比治山女子短期大学紀要，第28号，1993
Bul. Hijiyama Women's Jun. Col., No. 28, 1993

Microflora Undergoing on Putrefaction Process of Brown Algae, *Ishige foliacea, Ishige okamurai,* and *Kjellmaniela crassifolia*

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ABSTRACT

When some brown algae, *I. foliacea*, *I. okamurai* and *K. crassifolia* were mixed with sea sands, they were vigorously putrefied. At the first stage of the putrefaction bacterial species were dominanted and then with a lag time fungal ones were followed. Three species of bacterium and a fungi were isolated from the putrefying seaweeds. These microorganisms were seemed to be dominantly putrefied brown algae. The fungi isolated were well grown on a synthetic medium containing fucoidan.

INTRODUCTION

In the marine environment, on putrefactions of dead plant organisms, example dead seaweed fruting bodies or plant plankton, are concerned a lagre number of microorganisms. In the putrefaction process, many natural substances in seaweed bodies or plant plankton will be decomposed by the microorganisms. So far, there have been many reports on the abundant development of microorganisms on decomposing seaweeds or plant plankson.[1)~18)] Most of the seaweed-decomposing microorganisms have commonly the biochemical abilities so hydrolyze a variety of organic substances such as starch,[19),20)] cellulose,[20)] chitin,[20),21)] alginate,[20),22)] lignin,[23),24)] and fucoidan.[25),26)]

Fucoidan[30)] is a sulfated polysaccharide consisting of L-fucose as major constituent sugar, and ester-sulfate, and then is found typically in brown algae, and is one of decompositing-resistant materials in nature. Therefore, fucoidan-degrading microorganisms are potentially useful.

In order to collect the fucoidan-degrading microorganisms, microflora undergoing on brown algae, *Ishige foliacea*, *Ishige okamurai*, and *Kjellmaniella crassifolia*, putrefied at the artificial conditions, was investigated. At the same time, a morphological characters of fucoidan-degrading microorganisms obtained were examined.

MATERIALS AND METHODS

Seaweeds

Brown algae, *I. foliacea* and *I. okamurai*, were collected at Kurahashi island (in Hiroshima Japan, 1988). *K. crassifolia* was purchased from Naya Company (Muroran city in Hokkaido, Japan 1987). The seaweeds were desiccated under the sun and then stored at 25°C with shading until used.

Collection of Suspended matter

Seawater was collected by using a plastic type botle. The seawater was filtered through a grass fiber filter paper (Toyo Roshi GC-50) and then was sterilized at 121°C for 20 min. Sea sands were collected at inside 1 m from a

waterside. The sea sands were filtered through a metallic mesh filter having as advertised pore size of 2.0 mm. The seawater and sea sands were stored at 4°C until used.

Conditions for Putrefaction of Sesweed

i) *Apparutus:* Thermo- and lighting controlled incubator (LPH-200-RDS, Nippon Medical and Chemical Instruments Co., Ltd) was used for incubation of seaweeds.

ii) *Incubation of seaweed:* A seaweed (5 g) was inoculated on sea sands (10 g) in a petri dish, and then approximetric volume of natural sea water sterilized by autocrave at 121°C for 15 min was added. The Petri dish was shieled tightly with laboratory film (Parafilm, American National Comp.) and then was stored at 28°C and 80% of relative humidity with lighting at 12 hr intervals during the incubation. A seaweed (5 g) were also stored with 30 ml of natural seawater. The Petri dish was sampled at about one month interval during the incubation. The microorganisms developing on the stored seaweeds with sea sands were examined by the method of plate cultivation.

General analysis

NaCl concentration was measured by refraction method with Saliometer S-10 (RIKA Co., Ltd). Bacterial growth was monitored by measuring turbidity of the seawater at 660 nm.

Microflora examinations

In order to disperse microorganisms from a piece of putrefying seaweed into artificial seawater, vigorous mixing for 3 min by a vortex mixer was employed.

