Anaerobic Degradation of Carrageenan from the Red Macroalga *Eucheuma cottonii*†

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Anaerobic degradation of the sulfated polysaccharide carrageenan was investigated by batch digestion of the red macroalga *Eucheuma cottonii*. During a 10-week incubation, ca. 60% of the starting *E. cottonii* biomass was fermented to CO_2, methane, and volatile fatty acids (predominantly acetate). Carrageenan degradation paralleled the loss of total biomass, suggesting no preferential degradation or preservation. After 10 weeks of incubation, the carrageenan content of the remaining biomass was 51%, as opposed to 61% of the original *E. cottonii* biomass. Carrageenan recovered after 10 weeks of digestion had a lower average molecular weight (319,000 versus 510,000) and formed solutions with considerably lower viscosities than did intact carrageenan. The percent C and percent N content of the particulate material in the digestors increased over time, probably as a result of microbial growth. In contrast, the percent S content decreased continuously; the loss of sulfur was most likely a result of the hydrolysis of carrageenan. Results from this study indicate that it is not economically viable to process *E. cottonii* simultaneously for hydrocolloids and methane.

The search for alternative fuel sources has resulted in a significant research program with the goal of establishing the suitability of various seaweeds for use in biomethanation or methane production (15). For example, *Macrocystis pyrifera*, a brown macroalga, has been the subject of intensive mariculture and fermentation studies. It has been established that *M. pyrifera* can be readily fermented to methane and cultured in large marine farms (15, 16). In addition, it has been proposed that seaweeds might be processed for both methane and other valuable by-products, especially various hydrocolloids such as agar-agar (10). The production of hydrocolloids would presumably enhance the economics of energy production (10). This proposal is particularly intriguing, as the marketing of agar, carrageenan, or alginates could provide a measure of financial stability as well as a competitive edge over the marketing of other sources of biomass.

Based on earlier studies, it was suggested that agar could be recovered after fermentation of *Gracilaria tikvahiae* for up to 30 days, thus providing a potentially valuable by-product in addition to methane (10). However, in a later, more-detailed study, it was observed that agar was significantly degraded during anaerobic fermentation and that postfermentation recovery was not practical; in addition, the agar that remained after fermentation appeared somewhat reduced in molecular weight and in its ability to form gels (2). Therefore, it is not clear to what extent methane production and hydrocolloid production from postfermentation extractions are compatible.

We report here a study of the stability of carrageenan during anaerobic degradation of the macroalga *Eucheuma cottonii*. *E. cottonii* is a rhodophyte of the order Gigartinales that contains large quantities (>50% on a dry-weight basis) of κ-carrageenan (5). Carrageenan is a large, branched polysaccharide (molecular weight, >500,000) that is associated with the cell matrix and wall and that consists of repeating units of galactose linked $\alpha1\rightarrow3$, $\beta1\rightarrow4$. The pres-

ence of 3,6-anhydro-D-galactose and sulfation of the 2-, 4-, or 6-hydroxyl groups distinguishes the various forms of carrageenan and contributes to the solubility, gelation, viscosity, and protein reactivity properties of the polymer (5). This study was designed to address the potential for carrageenan recovery and its properties after anaerobic digestion. No attempt was made to optimize for methanogenesis, as this was considered of secondary importance initially; optimization for methanogenesis would prove useful only if carrageenan were relatively stable. Results of this study suggest that postfermentation production of carrageenan is not feasible and indicate that hydrocolloids may not generally be recoverable in commercially important quantities after anaerobic digestion.

MATERIALS AND METHODS

The stability of *E. cottonii* carrageenan during anaerobic digestion was investigated with 1-liter spinner flasks (Bellco Glass, Inc.) that had been modified to allow sampling of the flask gas and liquid phases. The flasks (fermentors) contained 800 ml of a simple mineral salts medium (pH 6.9) containing the following (g/liter, unless otherwise indicated): NaCl, 10.0; KCl, 1.0; NH_4Cl, 1.0; $MgCl_2 \cdot 6H_2O$, 1.0; $CaCl_2 \cdot 2H_2O$, 1.0; phosphate buffer, 0.1 M; and yeast extract, 5.0. The medium also contained ca. 40 g of *E. cottonii* tissue that had been washed to remove salts and sand, dried, and then ground to a 40-mesh size. An inoculum (200 ml) derived from an active methane-producing kelp digestor operating at pH 6.5 and room temperature was added to each fermentor to begin digestion. The original inoculum for the kelp digestor was anaerobic sewage sludge from a municipal waste system in Ellsworth, Maine. The fermentors were operated as a batch process at room temperature (20 to 25°C) for up to 10 weeks with continuous stirring. Experiments were performed twice, once with duplicate fermentors and once with quadruplicate fermentors.

