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Application of imaging Raman spectroscopy to study the distribution of *Kappa* carrageenan in the seaweed *Kappaphycus alvarezii*

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Abstract
Raman imaging spectroscopy has been applied to analyze carrageenan production in the red alga, *Kappaphycus alvarezii*. The Raman spectra of the sample suggested that the thallus of *K. alvarezii* mainly consists of cellulose and carrageenan. A partial least square regression prediction model for carrageenan semi-quantitative analysis was built with a simple two-material system and applied to visualize the three-dimensional carrageenan distribution in the algal body. The images clearly depicted the carrageenan distribution in carrageenan-rich and carrageenan-poor samples. Images stained for carrageenan with methylene blue showed results similar to those from analysis with Raman imaging spectroscopy. Our results suggest that Raman imaging spectroscopy, which is nondestructive and label free, is an accurate and useful method for detecting carrageenan distribution in algae.

Keywords *Kappaphycus alvarezii* · Carrageenan · Raman spectroscopy · Chemometrics

Introduction

The seaweed *Kappaphycus alvarezii* (Rhodophyta) is abundant in tropical seas, especially on the coasts of Indonesia, Philippines, China, and Vietnam (Hurtado et al. 2014, 2015; Msuya et al. 2014). The Food and Agriculture Organization (FAO) reported that Indonesia is a major producer of *K. alvarezii*, with a production capacity of up to 10 million tonnes in 2014. There are many places of seaweed cultivation in Indonesia, i.e., Karimunjawa, Bali, Sumba, and Sulawesi (Soegiarto and Sulustijo 1990; Poeloengasih et al. 2014; Manuhara et al. 2016; Adharini et al. 2018). *Kappaphycus alvarezii* contains a high-weight ratio of carbohydrates (Bixler 1996; Lechat et al. 1997). Carrageenan is a sulfated polysaccharide extracted from red algae (Normah and Nazarifah 2003). The most valuable carbohydrate extracted from *K. alvarezii* is *kappa* (κ)-carrageenan (Fig. 1), which has D-galactose 4-sulfate and 3,6 anhydrous D-galactose residues linked at the β(1,4) and α(1,3) carbons, respectively (Lechat et al. 1997; Rhein-Knudsen et al. 2015; Cunha and Grenha 2016). Carrageenan is in high demand in many industries, i.e., food, pharmacy, and cosmetics, owing to its stabilizing, gelling, and thickening properties (Azevedo et al. 2013; Necas and Bartosikova, 2013; Pereira et al. 2013). In the market, there are two types of carrageenan products that are differentiated by quality: semi-refined carrageenan (SRC) and refined carrageenan (RC). SRC and RC use different purification steps such as centrifugation, filtration, and alcohol precipitation (McHugh 2003).

The purpose of the present study is to demonstrate that Raman imaging combined with chemometrics are powerful tools for studying carrageenan distribution in algae. Although the quality and yield of carrageenan production generally depends on the seaweed itself, there is no clear standard for quality control. It is difficult to determine the quality of the seaweed only from its appearance and shape. Currently, extraction is the only method to examine the quality of seaweed cultivation, but it is destructive and not applicable to study the localization of carrageenan in the algal body. Thus, many researchers in carrageenan industries have keen interest in a

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tool that can investigate the quality and quantity of seaweed *K. alvarezii* in a raw material.

Vega et al. (2017) reported that fluorescence microscopy can be used to determine the distribution and type of carrageenan in *K. alvarezii*. This method, based on fluorescent labeling, allows the monitoring of microlocalization of the target molecule, even in a cell (Henriques et al. 2011; Smith et al. 2016). In contrast, Raman spectroscopy is a label-free technique that can detect chemical changes even within a single cell. Carrageenan and *K. alvarezii* have been extensively studied by Raman and IR spectroscopies (Pereira et al. 2003, 2009, 2013; Freile-Pelegrín et al. 2006; Dewi et al. 2012; Webber et al. 2012). Carrageenan is a sulfated polysaccharide, which gives specific bands in both Raman and IR spectra. Pereira et al. (2009) have assigned three strong bands in the range of 1240–1260, 1075–1085, and 845–850 cm^{-1}, due to the vibrational modes of the sulfate ester, galactose, and galactose 4-sulfate, respectively (Table 1).

