

## ANALYSIS OF NATIVE AND ALKALI-TREATED AGAR FROM SPECIES OF *Gracilaria* (GRACILARIACEAE, RHODOPHYTA) COLLECTED FROM MORIB, MALAYSIA.

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**ABSTRACT** Seaweeds are marine algae commonly found in brackish water to marine environments. The red seaweed *Gracilaria* (Gracilariaceae, Rhodophyta) is commonly exploited for the production of agar. Alkaline pre-treatment has been shown to increase the quality of agar in previous studies. The aim of this study was to compare the effect of native (untreated) and alkali treatment on the yield and physical properties of agar from *Gracilaria changii* (Xia & Abbott) Abbott, Zhang & Xia, *Gracilaria salicornia* (C.Agardh) Dawson and *Gracilaria edulis* (Gmelin) Silva collected from Morib in Malaysia. Compared to native samples (control), an addition of alkali pre-treatment step which involved soaking samples with 0.5M NaOH for 1 hour at room temperature prior to extraction was included for the treated samples. Comparative analysis showed that alkali-treated agar of *G. edulis* gave the highest agar yield (82.56 %) whereas the highest gel strength was obtained from native agar of *G. salicornia* ( $430 \pm 17.32$  g cm<sup>-2</sup>). Alkali treatment was found to be effective only for the increase of gel strength in *G. edulis* but not in *G. changii* and *G. salicornia*. The native and alkali-treated agars in all species gave comparable gelling and melting temperature which were in the range of (36-39°C) and (80-87°C) respectively. The overall results showed that *G. salicornia* had superior agar quality and is thus a potentially good source for industrial use. Analysis on chemical properties of agars can be performed in future to determine the agar composition of these species.

**(Key words:** agar; gel strength; *Gracilaria*; red seaweed; physical properties; yield)

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### INTRODUCTION

Agar is a colloid, or a gelatinous substance derived from seaweeds belonging to the Rhodophyceae which includes the orders *Gelidiales* and *Gracilariales* (Pereira-Pacheco et al. 2007). Agar is also known as a type of polysaccharide or a matrix component extracted from cell walls of red seaweeds (Li et al. 2008) which functions as the primary structural support for the algae cell walls. Agar consists of a mixture of neutral agarose and charged agaropectin (Santos 1990). The basic repeat of the polysaccharide is agarobiose (galactose) which consists of alternating 4-O-3,6-anhydro- $\alpha$ -L-galactopyranose and 3-O- $\beta$ -D-galactopyranose (Lahaye and Yaphe 1988). The yield and physical properties of agar as well as the chemical characteristics determine its value in

the industry (Marinho-Soriano 2001). Agar is important for industrial use due to its excellent thickening and gelling properties. The parameters which determine its quality includes the gel strength, gelling and melting temperatures, viscosity, sulphate content, 3,6-anhydrogalactose content and syneresis index (lost of water during ageing) (Pereira-Pacheco et al. 2007).

*Gracilaria* is the major world-wide agar source (Freile-Pelegrin and Murano 2005) and can be found in temperate as well as in tropical countries (Santos 1990). *Gracilaria* species have become an important source of agar due to the shortage of *Gelidium* species (Pereira-Pacheco et al. 2007). *Gracilaria changii* is the most

abundant agarophytic seaweed found in Malaysia and is recently cultured mainly for production of agar (Phang et al. 1996). This species can be found in west and east coast of peninsular Malaysia (Phang 2006). *G. changii* is a good source of high quality agar and agarose and has been experimentally cultivated in shrimp ponds, mangrove ponds and water supplying canals (Phang et al. 1996). Generally, native agars of *Gracilaria* species are low in quality due to high sulfate content which produces low gel strength of agar. Hence, alkali pre-treatment is used prior to the agar extraction step in order to eliminate the sulphate groups and enhance their gel forming ability (Pereira-Pacheco et al. 2007). However, the low gel strength of the native agars of this genus, together with their low syneresis, allow their use as gelling agents for spread foods, soft-texture confectionery and as emulsifier (Freile-Pelegrin and Murano 2005).

