Carbohydrate Polymers 92 (2013) 1555–1560

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Natural porous agar materials from macroalgae

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ARTICLE INFO

Article history:
Received 11 September 2012
Received in revised form 30 October 2012
Accepted 1 November 2012
Available online 10 November 2012

In memoriam of Prof. Juan M. Campelo and Dr Paquale Trotta, mentors, great inspiration and friends, who passed away in October and November 2012, respectively.

Keywords:
Gracilaria
Microwave extraction
Porous materials

ABSTRACT

Porous agar materials have been prepared from marine macroalgae species using a simple microwave-assisted extraction/drying methodology, providing a new family of polysaccharide derived porous solids. The microwave-assisted extraction allows a more efficient and less time-consuming extraction of the polysaccharide compared to conventional extraction protocols based on conventional heating. DRIFT and ^{13}C NMR results indicated that the internal agar structure (based on D-galactose and 3,6-anhydro-L-galactose linked units) was preserved after the extraction methodology, which opens a wide range of future possibilities and applications for this new family of porous polysaccharides. The extracted agar materials, which have already applications per se due to their high purities, could be subsequently transformed into a novel family of attractive mesoporous agar materials that could be used as natural templates for the production of nanocrystals of metal oxides.

1. Introduction

Nature inspires scientists to devise smart and innovative approaches that can mimic its miniaturisation and efficiency in the production of energy, biometabolites and materials. An advanced degree of innovation in research can therefore be introduced by designing processes with natural resources from a fundamental and rational understanding. Algae are sunlight driven factories able to photosynthetically convert atmospheric carbon dioxide into a plethora of metabolites and chemicals including methane, hydrogen, polysaccharides and/or lipids (Luque, 2010). The production of such compounds, especially lipids and valuable chemicals, has been mostly focused to date on microalgae (Wijffels & Barbosa, 2010), while macroalgal species have been comparably less investigated. Nevertheless, there are interesting research avenues to explore in the extraction of relevant metabolites and/or polysaccharides from macroalgae (Cardozo et al., 2007; van Ginneken, Helsper, de Visser, van Keulen, & Brandenburg, 2011).

The motivation of this work was derived from an important and widely spread environmental problem present in many lagoons and natural water sources, which is related to increasing quantities of nutrients (e.g. NO_3^-, NH_4^+, PO_4^{3-}) found in aqueous reserves in recent years. These are known to be derived from the dumping of effluents from nearby anthropic activities (agriculture and fish-farming), which is the particular case of the Lesina Lagoon in Italy. In this particular case, locals from the marina developed a growing macroalgae (*Gracilaria* (*ex*) *verrucosa* now *Gracilaria gracilis*) approach as bioremediation solution for the existing eutrophication problem which initially led to highly promising results in removal of N and P (Francavilla, 2008). Interestingly, the abundant biomass growing in excess in the lagoon has in principle a remarkable potential for further exploitation as interesting compounds can potentially be extracted from it.

Gracilaria species are in fact one of the most important natural source of agar polysaccharides (Bourgougnon & Stiger-Pouvreau, 2012). Agar is a biopolymer built on a disaccharide-repeating unit of 3-linked β-D-galactose (G) and 4 linked 3,6-anhydro-α-L-galactose (AG) residues, with possible occurrence of sulphate, methoxyl, and/or pyruvate substituents at various positions in the polysaccharide chain (Scheme 1) (Rees, 1970). This biopolymer has been extensively used in both food and pharmaceutical industries as gelling and stabilising agent. It exhibits also many beneficial biological activities including anticoagulant, antiviral, antioxidative, anticancer and

