPS in the thraustochytrids studied. It is likely that under

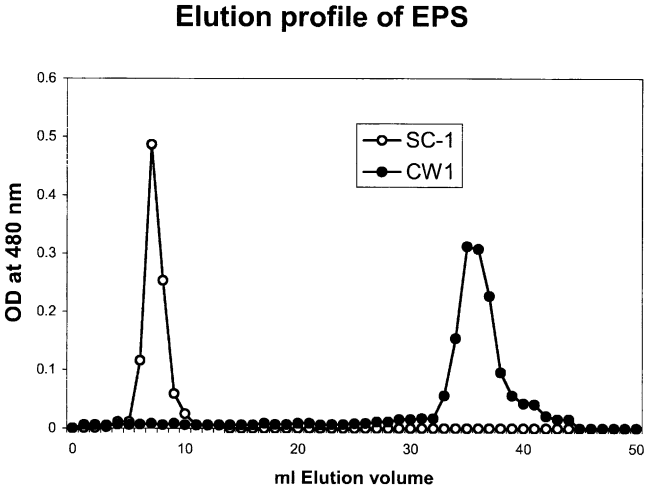

Fig. 4. Elution profile in an ion exchange column of the EPS produced by SC-1 and CW1.

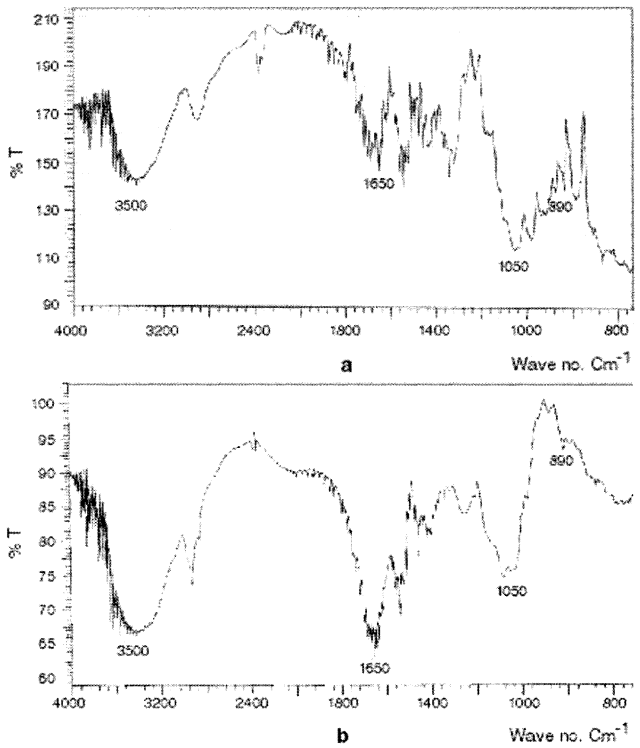

Fig. 5. IR spectra of the EPS produced by the thraustochytrids SC-1 (**a**) and CW1 (**b**).

ideal conditions they would produce much larger amounts of EPS than those produced in our experiments.

Exopolysaccharide production was observed at all stages of growth, but the concentration increased with age of the culture, reaching the highest values during the stationary phase (Figure 3). Similar observations were made in a *Vibrio* species and a diatom, *Navicula* species (Majumdar et al., 1999, Bhosle et al., 1995). In addition to sugars, the EPS produced by SC-1 and CW1 also contained uronic acids, sulfates, proteins, and lipids (Table 1). The composition of EPS of the thraustochytrids studied was similar to that of their cell walls in terms of the presence of glucose, galactose, mannose, proteins, and sulfates (see Ulken and Bahnweg, 1985; Bahnweg and Jäckle, 1986; Chamberlain and Moss, 1986). Although the cell walls contain much higher levels of galactose than glucose, EPS contained several times more glucose than galactose (Figure 6). Besides, the EPS did not contain xylose, which has been shown to be present in cell walls. The nonsugar moieties of EPS in thraustochytrids could be extremely important to the tertiary structure and physical properties of EPS. These components are most often in the form of residues and side groups of the polysaccharide chains and contain a variety of carboxyl, amino, and sulfate groups (Decho, 1990; Bhosle et al., 1995). The presence of uronic acids and sulfates confers an overall negative charge and acidic property to the polymer. Alcian blue staining suggested that the EPS was anionic in nature. Acidic polysaccharides are also reported from bacteria, cy-

