Cultivation of *Brevibacterium stationis var. iodininofaciens* and its Iodinin Production*

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INTRODUCTION

Since CLEMO and MCILWAIN first reported iodinin[1], several studies on chemistry, antibiotic activity and biosynthesis of iodinin have followed[2,3,4,5,6,7], but few are found on cultivation condition for iodinin producing microorganisms. The authors have carried out studies on cultivation conditions favoring pigments production, antibiotic activity of iodinin and its mechanisms and biosynthesis of iodinin, produced by the marine bacteria, *Brevibacterium stationis var. iodininofaciens* presented in the former reports[8,9]. This report describes the cultivation condition of *Br. stationis var. iodininofaciens* and its iodinin production.

MATERIALS AND METHODS

Organisms. *Br. stationis var. iodininofaciens* was isolated from the sea water sample collected by means of sterile J-Z bacteriological water samplers at a depth of 99 m at the station 7 (145°00'E, 39°03'N, Sept. 12, 9 : 00) in the Sea to the East of Japan in the Pacific Ocean[8]. Achromogenic mutants are derived from sectors, appearing in slant cultures kept at 30° to 40°C in an incubator. *Pseudomonas iodina* IFO 3558, employed for comparison with iodinin producing marine bacteria, was obtained from the type culture collection of the Institute for Fermentation, Osaka. *Br. stationis var. iodininofaciens* Po-35, Po-36, and Po-363 that was gained from the strain Po-36 by plating for microbiological investigations, and its achromogenic mutants have been maintained on the sea water agar medium *G55*, and transferred serially once a month.

Culture medium. Basal medium for cultivation consists of glucose, 5 g, polypepton, 5 g, and 75 % aged sea water, 1 L, pH 7.6 to 7.8 initial (abbreviation, *G55*), and was modified according to experiments. In many experiments, 1 ml of 2 day-preculture was inoculated into 50 or 60 ml of medium in a 300-ml Erlenmeyer flask. The flask was incubated on a reciprocal shaker operating at 95 times per minute with width of motion of 7.5 cm usually for 5 days at 30°C. Multiplication was estimated by turbidity of 50

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times-dilution of culture broth by HITACHI spectrophotometer Model 101, or by turbidity of culture broth by HITACHI photoelectric photometer Model EPO-B.

Isolation and Separation of the Pigments. Isolation of the pigments was carried out by extraction with chloroform at pH less than 2. Iodinin and its reduced compound, 1,6-phenazine-diol were separated each into the respective fractions very easily by means of extraction of chloroform solution of pigments with 1 N sodium carbonate and 1 N sodium hydroxide solutions, respectively, as outlined in Fig. 1. Sodium carbonate layer in which 1,6-phenazine-diol was recovered was washed with new chloroform repeatedly in order to remove contaminating iodinin and 1,6-phenazine-diol was removed again to chloroform from sodium carbonate layer at pH less than 2. This treatment was repeated several times. Iodinin was recovered in sodium hydroxide layer, and was removed

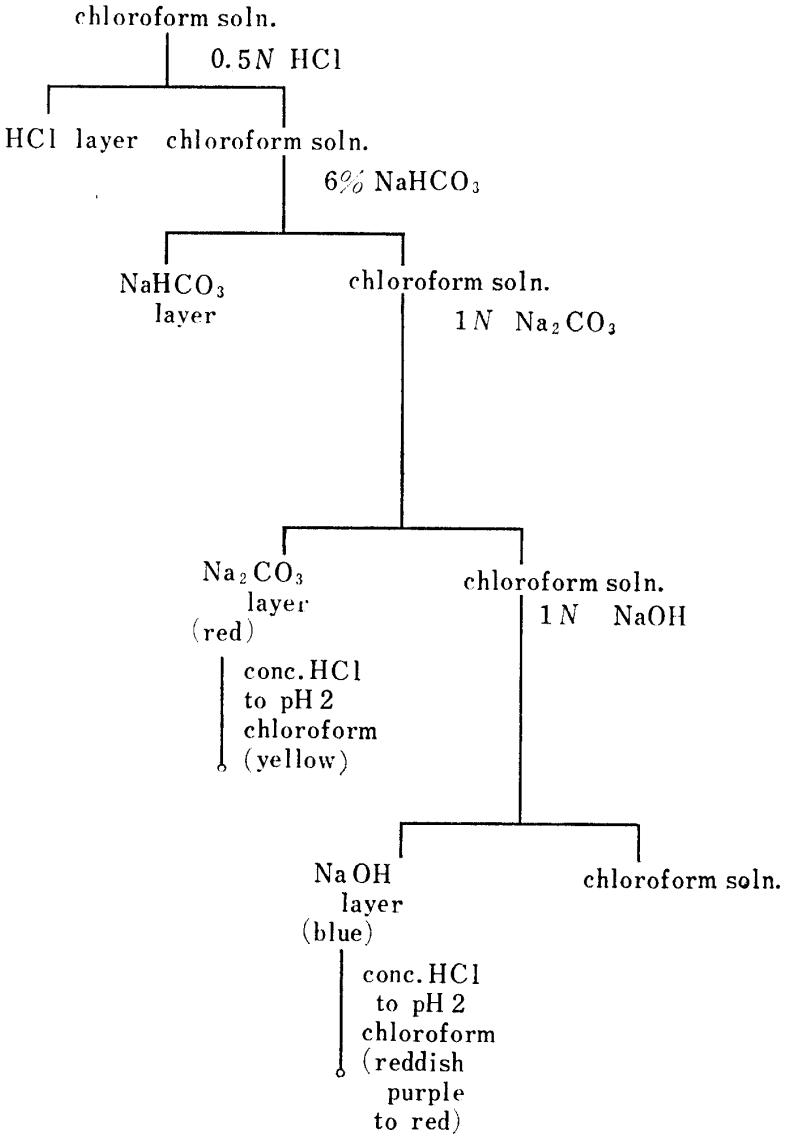

Fig. 1. Scheme for separation and purification of pigments extracted with chloroform from the culture of *Br. stationis var. iodininofaciens*. I.

again to chloroform from sodium hydroxide layer at pH less than 2. Iodinin chloroform solution was washed with 1 N sodium carbonate solution in order to take off contaminants. Further purification required, such a method as shown in Fig. 2. was applied in addition to the above treatments.

