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Studies on the Microbial Pigment (I)

—The Effects of Environmental Factors to the Pigmentation—

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微生物의 色素에 관한 硏究(第1報)

——색소형성에 미치는 환경요인의 영향——

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ABSTRACT

The bacteria of red colonies isolated from soil were identified as *Serratia marcescens*. The best solvent for pigment extraction was *n*-buthanol and the pigment was identified as prodigiosene.

The extracted pigment was stable on temperature and light but not on acidity. The redpigment color changed into red in alkaline solution. The maximum absorbancy of pigment was 466 nm in alkaline condition and 540 nm in acid condition. And the pigment formed single spot on the TLC(starch). By the result of infra red spectrum, the red pigment has the same absorption pattern comparing with, the prodigiosin produced by *S. marcescens* strain Nima.

It was confirmed that the pigment was secondary metabolite and that the maximal peak of production appeared at 30 hrs after the inoculation, when the bacterial growth was in stationary state.

Referring to the effect of temperature, the pigment was not formed at 36°C and the optimal temperature for both of bacterial growth and pigmentation was 30°C. The optimal range of *p*H for pigmentation was 5.0 and under the condition the bacterial growth was not affected at all. Examining the effects of light, the bacterial pigmentation was more increased in darkness than in visible light.

INTRODUCTION

It is generally accepted that the pigment of microorganisms is one of the important key on the microbial taxonomy. In *Streptomyces*, the sporulation is closely related with the pigmentation which is very important character of taxonomy (Pridham, 1965). In case of *Nocardia* and some other genera, the pigmentation is related with the photochromogenesis, photoinatoinactiv-

ation(Koyama *et al.*, 1973).

The microbial pigments which were elucidated their structure are melanoid pigments produced by *Streptomyces*, (Pridham, 1965), carotenoid pigments by *Staphylococcus aureus* (Hammond and White, 1970), *Micrococccus roseus* (Schwartzel and Cooney, 1970), *Rhodotorula glutinis* (Hayman *et al.*, 1974), *Xanthomonas juglandis* (Andrews *et al.*, 1973), Violacein by *Chromobacterim violaceum* (Sivendra *et al.*, 1975), pyocyanine and pyrorubrin by *Pseudomonas aerugenosa* (Calhoun *et al.*, 1972; Wahba, 1964) and prodigiosin by *Serratia marcescens* (Williams, 1967).

Serratia marcescens produces the red pigment, prodigiosin, and besides of it, produces the nor prodigiosin and di-pyrolyl-di-pyromethane prodigiosin(Williams, 1973). And also it is known that *Streptoverticillium rubrireticuli* produces the prodiginine (prodigiosinlike pigment), red pigmnt (Geber, 1975).

In this experiment, the pigment produced by *Serratia marcescens* was determined as secondary metabolite. It is already reported that prodigiosin is the secondary metabolite (Williams, 1976). The pigment of *Serratia marcescens* strain P was examined whether it was secondary metabolite or not and the optimal conditions for pigmentation was observed.

MATERIALS AND METHODS

1. Organism

The soil samples were inoculated on the solid nutrient media, and then cultured for 24 hrs at 30°C. The red colonies were isolated on fresh nutrient agar medium. The strain showing the best activity of pigmentation was described as strain P and identified as *Serratia marcescens* in according to the Bergey's Manual(1975). *Serratia marcescens* strain Nima from Prof. R.P. Williams was used as the control.

The non-pigmented bacteria was obtained by growing at 37°C for 12 hrs. and the non-pigmented bacteria reduced the pigment when cultured on nutrient medium at 30°C.

2. Media

Solid nutrient media were used as isolating media, and for the observations of effects on pigmentation, the liquid nutrient media were used. The nutrient medium was consisted of; peptone 5g, beef-extract 3g and NaCl 8g in 1.000m*l* of distilled water. Agar was into the liquid medium to solidify as the concentration of 1.5%.

3. Bacterial count

Bacterial counts were carried out by optical density in a spectrophotometer (Bausher and Lomb spectronic 20) at 350 nm comparing with the ready made standard curve. Below the 10^6 cells/m*l*, the bacterial counts were determined by standard plate procedure using the solid nutrient media. Above the 10^9 cells/m*l*, the bacterial counts carried out by the spectrophotometer after the samples were diluted to 10^{-1} with sterilized saline solution.

