RSC Advances

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Journal:	*RSC Advances*
Manuscript ID:	RA-ART-05-2014-004476
Article Type:	Paper
Date Submitted by the Author:	13-May-2014

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Surfactant-induced coagulation of agarose from aqueous extract of *Gracilaria dura* seaweed as an energy-efficient alternative to the conventional freeze-thaw process

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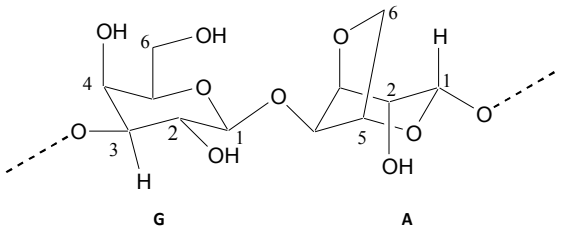
Surfactant-induced coagulation of agarose from alkali-treated *Gracilaria dura* seaweed extract (SE) is reported. The new approach, which was suitable for linear galactans with low sulphate content, dispensed with the traditional energy intensive process of "freeze-thaw" employed for product isolation from extract. Only nonionic surfactants were effective and detailed studies were undertaken with octyl phenol ethoxylate (Triton X-100). The coagulated product was washed successively with water and water-isopropyl alcohol (IPA) to yield a fine powder of agarose in 13-15% yield (with respect to dry biomass). The product exhibited excellent properties (sulphate content: 0.2% *w/w*); degree of electro-endosmosis: 0.13; gel strength: 2200

g cm^{-2} (1% gel, *w/v*); gelling temperature: 35 ± 1°C essential for demanding molecular biology applications, and the desired gel electrophoretic separation of DNA and RNA was demonstrated. It was further confirmed that there was no degradation of the nucleic acids in the gel. The agarose-depleted extract, along with water washings, was subjected to reverse osmosis to recover the surfactant in concentrated form for its subsequent reuse. An assessment was made of energy savings from the improved process.

Keywords**:** *Gracilaria dura*, Agarose, Surfactant, Preferential precipitation, Improved process, Nucleic acid separation

Introduction

Agarose is a purified linear galactan hydrocolloid isolated from agar or agar-bearing marine algae prepared by the purification of agar. Agar may be depicted by the structural formula shown below. The structure comprises alternating D-galactose sub-unit (G) and 3,6-



anhydro-L-galactopyranose sub-unit (A) linked by α-(1→3) and β-(1→4) glycosidic bonds. A small fraction of the hydroxyl groups at 4 position of G and/or 2 position of A is present in sulphated form.[1,2] Various grades of agarose are reported, with sulfate content ranging from 0.10 to 0.35% (*w/w*). Agarose forms a gel matrix in aqueous medium that is ideal for diffusion and electro-kinetic movement of biopolymers. This makes it suitable for

applications in molecular biology, electrophoresis and cell culture. Agarose is commonly prepared from superior quality agar or agar-bearing marine algae, such as *Gelidium spp., Gracilaria spp., Acanthopeltis spp., Ceramium spp., Pterocladia spp.*, and *Campylaephora spp.*[1] The red seaweed, *Gracilaria dura*, from Indian seawater has been reported recently as a promising bio-resource for its preparation.[1]

The process of agarose preparation involves (i) alkali pre-treatment of the seaweed followed by autoclaving, (ii) subjecting the aqueous extract to several cycles of freeze-thaw to isolate the product, and (iii) purifying the product further through solvent/chemical treatment and/or chromatography to eliminate residual impurities.[1,3-6] The energy intensive nature of the process, costly purification steps and long batch time are prime reasons behind the high cost of the product.