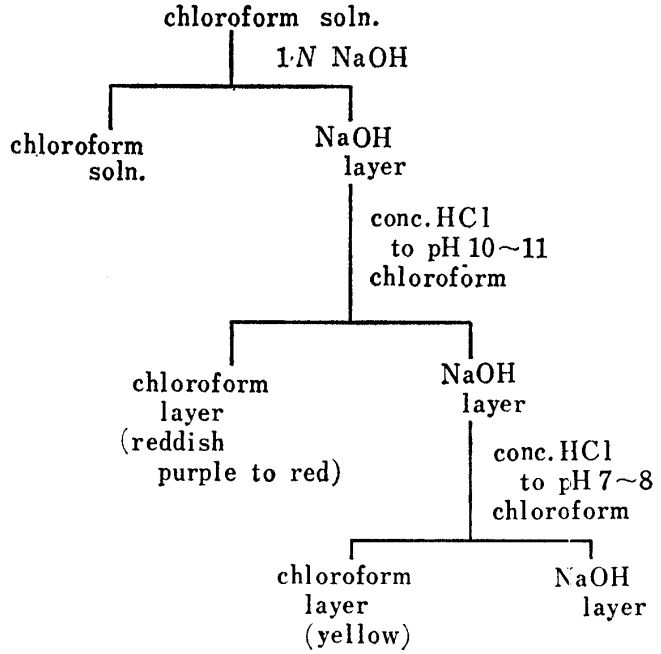

Fig. 2. Scheme for separation and purification of pigments extracted with chloroform from the culture of *Br. stationis var. iodininofaciens*. II.

Identification of the Pigments. Melting points determinations, coloration with ferric chloride, and reaction of lead acetate were carried out in the usual way[1]. Ultraviolet and visible light absorption spectra were recorded with HITACHI recording spectrophotometer Model EPS-3T, and infrared absorption spectra were recorded on potassium bromide tablets by using HITACHI grating infrared spectrophotometer Model EPI-G2. Microanalysis of pigments was carried out through the courtesy of Prof. Dr. M. Fujita, Kyoto University. The yields of iodinin and 1,6-phenazine-diol were determined by estimation at 535 mμ and 445 mμ in their chloroform solutions by HITACHI spectrophotometer Model 101, respectively.

RESULTS AND DISCUSSION

Identification of the Pigments

Chloroform solution of iodinin was concentrated to separate iodinin as dark violet needles with bronze lustre. A definite melting point of iodinin was not given, as GERBER and LECHEVALIER described[5]. At 217°C, gas evolution began and yellow crystals appeared by sublimation, and at 232.5° C, browning of iodinin powder, decomposition of iodinin, began and prevailed at 234° C. Ultraviolet and visible light absorption spectra

of yellow crystals were similar to those of 1,6-phenazine-diol, into which iodinin was reduced. Chloroform solution of 1,6-phenazinediol was concentrated to separate 1,6-phenazine-diol as yellow needles. The melting point of 1,6-phenazine-diol is not identical with published data[4,5], but the ultraviolet, visible light, and infrared absorption spectra were similar to published data[4,5]. Sublimation of this pigment began at 172° C, and at 236° C yellow pigment powder turned brown.

The ultraviolet and visible light absorption spectra of iodinin and 1,6-phenazine-diol