In the present study, we aimed to analyze the distribution of carrageenan in three different branch sizes of *K. alvarezii* using Raman imaging technique. Raman spectroscopy has been applied for the semi-quantitative analysis of specific materials in intact biological samples (Meksiarun et al. 2015, 2016). Since Raman spectroscopy is a vibrational spectroscopy, all molecules present in the biological matrix display fingerprint bands of their chemical component in the Raman spectrum, unlike in absorption or fluorescence spectroscopy. However, it is necessary to apply multivariate analysis to extract information on a specific molecule in the Raman spectrum. The classical least square (CLS) method or least square curve fitting method is often used for quantitative analysis. Since it is based on a linear combination of spectra of major components included in the sample, the analysis does not require any prediction model and sample preparation, except for measurement of the component spectra. CLS, however, requires full knowledge of all components in the training samples of the measured system (Nadler and Coifman 2005). In contrast, partial least square regression (PLSR) analysis is another powerful quantitative analysis technique, which builds a robust prediction model for targeted material. Unlike the CLS method, it works with only knowledge of the substance of interest. Therefore, PLSR analysis is useful for the analysis of noisy spectra. Raman imaging is useful for studying the distribution of a component in a sample (Ishigaki et al. 2017). In this imaging technique, the Raman spectrum could be obtained from each measurement point, which covers the whole sampling area with spot-to-spot spatial intervals. Consequently, one Raman image consists of many Raman spectra, which composes a hyperspectral image. To reduce the total measuring time, it is necessary to reduce the acquisition time at each sampling spot, resulting in the relatively noisy feature of the spectra. This suggests that the PLSR analysis is suitable in the present study, owing to the noise and perturbation compared to other multivariate analyses, such as CLS.

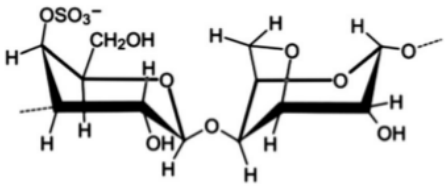


Fig. 1 Chemical structure of kappa (κ)-carrageenan (Necas and Bartosikova 2013)

Materials and methods

Sample preparation

Sun dried *Kappaphycus alvarezii* sampled in two cultivation areas (Karimunjawa and Sumbawa, Indonesia) was used. Samples were washed with purified water three times to remove dust, sand, and crystal salt, before soaking in 3.5% NaCl overnight. Several 2-mm-thick samples were sliced with a knife to obtain Raman images. Three samples were obtained from different places on each plant ((a) first, (b) second, and (c) third branches) of the seaweed as shown in Fig. 2. Refined κ-carrageenan was purchased (Wako Pure Chemical, Japan) and used without further purification. Powdered methylene blue was dissolved in purified water with 3.5% NaCl to prepare the 0.05% aqueous solution. The same sliced sample, often the Raman measurement, was soaked in the solution for 30 min, and then rinsed with purified water three times. Carrageenan distribution was observed using methylene blue, as previously described (Campo et al. 2009).

Raman measurement

The inVia Raman system (Renishaw Inc., UK) with a 785-nm excitation laser was employed to obtain 2D and 3D images. A homemade aluminum stage with water reservoirs was used for image measurement to keep the samples from drying. The cross-sectioned sample was fixed on the aluminum stage and soaked in 3.5% NaCl. The spectra of test samples for PLSR analysis were obtained with the streamline mode of the instrument, which employs a line-focused laser light for excitation with a 20 × magnification objective lens. The laser power was 190 mW at the sampling point. For the 2D and 3D imaging measurement, we used the confocal mode of the instrument. Data were collected at sampling points, with intervals of 250 μm for the X- and Y-axes and 50 μm for the Z-axis. The exposure time was 3 s at every sampling point. It took approximately 3–5 h for each sample to obtain 2D or 3D images.

