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Full Length Research Paper

Seasonal variation of the growth, chemical composition and carrageenan extracted from *Hypnea musciformis* (Wulfen) Lamouroux harvested along the Atlantic coast of Morocco

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Hypnea musciformis **is a common species of the Moroccan Atlantic coast. Both biology and chemical composition of the carrageenan extracted were studied on samples collected monthly during two years on the jetty of Mehdia in the Northwest of the country. The growth of the alga was at its maximum in summer and autumn. Chlorophyll a and R-phycoerythrin (maximum in winter) was the main pigments of the alga. The ash content presented significant fluctuations between 11 to 55% of dry matter. The carrageenan content could reach up to 41% of the dry matter in winter and then decreased in autumn. Its chemical composition did not show marked seasonal variations. Galactose and 3,6-anhydrogalactose were the main components of the carrageenan and varied respectively between 53.79 and 63.79 mole % for galactose and 31.69 and 41.41 mole % for 3,6-anhydrogalactose. The sulphates oscillated between 15 and 23% of carrageenan dry matter. I.R and NMR ^{13}C Spectra showed that the native phycocolloïd extracted from *H. musciformis* was formed by the repetition of the disaccharidic unit of kappa carrageenan.**

Key words: Biology, carrageenans, chemical composition, pigments, *Hypnea musciformis,* seasonal variation.

INTRODUCTION

Carrageenans are galactans extracted mainly from species belonging to Gigartinales (McCandless, 1978; Craigie, 1990). These sulfated galactans, consist of linear chains of D-galactopyranoses linked in α (1$\rightarrow$3) and β (1$\rightarrow$4) (Anderson and Rees, 1965). The classification of these polysaccharides is based on the presence and localisation of sulphate esters and on the presence of the 3,6-anhydro bridge on α-D-galactose linked in (1$\rightarrow$4) (Mc Candless and Craigie, 1979; Craigie, 1990).

In the past, the genus *Hypnea* was regarded as producing agar (Isaac, 1948; De Loach et al., 1946; Humm and Williams, 1948; Diaz-Piferrer and Caballer-de-Perèz, 1964; Rama Rao and Krishna-Murthy, 1968;Rama Rao, 1970). Since the works of Mollion (1973, 1978), Mshigeni and Mziray (1979) and of Smith et al. (2002), it was established that *Hypnea* contained carrageenan. Greer et al. (1984), Knutsen et al. (1995) and Miller (2003) have clearly defined that the galactan synthesized by this alga was of kappa type. This galactan is composed of β-D-galactose-4-sulphate linked in (1$\rightarrow$3) (symbolically represented by G4S), alternating with 3,6-anhydro-α-D-galactose linked in (1$\rightarrow$4) (AD) (Knutsen et al., 1995). The percentage of galactan can reach 40% of algal dry matter (Rama Rao and Krishna-Murthy, 1968). This alga is also characterized by a high growth rate. These characteristics are at the origin of its economic potential (Guist et al., 1982; Friedlander and Zeilikovitsch,

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Figure 1. Geographical situation of the station of study.

1984; Bravin and Yoneshigue-Valentin, 2002).

According to Mollion (1978) there was an opposite relation between carrageenan yield and total sugar content. The variations of other components (ash, proteins, lipids and sugars) have been also used to follow the biology of this species (Durako and Dawes, 1980).

The present work constitutes a first approach for the exploitation of this species, in Morocco, as an industrial source of raw material for carrageenan extraction. We report the growth and chemical composition of alga collected monthly in the north of the country during two years.

MATERIAL AND METHODS

Algal material

Along the Moroccan Atlantic coast, *Hypnea musciformis* lives on rocky shore from low tide level to 10 m depth. Sampling was carried out monthly from February 1997 to December 1998 on the jetty of Mehdia in the north of Morocco (Figure 1).

Biological survey

On samples collected monthly, 100 thalli were used to follow the growth and the fertility of the alga. The weight and length of each thallus were measured. The total number of fertile thalli was noted.