At weekly intervals, 20- to 40-ml aliquots were removed with a syringe via side ports in the fermentors for analysis of the total dry weight of solids (particulates), the total partic-
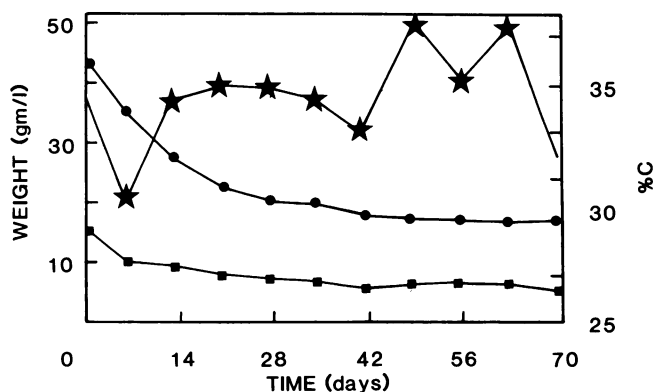
FIG. 1. Weight loss of particulates (in grams per liter) during fermentation of *E. cottonii* and changes in the percent carbon content of particulates. Symbols: ●, total weight loss; ■, loss of total carbon; ★, percent carbon.

ulate carbon, nitrogen, and sulfur content, the pH, and volatile fatty acids (VFA). Total gas production was measured with a bubble trap at 2- to 3-day intervals, and the gas-phase composition was measured on a weekly basis. Sulfohydrolase activity and dissolved sulfides were measured occasionally. The ester sulfate content, the carrageenan content, and the molecular-weight distribution and viscosity of extracted carrageenan were assayed for the original *E. cottonii* tissue and for the particulate material remaining after 10 weeks of digestion.

The total dry weight of particulates was measured by centrifugation of 20 ml of the fermentor contents at 31,180 × *g* for 15 min. After the supernatant was decanted for VFA analysis, the pellet was washed twice by suspension in deionized water followed by centrifugation. The remaining residue was dried to a constant weight for 24 to 48 h at 70°C and then ground with a mortar and pestle. Subsamples of the dried residue were analyzed for percent C and percent N with an elemental analyzer (model 1106; Carlo Erba). Total particulate sulfur was measured by digesting subsamples in boiling nitric acid and analyzing the resulting sulfate gravimetrically (19). Ester sulfate was measured by hydrolysis of *E. cottonii* tissue or particulates with 6 N HCl for 1 h at 121°C and 15 lb/in² followed by a turbidimetric assay of sulfate (13). The carrageenan content of *E. cottonii* and particulates was measured with toluidine blue O for total polyanions (3) and by isopropanol precipitation of carrageenan after extraction with boiling deionized water. Both techniques gave comparable results for the samples assayed. The molecular-weight distribution of extracted carrageenan was determined by gel permeation chromatography (22); viscosities were measured with a Brookfield-type viscometer operated at 12 and 60 rpm and 75°C with 1.5% solutions of carrageenan extracts.

Dissolved sulfide in the fermentors was determined by the methylene blue technique (7) with 10- to 50-μl aliquots diluted to 1 ml. The absorbance of the methylene blue formed by reaction of the sulfide with the phenylenediamine reagent was measured at 640 nm with an LKB Ultrospec (model 4050; LKB Instruments, Inc.) spectrophotometer. Sulfohydrolase activity was assayed with *p*-nitrophenyl sulfate as described by King and Klug (13). Subsamples of the fermentor contents were incubated at an ambient temperature in serum bottles with a nitrogen headspace. The absorbance of any *p*-nitrophenol released was measured at 407 nm.
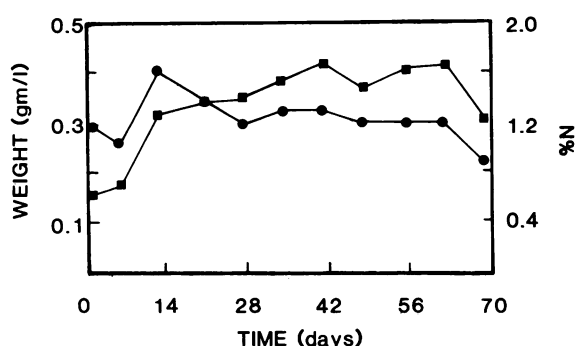

FIG. 2. Weight loss of particulate nitrogen (in grams per liter) during fermentation of *E. cottonii* (●) and changes in the percent nitrogen content of particulates (■).

