Bacterial dynamics associated with algal antibacterial substances during post harvest desiccation process of *Sargassum stolonifolium* Phang et Yoshida

Charles Santhanaraju Vairappan

Borneo Marine Research Institute, Universiti Malaysia Sabah, Locked Bag 2073, Kota Kinabalu, 88999, Sabah, Malaysia

Received 13 August 2002, revised 24 April 2003

Brown algae of genus *Sargassum* are known to produce relatively higher amount of alginic acid. Optimal extraction of this algalcolloid for local consumption requires in-depth studies on post-harvest treatment of the algal fronds. Present investigation endeavors to establish the dynamics and inter-relationship of moisture content and bacteria found on the surface of the algae and alginic acid content during post-harvest desiccation of *Sargassum stolonifolium* Phang et Yoshida. Harvested fronds were subjected to desiccation for 31 days and bacterial dynamics were monitored with relation to moisture content and water activity index (\(a_w\)). There was 85% decrease in moisture content, however, \(a_w\) showed a more gradual decrease. Total bacterial count increased during the first week and attained maximal value on day 7. Thereafter, a drastic decrease was seen until day 14, followed by a gradual decline. Six species of bacteria were isolated and identified, i.e. *Azomonas punctata*, *Azomonas* sp., *Escherichia coli*, *Micrococcus* sp., *Proteus vulgaris* and *Vibrio alginolyticus*. Calculated ratios for increase in alginic acid content and decrease in moisture content were almost the same throughout the desiccation process, implying that extracellular alginase-producing bacteria did not use the alginic acid produced by the algae as its carbon source. It became apparent that drastic decrease in bacterial count after day 7 could not be attributed to salinity, moisture content, \(a_w\), or lack of carbon source for the bacteria. The possible exposure of these bacteria to algal cell sap which is formed due to the rupture of algal cells was seen as the most likely reason for the drop in bacterial population. Scanning electron microscope (SEM) micrograph taken on day 10 of desiccation showed the presence of cracks and localities where bacteria were exposed to algal cell sap. *In vitro* antibacterial tests were carried out to verify the effect of algal extracts. Separation and purification of crude algal extracts via bioassay guided separation methodology revealed the identity of active compounds (i.e. glycolipids and free fatty acids) involved in this inherently available antibacterial defense mechanism during algal desiccation.

**Keywords:** Algal colloid, Anti-bacterial defence mechanism, Bacterial dynamics, *Sargassum*

Seaweeds have been utilized for human consumption since time immemorial. It has been used as salad, animal fodder, source of biogas, and extraction of commercially important chemicals. Main products of seaweed are gelling and viscous substances such as agar, carrageenan and alginic acid. Members of the brown algae family *Sargassaceae*, besides being a prolific source of alginic acid, are also known to contain several other bioactive substances. Perhaps the most intriguing of its potential is its antimicrobial activities.

In Malaysia, *Sargassum* grows in abundance along the coastal waters of Peninsula Malaysia, Sabah and Sarawak. Although *Sargassum* beds are known as good shelters and nursery grounds for fishes, it is generally not appreciated by the coastal communities and is often regarded as weeds. In an effort to optimize the utilization of marine resources, efforts are now being channeled to use *Sargassum stolonifolium* Phang et Yoshida, as a source of alginic acid for local needs. Hence, post harvest handling of collected algal fronds has been identified as an area that requires in-depth study. Algal fronds are usually harvested in large quantities for processing and in the absence of immediate processing; these fronds start to emit foul smell due to aerobic and anaerobic fermentation which is triggered by the presence of moisture, heat and bacteria. Such fermentation processes often occur due to the presence of fermentative bacteria. Some of these bacteria produce extracellular alginase enzymes, hence information on the surface bacteria's ability to utilize alginic acid as their carbon source is important to safe-guard the quality of alginic acid. Hitherto, no data is available on the dynamics of algal surface bacteria in relation to alginic acid content during post-harvest desiccation process of the alga. In an effort to understand this aspect, the present study was undertaken on the dynamics of surface bacteria and its interaction with...
the algal cell sap during desiccation. This phenomenon was elucidated by subjecting the alga to post-harvest desiccation process for 31 days, during which dynamics of total bacteria and bacterial species counts were monitored in relation to the decrease in algal weight and water activity index \((a_w)\). Total alginic acid content was monitored to verify its utilization by surface bacteria as their carbon source. Subsequently, SEM observation was carried out to understand the interaction between surface bacteria and algal cell sap. Crude algal extracts that were obtained via solid/liquid phase extraction with benzene, diethyl ether, chloroform:methanol:water (2:1:1.8) and buffer solution, were subjected to antibacterial bioassay against its surface bacteria. Further, bioassay guided separation of these extracts yielded active compounds that were subsequently identified using IR, \(^1\)H-NMR, \(^{13}\)C-NMR and GC.

