Isolation of *Laminaria*-frond Decomposing Bacteria from Japanese Coastal Waters

Motoharu Uchida* and Akihiko Nakayama*

(Received June 4, 1993)

A search was undertaken to isolate bacteria capable of decomposing *Laminaria* fronds directly. Among 89 strains isolated from seawater samples with an agar plate enriched with *Laminaria* powder, 62 strains showed a *Laminaria*-frond decomposing ability which was detected by a rheometer test after 4 weeks' incubation at 20°C. The *Laminaria*-frond decomposing bacteria (LDB) were isolated from 10 locations out of 11, suggesting their widespread distribution in the Japanese coastal waters. Most of the LDB were assigned to the *Vibrio* group according to Simidu's scheme and had an alginate-degrading ability. Gel filtration analysis of saccharides dissolved in the culture medium during the decomposition test showed that the algal polysaccharides were degraded, and that the decomposition products differed greatly in molecular size from strain to strain.

It is generally accepted that microbial decomposition plays a great part in the decay process of macrophytic brown algae in the marine environment.¹⁻³⁾ Research on the microbial decomposition of macroalgae elucidated an increase in the numbers of bacteria capable of utilizing algal substances not only on the surface of decaying fronds¹⁾ but also in water associated with algal vegetation.²⁾ Especially, bacteria able to degrade alginate compounds which are one of the major components of brown algae have been isolated by many researchers.⁴⁻¹⁶⁾ The authors are interested in bacteria which have a frond-decomposing ability not only from a bacteriological point of view but also for the reason that microbial decomposition of macroalgae contributes greatly to the detrital food chain in the marine ecosystem.¹⁷⁾ But an investigation on bacterial isolates with an ability to decompose macroalgal fronds directly^{2,18)} is limited. This is partly attributed to the reason that there was no good method for estimating the decomposing activity for macroalgal fronds besides the method of measuring their weight losses.¹⁻³⁾

In this study, we developed a method for estimating the frond decomposition activity by use of a rheometer, and attempted to isolate bacteria which have the ability to decrease the physical strength of frond tissue of brown alga *Laminaria japonica* from Japanese coastal waters. We called them *Laminaria*-frond decomposing bacteria

(LDB) and examined their bacteriological characteristics including a test for their alginate-degrading ability. Saccharide substances remaining after the frond decomposition test were analyzed by gel-filtration for some strains to elucidate the mode of decomposition processes.

Materials and Methods

Laminaria-fronds

Damp-dried fronds of *Laminaria japonica*, kombu, harvested off Hakodate were obtained. To prepare the isolation medium, fine *Laminaria* powder (200-mesh) made by milling the damp-dried fronds was used. Square pieces of frond (1.5 × 3.0 cm, 0.4-0.5 g dry weight) taken from a part between 30 and 100 cm from the basal part (Fig. 1) were used for a frond decomposition test.

Samples of Seawater

Samples of seawater were taken separately in a sterilized bottle at 11 locations (Table 1) off the coast of Japan between June 13th and August 11th, 1987. The seawater, cooled with ice, was brought to our laboratory and used for bacterial isolation. The water temperature was measured in situ, and pH and salinity were determined at the laboratory. The viable counts were determined on the following isolation medium.

* National Research Institute of Fisheries Science, Fisheries Agency, Fukuoka, Kanazawa, Yokohama 236, Japan (内田基晴, 中山昭彦: 水産庁中央水産研究所).

Fig. 1. Diagram showing the preparation of *Laminaria* frond (damp-dried *Laminaria japonica*: kombu) pieces for a frond-decomposition test.

Isolation of Bacteria with *Laminaria* Powder-Enriched Agar Plate

One milliliter each of the serial dilutions of the seawater sample was plated out with 10 ml of an upper medium: 10% *Laminaria* powder, 90% aged natural seawater, 1.5% agar (pH 7.6), which was laid on 10 ml of a lower medium: 90% aged natural seawater, 1.5% agar (KSA medium). After incubation at 20°C for 8–20 days, single colonies with good growth and/or hollow formation were picked up and purified on agar plates containing 0.5% pepton, 0.3% beef-extract, 1.5% agar, and 90% aged natural seawater (NAS medium). All isolates were subject to the following frond decomposition test.