The colony counting of aerobic hetero trophic microorgranisms was carried out by the spread plate method of Buck and Cleverdon, but the plating medium was replaced by a FP medium (final pH 8.0), consisting of polypeptone (Daigo Nutrition Chemistry Ltd.), 1 g; yeast extract (Oriental Yeast Ltd.), 0.5 g; gagome fucoidan (prepared by the method of Fujikawa[32]) from *K. crassifolia*, 5.0 g, and agar, 20 g in 1000 ml of sterilized natural seawater. All cultures were incubated at 25°C. The well grown and appearant different colonies were chosen. The microorganisms chosen were screened on the same medium by high dilution plates. Microbial utilization of fucoidan was tested in a SFC medium (pH 8.0), consisting of gagome fucoidan, 5.0 g; NH_4NO_3, 2.0 g; ferric citrate, 0.5 g in 100 ml of sterilized artificial seawater ($MgSO_4 \cdot 7H_2O$, 2.0 g; $MgCl_2 \cdot 6H_2O$, 2.0 g; $CaCl_2$, 0.05 g; Na_2PO_4, 0.5 g/1000 ml of distilled water) or a FPT medium (at 1 week's incubation clear zone appearing around colonies provides evidence for hydrolysis of fucoidan)[27].

RESULTS

Time course of Putrefaction of Seaweeds

Profiles of putrefying brown algae, *I. foliacea*, *I. okamurai*, and *K. crassifolia*, were shown in Photo. 1 and chemical analyses of them was summarized in Table I. All of the brown algae were severely putrefied when they were mixed with natural seawater or sea sand

Table I Chemical analyses of putrefying brown algae

Strain	pH				NaCl conc. (%)				OD of medium at 660 nm			
(day)	0	15	30	60	0	15	30	60	0	15	30	60
I. foliacea	8.01	7.99	7.95	7.80	35	34	36	42	0.05	0.45	0.36	0.23
I. okamurai	8.01	7.95	7.90	8.15	35	35	32	37	0.04	0.37	0.34	0.23
K. crassifolia	8.01	7.89	7.92	8.15	35	35	37	37	0.04	>2.0	>2.0	>2.0
Seawater (SW)	8.01	8.04	8.02	8.01	32	32	33	32	0.02	—	—	0.03
SW+Sea sand	8.00	7.98	7.95	7.97	34	34	35	35	0.04	—	—	0.09

—, not tested.