The present work emanated from a desire to explore alternative means of isolating agarose from seaweed extract. Rheological properties of agar sol and gel in presence of various cationic, anionic and nonionic surfactants have been reported previously.[7] The gel strength, viscosity, rigidity, gelling temperature and melting temperature were observed to increase in presence of ionic surfactants whereas nonionic surfactants had the opposite effect. The apparent weakening of gel network with nonionic surfactant prompted us to exploit the phenomenon towards the development of an alternative process of product isolation from seaweed extract. The present study reports the isolation of agarose from *G. dura* seaweed extract through spontaneous coagulation mediated by nonionic surfactants. Simplification of the downstream operations of purification and recycle of the surfactant were additional merits of the process. The efficacy of the product obtained through the new process was tested through gel electrophoresis of nucleic acids.

Experimental

Materials

Cultivated *G. dura* was collected from the south-east coast (Latitude: 9.28°N, and Longitude: 79.12°E, Mandapam, Tamil Nadu) of India. 45-days-old plants were harvested, air dried and stored in plastic bags. Two cationic [cetyl trimethyl ammonium bromide (CTAB); cetyl pyridinium chloride (CPC)], one anionic [sodium lauryl sulphate (SLS)] and four nonionic [Triton X-100 (octyl phenol ethoxylate; $C_{14}H_{22}O(C_2H_4O)_n$]; [Synperonic 91/6 (alcohol ethoxylate; C_8/C_{10}-CH_2O-$(C_2H_4O)_6$]; [Tween-80 (Polyoxyethylenesorbitan monooleate; $C_{64}H_{124}O_{26}$)]; [Atplus 245 (C_9/C_{11} alcohol ethoxylate/propoxylate)] surfactants were used in the present study. CTAB, CPC, SLS and Triton X-100 were procured from S. D. Fine Chemicals, India and the rest of the surfactants were gifted by ICI Uniqema (presently Croda), India. Agarose samples from Sigma–Aldrich, USA (Cat. No. A05066), Merck (Genei), and a sample prepared from *G. dura* in our own laboratory[1] served as controls.

Characterizations

Sulphate contents analyses (ICP) were carried out on a Perkin-Elmer ICP-OES Optima 2000DV machine.[1] Weight average molecular weight (M_w) was estimated as per literature procedure.[1] Morphology of agarose hydrogel samples before and after freeze drying was studied out using optical microscope (OLYMPUS, U. TV0.63XC, T_7 Tokyo, Japan). The dynamic light scattering (DLS) measurements were carried out using a Malvern instrument at a scattered angle of 90° and at temperature 30°C. The incident light was the 488 nm line of a argon laser GLS 3110. First, the solution was taken in a cell and the measurement was carried out at 30°C. Detailed procedures of gelling property measurement, DNA and RNA gel

electrophoresis experiments, electroendosmosis (EEO) measurement, and recovery of surfactant by reverse osmosis (RO) are provided as Electronic Supplementary Information (ESI, General Experimental Section). Quantification of surfactant in concentrate stream after RO was computed using the following equation:

$V_f \times C_f = V_p \times C_p + V_c \times C_c$

wherein V_f = volume of feed; C_f = surfactant concentration in feed (*w/v*); V_p = volume of permeate; C_p = surfactant concentration in permeate (*w/v*); V_c = volume of concentrate; and C_c = surfactant concentration in concentrate (*w/v*). Absence of surfactant (Triton X-100) in permeate stream was checked by HPLC (Waters Alliance 2996, USA) using C-18H column and PDA detector at 220 nm, and accordingly the first term in the right hand side of the equation was neglected.

Preparation of agarose

The alkali treated seaweed extract was prepared by modification of a previous process.[1] In the laboratory process, 0.20 kg of the dry seaweed having 9 ± 1 % moisture content was taken in 2 L of 10 % NaOH and the reaction mixture was heated to 80^{o}C to reduce the sulphate content of the linear galactan. This was followed by several water washes (4 x 2 L) to remove the excess alkali. The treated seaweed was then crushed and autoclaved (seaweed: water = 1: 35 *w/w*) at 120^{o}C for 90 min, and the resultant hot mass was subjected directly to centrifugation at 10,000 rpm to obtain a clear extract. After reaching a temperature of 70-80^{o}C, the extract was treated with surfactant (4 % *w/w*) under continuous stirring, while allowing the mass to cool gradually to room temperature. Phycocolloid precipitation was observed with the nonionic surfactants and the resultant solid mass was isolated by centrifugation. The solid mass was washed with water to remove excess surfactant followed by successive washing with 1: 1 (*w/w*) IPA: H_2O

