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Water soluble polysaccharides of marine algal species of *Ulva* (Ulvales, Chlorophyta) of Indian waters

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Cold and hot water extracts of four different species of *Ulva* viz. *U. reticulata*, *U. lactuca*, *U. rigida* and *U. fasciata* were studied for their polysaccharide (PS) contents. In both the cold (CWE) and hot water (HWE) extracts relatively higher yield of polysaccharides were obtained in *Ulva fasciata* (6.5 and 16% respectively). *Ulva lactuca* was found to contain higher amounts of protein (33.1% in CWE), uronic acid (35.7% in HWE) and sulfate (23.8% in HWE). Cold water extracts were found to be enriched with hexose sugars, comprising a part of structural polysaccharide, whereas the hot water extracts were rich in rhamnose, xylose as well as glucose. The average molecular weight of these polymers were found to be in the range 1.14 to $>2.0\times10^6$ Da. Seasonal variation of PS of *U. fasciata* were also studied alongside. For this, cold and hot water soluble polysaccharides (PS) were isolated separately from the samples of *Ulva fasciata* Delile, collected monthly from a single location during the season of algal growth (September-March) of the year 1995-96 from the west coast of India. Yield (17-21%) and viscosity (203 247 cps) of HWE were high during the active period of growth (October-February) of algae. Given the abundance of *Ulva* species in Indian waters coupled with the potential utilities of their polysaccharides, the results obtained in this investigation would be useful in product development and bioprospecting strategies.

Species of the marine algal genus *Ulva* Linnaeus (Ulvales, Chlorophyta) are known for their various uses like feed, manure, folk medicines, dietary fiber contents as well as for the rheological and gelling properties of their sulfated polysaccharides (SPS)[1-4]. Polysaccharides isolated from *Ulva lactuca* Linnaeus have also shown antiviral activity against influenza virus and the same from *Ulva conglobata* Kjellman are reported to have antiretroviral properties[5-7]. Intensive studies were done, since 1941 by various groups of researchers on the chemical nature, structure and rheological properties of these sulfated polysaccharides and potential industrial utilisation of these mucilagenous polysaccharides were suggested[8-12]. Cell-wall polysaccharides, isolated from different species of *Ulva* have been identified as sulfated polymers of rhamnose, xylose, and glucuronic acid (glucuronoxylorhamnan) along with small amounts of arabinose, galactose and mannose[13,14]. Various groups of workers have also reported glucan, a polymer of glucose units (1,3 linked) from *Ulva*[2,11,15]. Recently, Lahaye *et al.*[10] have also reported SPS containing iduronic acid residue from *Ulva*.

The species of the family Ulvaceae, particularly *U. fasciata* Delile and *U. lactuca*, occur abundantly on the Indian coasts. Though there are few reports on the cultivation, laboratory culture and growth studies of *Ulva* of Indian waters[16,17], there are no reports on the chemical nature of the polysaccharide (PS) contents of these species. Therefore, the studies on the PS of four different taxa of *Ulva viz. U. reticulata* Forssk., *U. lactuca*, *U. rigida* C. Ag. and *U. fasciata*, collected from the Indian coasts, were undertaken for the first time. *Ulva fasciata* being one of the most proliferating as well as poorly utilized algae, study on the seasonal changes in the biosynthetic process and physicochemical properties of its PS was undertaken in view of the potential utility of these phycocolloids.

Materials and Methods

Collection and extraction—Different species of *Ulva* collected were - *U. reticulata* Forssk. from Ervadi (lat. 9° 25′ N, long.79° 08′ E), in January 1997; *U. lactuca* Linnaeus from Porbandar (lat. 21° 38′ N, long. 69° 37′ E), in February 1997; *U. rigida* C. Ag. from Gopnath (lat. 21° 18′ N, long. 72° 14′ E), in January 1998; *U. fasciata* Delile from Diu (lat. 20°45′ N, long. 70° 58′ E), in September 1996. For the seasonal variation studies, *U. fasciata* was collected monthly during its season of growth i.e. September to March (1995-1996) from along the coast of Diu at the time of low tide. All the algal specimens were