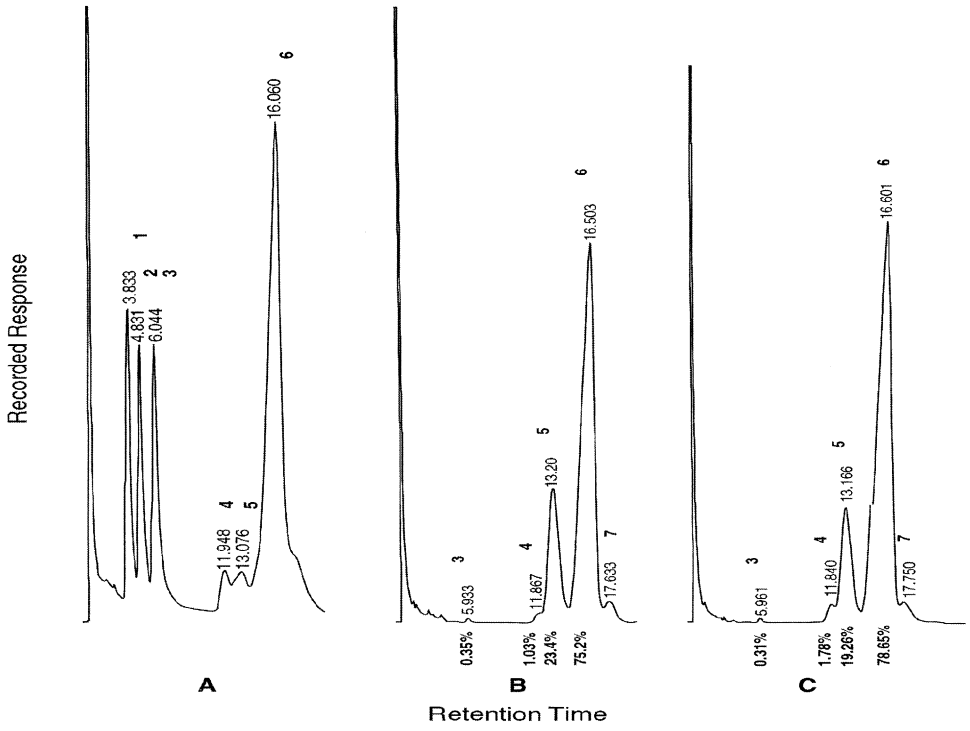

Fig. 6. GC profiles of component sugars in EPS of standards (**A**), SC-1 (**B**) and CW1 (**C**). Numbered peaks are 1, rhamnose / fucose; 2, xylose; 3, arabinose; 4, mannose; 5, galactose; 6, glucose; 7, inositol (internal standard).

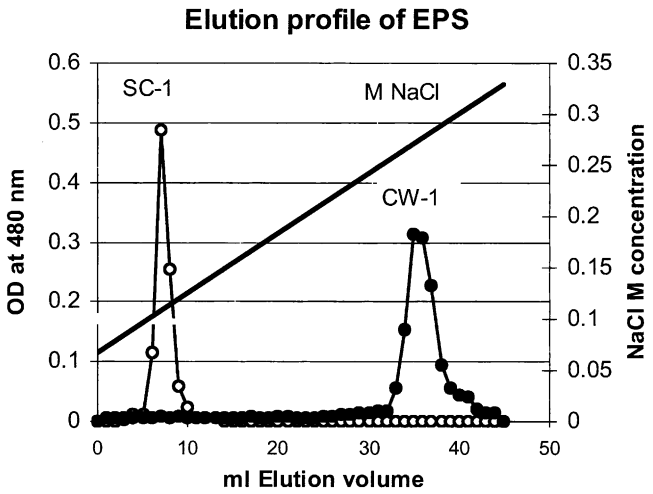

Fig. 7. Elution profile in a Sephacryl S-300 column of the EPS produced by SC-1 and CW1.

anobacteria, and diatoms and are common in marine organisms (Christensen et al., 1985; Philippis and Vincenzini, 1998; Khandeparkar and Bhosle, 2001).

Production of EPS could confer several biological benefits to thraustochytrids. First, the thraustochytrid cultures SC-1 and CW1 examined in this study were isolated from intertidal mangrove regions, which undergo periodic exposure and submergence to water. Hence, the microbial cells living here are likely to become desiccated when exposed to air during low tide. Sulfated polysaccharides that are hydrated are known to play an important role in offering protection from desiccation (Decho, 1990). Such polysaccharides in thraustochytrids could help in moisture retention and prevention of desiccation of thraustochytrid cells. As evidence, we observed that cells of all isolates still remained viable after drying for 48 hours (Figure 8). Isolates SC-1 and CW1, which produced the highest amounts of EPS, survived even up to 96 hours, further suggesting the role of these polysaccharides in protection against desiccation. The remarkable recovery of thraustochytrids in culture from dried herbarium specimens (Porter, 1990) may be cited as further evidence to support this.