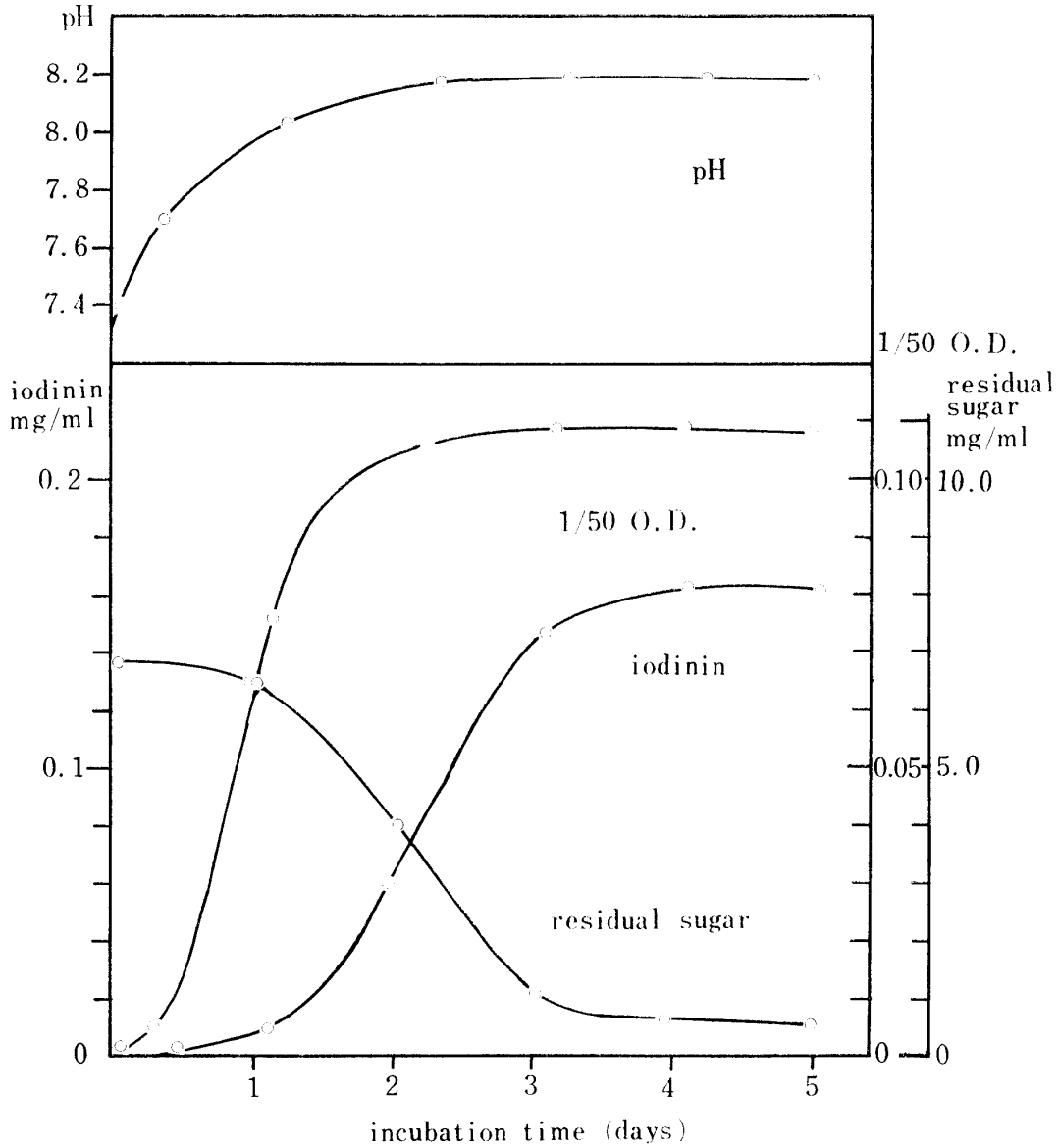

Fig. 3. Iodinin production in relation to growth and residual sugar. Cultivation conditions: 50 ml of the culture medium *G 55* in a 300-ml flask was inoculated with 1 ml of 2 day-culture. After incubation at 30° C, on a reciprocal shaker, samples were removed and assayed at the times specified.

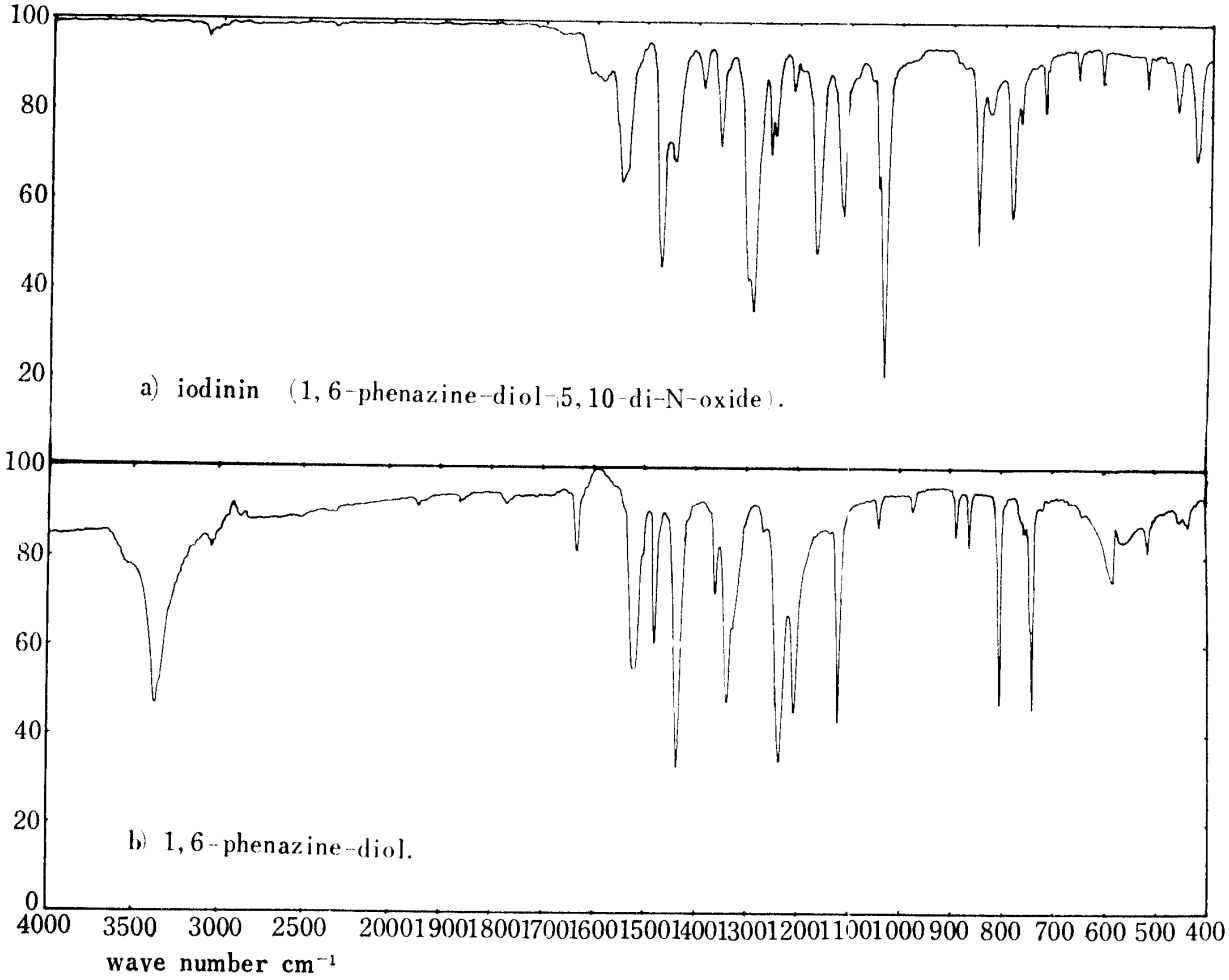

Fig. 4. Infrared spectra : a) iodinin from Br. stationis var. iodininofaciens (Found : C, 58. 89; H, 3.47 ; N, 11.19. $C_{12}H_8O_4N_2$ requires C, 59.02 ; H, 3.30 ; N, 11.47%) ; b) 1,6-phenazine-diol, to which iodinin was not reduced only chemically, but also biologically under anaerobic conditions (Found : C, 66.58, 66.38; H, 3.81, 4.05 ; N, 12.83. $C_{12}H_8O_2N_2$ requires C, 67.97 ; H, 3.80 ; N, 13.28 %).

