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Optimization of yield and quality of agar polysaccharide isolated from the marine red macroalga *Pyropia yezoensis*

Keiji Sasuga [a,b], Tomoya Yamanashi [a], Sigeru Nakayama [a], Syuetsu Ono [c] and Koji Mikami [d,*]

[a] Suzuyo Research Institute, Co. Ltd., 11-26 Tsukiji-cho, Shimizu, 424-0944 Shizuoka, Japan

[b]Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, 041-8611 Hakodate, Japan

[c] Miyagi Prefecture Fisheries Cooperative Association, 1-27 Kaisei, 986-0032 Ishinomaki, Japan

[d] Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, 041-8611 Hakodate, Japan

*Corresponding author:

Koji Mikami

Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, 041-8611 Hakodate, Japan

TEL/FAX: +81-138-40-8899

E-mail: komikami@fish.hokudai.ac.jp

Abstract

The marine red seaweed *Pyropia yezoensis* is grown on a large scale in Japan using mariculture for production of nori sheets. Here, we optimized the isolation of agar from *P. yezoensis* and evaluated its yield, physical properties, and product applications. An alkali pre-treatment of seaweeds before agar extraction increased the weight-average molecular weight of the agar and promoted the conversion of L-galactose sulfate to 3,6-anhydrogalactose, which in turn reduced the sulfate contents, thus altering the gel strength of the agar. This ability to adjust agar quality by altering the alkali concentration during material pre-treatment allowed production of agar with properties similar to those of agar from the red seaweed *Gelidium* sp., which is widely used for industrial agar applications. We demonstrate the suitability of *P. yezoensis* agar in production of solid plates for bacterial growth. In addition, the *P. yezoensis* agar was particularly useful as a gel material, with the capacity for excellent size separation of DNA by electrophoresis even without agarose purification, because of quite low contents of sulfate. These findings support the applicability and usefulness of *P. yezoensis* for agar production. The established large-scale cultivation methods for *P. yezoensis* can generate sufficient biomass to produce agar to support medical and biological studies.

Keywords: agar, physical property, extraction optimization, macroalga, red seaweed, *Pyropia yezoensis*

1. Introduction

Agar is a water-soluble, gel-forming polysaccharide that can be extracted from the cell wall of red seaweeds [1]. The capacity of agar to gel, with concomitant high water absorption, contributed to the epoch-making advance of using agar to solidify media in plates for bacterial culture [2-3]. Although not all red seaweeds contain agar, Gracilariaceae including *Gracilaria*, *Curdiea*, and *Hydropuntia* [4-17], as well as *Gelidium* and *Pterocladia* of the Gelideales [6,18-24] are major agar sources [25]. Agar from these seaweeds represents complex polysaccharide structures consisting of agarose and agaropectin [1]. Agarose is a linear polymer with a disaccharide repeat of alternating 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose residues without sulfates. Agaropectin is the non-gelling fraction of agar and is a sulfated polysaccharide [26-28]. In addition to its use in bacterial plates, Hjertén [29] put agar to use for gel electrophoresis after purifying the agarose by removing agaropectin. Agarose gel electrophoresis remains an essential technique in molecular biology research. Thus, agar is an indispensable material for many medical and biological studies.

Gracilariaceae, Gelideales, and other red seaweeds have been employed for extraction of agar. Agar yield from *Gracilaria cervicornis* decreases with increasing NaOH concentration [8] and those from other *Gracilaria* and *Curdiea* species vary from 10 to 50% depending on the species and season [4,5,7,9,15-17]. In addition, agar has been produced from Bangiophyceae such as *Pyropia haitanensis* [30-32] and *Porphyra capensis* [33]. Indeed, the marine red seaweed *P. haitanensis*, has been successfully

used in China for commercial and industrial applications. By contrast, there has been minimal use of Bangiophyceae to produce agar in Japan, despite enormous quantities, approximately 300,000 tons (wet weight) per year, of the *Pyropia yezoensis* maricultured to produce nori sheets. Although a few pioneer experiments have been performed using *P. yezoensis* for agar production, the utility of this species for agar production has not been established [34-37]. Development of methods to extract and produce agar from *P. yezoensis* is therefore an important step toward effective use of the large biomass already produced in Japan and enhancing the value of this macroalga beyond as a nori food source.

Here, we confirmed the feasibility of obtaining high quality agar from *P. yezoensis.* Evaluation of alkali treatment and extraction conditions identified a method that produced sufficient yield and met quality characteristics needed to routinely produce agar from *P. yezoensis*. The agar produced from *P. yezoensis* using our method was comparable in performance to agar used for industrial applications from the red seaweed *Gelidium* sp.

2. Materials and methods

2.1. Algal materials

The marine red seaweed *P. yezoensis* was maricultured in Shichigahama, Miyagi, Japan by the Miyagi Prefecture Fisheries Cooperative Association and harvested in January 2016. The harvested seaweed was manufactured to produce and provide us commercial nori sheets by members of the Shichigahama branch office of the Miyagi

Prefecture Fisheries Cooperative Association or air-dried at room temperature in our laboratory to obtain dry seaweed (Supplementary Fig. 1), both of which were used as materials for agar extraction. Partially dried *Gelidium* sp., harvested between June and September 2013, was purchased after import from Morocco and further dried at room temperature in the laboratory (Supplementary Fig. 1). These three types of materials were cut into small pieces (25 mm x 3 mm square) with a shredder (KPS-MX200, Kokuyo, Japan) just prior to agar extraction.

2.2. Agar extraction

For each source type, 13 g material was soaked in 390 g (30-fold weight per volume of sample) of 0, 4, 6, 8 or 10% w/v NaOH solution in distilled water in a cylindrical stainless container (D 72 cm x H 180 cm, 570 mL; Private Brand of Shimizu Akira Inc.). Algal materials in the containers were incubated for 2 h in an oil bath at 80°C. The alkali-treated materials were then washed with flowing tap water for 17 h to remove the excess NaOH and transferred to 260 mL (260 g, 20-fold weight per volume of sample) distilled water with pH adjusted to 6.8–6.9 with 0.1 N H_2SO_4 or 0.1 N NaOH. The samples were then autoclaved for 1 h at 130°C. Extracts were subsequently filtered through a cotton cloth (pore size ca. 0.3 mm) with pressing to obtain as much filtrate as possible, and poured into trays to promote gelling at room temperature. The resultant gels were sliced into cubes (approximately 7 cm x 7 mm with 5 mm height) and frozen at -20°C overnight. The pieces were subsequently defrosted with running water and dried at 60°C using a dry oven (Oven Dryer AFO-151, Iwaki, Japan) for 7 h. Yield of agar was calculated using the following equation: Yield = Dry weight of agar (g)/Dry

weight of seaweed (g)×100 (%).

2.3. Rheological analyses

Physical properties of a 1.5% w/v solution of agar from *P. yezoensis* and *Gelidium* sp. were measured in term of rupture stress, rupture strain, melting temperature, and gelling temperature. These rheological analyses were performed in triplicate for all three seaweed materials. All results are presented as the mean ± standard deviation (S.D.). All statistical analyses were performed using Statcel for Windows (OMS Ltd., Saitama, Japan). One-way ANOVA was followed by the Tukey–Kramer test for multiple comparisons. Differences were considered significant when the calculated *p* value was less than 0.05.

