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An extracellular sulfated fucose-rich polysaccharide produced by a tropical strain of *Cryptomonas obovata* (Cryptophyceae)

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Abstract

A tropical strain of *Cryptomonas obovata* Skuja, isolated from a shallow oxbow lake, released a sulfated fucose-rich polysaccharide. The polysaccharide is composed mainly of fucose (42%), N-acetyl-galactosamine (26%) and rhamnose (15%), with small quantities of glucuronic acid, mannose, galactose, xylose and glucose. Sulfate accounted for 1.7% total polysaccharide. Quantitative release was studied with cells exposed to optimal culture conditions contrasted with high irradiance and nitrate depletion. This latter set of conditions could simulate stress situations usually found in the place from which this strain was isolated. The monosaccharide composition of the polysaccharide was evaluated using PAD-HPLC and gas chromatography. The two irradiances tested (165 μmol m^{-2} s^{-1} and 2000 μmol m^{-2} s^{-1}) had no significant effect on amounts of polysaccharide released by the cells. Differences were observed when the nitrate availability was varied. In the nitrate-depleted situation, extracellular polysaccharide production was 2.5 times higher than replete cells after 6 h at 165 μmol m^{-2} s^{-1}, and 2.25 times higher at 2000 μmol m^{-2} s^{-1}.

Introduction

The extracellular carbohydrates from phytoplankton have been studied because of their ecological significance in aquatic systems (Fogg 1983; Wood and Van Vallen 1990; Zhou et al. 1998). Since the exopolysaccharides may be used as a substrate by bacteria (Hanisch et al. 1996; Freire-Nordi and Vieira 1998), these compounds could perform an important role in the possible specific algae/bacteria associations. They are also the principal component of the algal-released organic carbon, and are extremely variable in their monosaccharide composition and their stereochemistry. Another important function of extracellular polysaccharides with functional groups with negative charges is to form metallic complexes with toxic metals such as copper and lead. Surface-active extracellular polysaccharides of algae are one of the principal TEP (transparent exopolymer particles) producers in aquatic environments (Alldredge et al. 1993; Zhou et al. 1998). These particles would transport metals to sediments (Sigg et al. 1987; Cho and Azam 1988) and trophic web if eaten by zooplankton and other organisms.

Extracellular polysaccharides, such as sulfated fucose-rich polysaccharides, from some phytoplankters may also find important application in pharmaceutical sciences and industry. Administration in vivo of fucoidan, a sulfated fucose rich phaeophycean polysaccharide, inhibited the settling of metastatic sarcoma L-1 cells in the lungs of mice (Roszkowski et al. 1989). Sarcoma L-1 cells contain high amounts of L-fucose that apparently act in their metastatic capacity. The settling of metastatic cells should be by L-fucose specific lectin-like adhesion molecules situated on lung cells (Kieda and Monsigny 1986). Both L-fucose and fucoidan inhibited the adhesion process by competition. Highly purified sulfated fucose-rich

polysaccharides from several Phaeophyceae also have shown high blood-anticoagulant activities (Nishino et al. 1991). Other uses are related to algal extracellular sulfate-containing polysaccharides from non planktonic algae (Bourgougnon et al. 1993; Green et al. 1993; Damonte et al. 1994; Vanhoore and Vandamme 1999) or blue-green algae (De Philippis and Vicenzini 1998). However, data about sulfated fucose-rich polysaccharides from eukariotic planktonic microalgae, concerning either their applications or their release to surrounding environment and productivity, are rare.

L-fucose represents a rather rare and expensive sugar, so improved access to L-fucose and L-fucose containing oligosaccharides (Vanhoore and Vandamme 1999) is important. Several processes besides the extraction from seaweed have been tried to obtain L-fucose production with large yield. Enzymatic and chemical hydrolysis of L-fucose rich microbial polysaccharides, produced by micro-organisms such as bacteria, open up a new route towards efficient L-fucose production (Vanhoore and Vandamme 1999). *Cryptomonas obovata* may represent a suitable source of a sulfated fucose-rich polysaccharide due to its high content of this sugar, culture facilities and high rates of production of extracellular polymers under special conditions.

