Mem. Fac. Fish., Kagoshima Univ.
Vol. 33, No. 1, pp. 107~113 (1984)

Enzymes Digesting the Crude Fiber Isolated from Cultured Nori (*Porphyra* sp.)*1

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Abstract

Enzymes secreted from the bacteria isolated from cultured nori (*Porphyra* sp.) infected with Anaaki-disease digested the crude fiber isolated from *Porphyra*. Bacteria, two strains of P4 and P5, were identified as *Vibrio fisheri* by taxonomic studies.

The optimum temperatures for the growth of both strains were 25 to 30 °C against 12 hour-culture, 20 °C against 24 hour-culture, and 15 °C against 48 hour-culture.

When the both strains were cultured in the presence of crude fiber in the cultural medium, the enzyme activity increased by 2 to 9 times. The activity of the enzyme of P4 strain was the highest in the presence of 4% crude fiber in the cultural medium. Optimum pHs of enzymes from P4 were pH 6 and pH 8, whereas that from P5 was pH 8.

Recently the improvement of plant breeding has been carried out by cell-fusion of protoplast.[1-3] Although the attempt of the improvement of marine algae by cell-fusion is expected, it is difficult to prepare a large quantity of protoplast from marine algae. Enzymatic method is very useful for the preparation of a large quantity of protoplast. However, since the kinds of polysaccharides constructing the cell wall of red- or brown-algae is different from those of terrestrial plant, the commercially available enzymes for the preparation of protoplast of terrestrial plant is not useful for the preparation of protoplast of red- or brown-algae. It is needed to find out enzymes digesting the cell walls of such algae.

It is known that there are some bacteria which induce some diseases of cultured nori (*Porphyra* sp.). Anaaki-disease is one of diseases of *Porphyra* and the bacteria inducing Anaaki-disease attach to the thalli of *Porphyra,* digest their cells and make holes on their thalli. This suggests that the bacteria secret some enzymes to digest the cell wall of the algae. Then we investigated whether the medium after the culture of the bacteria digested the crude fiber prepared from *Porphyra,* and the enzymes digesting the crude fiber were found. In this paper the relationship between the conditions of growth of bacteria and the enzymes activity were described.

Materials and Methods

Reagents Polypepton and yeast extract were obtained from Daigo Eiyo Kagaku Co. Other

*1 This work was partly supported by the grant from Agriculture, Forestry, and Fisheries Research Council Secretariat.

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reagents were purchased from Wako Pure Chemical Industries Ltd.

Bacteria Marine bacteria, two strains of P4 and P5, were isolated from cultured nori (*Porphyra* sp.) infected with Anaaki-disease. These strains were cultured in Zobell 2216 E medium, composed of 0.5 g polypepton and 0.1 g yeast extract in 100 m*l* of artificial sea water (pH 7.6). The artificial sea water was the following composition: 20.1 mM KCl, 10.9 mM $CaCl_2 2H_2O$, 24.3 mM $MgSO_4 7H_2O$, 29.5 mM $MgCl_2 6H_2O$, and 462 mM NaCl.

Taxonomic Studies Taxonomic studies were done by investigating morphological and physiological properties according to the usual method.

Preparation of Crude Fiber from Porphyra The crude fiber of *Porphyra* was prepared according to the method of Iriki *et al*[4]. 500 g of dried *Porphyra* was cut into small pieces with scissors and dipped into 3*l* of 1% HCl-70% ethanol at room temperature for 3 hours. The material was filtered with cotton cloth and washed with water. The residue was added into 5*l* of 1.25% H_2SO_4 and heated at 100 °C for 30 min. After the material was filtered and washed with water, the residue was added into 5*l* of 1.25% NaOH and heated at 100 °C for 30 min. Then the material was filtered and washed with water. The residue was added into 5*l* of 1% sodium hypochlorite and allowed to stand for about 30 min. The material was filtered with Buchner type-funnel (ϕ 30 cm) laying filter paper (Toyo No.2) by suction. After washing with water the residue was added into 3*l* of 5% sodium sulfite and allowed to stand for 30 min. The material was filtered with Buchner type-funnel by suction and washed with water adequately. The residue was added into 2*l* of ethanol and allowed to stand overnight. Then the material was filtered and washed with acetone and diethylether. The residue was dried under the reduced pressure in a desicator. The average yield of crude fiber was 55 g.