The bacterial counts were measured with intervals of 30 minutes within the first 10 hrs and with 1 hr each after 10 hrs.

4. Extraction of pigment

Ten milliliter of the liquid culture was added by 0.1m*l* of 1 N H_2SO_4 and 5m*l* of a solvent(chloroform, benzen, petroleum ether, xylene, ether, *iso*-buthanol and *n*-buthanol) was added to vials of this solution, respectively. After the mixture were well shaken with tube stirrer, the solutions were centrifuged for 5 minutes at 1,000 rpm. The supernatants were taken carefully

and determined the specific absorbancy of the supernatant with the spectrophotometer. The quantitative test of pigmentation was carried out by using the extraction of *n*-buthanol, and read in 540nm, the maximum absorbancy.

The amount of pigment was measured with intervals of 1 hr within the first 10 hrs and 30 minutes after the 10 hrs.

5. The identification of the pigment.

The two kind of extracted pigments, from strain Nima as control and strain P were examined on TLC with the solvent mixture of *n*-buthanol, pertroleum ether, methanol and distilled water(1 : 1 : 1 : 1). The strip used was the starch(10×10cm), and the origin was 1cm from the lower end.

For the examination of the infra red spectrum, the two kind of pigments were dried in the vaccum evaporator, The dried pigments were resoluted in ethanol and measured with the infra red spectrophotometer(Perkin-Elmer model 700).

6. Environmental factors

To find the optimal conditions for pigmentations for pigmentation, the bacteria inoculated on the solid nutrient media and cultured at 25°C which is known the optimal temperature of mesophilic, 30°C which was the temperature of isolation, and 37°C which is known the optimal temperature of pathogens. And then the non-pigmented bacteria were inoculated in 500m*l* Erlenmeyer flaks containing 100m*l* of liquid nutrient medium and cultured at 25°C, 30°C and 37°C.

At the optimal temperature for pigmentation, 30°C. the optimal acidity was determined. The liquid nutrient media were controlled *p*H as of 5.0, 6.0, 6.5, 7.0, 7.5 and 8.0 with 0.1 *N* H_2SO_4 and 0.1*N* NaOH before the sterilization. The non-pigmented bacteria were inoculated in 500m*l* Erlenmeyer flasks containing 100m*l* of these media and cultured at 30°C in water bath shaker.

For the observation of the effects of various lights, the non-pigmented bacteria were inoculated in 500m*l* Erlenmeyer flasks containing 100m*l* of liquid nutrient media controlled to *p*H 4.0. The flasks were shaken in water bath shaker adjusted at 30°C illuminating at a distance of 50cm with two 300 W white, yellow, red, blue and green light lamps each and one in darkness.

RESULTS

1. Identification of the isolated bacteria

The strain P was idenditified by the key of Bergey's Manual(Bauchanan and Gibbons, 1974). As the results, the strain P was identified as *Serratia marcescens*. Table 1 shows the characteristics of the strain Nima as a control. The differences between strain P and strain Nima were Voges-Proskauer test and some of sugar fermentation like adonitol, inositol, raffinose, saccharose and xylose.

Red colonies may appear in *Escherichia coli*(Olden and Hemfling, 1973), *Pseudomonas extorquens* and *Protaminobacter ruber*(Hendrie and Shewan, 1972) but the most widely distributed red colonies of bacteria are known to the *Serratia marcescens*(Goldschmidt and Williams, 1968).

2. The characteristics of pigment

The best solvent for pigment extraction was *n*-buthanol. Table 2 shows the optimal densities of each pigment-solvent mixtures. Compared with the procedures of Williams (1972), the extraction yield with *n*-buthanol was more efficient than those of Williams' method.