(single cycle), 3: 1 (*w/w*) IPA: H_2O (single cycle), 17: 3 (*w/w*) IPA: H_2O (single cycle), and finally with neat IPA (single cycle). For each washing, the solvent weight was twice the weight of the solid mass taken. The product was finally subjected to vacuum drying at 50^{o}C to obtain a readily water soluble agarose powder. Experiments were subsequently conducted at bench scale with 1 kg of dry biomass, yielding similar results. The procedure for recovery of surfactant from agarose-depleted extract and water washings is described under supplementary material (ESI, General Experimental Section). Filtrate containing surfactant left behind after the recovery of agarose, along with the first water wash, was subjected to reverse osmosis (RO) (Hydronautics SWC5 LD4040 low fouling seawater RO membrane module; 150 psi applied pressure) for the recovery of the surfactant in concentrated form. In this process, 90 % of the water was removed, leaving a concentrated surfactant which, after removal of color through charcoal treatment, could be added directly into the next lot of seaweed extract to induce coagulation. Similarly, the IPA-water mixtures were collected from which IPA was recovered by distillation.

Results and discussion

Normally in a conventional extraction process 0.2 kg of *G. dura* gives ca. 4.5 L of extract containing only 0.50-0.75 % agar/agarose. Consequently, considerable amount of energy is expended in repeatedly freezing and thawing of the mass to isolate and purify the product by the widely used conventional means. Precipitation through addition of alcoholic solvent is also feasible but it requires twice the volume of solvent. It is therefore of interest to explore alternative solutions such as incorporation of additives to induce precipitation. In line with this approach, ionic and nonionic surfactants were probed in the concentration range of 2-5 % (*w/w*)

(Table 1). No effect was seen with up to 5 % (*w/v*) concentration of ionic surfactants added into hot seaweed extract. The lack of any coagulation with charged surfactants was consistent with literature reports that polymers bearing low charge do not interact with ionic surfactants.[7-9] When nonionic alkoxylate surfactants were evaluated, no effect was seen at 2 % (*w/v*) concentration. However some precipitation was observed at 3 % level while heavy precipitation was seen at 4 % concentration within 4 h upon gradual cooling of the mass to ambient temperature under continuous agitation. A study was also conducted to ascertain the relative ease of coagulation of three different seaweed extracts, namely *Gelidiella acerosa* and *Gracilaria edulis*, besides *G. dura*. No coagulation was observed with *G. edulis,* partial coagulation was seen with *G. acerosa*, and maximum coagulation with *G. dura*. This may be due to differences in the galactan charge arising from variations in the sulphate content, the value being lowest (0.2 %) in the case of *G. dura* and highest (> 1 % sulphate) in the case of *G. edulis*. Table 2 provides data on the properties of the product obtained upon purification

Table 1. Effect of treatment of seaweed extract with cationic, anionic and nonionic surfactants.

Surfactant	*% w/w*	Remarks
CTAB	5	No precipitation
CPC	5	No precipitation
SLS	5	No precipitation
Triton X-100	4	Precipitation observed
Synperonic 91/6	4	Precipitation observed
Tween-80	4	Precipitation observed
Atplus 245	4	Precipitation observed

156 through water wash followed by water-IPA wash. The best properties for molecular biology
157 applications in terms of gel strength (2200 ± 50 g cm^{-2}), sulphate content and EEO were
158 obtained with Triton X-100. Hence this surfactant was chosen for further studies although the
159 product yield was marginally lower than in the case of Synperonic 91/6 and Atplus 245.
160 Another important reason behind the choice was the proven applications of this surfactant in
161 biology. Product of similar quality in 14.9 % yield was obtained when 20 L of extract (prepared
162 at bench scale from 1 kg of dried *G. dura*) was processed similarly in a single lot. Table 3
163 shows that the gel strength of the bench scale product was 1.5 times that of A05066 Sigma
164 agarose employed for molecular biology applications, and the other critical properties were
165 comparable or superior. Indeed, the gel strength was even superior to that of "exceptionally
166 high gel strength" agarose from Sigma (A0576) employed for separation of high molecular
167 weight nucleic acids.

