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FOURIER TRANSFORM INFRARED (FT-IR) SPECTROSCOPIC INVESTIGATIONS OF FOUR AGAROPHYTES FROM NORTHERN ARABIAN SEA

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Abstract

The fresh specimens of *Champia compressa* Harvey, *Gelidium usmanghanii* Afaq-Husain and M. Shameel, *Gracilaria foliifera* (Forsskål) Børgesen and *Hypnea musciformis* (Wulfen) J. V. Lamouroux, were collected from the coastal areas of Karachi (Pakistan) and their yield of agar was observed. With pre-extraction technique the percentage of algal yield was found to range from 13 to 28%. High quality gel strength was determined by modulus of elasticity *i.e.* (550 – 612g/cm^2) and 1090 kPa and extraction time was 2 hrs. The structure of agar and agar contents were investigated by FT-IR spectroscopy for the first time from Pakistan. The results revealed some interesting characters (non sulphated β-D galacto pyranose residues, 3,6 anhydro galactose vibration), which were not reported earlier.

Introduction

The coastline of Pakistan is about 885 km long and rich in algal vegetation found either in growing and in drift form or attached to the rocks, occurring also in the sandy and rocky bottom of the pools (Shameel and Tanaka 1992). Algae produced a number of biologically and chemically active compounds such as aldehydes, fatty acids, halogenated compounds, sterols and terpenes (Aliya and Shameel 2003, Shahnaz and Shameel 2008, 2009). According to Bhadury and Wright (2004) algal metabolites have antibacterial, antifungal, and antibiofouling properties. Additionally, natural products isolated from seaweeds have pharmaceutical and medicinal uses in various parts of the world (Smit 2004).

Seaweeds the imperative revenue generating resources of the marine environment, have a fundamental and important position in traditional drug of all ancient civilizations of the world, i.e. Greek, Roman, Chinese and Indian and are exploited as vermifuges, aesthetics and antibiotics in the treatment of cough, wounds, gout, goiter, hypertension, cancer, venereal and a variety of other diseases (Smit 2004, South and Whittick 1987, Sridharan and Dhamotharan 2012). They also provide alginate, agar and carrageenan some of their carbohydrates (polysaccharides) are being used in the preparation of toothpastes, soaps, shampoos, and as a thickening agent in ice creams and milk (NAAS 2003). Particularly agar is widely used in leather, cosmetics, paper, textile, dairy and pharmaceutical industries. Moreover, it is the major component of culture media for microbiology laboratory (NAAS 2003).

A number of research work have been reported on sulfated polysaccharides commonly known as agar and cell wall constituents of red algae (Akahane and Izumi 1976, Villanueva and Montaño 1999, Freile-Pelegrin and Murano 2005, Laurienzo 2010, Villanueva *et al.* 2009, 2010a, b). The agarose is the major component of agar, the repeating agarobiose units blinking between 3-linked β-D-galactopyranosyl (G) and 4-linked 3,6-anhydro-α-L-galactopyranosyl (LA) units. The sulfate hemi esters and methyl esters in a variety of groupings and with a cyclic pyruvate ketal as 4,6-O-[(R)-1-carboxyethylidene] acetal are modified by substitution of hydroxyl groups in

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disaccharides, which forms the sustainable structure in the cell wall of several species of red seaweeds, and it is released on boiling (Praiboon *et al.* 2006, Hotchkiss and Trius 2007, Van *et al.* 2008).

In the present study the structure of agar extracted from the four different species of agarophytes i.e., *C. compressa, G. usmanghanii, G. foliifera* and *H. musciformis* by Fourier Transform Infrared (FT-IR) Spectroscopy. Previously no work has been reported about the vibrational band studies of agar from this region.