The pigment formed single spot on the

Table 1. The characteristics of strain P and Nima

teste	Strain P	Strain Nima
Gram stain	G−	G−
Motility	+	+
Indole	−	−
M. R. test	−	−
V. P. test	−	+
Citrate utilization	+	+
Gelatin liquefaction	+	+
Litmus milk	alkaline	alkaline
Coagulation	+	+
Ropiness	+	+
Growth on Hugh and Leifson media	fermentation	fermentation
Oxidase	−	−
Nitrate reduotion	+	+
Sugar fermentation		
Glucose	−/K	−/K
Arabinose	−/K	−/K
Adonitol	−/A	−/K
Fructose	−/K	−/K
Inositol	−/A	−/K
Lactose	−/K	−/K
Levulose	−/K	−/K
Maltose	−/A	−/A
Mannitol	−/A	−/A
Melibiose	−/K	−/K
Raffinose	−/K	−/A
Rhamnose	−/K	−/K
Saccharose	−/K	−/A
Xylose	−/K	−/A

*− : no gas in sugar fermentation, K: alkaline A: acid

TLC and the *Rf* value was 0.8. The pigment extracted with the vaccum evaporator was stable on temperature, even at 120°C and light independent But the pigment was unstable according to the change of acidity. For instance, the pigment soluted in methanol changed red to orange in alkaline solution. However, the orange pigment had the same absorbancy as of the prodigiosin and also the red pigment had the same absorbancy as of the prodigiosin-HCl(Merck Index, 1972). The peak of maximum absorbancy was appeared at 466nm in alkaline solution and 540nm in acid.

The parent nucleus of prodigiosin and prodigiosinlike pigments was named prodigisene(Williams, 1973). As shown in Table 3, pigments isolated and chemically identified as to structure all contain the prodigiosene nucleus. Therefore these pigments seem to be prodigiosene derivaties (Williams, 1973).

Only microorganisms of the class *Schiz-*

Table 2. The optical densities of pigment in the organic solvents

Organic solvent	Absorbancy at 540nm
Chloroform	0.23
Benzen	0.23
Petroleum ether	0.26
Xylene	0.29
Ethyl ether	0.34
iso-butanol	0.37
n-utanol	0.38
*Procedure of Williams(1972)	0.32

#Sample was taken from 4 day cultured broth.

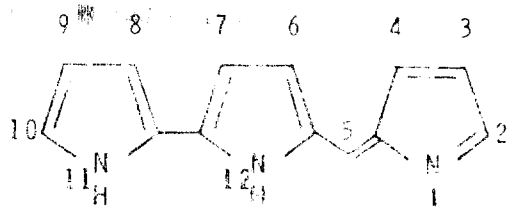

Fig. 1. Chemical structure of prodigiosene.

omycetes are known to produce prodigeiosines. These species of bacteria occur in two orders, Eubacteriales and Actinomycetales. Among the Eubacteriales, synthesis of prodigiosin is associated only with the genus of *Serratia* and two unidentified

