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Optimization of carrageenan production from Persian Gulf algae *Laurencia obtusa*: Purification and rheological characterization

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In this study, *Laurencia obtusa* (Yamada, 1931) species was investigated for carrageenan extraction. *L. obtusa* species was identified using the molecular barcoding method with two COI and rbcL genes. Rheological analysis was performed to determine the rheological properties of semi-refined carrageenans. Experimental design was carried out with Design Expert software to produce and optimize SRC extraction. The software presented 18 treatments based on temperature, cooking time and concentration of KOH. Treatments were prepared for rheometric analysis (i.e., viscosity measurements). Optimization was performed by the software with maximum viscosity. Also, two refined carrageenan extraction methods were performed, and the FTIR and NMR spectra were compared. Barcoding showed similarity values of 96 and 94% using the COI sequence and therbcL sequence, respectively. The optimal treatment included temperature of 77°C, 99 minutes, and 7% concentration of w/v KOH. The highest efficiency was associated with the dialysis method, which was 19%. Based on the rheometric analysis, the SRC solution was introduced as a pseudo plastic Non-Newtonian fluid. According to these two analyses, the refined carrageenans of *L. obtusa* were of the iota type.

Keywords: Barcoding, COI, Design-Expert, RbcL, Rheometric analysis

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Introduction

Algae have a critical function in aquatic ecosystems and play a significant role in producing economically valuable compounds. The cell wall of red algae consists of cellulose and a high percentage of mucilage and is the source of desirable commercial products, such as agar and carrageenans[1].

The *Laurencia* algae have a small to medium size and belong in the *Ceramiales phylum* and *Rhodomelaceae* family[2]. Many of the metabolites isolated from the *Laurencia* species have shown cytotoxic[3] as well as anti-tumor[4], antibacterial[5], antifungal and antiviral activities[6]. These Sea creatures are a promising source of anticoagulant compounds[7]. The *L. obtusa* algae are found in colours such as dark red and reddish or pinkish brown. These algae are usually massive in the middle area of the tidal range of rocky substrates. They have been widely dispersed throughout the world. The distribution of *Laurencia obtusa* chemical substances such as phenols, polyphenols, lignin, chitin, subbine and tannin has been analyzed for evaluation as a raw drug using histochemistry[8].

Carrageenans are structurally similar to cellulose in plants and chemically similar to sulfate galactos. There are six types of carrageenans: Iota, Kappa, Lambda, Mo, Nov, and Theta[9]. Of these, three types namely Kappa, Iota, and Lambda have commercial value[10]. Commercial carrageenans are available in the form of sodium, potassium, and calcium salts or often in the form of mixtures of them with different solubility and gelling properties. Kappa and Iota types are dissolved in hot water (over 70°C), and they can form gels in the presence of potassium and calcium cations, whereas lambda carrageenans do not form a gel[11].

The functionality of carrageenans in various applications depends on their rheological properties. Carrageenans are linear and water-soluble polymer and form highly viscous aqueous solutions. Viscosity is dependent on the concentration, temperature, presence of other salts, cariogenic type and molecular weight[12]. The viscosity of food carrageenans is less than 5 cps at 75°C for a solution of 1.5%; the concentration in food is equal to 0.005-2 % in weight[13].

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Carrageenans are used as emulsifiers (suspenders), stabilizers, colloids or glues. Many of the products used currently, such as soy milk, chocolate milk or other flavoured milk and dairy products, baby food and complementary drinks, are carrageenan-dependent to maintain their homogeneity. They cannot be produced, packaged and stored for long periods without using carrageenans that are used for gel thickening or suspension[14]. The production processes of antibiotics[15], aspartic acid[16], ethanol from glucose[17], the continuous production of acetic acid[18] and purification of industrial wastewater[19] have been improved by stabilizing different bacteria in Kappa carrageenan gels. Carrageenan is used as a thickener and stabilizer in toothpaste, especially for the treatment of dental caries. Carrageenans are utilized as a viscous material for transferring rheological properties to toothpaste and providing quality and beauty. In the US market, carrageenans are in intense competition with carboxymethylcellulose, which is a much cheaper gum. However, carrageenans have better quality and appearance than carboxymethylcellulose for use in toothpastes. Usually, about 1% Iota carrageenans are added to some toothpaste compounds[14]. Carrageenans have shown potential in antiviral activity at the laboratory level. Carraguard is a vaginal microbicide based on carrageenans that inhibits HIV and other sexually transmitted diseases at the laboratory level. In 2003, in North Africa, Carraguard entered phase III of clinical trials and was tested on 6,000 non-pregnant HIV negative women[20].

The algae species in the Persian Gulf are unique due to the difficult life conditions such as high salinity and temperature. There are several red algae species on the Persian Gulf coasts that have not been investigated for carrageenan presence. Considering the importance and applications of carrageenan and the presence of various red algae such as *L. obtusa* found on the coasts of the Persian Gulf in Bushehr province in all seasons, the authors conducted a study to screen the *L. obtusa* species for carrageenans. Efficiency is one of the important points in economically Extraction. *L. obtusa* extraction optimization tests have not yet been completed.