The aim of this study was to investigate polysaccharide release in a tropical freshwater strain of *Cryptomonas obovata*, including conditions combining high irradiance and nitrate depletion.

Material and methods

Organism and culture conditions

Cryptomonas obovata Skuja was isolated from Infernão Lake, a shallow oxbow lake at the margin of Moji-Guaçu River, São Paulo State, Brazil (21°33′ S, 47°55′ W). Axenic experimental cultures were obtained by repeated re-isolations under optical microscope and were grown in WC medium (Guillard and Lorenzen 1972) at pH 7.0. Tests to check for bacterial contamination were carried out with WC media enriched with glucose and peptone (250 mg l^{-1} each). The inocula were kept under 100 μmol m^{-2} s^{-1} for a photoperiod of 12:12 h and at a temperature of 21° ± 1 °C. The experimental irradiance levels were obtained using 500-W halogen lamps in combination with neutral glass filters. Scalar irradiance (E) was measured using a spherical quantum sensor (Biospherical Instruments™ QSL-100) in 1-l glass cultures flasks filled with distilled water.

Experiment I: growth at several concentrations of nitrate

Experiment I was carried out to determine the growth of *C. obovata* at four concentrations of nitrate. The aim was to obtain an optimum and a limiting concentration for growth and the period for which the culture could be kept in the absence of nitrate without cell death. Nitrate-free cultures, confirmed by the method described by Strickland and Parsons (1968), were inoculated in WC medium with nitrate modified to provide four different concentrations: 5, 10, 20, 50 μM. Since nitrate concentration in complete medium is 1000 μM, they were diluted 200, 100, 50 and 20 times, respectively, from the original concentration. Cell density was monitored for 13 days at each nitrate concentration by direct counting under a microscope, using a Fuchs-Rosenthal haemacytometer camera. Daily samples of each culture were filtered on 1.2-μm porosity acetate membrane (Microfiltration system™ Dublin, California) at low vacuum pressure (24 cm Hg) to determine chlorophyll *a*. The chlorophyll *a* extraction was performed with 90% acetone at −5 °C (Talling and Driver 1963).

Experiment II: nitrate-replete cells

The aim of the second experiment was to determine the release of carbohydrates by replete cells (without nitrate limitation) after 6 h at 165 or 2000 μmol m^{-2} s^{-1}. The lower value is near the light saturation point (E_k = Pm / α) (Sakshaug et al. 1997) for *C. obovata* (180 μmol m^{-2} s^{-1}) and the second is saturating (Giroldo and Vieira 1999). Experiment II was carried out with cells in the middle of the *log* phase of growth, using complete WC medium. After 8 days growth, the nitrate concentration decreased from 1000 to 106.3 ± 20.1 μM. The experimental culture (2600 ml, 2×10^4 cell ml^{-1}) was divided and used as follows: 300 ml was taken before the experiment to measure the initial chlorophyll *a* concentration, initial cell density and initial carbohydrate concentration. 900 ml was split into two 450-ml aliquots to determine the release of carbohydrates after 6 h exposure at 165 and 2000 μmol m^{-2} s^{-1}. After exposure to light the cells were filtered on 1.2-μm porosity acetate membranes (Microfiltration system™

Dublin, California). Carbohydrate concentrations in the media (polymeric carbohydrates and free dissolved monosaccharides), both initial and after 6 hours exposure, were evaluated by PAD-HPLC methodology (Jørgensen and Jensen 1994; Gremm 1997) and gas chromatography, described in the next section. The cell counts and the chlorophyll-*a* concentrations were measured as in experiment I.