Second, the production of EPS as extensive matrices around groups of cells under static growth conditions (Figure 1, a–d) could enhance their capacity to adhere to marine substrata. Bacteria that form primary films on inert marine substrata produce similar sheaths of EPS that help in adhesion (Tosteson, 1986). The EPS produced under shaken conditions by SC-1 were totally soluble, but this was not so in the case of CW1.

Finally, production of extracellular polysaccharides could be an ecological strategy for thraustochytrids to survive oligotrophic conditions (Decho, 1990). Thraustochytrids do not produce resting spores capable of withstanding starvation and are therefore likely to be perpetually in an active metabolic state. Extracellular polysaccharides may serve as a reserve energy source for such cells during conditions of oligotrophy. Our preliminary observations have shown that cells of 2 isolates could grow exclusively on EPSs provided as nutrients.

The EPS produced by thraustochytrids might play several other roles. It may buffer cells against quick ionic and environmental changes in pH, salinity, or nutrient levels. Secretions of EPS also sequester and concentrate nutrients and help to localize and maintain activity of exoenzymes. They may further provide protection against heavy metals and other toxins (Whitfield, 1988; Decho, 1990;

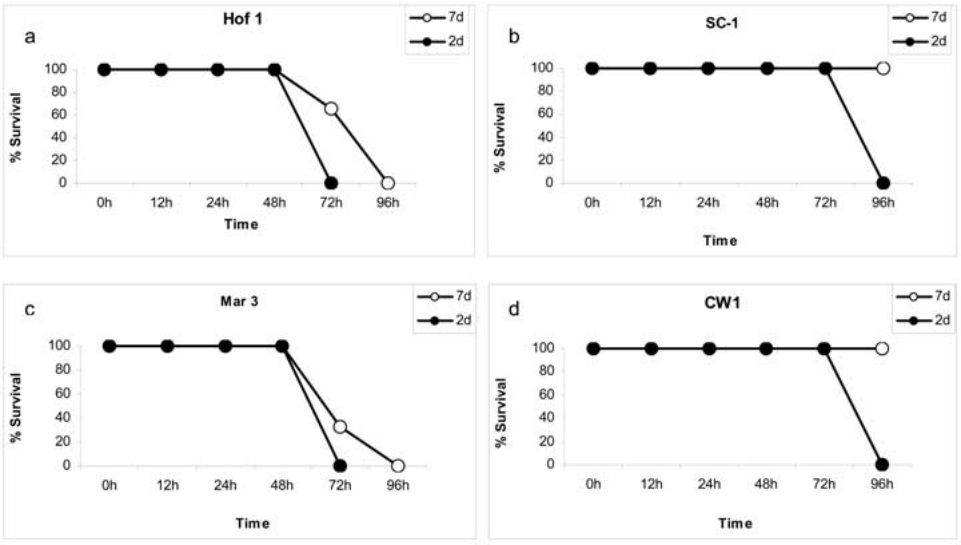

Fig. 8. Percentage of survival of Hof1 (**a**), SC-1 (**b**), Mar 3 (**c**) and CW1 (**d**) on air-drying the cells.

Philippis and Vincenzeni, 1998). Negatively charged cyanobacterial EPS containing uronic acids exhibited a high metal-complexing capacity (Philippis et al., 1998). Freshly decaying mangrove leaves leach out soluble phenolics, which are likely to inhibit growth of thraustochytrids (Raghukumar, 2002). EPS may afford protection in such situations.

Sulfated polysaccharides are also of biotechnological importance, for example, as blood anticoagulants and antiviral compounds (Sogawa et al., 1998). Several papers have described the application of extracellular sulfated polysaccharides from organisms such as the cyanobacterium *Aphanocapsa halophytia* and the red alga *Porphyridium* sp. (Geresh and Arad, 1991; Matsunaga et al., 1996). In view of this, studies on the biotechnological importance and ecological significance of the extracellular sulfated polysaccharides in thraustochytrids, reported for the first time in this paper, deserve further attention. Further studies to explore these are underway.

Acknowledgments

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