Chemometrics analysis

To analyze the concentration of carrageenan, a PLSR model was built with 11 test samples that were prepared with carrageenan

Table 1 Characterization of κ-type carrageenan by Raman spectroscopy

Wavenumbers (cm^{-1})	Bond(s)/group(s)	Letter code	Kappa
1240–1260	S–O (sulfate ester)		++
1075–1085	C–O (3,6-anhydrogalactose)	DA	+++
970–975	Galactose	G/D	+
925–935	C–O (3,6-anhydrogalactose)	DA	–
905–907	C–O–SO_4 (C2–3,6-anhydrogalactose)	DA2S	–
890–900	Unsulfated β-D-galactose	G/D	–
867–871	C–O–SO_4 (C6-galactose)	G/D6S	–
845–850	C–O–SO_4 (C4-galactose)	G4S	++
825–830	C–O–SO_4 (C2-galactose)	G/D2S	–
815–825	C–O–SO_4 (C6-galactose)	G/D6S	–
804–808	C–O–SO_4 (C2–3,6-anhydrogalactose)	DA2S	–

Adapted and modified from Pereira et al. (2009)

and cellulose powder (Mahardika et al. 2018). Carrageenan and cellulose powders were mixed and ground with a hand homogenizer. The mixed carrageenan-cellulose test sample was placed on a metal substrate and pressed to make a small tablet. One hundred Raman spectra points were obtained at each tablet. All Raman spectra from samples were processed by interpolation, background subtracted, sixth polynomial baseline corrected, normalized by CH band at 1470 cm^{-1}, and smoothed using a Savitzky-Golay second order polynomial with 25 points on both sides of the frequency. The PLSR model was built using Unscrambler 10.1 software (CAMO Software AS., Norway).

Results

The Raman spectra of the inner (a) and outer (b) layers of the algae branch were compared to those of cellulose (c) and refined carrageenan (d) in Fig. 3a. A bright-field image of the sample is shown in Fig. 3b, where the sampling point of the inner (a) and outer (b) layers are marked with arrows. The PLSR analysis was employed to quantify the concentration of carrageenan in the algal body. A calibration curve of the PLSR

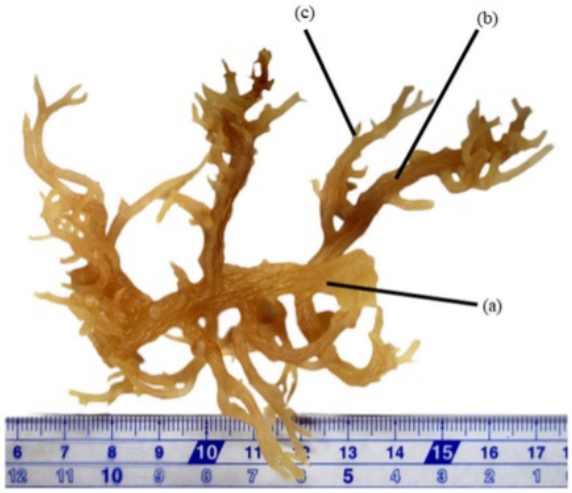


Fig. 2 A photo of the sample, *Kappaphycus alvarezii*. The arrows indicate the first (a), second (b), and third (c) branches