Extraction of the carrageenans

The algae were thoroughly cleaned with filtered seawater to eliminate any undesirable epiphytes attached to the thalli. The fresh material was washed quickly with distilled water, wiped out, weighed and weight. The algae were ground to a powder. The carrageenans were extracted according to a method adapted from Craigie and Leigh (1978). 10 g of algae were hydrated in 500 ml of distilled water for a night at 20°C under agitation. The algae were depigmented with acetone for 2 h under agitation. Then, the carrageenans were extracted at 90°C during 5 h in 500 ml distilled water with continuous stirring. The solution is purified by filtration using diatomite as filter aid, concentrated under vacuum and precipitated in three volumes of isopropanol. The coagulum is recovered by filtration on a nylon sieve, oven-dried at 60°C, and weighed to calculate the yield of the extraction.

Chemical analysis

The pigments were measured as soon as possible after harvest. They were extracted from 400 mg of fresh algae in a mortar with 5 ml of phosphate buffer 0.75 M pH 7 for phycobilins and with 10 ml of 80% acetone for chlorophyll a. The pigments were separated from the algal residue by centrifugation (5000 rpm at 4°C during 12 min). These extractions were carried out under attenuate light. The phycobilins content was calculated according to Kursar et al. (1983) and chlorophyll a from the absorbance at 664 nm with the method of Ziegler and Egle (1965). The results were expressed in mg g^{-1} of fresh alga.

Ash content was measured after incineration of 2 g of dry algal powder in a muffle furnace at 600°C during 6 h (Larsen, 1978).

The neutral sugars were quantified by gas chromatography after reductive hydrolysis of galactans and acetylation of the monomers (Stevenson and Furneaux, 1991). They were separated on a column dB 225 (J and W Scientific) eluated by hydrogen at 210°C. The monosaccharides derivatives were identified according to their retention time relating to myo-inositol hexacetate used as internal standard.

The sulphates contents of polysaccharide were measured according to the method of Quemener et al. (1997). The sulphates released by hydrolysis with 2 M trifluoroacetic acid were separated by HPAEC-conductimetry.

Infra-red spectroscopy

The infrared spectra were carried out on films prepared by evaporation of carrageenan aqueous solution of 0.5% (p/v) in polyethylene caps. The spectra were recorded on an IRS 25 FT (Bruker) spectrophotometer.

*^{13}C NMR spectroscopy

^{13}C NMR spectra were obtained at 90°C on 3% polysaccharide solutions in D_2O: H_2O (50: 50, V/V) using a Bruker ARX 400 instrument. Chemical shifts were calculated in part-per-million (ppm) from the Me_2SO line at 39.6 ppm as an internal reference.

RESULTS

Growth and reproduction

Analysis of length and weight variations of *H. musciformis* in natural environment showed that the two parameters were correlated in the time (r^2 = 0.6 and r^2 = 0.75 respectively in 1997 and 1998; $p \leq 0.05$) (Figure 2). Two annual periods of active growth were observed; one from May to August in 1997 and from March to June in 1998, the second between September and November for the two years of study. The species was fertile almost all

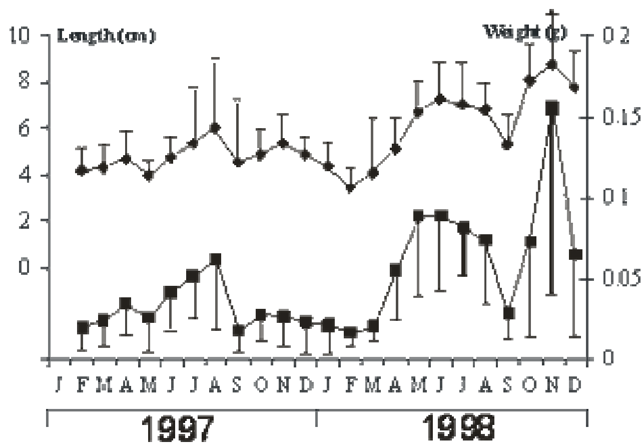

Figure 2. Seasonal variation of length (♦) and weight (■) of *Hypnea musciformis* thalli during the two years of study.