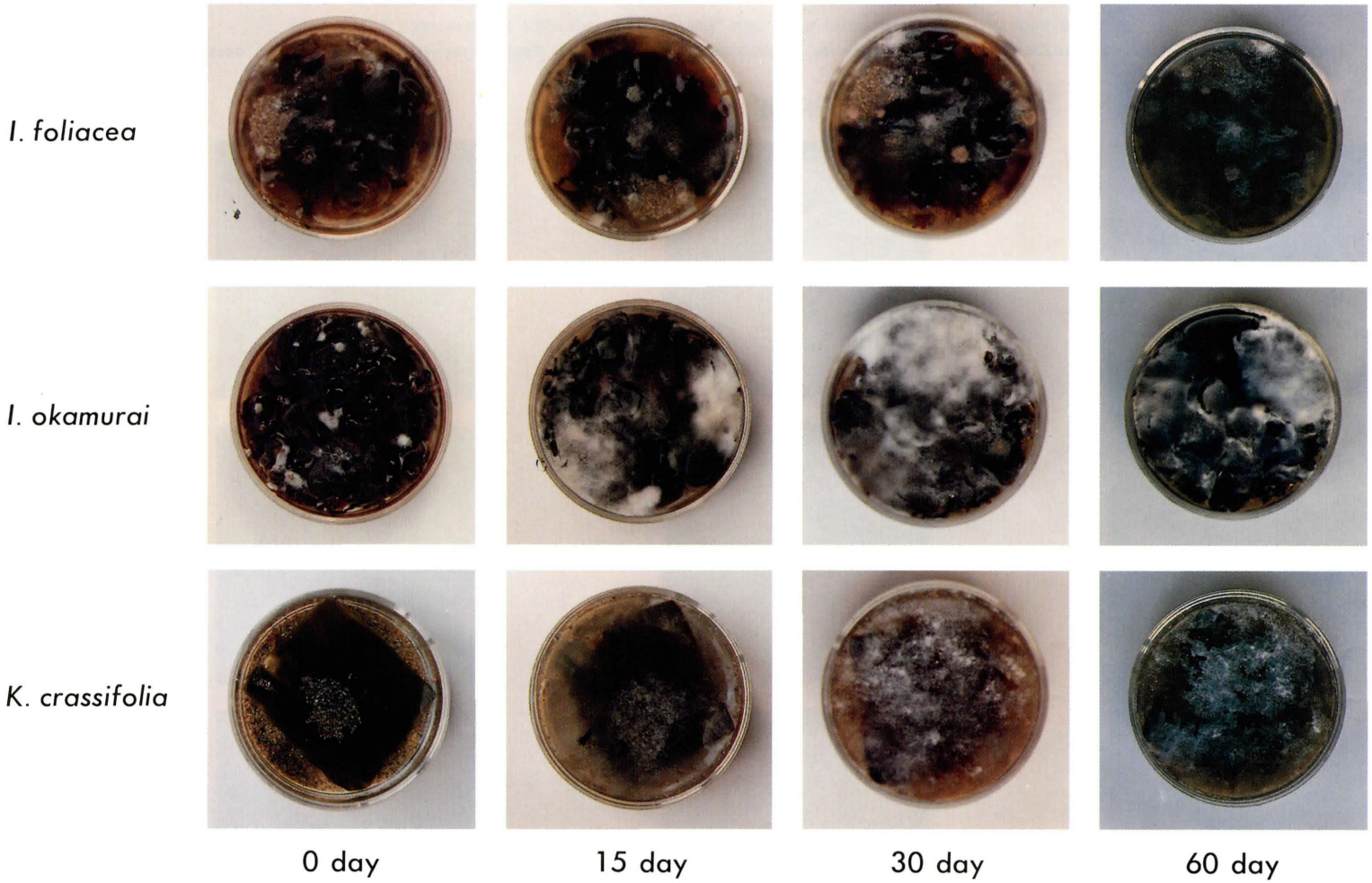

Photo. 1 Time course of putrefaction of seaweeds. Seaweeds mixed with sea sands in artificial seawater weredincu ated at 25°C and 80% of relative humidity. Incubation time, 60 days.