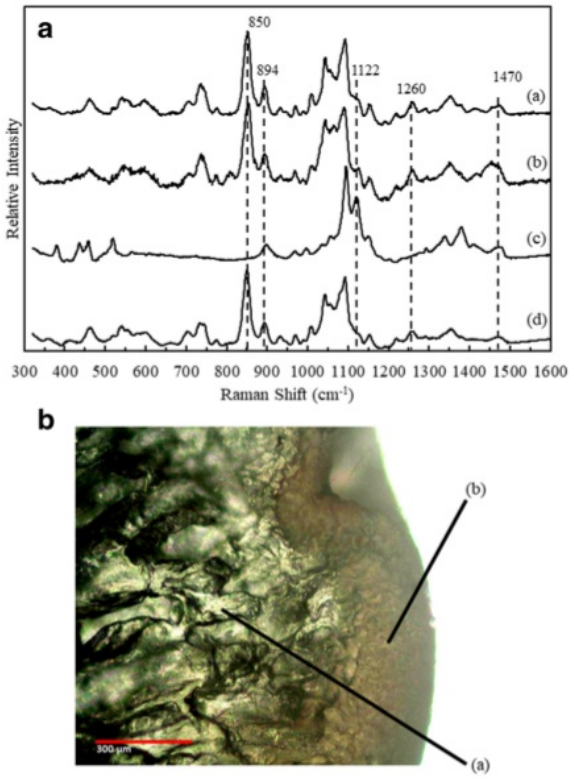


Fig. 3 Raman spectra (**a**) and bright-field image (**b**) of the cross-section of the first branch. The spectra are the medullary portion (a), outer surrounding portion (b), pure cellulose (c), and refined carrageenan (d). The arrows in **b** indicate selected points for Raman measurements of the medullary (a) and outer surrounding portion (b)

prediction model built with only one factor is shown in Fig. 4a. The loading plot of one factor shows bands from carrageenan in a positive direction and those from cellulose in a negative direction (Fig. 4b). The regression square (R^2) of the one-leave-out cross validation was 0.993 and its root mean square error (RMSE) was 0.027.

A hyperspectral Raman image was obtained to estimate the distribution and concentration of carrageenan in the seaweed branches. The measurement mode was changed to the confocal setup that has a high spatial resolution of 250 μm in the lateral direction and 50-μm depth. The PLSR prediction model was applied to the hyperspectral image to obtain a topological map of carrageenan distribution. Figure 5a depicts the carrageenan distribution in the three branches at − 100 μm depth. A bright field image of the cross sections of branches (Fig. 5b) shows no specific signal for carrageenan, indicating that it is impossible to estimate the concentration of carrageenan from its visual appearance. Figure 5c shows the carrageenan concentration in the cross section near 4000 μm on the Y-axis in the image (shown as a bright red line in Fig. 5a).

Figure 6a shows the carrageenan distribution in a carrageenan-poor branch. A bright field image (Fig. 6b) shows a similar feature to that of the carrageenan-rich branch (Fig. 5b), suggesting that it is very difficult to estimate the concentration of carrageenan with the shape of the branch. The carrageenan concentration in the cross section was near 3000 μm on the Y-axis in the image. The carrageenan concentration predicted by the PLSR analysis in this cross section was a maximum of 10% in both the first and second branches. The average carrageenan concentration in the first branch of the carrageenan-poor branch was only 4.5%.

Three-dimensional (3D) mapping images of the carrageenan concentration for the carrageenan-rich and carrageenan-poor samples are depicted in Fig. 7a, b. It should be noted that the carrageenan distribution was analyzed at 250 μm below the surface in a nondestructive manner. For the comparison of carrageenan distribution study, the MB staining methods were employed. The MB staining is a useful method for determining carrageenan and other anionic hydrocolloids (Soedjak 1994; Campo et al. 2009). MB has the characteristics of a cation; it easily binds to anions. The sulfate group in carrageenan has the characteristics of an anion because of its negative charge, which enables binding with MB. The MB staining of *K. alvarezii* of the first (a), second (b), and third (c) branches are shown in Fig. 8.

Fig. 5 Topographic image of carrageenan distribution (**a**) and bright-field image of the carrageenan-rich branches (**b**). The first, second, and third branches are shown with red, green, and yellow dotted lines, respectively. A graph (**c**) shows the carrageenan concentration along with the horizontal line at 3000 μm on the Y-axis