Materials and Methods

Sample preparation — *Sargassum stolonifolium* was harvested during low tides at the rocky shores of Batu Feringghi, Penang Island, Malaysia. The fronds were cleaned of barnacles, gastropods and other contaminants at the site. These were immediately transported to the lab in sterile polyethylene containers under cooled condition. The fronds were drained of excess seawater and remaining water was blotted using sterile Whatman No. 1 filter papers until negligible weight fluctuations were observed. Bacterial enumeration of the blotted and unblotted fronds was carried out to verify any significant loss of surface bacteria due to blotting. Algal fronds were then divided into four lots consisting of nine samples respectively, each sample weighing about 20-25 g. These samples were subjected to desiccation in a sterile chamber within a clean room at 28°C under 12 hr light (approximately 30-40 μmol photon m\(^{-2}\)s\(^{-1}\) illumination).

Relative humidity of the desiccation chamber was about 85-88% and there was free air exchange between the chamber and clean room. Padding of desiccation chamber was made of glass and could not bind any moisture from the exudates and thus affect the humidity measurements. Clean room was prevented from contamination through a one-way filter system and routine bacterial monitoring was carried out as warranted in any clean room facility. Desiccation of these algal fronds was monitored for 31 days. The first lot of sample was used to determine algal weight. The second lot of sample was used to monitor water activity index \((a_w)\), while the third lot was used to monitor total bacteria and bacterial species counts. The fourth lot of sample was used to monitor the content of total alginic acid during desiccation.

Sample weight — Algal weight was monitored periodically throughout the desiccation process, on day 0, 4, 7, 14, 21, 28 and 31, under aseptic conditions. After measurements, fronds were immediately returned to desiccate in sterile chamber. Each data represents an average of 27 readings \((n=27)\).

Water activity index \((a_w)\) — Water activity index \((a_w)\) was determined by using a camstat-type Durotherm hygrometer (hair hygrometer) according to Bone et al.,\(^6\). Since the sensitivity of this instrument is about \(+/-0.003 a_w\), a total of 27 readings were taken for 9 samples to represent one data point \((n=27)\). The hygrometer camstat was UV-sterilized before use. Thus, water activity index \((a_w)\) was measured indirectly, as Equilibrium Relative Humidity. Measurements were taken periodically throughout the desiccation process on day 0, 7, 14, 21, 28 and 31.

Bacterial enumeration — The algal surface \((1 \text{ cm}^2)\) was scraped using sterile scalpel. Precautions were taken so as not to injure the surface layer cells and exposing the surface bacteria to the algal internal fluids. Scrapings were then diluted into 10 ml of sterile-filtered seawater. Decimal dilutions were made of the algal scrapings in the range of \(1 \times 10^{-1}-1 \times 10^5\) and 0.1 ml from each dilution was spread on Spread Plate Agar \((3\% \text{ NaCl, Himedia})\). This scraping method was used for this study after a preliminary bacterial enumeration was done using two other methods, homogenization and cotton bud swab method. The homogenization and the cotton bud swab method showed a drop of 30 and 15%, respectively, in total bacterial count as compared to the scraping method.

The Spread Plate Agar was chosen for this study after a preliminary bacterial enumeration using-1) Nutrient agar in seawater; 2) Nutrient agar in aged seawater; 3) Nutrient agar + 3\% NaCl; 4) Spread Plate Agar + 3\% NaCl; and 5) Difco marine agar. The plates were incubated at 28 °C for 2 days. Bacterial colonies with different morphological characters were counted and isolated. Purified cultures were maintained on Nutrient agar \((3\% \text{ NaCl, Himedia})\) slants. Quantitative enumeration of total bacteria and bacterial species counts were recorded. Isolated pure cultures were identified using conventional methods.
described by Shewan et al., Chan and Mc Manus, Baumann et al., Baumann et al. and Lee et al.