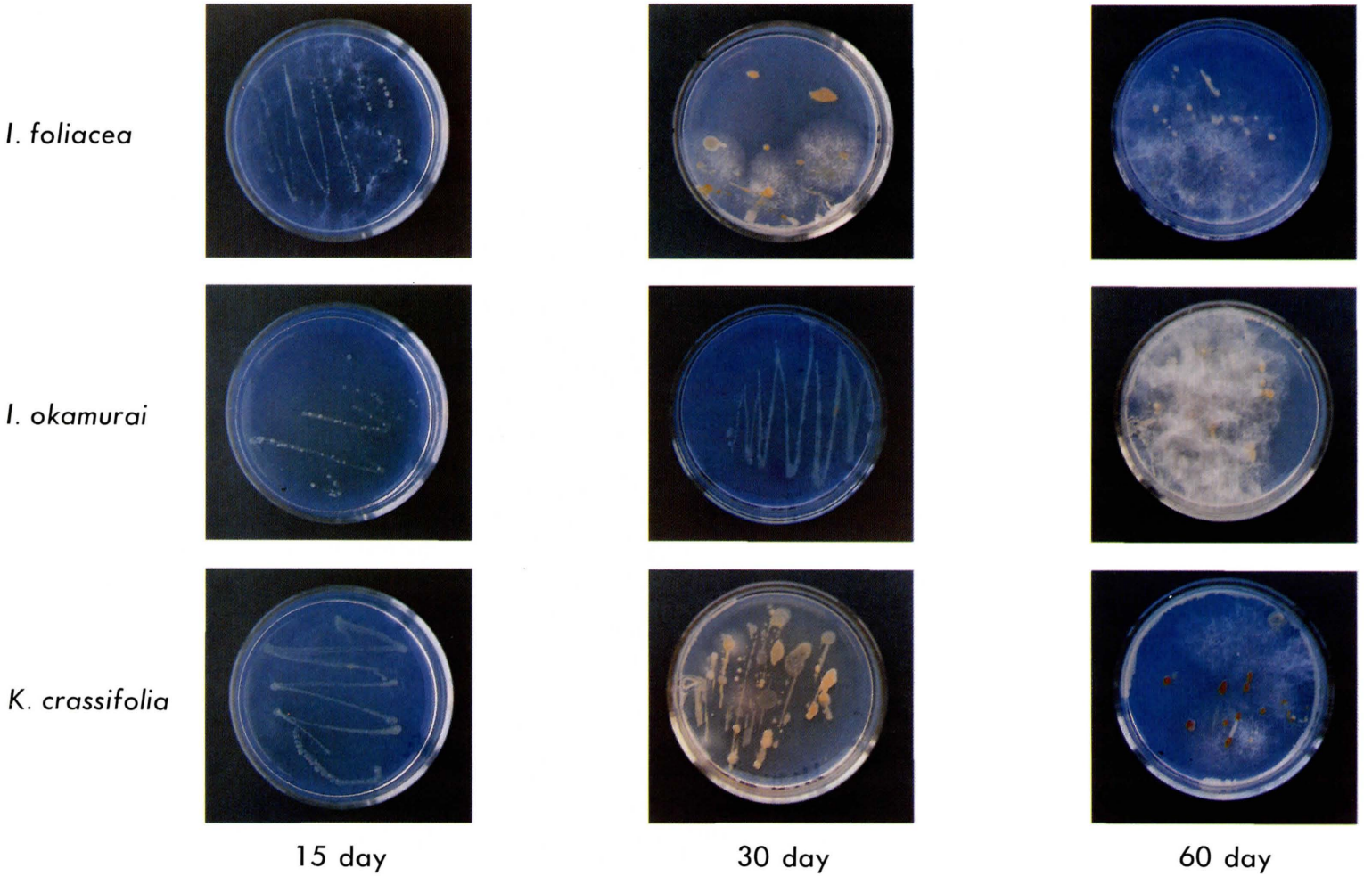

Photo. 2 Microoganisms developing on putrefaction precess of seaweeds. The microorganisms were sampled from putrefying seaweeds at appropriate interval, and then cultivated at a FP medium for 3 days.