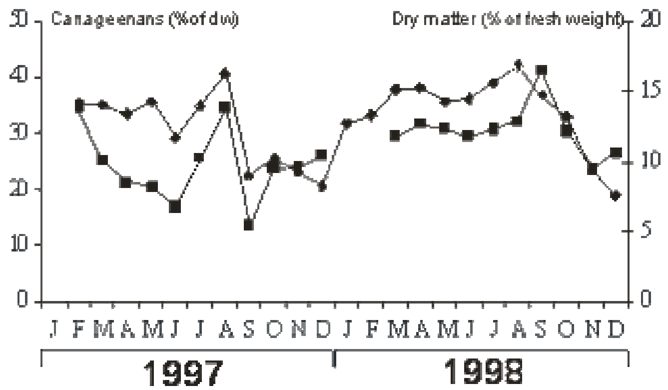

Figure 4. Seasonal variation of the dry matter (♦) and carrageenan yield (■) from *Hypnea musciformis.*

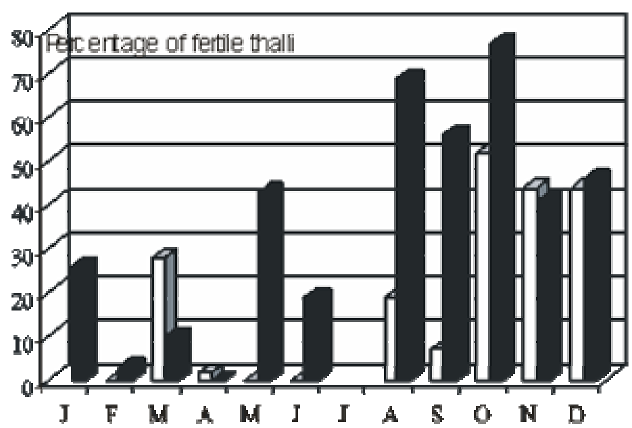

Figure 3. Seasonal variations of the percentage fertile *Hypnea musciformis* thalli during the two cycles of study: (■): 1998, (□): 1997.

Table 1. Ash content of Hypnea musciformis thalli expressed as a percentage of dry matteer.

Month	Ash	
	1997	1998
J	12.2	-
F	46.6	12
M	51.7	12.2
A	38.0	15.5
M	55.5	13.9
J	-	-
J	11.4	13
A	26.4	11
S	31.0	12.7
O	12	33.6
N	42.9	11.9
D	12.2	-

along the year, the maximum was noted in October (52 - 77%) and the minimum between April and June (0 - 2%) in 1997 and between February and April (3 – 10%) in 1998 (Figure 3).

Chemical composition of thalli

The percentage of dry matter presented a seasonal variation with a clear increase in summer, the maximum value (16.5%) was recorded in August and the minimum of 7.8% in December during the two years. The phycocolloid yield reached 34.5% in August 1997 and 41.0% in September 1998. Then, it decreased to 13.5% in September 1997 and 23.4% in November 1998 (Figure 4). The ash percentage presented significant fluctuations. The highest value was 55% of algal dry matter in June 1997 the lowest was 11% September 1998. The ash content presented important variations from year to year (Table 1).

The phycoerythrin content (major water-soluble pigments) was high in autumn and winter with a maximum of 2.85 mg g^{-1} of fresh matter noticed in November and low at the end of spring and at the beginning of summer with a minimum of 0.46 mg g^{-1} of fresh matter recorded in June (Figure 5). Phycocyanins were present in small amount (between 0.07 and 0.75 mg g^{-1} of the algal fresh matter). Chlorophyll a did not show marked seasonal variations, the highest value was measured in November (0.88 mg g^{-1} of fresh matter).

Chemical composition of the carrageenan

The gas liquid chromatographic analysis of the carrageenan revealed that it average composition (on 2 years) was: 67.7% of total carbohydrate, and 17% of sulphates.