Electron microscopy—Algal thalli were cut (ca. 0.5x0.5 cm pieces) and fixed for 24 hr in glutaraldehyde (4%) in 0.1M cacodylate buffer (pH 7.2). Specimens were then rinsed in 0.1M cacodylate buffer before post-fixation in 1% OsO₄ at 4°C for 2 hr, followed by dehydration with graded acetone series and finally subjected to critical point drying. Dehydrated specimens were mounted on stubs and coated with 10-30 nm layer of gold before observation with Leica Cambridge S360 electron microscope.

Alginic acid quantification—Alginic acid content was estimated on day 0, 4, 7, 14, 21, 28 and 31. Samples were oven dried to powder and treated with CaCl₂ (1%), approximately 3 times the sample volume, for about 4 hr to eliminate mannitol and other simple sugar. This was repeated 3 times before the residues were washed with distilled water. Then, 5% of HCl was added and predigested for 4 hr. Digested sample was washed 4 times with distilled water, mixed with Na₂CO₃ (3%) for 2 hr at 45°-50°C and stirred at room temperature for 8-12 hr at 200 rpm. This mixture was then diluted with an equal amount of distilled water and 2 ml of H₂O₂ was added as a bleacher before it was centrifuged at 4000 rpm for 20 min. The supernatant obtained was further concentrated to 1/3 of its original volume. Equal amount of absolute ethanol was added in order to precipitate the sodium alginate, centrifuged and freeze dried before its weight was taken.

Calculation of ratios
Ratio of moisture decrement = Wₙ₋₀ / W₀₋₀ (i)
Ratio of alginic acid increment = ΔCₙ₋₀/ ΔC₀₋₀ (ii)

Whereas, Wₙ₋₀ = Algal weight (g) at the beginning of desiccation process; W₀₋₀ = Algal weight (g) at the end of desiccation process; ΔCₙ₋₀ = Alginic acid content (%) at the beginning of desiccation process; and ΔC₀₋₀ = Alginic acid content (%) at the end of desiccation process.

Antibacterial activity bioassay

Extraction—Algal fronds were divided into two lots—(1) lot was partially dried (minimal weight fluctuation) under shade; and (2) lot was used as fresh algae for phosphate buffer (pH 7.8) and chloroform/methanol/water (2:1:1.8) extractions. First batch of fresh algae (100 g) was homogenized for ca. 60-90 sec. in 400 ml of sterile 0.2 M phosphate buffer (pH 7.8) and was kept at 4°-6°C for 24 hr under continuous agitation. Extract was filtered and centrifuged at 1000 g for 20 min, and the resulting clear supernatant was lyophilized to yield about 2g of freeze-dried powder. Second batch of fresh algae (100 g) was homogenized in Waring blender for 2 min with a mixture of 100 ml chloroform and 200 ml methanol. To the mixture is then added 100 ml of chloroform and after blending for 30 sec, 100 ml of distilled water is added and blending continued for another 30 sec. The homogenate was filtered and residues recovered. The above-described extraction was according to the method as suggested earlier.

Partially dried alga (100 g) was extracted with diethyl ether and benzene, respectively. The resulting solutions were concentrated in vacuum and partitioned between Et₂O and distilled H₂O. The Et₂O solution was washed with water, dried over anhydrous Na₂SO₄, and evaporated to leave dark green oil. These steps gave the non-polar crude diethyl ether and benzene extracts.

Bioassay—The extracts and isolated compounds were tested against pure bacterial isolates obtained from the algal surface. One loopful of each organism was precultured in 10 ml of peptone water (3% NaCl) overnight. The turbidity of precultured solution was adjusted to optical density (OD) of McFurland 0.5 (ref. 15). Then, 0.1 ml of each precultured suspension was spread on nutrient agar (3% NaCl) plates. Paper discs (Whatman, 6 mm) impregnated with 8.0 mg/disc of the respective extracts were placed on the agar and incubated at 28°C for 24 hr.