Experiment III: nitrate-depleted cells

The third experiment was performed exactly as the second, but with nitrate depleted cells. The experimental culture was prepared as follow: 600 ml of an 8 day old culture (end of the log phase), whose cells were growing in WC media diluted 10 times, were added to 2000 ml of nitrate-free WC media and remained for 4 days in a starvation process. The nitrate concentration after 8 days growing in WC N/10 was 0.016 μM ± 0.0008, and after 4 days in WC/N0 was 0.005 μM ± 0.0006. The experimental culture (2600 mL, 2×10^4 cell ml^{-1}) was kept in the same conditions of light and temperature as experiment II. Cell counts, chlorophyll-*a* concentration and analysis of polymeric and free monosaccharides in the fractions were performed as in experiment II. Carbohydrate analysis is described in the next section.

Carbohydrate analysis

The cells were filtered on 1.2 μm pore size acetate membranes (Microfiltration system™ Dublin, California) at low vacuum pressure (24 cm Hg) to obtain the carbohydrates of each experiment (Initial and final of experiment II and III). The integral filtered media was concentrated three times at 38 °C and the carbohydrates were fractionated in groups of similar molecular weight using gel filtration column chromatography (BioRad™ P-10 gel). The isolated fractions were detected by the phenol/sulfuric method (Dubois et al. 1956). Desalted samples without hydrolysis were analyzed directly on PAD-HPLC to detect and identify free monosaccharides dissolved in the media. For quantification and identification of the polymeric carbohydrates, dried samples were hydrolyzed with 1 ml of 90% formic acid for 6 h at 100 °C. Then 3 ml of distilled water added and the whole was left for two more hours at the same temperature. The samples were washed 3 times with methanol, to remove the formic acid, and finally diluted in distilled water.

PAD-HPLC analysis was performed on a Dionex™ DX500, consisting of a peek version GP40 gradient pump module, ED40 electrochemical detector, and a LC5 manual injector with a Rheodyne 9125 valve and a 25-μl peek sample loop. The ED40 was equipped with an amperometric flow cell, a gold working electrode, and a Ag/AgCl reference electrode. PA-10 (Dionex™) anion-exchange analytical column (4 × 250 mm), fitted with a corresponding guard-column (4 × 50 mm), was used to separate the monosaccharides. The eluent for the separation was NaOH 18 mM and for the recuperation of the column was NaOH 200 mM, at a flow rate of 1 ml min^{-1} (Gremm 1997). All samples (polymeric and free monosaccharides) were desalted on a Bio Rad™ ionic exchange resin (AG2X8–anion exchange and AG50W–cation exchange) to remove salts from culture medium.

Gas chromatography of the polysaccharide was performed after methanolysis of the dried samples using 1M HCl in methanol at 80 °C for 24 h. Mannitol was used as the internal standard. The samples were prepared by the method of Reinhold (1972); Paulsen et al. (1992) for analysis as the o-trimethyl silyl derivatives of the methyl glycosides. Gas-liquid chromatography separation were carried out on a HP 5890 Series II equipped with a DB-5 (J & W Scientific™) fused silica capillary column (30 m × 0.32 mm) and a hydrogen flame ionization detector (FID). The chromatograms were recorded and integrated with an Agilent A.08 ChemStation™ data system. Helium was used as the carrier gas (2.5 ml min $^{-1}$). Nitrogen flow was 28 ml min^{-1}; hydrogen, 30 ml min^{-1}; and air 360 ml min^{-1}. The injector temperature was 260 °C and the column temperature was initially set at 140 °C and programmed to rise 1 °C min^{-1} to 170 °C and 6 °C min^{-1} from 170 to 250 °C. The detector temperature was maintained at 300 °C.