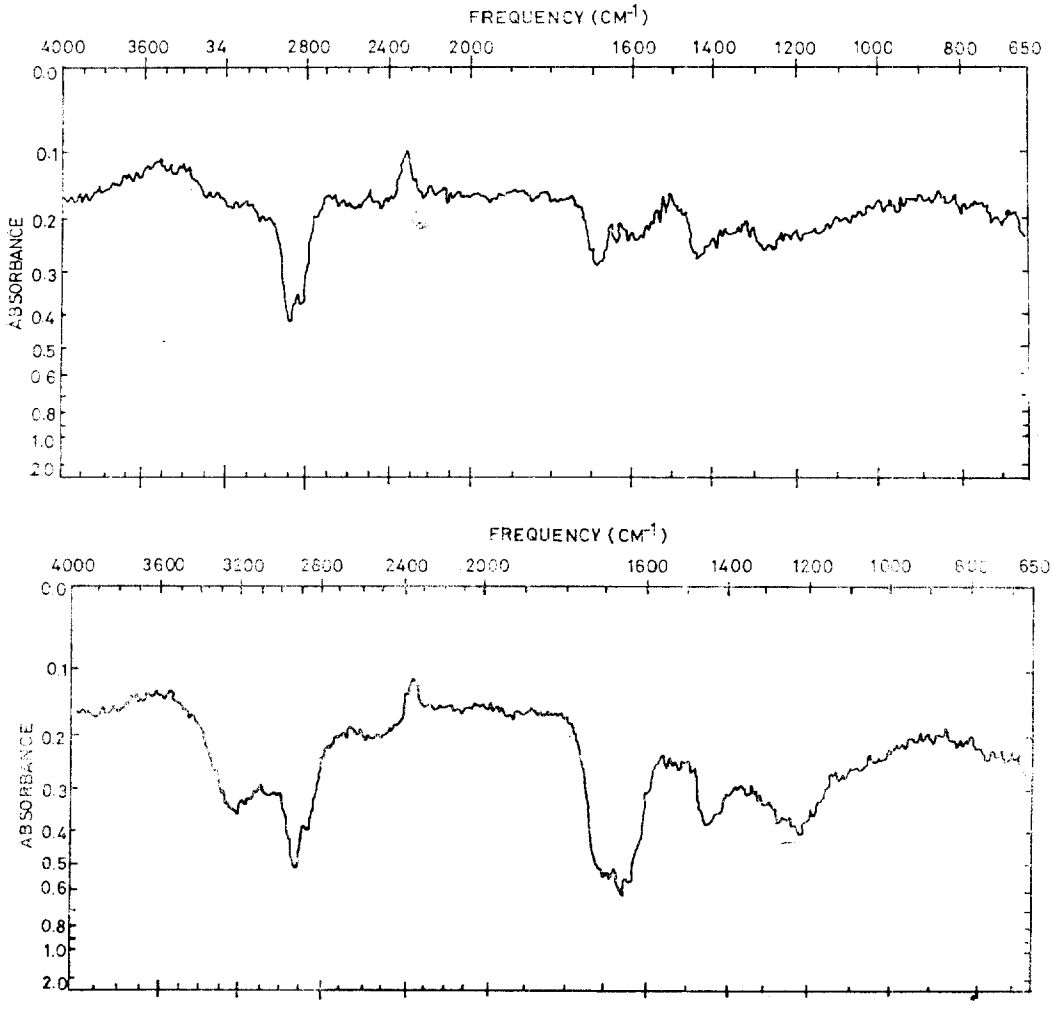

Fig. 2. The inira red spectrums of the pigments produced by *S. marcescens* strain Nima (upper) and strain P (lower).

marine bacteria. Among the Actinomycetales, two families sythesis the prodigiosene. Pigments of lower molecular weight are produced by *S. marcescens*, whereas *N. madurae*, *N. pelletieri*, and *S. longisporusruber* produce pigments of higher molecular weights(Williams, 1973).

The infra red spectrum of the two kind of pigments, from strain Nima and strain P are shown Fig. 2, The above is that of prodigiosinofrom strain Nima, and the below is that of pigment from strain P. These two absorbancy curves are closely similar to each other.

Table 3. *Schizomycetes* producing structually identified prodigiosenes

Microorganisms	Trivial name of pigment.	Formula
Serraitia marcescens	Prodigiosin	$C_{20}H_{25}N_3O$
S. marcescens	Norprodigiosin	$C_{19}H_{23}N_3O$
S. marcescens	Dipyrrolyl-dipyrromethene prodigiosin	$C_{19}H_{18}N_4O_2$
Nocardia madurae	Nonylprodigiosin	$C_{23}H_{31}N_3O$
N. madurae	Cyclononylprodigiosin	$C_{23}H_{29}N_3O$
N. pelletieri	Methylcyclodecylprodigiosin	$C_{25}H_{33}H_3O$
Stretomyces longisporusruber	Undecylprodigiosin	$C_{25}H_{35}N_3O$
S. longisporusruber	Metacycloprodigiosin	$C_{25}H_{33}N_3O$
**Streptoverticillium rubrireticuli*	Undecylprodiginine	$C_{25}H_{34}N_3O$
S. rubrireticuli	Butylcycloheptylprodiginine	$C_{25}H_{35}N_3O$

Williams, 1973; *Gerber and Stahly, 1975.

3. pigment as the secondary metabol

It was confirmed that the pigment produced by strain P was secondary metabolite. The data in Fig. 3 show the bacterial growth state and pigment formation of strain P. The sationary state of the bacterial grow appeared at 20 hrs after inoculation and the bacterial numbers reached 1.4×10^9 cells/ml. The bacterial pigment appeared at 12 hrs after inoculation, when the bacterial growth was in logarithmic state. And the pigment was formed explosively at 20 hrs when the bacterial growth was in stationary state. At 55 hrs, the pigment formation was reached the highest peak and the amount of pigment was 34μg/ml.