The gel strength and agar constituent vary with species, location, season and extraction method (Marinho-Soriano 2001). According to Marinho-Soriano (2001), gel strength of agar is species-specific although the samples were collected from the same location or share the same environmental conditions. In traditional extraction protocol of agar, a chemical is used as the bleaching agent (Li et al. 2008). Pre-treatment with alkali and acid on the raw material using 1N NaOH and 0.5% acetic acid before agar extraction has been applied (Roleda et al. 1997). Previous studies from Freile-Pelegrin and Murano (2005) reported that alkaline treatment strongly improved the gel strength and increased both gelling and melting temperatures of agar extracted from *Gracilaria crassissima*. The gel strength of this species was improved to 1390 g cm<sup>-2</sup> in 7% NaOH treated agar. The gelling temperature was also increased to 50°C while the melting temperature was increased to 93°C after the native agar was treated with 7% NaOH. However, the study from Freile-Pelegrin and Murano (2005) showed that the gel strength, gelling temperature and melting temperature of agar extracted from *Gracilaria cervicornis* and *Gracilaria blodgettii* were not affected by alkaline treatment. A study from Buriyo and Kivaisi (2003) also showed that alkali treatment reduced the yield of agar (% dry weight) by almost 25-56 % for *Gracilaria salicornia* in Tanzania.

This study was aimed to determine the quality of agars from *G. changii*, *G. salicornia* and *G.*

*edulis* collected from the mangroves at Morib, Malaysia and to study the effect of alkali pre-treatment during agar extraction, on the yield, gelling and melting temperatures as well as the gel strength of the extracted agars.

## MATERIALS AND METHODS

*G. changii*, *G. salicornia* and *G. edulis* were collected in August 2009 at Morib (02° 45.808' N; 101° 26.143' E), west coast peninsular Malaysia. The samples were washed thoroughly with tap water to remove the epiphytes and impurities. The samples were sun-dried and kept in a plastic bag to store at -80°C freezer until agar extraction in the laboratory. Samples of the three *Gracilaria* species were prepared as herbarium specimens for record.

### Agar extraction.

The native agar extraction was performed according to the method described by Phang et al. (1996) with some modifications. Dried samples of *G. changii*, *G. salicornia* and *G. edulis* (10g), respectively were immersed separately in 300 ml of distilled water for 10 minutes and heated for 15 min at 121°C in an autoclave. The hot solution was filtered through filter cloth (10 cm× 4 m cotton crepe bandage) (AnjiWande Medical Products Co. Ltd., China) to remove the algal residue. The filtrates were transferred into a beaker to gel at room temperature (24°C) and frozen overnight at -20°C. The frozen gel was thawed on the next day at room temperature. The thawed water was drained to remove the soluble impurities. The agar was then washed with 100 mL 95% isopropanol, followed by 100 mL 80% isopropanol. All the isopropanol was discarded and all the gel in the beaker was transferred into an empty petri dish (145/20 mm, Greiner bio-one, Germany) to dry at 50°C until the weight was constant for agar yield determination. Alkali-treated agar extraction was carried out where the sample was being immersed in 300 mL of distilled water for 10 minutes followed by a few drops of 0.5M NaOH being added into the soaked samples and incubated at room temperature for 1 hour. The pH of the agar suspension was then adjusted to pH 8. After that, the extraction and other remaining processes were carried out as in the native agar extraction. All the agar extractions were done in triplicate for each species. The agar yield of each replicate

was calculated as the percentage of dry matter based on the following formula:

$$\text{Agar yield (\%)} = \frac{\text{Dry weight of agar (g)}}{\text{Dry weight of seaweed (g)}} \times 100$$

