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Efficient Extraction of Agarose from Red Algae Using Ionic Liquids

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Abstract

We explored the possibility of using ionic liquids (ILs) as medium for efficient extraction of agarose via dissolution of red algae under varying conditions of heating or microwave irradiation. As compared to conventional methods, a very high extraction yield of good quality agarose (as high as 39 wt%) could be achieved depending upon the nature of used IL and applied experimental conditions. Purity of extracted agarose was confirmed from various spectral and analytical techniques, such as ^{1}H and ^{13}C NMR, FTIR, circular dichroism (CD), gel permeation chromatography (GPC) and thermogravimetric analysis (TGA). The physicochemical properties, such as gelling or melting temperature, viscosity and gel strength of extracted agarose hydrogels have been measured and compared with the agarose obtained from similar source reported in the literature. ILs were recovered after the extraction of agarose and were reused for further extraction experiments. % Recycling and extraction ability of recycled ILs in different cycles have been measured. The developed extraction process of utilizing ILs as medium is easy, simple and highly efficient as compared to the conventional methods of agarose extraction from algae.

Keywords

Red Algae, Ionic Liquids, Agarose, Thermal or Microwave Heating, Recycling, Extraction Ability

1. Introduction

Utilization of natural biopolymers has attracted increasing attention because of the consumption and over-exploitation of non-renewable resources, such as coal, fossil fuel and oil. The development of green processing of

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biomass (the most abundant bio renewable material on earth) is urgent from the viewpoints of both sustainability and environmental protection. Polysaccharides are widely distributed in nature and have been regarded as structural materials and as suppliers of water and energy [1]. Polysaccharide chemistry is presently receiving renewed attention due to the increase of role of biopolymers in various biochemical, biomedical and industrial applications. Due to fibril and rigid structure, the efficient dissolution and extraction of polysaccharides using organic solvents involve significant amounts of energy. Also, because of toxic, volatile, flammable and corrosive nature of organic solvents such processes are neither sustainable nor environmental friendly [2]. Therefore, the design of safe and environmentally benign extraction processes has an increasingly important role in the development of clean manufacturing processes and in the remediation of sites contaminated by an older generation of manufacturing technologies [3]. Utilization of comparatively greener solvents with high solvating ability towards biomass could be an alternative for the development of novel and efficient extraction processes of biopolymers from the plant species.

Ionic liquids (ILs) are compounds that contain only ions and have melting point below 100˚C [4]. ILs have been recognized as "greener" solvents compared to organic solvents mainly due to their negligible vapor pressure, high thermal and chemical stability, and their capacity for dissolving numerous polar and non-polar compounds. Due to high solvating ability, ILs are being used in different separation and extraction processes. After Swastloski [5] reporting the ILs as potential cellulose solvents in 2002, many research groups explored the utilization of these materials for dissolution and regeneration of biomass and some good reviews have been recently appeared [6]-[8]. In recent years the extraction of various chemicals or biopolymers from biomass has also been carried using ILs [9]-[30]. For example, the valuable chemicals, such as free *fatty acid* from soybean oil, *shikimic acid* from *Ginkgo biloba* leaves or star anise seeds, *lactones* from *Ligusticum chuanxiong*, *tannins* from plant materials and caffeine from bioresources guaraná seeds have been extracted employing different types of ILs [9]-[14]. As far as extraction of biopolymers from biomass utilizing ILs is concerned, various biomass fractions have been obtained by direct dissolution of wood biomass in ILs [15]-[19]. Lignin, which is another valuable biomass fraction, is also extracted from different wood sources utilizing ILs [20]-[25]. Besides the extraction of cellulose, hemicellulose or lignin from wood sources, there are other biopolymers, such as keratin [26], pectin [27], chitin [28] [29] and suberin [30] which have been successfully isolated from the bioresources using ILs.

Marine microalgae is another category of biomass that contains many useful and valuable chemicals, like lipid, oil, polysaccharides, fuels and chemical feedstock, etc. in abundance, but its poor solubility in conventional solvents restricts efficient extraction of these valuable chemicals. There are few reports where ILs have been used as extraction medium for the recovery of valuable chemicals from algae. Teixeira *et al*. [31] reported the extraction of fuels and other valuable chemicals from microalgae using ILs, while Kim *et al*. [32] investigated the extraction of lipids from algal biomass using ILs and methanol mixture. Ohno's research group has reported the extraction of polysaccharides from bran using phosphonate or phosphinate ILs in very short time at mild temperature [33]. Recently, the same research group reported dissolution of wet and saliferous marine microalgae (WSM) using polar ionic liquids without any heating [34].