Preparation of Crude Enzymes Cell- suspension after culture was centrifuged to remove cells at 6,000 rpm for 30 min. The supernatant was dialyzed against 10 mM $(NH_4)HCO_3$ (pH 7.7). Then dialyzed solution was centrifuged at 10,000 rpm for 30 min. The supernatant was used as crude enzymes.

Enzyme Assay The reaction mixture contained, in a final volume of 3 m*l*, 1.5 m*l* of 0.1M Tris-Cl buffer (pH 8.0), 60 mg of crude fiber, and 1.5 m*l* of crude enzymes.

The reaction was carried out at 30°C and stopped by heating at 100°C for 3 min. The reaction mixture was filtered with filter paper (Toyo No.2) and the reducing sugar in the filtrate was determined by the method of Somogyi-Nelson[5].

One unit of the enzyme was defined as the amount of enzyme which produced one μmole of reducing sugar being equivalent to glucose for one hour under the condition described above. Protein concentration was determined spectrophotometrically by Bio-Rad Protein Assay Kit.

Electron Microscope Copper grids (150 mesh) were coated with 0.7% collodion in amylacetate and carbon stabilized. Grids were inverted on drops of bacterial cell-suspension for 20 to 30 sec and then 1% phosphotungstic acid solution on ammonium acetate for 15 to 20 sec. After the prescribed time had passed, the excess solution was blotted with filter paper. Samples thus prepared were observed with a Hitachi H-300 electron microscope at 75 kV.

Results

Taxonomic Studies of the Isolated Bacteria

The isolated bacteria, two strains of P4 and P5, were oxidase positive, catalase positive, fermentative for glucose fermentation, sensitive to *Vibrio* static compound (o/129), grown in 3% NaCl,

Table 1. Taxonomic Studies of P4 and P5 Strains

Test	P4	P5	*Vibrio fisheri*
Motility	+	+	+
Gram straining	−	−	−
Cell form	Rod	Rod	Rod
Sporulation	−	−	−
Oxidase	+	+	+
Catalase	+	+	+
Hugh & Leifson test	F	F	F
Novobiocin sensitivity	+	+	+
o/129 sensitivity	+	+	+
Hydrolysis of			
Casein	+	+	+
Starch	+	+	d
Chitin	+	+	
Agar	+	+	
Salts requirement	H	H	
Growth with			
0% NaCl	−	−	−
3% NaCl	+	+	+
7% NaCl	+	−	+
10% NaCl	−	−	−
M.R. test	+	+	+
V.P. test	−	−	−
BTB-Teepol Agar	−	−	−
Swarming	−	−	−
Growth at 37°C	−	−	−
Flagella	M	M	

F: fermentative, H: halophilic, M: monofrichous

not grown at 37°C, and Gram-negative rod with monofrichous (Table 1). From these results both strains were identified as *Vibrio fisheri* comparing the description in BERGEY's Manual.

Optimum Temperature for the Growth of Bacteria and the Enzyme Activity

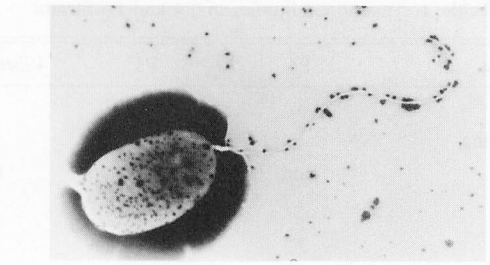
As shown in Fig. 2 the optimum temperature was changed according to the period of culture. The optimum temperature for the growth of both strains were 25 to 30°C against 12 hour-culture, 20°C against 24 hour-culture, 15°C against 48 hour-culture. The activities of the enzymes obtained from P4 strain cultured at 15°C for 48 hours and P5 strain cultured at 20°C for 24 hours were the highest (Table 2).