Separation, purification and isolation of active compounds—Most active crude extract was dissolved in CHCl₃ fractioned using SiO₂ gel column chromatography with CHCl₃/MeOH/H₂O gradient eluent system. The fractions were subjected to antimicrobial bioassay. Active fractions were checked with thin layer chromatography (TLC) and submitted to PTLC Preparative Thick Layer Chromatography (PTLC) to obtain pure compounds.

Characterization of active compounds—During isolation and purification, compounds were developed on silica gel 60 F₂₅₄ TLC plates in various solvent systems, visualized with molybdo(VI) phosphoric acid and orcinol-H₂SO₄ reagent. 1H-NMR (400 MHz) and 13C-NMR (100 MHz) spectra were measured in CDCl₃ (fatty acids) and DMSO-d₆/D₂O (98:2) (glycolipids) with trimethylsiline (TMS) as the internal standard by using a JEOL-JNN-EX-400 spectrometer.
GC Analysis of Fatty Acid Methyl Esters — Active fatty acids were determined by gas chromatography of methyl ester derivatives using Shimadzu GC-6AB with flame ionization detector. Fatty acid methyl esters were obtained by reaction of isolated active fatty acid with methanolic hydrochloride 
followed by silica gel chromatography with hexane-diethyl ether (9:1, v/v) as eluent. The conditions for GC measurements were as follows: column, RASCOT Silar 5CP (0.25 mm i.d. x 50m, Nihon Chromato Works Ltd.); injection and detector temperature, 230°C; column temperature, 180°C; carrier gas, H₂; flow rate, 1 ml/min.

Results
Post-harvest desiccation — As shown in Fig. 1, total algal weight showed decreasing tendency during post harvest desiccation process. A drastic 64% decrease in algal weight was observed in the first 7 days, followed by gradual decrease to a constant weight at the end of the desiccation process. The algal fronds lost about 85% moisture during entire process.

However, water activity index (a_w) showed a more gradual decreasing tendency. It decreased from 0.99 a_w at the beginning of the desiccation process, to about 0.89 a_w, on day 31. The correlation observed between water activity index (a_w) and algal weight decrement agrees with the suggestion by Troller and Christian on water content and water activity index (a_w). Hence, this finding showed that water content in seaweed thallus was not in direct equilibrium with water vapor in its surroundings.

Preliminary data on total bacterial count showed about 6% drop in the total bacterial counts for the blotted fronds as compared to the unblotted fronds. Blotting was vital to avoid possible bacterial contamination due to excess seawater on the algal fronds. Fig. 1 clearly shows unique pattern in the total bacterial count. Initial total surface bacterial count of 792 CFU/cm² increased until 7th day of desiccation, reaching a maximum at 1490 CFU/cm². However, this did not persist and was followed by a drop to 864 CFU/cm² on 14th day. After this point, the decrease in total bacterial count was more gradual for the next 2 weeks with a final count of 360 CFU/cm².

Fig. 2 shows the respective bacterial counts of the six bacterial species belonging to four families of bacteria (Vibrionaceae, Azotobacteraceae, Enterobacteriaceae and Micrococcaceae) isolated throughout this process. Isolated bacteria were identified using morphological, biochemical and specific sugar test, with reference to Bergey’s Manual. These bacteria were — Aeromonas punctata, Azomonas sp., Escherichia coli, Micrococcus sp., Proteus vulgaris and Vibrio alginolytica. The presence of extracellular alginate was detected in Aeromonas punctata, Micrococcus sp. and Vibrio alginolytica, hence suggesting their ability to digest alginic acid.

Initially, six species of bacteria were isolated from the algal surface with these relative abundance—

---

**Fig. 1** — Correlation of algal weight in relation to water activity index and total bacterial count of Sargassum stolonifillum (Phaeophyta), during desiccation at normal room temperature.

**Fig. 2** — Total bacterial and bacterial species count variation in Sargassum stolonifillum (Phaeophyta), during desiccation at normal room temperature.
**Electron microscopy**—General ultrastructure of the thallus has been shown in Fig. 4. The thallus is undergoing dehydration where its surface cell layers show areas that are under stress (SR). Besides these, there are also features that show cracks on its thallus (CR). Hence, at these vicinities, the surface bacteria are exposed to the internal fluid of the seaweed, thus resulting in the decrease in number of bacteria. The gap between the cracked layers of this thallus holds numerous dead bacteria (DB) which could be easily differentiated with the healthy ones (VB). This micrograph clearly suggested the possible presence of antibacterial substance in the internal fluid of this alga.