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deposited with CSMCRI Herbarium, Bhavnagar, India. The algae were washed with tap water to get rid of mud, dirt and sand and then the same was dried in the shade and powdered in a rotating ball mill. After depigmentation of the dry algal powder by methanol in a Soxhlet apparatus, 50 g of alga was extracted in 20 volumes (w/v) of distilled water (DW) at 4-5°C. The cold water extract (CWE) was first filtered, concentrated and precipitated with acetone (4 volume, v/v). The precipitate was dissolved in minimum DW, dialysed (MWCO 12,000 Da) against DW and lyophilized. The algal residue after cold water extraction was extracted in DW at 80°C for 3 hr. From the filtrate, called hot water extract (HWE) was obtained following the same procedure used for the CWE. The crude PS products obtained from cold and hot water extracts have been designated as REC/REH (*U. reticulata*), LAC/LAH (*U. lactuca*), RIC / RIH (*U. rigida*) and FAC/FAH (*U. fasciata*).

Fractionation and purification—Fractionation of hot water extracts (HWE) of four different species of *Ulva* were made on a DEAE cellulose anion exchange column (Cl^- form, 45 × 3 cm) by a stepwise gradient elution of NaCl (0.0, 0.5, 1.0, 1.5 and 2.0 *M*) at a flow rate of 25 ml/hr. Fractions were dialyzed and freeze-dried to obtain the products (A1, A2, A3 for *U. reticulata*; B1, B2, B3, B4 for *U. lactuca*; C1, C2, C3, for *U. rigida*; D1, D2, D3 for *U. fasciata*). Repeated ion-exchange chromatography of major fractions (product of 0.5 *M* NaCl eluent) were made by a stepwise gradient elution of NaCl (0.15, 0.30, 0.45, 1.5 and 2.0 *M*). Products obtained were designated as: (A2-1, A2-2, A2-3 for *U. reticulata*, B2-1, B2-2, B2-3 for *U. lactuca* C2-1, C2-2, C2-3, for *U. rigida*; D2-1, D2-2, D2-3 for *U. fasciata*. Molecular weight of major fractions obtained from the second DEAE cellulose column were determined by gel filtration on Seralose CL-4B column and standard dextrans as molecular weight markers (580,000-19,500 Da). Blue dextran (2×10^6 Da) was used to determine the bed volume of the column.

Chemical analyses—Moisture and ash contents were determined after oven-drying (120°C for 2 hr) and igniting (550°C for 6 hr) the polysaccharide samples, respectively. Total sugar and protein were estimated by the methods of Dubois *et al.*[18] and Lowry *et al.*[19] respectively. Sulphate content was determined[20] after hydrolysis with 1*N* HCl. Uronic acid was analysed colorimetrically on a Shimadzu UV-VIS 160A spectrophotometer using glucuronic acid as standard[21]. Neutral sugars were determined as their alditol acetates by GC-MS analysis on a BP-225 column[22] using a Hewlett-Packard 5890 Series II GC machine connected to 5971 MSD.

Viscosity—Viscosity of polysaccharide samples was measured by using a Brookfield viscometer at 30°C in 1.6% and 5% solutions of SPS using #1and #2 spindle. Measurements for each sample were done in duplicate and an average value was considered.

Results and Discussion

Chemical analyses data of the sulfated polysaccharides obtained from cold and hot water extracts of four different species of *Ulva* are presented in Tables 1 and 2 respectively. Analytical data of SPS fractions obtained from the DEAE cellulose column chromatography of crude polysaccharide samples are presented in the Table 3. Table 4 describes the monthly variations of physicochemical properties of the cold and hot water extracted product from *U. fasciata*. The constituent sugar ratio and viscosity (in cps) of hot water extracts are presented in Table 5. Percent yield has been calculated based on the dry weight of the alga taken for extraction. Other physicochemical parameters have been presented as the percentage of extracted polysaccharide.