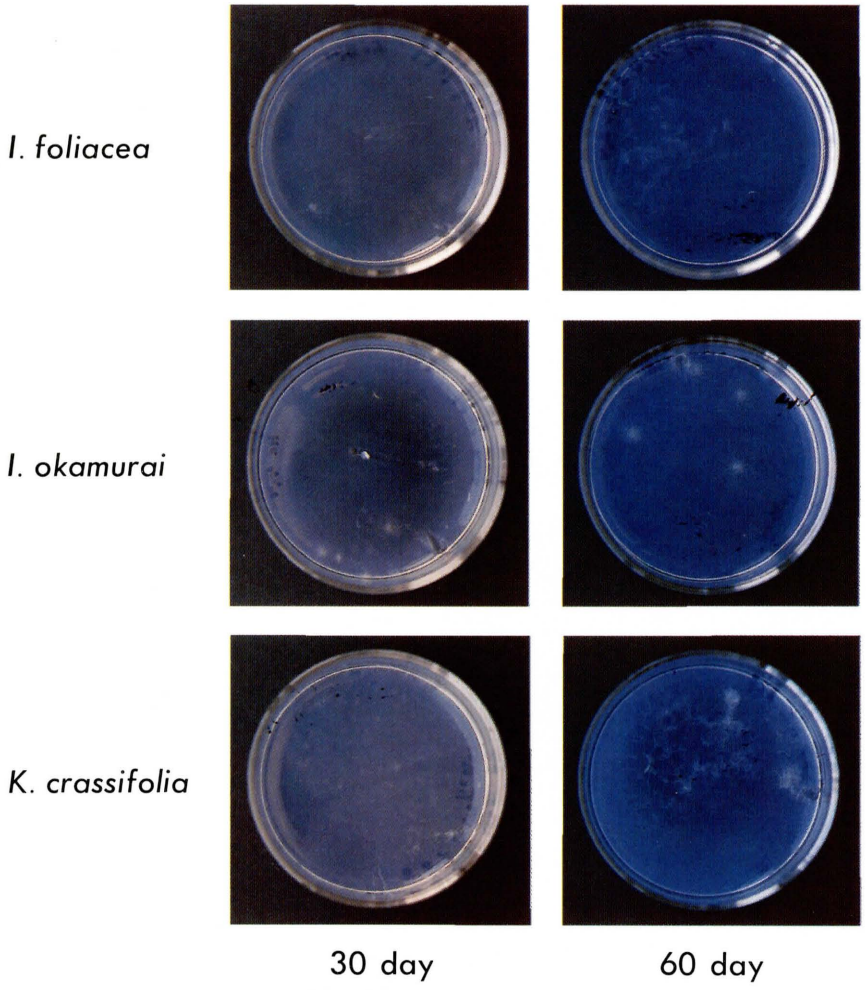

Photo. 3 Fucoidan-utilizing microorganisms developing on putrefying seaweeds.
The microorganisms were cultivated at a SFC medium for 1 week.

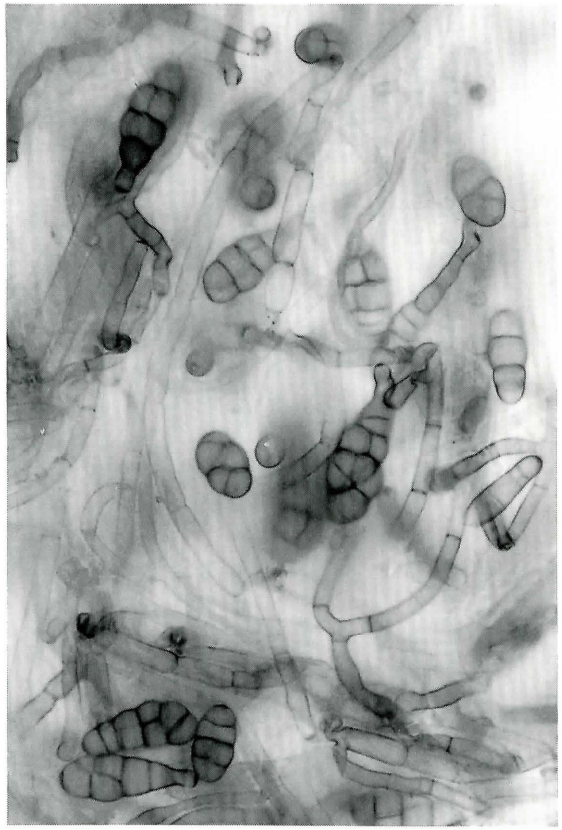

Photo. 4 Microscopic photograph of fucoidan-utilizing fungi type microorganism isolated from putrefying seaweeds.
Microscope scale : ×400.

and then incubated at 25°C, around pH 7, and 80% of relative humidity. On progression of the putrefaction, fungi type microorganisms were dominantly obserbed. Seaweeds stored with sea sands were putrefied faster than that in natural seawater. The pHs of the seawater were also scarcely decreased with putrefaction of seaweeds. The putrefaction appeared to be fast according to following order, *K. crassifolia* > *I. foliacea* > *I. okamurai*.