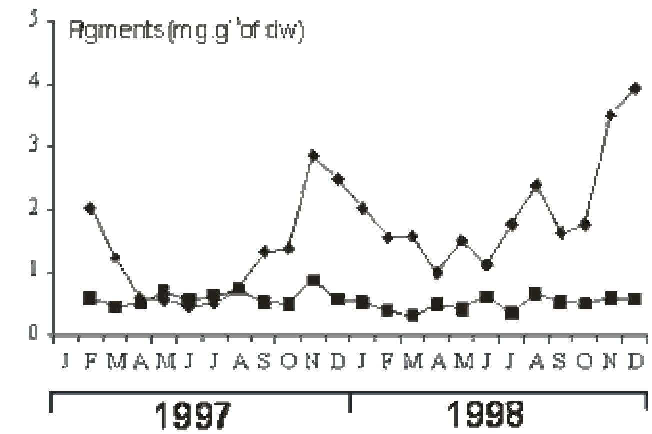

Figure 5. Seasonal variation of the R-PE (♦) and chlorophyll a (■) extracted from *Hypnea musciformis.*

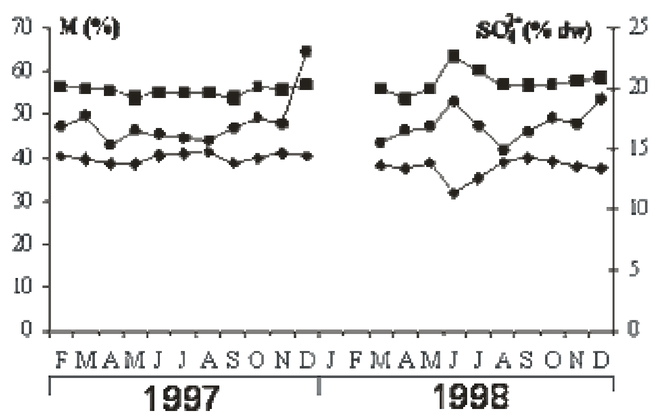

Figure 6. Variation of the chemical composition of the carrageenan extracts from *Hypnea musciformis.* Sulfate (●), galactose (■), and 3, 6-anhydro galactose (♦).

The phycocolloid percentage (total sugars and sulphates) varied along the year between 76 and 92% of the total extract. The carrageenan extracted from *H. musciformis* was mainly composed of D-galactose (56.5 mol %) and of 3,6 anhydro D-galactose (39 mol %) which were the 2 main sugars (Figure 6). Glucose was present as traces. The sulphate content varied between 15 and 23% of the dry carrageenan. The main constitutive sugars of the carrageenan did not vary significantly according to the season of harvest. However, the sulphate content presented a maximum of 23% in December 1997. Apart from this period, the sulphate content oscillated around 17%.

I.R. spectroscopy

I.R spectra of the extracts were identical to those of the literature for a Kappa-carrageenan (Santos and Doty, 1975; Chopin and Whalen, 1993; Liao et al., 1996; Caceres et al., 1997). They showed vibration at 1240 cm^{1} due to the esters sulphates (S = O), at 930 cm^{-1} characteristic of the 3,6 anhydrogalactose bridge and at 840 cm^{-1} for sulphate in axial position on the C-4 of galactose. The various peaks allocated to the structural elements of this fraction indicated that the carrageenan studied was of kappa type.

NMR spectroscopy

^{13}C NMR Spectra were in agreement with the structure of a kappa-carrageenan (Rochas et al., 1983; Usov 1984; Mollion et al., 1988; Matsuhiro and Urzua, 1992) with the resonances for the carbons of the galactose unit (G) G1: 102.5 ppm, G2: 69.7 ppm, G3: 79.2 ppm, G4: 74.1 ppm, G5: 74.8 ppm, G6: 61.4 ppm and of the anhydrogalactose unit (AD) A1: 95.2 ppm, A2: 69.7 ppm, A3: 79.2 ppm, A4: 78.3 ppm, A5: 76.8 ppm, A6: 69.7 ppm. These results showed that the native phycocolloïd of *H. musciformis* is formed by the repetition of the kappa-carrageenan disaccharidic unit.

DISCUSSION

The biological cycle of *H. musciformis* collected at Mehdia was characterized by two periods of growth. At the beginning of the first one (May in 1997 and March in 1998) the water content of thalli would explain the dry matter reduction and the low carrageenan content. Thereafter, the increment of algal dry matter and carrageenan yield mostly resulted from active growth. During the second phase of growth (September-November), the alga behave in a different way from one year to the other for carrageenan synthesis, that lets suppose the presence of a heterogeneous population by succession of generation or the polysaccharide synthesis was independent from growth during these months. From February to May, there was appearance of new shoots, the growth was slow, and the old thalli were fragmented under the effect of swell. After November the growth slowed down and the thalli worsen.