Test for Frond Decomposing Ability

A loopful of 3–7-day-old precultures of each strain on the NAS slant was inoculated into two kinds of 50 ml medium: one known as S-medium consisting of 2 frond pieces and 90% aged natural seawater (pH 6.9), and the other known as NS-medium made up of 2 frond pieces, 0.5% pepton, 0.3% beef-extract, and 90% aged natural seawater (pH 6.8). After 4 weeks' incubation at 20°C on a reciprocal shaker (70 rpm), frond pieces were taken out and cut into strips 10 mm in width. Cutting strength was measured 5 times for each piece with a rheometer using a plunger of steel wire (Fig. 2). The average value of the two pieces was calculated to determine the frond decomposing ability.

Fig. 2. Diagram of a rheometer test equipped with a plunger of steel wire.

Characterization of Isolates

The bacterial isolates were identified according to the scheme of Simidu.¹⁹⁾ All the isolates were examined for cell shape, gram reaction, motility, catalase activity, oxidase activity, and sensitivity to O/129 phosphate. An O/F test was carried out with a marine oxidation-fermentation medium proposed by Leifson.²⁰⁾ The ability to degrade sodium alginate was determined by Ando-Inoue's method.⁹⁾ Isolates were inoculated on to an agar plate consisting of 1% sodium alginate, 3.5% NaCl, 0.5% $(\text{NH}_4)_2\text{SO}_4$, 0.2% K_2HPO_4 , 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5% agar plate (pH 7.2), and incubated at 20°C for 7 days. The ability of alginate-degradation was judged by hollow formations around colonies. Halophilic tests were conducted by comparing the growth in ZoBell 2216E medium with that in the same medium but prepared with distilled water.

Gel Filtration of Solubilized Saccharides in the Culture Medium

The culture medium remaining after the frond-decomposition test were centrifuged at 8000 rpm for 20 minutes. The supernatant was filtrated through a 0.22 μm mesh. The filtrate (5 ml) was applied to a Sephacryl S-300 column 2.6 $\times$ 96 cm,

Table 1. Details of seawater samples

Sampling		Symbols	Temp. (°C)	pH	Salinity (‰)	Viable counts* (CFU/ml)
Locations	Date					
Yokosuka, Kanagawa	870613	AR	—	—	33.9	1.0×10^3
Shimizu, Shizuoka	870721	EN	26.5	8.3	29.8	2.2×10^5
Hakodate, Hokkaido	870720	HA	—	8.3	32.9	2.2×10^4
Kushiro, Hokkaido	870714	HO	12.7	8.3	32.5	4.6×10^5
Yosa, Kyoto	870714	KY	23.7	8.2	33.8	2.3×10^3
Saeki, Hiroshima	870730	NA	26.8	8.5	24.2	4.3×10^4
Niigata, Niigata	870722	NI	—	8.2	24.0	9.8×10^5
Hirado, Nagasaki	870804	SE	25.3	8.3	33.6	1.5×10^5
Shimonoseki, Yamaguchi	870803	SU	38.0	8.6	32.8	8.0×10^4
Miyagi, Miyagi	870713	TO	20.0	8.3	32.2	1.0×10^3
Watarai, Mie	870811	YO	26.8	8.3	33.8	1.8×10^5

* Counted on KSA agar plate (2% *Laminaria* powder, 90% aged natural seawater and 1.5% agar).

and eluted with 0.025 M sodium phosphate (pH 7.0) at a flow rate of 0.6–1.0 ml/min. Every 5 ml fraction was collected. The saccharide content in the fractions was measured according to the modified Carvazole-sulfuric acid method.²¹⁾ A medium with no bacterial inoculation was used as the control.