Microorganisms developing on Putrefying seaweed

Microorganisms developing on putrefying brown algae were examined by the method of plate cultivation uisng on FP medium and SFC medium (Photo. 2 and Photo. 3) and the species number of the microorganisms isolated was shown in Table II. The species number of microorganism developed did not vary according to the species of seaweeds. After 30 day incubation, 3 species of bacteria were isolated from *K. crassifolia* and *I. foliacea*, repectively, and 2 species of one were from *I. okamurai*. Fungi type microorganisms were isolated from all of the seaweeds used. On the other hand, no bacteria type microorganisms were done by the plate cultivation using a SFC medium. A fungi type one was commonly isolated from all of the seaweeds.

Microflora examinations

Morphological and biochemical properties of isolated bacteria were examined. The results were summarized in Table III. The isolated bacterium was named as strain If-1, If-2, If-3,

Table II Viable microbal counts of putrefying brown algae

Microorganism	FP medium			SFC medium		
	If	Io	Kc	If	Io	Kc
Bacteria type	3	2	3	0	0	0
Yeast type	0	0	0	0	0	0
Fungi type	1	2	2	1	1	1
Total	4	4	5(13)	1	1	1(3)

If, *Ishige foliacea*; Io, *Ishige okamurai*; Kc, *Kjellmaniella crassifolia*

Table III Morphological and biochemical properties of bacterial strains isolated from putrefying brown algae

	I. foliacea			*I. okamurai*		*K. crassifolia*		
	If-1	If-2	If-3	Io-1	Io-2	Kc-1	Kc-2	Kc-3
Shape	R	R	R	R	R	R	R	R
Gram stain	−	−	−	−	−	−	−	−
Motility	−	−	−	−	−	−	−	−
Hugh-Leifson's test	F	wF			F	F	wF	
Oxidase*	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Pigmentation	−	−	yo	yo	−	−	−	yo
Growth on isolation medium	+	+	+	+	+	+	+	+

*N,N,N', N'-Tetramethy-*p*-phenlenediamine dihydrochloride disk (1%) was used. R, rod; F, fermentation; wF, weak fermentation; yo yellow-orange; +, positive; −, negative.

Table IV Viable microbes undergoing on putrefying brown algae

Time (day) / Alga species	0	15	30	60
I. foliacea	*Vi.Fl.Ac>Fu*	*Vi.Fl.Ac>Fu*	*Vi.Fl.Ac>Fu*	*Fl.Vi.Ac<Fu*
I. okamurai	*Vi.Fl>>Fu*	*Vi.Fl.>Fu*	*Vi.Fl.>Fu*	*Fl.Vi.<Fu*
K. crassifolia	*Vi.Fl>Ac>Fu*	*Vi.Fl.Ac>Fu*	*Fl.Vi.Ac<Fu*	*Fl.Vi.Ac<Fu*

Vi, *Vibrio*; *Fl*, *Flavobacterium*; *Ac*, *Achsomobacter*; *Fu*, *Fungi*.

Io-1, Io-2, Kc-1, Kc-2, and Kc-2, respectively (abbreviation: If, *I. foliacea*; Io, *I. okamurai*; Kc, *K. crassifolia*). All of them were anaerobic, gram negative, nonmotile rods, and then were also oxidase and catalase positive. Strain If-3, Io-2 and Kc-2 formed yellow-orange colonies on a FP medium, respectively. The strain If-1, If-2, Io-2, Kc-1 and Kc-2 fermented glucose without no gass formation, respectively.

By taxonomic examinations according to the method of Simidu *et al*[33], it was estimated that strain If-1, Io-1 and Kc-1 were blong to the genus *Vibrio*, and strain If-2 and Kc-2 were to the genus *Achromobacter*, and strain If-3, Io-2 and Kc-3 were to the genus *Flavobacterium*, respectively.

On the other hand, fungi type microorganisms isolated on FS medium was examined with microscopy (Photo. 4). The fungi appeared to be homologous. The fungi has not be identified yet.