**Antibacterial activity**—Active anti-microbial principles in *S. stolonifolium* were extracted using the four different solvent systems, phosphate buffer (pH 7.8), C/M/W (2:2:1.8), diethyl ether and benzene. Net weight of the various crude extracts was 9, 7, 2 and 3% of the algal weight used during extraction, respectively.

Results for the antibacterial tests are given in Table 1. The benzene and diethyl ether crude extracts showed antibacterial activity against 50% of the tested bacteria with strong inhibition against *Aeromonas punctata* and *Vibrio alginolyticus*. Minimum inhibitory concentration (MIC) for these extracts varied between 10 and 80 μg disc⁻¹. Lowest MIC value for benzene and diethyl ether extracts was 10 μg disc⁻¹. Both the extracts showed best

---

**Fig. 3**—Variation in alginic acid content as compared to algal moisture decrement in *Sargassum stolonifolium* (Phaeophyta), during desiccation at normal room temperature.
antibacterial inhibition with low MIC values against *Vibrio alginolyticus*. On the other hand, chloroform: methanol: water (C: M: W; 2:1:1.8) crude extract inhibited 100% of the bacteria strains. Potent antibacterial activity was seen against *Aeromonas punctata* and *Vibrio alginolyticus* with a MIC value of 10 µg disc⁻¹. Phosphate buffer extract only inhibited about 33% of the tested bacteria with good inhibition against *Aeromonas punctata* and *Vibrio alginolyticus* with a MIC value of 100µg disc⁻¹.

**Antibacterial compounds**—Biosassay guided separation methodology indicated two groups of compounds as the active principles: inseparable mixture of fatty acids and MGDGs [β-galactopyranosyl (1'-3)']-1,2-diacylglycerols (Compound 1). The fatty acid mixture consisted of saturated fatty acids and mono-unsaturated fatty acids. Saturated fatty acids were palmitic acid (67%) and stearic acid (12%), while mono-unsaturated fatty acids were palmitoleic acid (C16: 1,5%), oleic acid (C18: 1,7%) and gadoleic acid (C20: 1, 9%). MGDGs showed characteristic dark red spots due to sugar moieties when visualized with orcinol-sulfuric acid reagent, suggesting that these compounds are glycolipids. ¹H-NMR spectrum of compound 1 exhibited signals of two terminal methyl groups at δ 0.85 and 0.92 (br t, J = 7.3 Hz), broad methylene groups at δ 1.24, two methylene groups at δ 2.27 (t, J = 7.3 Hz) flanked by a carbonyl function, indicating the presence of two acyl groups. The ¹H-¹H COSY spectrum showed a 5-spin ABX'AB' system at δ 3.61 (1H, dd, J = 11.2, 5.9 Hz)/3.80 (1H, dd, J = 11.2, 5.4 Hz), 4.14 (1H, dd, J = 11.7, 6.8 Hz)/4.32 (1H, dd, J = 11.7, 2.4 Hz) and 5.10 (1H, m), which are characteristic of signals due to a 1,2-diacylglycerol moiety. Detailed analysis of the remaining protons in ¹H-¹H COSY coupled with ¹³C NMR and HSQC spectra showed that sugar moiety of compound 1 was β-galactoside having an anomeric proton at δ 4.11 (1H, d, J = 6.8 Hz). Therefore, structure of compound 1 was identified as [β-galactopyranosyl (1', 3)']-1,2-diacylglycerols (MGDGs)⁷⁻¹².