Rupture stress and rupture strain, indicators of the gel strength and elasticity, respectively, were determined at 20°C using a creep meter (RE-33005B, Yamaden Co., Japan) equipped with a cylindrical probe of 8 mm diameter. Rupture tests were performed on 50 mL of 1.5% w/v agar solutions that were previously gelled at 20°C for 15 h in 100 mL beakers (sample diameter was 50 mm). Measurements of the two parameters were performed at 20°C under conditions where each sample was compressed to 80% of its original height with a crosshead speed of 1 mm/s. The melting temperature and gelling temperature for the agar products was determined as described in [11-12] with slight modifications. A 1.5% w/v agar with 70 mm height was prepared in a test tube (10 mm diameter, 90 mm height) and then an iron ball (5 mm diameter, 1 g) was placed on the surface of the gel and the tube was clamped in a water bath that was programmed to increase in temperature by 1°C /min. The melting temperature was

predefined as the temperature at which the iron ball dropped 70 mm by reaching the bottom of the gel. To determine the gelling temperature, hot 1.5% w/v agar solution (10 mL) was poured into a test tube (17.5 mm diameter, 130 mm height) that was capped with a rubber stopper attached to a thermometer. After reaching 40°C, the gel solution was mixed by gentle inversion at 30 s intervals. Gelling temperature was measured as the point at which the agar solution was completely congealed.

To determine standard agar properties, 18 different kinds of agars were sourced from the Agar Division of SSK Sales Co., Ltd. (Shimizu, Shizuoka, Japan) and similarly measured for the physical properties of rupture stress, rupture strain, melting temperature, and gelling temperature as described above. These results were used as reference standard for evaluation of *P. yezoensis* agar.

2.4. Quantification of sulfate contents

Sulfate contents in samples were determined using inductively coupled plasma optical emission spectrometry (ICP-OES) [38], in which agar was transferred into polytetrafluoroethylene (PTFE)-TFM^{TM} (a modified form of PTFE with improved mechanical and chemical properties) vessels (Perkin-Elmer, Shelton, CT, USA) and digested with 6 mL of HNO_3 (65%) by the microwave system (Anton Paar Multiwave3000, Graz, Austria). The reaction mixtures were then diluted to 10 mL with ultrapure water. A blank sample (only HNO_3) was treated in the same way. Then, sulfate quantification was performed using an ICP-OES Spectrometer (Perkin-Elmer Optima8300, Shelton, CT, USA).

2.5. Quantification of 3,6-anhydrogalactose content

3,6-anhydrogalactose (3,6-AG) content was determined colourimetrically using the method as described in [39]. Non-gelling solution, in which 200 µg agar powder was dissolved in 2.0 mL of distilled water by heating, was mixed stepwise with 0.5 mL of 5% thymol dissolved in ethanol and then with 5.0 mL of 0.5% ferric chloride dissolved in concentrated hydrochloric acid in test tubes with screw caps. After heating the resultant solution for 13 min at 80℃ in water bath, it was rapidly cooled to room temperature and diluted with ethanol (10 mL). Quantification of 3,6-anhydro-α-D-galactose was performed by measurement of the absorbance at 635 nm with a spectrophotometer (UV-2450; Shimadzu Corporation., Japan). A standard curve was made with methyl 3,6-anhydro-α-D-galactose (Dextra Laboratories Ltd., UK). A correction factor of 0.92 [39] was introduced to calculate the concentration of 3,6-AG.

2.6. Estimation of weight-average molecular weight and polydispersity index

Agar samples were dissolved with 50 mM lithium bromide, in dimethyl sulfoxide (DMSO) at 0.2% w/v, at 40°C and filtered through a 0.2 µm polytetrafluoroethylene (PTFE) membrane (DISMIC 13HP020AN, Advantec, Japan) to remove insoluble aggregates. Using these clear filtrates, the weight-average molecular weight (Mw) and polydispersivity index (Mw/ Mn, where Mn is the number-average molecular weight) of each agar sample, were determined by gel permeation chromatography (HPLC 1500 Series; Jasco Coporation, Japan) on two TSK gel GMH_{HR}-H columns (7.8×300 mm; Tosoh Corporation, Japan) eluted with 50 mM lithium bromide in DMSO at a flow rate of 0.5 mL/min at 40°C. Elution was monitored by the refractive index detector (RI-1530; JASCO Corporation, Japan). A calibration curve was obtained by measuring

pullulans (Shodex Standard P-82; Showa Denko, Japan) as standards. After obtaining the data for elution time of agar, Mw and Mw/Mn were calculated using ChromNaV GPC program (Jasco Coporation, Japan).

2.7. Bacterial growth

Bacterial strains including *Escherichia coli* (NBRC12713), *Staphylococcus aureus* (NBRC13276), *Pseudomonas aeruginosa* (NBRC13275), and *Bacillus subtilis* (NBRC3134) were cultured on slant agar medium for 18 h at 35°C and suspended in 0.9% NaCl to generate 10 to 100 colonies per plate. Bacterial suspensions were inoculated onto 0.75% agar plates composed with 5.0 g/L peptone from casein, 2.5 g/L yeast extract, 1.0 g/L D (+) glucose, and 7.5 g/L agar either from *Gelidium* sp. or *P. yezoensis.* Both agars were prepared with the same gel rupture stress of 1.8 x 10^5 Pa. After incubation for 24 h at 35°C, the number of bacterial colonies was determined and compared between the agar sources. In the present study, agar from seaweeds was not pretreated with diatomite filtration prior to use.

To compare the behavior of *B. subtilis* on three kinds of agar plates, protease activity was examined by comparison of the size of clear zones (halos) against the creamy background on 7.5 g/L agar medium containing 0.5 g/L skim milk with incubation at a temperature of 35ºC for 24 h.

2.8. Gel electrophoresis of DNA

Commercially purchased DNA size makers, 100bp DNA Ladder (Takara Bio, Japan)

and 1kb DNA Ladder (New England Bio Lab, UK) were separated on 1.0 % w/v gels made using agar from *Gelidium* sp. (rupture stress 2.0 x 10^5 Pa) or nori sheets (rupture stress 2.1 x 10^5 Pa) or using commercially supplied agarose (rupture stress 1.7 x 10^5 Pa). After electrophoresis at 100 volts for 30 min using Tris-acetate-ethylenediaminetetraacetic acid buffer, DNA bands in gels were stained with ethidium bromide and observed under UV light.

3. Results and Discussion

3.1. Agar yield

The agar extraction method used for this study was based on a protocol initially established for *Gracilaria* and *Curdiea* species in the Agar Division of SSK Sales Co., Ltd (Shimizu, Shizuoka, Japan). To validate this methodology in our hands, and apply it to other species, we first extracted agar from *Gelidium* sp. The resulting agar yield from *Gelidium* sp. was ca 23% and was not significantly altered by alkali treatment (Table 1).

When agar was extracted from *P. yezoensis* using this method, alkali concentration had a generally positive influence on the yield of agar from both nori sheets and dry nori (Table 1). Although no agar was extracted at 0% NaOH, the maximum agar yields of 12.8% and 16.8% for dry nori and nori sheets, respectively, were observed at 4% NaOH. It is possible that chopping into small pieces of *P. yezoensis* thalli in full automatic production process of nori sheets is responsible for best yield of agar by increasing surface area treated with alkali. Further increases in NaOH concentration reduced yield in both *P. yezoensis* materials. This finding is consistent with the agar

yields observed in a study of the marine red seaweed *Pyropia haitanensis*, where 4% alkali pre-treatment was also optimal [31]. Since Ogawa [34] reported a yield of 16% from nori sheets, we expected similar yield by our procedure. Indeed, maximum yield of agar was 16.8% from nori sheets, which is better than dry nori showing 12.8% yield (Table 1). These findings confirm the feasibility of our extraction method to isolate agar from *P. yezoensis* and suggest suitability of nori sheets for agar production.