Microflora undergoing

Microflora undergoing on putrefaction of the seaweeds were summarized in Table IV. In all of the seaweeds used, bacterial flora did not undergo specially accoding to the species of the seaweeds. The *Vibrio*, *Flavobacterium*, and non-identified fungi were commonly observed at putrefaction process of all the seaweeds. At first stage of the putrefaction, viable bacterial growth was observed, and then with a lag time fungi type microorganisms were well grown.

DISCUSSION

On microflora undergoing on putrefaction of the brown algae, at the first stage of putrefaction bacterial species were dominanted and with progress of putrefaction a fungi type microorganisms were well obserbed. At the final stage, fungi type microorganisms were remakable. This may be due to that potential bacteria in sea sands grew first by using released materials from seaweed, because seaweeds stored in artificial seawater mixed with sea sand were putrefied faster than that in natural seawater.

Of all the potential habitats and sources of microorganisms in coastal water, seaweeds are the most obvious due to their rich content and variety of carbohydrates.[34] So it is not mentioned that a large number of microorganisms will be concerned to purtefaction of seaweeds. In general, there have been few reports on microorganisms which are capable of decomposing brown algae. Of all the reports on microflora encountered on living thallus of seaweeds, in 1969 by examination of microflora developed on the surfaces of living thallus of *Ascophyllum nodosum* and *Polysiphonia lanosa*, seven species of bacterium and a yeast, *Vibrio*, *Flavobacterium*, *Escherichia*, *Pseudomonas*, *Sacrina*, *Staphylococcus*, *Achromobacter*, and *Rhodotorula* were isolated.[35] And in 1971 five species of bacterium, *Staphyrococcus*, *Bacillus*, *Eshierichia*, *Flavobacterium*, and *aeromonas* isolated from a thallus of *Porphyra leucostica* which was cultured *in vitro*.[36]

In this study, three species of bacterium, *Vibrio*, *Flavobacterium*, and *Achromobacter* were isolated from a seaweed putrefied in a Petri dish. These bacterial species were agrreed with that encountered on living thallus of *A.*

nodosum or *P. lanosa*, except the small number of bacterial species. This suggested that these bacterial species might be dominanted on putrefaction of brown algae. The small nummber of bacterial species may be attributed to the suitability of the medium used.

We previously reported that some species of fucoidan-utilizing bacterium were lived in sea sands around inshore water.[26)-28)] van Udern & Castero Branco reported that there were many yeast on *Macrocystis pyrifere* which had been thrown to the shore and then been putrefied.[17)] Of all the isolates, however, any fucoidan-utilizing bacterium and yeast were not observed. This may be due to the release of rich carbohydrates of the seaweeds and of inhibitory phenolic materials,[29)] respectively.

Whereas, the fungi type microorganism isolated also appeares to be donimanted on putrefaction on the brown algae. The microorganism was well grown on SFC medium, suggesting that it might be able to degrade a fucoidan. By morphological observation, the microorganism was differ from some fungi isolated from sea sands, previously.[28)] This microorganism will arouse an interest in avairable utilization of fucoidan which is a nonutilizing carbohydrate in brown algae.

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(Received October 5, 1992)

要　　約

褐藻，イロロ，イシゲおよびガゴメコンブの腐敗過程において遷移する微生物相

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褐藻類の腐敗過程において遷移する微生物相を検討するために，3種類の褐藻，イロロ，イシゲおよびガゴメコンブを海砂または海水と共に貯蔵し，腐敗させた。平面塗抹培養法により腐敗藻に棲息する微生物を経時的に分離し，調べた結果，次のことが明かとなった。

1）3種類の褐藻は全て海水よりも海砂においてより速く腐敗した。

2）腐敗藻より分離された微物物の種類は，褐藻の種類に依存しなかった。

3）イロロ，イシゲおよびガゴメコンブの腐敗には，*Vibrio* 属，*Flavobacterium* 属および *Achromobacter* 属の細菌とある種の糸状菌が主に関与するように考えられた。

（受理　平成4年10月5日）