Protein in the polysaccharides was determined according to Lowry et al. (1951). Sulfur content was determined by CHNS Automatic Elemental Analyzer (EA 1110 Carlo Erba)

Results

Figures 1 and 2 show, respectively, cell density and the chlorophyll *a* concentration during growth for 13 days. Growth was limited by nitrate at 5 μM, while at other concentrations, at least one cell division oc-

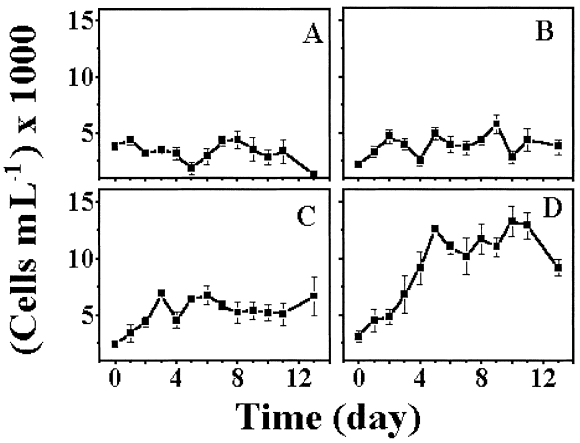

Figure 1. Cell density of *C. obovata* at four different concentration of nitrate (5, 10, 20 and 50 μM) during 13 days. The error bars correspond to the mean error (n = 6).

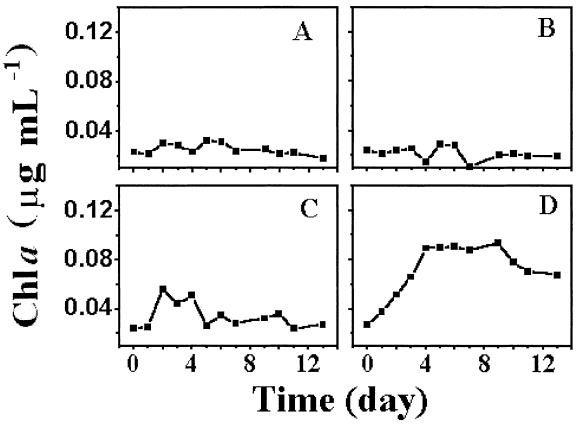

Figure 2. Chlorophyll-*a* concentration of *C. obovata* at four different concentration of nitrate (5, 10, 20 and 50 μM) during 13 days.

curred (ANOVA p = 0.0037). The growth was limited after 4 days at 10 μM and after 5 days at 20 μM and 50 μM. Also, *C. obovata* resisted to the absence of nitrate for at least 13 days without any decrease in cell density, except at 5 μM after 13 days, when the cellular density decreased to 1250 cells per ml. This result was needed to determine the period of time during which we could maintain the culture in the absence of nitrate without cell death, since dead cells may contaminate the extracellular carbohydrate measurements in the subsequent starvation experiment. In the P-10 gel filtration chromatography (Figure 3) polysaccharide (around 2×10^6 D) was observed as the principal component of the carbohydrates dissolved in the media, but two further groups of carbohydrates were detected by the phenol/sulfuric method. Besides high molecular weight (Ve/Vt = 0–0.2), intermediate (Ve/Vt = 0.3–0.6) and low (Ve/Vt = 0.7–1.0) molecular weight carbohydrates were found by P-10 gel filtration chromatography.

Figure 4 shows the PAD-HPLC analysis of the high molecular weight carbohydrates fraction (Ve/Vt = 0–0.2) released after six hours of exposure at 165 and 2000 μmol m^{-2} s^{-1}. The neutral monosaccharides identified after hydrolysis of the polysaccharide released by *C. obovata* were essentially fucose and rhamnose, with small amounts of galactose, glucose and mannose or xylose, as gas chromatography analysis also confirmed. Intermediate and low molecular weight carbohydrates occurred at low concentrations (< 0.1 mg l^{-1}); no qualitative and quantitative differences were detected between nitrate-depleted and nitrate-replete cells, nor between 165 and 2000