168
169 **Table 2.** Effect of nonionic surfactants on the properties of agarose.
170
171

	Triton X-100	Synperonic 91/6	Tween-80	Atplus 245
Yield (%)	13.2	13.6	13.0	14.6
Moisture (%)	7.0	7.0	8.0	8.0
Ash (%)	0.8	0.9	0.9	0.9
EEO	0.13	0.13	0.14	0.14
Sulphate (%)	0.20	0.21	0.23	0.24
Gel strength (g/cm^2)	2200	2000	2000	1900
Gelling temperature (ºC)	35 ± 1	35 ± 1	35 ± 1	35 ± 1
M_w/ g mol^{-1}	1.31 x 10^5	1.29 x 10^5	----	---

Table 3. Comparative data of agarose obtained at bench scale with Sigma agarose.

	G. dura agarose (present study)	Sigma agarose (A 05066)[a]	Sigma agarose (A0576)[b]
Gel strength (g cm^{-2})	2200 ± 50 (1 % gel)	≥1500 (1.5 % gel)	≥1800 (1 % gel)
Sulphate (% *w/w*)	0.20	≤0.6	≤0.12
EEO[c]	0.13 (± 0.01)	0.23-0.27	0.12
Melting Temp/ °C	89 (± 1)	N/A[d]	86 (± 2)
Gelling Temp/ °C	35 (± 1)	34-37 (± 1.5)	36 (± 1.5)
DNAse, RNAse activity	ND[e]	ND[e]	ND[e]

[a]Used for molecular biology; [b]High gel strength particularly suitable for separating high molecular weight nucleic acids at low gel concentrations; [c]≤0.13 considered as low EEO; [d]N/A = not available; [e]ND = None detected.

An experiment was undertaken to observe the gel networks after lyophilisation of the hydrogels. As can be seen from Fig. 1, agarose from *G. dura* revealed thinner strands of the linear galactan with denser network of the strands compared to A05066. The observed differences in the network properties possibly contribute to the observed variations in the gel strengths. Artifacts arising from the manner of freezing and sublimation cannot, however, be ruled out.[10] It is noted that gel strength is influenced by other factors also.[11]

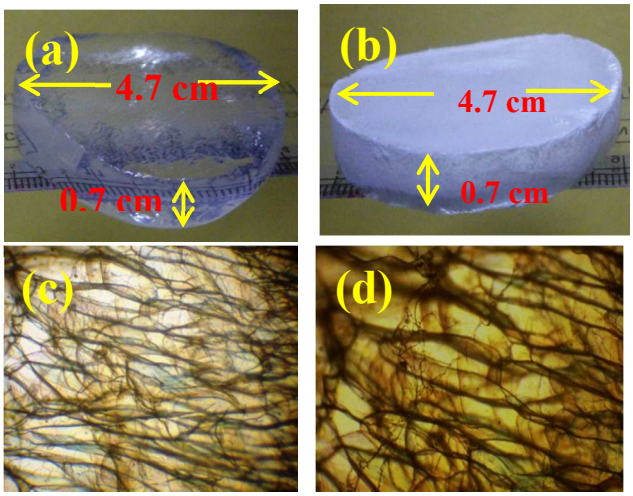
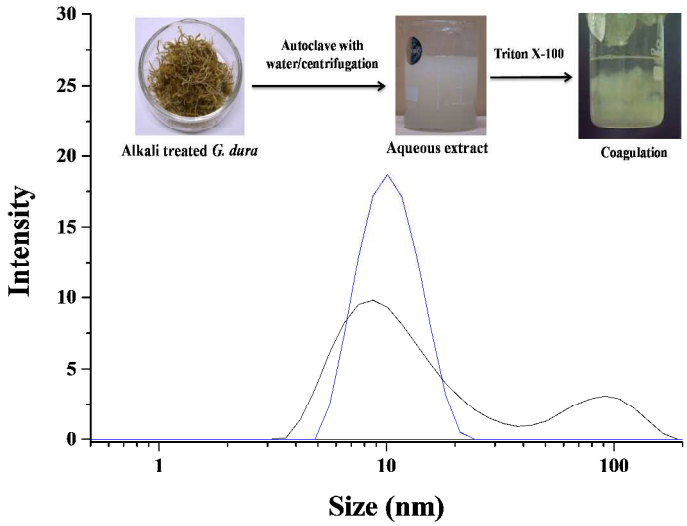