3.2. Physical properties of agar gels

Rupture stress, which is an indicator of gel strength, was increased in an alkali concentration-dependent manner, especially in *P. yezoensis* (Table 1). By contrast, the rupture stress of *Gelidium* sp. agar was quite low without alkali treatment, but responded to other NaOH concentrations similarly (Table 1). Interestingly, treatment with 8% and 10% NaOH resulted in rupture stress levels for *P. yezoensis* agar that surpassed those observed in *Gelidium* sp. agar, and were similar to those of agar from *Gracilaria cervicornis* [8], *Gracilaria tenuistipitata* [15], *Gracilaria dura* [16], *Gracilaria cornea* [17], whose gel strength also increases with higher NaOH concentration. The maximum rupture stress value was recorded in agar from nori sheets with 10% NaOH treatment. Rupture stress ranged between 0.6 to 1.4 x 10^5 Pa in the commercial agar that we tested (Supplementary Fig. 2a). Thus, 6 to 10% NaOH pre-treatment allows production of agar from *P. yezoensis* with the same quality as commercially prepared agar and agarose.

The elasticity of agar can be described using rupture strain. Agar from *P. yezoensis* nori sheets showed increased rupture strain with increasing NaOH pre-treatment up to

10%, at which the highest value (24%) was obtained. Alkali concentration did not influence the agar from dry nori and *Gelidium* sp. (Table 1). As commercial agar for bacterial growth had rupture strains ranging between 10 and 20% (Supplementary Fig. 2a), NaOH treatment is sufficient to produce agar from *P. yezoensis* with the same elasticity qualities.

The effects of alkali concentration differed between melting and gelling temperatures. For agar from *P. yezoensis*, increases in melting temperature and NaOH concentration were positively correlated (Table 1), while gelling temperature was similar among the 6 to 10% treatments (Table 1). Treatment with 4% NaOH produced agar with low melting and gelling temperatures in both *P. yezoensis* and *Gelidium* sp. Commercial agar had melting temperatures ranging from 78 to 92°C (Supplementary Fig. 2b); thus, agar from nori sheets treated with 4 to 10% NaOH were comparable in performance to commercial agar. There was no difference in gelling temperatures between *P. yezoensis* and *Gelidium* sp (Table 1). Commercial agar had gelling temperatures between 34 and 37°C (Supplementary Fig. 2b). Accordingly, agar from *P. yezoensis* treated with 6 to 10% NaOH is comparable in quality to commercial products in terms of gelling temperature. The lowest melting and gelling temperatures in *P. yezoensis* were measured on agar prepared from the 4% NaOH pre-treatment (Table 1). By contrast, melting and gelling temperatures were not significantly affected by increasing NaOH concentration for *Gelidium* sp. agar, although agar prepared with 0% NaOH treatment showed the lowest values (Table 1). *Gelidium* sp. agar generally melted at a higher temperature than *P. yezoensis* agar, but gelling temperature was not different between the two species when pre-treated with NaOH between 6 and 10% (Table 1).

3.3.Chemical properties of agar gels

The precursor of agar is porphyran, which is a sulfated linear galactan consisting of alternate 3-linked β-D-galactose and 4-linked α-L-galactose-6-sulfate residues, the latter of which are sometimes substituted with 3,6-AG and 6-*O*-methyl-D-galactose [40]. Thus, the majority of the native polysaccharide in red algae is present as L-galactose sulfate, which does not form a gel. However, alkali treatment of seaweeds before agar extraction changes the galactan sulfate in porphyran to agarose by converting L-galactose sulfate to 3,6-AG. This process increases the gel strength of the resultant agar [13,14,36,41]. Thus, the decrease in sulfate contents and the increase in 3,6-AG contents promote gelling of porphyran [41]. Accordingly, we quantified contents of sulfate and 3,6-AG to understand the relationship between physical properties and alkali treatment in the *P. yezoensis* agar.

As shown in Table 2, an increase in the alkali concentration resulted in a decrease in the sulfate contents in all three materials, which is consistent with previous observations in *P. yezoensis* [34,37], *P. haitanensis* [31,32], and *G. tenuistpitata* [15]. In addition, sulfate contents in two kinds of *P. yezoensis* agar under 6 to 10% NaOH were less than 0.12%, much lower than that in the *Gelidium* sp. agar showing ca 1% (Table 2) and comparable to commercially released agaroses [16]. By contrast, contents of 3,6-AG were increased in the *Gelidium* sp. agar when materials were treated with alkali (Table 2). Although the contents of 3,6-AG in the *P. yezoensis* agar was ca. 44%, which

was similar to the *Gelidium* sp. agar under all NaOH concentrations (Table 2), we were not able to quantify the changes in 3,6-AG contents under our conditions, because no gelling agar was obtained under the 0% NaOH condition. The contents of 3,6-AG in porphyran from *P. yezoensis* was previously determined as ca. 14% [42,43] and 7.9–10.8% [37]. Thus, it appears that the contents of 3,6-AG in the *P. yezoensis* agar are highly increased by NaOH treatment, as observed for the *Gelidium* sp. agar. These observations support the conclusion that the conversion of L-galactose sulfate to 3,6-AG in *P. yezoensis* by the alkali treatment resulted in gelling of porphyran.

Weight-average molecular weight (Mw) and polydispersity index (Mw/Mn), reflecting molecular weight distribution, are important chemical parameters influencing gel strength [6,22,44]. As shown in Table 2, there were differences in the Mw between the presence and absence of the pre-treatment with NaOH in *Gelidium* sp. agar, although Mw was similar under the presence of NaOH. By contrast, agar obtained from *P. yezoensis* exhibited an increase in Mw in concert with an increase in the NaOH concentration. Evaluation of Mw from agar prepared after 4% and 10% NaOH pre-treatments showed an increase from 1.8 to 2.6 x 10^5 g/mol in dry nori and from 1.9 to 4.3 x 10^5 g/mol in nori sheets. Although we were not able to measure the Mw of the *P. yezoensis* agar under no NaOH conditions, Isaka et al. [42] reported that Mw of porphyran is ca. 2.2 x 10^5 g/mol, suggesting an increase in Mw by alkali treatment in the *P. yezoensis* agar. These observations are different from those for *Melanothamnus somalensis* and *Gracilaria dura* agar, in which an increase in alkali concentrations resulted in a decrease in Mw [16,45]. Thus, the alkali-dependent increase in Mw is a unique characteristic of the *P. yezoensis* agar. In agreement, the dispersivity index (Mw/Mn) was increased in agar from nori sheets, although those of dry nori and

Gelidium sp. agar were largely unchanged (Table 2). Moreover, the molecular weight distribution curves of agar from nori sheets and dry nori shifted to higher molecular weights largely and slightly, respectively, along with an increase in alkali concentration, whereas the molecular weight distribution in *Gelidium* sp. agar was not different under all alkali conditions (Fig. 1). Since higher Mw/Mn values indicate higher heterogeneity of agar molecules with increasing molecular weight [22] as well as the molecular distribution curve, NaOH treatment seems to promote decomposition of agar molecules and elution of high molecular weight agar molecules from the alkali-damaged cell wall, which could result in an increase in the molecular weight of agar chains in *P. yezoensis*.