VFA concentrations were determined with the supernatants obtained from centrifugation of the fermentor contents as described above. The supernatants (1 ml) were acidified with 100 μl of 6 N phosphoric acid after hexanoic acid was added as an internal standard; preliminary analyses established that hexanoic acid was absent from or present in only trace amounts in the digestors. Approximately 1-μl aliquots of the resulting solutions were injected into a Varian 3700 gas chromatograph that was fitted with a flame ionization detector and an glass column (2 m by 4-mm outer diameter) packed with SP-1200 (Supelco Inc.) and that was operated at an oven temperature of 110°C with a nitrogen carrier gas flow rate of 30 ml/min. The gas-phase composition of the fermentors was analyzed by injection of 0.2-ml volumes of the fermentor headspaces into a Varian 3700 gas chromatograph that was fitted with a thermal conductivity detector and a stainless steel column (3 m by 0.32 cm, outer diameter) packed with Porapak Q (Waters Associates, Inc.) and that was operated at an oven temperature of 60°C with a helium carrier gas flow rate of 20 ml/min. Peak areas and concentrations for VFA and gas analyses were determined with a recording integrator (Hewlett-Packard Instruments, Inc.).

RESULTS

The particulate content of duplicate fermentors decreased significantly over time, with the most rapid loss occurring during the first 3 to 4 weeks of digestion (Fig. 1). Weight loss after 5 to 6 weeks was minimal, although a slow, continuous decrease was observed. After 10 weeks of digestion, ca. 40% of the added biomass remained. In contrast, the percent
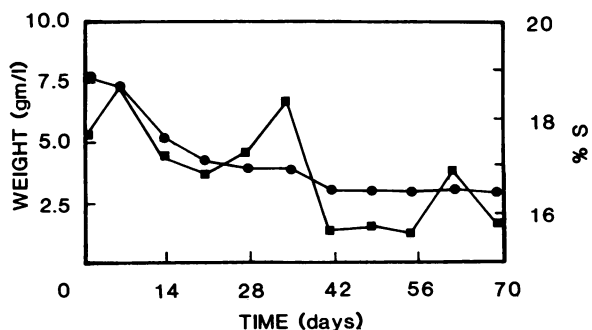

FIG. 3. Weight loss of particulate sulfur (in grams per liter) during fermentation of *E. cottonii* (●) and changes in the percent sulfur content of particulates (■).

TABLE 1. Total particulate sulfur content and sulfate ester content of *E. cottonii* tissue before and after 10 weeks of anaerobic digestion

Sample	Sulfur content (mmol/g [dry wt] ± SE)		% Sulfate ester
	Total[a]	Sulfate ester[b]	
E. cottonii	1.87	1.62 ± 0.04	86.6
Fermented *E. cottonii*	1.64 ± 0.11	1.28 ± 0.03	78.0

[a] The total sulfur content was determined by digestion in boiling nitric acid.
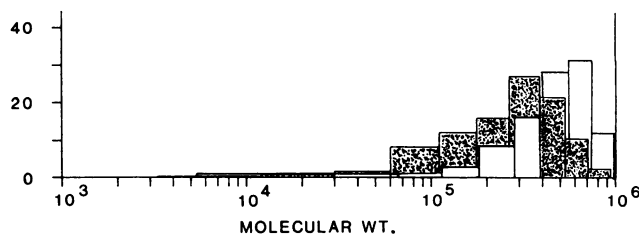[b] The sulfate ester content is defined as sulfate hydrolyzable by HCl.