Herein, we reported the extraction of agarose from red microalgae (Rhodophyta), mainly *Gracilaria dura* obtained from west coast of India using different ILs under varying conditions of heating and examined the effect of nature of different cations or anions of ILs on extraction efficiency. Extracted agarose has been characterized by means of spectroscopic and analytical methods. Physico-chemical properties of extracted agarose and its hydrogels have been measured and compared with agarose extracted earlier from the similar sources using conventional methods [35]. ILs were efficiently recovered and recycled in various experiments.

2. Experimental

2.1. Materials

ILs: 1-ethyl-3-methylimidazolium acetate, [Emim] [OAc], choline acetate, [Ch] [OAc] and 1-ethyl-3-methyl imidazolium diethyl phosphate, [Emim] [Dep] were purchased from Sigma Aldrich having higher than 99 wt% purity. Prior to use ILs were dried and stored in glove box. The red algae *Gracilaria dura* from family Gracilariaceae, Rhodophyta were collected from west Indian coastal regions. Algae were dried and crushed before use in experiments. Molecular structures of the repeating unit of agarose monomer and the constituent ions of the ILs used in this study are shown in **Figure 1**.

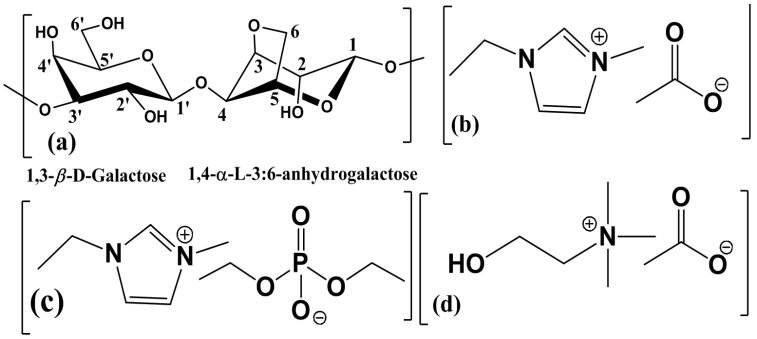


Figure 1. (a) Molecular structures of repeating unit of agarose monomer and the constituent ions of the ILs; (b) 1-Ethyl-3-methylimidazolium acetate, [Emim] [OAc]; (c) 1-Ethyl-3-methylimidazolium diethyl phosphate, [Emim] [Dep]; and (d) Choline acetate, [Ch] [OAc].