#### Physical properties determination.

Gelling and melting temperatures and gel strength of agar were determined on 1.5% (w/v) agar solutions. Gel strength determination was carried out using a Nikkansui Gel Strength tester on 1.5% gel. The gelling temperature was determined from the melted agar solution in the test tube (15×120 mm) which was allowed to gel slowly at room temperature (24°C). The test tube was then constantly moved (inclined to a 45° angle). A thermometer was inserted into the agar and the temperature was recorded as the gelling temperature when the solution was stopped

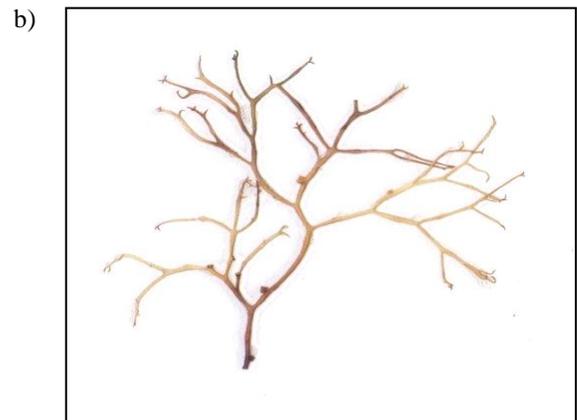
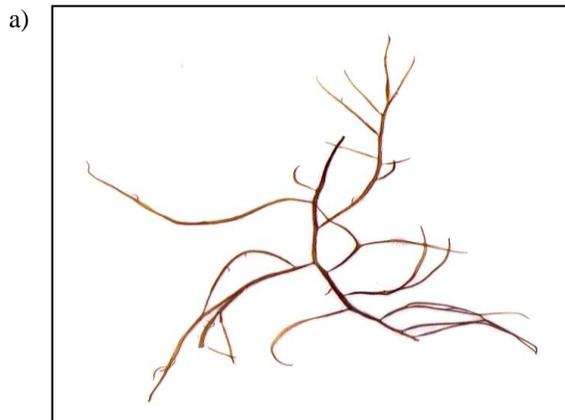
Herbarium specimens were prepared with different thalli of *G. changii*, *G. salicornia* and *G. edulis* on herbarium mounting paper for the comparison of morphological characteristics (Figure 1). The colour observed for *G. changii* was purplish while it was yellowish to reddish colour observed for *G. edulis*. The colour for *G. salicornia* was slightly darker than that of *G. changii*. *G. changii* had thicker and longer thalli compared to *G. edulis*. Both species had irregular branching and *G. edulis* was found to be attached closely to form thick bunches. *G. edulis* has fastigiated branches with bifurcate tips. There are segments separated by constrictions along the thallus of *G. salicornia* compared to *G. changii* which had constriction at point of insertion of branches.

moving or the agar did not flow back to form a normal meniscus.

The melting temperature was determined from the gelled agar in the test tube (15×120 mm) which was placed in a 60°C water bath. At the same time, a small metal bead (diameter 2 mm) was placed on the surface of each gelled agar sample. The temperature of the water bath was gradually raised at 1°C per minute and the temperature when the bead fell until the bottom of the test tube was recorded as the melting temperature.

The thallus of *G. salicornia* was also thicker than both *G. changii* and *G. edulis*. While it is difficult to separate the life – stages of *Gracilaria* plants in the field when they are immature, female plants are easily identified through presence of the cystocarp. Cystocarps are formed after sexual reproduction, and produce diploid carpospores which when released, germinate back into the diploid sporophyte. The samples used for this study could not be verified for the life-stage as no reproductive structures were observed. Male and female gametophytic plants were reported to be found in similar proportions throughout the year but tetrasporic plants were usually in greater numbers (Phang et al. 1996)