We also analyzed the relationship between gel strength, which is described by rupture stress and rupture strain [46], and chemical properties (Figs. 2 and 3). We found a positive correlation for rheological qualities like rupture stress and rupture strain with Mw and 3,6-AG content. In addition, these rheological qualities were negatively related to sulfate content. Thus, the high rheological qualities in nori sheets pretreated with 10% of NaOH is related to its high Mw in addition to high 3,6-AG and low sulfate content (Tables 1 and 2). It is worth noting that the sulfate content of the *P. yezoensis* agar is much lower than that of the *Gelidium* sp. agar, which is likely due to characteristics of porphyran from which sulfates are highly removed by alkali treatment [32].

These findings indicate that an alkali pre-treatment of seaweeds increases contents of high molecular weight agar chains and conversion of L-galactose sulfate to 3,6-AG, such that gelling is strengthened with the increase in NaOH concentrations in the *P. yezoensis* agar. These results are similar to those for characteristics of agar from *Gracilaria* [16]. In addition, a negative relationship between sulfate concentrations

and gel strength was also observed in *P. haitanensis* agar [31], although Mw and 3,6-AG content were not determined. Thus, these correlations suggest that the *P. yezoensis* agar could be adjusted to fit the criteria for commercial agar by appropriate alkali treatment.

3.4. Suitability of *P. yezoensis* agar for bacterial growth

We evaluated the growth of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* cultures on plates made with agar from dry nori and nori sheets of *P. yezoensis* in comparison to those made with agar from *Gelidium* sp. as a control. As summarized in Table 3, all bacterial strains grew similarly, without statistical differences in colony numbers, on plates from all agar sources, although the colony size of *B. subtilis* was smaller in the nori sheet plate than in the other two agar plates (Supplemental Fig. 3). These results are consistent with those of Ogawa et al. [34], who checked growth of *E. coli*, *S. aureus*, *P. aeruginosa*, and *Vibrio parahaemolyticus* on *P. yezoensis* agar plates. In addition, the size of halos produced by *B. subtilis* was the same among all three seaweed agar plates containing skim milk (Supplemental Fig. 4). Thus, agar from *P. yezoensis* does not appear to show general inhibitory effects on bacterial growth, and we conclude that it is suitable for use in bacterial culture plates.

3.5. Suitability of *P. yezoensis* agar for electrophoresis of DNA

For electrophoresis of nucleic acids, agarose is usually purified from agar by

removing agaropectin via washing with EDTA, isopropanol, $Al(OH)_3$, or DMSO [23,24,47,48], which involves complicated processes and costs. An important property of agarose is its low amount of sulfate in comparison with agar [23]. As mentioned above, sulfate contents in the *P. yezoensis* agar are very low (Fig. 2, Table 2), and in fact are comparable with those of commercially released agarose (data not shown). These findings lead us to evaluate the viability of using the *P. yezoensis* agar for DNA electrophoresis. Thus, we performed separation of 1 kb and 100 bp DNA marker ladders using 1% gels prepared with agar from nori sheets, agar from *Gelidium* sp., and commercially supplied agarose. Although the DNA ladders did not separate well in *Gelidium* sp. agar, fine separation of DNA was observed with the *P. yezoensis* agar, with even better resolution than that found with the commercial agarose gel (Fig. 4). Our results indicated that *P. yezoensis* agar is applicable for DNA electrophoresis without agarose purification. As far as we know, there are no previous reports of successful DNA electrophoresis using unpurified agar itself. Taken together, our results indicate that *P. yezoensis* agar could provide low cost and high quality performance for DNA electrophoresis.

4. Conclusion

We have demonstrated that *P. yezoensis* is useful as a material for agar production. The physical properties of *P. yezoensis* agar can be controlled by altering the NaOH concentration used on materials during pre-treatment so that the extracted agar has qualities similar to agar from *Gelidium* sp. and *Gracilaria* species. Indeed, we confirmed the utility of *P. yezoensis* agar as a plate medium for several bacterial species

and for DNA gel electrophoresis. It is also worth noting that purification of agarose from *P. yezoensis* agar was not necessary to obtain high resolution during DNA electrophoresis. These findings indicate that the large biomass of *P. yezoensis* produced by mariculture can be effectively utilized to obtain agar for medical and biological applications.

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Figure captions

Fig. 1. Molecular weight distribution curves from GPC experiments of three kinds of agar from *Gelidium* sp. (a), dry nori (b) and nori sheets (c). A value of dw/d(log molecular weight) on the Y axis refers the weight fraction indicating relative amounts of each size of molecules, which is calculated by differentiation of integrated weight fraction (w) from an integral molecular weight curve with respect to a logarithmic value of molecular weight showing on the X axis.

Fig. 2. Relationships between rupture stress and weight-average molecular weight (M_w), polydispersivity index (M_w/M_n), sulfate content, and 3,6-anhydro galactose (3,6-AG) content in agars obtained from *Gelidium* sp., dry nori, and nori sheets.

Fig. 3. Relationships between rupture strain and weight-average molecular weight (M_w), polydispersivity index (M_w/M_n), sulfate content, and 3,6-anhydro galactose (3,6-AG) content in agars obtained from *Gelidium* sp., dry nori, and nori sheets.

Fig. 4. Suitability of *P. yezoensis* agar for gel electrophoresis of DNA. Separation of 100 bp and 1 kb DNA ladder fragments was tested using 1% agar from *Gelidium* sp. or *P. yezoensis* as compared to commercially supplied 1% agarose.

Supplementary Fig. 1. Seaweed samples used in the present study. Dry nori and nori sheets were from Shichigahama, while *Gelidium* sp. was purchased from Morocco.

Supplementary Fig. 2. Comparison of rupture stress and rupture strain (a) and melting and gelling temperatures (b) among 18 commercially released agars. Each dot corresponds to an individual agar that was tested.

Supplementary Fig. 3. Suitability of *P. yezoensis* agar for bacterial growth. Colony formation and growth of *E. coli*, *S aureus*, *P. aeruginosa*, and *B. subtilis* were evaluated on plates containing 0.75% agar prepared from *Gelidium* sp. (left), dry nori (center) and nori sheets (right).

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Table 1

Effect of NaOH concentration during pretreatment of dry nori, nori sheets, and *Gelidium* sp. on yield and rheological properties of agar. Values are indicated with ± SD from triplicate experiments. Alphabetical letters denote statistically significant differences ($P<0.005$) as determined by one-way ANOVA.