Discussion

There were either no or very few features observed in the Raman spectroscopy at a frequency region higher than 1600 cm^{-1}, except for a broad band due to OH groups near 1640 cm^{-1} (Fig. 3a). This suggests that the algae contain very little lipids and proteins. Although *K. alvarezii* is a red alga that generally has carotenoids such as zeaxanthin and β-carotene, there were no carotenoid bands observed in the Raman spectra (Indriatmoko et al. 2015). Since the sample was dried under the sun, pigments in the sample would be reduced. Additionally, the excitation light at 785 nm has either no or a very weak resonance enhancement effect for carotenoids (Sato et al. 2001). Strong bands at 1122 and 850 cm^{-1} are good marker of cellulose and carrageenan (Fig. 3). Cellulose is a major component of the cell walls of the algae; therefore, the spectrum of the outer layer has a relatively strong contribution of cellulose, which is observed as a shoulder band. In contrast, a strong band at 850 cm^{-1} of carrageenan was observed in both the inner and outer layers of the branch. The spectra of the cross-sectioned branch have strong

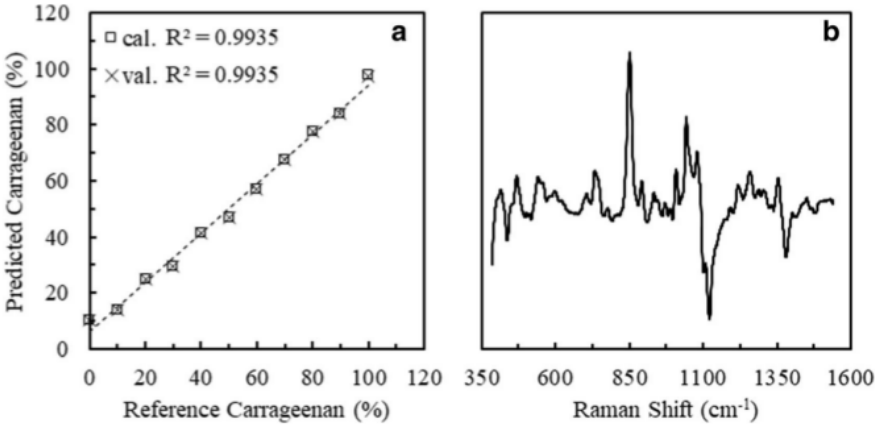
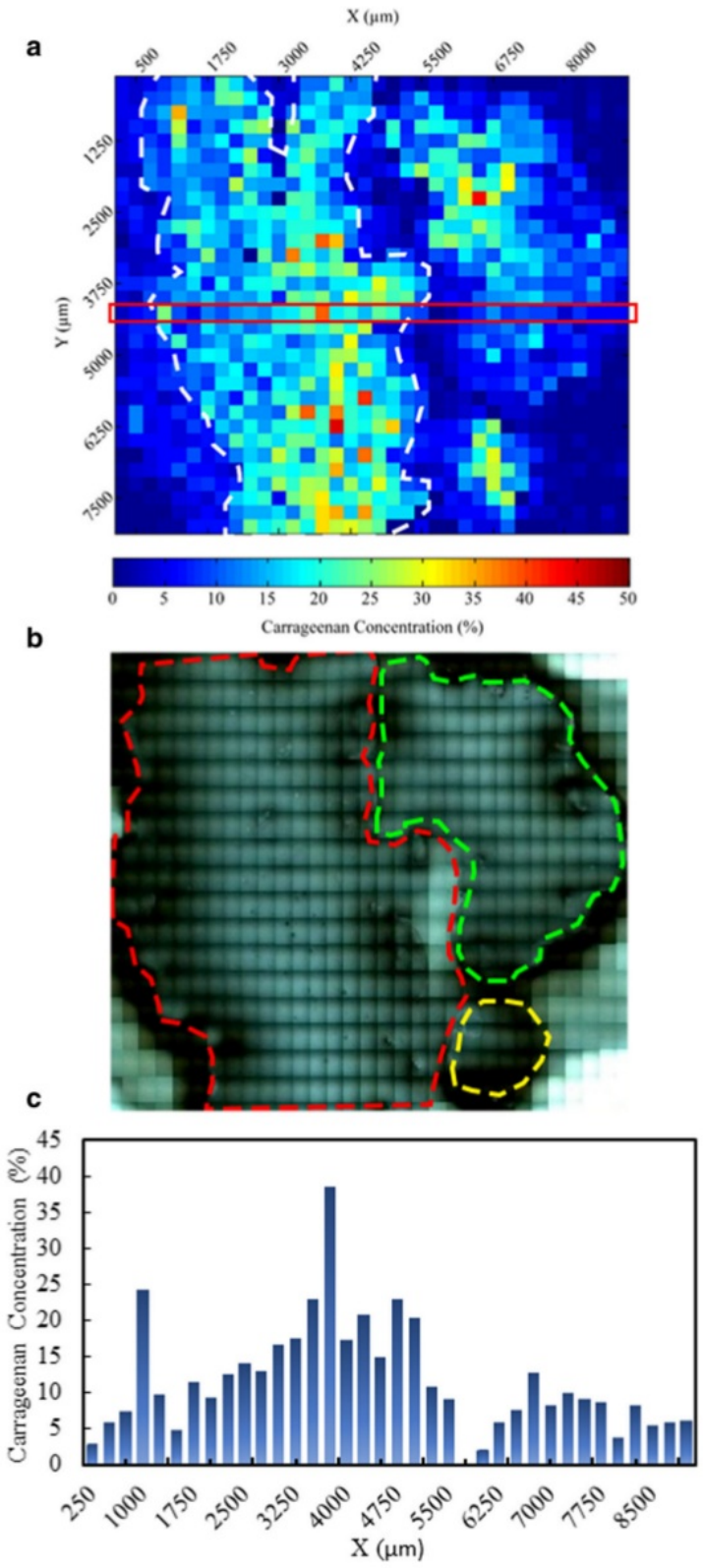