Since compound 1 consisted of a mixture of molecular species, the composition and positional distribution of fatty acids in compound 1 (MGDGs) were determined by enzymatic hydrolysis followed by alkaline hydrolysis. Lipase-catalyzed regioselective decylation at 1-position of 1 using lipase type XI from *Rhizopus delemar* in Triton X-100/boric-borax buffer (pH 7.7), produced exclusively 1a and mixture of fatty acids. The resulting fatty acids were converted to their corresponding methyl ester by treating with trimethylsilyldiazomethane in methanol-hexane. GC analysis of methyl ester confirmed that fatty acids at 1-position of 1 were 16: 0 (58%), 16: 1 (13%), 18: 0 (13%), 18: 1 (10%), 18: 2 (8%) and 20: 5 (2%). Alkaline treatment (NaOMe-MeOH) of 1a gave a mixture of methyl esters, GC analysis showed that fatty acids located at 2-position of compound 1 were 16: 0 (60%), 16: 1 (14%), 18: 1 (9%), 18: 2 (7%), 20: 1 (6%) and 20: 5 (4%).

Active compounds, fatty acids and MGDGs (I), were distributed in various extracts in different distinct quantities. Active fatty acids constituted 2% of benzene extract, 3% of diethyl ether extract, 6% of C/M/W extract and 2% of the buffer extract. On the other hand, MGDGs were only present in significant

<table>
<thead>
<tr>
<th>Bacteria tested</th>
<th>Buffer</th>
<th>Antibacterial activity</th>
<th>C/M/W</th>
<th>EtO</th>
<th>Benzene</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas punctata</em></td>
<td>Buffer</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas sp.</em></td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Micrococcus sp.</em></td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacteria tested</th>
<th>Crude extract MIC* values (µg/disc⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas punctata</em></td>
<td>100</td>
</tr>
<tr>
<td><em>Aeromonas sp.</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Micrococcus sp.</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Vibrio alginolyticus</em></td>
<td>100</td>
</tr>
</tbody>
</table>
quantities. 12 and 4% in C/M/W extract and phosphate buffer extract, respectively. Presence of these active substances was also detected in the exudates collected from the glass padding of the desiccation chamber. Detection of substances was done based on $R_t$ values of compounds in exudates and isolated pure active compounds, developed on silica gel F$_{254}$ TLC and visualized using specific sprays such as molybdo(VI) phosphoric acid and orcinol-H$_2$SO$_4$ reagent$^{16}$, as mentioned earlier.

**Discussion**

Net loss in algal weight could be attributed to the loss of moisture during desiccation process. Similar findings have also been reported by Gardner and Mitchell$^{23}$, and Vairappan and Suzuki$^{24}$ during their studies on seaweed drying. However, since this investigation involved monitoring of surface bacteria that lives on micro-structured algal thallus, a mere moisture determination was not sufficient. The algal surface with deposits of organic matters and algal exudates gives rise to a micro-structured surface with minute water capillaries within these layers. A mere moisture monitoring would not give much information pertaining to the forces that affect the surface bacteria which flourish on this micro-layered algal surface. Rather, water activity index ($a_w$) appears to be the most stable water content indicator$^{19}$. The water activity index would give a realistic figure of the moisture content available to the surface bacteria and could be defined as the readily available moisture in the algal thallus. In this study, solutes are NaCl, algal exudates, dissolved organic matter and other minerals present in seawater$^{25}$. Complexity of interactions between solutes and difficulties faced in quantifying these individual solutes rules out any possibility of determining the water activity index ($a_w$) through the usage of Gibbs-Duhen's integration equation as suggested by Bone$^6$ et al$^8$. Hence, water activity index ($a_w$) has proved to be a more appropriate parameter. Relationship between internal moisture content and its water activity index of *S. stolonifolium* in this study was comparable to those reported by Troller and Christian$^{13}$ and Vairappan and Suzuki$^{33}$.

Comparison of water activity index ($a_w$) with algal weight decrement gave good indication of the algal total moisture content. Gradual decline in water activity index also suggested the algal ability to harbor bacteria and sustain their growth. Water activity index decreased from 0.99 to 0.88 at the end of desiccation. Even then, water activity index was 0.88 $a_w$ and still ideal for the growth of moderately halophilic bacteria$^{12}$. Initial increase in bacterial count could be attributed to decrease in available surface for bacterial growth. This phenomenon was also illustrated by Zubell and Upham$^{26}$: Drop in total bacterial count and bacterial species count after 7th day of desiccation was drastic and could not be due to dehydration. Salinity has been regarded as one of the factors besides algal exudates, organic matters (dissolved) and minerals that govern the water activity index ($a_w$) of desiccated seaweed fronds$^{37}$. However, water activity index ($a_w$) was still ideal for bacterial growth. Influence of salinity has been taken into consideration based on Gibbs-Duhen equation$^8$ and was ruled out as the causative factor for the drop in the bacterial counts.