in chloroform shows maxima at 291 352.5 and 535 mμ, and at 275.5, 374 and 445 mμ, respectively. The infrared absorption spectra of iodinin and 1,6-phenazine-diol were identical with those published by Irie et al[4]., as shown in Fig. 4, and the ultraviolet, visible light, and infrared absorption spectra of iodinin were identical with those of iodinin produced by *Ps. indina* IFO 3558.

Iodinin gave a greenish blue coloration with ferric chloride and with lead acetate in alcali alcoholic solution, and no change in alcoholic solution, as it was difficult to dissolve in alcohol. The phenazine N-oxides have the property of liberating iodine from potassium iodide in acid solution[1]. With iodinin, liberation of much iodine from potassium iodide occurred. 1,6-Phenazine-diol gave a green coloration with ferric chloride and violet lake in its alcoholic solution, and did not liberate iodine from potassium iodide.

Iodinin forms blue solution in alkali, which is reduced to red by sodium hydrosulfite

and further reduced to colorless, if sodium hydrosulfite exists in excess If this colorless solution was separated from air with liquid paraffine layer, it shows no change, but it changes from colorless to red, color of 1,6-phenazine-diol in alkali, on exposure to the air. Auto-oxidation of further reduced form by air, mainly to 1,6-phenazine-diol, might become important in respect to respiration of iodinin producing bacteria in further investigations.

Factors Affecting Production of the Pigments

The components of culture medium. The time courses of growth, iodinin production, and consumption of glucose in shaking culture are shown in Fig. 4. These curves show that multiplication develops in proportion to the consumption of glucose and the consumption of glucose precedes iodinin production. In the medium *G55y-3* (glucose, 5 g, polypepton, 5 g, yeast extract, 3 g, and aged sea water, 1 L), bacterial population reaches the near-stationary phase in 2 days, and iodinin production also reaches the near-sta-

Table 1. Growth and iodinin production of *Br. stationis* var. *iodininofaciens* in various concentrations of glucose in the medium *GX5y-2*, at 30° C for 5 days.

glucose *g/L*	pH final	1/50 O.D.	iodinin *mg/60ml*
0	9.5	0.076	0.74
1	9.5	.091	1.03
3	9.5	.121	2.62
5	8.7	.149	4.92
10	7.7	.226	10.83
20	5.8	.362	16.49

Table 2. Growth and iodinin production of *Br. stationis* var. *iodininofaciens* on various sugars in the medium *S55y-2*. at 30° C for 5 days.

sugars	pH final	1/50 O.D.	iodinin *mg/60ml*
glucose	8.7	0.149	4.91
galactose	9.0	.185	6.45
maltose	9.5	.075	0.64
sucrose	9.5	.080	0.62
lactose	9.5	.072	0.71
xylose	9.5	.078	0.84
mannitol	9.5	.075	0.88
ethanol	4.0	.090	0.95
glycerol	8.0	.161	5.42
Na-acetate	10.0	.089	1.69
Na-p-hydroxy-benzoate		.126	0.26
no sugar	9.5	.076	0.74

tionary phase in 3 days, while original sugar has been used up. The fact shows that iodinin production may depend on consumption of glucose. Iodinin production was in proportion to increasing concentration of glucose in the medium *GX5y-2* (1, 3, 5, 10, and 20 g of glucose were included in 1 L, respectively), as shown in Table 1. Utilization of other sugars and alcohols in place of glucose observed, the better results for both growth and iodinin production were obtained evidently with galactose and glycerol than with glucose, as shown in Table 2. Various organic acids added by 10 mg for 5

Table 3. Growth and iodinin production on various organic acids added to 18 hr-growing culture of *Br. stationis* var. *iodininofaciens* in iodinin-production-restricted medium *G12*.

acids	O.D. 5 days	iodinin *mg/5ml* 5 days
adipic acid	0.985	0.320
Na-citrate	1.225	0.372
fumaric acid	1.070	0.265
Ca-gluconate	1.125	0.640
lactic acid	1.140	0.432
Na-malate	1.060	0.412
malonic acid	1.000	0.252
Na-propionate	1.095	0.428
Na-succinate	1.220	0.400
benzoic acid	0.924	0.280
Na-p-hydroxy-benzoate	1.150	0.452
Na-salicylate	0.947	0.284
D-glucose	1.275	1.040
no addition	0.960	0.310
18 hr-growing culture	(0.636)	(0.043)

Substrates were alcohol-sterilized, and added to 18hr-growing cultures in *G12* to 0.2%, respectively. Each culture was neutralized with sterile 1*N* Na_2CO_3 solution.