Fig. 4 Calibration curve (**a**) of the PLSR prediction model for cellulose-carrageenan mixed test samples and the loading plot of factor 1 (**b**)


a
X (μm)
500
1750
3000
4250
5500
6750
8000
1250
2500
3750
5000
6250
7500
Y (μm)
0
5
10
15
20
25
30
35
40
45
50
Carrageenan Concentration (%)
b
c
Carrageenan Concentration (%)
45
40
35
30
25
20
15
10
5
0
250
1000
1750
2500
3250
4000
4750
5500
6250
7000
7750
8500
X (μm)

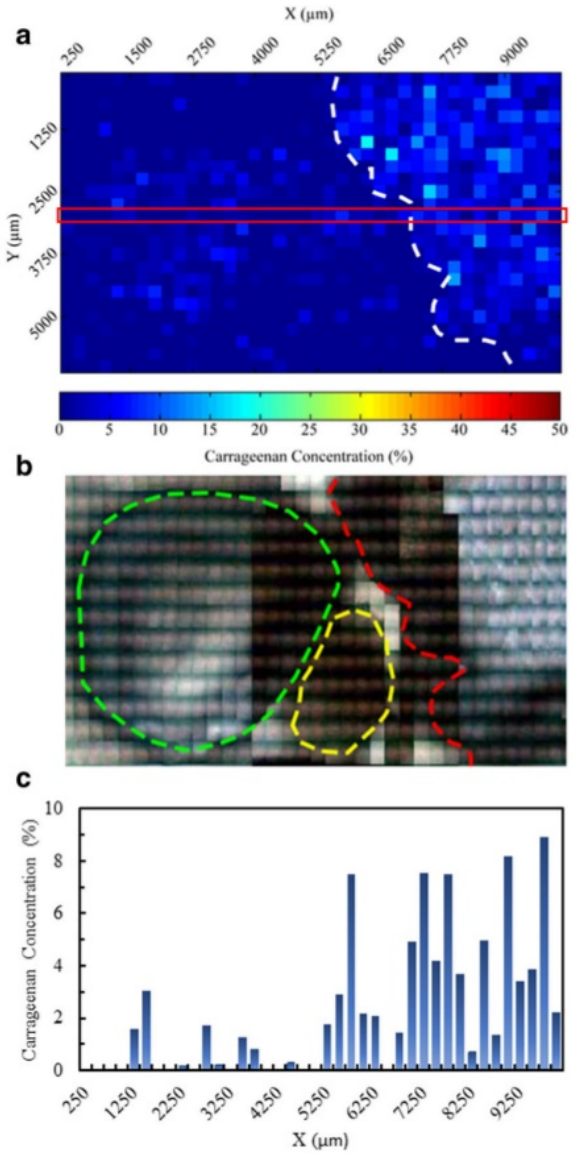


Fig. 6 Topographic map of carrageenan distribution (**a**) and bright-field image of the carrageenan-poor branches (**c**). The first, second, and third branches are shown with red, green, and yellow dotted lines, respectively. A graph (**c**) represents the carrageenan concentration for the horizontal line at 3750 μm on the lateral Y-axis

contributions from carrageenan, suggesting that *K. alvarezii* produces high concentrations of carrageenan. According to literature, the band at 850 cm^{-1} has been assigned to a vibrational mode of galactose 4-sulfate (G4S) (Pereira et al. 2009).

More than 50% of the dry weight of *K. alvarezii* comes from carbohydrates, including cellulose and carrageenan (Lechat et al. 2000; Vreeland and Kloareg 2000; Masarin et al. 2016). A PLSR prediction model was built with a series of 11 mixed κ-carrageenans and cellulose at different ratios (Fig. 4a) (Mahardika et al. 2018). Carrageenan is insoluble in both room temperature (25 °C) water and most organic solvents; it was not possible to make a homogenous mixture solution of the artificial test samples to build a calibration model, though they are microstructures in the algal body. Therefore, fine powders of materials were used to obtain the concentration-controlled mixed samples. Since the particle size was relatively large (~ 1 μm) compared to the spatial resolution of the objective lens, a line-excitation mode was employed instead of the confocal mode for the measurement of test samples. There were 11 test samples prepared, and 100 spectra obtained from each sample were used to build the PLSR model (Mahardika et al. 2018).