The Effect of Crude Fiber in the Cultural Medium on the Enzyme Activity

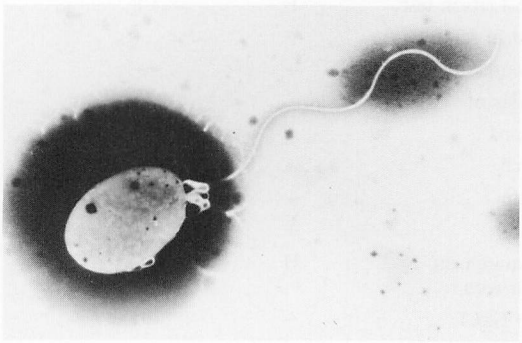
As shown in Table 3-A the addition of 2.9% crude fiber into the cultural medium increased the enzyme activity of P4 and P5 strains cultured at 20°C for 24 hours by 7 and 9 times, respectively.

When the amount of the crude fiber added into the cultural medium was changed, the addition of 4% of crude fiber induced the highest activity of the enzyme of P4 strain (Table 3-B).

The absence of polypepton affected the enzyme activity less than the absence of yeast extract



P4



P5

Fig. 1. Electron Micrograph of P4 and P5 Strains. Magnification: 10,000

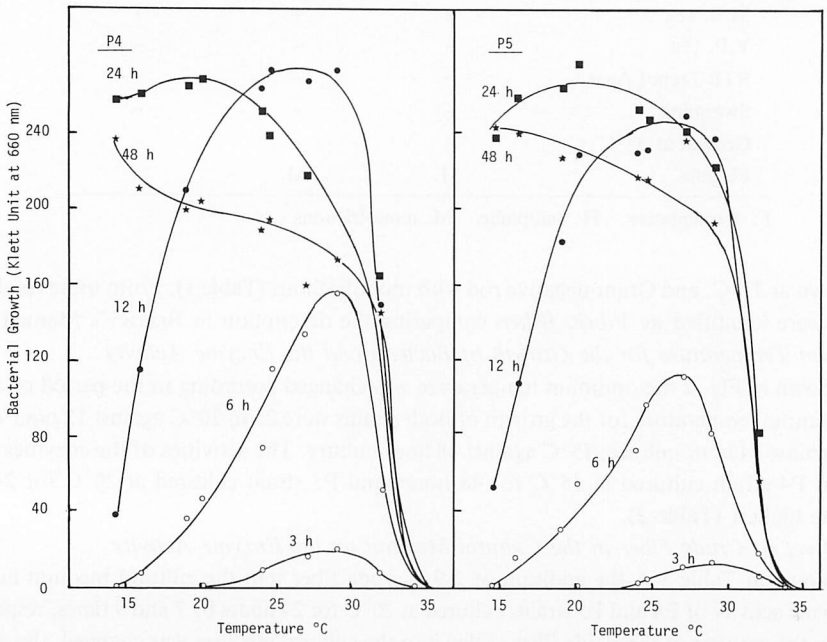


Fig. 2. Effect of Temperature on Growth. The composition of the medium was 0.5 g of polypepton and 0.1 g of yeast extract in 100 m*l* of artificial sea water (pH 7.6). Indicated hour in the figure represents the cultured time.

Table 2. Effects of Temperatures and Cultures Time for the Bacterial Growth on the Activities of Enzymes. P4 and P5 strains were cultured in 30m*l* of medium at the optimum temperature for each cultured time. Enzyme assay was carried out at 30°C for 2, 4, and 6 h as described in "Materials and Methods".

Temperature & Cultured Time		P4			P5		
		Activity (U/m*l*)	Protein (mg/m*l*)	S.A. (U/mg)	Activity (U/m*l*)	Protein (mg/m*l*)	S.A. (U/mg)
25°C	12 h	0.026	0.020	1.28	0	0.023	0
20°C	24 h	0.048	0.048	1.00	0.038	0.011	3.45
15°C	48 h	0.161	0.085	1.89	0.019	0.07	0.27

Table 3. Effect of the Addition of Crude Fiber into the Culture Medium on the Activity of Enzymes. The culture mediums of A and B were composed 0.5% polypepton and 0.1% yeast extract. Polypepton or yeast extract or both of them were removed from the culture medium of C. P4 and P5 strains were cultured in 30m*l* of the medium.