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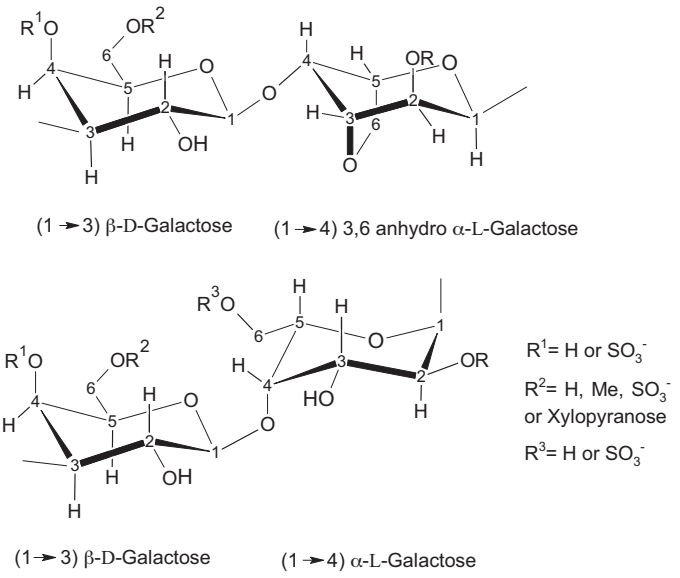
http://dx.doi.org/10.1016/j.carbpol.2012.11.005



Scheme 1. Structural motifs of agar polysaccharides showing carbon numbering (C_1–C_6).

inmunomodulating activities (Wijesekara, Pangestuti, & Kim, 2011). Furthermore, agar-based aerogels obtained from wet gels by using a suitable drying technology (e.g. supercritical drying process) have been proposed as promising biodegradable carriers for drug delivery systems (García-González, Alnaief, & Smirnova, 2011).

Following the recent endeavours of the group in the valorisation of biomass and residues to valuable products, materials and biofuels, we report herein an efficient methodology for the microwave-assisted aqueous extraction of agar from *G. gracilis* (a macroalgal species growing in the Lesina lagoon in Apulia region, Italy) which includes the additional possibility of the development of a novel family of porous agar materials with useful applications in food feedstuffs, catalysts supports and pharma/cosmeceutical industries.

In the course of our studies, we also found out there are various relevant compounds that could be extracted from such macroalgae species (e.g. lipids and phycobilinproteins) which are currently the subject of further investigations in our laboratories.

In our preliminary experiments, we focused on maximising the extraction of agar from macroalgae using a microwave assisted methodology as compared to conventional heating. Previous expertise from the group has demonstrated that microwaves can effectively extract and/or promote different compounds and chemistries as compared to conventional heating (Balu, Campelo, Luque, & Romero, 2010; Balu et al., 2008). Particularly related to the extraction of useful compounds from macroalgae, previous literature reports are also in good agreement with our proposed approach (Rodriguez-Jasso, Mussatto, Pastrana, Aguilar, & Teixeira, 2011). Temperatures and reaction conditions specified in Section 2 were selected based on previous work by Sousa, Alves, Morais, Delerue-Matos, and Gonçalves (2010) who performed microwave-assisted agar extraction from a different Gracilaria specie: *Gracilaria vermiculophylla*. There is to our knowledge no scientific papers regarding agar extraction from *G. gracilis* by using microwave. Moreover, in our opinion microwave-assisted polysaccharide extraction from seaweeds is a research branch about totally unexplored taking into account that only six papers were published in this topic (Navarro & Stortz, 2005; Rodriguez-Jasso et al., 2010, 2011; Sousa et al., 2010, 2012; Uy, Easteal, Farid, Keam, & Conner, 2005).

2. Materials and methods

2.1. Biomass sampling

G. gracilis was collected from the western area of the Lesina lagoon, where a stable assemblage of this seaweed was found (41.866470°N, 15.363350°E). About 1 kg of wet biomass was sampled in July 2011. Algal biomass was washed with distilled water and their epiphytes removed. The fresh seaweed was placed in a freezer (−20 °C) immediately after collection. The cleaned seaweed was freeze-dried at −110 °C for 3 days and then ground to fine powder and stored in airtight containers at −20 °C.