In the paper of Williams(1973), it was

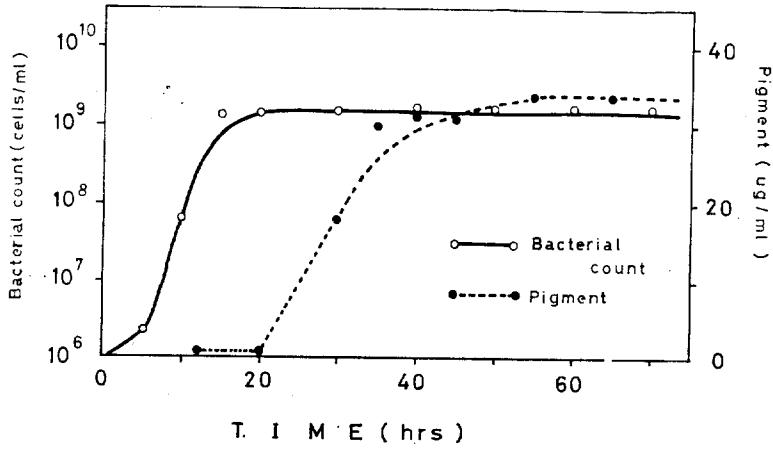

Fig. 3. The bacterial growth phase and the pigment formation during the cultivation at 30°C

reported that the maximal production of prodigiosin appeared in 96hrs after the inoculation. But the result of this experiment was slightly difference with that of Williams(1973) in time. It is generally accepted that prodigiosin produced by *Serratia marcescens* in secondary metabolite, and that other prodigiosin like pigments are also secondary metabolite. Calhoun *et al.*(1972) suggested that the pyocyanine of *Pseudomonas aerugenosa* is secondary metabolite.

4. Effects of environmental factors.

On solid nutrient media, the bacterial pigment was not produced at 37°C, weekly produced at 25°C but produced a large quantity of pigment at 30°C. Fig. 4 shows the differences of bacterial growth phase and pigment formation. The optimal temperatune for both of bacterial growth and pigment formation was 30°C. At 37°C, the bacterial growth proceeded slowly and the pigment was not formed. At 25°C, the bacteria also grew slowly and the amount of pigment was lower than that of 30°C. The maximal production of pigment at 25°C 22μg/ml.

The data in table 4 show no difference

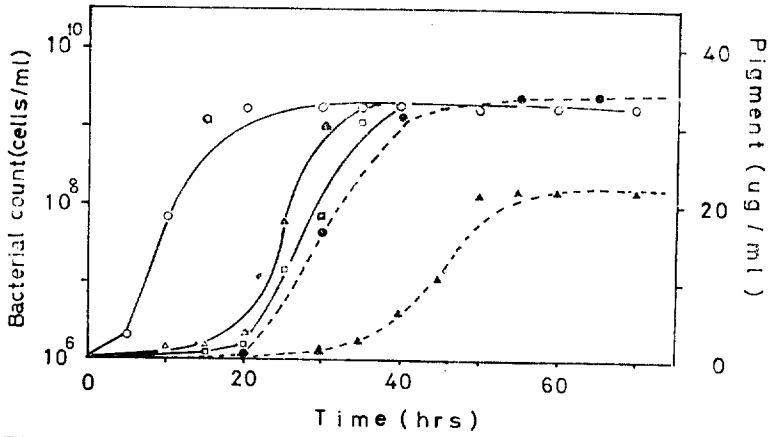

Fig. 4. The bacterial growth phase and pigment formation during the cult ivation with various temperature ·····; pigment ——; bacterial count
○ ; 30°C △ ; 25°C □ ; 37°C

Table 4. The bacterial counts of various initial *p*H

HRS	5.0	6.0	6.5	7.0	7.5	8.0
5	2.1×10^{6}	1.7×10^{6}	2.0×10^{6}	1.8×10^{6}	2.2×10^{6}	2.1×10^{6}
10	7.9×10^{7}	8.0×10^{7}	8.1×10^{7}	8.5×10^{7}	8.2×10^{7}	8.4×10^{6}
15	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}
20	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}
25	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}

*The initial bacterial numbers were 8.0×10^{5}.

between the changes of acidity even acid or alkaline condition. Fig. 5 shows the effect of initial *p*H on the formation of pigment during culture period. At the lower *p*H ranges, the more pigent was produced and earlier the maximum peak of production occured. At the above 7.0, the amount of pigment was decreased. And the final *p*H of all cultures were 8.0~8.5.