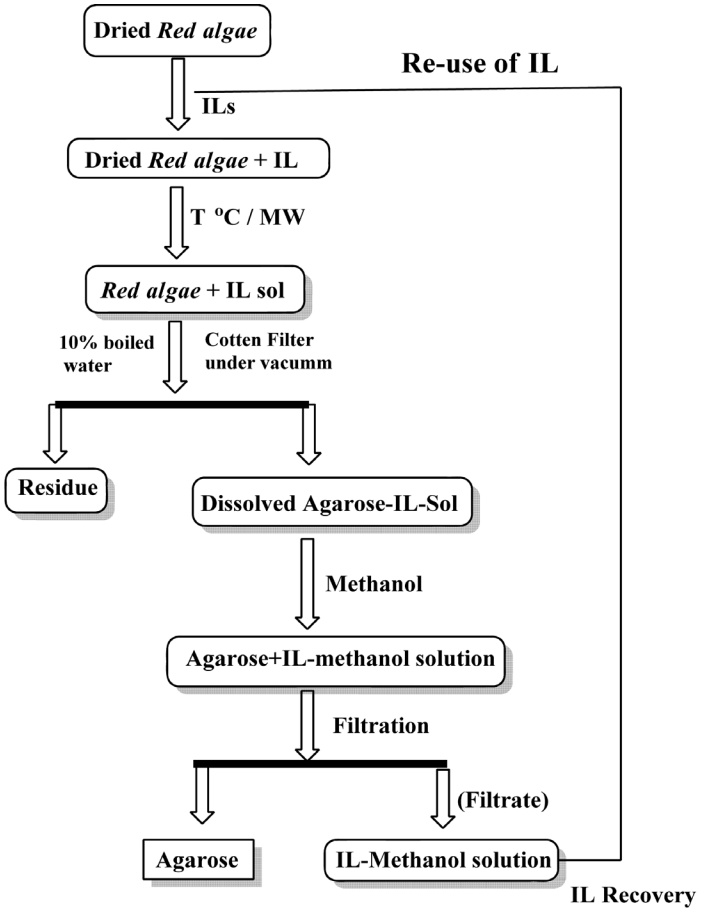
2.2. Methods

2.2.1. Extraction of Agarose from Algae Using ILs

Extraction of agarose from red algae using ILs was carried out according to the representative steps shown in **Scheme 1**. Extraction process was performed in 10 ml beakers with controlled temperature conditions such as temperature or micro wave (MW) heating coupled with normal heating. Typically, 0.5 g of dried and powdered algae was treated with 10.0 g of preheated ILs in beakers. The mixture containing (algae + ILs) was stirred at 80/100˚C for 2 h or MW treatment for 2 min at 3 s pulse was given prior to the normal heating. In order to reduce the viscosity of IL-algae solution, 10% boiled water was added. Hot water treated mixture was stirred until free flow homogenization, and was filtered using thin cotton under vacuum. Filtrate was treated with methanol to precipitate agarose dissolved in ILs. Several methanol washings were given to ensure the complete removal of ILs from extracted agarose. Agarose extracted from different experiments were dried under the vacuum and % yield was calculated on the basis of used dried algae weight. After the completion of agarose extraction, ILs were recovered from the methanol-water (filtrate) solutions using rotary evaporator and dried completely under reduced pressure and finally stored into desiccator for further use. Dried recovered ILs were characterized for their purity via analytical techniques (^{1}H and ^{13}C NMR) and were recycled in different extraction experiments. Extraction efficiency of recycled ILs up to four cycles was checked.

2.2.2. Characterization

The ^{1}HN MR and ^{13}C-NMR spectra of native and extracted agarose were recorded on a Bruker Advance-II 200 (Ultra Shield) Spectrometer, Switzerland, at 200 MHz. FTIR spectra of extracted agarose were recorded at room temperature using NICOLET 6700 FTIR spectrometer. Circular dichroism (CD) spectra of agarose solutions (0.05 wt%) in a wavelength range of 180 to 240 nm were recorded on a Jasco J-815 CD spectrometer. Experiments were carried out in a 1 cm path length cuvette at 25˚C, and were expressed as the average of five scans. The response time and the bandwidth were 2 s and 0.2 nm respectively. Samples for recording the spectra were taken in a quartz cuvette which was immediately sealed after sampling to avoid evaporation. The desired temperature was achieved with an inbuilt peltier device. Thermogravimetric analyses were performed on a TGA/SDTA851 Mettler Toledo under nitrogen atmosphere from 30˚C to 600˚C with a heating rate of 10˚C·min^{-1}. Molecular weight of the extracted agarose was determined through high temperature gel permeation chromatography (HT-GPC) using Waters 2695 separation module equipped with 2414 RI detector and having ultrahydrajel 500 and 120 columns in series. Columns were eluted with 0.1 M aqueous $NaNO_3$ at a flow rate of 0.5 ml/min. Calibration was performed using Dextran standard ranging from 401,000 to 4400 peak molecular weight. The concentration of agarose solutions in water was 0.02 wt%. Melting and gelling temperatures of agarose hydrogels (1.0% w/v) were determined following the method described by Craigie & Leigh [36]. For measurement of the gelling temperature, hot solution of agarose in water was allowed to cool at room temperature while immersing a thermometer in the agarose sol and holding the test tube at an inclined position to have a greater surface area on the top. Gel strength (g·cm^{-2}) was measured using a Nikkansui type gel tester (Kiya Sei Sakusho



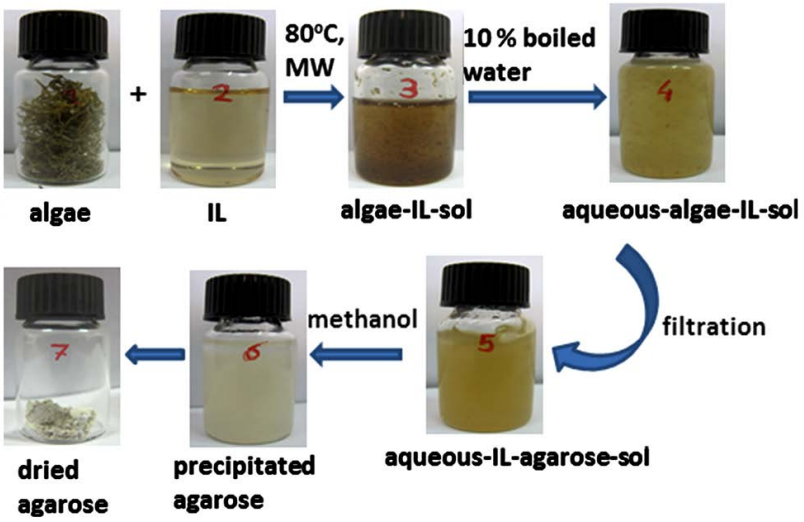
Scheme 1. Schematic of process for the extraction of agarose from red algae *Gracilaria dura* using ionic liquids (ILs).