2.2. Agar extraction and analysis

2.2.1. Conventional extraction of native (NA) and alkali-treated agar (ATA)

Native agar (NA) extraction was performed using the method proposed by Marinho-Soriano and Bourret (2003) with slight modifications. Three samples of ground freeze dried biomass (1 g) were immersed in 40 mL of deionized water (pH 6.5) and heated in an autoclave for 1.5 h at 120 °C (optimum temperature for extraction). The mixture was then homogenized in a grinder mixture and filtered trough paper filter under vacuum at 70 °C. The filtrate was held at room temperature for gel formation, and the gelled material was then frozen in the freezer at −15 °C overnight to obtain the native extracted agar. The frozen gel was thawed, washed with deionized water and dried for 24 h at 60 °C. The agar yield was then calculated as the percentage of dry matter. The extraction was repeated three times.

Alkali-treated agar (ATA) was extracted under identical conditions, aiming to compare results between both batches, but the freeze-dried biomass was previously treated with an alkaline water solution (100 mL, 6% NaOH) for 2 h at 60 °C and then washed repeatedly with deionized water (at room temperature) until pH 7 is reached. The reason why such alkali-treatment was performed is related to the removal of some SO_4^{2-} moieties which is related to an improved quality of the gel formed as well as of the final agar (Rodriguez-Jasso et al., 2011; Sousa et al., 2010).

2.2.2. Microwave assisted extraction of native & alkali-treated agar

Microwave-assisted extractions (MAE) were performed in a Ethos 1 (Microwave Accelerated Reaction System for Extraction and Digestion, Milestone, USA) configured with a 24 position carousel. One-gram of dried sample (not treated or alkali-treated) was transferred to a teflon extraction vessel containing 40 mL distilled water; then the vessel was closed upon introduction of a fibre optic probe to measure the temperature in the systems. The operational parameters employed in the MAE apparatus were the following: magnetron power 100%, ramp temperature time, 10 min. During operation, both temperature and pressure were monitored in a single vessel (control vessel). Three temperature programmes were found to be the optimum for extractions: 140 °C for 15 min; 110 °C for 20 min; and 100 °C for 10 min. After the extraction, the vessels were opened still warm because of the agar gelling properties. The mixture was filtered using paper filter under vacuum at 70 °C. Agar gel was then worked as above described. The extractions were repeated three times.

2.3. Characterization of porous agar materials

Materials were characterized using nitrogen physisorption, scanning electron microscopy (SEM), X-ray photoelectron

spectroscopy (XPS), diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS), and nuclear magnetic resonance spectroscopy (CP-MAS ^{13}C NMR).

Nitrogen adsorption measurements were carried out at 77 K using an ASAP 2010 volumetric adsorption analyzer from Micromeritics. The samples were outgassed for 2 h at 100 °C under vacuum ($p < 10^{-2}$ Pa) and subsequently analysed. The linear part of the BET equation (relative pressure between 0.05 and 0.30) was used for the determination of the specific surface area. D_{BJH} = mean pore size diameter; V_{BJH} = Pore volumes obtained from porosimetry data.

Scanning electron micrographs (SEM) and elemental composition of the calcined samples were obtained using a JEOL JSM-6300 Scanning Microscope with energy dispersive X-ray analysis (EDX) at 20 kV. Samples were coated with Au/Pd on a high resolution sputtering SC7640 instrument at a sputtering rate of 1.5 kV per minute, up to 7 nm thickness.

XPS measurements were performed in a ultra high vacuum (UHV) multipurpose surface analysis system (SpecsTM model, Germany) operating at pressures $<10^{-10}$ mbar using a conventional X-ray source (XR-50, Specs, Mg Kα, 1253.6 eV) in a "*stop-and-go*" mode to reduce potential damage due to sample irradiation. The survey and detailed Fe and Cu high-resolution spectra (pass energy 25 and 10 eV, step size 1 and 0.1 eV, respectively) were recorded at room temperature with a Phoibos 150-MCD energy analyser. Powdered samples were deposited on a sample holder using double-sided adhesive tape and subsequently evacuated under vacuum ($<10^{-6}$ Torr) overnight. Eventually, the sample holder containing the degassed sample was transferred to the analysis chamber for XPS studies. Binding energies were referenced to the C1s line at 284.6 eV from adventitious carbon. Deconvolution curves for the XPS spectra were obtained using software supplied by the spectrometer manufacturer.