Raman spectroscopy is a type of light scattering spectroscopy where the spectral intensity is affected by interference and sample conditions, such as scattering and absorption of the sample, and by instrumental instability, such as the distance between the sample and objective lens, laser power, and stray light. Therefore, an independent internal standard is necessary for Raman spectroscopy to obtain the absolute concentration of a sample. Instead, we employed a semi-quantitative analysis in this study that gave the relative concentrations of the targeted material. Intensity correction was performed on all Raman spectra in the hyperspectral image with a standard band at 1470 cm^{-1}, that was assigned to a CH bending mode of which represents all bio-organic materials, because they generally have CH groups. In the present test sample system, the concentration of carrageenan had a high collinearity with that of cellulose, which is usually avoided when building a robust theoretical prediction curve. However, it is acceptable in the case of semi-quantitative analysis when the system is composed of only two materials (Mahardika et al. 2018). In this case, the increment of the first material is negatively correlated with a decrease in the second material. Therefore, only one factor explains all variables and the component of the factor that has contributions from the spectrum of the first material is in a positive direction, and from that of the second material is in a negative direction (Fig. 4b).

The Raman image in Fig. 5a shows that carrageenan, located in the algal cell wall, is mostly distributed in the medullary (inner) portion in all branches corroborating the literature data (Vreeland et al. 1992). In contrast, the carrageenan distribution in Fig. 5a seems to have correlation with the size of the branch (Fig. 5b). The carrageenan concentration in the medullary portion has been found to be the highest in the largest branch and has gradually been reduces towards the peripheral area. These results indicate that the PLSR model can predict carrageenan concentration and distinguish between sample and interval space. The highest carrageenan concentration shown in this cross section was up to 40%, while the average carrageenan concentration in the first branch (enclosed by the white dashed line) in Fig. 5a was 16%. According to the