Results

Isolation of Bacteria with *Laminaria* Powder-Enriched Agar Plate

Details of seawater samples are given in Table 1. The salinity and pH were within the normal range, but only the samples taken at Saeki (NA) and Niigata (NI) had a low salinity of 24‰. Viable counts on KSA plates ranged from 10^2 to 10^5 cfu/ml. Some colonies formed clear hollows.

About 8 colonies with good growth and/or hollow formation were picked up for each location. In all 89 strains were isolated.

Test for Frond Decomposing Ability

The isolated 89 strains were tested for frond decomposing ability using two kinds of medium. The *Laminaria* frond pieces in the medium were autoclaved before the decomposition test, but hardly changed in their outer appearance or physical strength. In some cases, the frond pieces gradually decayed and lost their squarish appearance, followed by their partial fragmentation during incubation. To estimate the overall ability to decompose the frond piece, the decreasing physical strength of the frond piece was followed by measuring the cutting strength with a rheometer. As shown in Fig. 3, the cutting strength measured after 4 weeks' incubation with bacteria ranged

Fig. 3. Distribution of cutting strength of *Laminaria* frond pieces after the decomposition test.

A, in S-medium (two frond pieces in 90% aged natural seawater) and B, in NS-medium (two frond pieces in 90% aged natural seawater, 0.5% beef extract, and 0.3% polypepton) with bacterial inoculation (closed bar, $n=89$) and without inoculation (open bar, $n=10$). Cutting strengths of 0–200, 201–500, and >500 g were tentatively defined as decomposition strength ++, +, and –, respectively.

Table 2. Numbers of isolates having a decomposing ability for *Laminaria* frond in S and NS medium

		Frond-decomposing ability in NS medium***		
		±*	+	-*
Frond-decomposing ability in S medium**	++*	41	1	5
	+	4	0	0
	-*	7	4	27

* Decomposition ++, +, and - means cutting strength 0-200, 201-500, and > 500 g, respectively.

** Two *Laminaria* frond pieces in 90% aged natural seawater.

*** Two *Laminaria* frond pieces in 0.5% pepton, 0.3% beef-extract, and 90% aged natural seawater.

Table 3. Number of isolates having a *Laminaria* frond decomposing ability and a hollow forming ability on KSA plate

		Hollow forming ability on KSA*	
		Positive	Negative
Frond-decomposing ability	++/**	53	9
	-***	25	2

* 2% *Laminaria* powder, 90% seawater, and 1.5% agar.

** Frond decomposing ability is positive in either (S or NS) medium.

*** Frond decomposing ability is negative in both (S and NS) media.

from 10 to 1,750 g, while that of an axenic control was distributed between 500 and 1,300 g. We tentatively defined cases giving a cutting strength of 0-200, 201-500, and > 500 g as marked decomposition (++) , weak decomposition (+), and no decomposition (-), respectively. Fifty one of the 89 strains showed decomposing ability in the S-medium, and 57 in the NS-medium, while in total 62 of the 89 strains showed the ability in either medium (Table 2). However, not all of the strains which formed a hollow on the KSA plate decomposed the frond pieces; of the 78 strains which formed hollows, 25 did not decrease the cutting strength at all, while on the contrary, of the 11 strains which did not form hollows, 9 decomposed the frond pieces (Table 3). This indicates that the hollow is not always a useful marker for *Laminaria*-frond decomposing bacteria. The presence of LDB was found in the seawater taken at 10 of the 11 locations (Fig. 4).

Characterization of Isolates

The isolated 89 strains were tested for identification according to Simidu's scheme (Table 4). All of the isolates were gram-negative and rod bacteria,

Fig. 4. Numbers of *Laminaria* frond-decomposing bacteria isolated at 11 locations in S-medium (A) and NS-medium (B).

Bacteria showed marked decomposition (closed bar), weak decomposition (crossed bar), and no decomposition (open bar). See Table 1 for the symbols of sampling locations.

and were categorized into 3 groups. Of the 89 strains, 84 (94%) were identified as *Vibrio* (Group I), three as *Aeromonas* (Group II), and two as *Pseudomonas* or *Cytophaga* (Group III). It is noteworthy that 61 (98%) of the isolated 62 LDB strains were grouped into *Vibrio*, which corresponds to 73% of the total of 84 *Vibrio* strains isolated (Table 5).