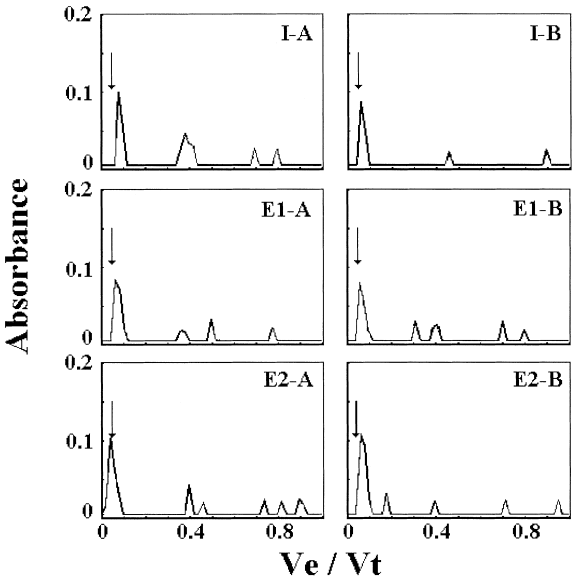

Figure 3. Fractions of extracellular carbohydrates released initially (I) and after 6 hours of exposure at 165 (E1) and 2000 (E2) μmol m^{-2} s^{-1} by nitrate replete (A) and depleted cells (B). P-10 gel (Bio-Rad™) was used, the eluent was distilled water plus 2% butanol at a flow rate of 1 ml per 7 minutes. Absorbance of fractions was measured at 487 nm. Ve/Vt is the relation between eluted and total volume of the column. Arrows show blue dextran (2×10^4 D) calibration.

μmol m^{-2} s^{-1}. Nitrate-replete cells (experiment II) increased the initial concentration of polysaccharide by about 42% at 165 μmol m^{-2} s^{-1} and 18% at 2000 μmol m^{-2} s^{-1}, after 6 h (Table 1). Nitrate-depleted cells (experiment III) increased the initial concentration of polysaccharides by about 148% at 165 μmol m^{-2} s^{-1} and 125% at 2000 μmol m^{-2} s^{-1}. ANOVA (p = 0.0001) and Tukey test (165 depleted >

Table 1. PAD-HPLC identification and quantification of the monosaccharides (including average error, n = 3) after hydrolysis of the high molecular weight carbohydrates released after 6 hours under 165 (E1) and 2000 μmol m^{-2} s^{-1} (E2) by nitrate-replete and depleted cells of *C. obovata*.

Sample	Nitrate	Monosaccharides (mg l^{-1})						Increase
		Fuc	Rham	Gal	Glu	Man/Xy	Total	
Initial	Replete	1.51 ± 0.006	0.54 ± 0.005	0.077 ± 0.01	0.036 ± 0.006	0.023 ± 0.001	2.21 ± 0.13	–
	Depleted	1.30 ± 0.21	0.40 ± 0,03	0.011 ± 0.002	0.009 ± 0.003	0.006 ± 0.001	1.72 ± 0.24	–
E1-6 h	Replete	2.18 ± 0.03	0.78 ± 0.06	0.087 ± 0.001	0.048 ± 0.001	0.053 ± 0.006	3.15 ± 0.038	42.5%
	Depleted	3.06 ± 0.05	1.02 ± 0.03	0.094 ± 0.006	0.040 ± 0.006	0.070 ± 0.008	4.28 ± 0.08	148.8%
E2-6 h	Replete	1.60 ± 0.04	0.80 ± 0.06	0.098 ± 0.018	0.057 ± 0.014	0.067 ± 0.012	2.62 ± 0.18	18.5%
	Depleted	2.74 ± 0.11	0.97 ± 0.07	0.050 ± 0.007	0.035 ± 0.003	0.085 ± 0.002	3.88 ± 0.17	125.6%