Ltd., Tokyo, Japan). Measurements were performed using a solid cylindrical plunger 1.127 cm in diameter. Optical rotation was measured in 0.1 wt% agarose solutions at 30˚C on a Rudolph Digipol 781 Polarimeter (Rudolph Instruments Inc., NJ, USA). Apparent viscosity of 1 wt% agarose solutions was measured on Brookfield viscometer (DV-II + Pro) using SC4-18 spindle at 60 rpm and 80˚C. Sulphate content analysis was performed on Perkin-Elmer ICP-OES optima 2000DV machine following the method described by Wolnik [37].

3. Results and Discussion

3.1. Agarose Extraction

Imidazolium based ILs having high basicity anions such as $[OAc]^-$ or $[Dep]^-$ have been found good dissolution ability for biomass [16] [34]. Such ILs are suitable for biomass processing because having low melting point and viscosity. Besides these, the bio-based IL containing choline as cation counter ion having same anions analogue have also shown the biomass dissolution ability [38]. Therefore, we have chosen three different ILs having high hydrogen bond basicity *i.e.* [Emim] [OAc], [Ch] [OAc] and [Emim] [Dep] for extraction of agarose from algae (*Gracilaria dura*) (molecular structure shown in **Figure 1**) via dissolution process and investigated the effect of nature of cations and anions of ILs on extraction ability towards agarose. Detailed procedural steps carried out for extraction of agarose are summarized in **Scheme 1** and **Scheme 2**. Various parameters such as increase in temperature or microwave heating before normal cooking have been found to assist in rapid and higher dissolution of algae and efficient agarose extraction. **Table 1** shows the % yield (calculated on the basis of used dried



Scheme 2. Schematic representation of different steps carried out for efficient extraction of agarose using ionic liquids (ILs).

Table 1. ILs, treatment condition, % yield, gelling temperature (T_{gel}), melting temperature (T_m), gel strength, apparent viscosity, ash and sulfate content for and standard agarose.

ILs	Treatment condition	% Yield	T_{gel} (˚C)	T_m (˚C)	Gel strength g·cm^{-2} (30˚C)	Optical rotation $[\alpha]_{580}$	Ash (%)	Sulpahte content (%)
[Emim] [OAc]	80˚C; 2 h	28.5						
	MW; 80˚C; 2 h	39	29	80	600	−22	6.4	1.95
	100˚C; 2 h	35						
[Ch] [OAc]	80˚C; 2 h	2.5						
	MW; 80˚C; 2 h	12.5	28	77	580	−23	7.3	2.70
	100˚C; 2 h	7.2						
[Emim] [Dep]	80˚C; 2 h	7.6						
	MW; 80˚C; 2 h	18.5	29	78	570	−24	7.5	2.75
	100˚C; 2 h	17						

algae weight) of extracted agarose under applied different conditions of heating or combination of microwave heating followed by normal heating. Nearly 2.5 and 7.6 wt% of agarose could be extracted using [Ch] [OAc] and [Emim] [Dep] respectively by simple heating at 80˚C for 2 h, whereas the yield was dramatically higher (28.5 wt%) for [Emim] [OAc] under the similar conditions. It was also observed that by increasing reaction temperature from 80˚C to 100˚C, % yield for agarose extraction increased from 2.5% to 7.5% in [Ch] [OAc], 7.6% to 17% in [Emim] [Dep] and 28.5% to 35% in [Emim] [OAc]. Application of microwave irradiation for 2 min at 5 s pulse prior to the conventional heating at 80˚C increased % yield of agarose in all the ILs. Under coupled conditions of microwave and normal heating, the agarose yield enhanced from 2.5% to 12.5% in [Ch] [OAc], 7.6% to 18.5% in [Emim] [Dep] and 28.5% to 39% in [Emim] [OAc]. The extraction efficiency of used ILs followed the order: [Emim] [OAc] > [Emim] [Dep] > [Ch] [OAc] suggesting that the extraction ability depends on both viscosity and nature of cation/anion of ILs. It is well known that strong hydrogen bonding basicity is effective weakening the hydrogen bonding network of polymer chain, which conclude that higher the β value higher is the dissolution and extraction efficiency [33] [39]. Higher extraction efficiency of imidazolium based ILs containing OAc and Dep anions is due to their high solvating ability, hydrogen bond basicity (β) and low viscosity, while the low extraction in [Ch] [OAc] may be due to the nature of cation of the IL.