During the vegetative cycle of *H. musciformis*, the dry matter and the carrageenan contents presented a positive correlation ($r^2 = 0.54$; $p \leq 0.05$). These results agreed with those of Zinoun and Cosson (1996) for *Calliblepharis jubata.* However, Durako and Dawes (1980) showed that the weight variation of *H. musciformis* studied in Florida was inversely proportional to the galactan content. In addition, the growth of this species showed fluctuations due to the variations of the environmental factors such as salinity, temperature and prolonged dessication (Mshigeni, 1979; Bravin and Yoneshigue-Valentin, 2002), light intensity (Friedlander and Zelikovitch, 1984) and air humidity (Murthy et al., 1989). Seasonal variations of the carrageenan contents were observed for the same species (Rama Rao, 1970; Mshigeni, 1979; Schenkman, 1980).

On the other hand, Neish et al. (1977); Friedlander et al. (1987) and Zinoun and Cosson (1996) established an opposite relation between periods of active growth and the phycocolloid production. The carrageenans content would increase with the age of the thalli. Similar results were reported at *Gracilaria tikvahiae* by Craigie and Wen (1984). Mouradi et al. (1992) measured high agar contents from old thalli of *Gelidium latifolium* (Greville) Bornet and Thuret. The analysis of phycobilins indicated that phycoer-ythrins were the main biliproteic pigments. The R-phyco-erythrin (R-PE) content remained higher than chlorophyll a during most of the year. A slight predominance of chlorophyll a on phycoerythrins was observed in summer period, this could be due to a degradation of R-PE by strong light intensities. This was found for *Gracilaria verrucosa* by Kosovel and Talarico (1979) and for *Gelidium sesquipedale* at the end of summer (Mouradi et al., 1999). However, for *Gracilaria multipartita*, harvested nearby *Hypnea* the R-PE contents remained always higher than chlorophyll a (Givernaud et al., 1999).

Ashes presented significant year-to-year variations and fluctuate between 11 and 55% of algal dry matter. High contents were also recorded for *H. musciformis* studied by Bi and Iqbal (1999) and Durako and Dawes (1980), for *G. tikvahiae* (Peniman and Mathieson, 1987), for *Gigartina pistillata* (Amimi et al., 2007) and for *G. multipartita* (El Gourji, 1999). The high ash percentages could be related to the accumulation of great quantities of mineral residues by the species in its tissues or come from adsorption on the surface of the thalli without biological accumulation phenomenon.

The chemical composition of the carrageenans was not affected by seasonal variations. D-galactose and 3,6-anhydrogalactose were the main components of the polysaccharide. IR and NMR ^{13}C spectra of carrageenans extrac-ted from *H. musciformis* were in conformity with those of the literature (Santos and Doty, 1975; Chopin and Whalen, 1993; Liao et al., 1996; Caceres et al., 1997; Rochas et al., 1983; Usov, 1984; Mollion et al., 1988; Matsuhiro and Urzua, 1992; Mtolera and Buriyo, 2004). They did not present seasonal variations. This stability of the chemical composition of the carrageenan extracted from *H. musciformis* was observed for other species of red algae such as *G. sesquipedale* (Mouradi et al., 1999) and *G. pistillata* (Amimi et al., 2007). Miller and Furneaux (1982), working on several species of Gelidia-ceae, had comparable results, contrary to the variations of chemical composition noted for *G. Multipartita* (Givernaud et al., 1999). On the other hand, the sulphate content presented some fluctuations. According to Doty and Santos (1983) the sulphates are influenced by the environmental factors. A slight increase was recorded in cold period characterized by better nutrients availability.

Conclusion

H. musciformis studied along the Moroccan Atlantic coast is an alga, which produces a kappa carrageenan regarded as a phycocolloid of quality that could be used for various utilisations. The most favourable period for the harvest of this species would be in summer when the carrageenan content and biomass of the alga are at their maximum values. However, to protect the natural resource, it would be preferable to cultivate this species.

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