* after 6 h

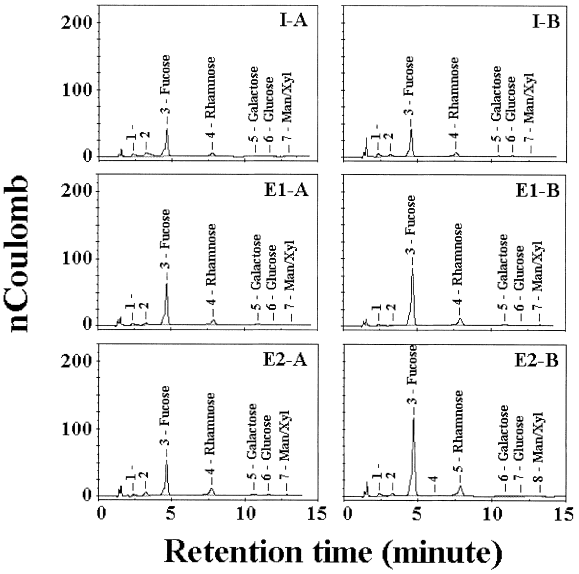

Figure 4. : PAD-HPLC analysis of the monosaccharides composing the high molecular weight fraction of the carbohydrates released by *C. obovata* initially (I) and after 6 hours of exposure at 165 (E1) and 2000 (E2) μmol m^{-2} s^{-1}; (A) nitrate-replete cells, (B) nitrate-depleted cells.

2000 depleted >>> 165 replete > 2000 replete) confirmed the differences between polysaccharide release from depleted and nitrate-replete cells. Although the intracellular nitrate concentration was not performed, the data shown in Figures 1 and 2 eliminate the possibility of cell division occurring with less than 5 μM extracellular nitrate. All the comparisons between replete and depleted cells were justified, because cell density was the same (ANOVA, p = 0.867).

Gas chromatography (Figure 5, Table 2) has confirmed fucose, rhamnose, galactose, glucose and that both mannose and xylose occur, though not well separated by column PA-10 at PAD-HPLC. Glucuronic acid and N-acetyl-galactosamine also occur, but were not detected at PAD-HPLC, because they were lost in the desalinization resins. Fucose constituted 41.6%, acetyl-galactosamine 26% and rhamnose for 15.3% total carbohydrate component of the polysaccharide. Protein and sulfate accounted for 2.2% and 1.7% of the high molecular weight content, respectively.

Table 2. Relative monosaccharide composition of the extracellular polysaccharide released by C. obovata determined by gas chromatography as the TMS-derivatives of the corresponding methyl-glycosides.

Monosaccharides	% Total Polysaccharide
fucose	41.6
N-acetyl-galactosamine	26.0
rhamnose	15.3
galactose	4.4
glucuronic acid	4.3
mannose	3.6
xylose	2.7
glucose	2.1

Discussion

Polysaccharide release, although ubiquitous, could be very specific and diversified by evolution in regard to its role and chemical composition. The monomeric composition of a polysaccharide isolated from a soil *Cryptomonas* sp (Paulsen et al. 1992) is very distinct from that found in this work. Thus, the functional role of these compounds, that in their origins may have had another function, could have been quite diversified with evolution.

The tolerance of *C. obovata* to levels of irradiance up to 2000 μmol m^{-2} s^{-1}, even in a depleted condition, and the ability to survive for a considerable time