DRIFTS experiments were conducted in a Perkin Elmer Spectrum 100 Infrared Spectrometer equipped with an attenuated total reflectance (ATR) module. Attenuated total reflectance infrared (FTIR-ATR) spectra of dried and ground algal material and polysaccharide standards were recorded using the Perkin Elmer® Spectrum™ 400 FT-IR/NIR spectrometer (Perkin Elmer Inc., Tres Cantos, Madrid) in mid-IR mode, equipped with a Universal ATR (attenuated total reflectance) sampling device containing diamond/ZnSe crystal. Besides, for powdered samples an extra accessory plate with a conic awl was used which required only a few milligrams without any previous sample preparation. The pressure applied to squeeze the powdered sample towards the diamond was approximately 148 ± 1 N. Spectra were acquired and then processed with the Spectrum software version 6.3.2. The spectra were scanned at room temperature in absorbance mode over the wave number range of 4000–650 cm^{-1}, with a scan speed of 0.20 cm/s, and 30 accumulations at a resolution of 4 cm^{-1}. A background spectrum of air was scanned under the same instrumental conditions before each series of measurements.

Cross polarization magic angle spinning (CP-MAS) ^{13}C NMR spectroscopy has been used in this study for characterizing agar polymer. ^{13}C NMR spectroscopy was carried out on a Bruker CXP 300 spectrometer. The solid state spectroscopy used magic angle spinning of 4 kHz and cross-polarization techniques employing contact and repetition times of 2 ms and 5 s, respectively. Samples were used directly and were run at ambient temperature. Spectra involved collection of 400–2000 scans and 11,000 scans for the dregs. Chemical shifts were referenced to adamantane run as an initial sample and are quoted relative to tetramethylsilane (TMS).

Table 1
Yields (% d.w.) of native (NA) and alkali-treated agar (ATA) extracted from *Gracilaria* biomass using conventional and microwave-assisted extraction (MAE) methodologies at different temperatures.

Conventional extraction		Microwave assisted extraction	
Sample	Agar yield (%)	Sample	Agar yield (%)
		NA-100 °C	25.7
NA-120 °C	21.3	NA-110 °C	25.4
		NA-140 °C	<1
		ATA-100 °C	2.1
ATA-120 °C	14.2	ATA-110 °C	6.6
		ATA-140 °C	16.0

100, 110 and 140 °C refer to temperatures used in MAE.

3. Results and discussion

3.1. Agar extraction

Results of the microwave assisted methodology employed for the extraction of agar from *G. gracilis*, as compared to those obtained using conventional heating, have been included in Table 1. Data show that MAE methodology was able to provide improved yields of agar even at a lower temperature (100 °C) compared to a conventional extraction under heating (25.7 vs 21.3%). Most importantly, a more efficient extraction could be achieved at remarkably reduced times of extraction (10 min) as compared to 1.5 h required with conventional heating extraction methodologies.

In the case of alkali-treated agar, a lower agar yield was obtained upon extraction under microwave at 110 °C, which in any case could be improved operating at 140 °C (Table 1). Interestingly, the lower agar yields obtained at temperatures above 120 °C compromised an effective agar extraction for algae as pyrolysis processes started to compete against biopolymer extraction (as seen by a significant evolution of gaseous products in the system). These results were in good agreement with previously reported work by Budarin et al. (2011). The opposite trends observed in agar yields extracted for alkali-treated polysaccharides pointed to a significant change in the structure of the biopolymer upon NaOH treatment, which will be shown in subsequent experiments (e.g. SEM, DRIFTS data). This finding suggested that efficiency of microwave-assisted extraction was not only related to the fact that microwave radiation was much more efficiently directed and strongly absorbed by water than infrared (in case of conventional heating), but it was related also to the molecular structure of polysaccharide. In our case, the decrease in sulphate substituents and the increase of 3,6-anhydro groups by means of alkali treatment seemed to cause a different absorbance of microwave radiation by agar polysaccharide. Therefore, differences in molecular structure of polysaccharide seemed to affect extrusion of agar polymer from algae cells by means of microwave irradiation. Optimum values were in any case found

Table 2
Porosity analysis of non-treated and alkali-treated agar extracted from *Gracilaria* biomass using MAE.