Polysaccharides of the species of Ulva

In cold water extracts, the yields of polysaccharides are in the range of 1.2-6.5%. Low yields of these products precluded further purification and hence characterization of these CWEs were done using the crude products. The yield as well as the sugar contents are higher in FAC (cold water extract of *U. fasciata*). High protein content (17-33%), which is a characteristic of *Ulva* polysaccharides[23], was found in all the polysaccharide samples except in *U. fasciata* containing low protein (9%). Uronic acid content was found to be high in LAC (cold water extract of *U. lactuca*). Ash content of RIC (cold water extract of *U. rigida*) was high in comparison to the others, which appears to be due to the higher sulfate and uronic acid contents that are associated with metal ions. Galactose, mannose and glucose contents were found to be relatively higher than xylose content in CWE samples which indicate that the presence of neutral polysaccharides as well as 'glucan' in these products (Table 1).

In all the hot water extracts, yields of polysaccharide products were higher, compared to

their cold water counterparts. Uronic acid, sulfate and ash contents of LAH were found to be higher than that of other PS samples. There was little variation in the sugar contents of these PS products and their protein contents were relatively low in all the samples compared to the cold water extracts. The latter may be due to the denaturation of protein during the hot water extraction. In HWEs, xylose was detected in higher quantity than that in CWE, the hexose sugars (galactose and glucose) being present in relatively lower amounts. Except in *U. fasciata*, small amount of arabinose was detected in the HWEs (Table 2) whereas mannose was detected in very small amounts in all the CWEs (Table 1).

Water-soluble polysaccharides of *Ulva* were reported to produce viscous solution when dissolved in water and forms gel, in presence of borate and calcium ions[3,4]. Haug[3] proposed that the gelation occurred due to the associations between borate and free hydroxyl groups of the polysaccharides and the chelation of calcium ion to the borate. Gel formation was described to be a function of *p*H. Lahaye & Axelos[4] reported that the SPS isolated from a proliferating *Ulva* spp., was of neutral *p*H (7.5) when dissolved in water and forms gel effectively in phosphate buffer (*p*H range 7.5) in presence of borate ion (4.5 m*M*)[4]. However, it was observed that *p*H of the 1.6% solutions of REH, LAH, RIH and FAH in the deionised water were acidic in nature (5.7-6.9 *p*H) and no change in viscosities were observed after 6-10 hr incubation of the SPS solutions in three different phosphate buffers of *p*H 7.0, 7.6 and 8.0. This is presumably due to the high uronic acid content (27-35%; *cf.* Table 2) of these SPS in comparison to the gel forming polysaccharides of *Ulva* (containing 19% uronic acid) reported earlier[4]. The viscosity of the 1.6% solution of RIH was found to be more than those of the other three SPS samples (Table 2).

The DEAE cellulose column chromatography of REH, LAH, RIH and FAH afforded the major products A2, B2, C2 and D2 respectively from the 0.5 *M* NaCl eluates. Further purification of these PS fractions on DEAE cellulose column yielded major products in the 0.15 *M* NaCl fractions (B2-1, C2-1 and D2-1) except in *U. reticulata*, from which the major product was obtained in 0.30 *M* NaCl (A2-2) fraction (Table 3). The total sugar and sulfate contents increased and protein contents decreased with the increasing molarity of the eluents as expected. Except in PS samples of *U. reticulata* the uronic acid content of the purified fractions was found to be decreasing with increasing molarity of eluents while sulfate contents increased, the major products (B2-1, C2-1 and D2-1) being obtained in the low molarity eluates. In all the PS samples, sugar constituents of the major products were found to be rhamnose, xylose and glucose (Table 3). The molecular weights of A2-2, B2-1 and D2-1 were found to be in the range 1.14 - 1.78 $\times 10^6$ Da whereas C2-1 having the molecular wt. higher than 2.0 $\times 10^6$ Da (Table 3). This result agrees with that reported (6.0 $\times 10^4$ to 1.5 $\times 10^6$ Da) by Yamamoto & Mita [24].