FIG. 4. Weight class distribution (percent; ordinate) of carrageenan molecular weights. Stippled bars represent carrageenan extracted from *E. cottonii* after 10 weeks of fermentation; nonstippled bars represent carrageenan extracted from *E. cottonii* before fermentation.

carbon content of the particulate material increased over time from initial values of 33.9% to 37.6% after ca. 7 weeks; the percent carbon content tended to decrease thereafter to 32.1% (Fig. 1). Total particulate carbon, calculated from the percent carbon content, decreased over time in parallel with the total particulate material, except for a slight increase during week 7 associated with the maximum percent carbon value. After 10 weeks, however, total carbon was ca. 38% of that originally present, in close agreement with the particulate loss.

The percent nitrogen content of the particulate material followed a pattern similar to that for the percent carbon content; values increased from ca. 0.7% initially to a maximum of 1.8% after 6 weeks, with a decline to 1.3% thereafter (Fig. 2). However, C/N ratios based on percent carbon and percent nitrogen generally declined over time from initial values of 49.0 to values that ranged between 18.3 and 24.8 during the last 5 weeks of digestion. Unlike total particulate carbon, total particulate nitrogen increased initially and then remained relatively constant after a slight decrease.

The time course of the percent sulfur content differed markedly from that of carbon and nitrogen. The percent sulfur content decreased continuously from initial values of 6.0% to values of 5.3% after 10 weeks (Fig. 3). In contrast, C/S ratios increased from initial values of 4.7 to a maximum of 7.1. Total particulate sulfur decreased steadily over time, with only ca. 37% of that initially present remaining after 10 weeks of digestion (Fig. 3). The contribution of ester sulfate to total particulate sulfur also decreased slightly over time; ester sulfate accounted for 87% of the total sulfur initially and 78% after 10 weeks (Table 1).

The carrageenan content of the particulate material as well as the molecular weight and viscosity of extracted carrageenan changed over time (Table 2). In the original *E. cottonii* tissue, carrageenan accounted for 72.9 and 61.1% of the total weight, as determined by the toluidine blue O and isopropanol precipitation techniques, respectively. After digestion for 10 weeks, carrageenan accounted for 59.6 and 49.8% of the total particulate weight, as determined by the respective techniques (Table 2). Average molecular weights were ca. 510,000 and 319,000, respectively, for nondigested *E. cot-*

tonii carrageenan and carrageenan remaining after 10 weeks of digestion (Table 2). The lower molecular weight of digested carrageenan was the result of an increase in the fraction of the polysaccharide in lower-molecular-weight classes and of a decrease in the maximum molecular weight (Fig. 4). The viscosity of carrageenan solutions also decreased after digestion; carrageenan from nondigested *E. cottonii* yielded values considerably higher than carrageenan recovered after 10 weeks of digestion (Table 2).

VFA increased markedly over time, with the most rapid increases occurring during the first 4 weeks of digestion and with somewhat slower increases occurring thereafter (Fig. 5). Coincidentally with increasing VFA pools, the pH declined from 6.9 to 5.2; after readjustment to pH 6.2, the pH decreased minimally to 6.0 (Fig. 5). The VFA pools were dominated throughout the fermentation period by acetate, which reached concentrations in excess of 200 mmol/liter after 10 weeks (Fig. 5). Propionate and butyrate, the only other significant VFA, reached maximal concentrations of only 16.4 and 14.6 mmol/liter, respectively. Generally, propionate and butyrate each represented <10% of the acetate concentration (Fig. 5). Isobutyrate, isovalerate, and valerate were typically <2 mmol/liter. Total VFA pools accounted for about 34% of the total added carbon. Formate concentrations were not determined; however, formate probably contributed only minor amounts, as total carbon recoveries without considering formate were >99%.

Total gas production paralleled VFA production. The greatest accumulation of gas occurred within the first 4 weeks of digestion, with a lower accumulation thereafter (Fig. 6). Gas production was dominated by CO_2 rather than methane, with the methane content of the fermentors increasing rapidly during the first 2 weeks to a maximum of ca. 25% and then declining more slowly to final concentrations of ca. 10%. Total gas production amounted to 2.4 liters after 10 weeks and accounted for ca. 11% of the added carbon as CO_2 and methane.