Table 4. Growth and iodinin production of *Br. stationis* var. *iodininofaciens* in various concentrations of polypepton in the media *G1X* and *G5X*, at 30° C for 5 days.

culture media	pH final	1/50 O.D.	iodinin *mg/60ml*
G 11	6.2	0.024	1.22
G 12	7.8	.038	2.62
G 15	8.6	.069	3.71
G 51	5.0	.052	3.83
G 52	5.4	.0935	10.28
G 55	7.8	.1385	15.85
G 510	9.0	.2055	8.06

ml of the growing culture that was incubated for 18 hr in the iodinin production-restricted medium *G12*. Calcium gluconate and sodium acetate were inferior to glucose in growth and iodinin production, but a little better than a control, as shown in Table 3. If addition of polypepton increases in well proportion, iodinin production also increases as shown in Table 4. Accordingly, a glucose-polypepton ratio in the culture medium seemed to restrict iodinin production. Iodinin production was observed to increase with increasing addition of glucose and polypepton, as shown in Table 5. Good iodinin yields were obtained from the media, *G31*, *G53*, *G103*, *G105*, *G203*, *G205*,

Table 5. Iodinin production, increasing with increasing addition of glucose and polypepton, in the media *GXX* for 5 days.

culture media	1/50 O.D.	iodinin *mg*/10*ml*	iodinin (*mg*) for g of glucose	iodinin (*mg*) for g of polypepton
G 11	.026	.384	38.4	38.4
G 13	.036	.488	48.8	16.3
G 15	.050	.684	68.4	13.7
G 17	.063	.736	73.6	10.5
G 110	.070	.772	77.2	7.7
G 31	.042	.880	29.3	88.0
G 33	.065	1.272	42.4	42.4
G 35	.075	1.504	50.1	30.1
G 37	.094	1.328	44.2	19.0
G 310	.102	1.343	44.8	13.4
G 51	.039	.428	8.6	42.8
G 53	.087	1.840	36.8	61.3
G 55	.102	1.940	38.8	38.8
G 57	.111	2.100	42.0	30.0
G 510	.128	1.940	38.8	19.4
G 101	.040	.372	3.7	37.2
G 103	.095	1.680	16.8	56.0
G 105	.154	2.380	23.8	47.6
G 107	.185	2.700	27.0	38.6
G 1010	.205	2.860	28.6	28.6
G 201	.029	.165	0.8	16.5
G 203	.096	1.620	8.1	54.0
G 205	.136	2.420	12.1	48.4
G 207	.187	2.720	13.6	38.9
G 2010	.234	3.260	16.3	32.9
G 301	.019	.052	0.2	5.2
G 303	.081	1.400	4.7	46.7
G 305	.138	2.240	7.5	44.8
G 307	.180	2.880	9.6	41.4
G 3010	.227	3.580	11.9	35.8

and *G303*, for g of polypepton employed, and from the media, *13*, *G15*, *G35*, *G53*, *G55*, *G57*, and *G510*, for g of glucose employed. This fact indicates that good iodinin production depends on a correct ratio of glucose-polypepton, and a correct ratio is 5: 3 to 1:1, in respect to an efficiency of utilization of glucose and polypepton.

Though various nitrogen sources in place of polypepton investigated, any nitrogen sources employed could not take the place of polypepton in both growth and iodinin

Table 6. Growth and iodinin production of *Br. stationis* var. *iodininofaciens* on various nitrogen sources in the medium *G5X*.at 30°C for 5 days.

N sources	pH final	1/50 O.D.	iodinin *mg*/10 *ml*
$NaNO_3$ 2*g*/L	6.5	.002	0.00
NH_4NO_3 〃	6.8	.009	0.25
NH_4Cl 〃	6.5	.009	0.33
urea 5*g*/L	6.8	.003	0.00
polypepton 〃	8.7	.148	4.92
amino acids and bases for lactic acid bacteria*	7.8	.004	0.09
vitamin-free casamino acid 5*g*/L	8.2	.122	0.95
〃 enriched with cys. and try.**	7.2	.101	1.25
Bacto casamino acid 5*g*/L	6.8	.069	0.33
〃 enriched with cys. and try.**	8.6	.088	0.67
malt extract*** 10*g*/L	5.0	.016	0.01
polypepton 5*g*/L	7.8	.384	3.11

* DL-alanine, 200, L-aspartic acid 200, L-arginine-HCl, 200, L-cystine-HCl. 100. Na-L-glutamate, 500, glycine, 100, L-histidine-HCl, 100, DL-isoleucine, 200, L-leucine, 100, L-lysine-HCl, 200, L-methionine, 100, L-phenylalanine, 200, L-proline, 100, DL-serine, 100, DL-threonine, 200, DL-tryptophan, 100, L-tyrosine, 100, DL-valine, 200, adenine, 10, guanine, 10, hypoxanthine, 10, and xanthine 10, *mg*/L.

** cys.: L-Cystine-HCl, 100 *mg*/L. try.: DL-Tryptophan, 100 *mg*/L.

*** To malt extract medium was not added glucose.

production, as shown in Table 6. Various amino acids were added by 10 mg for 5 ml of the growing culture, which was incubated for 18 hr. in the iodinin production-restricted medium, *G31*. Thus, it was distinguished whether any of the amino acids employed were responsible only for growth, for both growth and iodinin production, or for restriction of iodinin production. Amino acids, which presented better growth and iodinin production than no addition and polypepton, were *L*-leucine, *DL*-isoleucine, *L*-aspartic acid, and *L*-phenylalanine, as shown in Table 7. Amino acids which presented better results than no addition, but less than polypepton were *L*-tyrosine and *L*-glutamic acid. It is worth noting that they have branching carbon chain, or carbon chain that benzene ring is joined to. As *L*-leucine was the best nitrogen source in iodinin production, and ammonia salts cannot serve as nitrogen source for growth, but presumably for iodinin synthesis, it is estimated that iodinin may be synthesized through mevalonic acid. It was betaine, *L*-cysteine, *L*-cystine, *L*-histidine, *DL*-methionine, *L*-methionine, *DL*-tryptophan, *DL*-threonine, o-aminobenzoic acid, and p-aminobenzoic acid that restricted growth and iodinin production. It should be noted that a great majority of them were sulfur-containing amino acids.

