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RESEARCH ARTICLE

Sonication and grinding pre-treatments on *Gelidium amansii* seaweed for the extraction and characterization of Agarose

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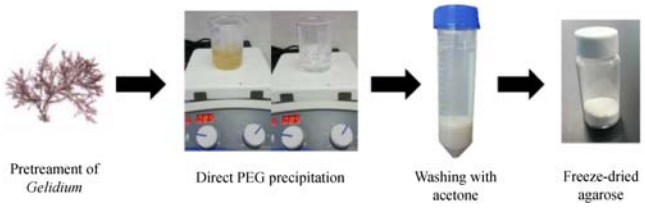
HIGHLIGHTS

- Effects of sonication and grinding pretreatment on agarose quality were observed.
- Successful agarose extraction with direct PEG method without the need to dry agar.
- FTIR spectra and the characteristics peaks in agarose are explained.
- Improvements in gel strength and sulfate content properties in agarose samples.

GRAPHIC ABSTRACT



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ABSTRACT

Various pretreatments methods including sonication and grinding were performed on red seaweed *Gelidium amansii* for the subsequent extraction of agarose. The agarose products are usually extracted from agar powder products from seaweeds. In this study, the agarose was extracted using a direct polyethylene glycol (PEG) method without the need to first process the agar from seaweed. The agar extract was frozen then thawed and mixed directly with PEG solution to precipitate the agarose. The quality of agarose obtained was evaluated through physico-chemical properties analysis which includes spectral technique (FTIR), melting and boiling point, gel strength and sulfate content. These properties were compared with a non-pretreated sample and it was found that the addition of pretreatment steps improved the quality of agarose but gave a slightly lower yield. The gel strength of pretreated samples was much higher and the sulfate content was lower compared to non-pretreated samples. The best pretreatment method was sonication which gave gel strength of 742 g cm^{-2} and sulfate content of 0.63%. The extraction of agarose can be further improved with the use of different neutralizing agents. Pretreating the seaweed shows potential in improving the quality of agarose from seaweed and can be applied for future extraction of the agarose.

1 Introduction

Marine macroalgae contains many beneficial and valuable products, such as agar, alginates, polysaccharides, food, cosmetics, pharmaceuticals and specialty feeds. The

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growing interest in biodegradable polymers is becoming more apparent due to the issues of environmental pollution caused by waste disposal and fossil fuels depletion. This has led to developments on the synthesis of natural polymers from renewable sources such as polysaccharides and other compounds. Agar is a gel-forming polysaccharide obtained from red algae which is commonly used as gelling and stabilizing agents, cosmetics and pharmaceuticals products as well as in biotechnological and medicinal researches (Guerrero et al., 2014). The value of agar is dependent on its physico-chemical properties such as gel strength, gelling temperature, melting temperature and sulfate content. Agars with very low gel strength are not desired for industrial applications while agars with too high gel strength will not always be desired for certain applications (Pereira-Pacheco et al., 2007). Different seaweed sources and extraction conditions will also produce agar of different gel properties. Typically, *Gelidium* sp. tends to yield agar of better quality in terms of gel strength, but the extraction costs will be higher (Wang et al., 2017).

Agarose is a polysaccharide polymer material created through the purification of agar. It is composed of repeating units of agarobiose, which is a disaccharide unit made up of (1,3) linked β-D-galactopyranose and (1,4) linked α-3,6-anhydro-L-galactopyranose (Araki, 1966). Agarose is commonly extracted from red seaweed species such as *Gracilaria*, *Ceramium*, *Gelidium*, *Pterocladia*, *Acanthopeltis* and *Campylaephora* (Cole and Sheath, 1990). The demand for agarose is high as it can be used in various applications such as gelling agent, protein purification, thickeners, wastewater treatment, bone filler and particularly gel electrophoresis (Kolanthai et al., 2016; Seow and Hauser, 2016). The poor yield and difficulty in quality maintenance of agarose products using conventional methods restricts the efficient extraction of this valuable polysaccharide. Conventional methods like quaternary ammonium precipitation would give good yield and quality but is infeasible for large scale production as a tedious washing procedure is required (Hjertén, 1962). Other methods like the extraction with polyethylene glycol medium or dimethyl sulfoxide utilize the solubility properties of agarose, where a faster production rate can be achieved but with lower yields (Duckworth and Yaphe, 1971; Jeon et al., 2005). Furthermore, alternative methods such as the use of ionic liquid to selectively precipitate agarose has been conducted in recent studies (Sharma et al., 2015; Trivedi and Kumar, 2014). This extraction technique is more cost effective and environmental friendly as the ionic liquid can be recycled and reused for subsequent batches of agarose extraction (Chew et al., 2018). Hence, the focus is on the discovery of more efficient and eco-friendly agarose extraction processes from algae.