All of the isolates were also tested for alginate-degrading ability, since alginate is the major structural material of the *Laminaria*-frond tissue. As seen in Table 6, there was a good correlation between *Laminaria*-frond decomposing ability and alginate-degrading activity: 48 of the 62 LDB strains formed hollows on alginate-enriched agar plates, whereas only one of the 27 non-LDB strains did.

Gel Filtration of Saccharides Solubilized from *Laminaria*-frond along with Frond Decomposition

It was supposed that the decrease in physical strength of the *Laminaria*-frond was attributed to the degradation of structural polysaccharides such

Table 4. Bacteriological characteristics of isolates

Group	Gram stain	Cell shape	Pigment	OF	Oxidase	O/129	Halophilic	Motility	Numbers
I	—	Rod	C/Y/B	F	+	S	H	+/-	82
	—	Rod	C	F	—	S	H	+	1
	—	Rod	C	F	—	S	H T	—	1
II	—	Rod	C/Y	F	+	R	H	+/-	3
III	—	Rod	Y	—	+	S	H	+/-	2

Symbols: +, positive; —, negative; C, cream-white; Y, yellow-orange; B, blue-black; F, fermentative; S, sensitive; R, resistant; H, halophilic; HT, halotolerant; /, or.

According to the Simidu's scheme, group I, II, and III is assigned to the genus *Vibrio*, *Aeromonas*, and *Pseudomonas* or *Cytophaga*, respectively.

Table 5. Grouping of isolates and their frond decomposing ability

Frond-decomposing ability			
		+/+**	—***
Group I *		61	23
Group II *		1	2
Group III *		0	2

* According to the Simidu's scheme, Group I, II, and III are assigned to the genus *Vibrio*, *Aeromonas*, and *Pseudomonas* or *Cytophaga*, respectively.

** Frond decomposing ability is positive in either (S or NS) medium.

*** Frond decomposing ability is negative in both (S and NS) media.

Table 6. Numbers of isolates having a frond decomposing ability and an alginate-degrading ability

		Alginate-degrading ability*	
		Positive	Negative
Frond-decomposing ability	+/+**	48	14
	—***	1	26

* Determined by Ando-Inoue's method.

** Frond decomposing ability is positive in either (S or NS) medium.

*** Frond decomposing ability is negative in both (S and NS) media.

Fig. 5. Gel filtration chromatograms of saccharides released into the S-medium of 4 samples, an axenic control and 3 LDB (HA-1, HO-2, and TO-1) after incubation.

Filtration conditions: gel Sephacryl S-300; column size 2.6×95 cm; eluent, 25 mM sodium phosphate (pH 7.0); flow rate, 0.6–1.0 ml/min; sample volume, 5 ml; fraction volume, 5 ml. Vo: void volume. Vt: total volume.

as alginate and cellulose. To make clear it that the polysaccharides were actually degraded, saccharides released from the frond piece into the S-medium during the decomposition test were analyzed by gel filtration. Elution patterns obtained from three different LDB strains (HA-1, HO-2, and TO-1) which possessed ++ decomposing ability and were characterized to *Vibrio* group are shown in Fig. 5 together with that of an axenic control. Only a single peak appeared at the void volume in the elution pattern of the control experiment, but some different peaks were observed in those of the three strains. The pattern of HA-1 had a broad peak corresponding from middle to low molecular weight substances. That of HO-2 is characterized by two large peaks of lower molecular weight substances. A relatively large peak of high molecular weight substance was observed in the case of TO-1. The viscosity of the S-medium incubated with TO-1 increased markedly according to the accumulation of the high molecular polysaccharides in the medium. These findings implied the degradation of algal polysaccharides by the LDB during the frond decomposition, since no such peak was observed in the cases of non-LDB strains. Although the decomposed and fragmented fronds were not macroscopically discernable from each other, the elution patterns showed a wide variation.