Addition	Culture	P4			P5		
		Activity (U/m*l*)	Protein (mg/m*l*)	S.A. (U/mg)	Activity (U/m*l*)	Protein (mg/m*l*)	S.A. (U/mg)
A None	20°C, 24 h	0.072	0.046	0.63	0.038	0.073	0.52
	15°C, 48 h	0.038	0.018	2.11	0.019	0.068	0.28
Crude Fiber (2.9%)	20°C, 24 h	0.144	0.034	4.20	0.194	0.044	4.40
	15°C, 48 h	0.144	0.050	2.88	0.048	0.060	0.80
B Crude Fiber	20°C, 24 h						
0%		0.019	0.020	0.97			
1		0.048	0.037	1.30			
2		0.34	0.043	7.91			
4		0.36	0.030	12.0			
8		0.30	0.032	9.37			
C Crude Fiber alone	20°C, 24 h	0.038	0.019	2.00			
Crude Fiber + Yeast Extract		0.24	0.027	8.99			
Crude Fiber + Polypepton		0.26	0.058	4.48			

(Table 3-C). P4 strain could grow in the medium containing only crude fiber as carbon source, but the growth and the enzyme activity was very low.

Optimum pH of the Enzymes

Enzyme of P4 strain had two optimum pHs, pH 6 and pH 8 (Fig. 3). Whereas optimum pH of the enzyme of P5 strain was pH 8.

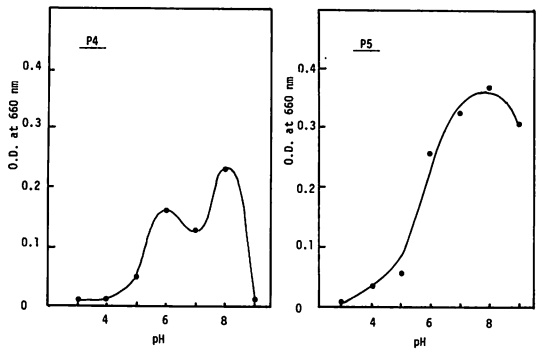


Fig. 3. Optimum pH of the Enzymes. Buffers used were as follows: pH 3, 4, and 5, 0.1 M Citrate-phosphate buffer; pH 4 and 5, 0.1 M Acetate buffer; pH 6, 7, and 8, 0.1 M Phosphate buffer; pH 8 and 9, 0.1 M Tris-Cl buffer. Enzyme assay was carried out at 30°C for 6 h.

Discussion

Two strains isolated from *Porphyra* infected with Anaaki-disease were identified as *Vibrio fisheri*. Both strains could digest agar, chitin, and starch. The crude fiber isolated from *Porphyra* was digested by enzymes secreted from these two strains. These enzymes had their optimum pH at pH 8, which is close to pH of the sea water. These results suggest that the bacteria attached to the thalli of *Porphyra* secret enzymes, and then the enzymes produced by the bacteria digest the cell wall of the algae in the sea water.

When P4 and P5 strains were cultured, the addition of crude fiber of *Porphyra* in the cultural medium increased the enzyme activity to digest crude fiber by 2 to 9 times. This suggests that the enzymes were one of the inducible enzymes. The activity of the enzyme of P4 strain was the highest in the presence of 4% crude fiber in the cultural medium. P4 strain could grow in the medium containing only crude fiber as carbon source, but the growth and the enzyme activity was very low. Optimum condition for the growth and the enzyme production needs the presence of polypepton and yeast extract besides crude fiber in the medium.

It is under the investigation whether the enzymes were available to prepare the protoplast of *Porphyra*. It is reported that the protoplast of *Porphyriodium*, one of the red algae, was prepared by using cellulase[6]. However, the protoplast of *Porphyra* could not be prepared by cellulase. Recently the isolation of protoplast from *Laminaria* and *Porphyra* has been tried by using sea urchin gut juice[7]. However, the yield of them were less than 10^3cells/m*l* and the viabilities of them were less than 10%.

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