## RESULTS



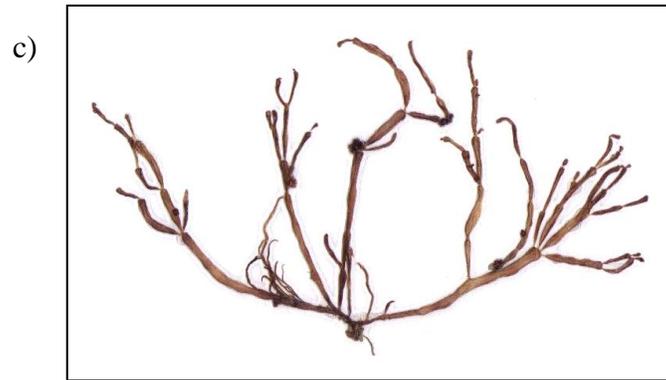


Figure 1. Herbarium of *Gracilaria* species. a) *G. changii*. b) *G. edulis*. c) *G. salicornia*

Table 1. Yield and physical properties of 1.5% (w/v) agar extracted from *G. changii*, *G. salicornia* and *G. edulis* using different extraction methods. (Average  $\pm$  SD, n=3)

	<i>G. changii</i>		<i>G. salicornia</i>		<i>G. edulis</i>	
	Native	Alkali-treated	Native	Alkali-treated	Native	Alkali-treated
<b>Agar Yield (% Dry Wt)</b>	39.33 $\pm$ 16.40	40.79 $\pm$ 4.49	63.89 $\pm$ 2.41	79.17 $\pm$ 5.01	69.75 $\pm$ 5.82	82.56 $\pm$ 5.40
<b>Gelling Temperature (<math>^{\circ}</math> C)</b>	38.7 $\pm$ 1.2	36.3 $\pm$ 3.0	37.7 $\pm$ 1.5	39.0 $\pm$ 1.0	38.3 $\pm$ 0.6	37.7 $\pm$ 0.6
<b>Melting Temperature (<math>^{\circ}</math>C)</b>	80.0 $\pm$ 1.7	80.3 $\pm$ 2.3	81.0 $\pm$ 3.6	84.3 $\pm$ 3.1	86.3 $\pm$ 0.6	81.0 $\pm$ 3.0
<b>Gel Strength (g cm<sup>-2</sup>)</b>	400 $\pm$ 17	370 $\pm$ 62	430 $\pm$ 17	385 $\pm$ 61	295 $\pm$ 85	365 $\pm$ 31

In the present study, alkaline treatment markedly improved the agar yield from *G. salicornia* and *G. edulis* but had little effect on agar fractions from *G. changii*. All the alkali-treated agar of these three species had higher yield as compared to the native samples (Table 1). However, the agar yield obtained from all the three *Gracilaria* species in this study were higher than those found in the literature. The minimum agar yield shown in samples of *G. changii* ( $39.33 \pm 16.40$  %), *G. salicornia* ( $63.89 \pm 2.41$  %) and also in *G. edulis* ( $69.75 \pm 5.82$  %) were considered very high compared with the results obtained from Buriyo and Kivaisi (2003) on native samples of *G. salicornia* (25 %), *G. changii* (12-25 %) and *G. edulis* (17.9 %) from Malaysia (Phang et al. 1996).

The highest gelling temperature ( $39.0 \pm 1.0^\circ\text{C}$ ) was measured on agar gels from alkali-treated *G. salicornia* while the lowest gelling temperature ( $36.3 \pm 3.0^\circ\text{C}$ ) was measured on agar gels from alkali-treated sample of *G. changii*. The higher gelling temperature is suitable for laboratory applications since the liquid agar does not require low temperature and can save time for it to solidify. There was no marked difference for gelling temperature between the three species with the range of ( $36-39^\circ\text{C}$ ). This range of gelling temperature is acceptable for agar according to the United States Pharmacopoeia (USP) (Li et al. 2008). However, there was a slight decrease in the gelling temperature after alkaline treatment on *G. changii* and *G. edulis*, similar to the finding from Praiboon et al. (2006) who reported that gelling temperatures were generally lower for alkali-treated agar especially on *G. edulis*. The results for all of the species in this study were considered lower than expected where agars extracted from *Gracilaria* species will usually gel at  $30-50^\circ\text{C}$  (Santos 1990).