Figure 1. (a) Typical appearance (camera photo) of 0.6 % (*w/v*) agarose gel prepared from *G. dura* (Table 3); (b) of solid foam after freeze drying (camera photo); optical micrographs of solid foam prepared from (c) *G. dura* agarose of Table 3 and (d) A05066 Sigma agarose.

The effect of Triton X-100 was studied next. Fig. 2 shows the DLS profiles of the surfactant in water and in *G. dura* extract at 4% (64 mM) concentration. The observed peak at 10 nm in water may be ascribed to micelle formation since the concentration of the surfactant was far in excess of the critical micelle concentration (cmc) of 0.28 mM.[12] Upon addition of the surfactant into seaweed extract, the intensity of the peak at 10 nm decreased markedly with corresponding formation of a peak at 100 nm due to formation of a fine suspension. When the agitation was stopped and the suspension maintained under ambient conditions, settled mass was seen at the bottom as shown in the inset. Fig. 3 shows a possible mechanism of the coagulation induced by Triton X-100. In the typical micellar structure, the hydrophobic aromatic group is located in the micellar core whereas the hydrophilic ethyoxylate chains project outward into the bulk of the solution. It is presumed that the micelle served as a template for the galactan chains, and H-bonding with water was partly substituted with H-bonding with the ethyoxylate chains of Triton X-100. Agarose polymer molecules may have also interacted among themselves as shown in the Fig. 3. Plausibly, the above promoted coagulation.



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214 **Figure 2.** DLS traces of solution of Triton X-100 (4% (w/v) in water (blue trace) and in
215 seaweed extract at the same concentration (black trace). The inset shows the alkali treated
216 seaweed, the aqueous extract prepared from the seaweed and subjecting to centrifugation, and
217 of the suspension obtained after Triton X-100-induced coagulation.

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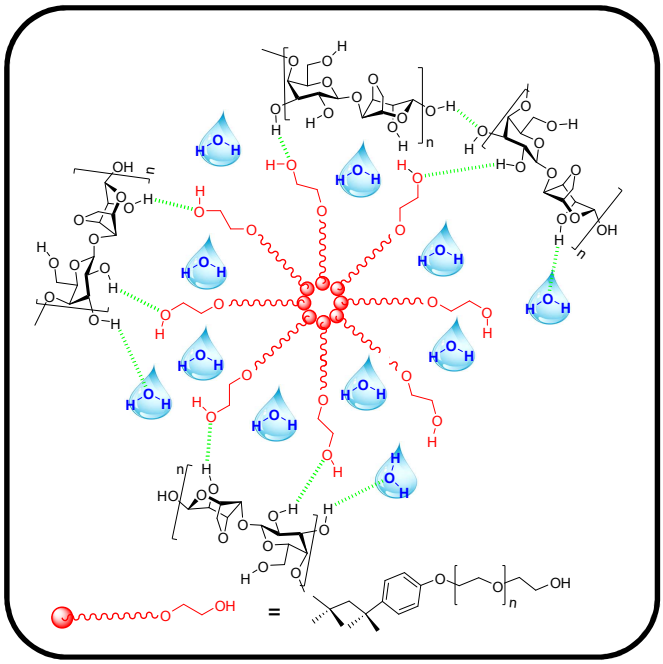