The objectives of this research were to extract carrageenan from red algae, *L. obtusa* and optimize the parameters affecting the quality of carrageenan gel using the Design Expert software. Purification of carrageenan and control of the efficiency of the various methods and identification of the type of extracted carrageenan were also performed.

Material and methods

Sampling

Samples of red algae were collected from the tidal zones of the Persian Gulf coast in Bushehr province at the low tides. The collected algae were packed in a plastic bag containing sea water to prevent evaporation and were transferred to the laboratory. A portion of each sample was isolated for molecular and morphological identification and was stored at -20°C. The remaining samples were dried in the shade. Samples were transferred to the laboratory and were washed with tap water for about 2 minutes to remove the salts. Then, the algae were dried in an oven at 50°C overnight. Algae samples were weighed. The dried algae were powdered using a blender[21].

DNA extraction

The DNA extraction method was used to optimize the method used Siemer *et al.*[22]. This procedure was performed as follows:

The dry algae samples were grinded by Mortar & Pestle. 900 µL of the extraction buffer containing 2% CTAB (containing 2% of sarcosyl and 0.2% of PVP) was added to the sample and vortexed[22]. Then, 100 µL of SDS 20%[23], 12 µL of betamercaptophenol and 5 µL of proteinase K were added to the samples[22]. After vortexing, the sample was incubated at 65°C overnight in a water bath. 500 µL of chloroform: isoamyl alcohol (24:1) was added to the sample, which was mixed for 2 minutes. The sample was centrifuged at 12000 rpm for 20 minutes (4°C). The supernatant was transferred to a new tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added, and then it was centrifuged again. These two steps were repeated until there was no contamination in the middle phase[18]. The supernatant was transferred to the new tube, 800 µL of chloroform: isoamyl alcohol (24:1 ratio) was added to it, and they were mixed for 5 minutes. The centrifuge was run at 12000 rpm for 20 minutes (15°C). The supernatant was collected, and the same volume of pure cold ethanol (-20°C) was added[24]. After several times of inverting, the sample was kept in ice for 30 minutes. The sample was centrifuged at 12000 rpm for 15 minutes. The supernatant was discarded, and the DNA pellet was washed with 70% alcohol. Then the centrifuge was run at 8000 rpm for 5 minutes. The dried DNA was dissolved in 100 µL of deionized distilled water and was stored at –20°C[25]. Electrophoresis with 1% agarose gel was used for the measurement of DNA quality[26].

Polymerase Chain Reaction (PCR) amplification

In this study, the COI-5P DNA barcode marker and the rbcL plastid gene have been used for molecular identification. The sequences of the selected primers for COI-5P were: Forward: (GAZF1) TCA ACA AAT, Reverse: (GAZR1) ACT TCT GGA TGT CCA AAA AAY CA, and for rbcL were: Forward: (F.7) AAC TCT GTA GTA GAA CGN ACA AG, Reverse: (R.753) GCT CTT TCA TAC ATA TCT TCC. The materials used in PCR (50 µL) contained 25 µL of Master Mix PCR, 16 µL of Buffer PCR, 4 µL of nuclear DNA, and 2.6 µL of each primer. The PCR programs for the two genes were different and adjusted as follows: the PCR program for COI-5P was 300 s at 95°C, followed by 35 cycles of 30 s at 94°C, 60 s at 50°C, 120 s at 72°C and a final extension cycle at 72°C for 60 s. The PCR program for rbcL was 300 s at 95°C, followed by 35 cycles of 30 s at 94°C, 60 s at 51°C, 120 s at 72°C and a final extension cycle at 72°C for 60 s[27].

Rheological analyses of carrageenans

Due to the absence of any reports regarding the rheological properties of carrageenans extracted from *L. obtusa*, rheological analyses were performed to determine the rheological properties of the extracted carrageenans. 10 treatments were planned (section 2.5), and the carrageenans were extracted (according to section 2.6). Then, an semi refined carrageenans (SRC) solution was prepared at weight of 1.5% in water bath at 80°C for 20 minutes[22]. Rheological analyses (tension, elongation, cutting rate, etc.) of the product were performed using an ANTON Parr MCR301 rheometer. Then graphs related to the rheometric results were drawn with Excel software (2013).

Semi refined carrageenans optimization

The Design-Expert (DX) software used in this study is one of the most specialized software applications for designing tests. 18 experimental runs with varying parameters of KOH concentration (7-11% w/w), cooking temperatures (60-80°C) and cooking times (40-100 min) were proposed by the Central Composite Design (CCD) of RSM in DX, which consisted of 4 replications at the central point in order to determine the experimental error by the software. Finally, the results were analyzed using DX.

SRC preparation

The stages of SRC powder production are as follows:

The KOH solution was prepared according to the experimental runs and 1.5 g of algae powder was added to it. The solution was placed in the water bath according to the cooking temperature and time determined for each experimental run. After a certain period of time, the sample was filtered using filter paper. The remaining material on the filter paper was placed in an oven at 60°C overnight to obtain a SRC powder[28].