The melting temperatures for agar from both of the species were in the range of ( $80-86^\circ\text{C}$ ). Native sample of *G. edulis* gave an unexpected high melting temperature ( $86.3 \pm 0.6^\circ\text{C}$ ) among all the samples in this study. The lowest melting temperature ( $80.0 \pm 1.7^\circ\text{C}$ ) was measured on agar from native sample of *G. changii* and the melting temperature of this species was not affected by alkaline treatment which gave almost the similar value ( $80.3 \pm 2.3^\circ\text{C}$ ). Alkaline treatment increased the melting temperature of agar from *G. salicornia* but there was a decrease in melting temperature observed in *G. edulis* after alkaline treatment.

The gel strength of agars extracted from *G. changii* ranged between  $300-420 \text{ gcm}^{-2}$ , *G. salicornia* range from  $330-450 \text{ gcm}^{-2}$  while the range in agars extracted from *G. edulis* was between  $225-390 \text{ gcm}^{-2}$ . The highest gel strength was obtained from native sample of *G. salicornia* while the lowest value was obtained from native sample of *G. edulis*. Alkaline treatment was effective in increasing the gel strength in *G. edulis* but not in *G. changii* and *G. salicornia*.

## DISCUSSION

The *Gracilaria* used in this study were collected during the rainy season and this may be the reason that the plants were smaller, and may have lower agar yield and gel strength. According to Phang et al. (1996), thalli of *Gracilaria changii* in Thailand were found throughout the year at one locality but seasonal variation occurred at two other locations where thalli were only found in two periods (January to July and March to September). This compared well with the condition in Philippines by Marinho-Soriano (2001) where *G. changii* was only present for six month especially during the dry season in a year compared to other *Gracilaria* species. According to them, except for rainfall, all other environmental parameters (temperature, salinity, pH, turbidity) showed insignificant relationship with the biomass of *Gracilaria* species (Pondevida and Hurtado-Ponce 1996; Marinho-Soriano 2001). This showed that seasonal variation has significant effect on the biomass and growth of *G. changii* in Malaysia, which may then affect agar yield and gel strength.

In the present study, alkaline treatment had markedly improved the agar yields from all three *Gracilaria* species. This is contrary to, previous researchers who reported that alkali treatment generally reduced agar yield by 25–56% (Buriyo and Kivaisi, 2003). The decrease in agar yield after alkali treatment was related to yield loss due to degradation of polysaccharides caused by alkaline hydrolysis during treatment and also agar loss by diffusion during the processing (Armisen and Galatas 1987; Freile-Pelegrin and Murano 2005; McHugh 2003). Hence, the increase of the agar yield in all three *Gracilaria* species in this study was most probably due to the effectiveness of the alkaline treatment process on more efficient but gentle extraction of

the agar from the cell walls, which caused no degradation of the polysaccharides. This result was similar as that obtained by Roleda et al. (1997) who reported that alkali pre-treatment *Gelidiella acerosa* gave high agar yields compared to untreated samples because alkali treatment was observed to soften the tough thalli. Besides that, agar yield from seaweed samples extracted by autoclaving at 121°C was slightly higher than that from the seaweed samples extracted in a water bath at 95°C (Buriyo and Kivaisi 2003). This showed that the extraction method was important in producing high yield of agar. Variation in agar yield was not only affected by pre-extraction treatment but also related to the extraction time, boiling and applying steam pressure during extraction of the agar (Roleda et al. 1997).