Various pretreatments have been performed on seaweed to enhance the production of valuable biomolecules and improve the quality of the products obtained from seaweed. Pretreatments such as acid hydrolysis were found to increase polyhydroxybutyrate (PHB) production in brown macroalgae (Azizi et al., 2017). The washing of seaweeds with hydrochloric acid, sulfuric acid and phosphoric acid pretreatments were also tested to observe its effect on the thermal degradation of seaweed biomass. It was reported that the use of selective acid pretreatments could influence the algae biomass pyrolysis characteristic and promote the thermal conversion of the biomass (Hu et al., 2016). Furthermore, the use of ultrasound assisted extraction reported higher yields for biomolecules such as phycobiliproteins from marine macroalgae. The compression and decompression mechanism created by the sound waves resulted in the disruption of the cell walls, providing higher yields at a lower process time (Chemat et al., 2017; Mittal et al., 2017). The use of mechanical pretreatment like beating also has potential to increase the methane production from brown seaweeds. The beating pretreatment will reduce the particle size of the biomass and allow better accessibility of the anaerobic microorganisms to the organic matter. Montingelli et al. (2017) reported that the use of mechanical treatment on brown seaweed led to higher methane production compared to the untreated sample (Montingelli et al., 2017). Besides that, microwave-assisted extraction of agar showed better quality agar compared to traditional extraction, and at the same time reducing the extraction time, solvent consumption and waste disposal requirements (Sousa et al., 2010). The use of alkali pre-treatment has also been found to improve the gel strength of agar through the desulphation reaction, leading to agar products of higher quality (Meena et al., 2011).

There are many research that have reported the impact of pretreatments on the yield and quality of agar. However, little information has been described for the characterization of agarose that can be potentially extracted from the pretreated seaweed. Therefore, the objective of this research was to investigate the effect of various pretreatment methods on the agarose yield and physico-chemical properties. The pretreatment methods tested include HCl treatment, grinding of seaweed, sonication and grinding-sonication combination. The extracted agarose was characterized by analytical methods and its physico-chemical properties were measured and compared with commercial grade agarose product.

2 Materials and methods

2.1 Materials

Gelidium amansii red algae was obtained from an algae culture laboratory. The seaweeds were washed with tap water and distilled water consecutively to remove sand particles and impurities. The washed seaweeds were dried

at 50°C for 3 to 4 days using an air drying oven (Memmert, Germany). The dried seaweeds were peeled to smaller strips for extraction purposes. Polyethylene glycol (PEG), hydrochloric acid [HCl], sodium hydroxide [NaOH], barium chloride [$BaCl_2$], potassium sulfate [K_2SO_4], gelatin, trichloroacetic acid [TCA], ethanol and acetone were obtained from R&M Chemicals (Malaysia). All chemicals used were of analytical grade.

2.2 Pretreatment of seaweed

The *G. amansii* seaweed was pretreated by four different pretreatment methods and the resulting agarose product quality was compared with a non-pretreatment extraction (NPT).

2.2.1 HCl treatment (HCT)

The seaweed samples (5 g) were immersed in beaker containing 300 mL of 0.1 M HCl solution and placed inside a refrigerator at 4°C for 30 min. The acid treated seaweeds were washed thoroughly with water and then neutralized using NaOH solution until pH of about 7. After the washing, the seaweeds were prepared for extraction.

2.2.2 Grinding with HCl treatment (GRD)

The seaweed samples were grinded into smaller fractions using pestle and mortar for about 5 min. The grinded seaweeds were then immersed in a beaker containing 300 mL of 0.1 M HCl solution and placed in a refrigerator at 4°C for 30 min. After the acid treatment, the seaweeds were washed thoroughly with water, neutralized with NaOH and prepared for extraction.