TABLE 2. Characterization of carrageenan extracted from dried, ground *E. cottonii* and from *E. cottonii* that had been fermented for 10 weeks

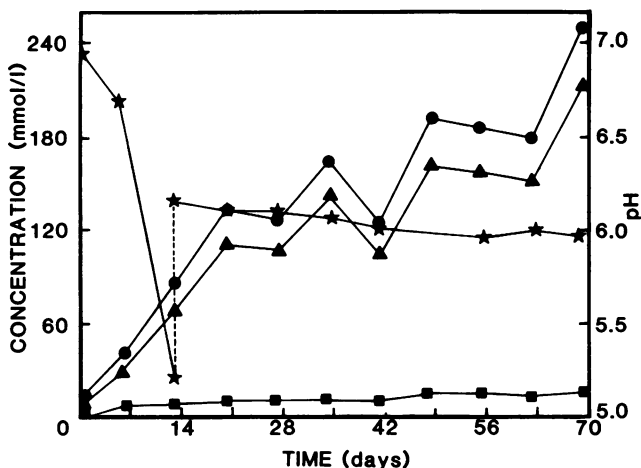| Sample	% Carrageenan content as determined by:		Mol wt		Viscosity (cps at 12 rpm)	% Sulfur content	% Toluidine blue O recovered
	Isopropanol precipitation technique	Toluidine blue O technique	Maximum	Mean			
E. cottonii	61.1	72.9	949,500	509,773	137.5	7.62	92.57
Fermented *E. cottonii*	49.8	59.6	870,250	319,088	6.25	7.17	90.53

FIG. 5. Time courses of total VFA production (●), acetate production (▲), propionate production (■), and pH changes (★) during fermentation of E. cottonii. VFA concentrations are given in millimoles per liter. ---, Point at which pH was readjusted.

DISCUSSION

The time courses for the weight loss of particulates and for VFA and gas production (Fig. 1, 5, and 6) established that E. cottonii tissue was readily degraded. The losses in particulate material were presumably caused by polymer hydrolysis and leaching. The formation of high concentrations of VFA, sulfide, CO_2, and methane demonstrated that particulate hydrolysis or dissolution was closely coupled to fermentation. Changes in total particulate carbon were also influenced by the formation of bacterial biomass. The increases in the percent carbon content in the particulate material relative to the starting material (Fig. 1) were presumably the result of the loss of noncarbon (e.g., sulfur)- or low-carbon-containing components of the E. cottonii tissue coupled with growth of the microflora.

The observed patterns of nitrogen accumulation in the particulate material (Fig. 2) were also consistent with growth of the microflora. Nitrogen increased both as a percentage of the particulate material and in absolute value during the first 3 to 4 weeks of digestion. Over this same period, C/N ratios dropped by a factor of ca. 2 (although final values were still within a range considered to indicate possible nitrogen limitation (6, 9). At present, it is not possible to estimate the fraction of the particulate material accounted for as microbial biomass from either the carbon or the nitrogen values. However, it is certain the microbial growth contributed significantly to quantitative and qualitative changes in the composition of the starting material.

The most important changes with respect to the recovery of commercially valuable polysaccharides were observed for the sulfur content and the carrageenan content of the particulate material. The continuous decreases in both the percent sulfur content as well as the absolute value of sulfur indicated that sulfated organics were degraded along with bulk organics. As the majority of E. cottonii sulfur is present as sulfated residues in carrageenan (Table 1), the extent of carrageenan metabolism can be calculated from the total particulate weight loss and carrageenan recovery (Fig. 1 and Table 2). Yields from the isopropanol precipitation technique suggest a loss after 10 weeks of digestion of 67% of the carrageenan originally present. This is slightly greater than but generally consistent with the observed particulate weight

loss of 60% and indicates that carrageenan was neither preferentially degraded nor enriched.

As significant as the loss of carrageenan were the observed changes in the physical properties of the material recovered after digestion. Both maximum and mean molecular weights decreased during fermentation; in addition, the proportion of digested carrageenan molecules with a molecular weight of <100,000 was significantly greater than that of carrageenan extracted from nondigested E. cottonii (Fig. 4 and Table 2). The viscosity of digested carrageenan solutions was also markedly reduced relative to that of nondigested carageenan solutions (Table 2). The decrease in viscosity during fermentation occurred quite rapidly; within 1 week, viscosity was depressed over 50-fold (data not shown). Interestingly, the percent sulfate content and the percent total polyanion content (toluidene blue O) of digested carrageenan and nondigested carrageenan were quite similar (Table 2), suggesting that neither of these parameters played a major role in determining viscosity or changes in viscosity.