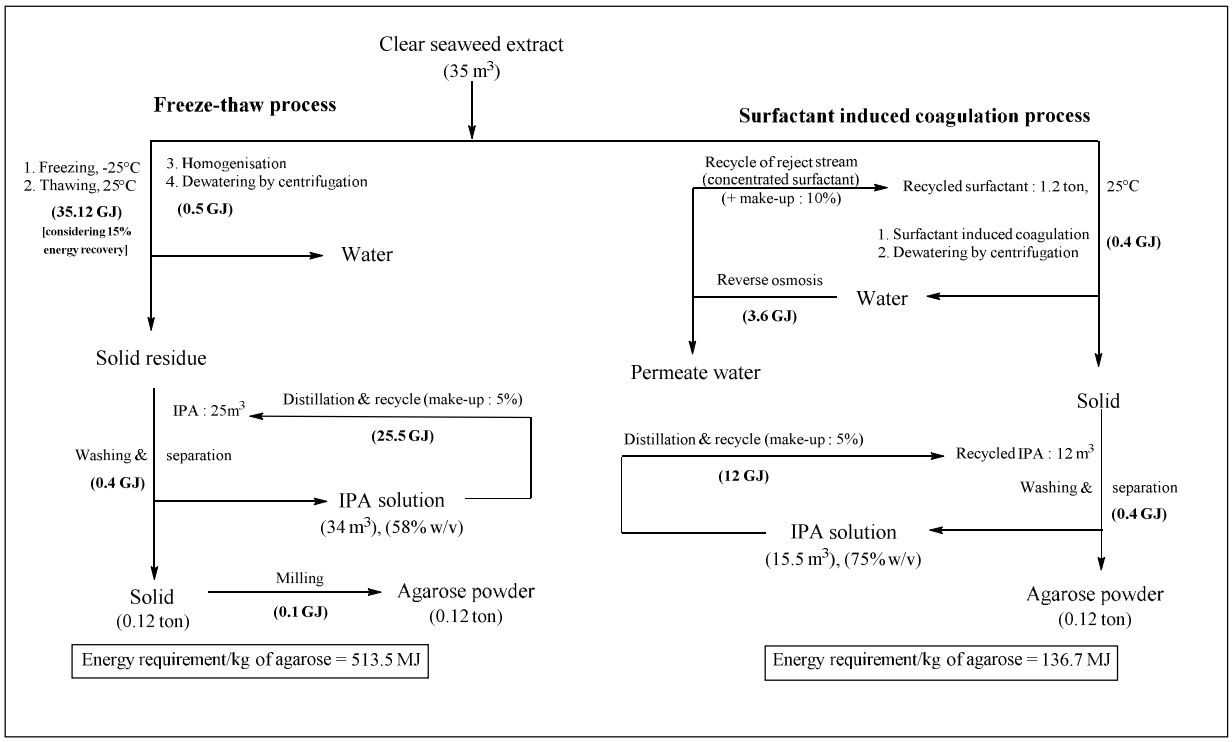
219

220 **Figure 3.** Possible mechanism of coagulation arising from the interaction of agarose with
221 micelles of Triton X-100.