Refined Carrageenan (RC) extraction

There are several methods for RC extraction. Two methods, dialysis bag and hydrogen peroxide, were selected for the extraction of RC from *L. obtusa* species.

The first method was dialysis bag, which had been optimized before[29]. This procedure was performed in the following way: 5 g of dried algae powder was added to 500 mL of a 4% w/v NaOH solution and 0.25% NaBH4 w/v. The mixture was incubated at 80°C (Incubator) at the rate of 140 for 3 h. The PH of the solution was adjusted to 7 with acetic acid. The neutralized solution was inserted into the dialysis bag[30] and placed in a sterile distilled water kettle on stirrer at the rate of 4.5 for 24 h to complete the dialysis process. After 24 h of dialysis, the solution was poured into a petri dish and was subjected to freeze-drying to obtain dry carrageenan powder[23].

The second method was hydrogen peroxide, which had similarly been optimized before[31]. This procedure was performed as follows: 20 g of shade-dried algae was added to 250 mL of sterile distilled water and was placed on a stirrer to be mixed a little. In the next step, 100 mL of 18% w/v calcium hydroxide solution was added and placed on a stirrer at 80°C at the rate of 4.5 for 10 minutes. 2 mL of 3.5% hydrogen peroxide solution was added to the material. After 5 minutes, 100 mL of sodium sulfite solution (8% w/v) was added and stirred for 2 h. The solution was filtered through the cloth and passed through the filter paper. The PH of the solution was adjusted at 6.8-9. Then, 2 volumes of the isopropanol were added to the solution to precipitate the carrageenans. The clear supernatant liquid was removed, and the remaining residue was placed in an oven at 50°C to be dried completely[25].

FTIR and HNMR analysis

FTIR analysis has been conducted to identify the type of carrageenan. For this purpose, the algae powder was mixed with potassium bromide and was

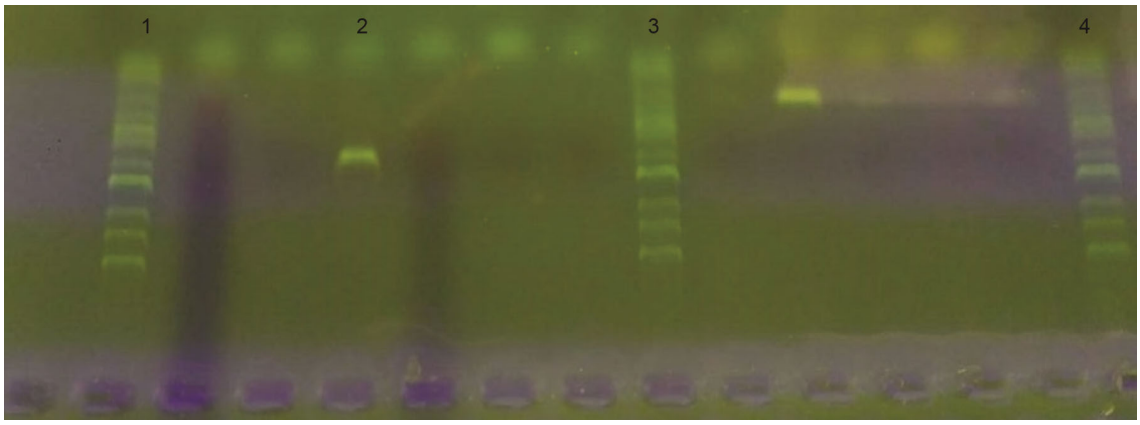

Fig. 1 — Product of polymerase chain reaction on agarose gel. Columns 1 and 3 represent the 100 bp ladder. Column 2 corresponds to the COI gene, and column 4 indicates the rbcL gene.

placed in a measuring plate. The FTIR was performed with a Bruker Vectorat a distance of 500-4000 cm^{-1} and at room temperature in the Postgraduate Studies Center of Zanjan University.

The HNMR spectrum was performed with a BrukerAvance 3 (400 MHZ) manufactured in Germany. The 1H spectra were recorded using 32 scans, relaxation delay of 5 s and acquisition time of 0.9999 s. The analysis was carried out at a temperature of 55 °C with a D2O solvent.

Statistical analysis

ANOVA analysis, determination of equation and optimization based on viscosity were done using DX. Initial analysis charts were drawn with Excel, and 3D graphs were drawn using DX.

Results and Discussion

Molecular identification

Morphological identification was difficult due to the great similarity between different *Laurencia* species. Therefore, molecular identification was performed. Molecular identification was carried out through amplification of COI (610 base pair) and rbcL (1300 base pair) fragments. After sequencing, the sequencing results of two readings were compared with the sequences recorded at the Gene Bank using BLAST software. The species was confirmed as *L. obtusa* with 96% and 94% of similarity using the COI sequence and therbcL sequence, respectively. Wattier *et al.* used a 20% SDS and proteinase K solution for DNA extraction of 12 red algae species[32]. Hu *et al.* also used SDS and EDTA extraction buffers and 0.2% PVP to extract the DNA of 15 red algae species[25]. Different methods were used for DNA extraction in our study, but due to the presence of high polysaccharides in red algae, only the above-mentioned method was successful. In this study, the presence of DNA in the extracted product was not detectable with an agarose gel. However, the product of the PCR was well observed in the agarose gel (Fig. 1). The success of cox1 in animals has led to its use as a DNA barcoding marker in red algae. Also, the rbcL sequence is another locus used to identify red algae following a barcoding strategy[33]. Nauer *et al.*[27] used DNA barcodes for identification of red algae.