	NaOH concentration (%)				
	0	4	6	8	10
Agar yield (%)					
Gelidium sp.	22.3±1.4^{a}	23.7±1.1^{a}	23.3±0.5^{a}	23.6±0.5^{a}	22.0±2.0^{a}
Dry nori	0	12.8±0.7^{b}	11.9±0.2^{bd}	10.6±0.7^{df}	10.4±0.5^{f}
Nori sheets	0	16.8±0.6^{c}	15.5±0.3^{e}	14.0±0.3^{g}	10.5±0.7^{f}
Rupture stress (×10^{5} Pa)					
Gelidium sp.	0.31±0.03^{a}	1.53±0.07^{b}	1.82±0.14^{bd}	1.72±0.06^{bd}	1.98±0.26^{df}
Dry nori	—	0.61±0.07^{c}	1.46±0.09^{e}	2.13±0.21^{f}	2.28±0.09^{f}
Nori sheets	—	0.47±0.06^{c}	1.60±0.12^{de}	2.17±0.13^{f}	2.80±0.13^{g}
Rupture strain (%)					
Gelidium sp.	8.2±0.7^{a}	18.8±1.1^{b}	19.5±1.1^{b}	18.9±0.7^{b}	19.0±1.7^{b}
Dry nori	—	17.7±1.0^{bc}	16.9±0.8^{c}	19.3±1.7^{bc}	19.7±0.2^{bc}
Nori sheets	—	16.1±1.1^{c}	17.8±0.1^{bc}	19.9±1.0^{b}	23.8±1.2^{d}
Melting temperature (℃)					
Gelidium sp.	82.8±0.7^{a}	94.0±0.6^{b}	95.8±0.9^{b}	95.0±0.5^{b}	96.6±2.4^{b}
Dry nori	—	80.1±0.5^{c}	87.7±0.4^{d}	90.5±0.6^{f}	91.5±0.4^{f}
Nori sheets	—	79.5±0.9^{c}	89.7±0.7^{e}	92.5±0.8^{g}	96.0±0.6^{b}
Gelling temperature (℃)					
Gelidium sp.	33.2±0.8^{a}	35.3±0.7^{b}	36.0±0.5^{b}	35.7±0.3^{b}	35.2±0.8^{b}
Dry nori	—	32.5±0.5^{a}	35.5±0.5^{b}	35.6±0.5^{b}	35.8±0.3^{b}
Nori sheets	—	31.8±0.3^{ac}	35.7±0.2^{b}	36.3±0.4^{b}	36.2±0.1^{b}

Table 2

Effect of NaOH concentration during pretreatment of dry nori, nori sheets, and *Gelidium* sp. on molecular weight (Mw), polydispersivity index (Mw/Mn), sulfate content, and 3,6-AG content of agar. Values are indicated with ± SD from triplicate experiments. Alphabetical letters denote statistically significant differences (P<0.005) as determined by one-way ANOVA.

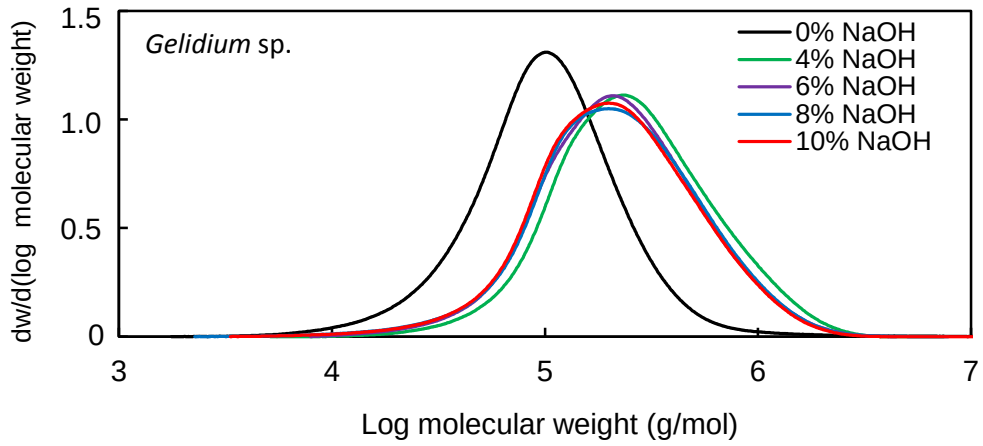
	NaOH concentration (%)				
	0	4	6	8	10
M_w ($\times 10^5$ g/mol)					
Gelidium sp.	1.54±0.12^{a}	3.43±0.20^{b}	3.42±0.25^{b}	3.22±0.18^{b}	3.83±0.68^{be}
Dry nori	−	1.85±0.11^{c}	1.86±0.14^{c}	2.23±0.20^{d}	2.59±0.03^{f}
Nori sheets	−	1.90±0.28^{c}	2.32±0.20^{cd}	2.86±0.36^{bd}	4.28±0.38^{e}
M_w/M_n					
Gelidium sp.	2.02±0.19^{ab}	2.16±0.24^{ab}	2.21±0.14^{ab}	2.25±0.03^{ab}	2.38±0.19^{ac}
Dry nori	−	1.89±0.05^{b}	1.93±0.21^{bc}	1.95±0.03^{b}	2.04±0.06^{bc}
Nori sheets	−	2.02±0.14^{bc}	1.93±0.03^{b}	2.19±0.26^{bc}	2.38±0.18^{ac}
Sulfate content (%)					
Gelidium sp.	2.03±0.12^{a}	1.54±0.10^{b}	1.14±0.07^{d}	1.13±0.16^{d}	1.18±0.19^{d}
Dry nori	−	0.59±0.05^{c}	0.10±0.01^{e}	0.03±0.01^{f}	0.03±0.01^{f}
Nori sheets	−	0.60±0.04^{c}	0.11±0.01^{e}	0.04±0.01^{f}	0.03±0.01^{f}
3,6-AG content (%)					
Gelidium sp.	39.8±1.1^{a}	44.5±1.0^{b}	43.4±0.7^{b}	43.6±1.2^{b}	44.7±0.3^{b}
Dry nori	−	44.7±1.2^{bc}	48.0±0.8^{c}	46.5±2.1^{bc}	46.5±1.6^{bc}
Nori sheets	−	44.8±0.1^{bc}	46.5±2.0^{bc}	46.9±1.1^{bc}	47.2±1.2^{bc}

Table 3

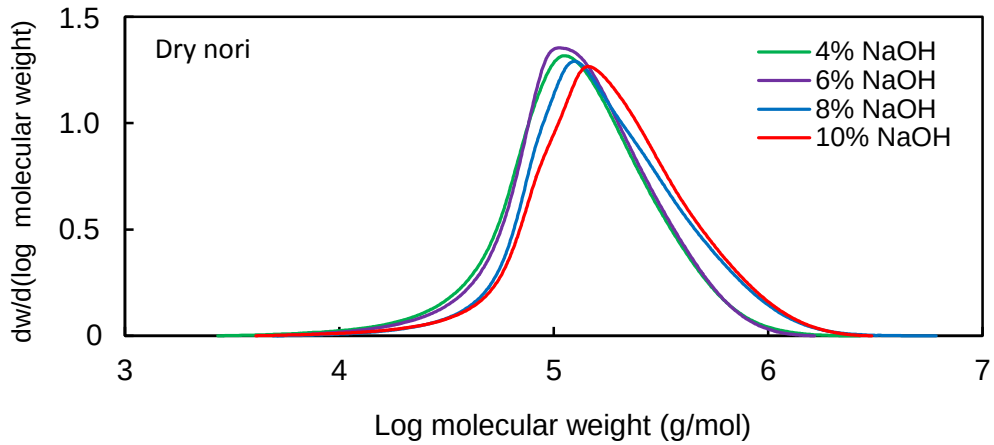
Number of colonies observed from four bacterial cultures grown on solid plates containing 0.75% agar from *Gelidium* sp., dry nori and nori sheets. Values are indicated with ± SD from three independent experiments. No statistically significant differences ($P<0.005$) of colony numbers was obtained among three kinds of plates by one-way ANOVA.