Materials and Methods

The specimens of *C. compressa, G. usmanghanii, G. foliifera* and *H. musciformis* were collected from the sandy bottom rocky pools of mid-littoral rocks at Buleji in the month of March 2012, from epilithic in mid to sub-littoral zones at Manora, Buleji and Paradise Point during August and September 2012, from rocky pools of mid-littoral rocks at Manora during January to April 2011, and as drift material from Manora and Buleji in the month of December 2012, respectively. The fresh algal specimens were washed with fresh water to remove the epiphytes, epizoons and attached sand particles and then with distilled water. The algal material in bulk was dried under shade.

Fifteen g dried thalli of each species were first soaked in 200 ml NaOH solution of 0, 1, 2, 3, 4 and 5% concentrations, respectively at 80 - 85°C for 1, 2, 3 and 4 hrs. Whole thalli of *C. compressa, G. usmanghanii, G. foliifera* and *H. musciformis* were used in the extraction for alkali pretreatment process (Freile-Pelegrin and Robledo 1997, Villanueva *et al.* 2010 a,b). The NaOH solution was discarded and the pretreated samples were hydrated by soaking in 350 ml of distilled water at room temperature, the same procedure was followed 2 - 3 times for washing. Next they were soaked in dilute acetic acid solution of 0.5% for 1.5 hr at room temperature, acetic acid solution was then decanted and again each species was washed with fresh water. Agarophytes were extracted from 350 ml of distilled water at boiling temperature for 1.5 hr. Each species of all agar were grinded in blender to homogenize and filtered with the help of cotton cloth. The freeze thawing method was followed for the extraction of agar from the blended mixture of *C. compressa, G. usmanghanii, G. foliifera* and *H. musciformis.*

The extracted agar was then washed and treated with alcohol (ethanol) to reduce the water content from the freshly extracted agar, finally oven dried at 60°C and collected in air tight bottles. The best quality agar was extracted from the solution of 5% NaOH solution with time duration of 4 hrs pretreatment process, Secondly the intense studies were carried out for the gel quality and the extraction time of each agar extracted from *C. compressa, G. usmanghanii, G. foliifera* and *H. musciformis*. After pretreatment process of (NaOH) the solution was neutralized by acetic acid and seaweeds were extracted with 350 ml distilled water at boiling temperature for 2, 3 and 4 hrs. The whole procedure proceeded in the alkali treatment and agar extraction section. The agar weight was determined by the percentage calculation of agar dry weight divided by seaweed dry weight mentioned in the following equation, (Armisen 1995, Villanueva *et al.* 2010 a,b).

$$\text{Agar yield} = \frac{\text{Agar dry weight (g)}}{\text{Seaweed dry weight (g)}} \times 100$$

In distilled water 2% w/w agar solution was prepared by boiling with continuous stirring, approximately 15 g of the hot solution was added to the cylindrical container (30 mm in diameter), allowed it to settle for 24 hrs. at room temperature and the solution was covered with aluminum foil. The depth of the gel was maintained in between 18 and 22 mm.

The consistency and quality of the gel and its apparent young's Modulus were determined by the Stable Micro System model TAXT2, Surrey, England. The methodology for the study of stress strain curve was followed by Hilliou *et al.* 2006 and (Villanueva *et al.* 2009, 2010a, b). The CSL rheometer was used for the rheological measurement with 3 mm thick agar gel slab loaded on pre heated up to 50 - 55°C Peltier plate and temperature was elevated up to 96 - 98°C to change the texture in the almost liquid state.

The overload sample was discarded and to minimize the probability of evaporation the sample was covered with paraffin oil. The cooling to heating scan was measured the storage (G') and loss (G″) moduli were measured at 5.91 rad s^{-1} with 0.1% strain and the temperatures at which crossover of the moduli occurred (G´ = G″ or tan d = 1), the gelling and melting temperatures were considered as the cooling and heating scans, respectively. Typical curves for these rheological tests are presented by Villanueva and Montaño 1999, Villanueva *et al.* 2009, 2010b.

The statistical analysis was carried out by SPSS Statistics version 17.0. The two-way ANOVA applied for agar yield, gel strength and texture and to measure the considerable differences among the properties of different agars of all four species extracted in different time intervals.