Moisture calculation

In this study, washing was performed with tap water for 2 minutes, following Normah and Nazarifah, to minimize the amount of salts. The samples were dried in the shade and then were dried in the oven at 50°C overnight in order to make sure regarding the non-degradation of algae, according to Normah and Nazarifah[21]. *L. obtusa* lost almost 82.57% of its moisture. According to the study by Vairappan *et al.*, the amount of lost moisture was suitable for carrageenan extraction[29].

Rheological analysis

The following graphs are plotted using the rheometric results of treatment number 10. The shear stress-shear rate diagram determines the type of fluid (Fig. 2). Since the slope of the shear stress-shear rate is not constant and meets the y axis at a point above the origin point, the SRC generated in this study is a non-Newtonian Bingham pseudo plastic fluid.

Fig. 3a shows the viscosity-shear rate diagram, which indicates the shear-thinning or the shear-thickening of the fluid. Fig. 3a shows that viscosity decreases with increasing shear stress, so the fluid is a shear-thinning product.

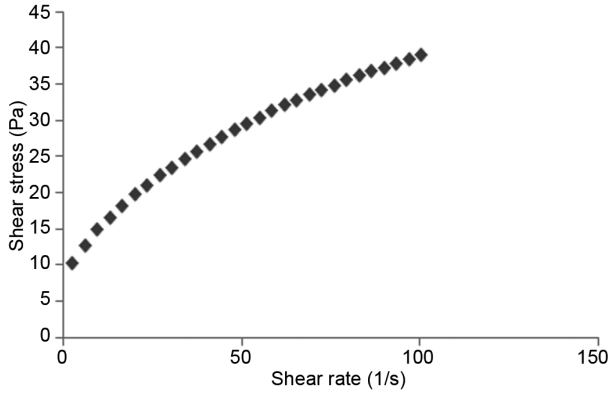

Fig. 2 — Shear stress - shear rate diagram.

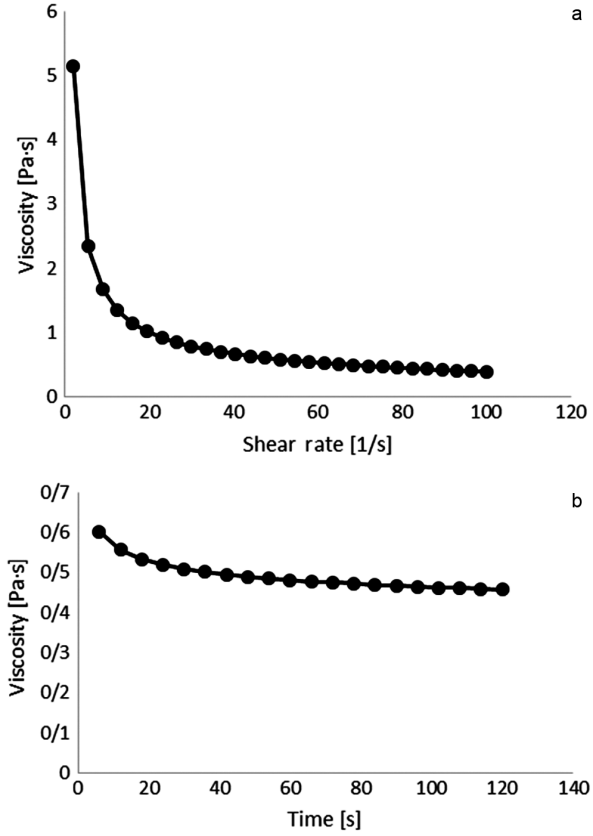

Fig. 3 — a) Viscosity - shear rate diagram; and b) Viscosity - time diagram.

Fig. 3b (viscosity - time) indicates the rheopectic or thixotropic fluid which changes through increasing or decreasing the viscosity. Fig. 3b shows that the viscosity decreased over time, and subsequently, a time-dependent and thixotropic fluid was produced.

The modulus-strain per cent diagram shows the critical strain (Fig. 4). In this diagram, the change in the elastic modulus (G') determines critical strain. In Fig. 4, the amount of critical strain for *L. obtusa*

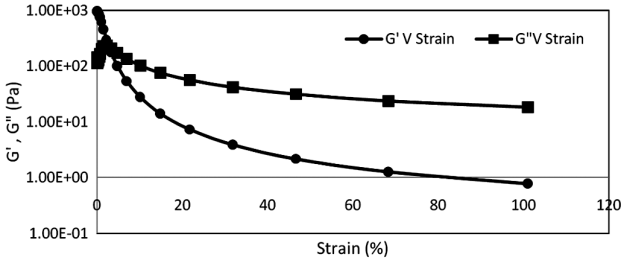

Fig. 4 — Modulus - Strain diagram.