Bacterial species	*Gelidium* sp. agar	Dry nori agar	Nori sheet agar
E. coli	59±3.0	64±4.0	56±7.2
S. aureus	63±2.1	58±6.2	48±13.4
P. aeruginosa	22±2.9	21±2.3	26±4.7
B. subtilis	60±4.9	49±15.1	68±9.0

a


Gelidium sp.
0% NaOH
4% NaOH
6% NaOH
8% NaOH
10% NaOH
dw/d(log molecular weight)
1.5
1.0
0.5
0
3
4
5
6
7
Log molecular weight (g/mol)

b


Dry nori
4% NaOH
6% NaOH
8% NaOH
10% NaOH
dw/d(log molecular weight)
1.5
1.0
0.5
0
3
4
5
6
7
Log molecular weight (g/mol)

c

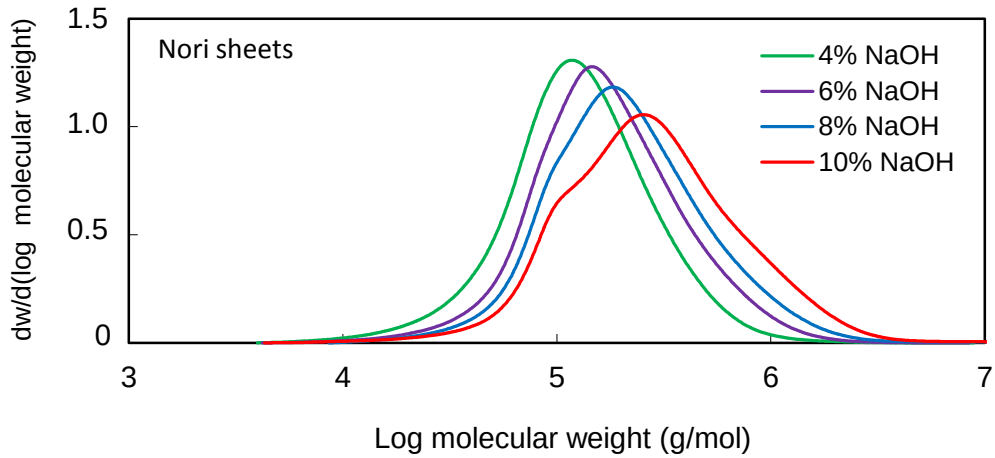

Nori sheets
4% NaOH
6% NaOH
8% NaOH
10% NaOH
dw/d(log molecular weight)
1.5
1.0
0.5
0
3
4
5
6
7
Log molecular weight (g/mol)

Fig. 1

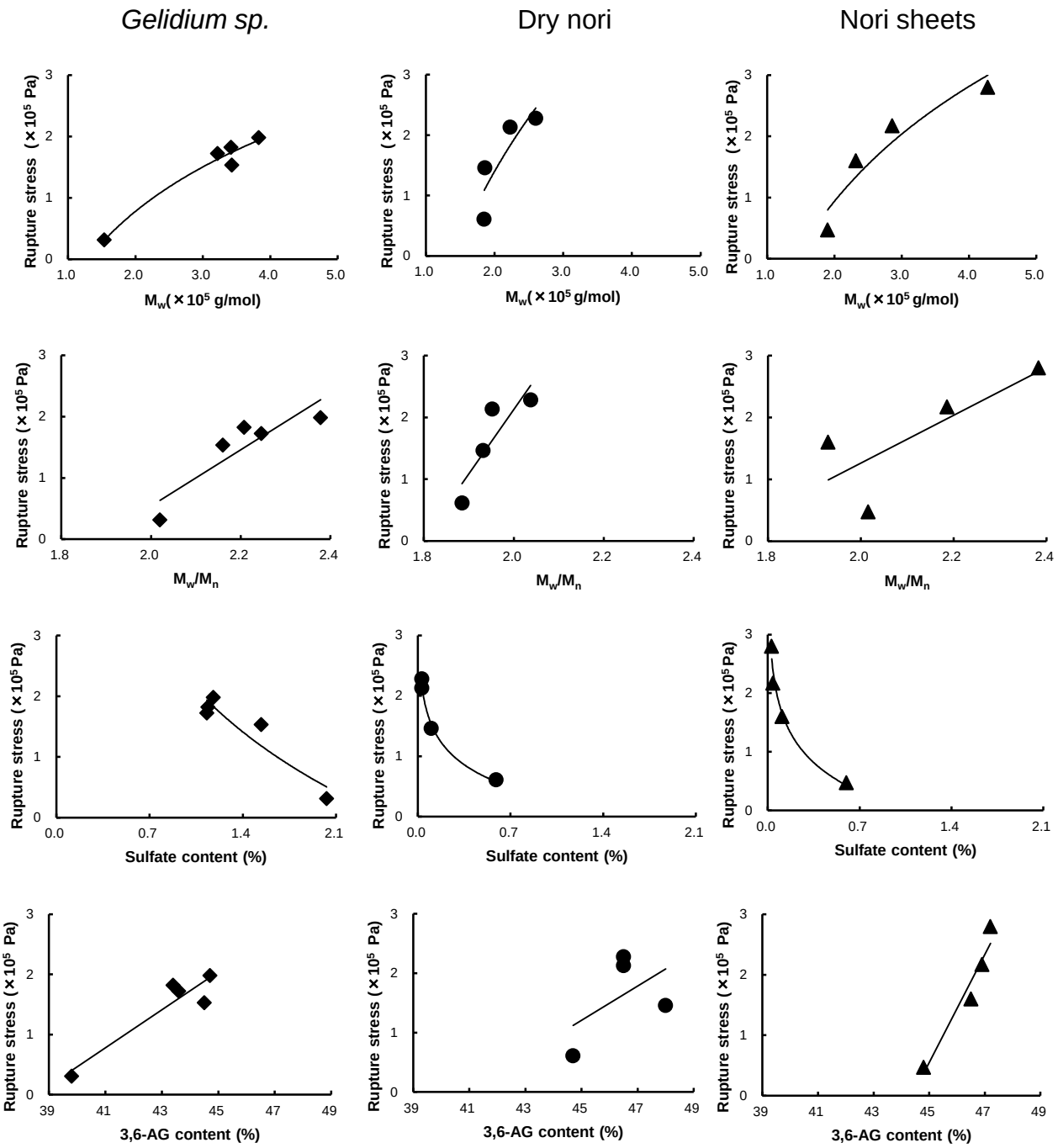

Gelidium sp.
Dry nori
Nori sheets
Rupture stress (×10⁵ Pa)
Mw(×10⁵ g/mol)
Mw/Mn
Sulfate content (%)
3,6-AG content (%)