The IR spectra were monitored on Shimadzu prestige-21 200 VCE coupled to a PIV-PC and loaded with IR solution version 1.2 software (potassium bromide disks). The dried powder 1 mg each was mixed in a mortar with 99 mg of KBr. A thin KBr disc was obtained by pressing the powder in French press and FT-IR spectra were recorded (Villanueva and Montaño 1999, Rajasulochana *et al*. 2008, Mulbry *et al*. 2012).

Results and Discussion

The preliminary studies reveal that the agar extraction from 5% sodium hydroxide solution has the best quality results from all the four species studied which have been under investigation for these extraction studies (Yaphe and Arsenault 1965, Rajasulochana *et al*. 2008).The effect of alkali concentration (Y), agar yield (Z), gel strength, agar's melting point, apparent Young's Modulus and melting temperature of agar is presented in Table 1.

The treatment and extraction from different solutions of sodium hydroxide during pretreatment procedures significantly influenced the yield and quality of agar extracted from the species of different seaweeds. The statistical parameters of F-static and p-value reveal that the values are comparatively high, the values interaction endorsed that all the parameters are significant with high F-values (Table 1). The low agar yield was observed in 0% NaOH solution from *C. compressa* (11%), *G. usmanghanii* (14%), *G. foliifera* (15%) and *H. musciformis* (13%). The best gels extracted from *C. compressa*, *G. usmanghanii*, *G. foliifera* and *H. musciformis* were 21, 23, 19, and 21%, respectively when treated with 4% NaOH solution and 36, 33, 35and 34% respectively when treated with 5% NaOH solution. On the other hand the weak gels extracted (11, 14, 15, and 13%, respectively from the above species) from the solution of zero per cent alkali with very fragile polymeric capacity even was difficult to recover after washing with alcohol (Villanueva and Montaño 1999, Trivedi and Kumar 2014).

The Young's modulus results and agar gel strength have same responses in all the four species of algae, lowest agar gel strength was observed at 0% NaOH pretreatment conditions whereas the agar gel quality was increased as the concentration of NaOH increased during pretreatment conditions. From the 5% NaOH, the best agar gel extracted has the highest value of 589 g/cm^2 in *C. compressa*, 612 g/cm^2 in *G. usmanghanii*, 550 g/cm^2 in *G. foliifera*and 582 g/cm^2 *H. musciformis*, so the 5% NaOH was the best concentration of the alkali solution for excellent agar extraction technique.

The representative local agar when compared with alkali treated agars extracted from the *C. compressa*, *G. usmanghanii*, *G. foliifera* and *H. musciformis* possess almost similar FT-IR bands and they are in close resemblance with that of standard Difco Agar from Merck. The FT-IR spectroscopic studies mentioned in Table 2 while the collected seaweeds were preserved in the form of herbaria (Villanueva *et al*. 2010 a,b).

Table 1. The SPSS statistics on the different properties of agar extracted from *Champia compressa, Gelidium usmanghanii, Gracilaria foliifera* and *Hypnea musciformis.*

Agar property	NaOH concentration (X)		Pretreatment duration (Y)		Y × Z	
Champia compressa	F	p	F	p	F	p
Agar yield	110.54	0.0001	7.58	0.0001	4.12	0.0039
Gel strength	70.12	0.0012	30.69	0.0001	11.25	0.0002
Apparent Young's modulus	61	0.0001	17.89	0.0019	2.99	0.0018
Gelling temperature	141.41	0.0001	9.01	0.0188	8.21	0.0001
Melting temperature	30.12	0.0001	8.88	0.0031	3.89	0.0003
Gelidium usmanghanii						
Agar yield	121.60	0.0017	8.78	0.0042	3.05	0.0031
Gel strength	69.14	0.0001	32	0.0001	10.02	0.0018
Apparent Young's modulus	58.54	0.0011	16.99	0.0002	3.77	0.0014
Gelling temperature	137.41	0.0001	9	0.0002	8.5	0.0001
Melting temperature	32.78	0.0001	8.88	0.0014	3.02	0.0065
Gracilaria foliifera						
Agar yield	119.25	0.0023	8.19	0.0045	4.12	0.0029
Gel strength	72.47	0.0011	28.77	0.0001	11.25	0.0001
Apparent Young's modulus	59.5	0.0001	18	0.0001	2.99	0.0015
Gelling temperature	133.2	0.0001	9.3	0.0178	8.21	0.0003
Melting temperature	29.2	0.0001	8.45	0.0001	3.89	0.0074
Hypnea musciformis						
Agar yield	122	0.0014	6.98	0.0003	3.18	0.0031
Gel strength	68.47	0.0015	33.12	0.0014	1.89	0.0001
Apparent Young's modulus	57	0.0011	20.14	0.0001	3.58	0.0013
Gelling temperature	139	0.0014	8.66	0.0002	7.45	0.0069
Melting temperature	28.77	0.0001	7.78	0.0036	4.18	0.0010