Sample	S_{BET} ($m^2\ g^{-1}$)	D_{BJH} (nm)	V_{BJH} (ml/g)
NA-100 °C	5	Macroporous	0.02
NA-100 °C_SOLEX	12	>20, macroporous	0.03
ATA-100 °C	41	Macroporous	0.17
ATA-100 °C_SOLEX	23	Macroporous	0.13
ATA-110 °C_SOLEX	15	Macroporous	0.11
ATA-110 °C	25	Macroporous	0.12
ATA-140 °C	26	Macroporous	0.11
ATA-140 °C_SOLEX	10	Macroporous	0.03

NA, native agar; ATA, alkali-treated agar; SOLEX, solvent extracted material (using ethanol as solvent). V_{BJH}, pore volume. Value in brackets represent the main contribution to pore diameter.

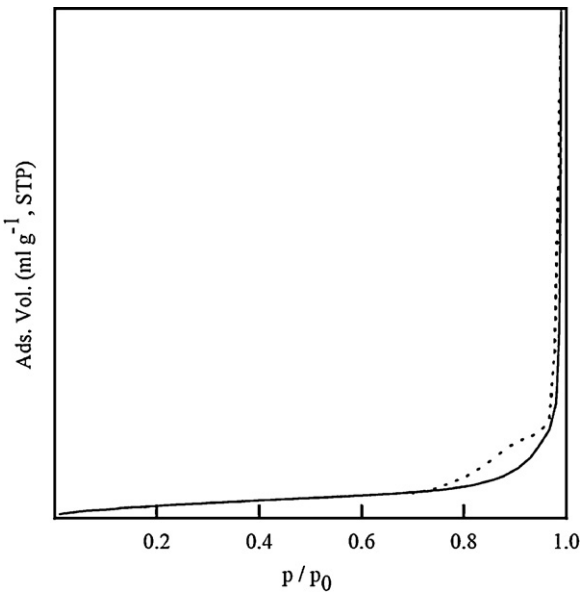


Fig. 1. Isotherm profile of material ATA-140 °C_SOLEX (surface area 10 $m^2\ g^{-1}$; pore diameter > 20 nm; pore volume 0.15 $mL\ g^{-1}$).

for non-treated agars microwave-extracted in water at 100 °C for 10 min.

3.2. Agar analysis and characterization

Upon optimisation of agar yields in the microwave-assisted extraction process, the preparation of porous agars derived from such extracted polysaccharides was subsequently investigated. In this regard, agar samples were either directly dried from the gel formed upon extraction or solvent-exchanged with lower surface-tension solvents than water (e.g. ethanol, acetone) to yield light white powders upon drying.

Samples were then characterized to investigate the textural properties of the prepared materials. Table 2 and Fig. 1 summarise some of the key textural properties of the porous agars as well as a typical isotherm.

The optimum non-treated agar obtained at 100 °C exhibited a borderline porosity (5 $m^2\ g^{-1}$, almost non porous), with a low pore volume (0.02 $mL\ g^{-1}$). Comparably, the alkali-treated counterparts (extracted also at 100 °C) showed a remarkable increase in surface area (12–41 $m^2\ g^{-1}$) with particularly enhanced pore volumes (0.1–0.17 $m^2\ g^{-1}$) and a predominantly macroporous character (Table 2 and Fig. 1). Higher temperatures for both non-treated and alkali-treated agars did not seem to have any