Fig. 2

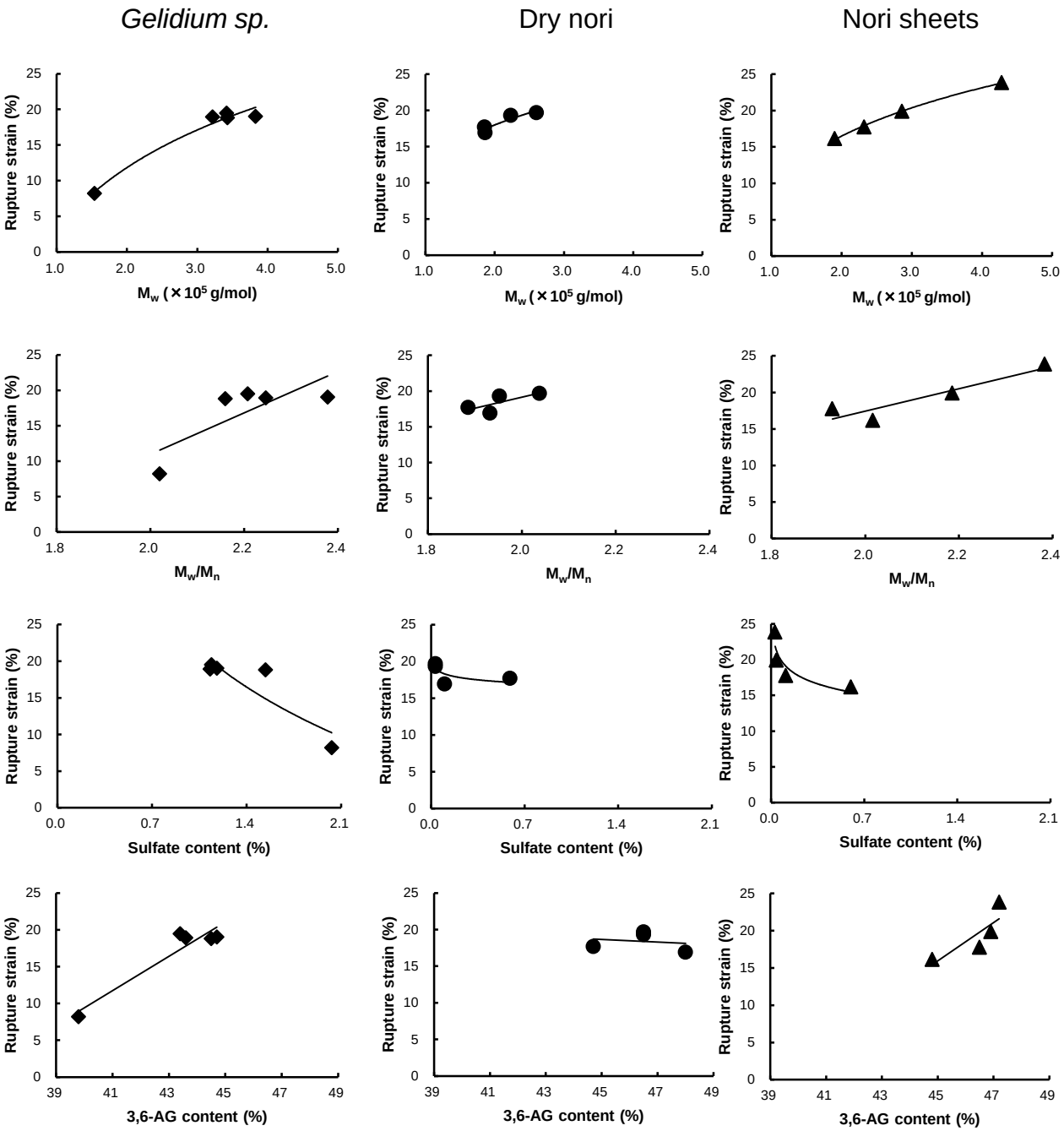

Gelidium sp.
Dry nori
Nori sheets
Rupture strain (%)
Mw (×10^5 g/mol)
Mw/Mn
Sulfate content (%)
3,6-AG content (%)

Fig. 3

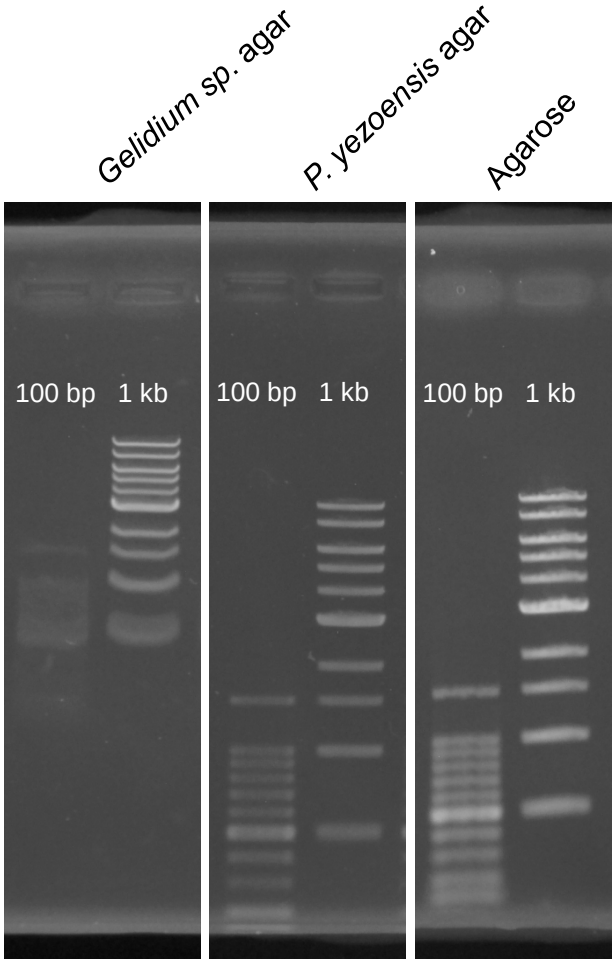
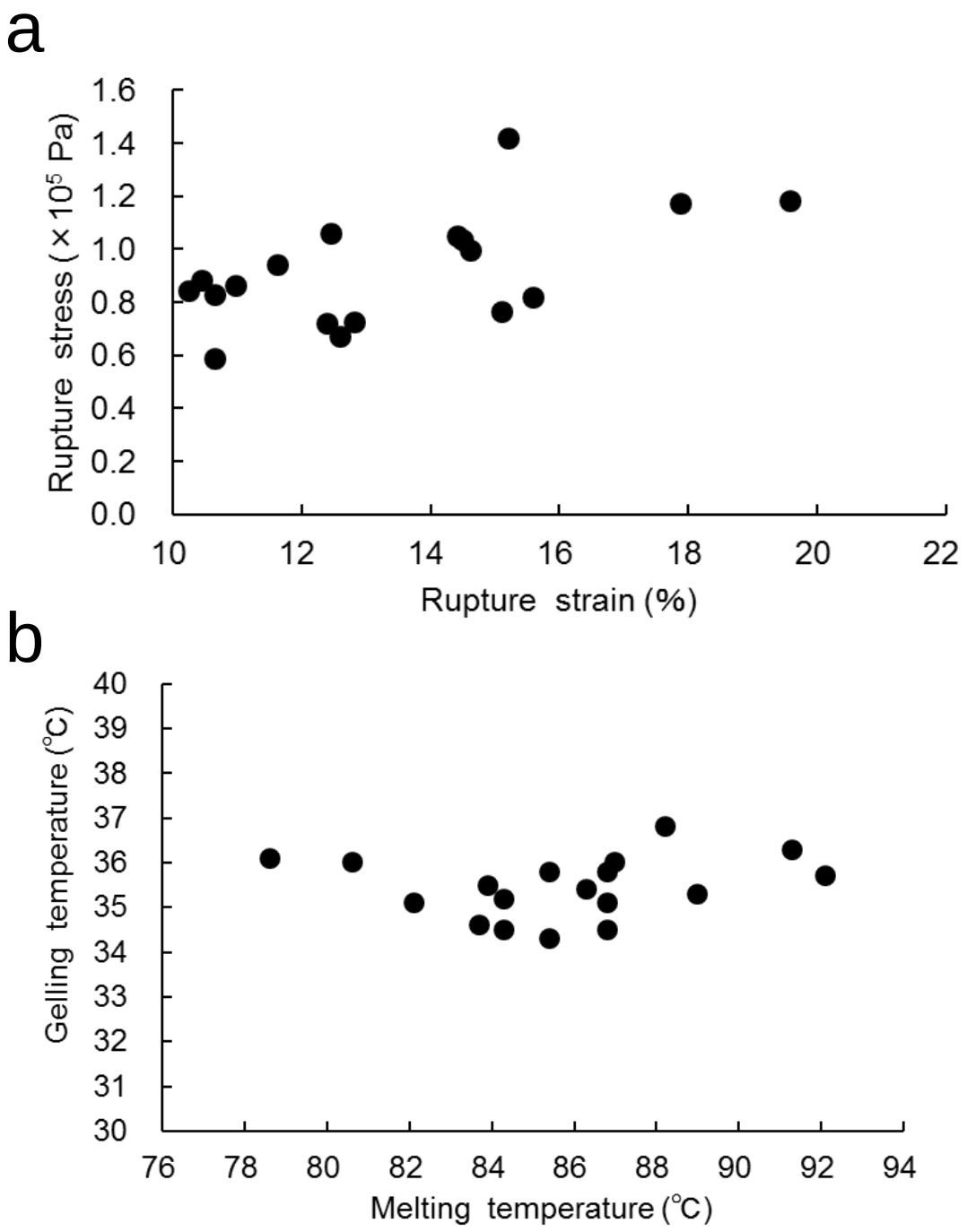