Table 1 — Treatment viscosity of *L. obtusa*

Run	Factor 1 A: KOH W/V	Factor 2 B: Temprature C	Factor 3 C: Time Min	Response 1 Viscosity Pa
1	7	80	100	173
2	9	70	120.454	49.4
3	11	80	100	26.1
4	7	60	100	92.4
5	9	70	70	21.5
6	9	53.1821	70	24.9
7	9	86.8179	70	0.115
8	9	70	70	75.5
9	7	80	40	7.06
10	11	80	40	26.8
11	5.63641	70	70	154
12	12.3636	70	70	1.54
13	11	60	100	51.6
14	9	70	70	45.1
15	9	70	70	73.2
16	7	60	40	56.5
17	11	60	40	5.45
18	9	70	19.5462	66.5

species is 3.23%, which means that in stretching less than this critical value, the behaviour of the fluid resembles the solid, and in stretching higher than that, it behaves like a liquid.

The results of the rheological analyses of carrageenan were similar to those obtained in the study by Marcotte *et al.*[34] and Garrec *et al.*[35].

DX software

DX is one of the most practical applications for designing and optimizing experiments. Bono *et al.* investigated the effect of three parameters including KOH concentration, temperature and cooking time on the viscosity of carrageenans obtained from *Karpaphycus alvarezii* algae using DX. This research was the only one that optimized the rheological properties of carrageenans using DX[28].

In our study, Rheology analyses were done with a rheometer for all treatments of the species *L. obtusa* at the frequency of 15 and at 80°C. The data were entered into the DX software (Table 1). Formula 1 shows the third-order equation of the *L. obtusa* species, which was

proposed by DX software. In this equation, the coefficients in the left column and the variable parameters in the right column are shown, respectively.

Viscosity = 53.55+ (-45.33) × KOH-7.37× Temprature-5.08× Time -4.41× KOH × Temprature-19.55 × KOH×Time+10.40× Temprature × Time+9.70 × KOH^2-13.37× $Temprature^2$ +2.70× $Time^2$-22.11 × KOH × Temprature × Time+10.74× KOH^2× Temprature+36.00× KOH^2×Time+17.95× KOH × $Temprature^2$ (Formula 1)

Fig. 5 shows the factors' interaction with viscosity in the form of a 3D graph. In the temperature-KOH diagram (Fig. 5a); it is observed that viscosity decreases with the increase in KOH at a constant temperature. In a constant KOH, at temperature below 70°C, viscosity has a direct relationship with temperature, but at temperatures above 70°C, the amount of viscosity decreases as temperature increases.

In the time-KOH graph (Fig. 5b), viscosity is inversely proportional to the KOH over a constant time period, but the effect of time varies in different KOH concentrations. At a KOH concentration less than 9%, viscosity has a direct relationship with cooking time, and above 9%, increasing cooking time leads to a decrease in viscosity.

In the temperature-time graph (Fig. 5c), at temperatures below 70°C, viscosity is inversely proportional to time, and at temperatures above 70°C, viscosity has a direct relationship with cooking time. At the time points less than 70 minutes, viscosity is inversely proportional to temperature. But after 70 minutes, temperature changes do not follow a specific pattern.

The first optimal treatment offered by the software was a 7.003% w/v KOH concentration at 77.673°C and 99.896 minutes with a 173.813 viscosity.

Refined carrageenan

RC output

The method of dialysis used in this study was carried out by applying variations including temperature (80°C) and filtration (paper-filter) according to the procedure employed by Vairappan *et al.*[29]. The output was 19%. The Hydrogen peroxide method was carried out according to the study by Gordon and Jonas[31]. The efficiency of the hydrogen peroxide method was 9.4%. Thus, the efficiency of the dialysis method proved higher.

Vairappan *et al.* used the dialysis method for carrageenan extraction from *K. alvarezii* Doty algae