The improved agar yield could be due to other various factors such as the conditions of the environment (Bird 1988; Buriyo and Kivaisi 2003), physiological factors (Lahaye and Yaphe 1988), extraction methods (Freile-Pelegrin and Robledo 1997; Marinho-Soriano 2001) which affect the proportion of the seaweed constituents. Environmental factors which caused higher yield included higher insolation, salinity, temperature and low nitrogen supply (Buriyo 1999; Buriyo and Kivaisi 2003). Another reason for higher agar yield may be the presence of solvents such as ethanol in the seaweed species. Lahaye and Yaphe (1988) showed that sequential solvent extraction with ethanol could prevent the loss of polysaccharides associated with freeze-thaw and alcohol precipitation procedure during the extraction of the agar.

The gelling temperature of agars for all the three species in this study were considered lower than the expected results, where agars extracted from *Gracilaria* species will usually gel at 40-42°C (Armise'n 1993; Phillips and Williams 2000). The low gelling temperatures were probably due to the low methoxyl content of agar (Guiseley 1970; Youngs et al. 1998) although this chemical composition was not established in this study. Besides that, the molecular weight and molecular weight distribution are also parameters which could affect the agar gelling behaviour (Freile-Pelegrin and Murano 2005). Furthermore, low gelling temperatures might also be due to the long storage of the samples. Enzymatic processes of endogenous and microbial origin might be the cause of agar hydrolysis due to prolonged storage of the samples (Freile-Pelegrin 2000).

There was a decrease in melting temperature to  $81.0 \pm 3.0^\circ\text{C}$  observed in *G. edulis* after alkaline treatment. Hisashi et al. (1999) reported that the decrease in melting points of agar were correlated to the shifting of the molecular weight distribution to the lower molecular weight side and with a decrease of the agar molecular weight. The decrease in the melting temperature after alkali treatment may be due to alkali hydrolysis which reduced its molecular weight (Praiboon et al. 2006). The results also showed that native agar of *G. edulis* had the highest melting temperature compared to all the samples. Nevertheless, the agar gel strength of both *G. changii* and *G. salicornia* were higher than *G. edulis*. Hence, the results obtained in this study were in contrast with the study from Lewis and Hanisak (1996) who mentioned that melting temperatures were higher in agars with higher gel strengths.

Different gel strengths were also observed for the native and alkali-treated agar from the samples of the same species. The results were in agreement with the study from Marinho-Soriano (2001) who indicated that gel strength is specific species where the three species of the red seaweeds namely *G. gracilis*, *G. dura* and *G. bursapastoris* used in his study were collected from the same location that shared the same environmental conditions but had different gel strengths.

Alkali treatment using sodium hydroxide can improve the quality of agar by converting L-galactose-6-sulfate to 3,6-anhydro-L-galactose (Armisen, 1995; Marinho-Soriano and Bourret 2005). Thus, alkali-treated agar will have lower sulfate content than the native agar (Praiboon et al. 2006). However, gel strength of agars from *G. changii* and *G. salicornia* in this study was not improved by alkali treatment and was even lower than that found for gel strength of native agar (Table 1). One of the possibilities was that the alkali treatment had slightly reduced the 3,6 anhydrogalactose content but had only little effect on the sulfate content. Moreover, the presence of high sulfate content in tropical agarophytes have been reported to have reduced the agar quality (Buriyo and Kivaisi 2003). The alkali treatment used in the present study should be intensified to result in sulphate reduction in the agar.

However, alkali treatment in this study had improved the gel strength of the agar from *G. edulis* similar to the report by Praiboon et al. (2006) on agar of *G. edulis* from Thailand which the native agar had the gel strength of  $197.08 \pm 72.87 \text{ g cm}^{-2}$  and was subsequently increased to  $239.95 \pm 28.35 \text{ g cm}^{-2}$  in alkali-treated agar. The results in this study was also similar with the results of wild *G. edulis* but was in contrast with the results of cultured *G. edulis* from Malaysia as being reported by Phang et al. (1996) that alkali treatment was not effective for cultured *G. edulis* but had slightly improved the gel strength in wild *G. edulis* from  $276 \pm 7 \text{ g cm}^{-2}$  to  $331 \pm 20 \text{ g cm}^{-2}$ . The *G. edulis* in this study was obtained from the wild and was not the cultured type of seaweed.