3.2. Physicochemical Property of Extracted Agarose and Hydrogels

Structural confirmation and purity of extracted agarose was confirmed by analytical testing as well as by comparison of physicochemical properties with a reference agarose [35]. ^{1}H and ^{13}C NMR spectra of extracted and reference agarose performed in DMSO-d_6 solvent at 70˚C are compared in **Figure 2** and **Figure 3**. Reference agarose shows peaks in ^{1}H NMR at δ = C1'-5.21, C1-5.07 and for agarose skeleton (C2-C6) and (C2'-C6')-4.84-3.8 ppm, while ^{13}C NMR shows peaks at δ = C1'-102.85, C1-98.09, agarose skeleton (C2-C6) and (C2'-C5')-81.68-68.62, and C6'-61.29 ppm. ^{1}H NMR and ^{13}C NMR spectra of extracted agarose show the peaks at the values similar to that of reference agarose confirming its high purity. A comparison of chemical shift values (^{13}C NMR) of extracted agarose in a representative IL, [Emim] [OAc] with the literature is shown in **Table 2**.

FTIR spectra of the extracted and reference agarose are compared in **Figure 4**. The FTIR spectra show that extracted agarose match with original structure. Characteristic peaks of agarose from IR bands at 773, 894, and 932 cm^{-1} because of 3,6-anydro-β-galactose skeletal and 1158 and 1071 cm^{-1} corresponding to -C-O-C- and glycosidic linkage respectively are present in the extracted agarose [40]-[43]. Circular dichroism (CD) spectra are recorded for examination of conformations in extracted agarose and is compared with confirmations of reference agarose shown in **Figure 5**. CD spectrum of agarose indicate the overall secondary structure with a positive band centered at ~185 nm [44]. In solution, low intensity of band is the consequence of highly extended chain geometries. Analysis of CD spectra (**Figure 5**) reveals distinct conformational preferences in the chains of extracted agarose similar to that observed for reference agarose. Extracted material was also checked for thermal stability by performing thermo gravimetric analysis (TGA) and the profiles are shown in **Figure 6**. The decomposition of extracted and reference agarose is characterized by a narrow temperature range from 275˚C to 300˚C with same onset decomposition temperature (T_{dec}). No significant weight loss was observed in TGA curves of agarose extracted using [Emim] [OAc] and [Emim] [Dep] before the decomposition temperature suggesting that the extracted agarose had less moisture as compared to reference agarose, whereas in case of agarose extracted using [Ch] [OAc], the moisture content is nearly same to that of reference agarose. Molecular weight of agarose extracted using different ILs was determined from GPC. Molecular weight, polydispersity index and degree of