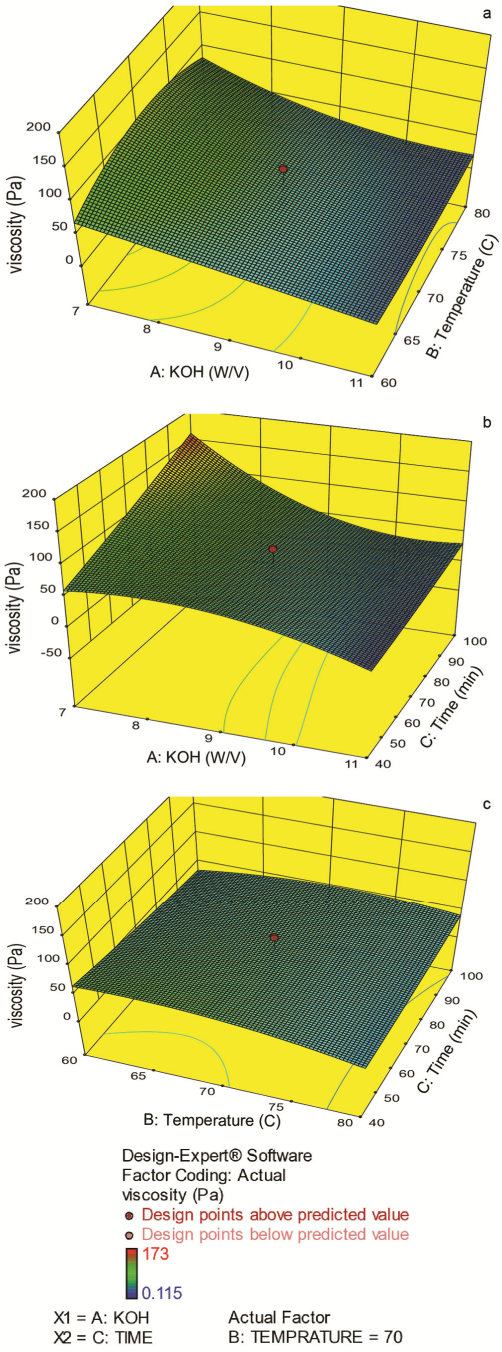

Fig. 5 — 3D graph of factors' interaction with viscosity. a) Temperature-KOH diagram; b) Time-KOH graph; and c) Temperature-time graph.

with a yield of 58%[29]. Gordon *et al.* extracted carrageenans with the calcium hydroxide method from *Chondrus crispus*. The method yield was 39.7%[31]. There are different methods for carrageenan extraction, but we used two of these methods in our study and extracted carrageenans by changing and optimizing them.

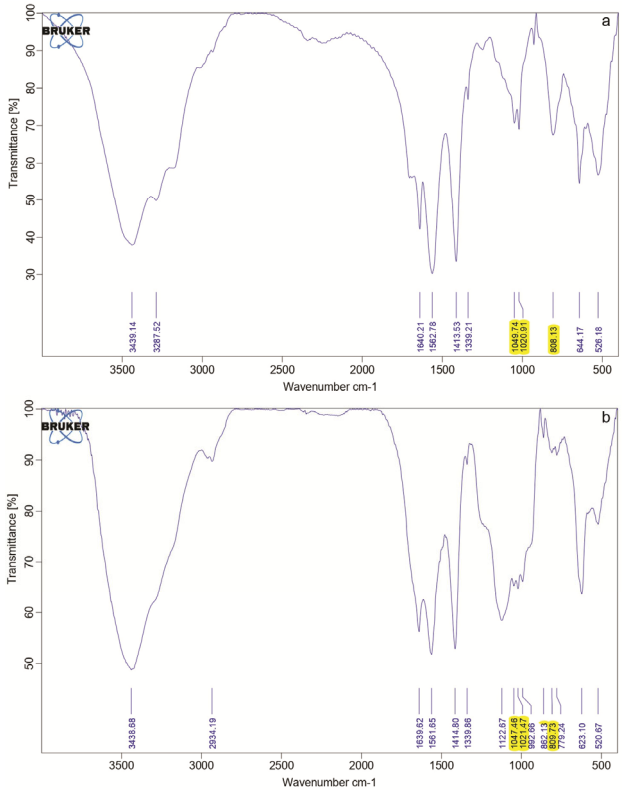

Fig. 6 — a) FTIR of dialysis method; and b) FTIR of hydrogen peroxide method.

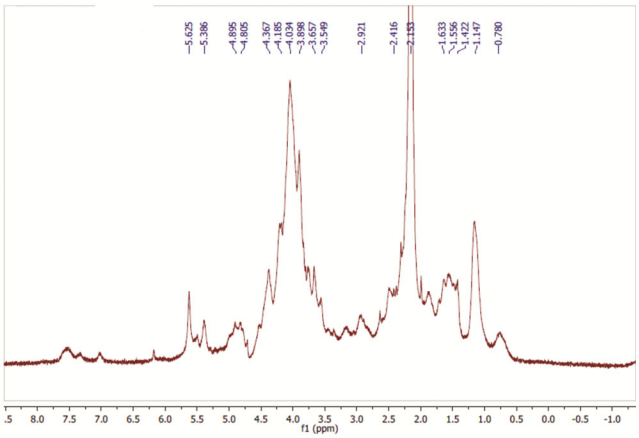

Fig. 7 — H-NMR analysis for hydrogen peroxide method of *L. obtusa*.

FTIR analysis of RC samples

FTIR analysis was done to identify the type of carrageenans. Fig. 6a shows the FTIR analysis for *L. obtusa*, which was performed with dialysis. In Fig. 6a, a band is seen at 808.13 cm^{-1} for the sulfate group in the 3, 6-D-galactose-2-sulfate, which indicates the presence of Iota carrageenans. Two bands are observed at 1020.91 cm^{-1} and 1049.74 cm^{-1} (glycosylated grafts) in all types of carrageenans.