2.2.3 Sonication with HCl treatment (SCT)

The seaweed samples were soaked in a beaker containing 300 mL of 0.1 M HCl solution and sonicated at 30 kHz, 40% amplitude for 30 min (Elma, Germany). Ice was added during the sonication process to prevent overheating. After the sonication process, the seaweeds were washed thoroughly with water, neutralized with NaOH and prepared for extraction.

2.2.4 Sonication with grinding treatment (SGD)

The seaweed samples were first grinded into smaller fractions, then soaked in a beaker containing 300 mL of 0.1M HCl solution and sonicated at 30 kHz, 40% amplitude for 30 min. Ice was added during the sonication process to prevent overheating. After the sonication process, the seaweeds were washed thoroughly with water, neutralized with NaOH and prepared for extraction.

2.3 Preparation of agarose

For the pretreated samples, after each treatment, the seaweed was heated in distilled water at 99°C for 3 h, following a ratio of seaweed to water of 1:25. For the NPT sample, the seaweeds were heated in distilled water at 99°C for 3 h directly after washing. The agar solution produced was filtered using cellulose nitrate membrane (3 μm) under gravity to remove impurities and insoluble particles. The filtrate was left to cool to room temperature and later stored at 4°C overnight. A 40% w/w PEG solution was prepared by dissolving PEG in ethanol and kept at the temperature of 70°C–75°C. The agar solution was left at room temperature and then mixed with the PEG solution at a volume ratio of 1:1 under vigorous stirring. After stirring for 5 min, white precipitates will appear and the mixture was left to cool at room temperature. The suspension was centrifuged at 5500 r/min for 15 min and the white precipitate was washed with distilled water at least three times to remove the PEG residue, followed by washing with acetone. Lastly, the recovered agarose was lyophilized for 24 h using a freeze dryer (Christ, Germany). The freeze-dried agarose was milled into powder for characterization purposes. The yields of agarose were expressed as % w/w on the dry basis of *G. amansii* seaweed taken.

2.4 Characterization of agarose

Fourier transform-infrared (FTIR) spectra were measured at room temperature using the agarose powder in the range of 4000–400 cm^{-1} (Perkin Elmer, Germany). Gelling temperature was measured by preparing hot agarose solution (1.0% w/v) in a test tube and allowing it to cool to room temperature while immersing a thermometer in the agarose solution. The test tube was held at an inclined position once every minute and the gelling temperature was recorded when the meniscus of the agarose solution stopped returning to its initial position. Melting temperature was determined by preparing a 1.0% w/v agarose solution and kept overnight at room temperature. A plastic bead was placed on the gel surface and the test tube and thermometer was placed in a water bath while increasing its temperature. The melting temperature was recorded when the plastic bead dropped to the bottom of the test tube. Agarose gel strength (g/cm^{-2}) was measured by making 1.0% w/v cylindrical agarose hydrogels of approximately 8–10 mm depth and 12–13 mm in diameter. A TA.XT plus texture analyzer (Stable Micro System, UK) was used to compress the agarose hydrogels at 1 mm/sec to approximately 40% of their original thickness. Stress-strain plot data was obtained and the gel strength was calculated based on the initial slope of the stress-strain plot. To determine the sulfate content of agarose, the method by Dodgson (1960) was used. A gelatin reagent was prepared

by dissolving 2 g of gelatin in 400 mL of hot water (60°C–70°C) and allowed to stand at 4°C overnight. 2 g of barium chloride was dissolved in the semi-gelatinous fluid and the resultant solution was allowed to stand for 3 h. The reagent was stored at 4°C and could be used for one week. A reagent blank was prepared by using 0.2 mL of distilled water added with 3.8 mL of 4% w/v TCA followed by 1 mL of the $BaCl_2$-gelatin reagent. The mixture was mixed and left at room temperature for about 10–20 min before measured using a UV-Vis Spectrophotometer (Shimadzu, model UV-1800) at absorbance of 360 nm. The sulfate-containing solution will replace the 0.2 mL distilled water for the determination of sulfate content. A calibration curve was prepared with solutions of K_2SO_4 containing between 20 and 400 μg of SO_4^{2-} ions. Each experimental test was done in triplicates.