Table 1—Composition of the CWE of the *Ulva* polysaccharides*

Algae	SPS	Yield	Moisture	Ash	Protein	Sulfate	Uronic acid	Sugar	Constituent sugars[δ] (Rhm : xyl : man : gal : glu)
U. reticulata	REC	1.2	20.9	19.3	20.8	14.8	26.2	46.1	1.00: 0.09: 0.16: 0.19: 0.20
U. lactuca	LAC	1.2	24.2	15.2	33.1	10.3	30.2	43.6	1.00: 0.10: 0.17: 0.21: 0.20
U. rigida	RIC	2.1	19.7	21.7	17.4	17.6	28.1	44.4	1.00: 0.12: 0.34: 0.33: 0.04
U. fasciata	FAC	6.5	12.6	13.9	9.2	16.9	24.5	50.1	1.00: 0.09: 0.09: 0.14: 0.16

*Yield in % dry weight of alga; other constituents in % weight of CWE
[δ]Ratios were calculated from GC-MS chromatogram of alditol acetates

Table 2—Composition of the HWE of the *Ulva* polysaccharides*

Algae	SPS	Yield	Moisture	Ash	Protein	Sulfate	Uronic acid	Sugar	Constituent sugars[δ] (Rhm : arab : xyl : gal : glu)	Viscosity[†] (cps)
U. reticulata	REH	5.2	21.4	19.7	9.1	17.8	27.4	43.0	1.00: 0.02: 0.07: 0.02: 0.05	23
U. lactuca	LAH	6.3	14.8	26.0	8.5	23.8	35.7	47.4	1.00: 0.01: 0.16: 0.02: 0.05	18
U. rigida	RIH	8.0	23.7	18.1	10.0	19.1	29.8	48.3	1.00: 0.01: 0.09: 0.02: 0.04	100
U. fasciata	FAH	16.9	17.8	18.4	5.1	18.6	27.0	47.8	1.00: 0.0: 0.681: 0.060: 0.0	41

*Yield in % dry weight of alga; other constituents in % weight of HWE
[δ]Ratios were calculated from GC-MS chromatogram of alditol acetates
[†]Viscosities were measured in 1.6% solution of polysacharides

Seasonal variation in the PS contents of U. fasciata

Good growth of *Ulva fasciata* Delile, was observed during September to March. Rest of the season is unfavourable for the growth of the plant due to high temperature, turbidity and physical disturbance when rocks get covered by the sand during the rainy season. Over the span of a year, the plants attain peak period of its growth for the first time during December-February (seawater temperature 21-24°C) and for the second time during September-October (seawater temperature 25-30°C). Each period is followed by defoliation of the branches of the thallus except holdfast, which remains attached to the substrate, and survives throughout the year for the perennation of the plant.

It has been observed that yield of PS is low (0.5-6.5%) in CWE compared to that of HWE (16-22%) all through the period under investigation (Table 4). In CWE protein content is more throughout the season than those in HWE, whereas sulfate, uronic acid and moisture contents in HWE are higher than that in CWE (Table 4). Cold water extract of algae generally contains smaller molecules compared to hot water counterpart (unpublished observation) and these in turn should have some effect on their viscosity. For this, the cold water soluble polysaccharides have been extracted prior to hot water extraction in this experiment to see the difference of viscosity of these two different extracts. As expected, the HWE has shown relatively high viscosity whereas viscosity was too low to be measured for CWE in the same experimental conditions.

The total sugar and polysaccharide yield were found to be maximum in October and November, respectively, in CWE while in HWE, highest values for these were obtained during December-January. The second peak of sugar contents of CWEs was observed, in January, the active growth period when plants are fully matured. In HWE, peak values of ash contents were observed in September and again in November which characterized the onset and decline of the first peak period of growth of the plant when basal portion predominates over the young thallus. In CWE, the ash contents are generally lower than those in HWE, showing maximum value in September and another peak in February marking the beginning and the end of the growing season respectively. Sulfate