FTIR analysis for the hydrogen peroxide method is shown in Fig. 6b. One band is seen at 808.73cm^{-1} for the sulfate group in the 3, 6-D-galactose-2-sulfate, which indicates the presence of iota carrageenans. Two bands are observed at 1021.47 cm^{-1} and 1047.46 cm^{-1} (glycosylated grafts) in all types of carrageenans.

Weber *et al.* confirmed the existence of 805 cm^{-1} band in Iota, which was seen in our study in 808 cm^{-1}[Ref. 36]. Volery *et al.* state that this band is the only difference between Iota and Kappa[37]. The bands of the glycoside linkage were confirmed by Pereira *et al.*[38].

FTIR results were very similar for both methods, but the hydrogen peroxide method showed a better separation, so the NMR analysis was performed for this method.

H-NMR analysis

The study on the NMR spectrum of the RC samples (obtained through the hydrogen peroxide method) at 55°C was confirmed by the results of the FTIR spectrum. According to previous studies, the temperature has affected the location of NMR bands. So that, in Fig. 7, it was observed to be 5.62 ppm for Iota carrageenans (hydrogen AG1). The observed band of iota was confirmed using the results obtained by Tojo *et al.*[39].

Conclusion

The presence of Carrageenans in the *L. obtusa* species was examined in this study for the first time. Therefore, further investigations on this group of algae are needed to fully confirm the presence and measure of carrageenans. further purification procedures are needed to obtain higher purity of this material to confirm its economic justification. the carrageenan structures should be examined more carefully. The rheology studies are preliminary, so more detailed studies can be done to know more about the carrageenans properties. In addition, after further analysis of the carrageenan types and properties and purity, researchers can focus on optimizing pure carrageenans.

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Conflict of interest

The authors report no conflicts of interest.

References

1 Pereira L and Neto J M, *Marine algae: biodiversity, taxonomy, environmental assessment, and biotechnology*, (CRC Press), 2014.

2 Lewis J A, Checklist and bibliography of benthic marine macroalgae recorded from northern Australia. I Rhodophyta, Materials Research Labs Ascot Vale (Australia), 1984,

3 Lhullier C, Falkenberg M, Ioannou E, Quesada A, Papazafiri P, *et al.*, Cytotoxic halogenated metabolites from the Brazilian red alga *Laurencia catarinensis*, *J Nat Prod*, 2009, 73(1), 27-32.

4 Melillo G, Targeting hypoxia cell signaling for cancer therapy, *Cancer Metastasis Rev*, 2007, 26(2), 341-352.

5 Chenniappan S, Durairaj G and Kumaran A V, Antibacterial activity of *Jania rubens* from Gulf of Mannar, south coast of India, *Indian J Nat Prod Resour*, 2021, 12(3), 451-458.

6 Sakemi S, Higa T, Jefford C W and Bernardinelli G, Venustatriol. A new, anti-viral, triterpene tetracyclic ether from *Laurencia venusta*, *Tetrahedron Lett*, 1986, 27(36), 4287-4290.

7 Dube A, Singh N, Saxena A and Lakshmi V, Antileishmanial potential of a marine sponge, *Haliclona exigua (Kirkpatrick)* against experimental visceral leishmaniasis, *Parasitol Res*, 2007, 101(2), 317-324.

8 Freile-Pelegrin Y, Robledo D, Chan-Bacab M J and Ortega-Morales B O, Antileishmanial properties of tropical marine algae extracts, *Fitoterapia*, 2008, 79(5), 374-377.

9 Campo V L, Kawano D F, da Silva Jr D B and Carvalho I, Carrageenans: Biological properties, chemical modifications and structural analysis– A review, *Carbohydr Polym*, 2009, 77(2), 167-180.

10 Pereira L and van de Velde F, Portuguese carrageenophytes: Carrageenan composition and geographic distribution of eight species (*Gigartinales, Rhodophyta*), *Carbohydr Polym*, 2011, 84(1), 614-623.

11 Stanley N, Production, properties and uses of carrageenan. *Production and utilization of products from commercial seaweeds*, *FAO Fisheries Technical Paper*, 1987, 288, 116-146.

12 Lai V M F, Wong P L and Lii C Y, Effects of cation properties on Sol-gel transition and gel properties of κ-carrageenan, *J Food Sci*, 2000, 65(8), 1332-1337.

13 Tobacman J K, Review of harmful gastrointestinal effects of carrageenan in animal experiments, *Environ Health Perspect*, 2001, 109(10), 983.

14 Necas J and Bartosikova L, Carrageenan: A review, *Vet Med*, 2013, 58, 187-205.

15 Teruel M A, Gontier E, Bienaime C, Saucedo J N and Barbotin J N, Response surface analysis of chlortetracycline and tetracycline production with K-carrageenan immobilized *Streptomyces aureofaciens*, *Enzyme Microb Technol*, 1997, 21(5), 314-320.

16 Van de Velde F, Lourenço N D, Pinheiro H M and Bakker M, Carrageenan: A food-grade and biocompatible support for immobilisation techniques, *Adv Synth Catal*, 2002, 344(8), 815-835.