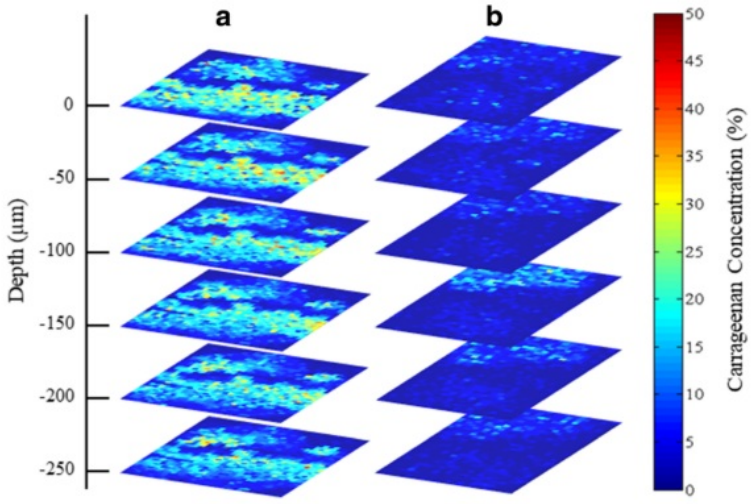


Fig. 7 3D Raman images of carrageenan-rich (**a**) and carrageenan-poor (**b**) branches

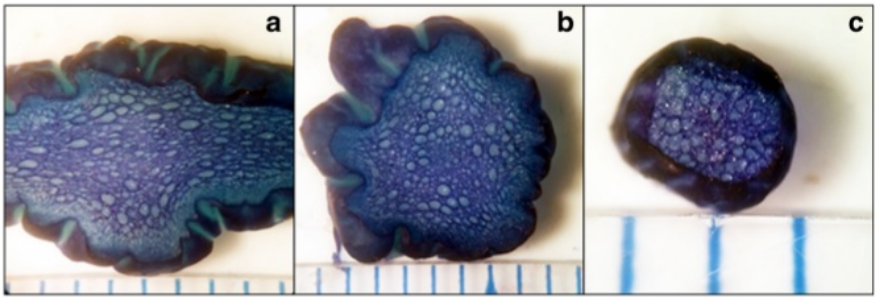


Fig. 8 Methylene blue-stained images of carrageenan on the first (a), second (b), and third (c) branches

carrageenan distribution images (Figs. 5 and 6), the average carrageenan concentration was 5–20% in the samples. Masarin et al. (2016) reported that extraction of carrageenan as a sulfated galactan from *K. alvarezii* was in the range of 30–40%. The present sample seems to have relatively lower concentrations compared to these samples.

The advantage of Raman 3D imaging has been demonstrated in the study of carrageenan distribution in the z-direction. The images in Fig. 7 illustrate that the carrageenan distribution is significantly altered as the depth increases along the branch. The carrageenan concentration increases at 150 μm in the z-direction, even in the carrageenan-poor branch (Fig. 7b). The 3D images suggest that carrageenan distribution is concentrated in the medullary space, as suggested in the 2D image, but the distribution throughout the depth of the branch shows diversity. This suggests that there are complicated structures for production and accumulation of carrageenan in the longitudinal direction of the branches and transportation of carrageenan is not active in the algae stem. It may be due to lack of phloem in the organ.

The images of the MB staining (Fig. 8) show similar distributions of carrageenan to those estimated by Raman observation. The first branch shows a deep blue color near the medulla portion and less at the cortex portion. Carrageenan seems to be distributed evenly across the second and third branch. The blue color at the outer cortex is the indication of sulfate anion from iota carrageenan (Vreeland and Kloareg 2000). Unfortunately, it is difficult to analyze the concentration and 3D distribution of carrageenan with the MB staining method.

Conclusion

Raman imaging spectroscopy is a nondestructive and label-free analytical tool that can be applied to investigate carrageenan production in *K. alvarezii*. No fluorescence and strong resonance Raman bands interference was observed due to pigments in the sun-dried seaweed when the Raman measurement has been performed. The PLSR prediction model, made of simple test samples of only carrageenan and cellulose, successfully illustrates carrageenan concentrations in the 3D images of seaweed. The present results demonstrate that Raman imaging spectroscopy has the potential to investigate saccharide present in the algal body and to evaluation the quality of carrageenan-producing seaweed plantations.

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