Table 3—Analytical data of the SPS fractions of *Ulva* polysaccharides*

Algae	SPS	Eluent (*M* NaCl)	Yield	Protein	Uronic acid	Sulfate	Sugar	Constituent sugars (Rhm : xyl : glu)	Molecular weight (range in Dalton)
U. reticulata	A1	0.0	9.1	10.5	9.3	1.5	40.5	ND	-
	A2$^{\delta}$	0.5	66.6	3.2	30.4	21.9	54.9	1.00: 0.33: 0.18	-
	A3	1.0	1.5	tr	5.6	24.1	52.5	ND	-
	A2-1	0.15	3.2	tr	19.7	16.7	52.4	ND	-
	A2-2†	0.30	55.2	tr	23.1	19.9	50.6	1.00: 0.27: 0.04	$1.29\text{-}1.54\times10^{6}$
	A2-3	0.45	30.0	tr	26.3	21.6	41.3	ND	-
U. lactuca	B1	0.0	8.3	8.0	8.9	1.8	45.9	ND	-
	B2$^{\delta}$	0.5	66.6	4.7	30.4	25.4	48.2	1.00: 0.47: 0.27	-
	B3	1.0	4.1	3.0	10.0	26.8	41.7	ND	-
	B4	1.5	0.8	ND	ND	ND	38.1	ND	-
	B2-1	0.15	57.1	tr	30.1	19.4	46.2	1.00: 0.42: 0.05	$1.14\times1.40\times10^{6}$
	B2-2	0.30	14.2	tr	18.5	19.8	60.9	ND	-
	B2-3	0.45	11.4	tr	19.5	22.0	64.0	ND	-
U. rigida	C1	0.0	41.6	6.1	15.9	3.5	41.3	ND	-
	C2$^{\delta}$	0.5	37.5	5.3	27.2	22.7	52.5	1.00: 0.61: 0.30	-
	C3	1.0	2.0	3.1	12.0	24.8	52.1	ND	-
	C2-1†	0.15	48.7	tr	21.7	20.9	51.0	1.00: 0.44: 0.13	$> 2.0\times10^{6}$
	C2-2	0.30	22.6	tr	19.9	22.3	59.6	ND	-
	C2-3	0.45	11.3	tr	17.8	24.6	59.6	ND	-
U. fasciata	D1	0.0	36.6	9.2	13.3	2.3	47.8	ND	-
	D2$^{\delta}$	0.5	44.1	4.1	24.2	22.8	48.8	1.00: 0.66: 0.01	-
	D3	1.0	2.2	2.1	12.0	12.0	46.7	ND	-
	D2-1†	0.15	46.0	tr	18.2	17.7	45.3	1.00: 0.27: 0.04	1.37×10^{6}
	D2-1	0.30	18.3	tr	14.9	20.6	64.9	ND	-
	D2-3	0.45	11.0	tr	15.6	25.4	66.0	ND	-

* Yield in % of SPS taken for column chromatography, other constituents in % weight of SPS product;
$^{\delta\,\dagger}$ major fraction of first ($^{\delta}$) and second (†) DEAE column respectively (cf. Materials and Methods); ND: not determined; tr : trace

contents of HWE increases during the late winter months followed by a decline in March when the algal growth process subsides. In CWE, sulfate contents maintained a level above 15% during the early winter, decreasing in December and then increased to reach another peak at the end of the season (Table 4). This may be due to the fact that highly sulfated polymers are required to maintain the metabolic balance within the plant during the minimum algal growth period as well as to provide structural strength required for the survival of the plant[2].

Rhamnose and xylose were detected as the two major components of the crude polysaccharides of HWE by GC-MS analysis of their aliditol acetates, throughout the season (Table 5). It was observed that in HWE, the proportion of xylose maintained a high during November-February and the viscosities were also high during this period (Table 5). Although two peaks of glucose contents were obtained in November and February, the variation lacked a definite pattern. This is presumably due to the fact that a part of the glucose detected in the GC-MS might have been derived from the glucan polymer, which is formed separately alongside the major glucuronoxylorhamnan[2,11].