FT-IR has generally used in biological materials as discussed and represented in various research articles, the spectra of the samples of *C. compressa, G. usmanghanii, G. foliifera* and *H. musciformis* are presented in (Tables 1 - 2) and their probable assignments on the vibrational frequencies are arranged as follows:

The FT-IR spectra of the samples exhibited at 3450 cm^{-1} and confirm the observation (South and Whittick 1987).

Table 2. FT-IR band assignment of standard agar and agars obtained from *Champia compressa, Gelidium usmanghanii, Gracilaria foliifera* and *Hypnea musciformis* (frequency/cm).

Band assignments	Standard agar	*Champia compressa*	*Gelidium usmanghanii*	*Gracilaria foliifera*	*Hypnea musciformis*
C-S linked vibration/S-O stretch/C-S deformation	690 s	688	672	687	669
C-S linked vibration/S-O stretch/C-S deformation	780 s	762	780	785	769
Ester sulphate in C-2 link vibration/C-C/C-O/C-O-S stretch		832	850	845	849
Non sulphated β-D galacto pyranose residues/C-C/C-O stretch	885 s	895	880	892	878
3,6 anhydro galactose vibration/C-C/C-O/C-O-S stretch	935 s	936	930	925	931
Ester sulphate link vibration/C-C/C-O/C-O-S stretch	1045 vs	1048	1051	1039	1050
Ester sulphate link vibration/C-C/C-O/C-O-S stretch	1165 s	1161	1162	1170	1175
Methyl group vibration/S=O symmetric stretch	1375	1381	1383	1385	1380
Methyl group vibration/S=O asymmetric stretch	1438	1435	1442	1445	1439
C=O symmetric stretch/N-H deformation		2880	2865	2868	2885
C=O symmetric stretch/N-H deformation	1650				
N-H deformation	1555	1551	1559	1549	1558
C=O stretch	1737				
Methyl group vibration/C-H symmetric stretch		2875	2874	2868	2871
Methyl group vibration/C-H asymmetric stretch	2932, 3420	2940, 3431	2945, 3433	2939, 3425	2928, 3445
OH/N-H stretch	3475	3481	3478	3470	3469

In all the four spectra the N-H stretching appeared as a combination band with stretching band of –OH *G. usmanghanii* showed absorption at 3545 cm^{-1}, *in G. foliifera* the N-H stretching appeared at 3457 cm^{-1}, *C. compressa* and *H. musciformis* showed their absorption band at 3461 cm^{-1}.

Methyl group has two types of stretching vibrations. In alkane C-H asymmetric stretching band occurs at 2939, 2940, 2928 and 2945 cm^{-1} and symmetric stretching vibrations observed at 2885 cm^{-1}. The FTIR spectra of *G. usmanghanii* has weak bands were exhibited for methyl group for asymmetric stretching at 2878 cm^{-1}, for *G. foliifera* the asymmetric stretching was observed at 2889 cm^{-1} with very weak bands appeared due to methyl substituent, the asymmetric vibration of

C. compressa was appeared at 2884 cm^{-1} and the asymmetric vibration frequency of *H. musciformis* was appeared at 2880 cm^{-1} (Freile-Pelegrin and Murano 2005).