Calculated ratios of alginic acid increment and moisture decrement also gave valuable information pertaining to dynamics of surface bacteria. Since the ratios were almost same, it became apparent that surface bacteria were not utilizing alginic acid as their carbon source although *Aeromonas punctata*, *Micrococcus* sp. and *Vibrio alginolyticus* are known to produce extracellular alginate$^{11}$, $^{12}$. Zubell and Upham$^{26}$ have suggested that alginate producing marine bacteria, particularly bacteria from the genus *Vibrio*, are able to digest and utilize alginic acid as its carbon source. Hence, at this juncture, it is reasonable to suggest that drastic drop in surface bacterial counts can not be attributed to salinity, water activity index ($a_w$) or lack of carbon source. Therefore, drop in the bacterial count could be due to the release of intracellular antibacterial compounds as algal thallus cells rupture.

The presence of exudates was also observed on glass padding of desiccation chamber during first two weeks of desiccation. However, exudates could not be observed in SEM micrographs since the specimens were preserved in fixative and were dehydrated prior to coating. Direct freeze-drying of samples without use of fixative resulted in appearance of excess salt crystals and failed to produce good SEM images.

SEM micrograph also suggested that algal exudates could have leaked from the cracked algal thalli as shown in Fig. 4. There were two morphologically different bacterial cells in the SEM micrograph (1) VB-normal bacterial cells; and (2) DB-deteriorated bacterial cells. Since the whole specimen was processed in the same manner, this implies that the differences in the bacterial cell morphology refer to
dead and viable bacterial cells prior to processing. SEM specimen processing protocol could not have caused these effects because the specimens were fixed prior to dehydration. Therefore, it has been suggested that most of the surface bacteria were affected by the exudates that could have caused the drop in bacterial counts.

Further investigation, using in vitro antibacterial bioassay of algal extracts against its surface bacteria, confirmed the presence of such an inherently available antibacterial potential. Results obtained from in vitro antibacterial bioassay test of four different extracts with varying solvent polarity suggested that antibacterial mechanism could be operating on a multi-compound basis. This was obvious since extracts of different polarity showed inhibition towards different bacteria. Compounds could be bacterium specific and C/M/W extract showed the best activity. Bioassay guided separation methodology and purification of active metabolites yielded 6 active compounds. Active metabolites isolated in this study have been shown to exhibit biological activities in other brown algae. In an effort to verify the antibacterial activities of fatty acids, pure commercially available corresponding fatty acids were tested against the same range of bacteria. Tested fatty acids exhibited anti-bacterial activity at various intensities. Investigation conducted by Rosell and Srivastava have also reported saturated and unsaturated free fatty acids as antibacterial principal in nine species of brown algae harvested from Vancouver Island, British Columbia. MGDGs occur widely throughout the plant kingdom and it constitutes a major class of lipids in photosynthetic tissues of terrestrial and marine plants. Studies on runner-bean leaves have shown that MGDGs are actively utilized as a means of defense response when mechanical damage is inflicted to the plant membrane. MGDGs isolated from Phormidium tenue have also shown to exhibit autolytic activity towards algal cells. Recently, researchers are discovering biological activities of glycolipids, and its importance is not limited as a constituent of plant cell component.

In conclusion, findings of this investigation suggest that seaweeds have inherently available antibacterial protection ability to protect themselves against intrusion of surface/environmental bacteria. Since isolated active metabolites consisted of glycolipids and fatty acids, it is suggested that seaweed's defense is located in its cell membrane where glycolipids form the major component. This study could be considered as another milestone in our effort to fully understand algal intricate defense mechanism against marine microorganisms. Hence, it has become apparent that desiccation of S. stolonifolium upon harvest does not contribute to fermentation or other processes that would reduce algal’s alginate quantity.

Acknowledgment

The author wishes to thank Dr Suzuki Minoru, Graduate School of Environmental Earth Science, Hokkaido University, Japan, for valuable advice and suggestions in spectroscopic data analysis.

References