Although the major polysaccharide was found to be same for different *Ulva* species some differences were observed in their other physical and chemical properties. The yield, uronic acid and viscosity of the polysaccharides which had been reported in the seasonal variation study of *U. lactuca*[2] (yield 13-20%, uronic acid 15-23%, viscosity 52-215 cps; 10% solution) were found to be lower than that of *U. fasciata* (yield 16-21%, uronic acid 27-29%, viscosity 168-247 cps; 5% solution) whereas the sulfate content of the PS of *U. fasciata* (18-21%) was found to be lower than that of *U. lactuca* (20-27%). Two distinct peak periods of growth (September-October and January-February) have been observed for *U. fasciata* of Indian waters during a season. This is in accordance to that observed for *U. lactuca*[2] (June-July and September-October). During the first peak period of growth of

Table 4—Analytical data of the CWE and HWE of *Ulva fasciata**

Extract	Month of collection	Yield	Sugar	Sulfate	Uronic acid	Protein	Ash	Moisture
Cold water extract (CWE)	September	0.5	40.9	17.4	18.8	11.8	20.9	10.6
	October	2.7	53.1	15.2	22.4	6.8	13.7	14.7
	November	6.5	50.1	16.9	24.5	9.2	13.9	12.6
	December	3.9	41.0	13.7	21.0	10.3	14.8	15.2
	January	2.5	43.5	14.9	20.4	9.4	13.6	13.5
	February	3.0	42.5	17.2	21.0	7.0	16.9	14.2
	March	1.6	42.0	18.9	20.1	7.3	12.9	14.8
Hot water extract (HWE)	September	15.9	52.2	21.8	26.9	4.6	17.2	16.2
	October	18.3	52.8	19.3	27.6	3.9	12.4	17.6
	November	16.9	47.8	18.6	27.0	5.1	18.4	17.8
	December	21.4	52.9	20.2	28.8	4.4	15.2	17.6
	January	18.6	52.1	21.3	28.3	4.2	14.8	16.9
	February	17.1	47.3	21.0	29.8	4.9	15.9	16.7
	March	17.1	50.8	18.3	27.9	2.6	15.0	19.7

* Yield in % dry weight of alga; other constituents in % weight of CWE and HWE

Table 5—Viscosity and constituent sugar ratio of HWE of *Ulva fasciata*

Month	Viscosity (cps)$^{\delta}$	Constituent sugars[a] (Rham : Xyl : Gluc: Galac : Man)
September	168	1 : 0.105 : 0.035 : 0.007 : 0.006
October	157	1 : 0.210 : 0.058 : 0.009 : 0.019
November*	222	1 : 0.681 : 0.060
December	203	1 : 0.330 : 0.031 : 0.008 : 0.010
January	207	1 : 0.389 : 0.040 : 0.023 : 0.016
February	247	1 : 0.955 : 0.063 : 0.011 : 0.005
March	185	1 : 0.176 : 0.055 : 0.006 : 0.003

[a] Ratios were calculated from the GC-MS chromatogram of the alditol acetates
*Galactose and mannose contents were negligibly small
$^{\delta}$ Viscosities were measured in 5% solution of polysaccharide

U. fasciata (in October), the sugar content and yield were found to be high for HWE . However, it appears that plants were not matured enough in terms of their phycocolloid contents as indicated by relatively low sulfate, uronic acid contents as well as by their low viscosity. The second peak period of growth started from November and the plants achieved complete maturity during January-February. In February, the viscosity of the phycocolloid was found to be the highest with relatively high sulfate and uronic acid contents, although the yield and sugar contents were low. These findings may be useful in defining bioprospecting strategies of this alga which has been reported to be a good source of dietary fibers and useful mucilagenous phycocolloids[4,25].

Water-soluble sulfated polysaccharides isolated from various Ulvaceae species are of similar nature as described earlier. Rhamnose, xylose and glucuronic acid being the main constituents, these acidic polymers are primarily known as sulfated glucuronoxylorhamnan[2]. This has been largely substantiated by the results of present investigation. Given the abundant availability of *Ulva* species, more studies are required on these polysaccharides, involving studies in the changes of viscosity with acidity (uronic acid content), temperature and sulfate contents eventually correlating with their conformational geometry for exploring their potential as useful products.

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