Attention was focused next on the recovery and reuse of the surfactant. Although the weight percentage of the surfactant in solution was similar to that of dissolved salts in seawater, the osmotic pressure in the former case would be much lower in view of the higher molecular weight (MW = 624) of the surfactant and formation of micellar aggregates. With the above reasoning, an attempt was made to concentrate the surfactant in the supernatant seaweed extract by reverse osmosis (RO) at low-to-moderate pressure (150 psi). Table 4 provides data on processing of ca. 105 L of supernatant and water wash. Permeate stream free from surfactant ($C_p \sim 0$) was recovered in ca. 90% yield (ESI, Fig. S1) and, based on mass balance equation, C_c was estimated to be ca. 40 %. The latter was subjected to charcoal treatment to remove color and utilized thereafter in a subsequent cycle of coagulation. Product of desired quality was obtained in 12.7% yield. IPA was also recycled and reused to give the desired quality of product in 13.2% yield. Scheme 1 presents schematically the differences between the freeze-thaw (considering a single cycle) and surfactant-induced coagulation processes, along with the energy consumption in critical steps. It can be seen from the scheme that the energy savings works out to 376.8 MJ/kg of agarose. Other important advantages of the new process are (i) shorter process time and (ii) lower capital expenditure.

Table 4. Data on permeate flow rate upon concentration of spent seaweed supernatant by RO.

Time/min	Permeate Flow/Liters per minute	Temperature/^{o}C
0	1.59	26.8
10	1.395	27.2
20	1.290	27.7
30	1.245	28.0
40	1.160	28.2
50	1.04	28.6
60	0.940	28.9
70	0.730	29.0
90	0.50	29.2



Scheme 1. Energy computations for "freeze-thaw" and "surfactant-induced coagulation" processes of agarose preparation starting from aqueous extract derived through autoclaving of 1 ton of dry *G. dura*.

Biological evaluation

Agarose prepared through the surfactant-mediated route was compared initially against the product prepared through conventional processing. The results are presented in ESI, Fig. S2. The results revealed that resolutions of DNA were identical in the two gels, confirming that both the agarose products were of similar quality. Comparative evaluation was subsequently conducted with Sigma agarose A05066. *G. dura* agarose prepared in the present work gave similar performance to that of the commercial sample when the gels were prepared with 0.7% (*w/v*) and 1.0 % (*w/v*) agarose, respectively (Fig. 4a). The separation of DNA bands both in the

100 bp and 1 kb DNA ladders showed near equivalence with sharp resolution. DNA recovery from the gels was >50% in both cases. PCR amplification of the extracted DNA samples showed similar intensity of amplicons, confirming that the agarose obtained through surfactant-induced flocculation can be used for molecular biology applications. Similarly, in the case of RNA, both sub-units (28S rRNA and 18S rRNA) were clearly resolved and no smear was observed in the gel, confirming the absence of degradation in the gel. Gel electrophoresis of DNA and RNA was carried out several times during process development and it was consistently observed that RNA and DNA do not degrade in the gel, confirming reproducible absence of DNAse and RNAse activity. A comparison was also made of the performances at higher gel concentration. 2% *w/v* Genei agarose from Merck was employed as benchmark. 1.5% *w/v G. dura* agarose of Table 3 gave equivalent DNA band resolution patterns (Fig. 4b).

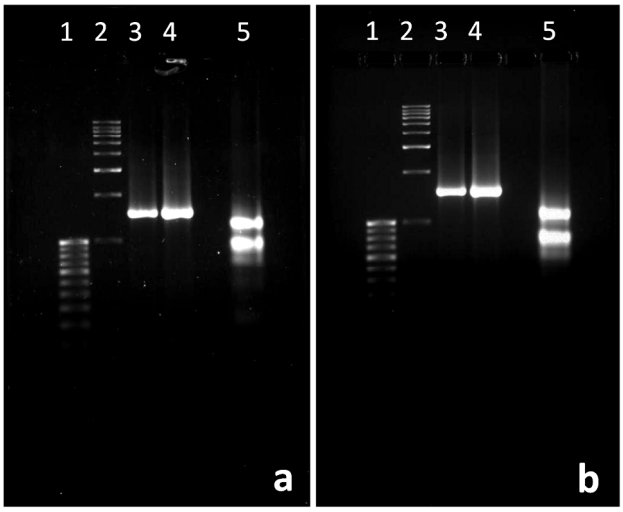


Figure 4a. Gel electrophoresis: gel was prepared in 1x TBE buffer and DNA and RNA was electrophoresed at 50 V in (a) 0.7% CSMCRI agarose gel and (b) 1.0% A05066 Sigma agarose gel. Lane 1: 100 bp ladder; Lane 2: 1 Kb ladder; Lanes 3 & 4: DNA band; Lane 5: RNA [28S rRNA (top); 18S rRNA (bottom)].