The gel strength of agar for both native and alkali-treated agars determined in this study was comparable to values reported in the literature. Praiboon et al. (2006) reported that agar of *G. edulis* from Thailand had gel strength of  $197.08 \pm 72.87 \text{ g cm}^{-2}$  and  $239.95 \pm 28.35 \text{ g cm}^{-2}$  observed in native agar and alkali-treated agar respectively. Buriyo and Kivaisi (2003) reported that the gel strength of *G. salicornia* ranged from 205 to  $220 \text{ g cm}^{-2}$ , which was about 40% lower than the result obtained for this species in the present study where the gel strength for native and alkali treated *G. salicornia* was  $430 \pm 17.32 \text{ g cm}^{-2}$  and  $385 \pm 60.62 \text{ g cm}^{-2}$  respectively. Phang et al. (1996) reported that gel strength of native agar from *G. changii* was in the range of 294-563  $\text{g cm}^{-2}$ . For the seaweeds collected in August 1990, the gel strength for *G. changii* was  $368.21 \pm 37.17 \text{ g cm}^{-2}$ , which was slightly lower than the gel strength of the same species obtained in this present study which was  $400 \pm 17.32 \text{ g cm}^{-2}$ .

Seasonal variation plays an important role although the focus of this research was not in this area. Increased temperatures at the mangrove in Morib during sample collection could cause the increase in sulfate content in agar molecule thus lowering the gel strength (Armisen 1995; Freile-Pelegrin and Murano 2005; Marinho-Soriano and Bourret 2005). However, rainy seasons are related to low salinity which may cause increase of osmotic gradients such that ions like  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  leave the cell into the medium and is also another factor that contributes to the lower gel strength (Lobban and Harrison 1994; Buriyo and Kivaisi 2003). These ions are responsible to increase the gel strength of some species of red seaweeds (Buriyo and

Kivaisi 2003). The seaweed samples used in this study were not dried prior to agar extraction; instead fresh samples were used. According to Santos (1990), the moisture content of the dry sample must be in the range of 15-20 % because very moist samples may be expose to rapid deterioration and thus produce low quality agar.

The gelling and melting temperature specifications for bacteriological agar according to the American Society of Microbiology Specification (Meer 1980; Roleda et al. 1997) are 33-38°C and  $80 \pm 5^\circ\text{C}$  respectively with the temperature hysteresis of 42- 52°C. Therefore, the results in this study showed that the agar extracted from *G. salicornia* having the gelling temperature of 37-39°C and melting temperature of 81-84.3°C with temperature hysteresis range between 43.3-45.3°C for both the native and alkali-treated agar were the best source among the all the samples in this study applicable for bacteriological grade agar. In addition to that, the agar yield obtained from this species was quite high although overall the highest agar yield was observed from *G. edulis*. Furthermore, the gel strength of native agar from *G. salicornia* was the highest among the three species in which alkaline treatment was not effective to increase the gel strength of agar from this species.

For future studies, variables and parameters such as the concentration of NaOH, temperature and time of extraction on native and alkali-treated agar should be studied in order to determine optimum extraction conditions for the yield, gel strength, gelling temperature and melting temperature of agar. The extraction method still can be improved for the increase in gel strength since alkaline treatment has been observed to have no significant effect on the agar quality in *G. changii* and *G. salicornia*. For example, extraction with hot alkaline treatment (Phang et al. 1996) and prolonged incubation time. Further research is required to collect samples in various seasons and with more replicates to obtain more accurate results. Further chemical analysis on the content of 3,6-anhydro-L-galactose, sulphur content, methoxyl and pyruvic acid ketal content in agar and NMR spectroscopic studies are also needed so that the chemical structure of the agar can be explained in detail.

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