Yeast extract supplies microorganisms with vitamins as well as organic nitrogens. Iodinin production decreases with increasing yeast extract added, if it exceeds a certain amount, as shown in Table 8. The fact indicates that certain vitamins would restrict

Table 7. Growth and iodinin production on various amino acids added to 18hr-growing culture of *Br. stationis* var. *iodininofaciens* in iodinin production-restricted medium *G31*.

N sources*	pH final	O.D. 5 days	iodinin *mg/5ml* 5 days
DL-alanine		1.10	0.460
L-alanine		1.165	0.424
β-alanine		1.09	0.496
L-arginine		1.10	0.456
L-asparagine		1.08	0.370
L-aspartic acid		1.225	0.912
betaine		0.845	0.290
DL-citrulline**		1.12	0.460
L-cysteine		0.75	0.264
L-cystine		0.90	0.198
L-glutamic acid		1.14	0.596
D-glutamic acid		1.18	0.420
L-glutamine		1.13	0.404
glycine		1.22	0.440
L-histidine		0.865	0.112
L-hydroxyproline		1.06	0.350
DL-isoleucine		1.22	0.940
L-leucine		1.11	1.104
L-lysine		1.055	0.488
DL-methionine		0.765	0.048
L-methionine		0.735	0.051
L-phenylalanine		1.175	0.904
L-proline		1.13	0.480
DL-serine		1.07	0.420
DL-threonine		0.995	0.244
L-threonine		1.045	0.452
DL-tryptophan		0.870	0.051
L-tyrosine		1.18	0.652
DL-valine		1.075	0.484
o-aminobenzoic acid		0.505	0.041
p-aminobenzoic acid		0.513	0.037
polypepton		1.27	0.744
no addition		1.02	0.504
18hr-growing culture		(0.44) (1/50 O.D.)	(0.026)
NH_4Cl	5.2	0.052	0.30
polypepton	8.2	0.122	0.80
no addition	5.5	0.051	0.25

* Substrates were alcohol-sterilized, and added to 18 hr-growing culture in *G31* to 0.2%, respectively. Each culture was neutralized with sterile 1*N* Na_2CO_3 solution.

** 0.1%.

iodinin production. At such the concentrations as shown in Table 9, calcium pantothenate promoted growth, and pyridoxine restricted growth. Vitamins employed, except biotin and p-aminobenzoic acid, restricted iodinin production more or less, especially niacin and pyridoxal with considerable restriction of iodinin production and thiamin following these.

pH control. Media were adjusted to pH 5, 6.6, 7.8, or 10, respectively at starting,

Table 8. Growth and iodinin production of *Br. stationis* var. *iodininofaciens* on various concentrations of yeast extract in the medium *G55y-x* at 30°C for 5 days.

yeast extract *g/L*	pH final	1/50 O.D.	iodinin *mg/60ml*
0	8.8	0.153	8.82
0.1	8.8	0.150	9.12
1	9.0	0.139	6.34
2	9.0	0.151	3.38
5	9.0	0.174	3.31

Table 9. Growth and iodinin production of *Br. stationis* var. *iodininofaciens* on various vitamins employed in place of yeast extract, in the medium *G55v* at 30 C for 5 days.

vitamins 2*μg/ml*	1/10 O.D.	iodinin *mg/10ml*
thiamine	0.446	2.05
riboflavin	0.444	2.45
pyridoxine	0.400	2.41
pyridoxal	0.447	1.06
Ca-pantothenate	0.493	2.38
niacin	0.439	0.96
p-aminobenzoic acid*	0.465	2.56
biotin*	0.449	2.60
folic acid*	0.451	2.36
no addition	0.453	2.59

* 0.2*μg/ml*.

Table 10. The effect of starting pH on growth and iodinin production of *Br. stationis* var. *iodininofaciens* in the medium *G55y-0.1* for 5 days.

pH starting	pH final	1/50 O.D.	iodinin *mg/60ml*
5.0	7.4	0.141	14.75
6.6	7.8	.1385	15.60
7.8	8.0	.138	14.27
10.0	8.0	.1485	14.59

but there were little differences among them in growth and iodinin production, as shown in Table 10. Relations between iodinin production and pH control were also observed by the method that cultivation was continued for 3 days with adjustment of pH of culture media with sterile 0.1 *N* or 1*N* sodium hydroxide or hydrogen chloride twice a day. There was little variations in growth at pH 6 to 9, as shown in Table 11, and the best iodinin production was given at pH 7, pH 8 following this. Iodinin

Table 11. Iodinin production under pH control during cultivation on reciprocal shaker.

pH	pH ranges	for adjustment 1N NaOH *ml*/20*ml*	1N HCl *ml*/20*ml*	1/50O.D. 20hr	3days	iodinin *mg*/20*ml* 3 days
start*				(.76)		(.368)
3	3.0～ 3.2			..69	.065	.384
4	4.0～ 4.1			.069	.063	.378
5	5.0～ 6.2		.072	.087	.079	.480
6	5.2～ 6.4	.014	.025	.116	.116	1.860
7	7.0～ 7.5		.031	.1135	.118	2.350
8	8.0～ 8.4			.115	.119	2.080
9	8.2～ 9.0	.237		.112	.114	1.590
10	8.8～10.0	.526		.1005	.105	.746
11	8.8～11.0	.888		.0855	.080	.444

* 2 day-culture in the medium *G55* was divided into 100*ml* of flasks by 20*ml*. Cultivation was carried out for 3 days under control.

Table 12. Growth and iodinin production in sea water and mineral solution in the medium *G55y-0.1* for 5 days.

medium	pH final	1/50 O.D.	iodinin *mg*/60*ml*
75% sea water*, dr. w., 16.9*mg*/*ml*	7.8	0.1385	15.85
mineral salts solution, dr. w., 1.82*mg*/*ml*	7.8	0.137	3.82

* collected at Tempozan, Kagoshima city.

was not produced at pH 3 and 4, and little at pH 5 and 11.