It is revealed that the light did not aff-

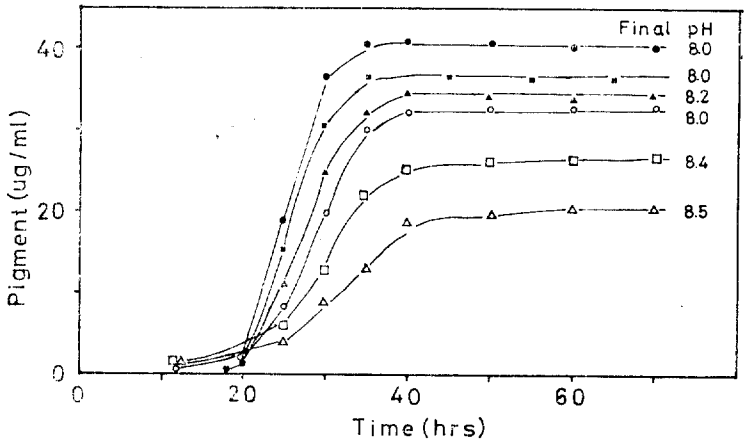

Fig. 5. The formation of pigment during the cultivation with the various pH conditions.
●; *p*H55.0, ■; 6.0, ▲; 6.5, ○; 7.0, □; 7.5, △; 8.0

Table 5. The bacterial counts of various light source

Hrs	Darkness	Green	Blue	Red	Yellow	White
5	1.8×10^{6}	2.1×10^{6}	2.4×10^{6}	1.9×10^{6}	2.1×10^{6}	2.4×10^{6}
10	7.5×10^{7}	7.8×10^{7}	8.2×10^{7}	8.1×10^{7}	7.8×10^{7}	7.9×10^{7}
15	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}
20	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}
25	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}	1.4×10^{9}

*The initial bacterial numbers were 8.0×10^{5}

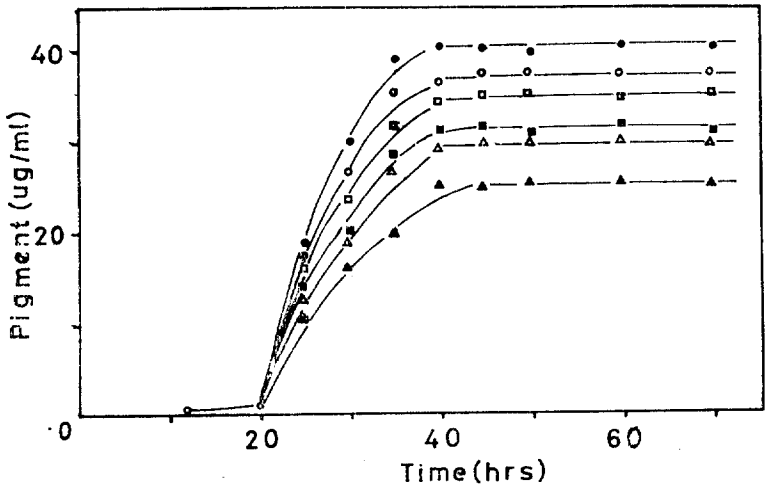

Fig. 6. The formation of pigment durirg the cultivation with the various light sources.
●; darkness, ○; green, □; blue ■; red, △; yellow, ▲; white

ected to the bacterial growth. The data in Table 5 show the bacterial numbers during the cultivation under the various light sources. The bacterial growth was not affected by the lights. Fig. 6 shows the effect of light on the formation of pigment. In darkness, the microbial pigment was produced mostly. However, the amount

of pigment were different with the light sources. The order of suitable light source for pigmentation was darkness, green, blue, red, yellow light and white light was poor for the pigmentation.

Kayama *et al.*(1973) suggested that some species of the *Nocardia*, *Corynebacterium*, *Arthrobacter*, *Brevibacterium* and *Flavobacterium* produced the pigment for photochromogenesis. For instance, *Nocardia brasiliensis* developed rich orange pigment when it was cultured under diffused sunlight, but entirely unpigmented under darkness. The result of this experiment, as it was reversed, showed rich red pigment was produced vigorously under darkness, while poor pigmentation was under white light.

DISCUSSION

The red pigment produced by *S. marcescens* strain P is the one of the prodigiosene because of its maximum absorbancy and the fact that the infra red specturum was similar to the prodigiosin of the strain Nima(Fig. 2).