17 Nigam J, Continuous ethanol production from pineapple cannery waste using immobilized yeast cells, *J Biotechnol*, 2000, 80(2), 189-193.

18 Tosa T and Shibatani T, Industrial application of immobilized biocatalysts in Japan, *Ann N Y Acad Sci*, 1995, 750(1), 364-375.

19 dos Santos V M, Bruijnse M, Tramper J and Wijffels R H, The magic-bead concept: An integrated approach to nitrogen removal with co-immobilized micro-organisms, *Appl Microbiol Biotechnol*, 1996. 45(4), 447-453.

20 Spieler R, Seaweed compound's anti-HIV efficacy will be tested in southern Africa, *Lancet*, 2002, 359(9318), 1675.

21 Normah O and Nazarifah I, Production of semi-refined carrageenan from locally available red seaweed, *Eucheuma cottonii* on a laboratory scale, *J Trop Agric Food Sci*, 2003, 31(2), 207.

22 Siemer B L, Stam W T, Olsen J L and Pedersen P M, Phylogenetic relationships of the brown algal orders *Ectocarpales, Chordariales, Dictyosiphonales*, and *Tilopteridales* (Phaeophyceae) based on RUBISCO large subunit and spacer sequences, *J Phycol*, 1998, 34(6), 1038-1048.

23 Tan H, Huang H, Tie M, Ma J and Li H, Comparative analysis of six DNA extraction methods in cowpea (Vigna unguiculata L. Walp), *J Agric Sci*, 2013, 5(7), 82.

24 Leach J, Finkelstein D B and Rambosek J A, Rapid miniprep of DNA from *filamentous fungi*, *Fungal Genet Newslett*, 1986, 33(1), 32.

25 Hu Z, Zeng X, Wang A, Shi C and Duan D, An efficient method for DNA isolation from red algae, *J Appl Phycol*, 2004, 16(3), 161-166.

26 Fulton T M, Chunwongse J and Tanksley S D, Microprep protocol for extraction of DNA from tomato and other herbaceous plants, *Plant Mol Biol Rep*, 1995, 13(3), 207-209.

27 Nauer F, Cassano V and Oliveira M C, Description of Hypnea pseudomusciformis sp. nov., a new species based on molecular and morphological analyses, in the context of the H. musciformis complex (*Gigartinales, Rhodophyta)*, *J Appl Phycol*, 2015, 27(6), 2405-2417.

28 Bono A, Anisuzzaman S M and Ding O W, Effect of process conditions on the gel viscosity and gel strength of semi-refined carrageenan (SRC) produced from seaweed (*Kappaphycus alvarezii*), *J King Saud Univ-Eng Sci*, 2014, 26(1), 3-9.

29 Vairappan C S, Razalie R, Elias U M and Ramachandram T, Effects of improved post-harvest handling on the chemical constituents and quality of carrageenan in red alga, *Kappaphycus alvarezii Doty*, *J Appl Phycol*, 2014, 26(2), 909-916.

30 Frankowski J, Lorito M, Scala F, Schmid R, Berg G, *et al.*, Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48, *Arch Microbiol*, 2001, 176(6), 421-426.

31 Gordon A L and Jonas J J, Method of extracting carrageenan in the presence of hydrogen peroxide, *U S Pat 3,236,833*, 1966.

32 Wattier R A, Prodöhl P A and Maggs C A, DNA isolation protocol for red seaweed (*Rhodophyta*), *Plant Mol Biol Rep*, 2000, 18(3), 275-281.

33 Freshwater D W, Tudor K, O'shaughnessy K and Wysor B, DNA barcoding in the red algal order Gelidiales: Comparison of COI with rbcL and verification of the" barcoding gap", *Cryptogam Algol*, 2010, 31(4), 435.

34 Marcotte M, Hoshahili A R T and Ramaswamy H S, Rheological properties of selected hydrocolloids as a function of concentration and temperature, *Food Res Int*, 2001, 34(8), 695-703.

35 Garrec D A and Norton I T, Kappa carrageenan fluid gel material properties. Part 2: Tribology, *Food Hydrocoll*, 2013, 33(1), 160-167.

36 Webber V, Carvalho S M D, Ogliari P J, Hayashi L and Barreto P L M, Optimization of the extraction of carrageenan from *Kappaphycus alvarezii* using response surface methodology, *Food Sci Technol*, 2012, 32(4), 812-818.

37 Volery P, Besson R and Schaffer-Lequart C, Characterization of commercial carrageenans by Fourier transform infrared spectroscopy using single-reflection attenuated total reflection, *J Agric Food Chem*, 2004, 52(25), 7457-7463.

38 Pereira L, Sousa A, Coelho H, Amado A M and Ribeiro-Claro P J, Use of FTIR, FT-Raman and 13 C-NMR spectroscopy for identification of some seaweed phycocolloids, *Biomol Eng*, 2003, 20(4), 223-228.

39 Tojo E and Prado J, A simple 1H NMR method for the quantification of carrageenans in blends, *Carbohydr Polym*, 2003, 53(3), 325-329