Salt concentration. As shown in Table 12, better results were given in 75 % sea water medium than in mineral salt solution medium. Serial concentrated and diluted sea waters were prepared, with sea water with five times the concentration as a base. Of serial concentrated and diluted sea waters, the best growth and iodinin production were given in the sea water medium and the medium of sea water with three fourths times the concentration, equivalent to 75 % sea water medium, and the media of sea water with two times the concentration, and of sea water with half the concentration following these. In the medium of sea water with three times the concentration, growth was restricted, but iodinin production seemed not to be restricted as shown in Table 13. Media with various concentrations of sodium chloride were prepared by such a method as serial concentrated and diluted sea water media. As shown in Table 14, serial concentrated and diluted sea waters were considered not to be replaced each by any solutions with corresponding concentration of sodium chloride.

Relation to oxygen. The more iodinin production was given under the more supply of oxygen to cultures, as was evident from relation between aerobic cultivation (60

Table 13. Growth and iodinin production in concentrated or diluted sea waters.

concentration of sea water*	dry weight g/L	1/50 O.D. 1 day	1/50 O.D. 2 days	1/50 O.D. 5 days	iodinin mg/10ml 2 days	iodinin mg/10ml 5 days
5×	192.875	.002	.006	.022	.000	.059
4×	154.300	.006	.0175	.035	.000	.247
3×	115.725	.014	.048	.079	.304	.530
2×	77.150	.032	.0785	.118	.732	1.432
1×	38.575	.059	.102	.106	.912	1.604
3/4×	28.932	.0645	.105	.103	.818	1.560
1/2×	19.288	.063	.0985	.1075	.680	1.240
1/4×	9.644	.061	.096	.121	.500	.952

* collected at Ryû-ga-mizu, Kagoshima city.

Table 14. Growth and iodinin production in sodium chloride solutions in place of sea water.

NaCl %	1/50 O.D. 1 day	1/50 O.D. 2 days	1/50 O.D. 5 days	iodinin mg/10ml 5 days
28.110	.001		.001	.000
23.425	.001		.001	.000
18.740	.001		.001	.002
14.055	.0015	.0025	.0028	.004
9.370	.005	.008	.0115	.032
4.685	.017	.030	.042	.080
2.811	.021	.036	.060	.050
1.874	.021	.036		.044
0.937	.0205	.0325	.055	.030
0.000	.0185	.029	.044	.018

Table 15. Relation between oxygen and pigments production in the medium *G55y-2*.

cultivation	iodinin μg/ml	1, 6-phenazine-diol μg/ml
under aerobic condition for 2 days	142.7	0.0
under anaerobic condition for 5 days	2.8	1.2
for 10 days	2.0	0.5

ml of culture medium in 300-ml Erlenmeyer flask on reciprocal shaker for 2 days) and anaerobic cultivation (800 ml of culture medium in 1 L Erlenmeyer flask, inoculated with 50 ml of shaking preculture, for stationary cultivation with slow stirring) as shown in Table 15. If culture was kept under anaerobic condition after cultivated aerobically on reciprocal shaker for 3 days, an amount of iodinin produced decreased and such reduced compounds as 1,6-phenazine-doil appeared, as shown in Table 16. On the other hand, 8.55 mg of 1,6-phenazine-diol was recovered from chemical reduction of

Table 16. The effect of anaerobic condition on pigments production of *Br. stationis* var. *iodininofaciens* in the medium *G55y-2.5*.

cultivation	iodinin *mg/360ml*	1, 6-phenazine-diol *mg/360ml*
3*	51.40	0.00
3-I**	25.47	6.79
3-II**	18.97	9.76
3-III**	14.43	15.89

* for 3 days under aerobic condition, *60ml/300ml* flask.
** for 1, 2, and 3 days, under anaerobic condition, *360ml/300ml* flask, after cultivated on reciprocal shaker in the medium, *60ml/300ml* flask.

13.9 mg of iodinin with about 1g of sodium hydrosulfite. If 1,6-phenazine-diol was added to 24 hr. culture in iodinin production-restricted media *G11 y-1* and *G51*, most of 1,6-phenazine-diol was recovered as iodinin after 1 hr. on reciprocal shaker as shown in Table 17. The facts indicate that the reduction of iodinin may proceed under anaerobic conditions, and the oxidation of 1,6-phenazine-diol proceed under aerobic conditions.

Table 17. Transformation of 1, 6-phenazine-diol to iodinin by *Br. stationis* var. *iodininofaciens* and its achromogenic mutants.

microorganisms	media	start 1, 6-phenazine -diol *mg/50ml*	start iodinin *mg/50ml*	1hr 1, 6-phenazine -diol *mg/50ml*	1hr iodinin *mg/50ml*
Br. stationis var. *iodininofaciens* P_0-363					
	G11y-1	0.00	0.53	-	0.70
		△1.00		-	1.92
	G51	0.00	0.25	-	0.22
		△1.00		0.12	0.98
Control (not inoculated)	*G51*	△1.00	0.00	0.98	0.00

microorganisms	media	18hr 1, 6-phenazine-diol *μg/5ml*	iodinin *μg/5ml*
Br. stationis var. *iodininofaciens* achromogenic mutants			
P_0-363-2	*G55*	4.00	0.46
〃		△ 97.65	4.00
P_0-363-12	*G55*	2.46	0.20
		△102.35	8.30

* To 50*ml* of the 24 hr-growing culture in the iodinin production-restricted media *G11y-1* and *G51* was added 1*mg* of 1, 6-phenazine-diol.
** To 5 *ml* of the 27 hr-growing culture in the medium *G55* were added 100 *μg* of 1, 6-phenazine-diol.

1,6-phenazine-diol was detected early in cultivation in a small amount, but not detected later at all. Accordingly, 1,6-phenazine-diol seemed to be precursor of iodinin in its biosynthesis. With the significancance of iodinin, iodinin seemed to be one of the vestiges of devices for adaptation, gained by primitive cells in biological evolution, living anaerobically, for survival under the presence of oxygen, beginning to appear in the atmosphere.