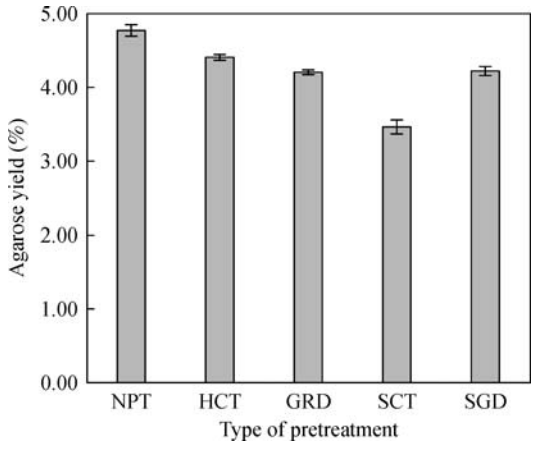

Fig. 2 Agarose yield obtained from different pretreatment methods

3 Results

The FTIR spectra of the extracted agarose are shown in Fig. 1. The FTIR spectra shows the characteristics peaks of agarose from IR bands at 3430 cm^{-1}, which is attributed to the stretching band of hydroxyl group, 1071 cm^{-1} attributed to the -C-O-C- and glycosidic linkage, while the spectra 932 and 890 cm^{-1} indicates the -C-O-C- bridge of 3,6-anhydrogalactose unit. For both the NPT and SGD, there is a slightly higher peak at the absorption band of 1258 cm^{-1}, indicating the presence of asymmetric stretching of S = O ester sulfate (Meena et al., 2007; Trivedi and Kumar, 2014; Sharma et al., 2015).

The agarose yield obtained is shown in Fig. 2. The overall agarose yield is calculated on the basis of dry seaweed. The fractionation of agarose from agar using PEG involves the mixing of an agar solution with a PEG solution at a temperature between 70°C and 100°C. After cooling, the precipitate settles out and a yield of 30% to 45% can be obtained (Alfred, 1967). This extraction method was selected for its relatively high agarose yield. For the direct PEG modification, the agar gel obtained directly from the boiling process of seaweed was thawed and added into the PEG solution and then mixed. Usually, the agar would need to be dried and milled into powder, this would be followed by the dissolving of the agar powder in water and then extracting the agarose from the agar solution. This direct PEG method applies the frozen agar gel directly into the PEG solution for agarose extraction. The yield of the NPT seaweed samples appears to be the highest, while the SCT samples showed the lowest yield.

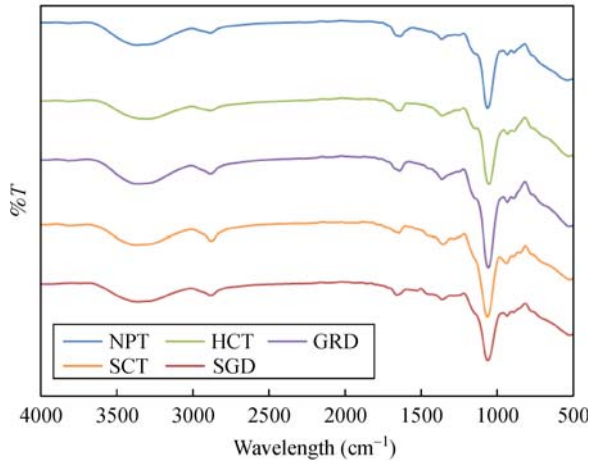

Fig. 1 FTIR spectra of agarose products from different pretreatment methods

The gelling and melting temperatures of agarose are shown in Table 1. Comparing the temperature values of commercial agarose, the typical gelling and melting temperature was found to be approximately 37°C and 90°C, respectively (Sharma et al., 2015; Wang et al., 2012). The gelling and melting temperatures of agarose obtained from this study are about 24.0°C–28.1°C and 76.7°C–77.9°C, respectively. These temperature values are lower by about 10°C for both the gelling and melting temperatures when compared with commercial and agarose from different extraction processes, making the agarose products from this study slightly suited toward low-melting agarose products, which is particularly useful

Table 1 Gelling and melting temperatures of agarose products from different pretreatment methods

Treatment	Gelling temperature(°C)	Melting temperature(°C)
NPT	26.1±0.1	77.7±0.4
HCT	24.9±0.8	76.9±0.2
GRD	25.8±0.1	76.8±0.1
SCT	28.1±0.4	77.9±0.4
SGD	24.0±0.4	76.7±0.6

in molecular biology for isolating nucleic acids from gels without prior purifications.