Ester-sulphate link vibration of *G. usmanghanii* was manifested at 1080 cm^{-1}, for *G. foliifera* vibration observed at 1180 cm^{-1} and for *C. compressa* and *H. musciformis* they appeared at 1202 and 1156 cm^{-1}, respectively (Akahane and Izumi 1976). The intensity of the absorptions vary was found to from medium strong to weak or even very weak and it depends up on the species. The bands at 1075 to 1125 cm^{-1} are due to symmetric stretching of S-O/C-O and asymmetric vibrations occurred at 1245 - 1370 cm^{-1}. The sulphate ester of C-6 linkage was observed at 880 cm^{-1} in *G. usmanghanii,* in *G. foliifera* it appeared at 885 cm^{-1} in *C. compressa* this peak appeared at 895 cm^{-1} and in *H. musciformis* this absorption was appeared at 865 cm^{-1}. The β-D-galactose pyranose residues are well known agar peak according to the literature survey (Troung *et al*. 1988).The additional peaks 830, 846 and 821 cm^{-1} were noticed in the spectrum of rest of the samples of *G. foliifera, C. compressa* and *H. musciformis* which is the representative peak of sulphate ester C-2 link absorptions. The linkage of C-S absorption of polysaccharides has been reported in literature in the region of 670 - 770 cm^{-1}. The spectra of present study showed the strong bands to weak bands over the range of 670-770 cm^{-1} which may be attributed to the deformation of the sulphate linkage.

The strong to weak bands occurring at 936 - 925 and 1039 - 1051 cm^{-1} in spectra of the present study are attributed to C-O ether bond of 3, 6 anhydro galactose vibration as observed in the previous work these two peaks are archetypal vibrations of phycocolloides.

In the present work, the strong absorption bands at 1650 cm^{-1} was assigned for carbonyl vibrations of carboxylic group (-COO) due to asymmetric stretching of C=O band. The medium to weak intensity band of C=O of symmetrical stretching was observed at 1545 cm^{-1} which was also reported previously (Mulbry *et al*. 2012).

The presence of protein in polysaccharides shows carbonyl absorption as amide group. Primary and secondary amides display a band in the region 1640 cm^{-1} and very weak band of 1540 cm^{-1} has been reported which also been discussed by (Mulbry *et al*. 2012). A sharp peak appeared in the region of 3469-3481 cm^{-1} due to N-H stretching vibration and all these vibrations have been presented first time in this study.

The C-C vibrations are weak in the region of 1200-800 cm^{-1} all the bands in this region are in the overlapping of C-C and C-H stretching that's why it is difficult to differentiate between these weak and small bands in this region.

This linkage of C-S polysaccharides band was observed in between the range of 670 - 770 cm^{-1} and the strong to weak bands has been recorded for the deformation of sulphate in all the four species of the *C. compressa, G. usmanghanii, G. foliifera* and *H. musciformis.*

The results revealed that the alkali treatment was the ideal method for the extraction of better quality of agar from the different species of red algae *i.e. C. compressa, G. usmanghanii, G. foliifera* and *H. musciformis*, while from non-alkali treatment method the poor-quality agar has been extracted. Gel strength and apparent Young's modulus results showed that there was a marked difference between all the treatments and the shorter extraction time was ideal for the better gel production. The intensity of bands varies with algal resources. The spectra of all the four samples exhibited consistent observations as similar to those shown by standard agar. It is interesting to note that a broad band is exhibited in the studied samples at 1245 cm^{-1}, which is specific peak of sulphate esters. The peak is corresponding to as hump in standard agar, point to that the phycocolloids from these species contain a greater amount of sulphate esters as compared to the standard agar. The quality of agar is the indication of measure of substitution patterns, which differ in general with basic bands. In the present study, agar obtained from *C. compressa,*

G. usmanghanii, G. foliifera and *H. musciformis* are more sulphated than standard agar, and consequently provide soft gelling agars which could be suitable for food industries.

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