Temperature. On the sea water medium, *G55*, good growth and iodinin production were given for 3 days at 30° C, for 1 week at 20° C, and for 3 weeks at 7° C. At 35° C, good growth and iodinin production were given for 3 to 4 days, but many achromogenic sectors appeared in culture. No growth was given at 40° and 45° C.

Stability of production of pigments. It was observed that a given culture medium gave corresponding growth and iodinin production, as shown in Table 18. In respect to the temperature, achromogenic sectors that have losed the ability of iodinin production at all or little, often appeared in cultivation at 35° C, and also in cultivation in nutrient

Table 18. Stability of growth and iodinin production on serial transfer of *Br. stationis* var. *iodininofaciens* in various culture media.

serial transfers	pH final	1/50 O.D.	iodinin *mg/60 ml*
*G55**	7.5	.136	8.23
*G55-G55**	7.8	.127	7.64
*G55-G55-G55**	7.6	.125	8.00
*G55y-0.1**	7.2	.1365	7.81
*G55y-0.1-G55y-0.1**	7.8	.1275	8.03
*G11y-1**	6.2	.036	0.33
*G11y-1-G11y-1**	7.2	.0365	0.06
*G510y-0.1**	9.0	.2055	8.06
*G510y-0.1-G510y-0.1**	9.0	.190	7.71
*G55y-0.1-G11y-1**	7.2	.036	0.27
*G11y-1-G55y-0.1**	7.6	.1315	7.84
*G11y-1-G510y-0.1**	9.5	.194	6.18

* 5 days cultures were employed for experiments.

Table 19. Effects of the temperature of cultivation on pigment formation on the agar plate *G55*.

temperature	dilution	color of colony				total
		yellowish gray	pale brown	grayish brown~ brownish gray	dark red purples*	
30°C	$\times 10^8$	1	2	38	2	43
	〃	0	2	60	4	66
37°C	〃	14	30	15		59
	〃	46	26	13		85

* color of iodinin crystals.

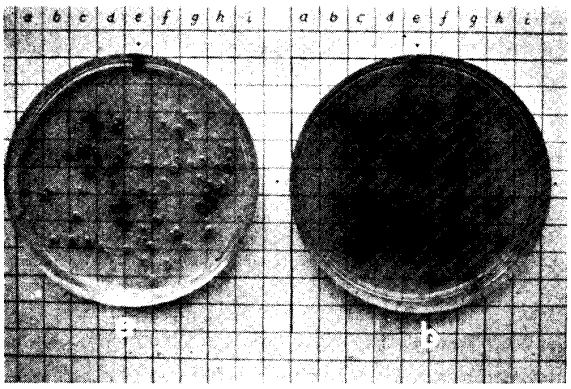

Fig. 5. Sea water agar plates, incubated a) at 37 C. and b) at 30 C, after smeared with a dilution of culture.

agar at 30° C, as shown in Table 19. Plates, smeared with cultures incubated on a shaker or their dilutions for 2 days, were incubated at 30° and 37° C respectively. As shown in Table 19 and Fig. 5, there were little differences in plate counts, but evident differences only in pigment production. A plate, if put in 30°C incubater after incubated at 37° C for 1 week, presented such distribution of pigment production as that in incubation only at 30° C presented. Iodinin production was restricted in incubation at more than 35° C and it would be caused by stop of iodinin biosynthesis at high temperature and partially by achromogenic mutants, selected and prevailing with high temperature. Growth and iodinin production of achromogenic mutants were as shown in Table 20. Achromogenic mutants have little ability of oxidation of 1,6-phenazine-diol to iodinin as shown in Table 17.

Table 20. Growth and iodinin production of achromogenic mutants of *Br. stationis* var. *iodininofaciens*, in the medium *G55* (glucose, 5.27 *mg/ml* at start), for 5 days.

microorganisms	pH final	1/50 O.D.	residual glucose *mg/ml*	iodinin *mg/10ml*
P_0-363	8.6	.166	0.20	2.01
P_0-363-11	8.6	.140	.19	0.08
P_0-363-12	8.6	.1575	.14	0.12
P_0-363- 2	8.4	.162	.14	0.00
P_0-363- 3	8.6	.1455	.23	0.00
P_0-363- 4	8.4	.157	.18	0.00
P_0-363- 5	8.6	.149	.18	0.00

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SUMMARY

Cultivation conditions for growth and pigments production of *Brevibacterium stationis var. iodininofaciens* were observed. Pigments produced were iodinin and its reduced form, each identified with 1,6-phenazine-diol-5,10-di-N-oxide, and 1,6-phenazine-diol, respective-

ly. Growth and iodinin production were in proportion to increasing concentration of glucose and polypepton in the sea water medium, good iodinin production depended on a correct ratio of glucose-polypepton, 5 : 3 to 1 : 1, under such condition as glucose and polypepton were contained in the medium to 30 g for liter, respectively. Amino acids, with which good growth and iodinin production were presented, were *L*-leucine, *DL*-isoleucine, *L*-aspartic acid, *L*-phenylalanine, *L*-tyrosine, and *L*-glutamic acid, arranged in order. Ammonia salts cannot serve as a nitrogen source for growth, but presumably for iodinin production, and sulfur-containing amino acids restricted iodinin production. The more iodinin production was given under the more supply of oxygen to cultures. In the culture under anaerobic condition, the reduction of iodinin to 1,6-phenazine-diol proceeded, while in the culture under aerobic condition the oxidation of 1,6-phenazine-diol to iodinin proceeded. Relations between iodinin production and cultivation conditions, such as amounts of vitamin and yeast extract added in the medium, pH control, salt concentration in the medium, and temperature of cultivation, and stability of production of pigments were given.

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