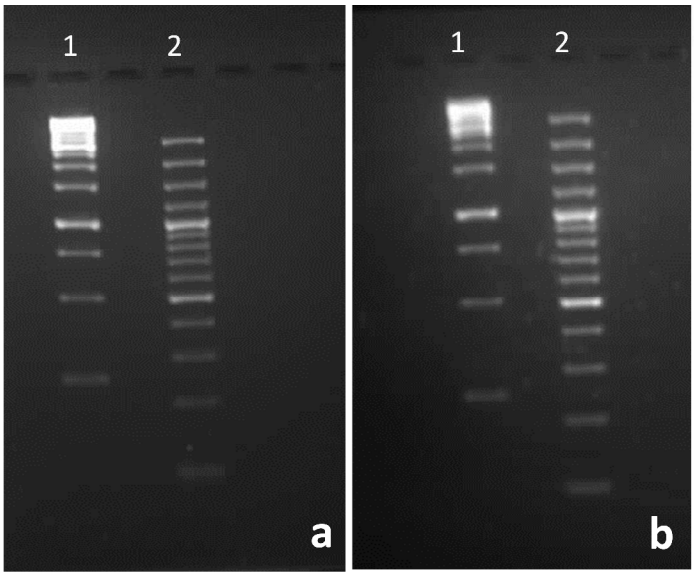


Figure 4b. DNA resolution pattern: Gel was prepared in 1x TBE buffer and DNA was electrophoresed at 50 V using (a) 1.5% CSMCRI agarose gel and (b) 2 % Genei agarose (Product No. 6126001, lot no. 124517) (run time: 2 h; Lane 1: 1 Kb DNA ladder; Lane 2: 100 bp plus DNA ladder).

Conclusions

The present study reports an alternative process of isolation of agarose from *G. dura* seaweed extract that helped dispense with the energy demanding and clumsy process of "freeze-thaw." The improved process relied on spontaneous coagulation of agarose from the extract through addition of a nonionic surfactant. It is presumed that the micellar aggregate of the surfactant molecules above the critical micelle concentration served as template for the linear galactan chains, replacing in part hydrogen-bonding to water molecules with hydrogen bonding to the polar ethoxylate chain of the surfactant. This may have caused the breakdown of the gel network. The micellar aggregate may also have helped to promote hydrogen bonding between the agarose molecules themselves. The process could be practiced most advantageously with

linear galactan hydrocolloids having low sulphate content such as the product from *G. dura*. Recycling of the surfactant through RO-based concentration raised the green quotient of the process, although further studies are necessary to address the issue of membrane fouling upon prolonged exposure. A minimum energy savings of 376.8 MJ/kg of agarose was computed for the improved process but actual savings would be higher if multiple freeze-thaw cycles are to be considered for the conventional process, as is normally the case in practice. Another advantage was that the product was obtained directly in powder form, making it easier to remove the impurities through washing and obviating the need for milling. The multiple advantages of the present process, without any compromise on the quality of the product essential for molecular biology applications, provide the motivation for further studies in this area.

Acknowledgement

The authors are thankful to: K. Eswaran for supply of the dry seaweed; A. Gogda and N. Vadodariya for help with seaweed processing; Analytical Discipline and Centralized Instrument Facility for analytical data; M. Dinda for helpful assistance; DST, New Delhi for financial assistance under a project (No.SB/EMEQ-052/2013); CSIR, New Delhi for generous support towards infrastructure development under network (CSC0130) project.

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Graphical abstract