These results suggest substantial carrageenan hydrolysis, presumably by carrageenases such as have been previously described for several marine pseudomonads (1, 26, 27). Curiously, no sulfohydrolase activity was observed in this study, even though carrageenase is reported to possess sulfohydrolytic capabilities (18, 26). It is possible that such activity cannot be measured with p-nitrophenyl sulfate as a test substrate. Nonetheless, arylsulfohydrolase activity has been readily observed in a variety of anaerobic systems (13, 20), and the absence of any such activity here appears to be unusual.

The observed changes in molecular weight and viscosity are particularly important, as they indicate that carrageenan recovered from anaerobic digestion may not be of significant commercial value. For most industrial purposes, carrageenan must have a molecular weight in excess of 100,000, and solutions must have viscosities in excess of 100 cps. Thus, even if carrageenan could be recovered at a high yield after fermentation of carrageenophytes, it probably would not be usable. Similar observations have been reported by Bird et al. (2), who noted that agar from G. tikvahiae was degraded significantly during fermentation and that the gelling capacity of agar recovered after fermentation was markedly reduced.

A final aspect of this study concerns the observed patterns of fermentation and methanogenesis. During the course of the incubation, acetate dominated the VFA pools, indicating that fermentation was closely coupled to hydrogen uptake (via interspecies hydrogen transfer). In this respect, the
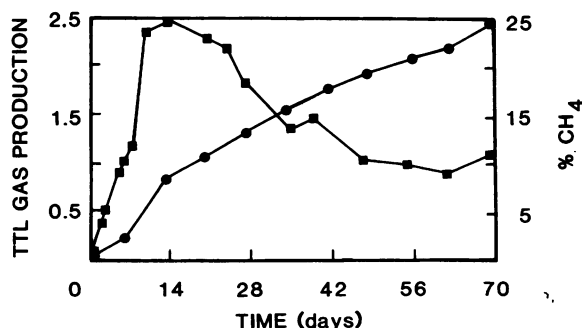

FIG. 6. Total (TTL) gas production (●) during fermentation of E. cottonii (given in liters) and time course of the percent methane composition of the gas phase (■).

digestion of *E. cottonii* appeared to be similar to that described for sewage sludge (12) and lake sediments (14, 17), and to digestions based on algal biomass (8, 9). However, yields of methane from *E. cottonii* were considerably lower than those reported for other systems (e.g., reference 16), with maximum methane concentrations being only 10 to 25% of the fermentor gas phase (Fig. 6), in contrast to more typical values of 40 to 50% (24).

Several explanations may account for the relatively low methane yields. First, incubation conditions (e.g., pH and temperature) may have been suboptimal, resulting in the relatively poor growth of methanogens. The methanogenic populations were replaced by other hydrogen utilizers, as VFA patterns were dominated by acetate and not by other, more-reduced fatty acids. Second, *E. cottonii* tissue contains a significant concentration of sulfur as sulfate (5% sulfate by weight [Table 1]), of which over 60 mmol was lost from the added biomass. The reduction of this sulfate to sulfide would have consumed the equivalent of 240 mmol of hydrogen and significantly decreased potential methane production. Finally, low methane concentrations could have been the result of a third factor. The reduction of sulfate resulted in the accumulation of very high concentrations of dissolved sulfide (~50 mmol/liter). Concentrations of this magnitude could have inhibited methanogenesis, particularly methanogenesis from acetate. Some acetate-fermenting methanogens are sensitive to sulfide, especially at the levels observed in this study (4, 25). Acetate-fermenting methanogens are also sensitive to the availability of iron in both pure cultures and sludge digestors (11, 21); dissolved iron concentrations are in turn limited by the presence of sulfides (23). The reduction of *E. cottonii* sulfate could therefore have affected the availability of reducing equivalents as well as the physiology of acetate utilization.

In summary, postfermentation recovery of commercially valuable quantities of *E. cottonii* carrageenan does not appear to be feasible. The poor recovery of carrageenan appears to be caused by hydrolysis of the molecule, resulting in lower average molecular weights and markedly reduced solution viscosities. As a result, digested carrageenan is not suitable for industrial needs. Prefermentation processing of *E. cottonii* for carrageenan followed by fermentation of the residual or waste organic matter may provide a possible solution to these problems. However, in carrageenophytes with a high carrageenan content, such as *E. cottonii*, even fermentation of the residual biomass does not appear to be economically feasible.

ACKNOWLEDGMENTS

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