The gel strengths of the agarose extracted are shown in Fig. 3. A large variation between SCT and other treatments are observed, where SCT shows the gel strength of 742 g/cm^2, which is about two or more times higher than NPT, HCT and GRD. The other treatments showed the gel strength of 192, 227, 364 and 610 g/cm^2 for NPT, HCT, GRD, and SGD, respectively. All the pretreatment methods yielded a higher gel strength compared to NPT, although only a slight increase is seen for HCT. SCT and SGD samples showed much higher gel strength compared to the rest. As for the sulfate content, the use of a non-enzyme hydrolysis method was selected, which is deemed suitable for carbohydrate sulphates. The absorbance at 360 nm was used as greater sensitivity was found at this wavelength range (Dodgson, 1961). The results of the sulfate content analysis for the pretreatment methods are shown in Fig. 4. The sulfate content of the agarose samples range from 0.61% to 1.40%. NPT samples showed the highest sulfate content (1.40%) compared to all pretreated samples, where HCT, GRD, SCT, and SGD samples showed a sulfate content of 0.86%, 0.88%, 0.63%, and 0.61% respectively. SCT and SGD showed among the lowest sulfate content.

4 Discussion

The use of direct PEG method was successful in obtaining agarose, and this method can avoid the lyophilization of agar by directly adding the gelatinous agar solution into the PEG solution after maintaining the temperature of both solutions at 70°C–75°C. The overall yield of agarose ranges from 3.5%–4.8% and the lower yield observe by all the pretreated samples is likely due to the degradation of polysaccharides during the alkali treatment and also by agar losses through diffusion during the processing (Freile-Pelegrın and Murano, 2005). SCT pretreatment shows the lowest yield as the ultrasonication of the seaweed was found to promote the extraction of polysaccharides other than sulphated polysaccharides, which includes agarose, and this means than no benefit is obtained from ultrasonication in terms of agarose yield (Fidelis et al., 2014). As for SGD, the higher yield may be a result of the better dispersion of the grinded seaweed which enabled better extraction of agarose from the seaweed. Besides that, the yield of agarose is also dependent on factors such as the boiling time and temperature, where prolonged boiling of seaweeds at high temperature can lead to the degradation of agar and agarose compounds. The environmental conditions such as light availability, sea temperature and salinity also affects the quality and yield of agar (Hurtado et al., 2011; Chirapart and Praiboon, 2018).