Gelidium sp. agar
P. yezoensis agar
Agarose
100 bp
1 kb
100 bp
1 kb
100 bp
1 kb

Fig. 4

Nori sheets | Dry nori | *Gelidium* sp.

Supplementary Fig. 1. Seaweed samples used in the present study. Dry nori and nori sheets were from Shichigahama, while *Gelidium* sp. was purchased from Morocco.



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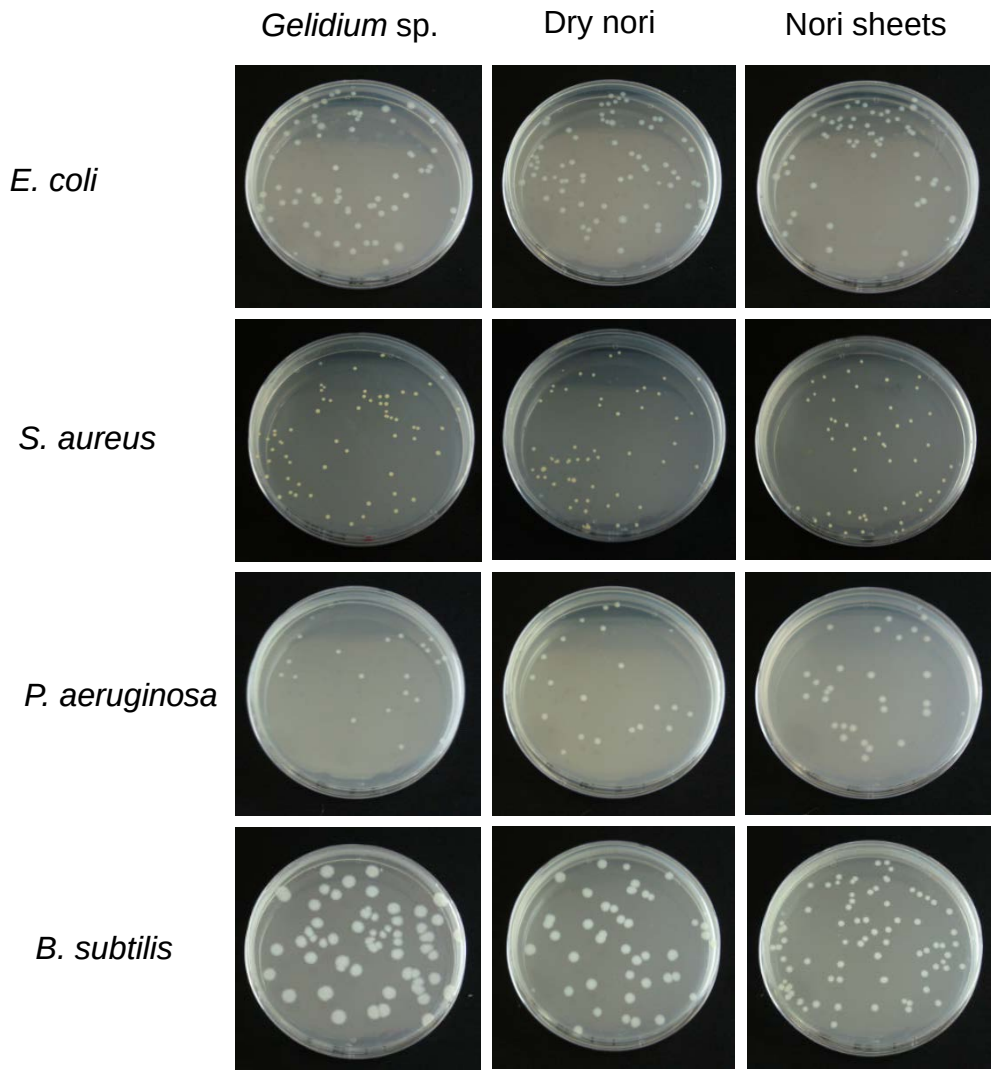

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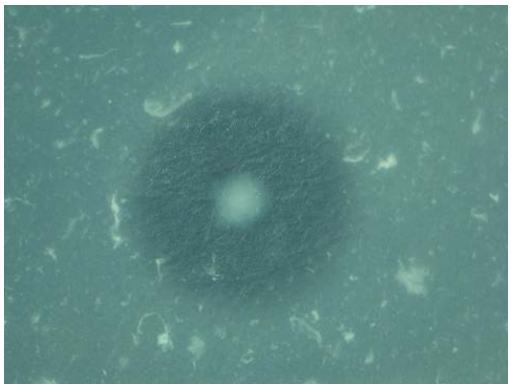
a



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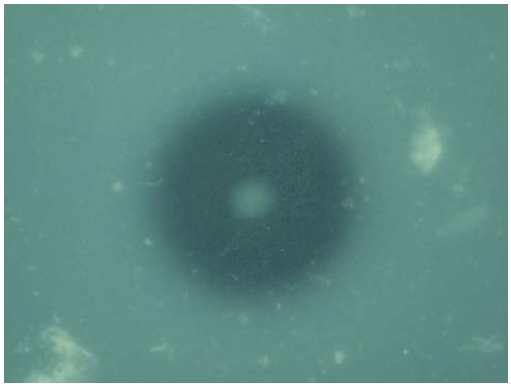
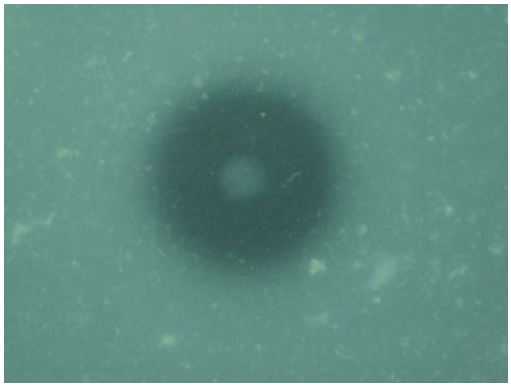


c



Supplementary Fig. 4. Comparison of the size of halos produced by *B. subtilis* on skim milk plates made with agar from *Gelidium* sp. (a), dry nori (b) and nori sheets (c).