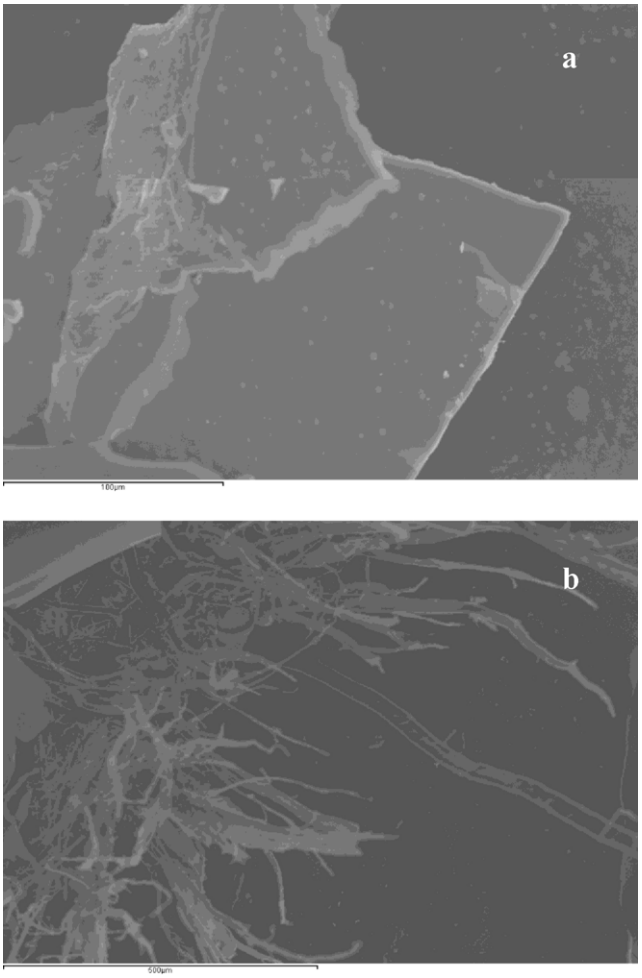


Fig. 2. SEM micrographs of non-treated (a) and alkali-treated (b) mesoporous agar materials.

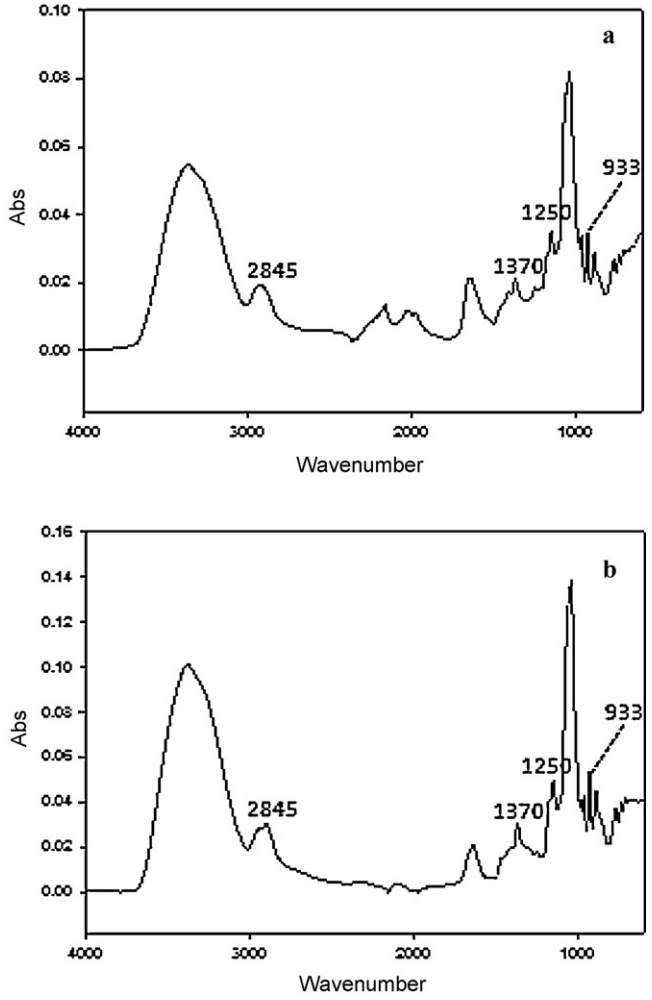


Fig. 3. Attenuated total reflectance infrared (FTIR-ATR) spectra of non-treated (a) and alkali-treated (b) agar extracted from *Gracilaria gracilis*.