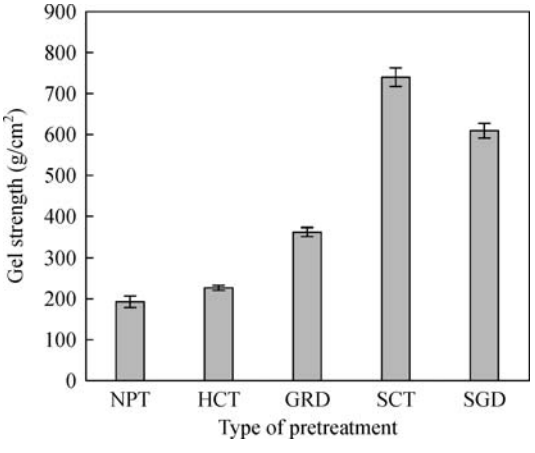

Fig. 3 Gel strength of agarose obtained from different pretreatment methods

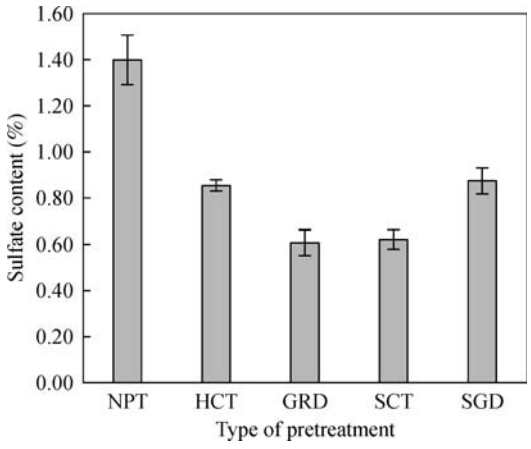

Fig. 4 Sulphate content of agarose obtained from different pretreatment methods

The gelling and melting temperature of the agarose obtained was lower than the typical commercial agarose. This is likely due to the hydroxyethylation of agarose as PEG, also known as polyethylene oxide, was mixed and reacted with agar solution to extract agarose. The mixing of PEG with agar solution might have caused the hydroxyethylation reaction and this will reduce the packing density of agarose bundles and effectively reduce its pore size. Hence, its gelling and melting temperature has decreased, making it more suitable for gel electrophoresis (Lee et al., 2012).

The gel strength is an important indication of the agarose quality and can depend heavily on the chemical structure of the polysaccharide. The use of hydrochloric acid (HCl) in low concentrations has been tested and proven to increase gel strength. However, too high concentrations of HCl will lead to agar of poorer quality. The concentration of HCl used in this study is 0.1 M which is sufficient to improve the gel strength (Matsuhashi, 1977). The high gel strength by SCT and SGD is likely due to effect of ultrasonication on the seaweed which has been found to

improve the mechanical strength of agarose (Żyła et al., 2017). The gel strength for HCT and GRD are not very high as compared to the samples which have undergone sonication, this is likely due to the effect of alkali treatment which will decrease the gel strength of agar, leading to consequence reduction during agarose extraction. The overall gel strength may also be affected by the insufficient transformation and conformation of the substitute groups in the agarose polymer, as well as the alkali treatment time (Arvizu-Higuera et al., 2008).

The overall sulfate content of the agarose samples was found to be higher than commercially and other agarose products (Jeon et al., 2005; Sharma et al., 2015). It is believed that the technique used for determining the sulfate content could be a factor to the high sulfate content results obtained. A work by Al-Nahdi et al. (2015) used the similar sulfate determination method for agar and yielded sulfate contents of around 5.4 to 10.1%. The high sulfate content in agarose could also be due to the environmental conditions at the seaweed collection site, where elevated temperatures will result in agarose with higher sulfate content (Al-Nahdi et al., 2015). The focus of this study is to determine the effect of pretreatment on the physico-chemical properties of agarose, which includes sulfate content, and it was discovered that the use of pretreatment was able to reduce the sulfate content significantly through SCT and SGD. These results indicate that apart from the addition of HCl, the sonication treatment of seaweed could potentially reduce the sulfate content. This is likely due to the effect of sonication which can induce sulfate precipitation (Davies et al., 2015; Dodds et al., 2007). The sulphated polysaccharide portion in agar, which is agaropectin, is possibly extracted and separated more efficiently after sonication during the PEG extraction, resulting in agarose with lower sulfate content. Nonetheless, the combination of both grinding and sonication treatments does not yield a lower sulfate content compared to just sonication treatment.

Among the treatment methods performed, SCT was found to be the most advantageous as this treatment has successfully increased the gel strength and reduced significantly the sulfate content of agarose, despite its lower yield. The low yield can be increased through the use of different neutralizing agents. The discrepancies in values of physico-chemical properties of agarose reported by various studies with this present study may be caused by the distinctive methods used for measurement of the properties. Since no standard protocols have been made for agarose characterization, it is inaccurate to compare the existing results with the specifications provided by industries and manufacturers (Wang et al., 2012). This study serves the purpose to evaluate the potential of pretreatment addition for the enhancement of agarose product quality, as well as to assess the feasibility of using direct PEG method for agarose extraction from seaweed.

5 Conclusions

In this work, agarose was successfully extracted from algae using a direct PEG extraction method with pretreatment. The yield obtained from agarose samples with pretreatment were slightly lower compared to non-pretreated sample but the physico-chemical properties of the resulting agarose with pretreatment are much better. The use of direct PEG extraction can reduce the time and energy needed for agarose extraction without the need to first prepare and dry the agar products. Direct PEG extraction for agarose will also likely result in agarose products with lower melting and boiling points. The sonication treatment is effective in improving the gel strength of agarose and the HCl treatment has successfully reduced the sulfate content of agarose. Additions of pretreatment step on seaweed will be beneficial in enhancing the quality of the agarose products.

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