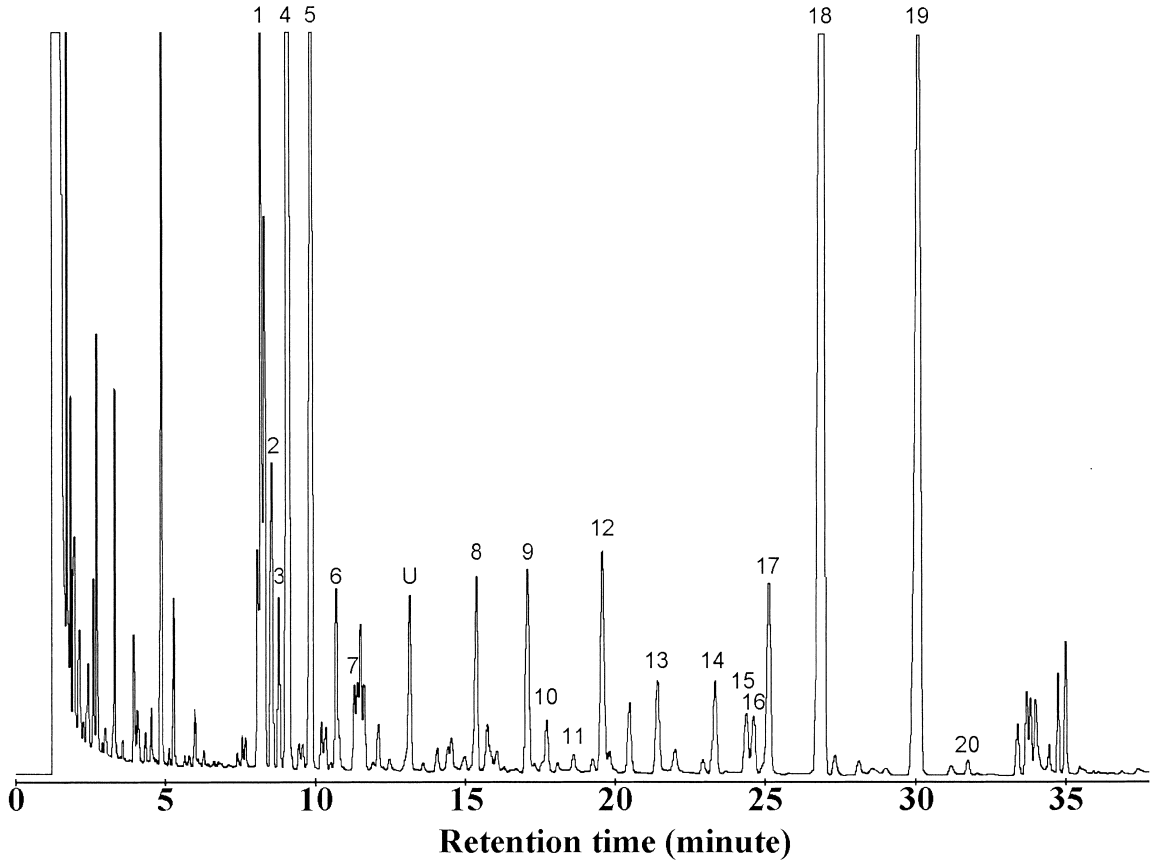

Figure 5. Identification of the monosaccharides that form the polysaccharide released by *C. obovata* using gas chromatography. **1**–Rhamnose 1, **2**–Fucose 1, **3**–Rhamnose 2, **4**–Fucose 2, **5**–Fucose 3, **6**–Xylose 1, **7**–Xylose 2, **8**–Glucuronic acid 1, **9**–Mannose 1, **10**–Galactose 1, **11**–Mannose 2, **12**–Galactose 2, **13**–Galactose 3, **14**–Glucose 1, **15**–Glucose 2, **16**–Glucuronic acid 2, **17**–Glucuronic acid 3, **18**–Mannitol, **19**–N-acetyl-galactosamine 1, **20**–N-acetyl-galactosamine 2. U = unknown.

under nitrate starvation are evidence of the adaptation of *C. obovata* to different stress situations and may explain the occurrence of this strain all year round on the Infernão Lake (Dias 1990). In fact, Giroldo and Vieira (1999) showed that carbon assimilation was not inhibited up to 2000 μmol m^{-2} s^{-1} and an acclimation to low irradiance was also observed.

The release of polysaccharides by depleted cells was higher than by replete cells, corroborating the "excess of carbon" hypothesis (Fogg 1983). Since there is no cell division in nitrate-depleted conditions, the carbon assimilated via photosynthesis would continuously be directed to the production and release of polysaccharides. Nitrate depletion would simulate a stationary growth phase, when the release of carbohydrates is increased (Smith and Underwood 2000). Nitrogen limitation associated with high irradiance results in a decrease of cytokinesis in most planktonic microalgae, but if photosynthesis is not inhibited, the cellular ratio C/N is increased by production of lipids, such as triacylglycerols (Mayzaud et al. 1989), and also carbohydrates which can be released by the cells in a polymeric form.

Adaptation to nitrate starvation and high irradiance, together with associated high rates of polysaccharide release, make this species suitable for easy cultivation when the purpose is to obtain extracellular sulfated fucose-rich polysaccharide.

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