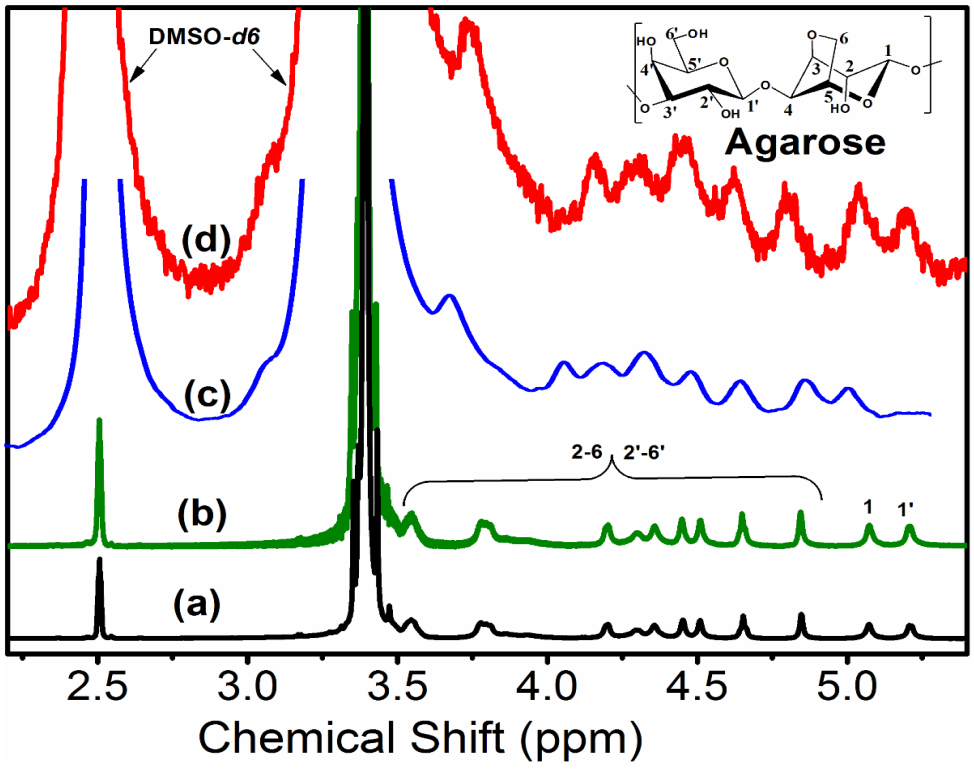


Figure 2. ^{1}H NMR spectrum of (a) reference agarose and agarose extracted using different ionic liquids (b) [Emim] [OAc], (c) [Ch] [OAc], and (d) [Emim] [Dep].

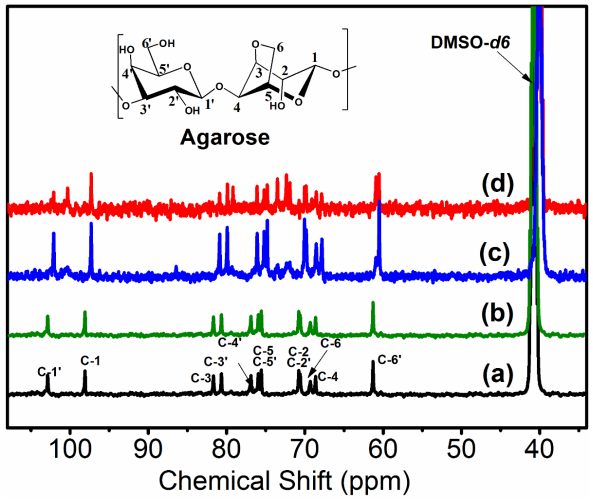


Figure 3. ^{13}C NMR spectrum of (a) reference agarose and agarose extracted using different ionic liquids (b) [Emim] [OAc], (c) [Ch] [OAc], and (d) [Emim] [Dep].

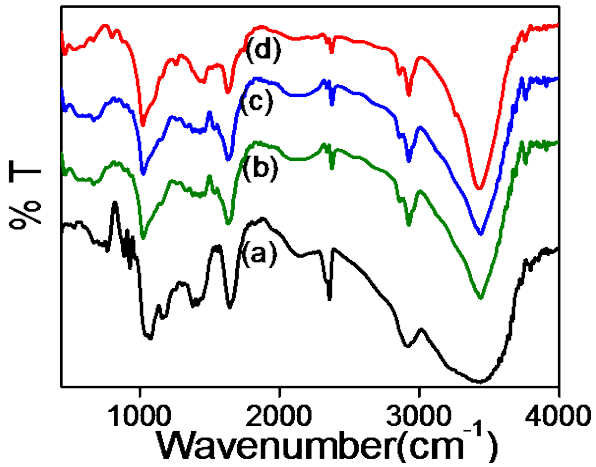


Figure 4. Comparison of FTIR spectrum of (a) reference agarose and agarose extracted using different ionic liquids (b) [Emim] [OAc], (c) [Ch] [OAc], and (d) [Emim] [Dep].

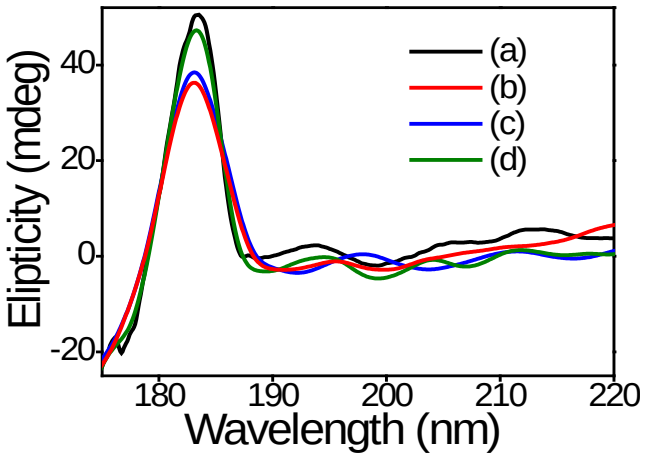


Figure 5. Comparison of CD spectra of (a) reference agarose and agarose extracted using different ionic liquids (b) [Emim] [OAc], (c) [Ch] [OAc], and (d) [Emim] [Dep].