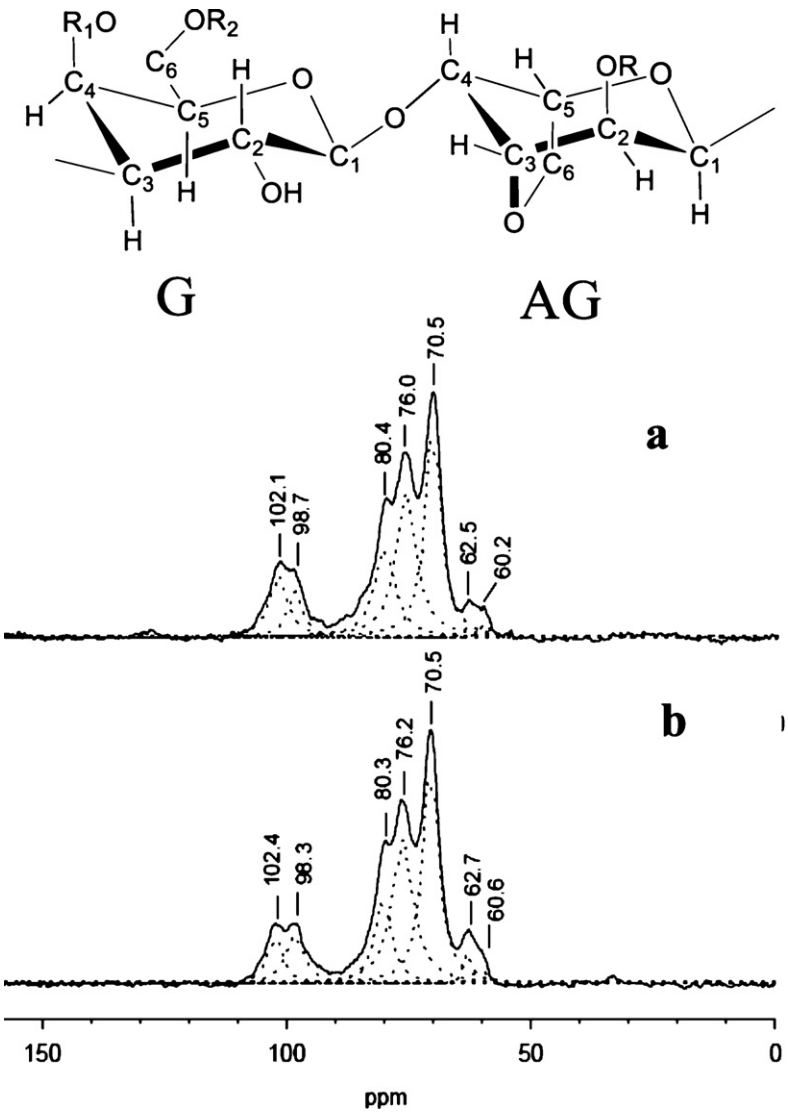


Fig. 4. CP-MAS ^{13}C NMR spectra of native agar (a) extracted at 100 °C and alkali-treated agar (b) extracted at 140 °C from *Gracilaria*. Top structure depicts the various carbons (C_1–C_6 from G and AG) associated to the different NMR peaks.

remarkable effects on the porosity of the materials, following similar trends to those observed for NA and ATA-100 °C materials.

Previous experience from our group in the development of novel families of biomass-derived mesoporous materials from biomass pointed out a solvent-exchange step (using low surface tension solvents than water) could in principle improve the porous properties of the final materials upon drying (Budarin et al., 2006). However, as indicated in Table 2, the introduction of the solvent-exchange step did not have any influence in the textural properties of the materials apart from a significant contribution in the formation of large mesopores (>20 nm).

The differences observed between non-treated and alkali treated materials were also noticeable in SEM micrographs of both types of materials (Fig. 2). These images illustrate the structural changes that take place in the polysaccharide upon alkali-treatment. Non-treated materials exhibited a characteristic squared shaped morphology (Fig. 2a) compared to fibre like shapes observed for alkali-treated materials (Fig. 2b). This morphology change could explain the drastic increase in porosity and textural properties observed for alkali treated samples as compared to non-treated agar (Table 2). Comparably, the solvent-exchange step introduced in the process did not influence the morphology of the final materials (data not shown).