Discussion

Many kinds of bacteria able to utilize algal substrates such as alginate, cellulose, laminaran, and mannitol have been found on the surface of fronds of macroalgae¹⁻³⁾ and are considered to be involved in the decay process of algal fronds. But it was not clear which kind of bacteria played an important role for the direct decomposition of macroalgal fronds, by which frond tissue is softened, fragmented, and carried into the detrital food chain in the marine environment. This is partly attributable to the fact that there was no good method for estimating the microbial decomposing ability for algal fronds. Measuring the physical strength of frond pieces with the use of a rheometer, we found bacteria which had the ability to decrease the cutting strength of frond tissue during incubation, and called them LDB. The present method was considered to be effective because the frond pieces incubated with LDB showed a definite decrease in cutting strength less than 200 g (Fig. 3; and about 90% of the LDB

were easily discerned from non-LDB. The LDB were isolated from 10 did out of 11 locations, even from the sea where *Laminaria* did not vegetate, suggesting that LDB is commonly distributed in the waters. No LDB was found at location Niigata, and only one at location Saeki. This might be related to the fact that salinity was as low as 24‰ (Table 1). Bacteria forming hollows on *Laminaria* powder-enriched agar plates did not always decrease the cutting strength of the *Laminaria* frond pieces. This may be due to the fact that hollows could be made by the degradation of agar or some other low molecular compounds which did not participate in the physical strength of the frond pieces. Nutrition enrichment to the culture medium had little influence on the assessment of the frond-decomposing ability (Table 2), but rapid decomposition was often observed in the NS-medium.

Hollohan *et al.*³⁾ reported that *Vibrio* dominated on the decaying fronds of a brown alga. Alginate-degrading bacteria isolated so far were often also identified as genus *Vibrio*.^{9,14)} With the present result that the major part of the LDB isolated was characterized as *Vibrio* (Group I in Table 5), it is confirmed that this kind of bacteria plays an important role in the microbial decomposition processes of macroalgal fronds in the marine environment. It was also found that LDB commonly possessed an alginate-degrading activity (Table 6), but degrading activities against other kinds of algal polysaccharides remain to be studied. It is, however, clear that the frond decomposition detected by the use of a rheometer was due to the degradation of polysaccharides. Though components in each peak were not identified, the peak which appeared at the position of void volume in case of the medium of TO-1 was thought to represent that of alginate compounds released by alginate-degrading enzymes. This was because its molecular weight was over 10^4 and its total amount accounted for approximately 10% of the *Laminaria* frond pieces by dry weight (on the basis of sodium alginate). As for the other strains of LDB, the information about the frond decomposing mechanism is scanty. However, when we remember the significance of seaweed decomposition in the natural environment as a source of nutrients for offshore areas,²⁰⁾ it is an interesting finding that the saccharides released from frond tissue, though under experimental conditions, would differ greatly according to the kind of bacteria involved in the frond decomposition.

Acknowledgments

We express our gratitude to Dr. B. Kimura, Dr. K. Kato, Dr. A. Sugiyama, Dr. A. Shiimoto, Mr. A. Fujii, Mr. H. Saito, Mr. S. Yamada, Mr. K. Kimoto, and Mr. S. Itakura for helping us in seawater sampling. This work was partly supported by a grant (Biomass conversion program) from the Research Council of the Ministry of Agriculture, Forestry and Fisheries, Japan.