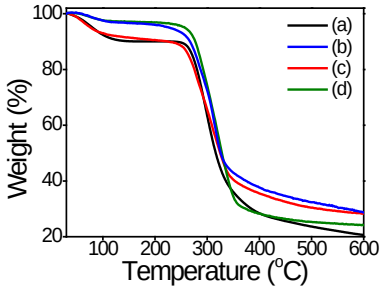


Figure 6. Comparison of thermograms for (a) reference agarose and agarose extracted using different ionic liquids (b) [Emim] [OAc], (c) [Ch] [OAc], and (d) [Emim] [Dep].

Table 2. ^{13}C NMR chemical shift comparison of reference and extracted agarose.

Source	Unit	^{13}C chemical shift (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
Reference agarose	G	102.05	69.76	81.70	68.23	74.94	60.92
	A	97.66	69.59	79.72	76.75	75.13	68.90
Extracted agarose	G	102.09	70.05	80.89	67.83	75.22	60.51
	A	97.30	69.83	79.92	79.92	74.80	65.55

G—1,3-*β*-D-galactose; A—1,4-*α*-L-3-6-anhydrogalactose.

polymerization of the extracted material are noted in **Table 3**. Results show that extracted agarose have M_w ranging from 115 to 118 KDa and polydispersity from 2.55 to 2.65 depending upon the IL used. Ash and sulfate content present in the extracted agarose are given in **Table 1** and are comparable to that of 3% NaOH treated reference agarose [35].

Physiochemical property of extracted agarose hydrogels are compared with reference agarose hydrogel in **Table 1**. Gelling and melting temperature of extracted agarose hydrogels (1 wt%) are slightly lower as compared to the reference agarose, whereas the gel strength is also somewhat lower (~570 to 600 g·cm^{-2}) as compared to the 3% NaOH treated reference hydrogel (700 g·cm^{-2}) [35]. This may be due to comparatively higher sulphate content present in extracted agarose. Optical rotation and apparent viscosity of extracted agarose ranged between −22 to −24 and 3.6 to 4.5 cP respectively depending upon the IL used and the values are noted in **Table 1** and **Table 3**.

3.3. % Recycling and Extraction Ability of Recovered ILs

Recycling of used solvents from the reaction mixture after materials extraction is important for the development of an energy-saving process. After the extraction of agarose, as shown in **Scheme 1**, the filtrates containing a mixture of ILs, methanol and water were evaporated under reduced pressure using rotary evaporator to get pure ILs. Recovered ILs were vacuum dried at 80˚C and were characterized for purity via ^{1}H and ^{13}C NMR (**Figure 7**). Recycling efficiency and extracting ability was checked for a representative IL, [Emim] [OAc]. Extraction reactions were performed at 80˚C; 2 h for up to four cycles and results of extraction are illustrated in **Table 4**. Result shows that the extracting of agarose was reduced from 28.5% to 21% after fourth cycle indicating a reduction of about 27% efficiency of extraction. % Recovery of ILs decreased from 87.5% to 46.23% in the fourth cycle which is mainly because of experimental and handling error after each experiment as well as rigidness of the process.

4. Conclusion

In this work, algae (*Gracilaria dura*) were successfully dissolved in the ILs; [Emim] [OAc], [Ch] [OAc] and