DRIFTS of both types of materials have also been presented in Fig. 3. The presence of shoulder at 2845 cm^{-1} is indicative of the presence of C—C saturated bonds from the polysaccharide backbone (Scheme 1). Bands at 1370 and 1250 cm^{-1} are characteristic of sulphate substituents in the agar structure, while the band at 933 cm^{-1} indicated the presence of 3,6 anhydrogalactose (AG). The $A_{933}/A_{1.250}$ ratio of native agar (1.82 ± 0.25, mean ± s.d.) is significantly lower than that of alkali-treated agar (2.66 ± 0.35), which is in good agreement with a higher relative concentration of sulfur-free AG upon alkali treatment (Villanueva, Sousa, Gonçalves, Nilsson, & Hilliou, 2010). The region between 900 and 700 cm^{-1} gives information about the position of sulphate group in the agar structure. In our samples we found some weak signals (shoulder) at 868 cm^{-1} indicative of the presence of sulphate group on C_6 of galactose. The signals at 850 cm^{-1} (weak shoulder) and 705 cm^{-1} (pick) indicated the presence of sulphate group on C_4 of galactose (3 linked D-galactose). The absence of signals at 905 and 805 cm^{-1} excluded the presence of sulphate group on C_2 of 3,6-anhydrogalactose, while the absence of signal at 830 cm^{-1} excluded the presence of sulphate group on C_2 of galactose (Scheme 1).

CP-MAS ^{13}C NMR spectrum of native agar extracted from *Gracilaria* biomass exhibited eight major peaks at 173.5, 102.1, 98.7 80.4, 76.0, 70.5, and 62.5 ppm and a peak shoulder at around 60 ppm (Fig. 4). According to previous work (Gordon-Mills, Tate, & Hounslow, 1990; Rochas, Lahaye, & Yaphe, 1986) signals at 102.1 and 98.8 ppm can be attributed to C_1 of G and C_1 of AG, respectively, thereby confirming the agarose structure of the extracted material. The signal at 80 ppm is associated to carbons C_3 of G and C_3 of AG, while the signal at 76 ppm is attributed to carbons C_4 of AG, C_5 of G and C_5 of AG (Fig. 4 and Scheme 1).

Carbons C_2 of G, C_2 of AG, C_6 of AG and C_4 of G gave resonance at 70.5 ppm and carbon C_6 of G at 62.7 ppm. The spectrum of native agar shows one minor peak at 60 ppm due to the methyl carbon on a 6-O-methylated galactose residue. XPS of the materials also confirmed the presence of the different carbon signals and a main oxygen component (with two minor O peaks, see ESI) as well as the presence of a range of impurities (K, Ca, N) as well as some S (from sulphate substituents) present in the materials. S content in samples was difficult to quantify by XPS due to its small content.

In any case, both DRIFTs and ^{13}C NMR data demonstrate that the agar structure was fully preserved upon MAE, which has important consequences in future applications of such materials in various fields including catalysis and separation technologies as well as their use as natural templates for nanocrystals preparation, currently under investigation in our groups.

4. Conclusions

We report a simple microwave-assisted methodology for the extraction of agar from *G. gracilis*. It allows a more efficient and less time-consuming extraction of agar compared to conventional extraction protocols. Interestingly, the extracted biopolymer could be subsequently transformed into a novel family of mesoporous agar materials with interesting textural properties which may find suitable applications as catalysts supports and templates for the preparation of metal oxides.

Acknowledgements

This work was supported by a research grant for the project "*Macroalghe della laguna di Lesina: potenziali sorgenti di prodotti naturali di uso farmaceutico e alimentare* (*MADLENA*)" funded by Fondazione Cassa di Risparmio di Puglia (Italy). R.L. gratefully acknowledges support from the Spanish MICINN via the concession of a RyC contract (ref. RYC 2009-04199) and funding under projects P10-FQM-6711 (Consejeria de Ciencia e Innovacion, Junta de Andalucia) and CTQ2010-18126 and CTQ2011 28954-C02-02 (MICINN). Dr. Juan Carlos Serrano-Ruiz is gratefully acknowledged for his support, contribution, comments and revisions during the preparation of this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.11.005.

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