References

- 1) R. A. Laycock: The detrital food chain based on seaweeds. I. Bacteria associated with the surface of *Laminaria* fronds. *Mar. Biol.*, **25**, 223-231 (1974).
- 2) M. R. Kirchner: Microbial degradation of North Sea macroalgae: Field and laboratory studies. *Bot. Mar.*, **32**, 241-252 (1989).
- 3) B. T. Hollahan, P. E. Dabinett, and J. A. Gow: Bacterial succession during biodegradation of the kelp *Alaria esculenta* (L.) Greville. *Can. J. Microbiol.*, **32**, 505-512 (1986).
- 4) S. A. Waksman, C. L. Carey, and M. C. Allen: Bacteria decomposing alginic acid. *J. Bact.*, **28**, (1934).
- 5) S. A. Waksman and M. C. Allen: Decomposition of polyuronides by fungi and bacteria. II. Decomposition of alginic acid by bacteria and formation of the enzyme alginase. *J. Am. Chem. Soc.*, **56**, 2701-2705 (1934).
- 6) P. Kooiman: Enzymic hydrolysis of alginic acid. *Biochemica et Biophysica Acta*, **13**, 338-340 (1954).
- 7) K. Inoue and Y. Ando: Decomposition of alginic acid by microorganisms. Part I. Decomposition of alginic acid by *Aerobacter aerogenes* type Y-11 strain and adaptive formation of alginase. *Nippon Nogeikagaku Kaishi*, **30**, 742-746 (1956) (in Japanese).
- 8) K. Inoue: Decomposition of alginic acid by microorganisms. Part II. On the "alginase" action of *A. aerogenes* Y-11. *Nippon Nogeikagaku Kaishi*, **31**, 798-801 (1957) (in Japanese).
- 9) Y. Ando and K. Inoue: Decomposition of alginic acid by microorganisms-IV. on the *Vibrio*-type bacteria, newly isolated from the decaying *Laminaria*. *Nippon Suisan Gakkaishi*, **27**, 339-341 (1961).
- 10) Y. Ando and K. Inoue: Decomposition of alginic acid by microorganisms-VI. on the modes of action of two alginases. *Nippon Suisan Gakkaishi*, **31**, 552-557 (1965).
- 11) K. Yamaguchi: Studies on the alginic acid destroying bacteria Part I. On the identification of one isolated strain and its alginase. *Nippon Nogeikagaku Kaishi*, **32**, 483-486 (1958) (in Japanese).
- 12) R. S. Doubet and R. S. Quatrano: Isolation of marine bacteria capable of producing specific lyases for alginate degradation. *Appl. Env. Microbiol.*, **44**, 754-756 (1982).
- 13) T. Romeo and J. F. Preston III: Depolymerization of alginate by an extracellular alginate lyase from a marine bacteria: Substrate specificity and accumulation of reaction products. *Biochemistry*, **25**, 8391-8396 (1986).
- 14) M. Kitamikado, C. H. Tseng, T. Aoki, K. Yamaguchi, and T. Araki: Isolation of bacteria capable of producing alginate-degrading enzyme from natural environment. *Nippon Suisan Gakkaishi*, **55**, 709-713 (1989).
- 15) T. Sawabe, Y. Ezura, and T. Kimura: Characterization of an alginolytic marine bacterium from decaying Rishiri-kombu *Laminaria japonica* var. *ochotensis*. *Nippon Suisan Gakkaishi*, **58**, 141-145 (1992).
- 16) C. H. Tseng, K. Yamaguchi, and M. Kitamikado: Two types of alginate lyase from a marine bacterium *Vibrio* sp. AL-9. *Nippon Suisan Gakkaishi*, **58**, 743-749 (1992).
- 17) K. H. Mann: Seaweeds: their productivity and strategy for growth. *Science*, **182**, 975-981 (1973).
- 18) T. Sekine: Studies of softening and decomposition of seaweeds (*Phaeophyceae*) by microorganisms and their utilization I. On general characteristics of bacillus. *Nippon Suisan Gakkaishi*, **16**, 43-45 (1951).
- 19) U. Simidu: In "Methods on marine microbiology" (ed. by H. Kadota and N. Taga), Gakkaishuppan Center, Tokyo, 1985, pp. 228-233 (in Japanese).
- 20) E. Leifson: Determination of carbohydrate metabolism of marine bacteria. *J. Bact.*, **85**, 1183-1184 (1963).
- 21) T. Bitter and H. M. Muir: A modified uronic acid carbazole reaction. *Anal. Biochem.*, **4**, 330 (1962).
- 22) A. I. Robertson and J. A. Hansen: Decomposing seaweed: a nuisance or a vital link in coastal food chains? *C/SIRO Division of Fisheries and Oceanography Report 1980-1981*, 75-83 (1982).