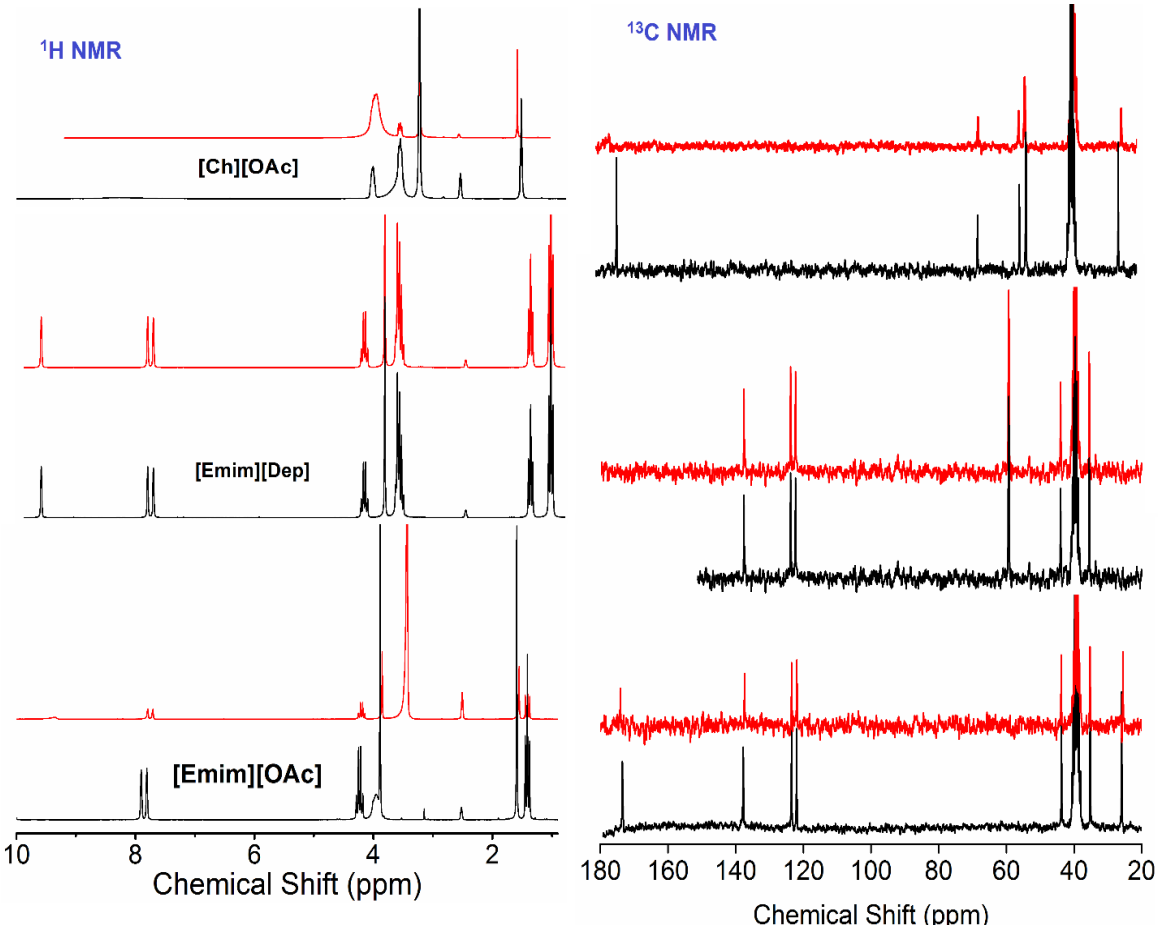


Figure 7. Comparison of NMR spectrum of (a) pure and (b) recycled ionic liquids (ILs) after agarose extraction.

Table 3. Molecular weight (M_n and M_w in kDa), polydispersity (P), degree of polymerization, decomposition temperature (T_{dec}) and apparent viscosity (cP) of agarose extracted using different ILs.

No.	IL	M_w	M_n	P	D_p	T_{dec}	cP
1	[Emim] [OAc]	118.56	46.22	2.56	388	308	3.68
2	[Ch] [OAc]	115.78	45.03	2.57	387	315	4.52
3	[Emim] [DEP]	117.10	44.27	2.64	386	316	3.76

Table 4. Extraction ability and recovery of 1-ethyl 3-methyl imidazolium acetate in different cycles.

No. of cycles	% Yield	% IL recovery
First	28.5	87.56
Second	28	79.31
Third	24.97	65.30
Fourth	21.66	46.23

[Emim] [Dep] and high purity agarose could be extracted from algae-IL solutions through precipitation using methanol. In particular, [Emim] [OAc] was found highly efficient for the extraction of agarose. It was observed that the increase in temperature or assistance of microwave heating increased extraction efficiency of agarose

from algae. As high as 39 wt% of agarose could be extracted, which is very high as compared to the agarose extracted from conventional methods. ILs could be easily recovered and recycled after the completion of extraction process. Extracted agarose were fully characterized in terms of structural purity and physicochemical properties. The method of extraction developed herein utilizing the ILs is economic and environmental friendly, and looking at the abundance of marine resources, will be highly useful for extraction of value added byproducts.

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