The red pigment produced by *S. marcescens* strain P is the secondary metabolite according to the fact that the pigment occured when the bacterial growth was in stationary state(Fig. 3). The pigment is the metabolite which is not related to the bacterial growth and multiplication. The prodigiosin produced by *S. marcescens* strain Nima is also the secondary metabolite and the amount of pigment was decreased after the stationary state, and this phenomenon was believed due to the enzymatic degradation of pigment(Qadri and Williams, 1972). But in this experiment, the amount of pigment was not decreased after the stationary phase.

Almost the microbial pigments are the secondary metabolite (Anderews *et al.*, 1973; Calhoun *et al.*, 1972; Geber, 1975; Hammond and White, 1970; Kluyver, 1956; Stephens and Starr, 1963), and secondary metablites may be located in the cell envelope of microorganisms(Sthephens and Starr, 1963; cited from Williams, 1973), as it is true of prodigiosin(Williams, 1973). The pigment of *S. marcescens* strain P was located in the cell envelope and not released into the medium.

The quantitative data(Fig. 4) confirm the common belief that maximal pigmentation of *S. marcescens* strain P occured at 30°C. Some strains of *S. marcescens* can produce red pigment when incubated at 37°C(cited from Williams *et al.*, 1971), but the strain Nima and strain P could not produce the pigment at 37°C. Since the bacteria which were cultured at 37°C could form the pigment when incubated at 30°C, it is confirmed that the metabolism of pigmental intermediates might be depressed by temperature. Qadri and Williams (1972) suggested the hypothesis that certain cellular metabolites must be preserved for the induction of pigmentation after the temperature shift-down.

S. marcescens synthesized the pigment under alkaline conditions(Williams, 1973). And the alkaline conditions were also characteristics for production of other secondary metabolites(cited from Williams, 1973),

The results of the effects of acidity to *S. marcescens*(Table 4, Fig. 5) show the initial *p*H is very important to pigmentation, and the longer to be alkaline of medium, the more pigment was formed. For instance, above the range of *p*H 7.0, the time to be *p*H 8.4 was shorter than others, so the amount of pigment was smaller.

The pigment did not change the color among with the change of acidity. Wood *et al.*(1971) reported that the red pigment of *S. marcescens* varied from pink(*p*H 5.0 ~6.0) to orange(*p*H 7.0~8.0) along with the change of medium acidity.

By the results of the various light sources (Table 5, Fig. 6), it is confirmed that the pigment of *S. marcescens* strain P is the pigment of non-photochromogenesis. As the purified pigment was stable on the lights, the pigment was not destroyed during the cultivation, and it is also confirmed that the formation of pigment was depressed by lights. And the longer waves of lights, such as red and yellow light, are the more potent depressor of pigmentation.

적 요

적색색소를 분비하는 세균은 *Serratia marcescens*로 동정되었으며, 이 적색색소의 추출에는 *n*-buthanol이 가장 좋았으며, 추출된 색소는 prodigiosene으로 동정이 되었다.

추출된 색소는 온도와 빛에 대해서는 안정하였으나, 산성용액에서는 매우 불안정하며, 색소의 색깔은 alkaline 상태에서는 오렌지 색을 띠고, 산성 상태에서는 적색을 띠었다. 색소의 최대흡광파장은 alkali 상태에서는 466nm이였고, 산성상태에서는 540nm이였으며 TLC(starch)에서 분리되지 않아 단일물질로 추정되며, 적외선 흡광분석의 결과 비교 균주인 *S. marcescens*에서 추출한 prodigiosin과 거의 같은 흡광 파장을 가졌다.

적색색소는 2차대사 물질이였고, 색소생성은 접종후 30시간째에 최대에 이르렀으며, 이때 세균의 생장곡선은 휴지기에 있었다. 37°C에서는 색소는 형성되지 않았으며, 세균의 생장과 색소형성에 제일좋은 온도는 30°C이였다. 색소형성의 최적 *p*H는 *p*H 5.0이였으며 산성도에 의해서 세균의 생장은 전혀 변화되지 않았다. 광선에 의한 영향은 암소가 가장 좋은 조건이였으며, 역시 세균의 생장에는 아무런 영향을 미치지 못했다.

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