Studies on the Biosynthesis of Pyocyanine. (I)*

On the Cultural Condition for Pyocyanine Formation

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In order to decide a suitable cultural condition for pyocyanine formation and to find out a clue for the mechanism of biosynthesis of pyocyanine, various experiments were carried out.

It has been observed that pyocyanine formation was easily affected by various cultural conditions such as the composition of culture medium, especially the kind or the concentration of carbon source, the pH of the medium, the incubation temperature, *etc.*

On the other hand, it has been noted that in the organic medium prepared from natural materials such as bouillon, yeast or malt extract and a certain commercial peptone, pyocyanine formation was hardly revealed owing to the existence of the substance considered to inhibit the enzyme action in pyocyanine synthesis system.

INTRODUCTION

Pyocyanine has widely been known as a blue pigment or an antibiotic substance formed by *Pseudomonas aeruginosa.* Since pyocynaine was first found by Fords in 1860, who obtained it from a purlent matter, various investigations have been made concerning this pigment from the chemical, physical or biological point of view.

Wrede and Strack[2] demonstrated by chemical synthesis of this pigment that pyocyanine is a phenazine derivative, *i.e.* α-hydroxy-N-methylphenazine.

Pyocyanine is reversibly reduced and oxidized by the bacterial cell in culture medium and is believed, therefore, to be assigned a useful role in physiology of the bacteria by conjunction with cytochrome system to increase the cell respiration.[3~11]. However, there observed various strains incapable of pyocyanine formation or the phenomenon that the normal strain capable of pigmentation reveals no pyocyanine formation independent of the bacterial growth, according to the cultural conditions. On the other hand, it has been observed that even in a physiological concentration pyocyanine exhibited rather inhibitory action on the growth of bacteria themselves. Therefore, the view stated above may not be so much appropriate to understand the essential physiological role of pyocyanine as to elucidate a role as an accessory in physiology. But, at any rate, there must be some significance for the bacterial activity in revealing the re-

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markable pigmentation. Not only from a physiological but from a biochemical viewpoint another interesting problem will be proposed : the mechanism of pyocyanine biosynthesis, especially of biosynthesis of phenazine nucleus.

At present, however, the information relating to this field has not yet been furnished. Lately, the author has designed to collect the acquirements concerning the biosynthesis of pyocyanine and obtained some results on the conditions for the formation of this pigment.

MATERIAL AND METHOD

Bacterial Strain

Bacteria used were the strain isolated from cow's milk in author's laboratory, which had been identified as *Pseudomonas aeruginosa* variant. Since the present experiment had been charged with the mission rather to find out the condition suitable for obtaining a considerable amount of pyocyanine than to search for the clue for the mechanism of its biosynthesis, one strain was throughout supplied to the experiment except the special case.

Culture of Bacteria

Bacterial culture was carried out as follows : so as to extend the surface of cultural solution, 5 ml of nutrient medium was taken into a test tube or 100 ml, in 500 ml Erlenmeyer flask and the bacteria inoculated were incubated in static state at 37° for desired length of time (2 to 6 days).

Determination of Pyocyanine

Method for the determination of pyocyanine has not been established as yet. For the determination, therefore, a temporary method which represents merely the relative amount of pyocyanine produced was obliged to employ : 5 ml of cultured solution was extracted with chloroform several times and the chloroform extract was shaken with dilute HCl. After the red solution of pyocyanine was neutralized with Na_2HPO_4 solution, the blue colored solution obtained was colorimetrically compared with the standard solution of $CuSO_4$.

EXPERIMENTAL AND DISCUSSION

Medium

In view of the fact that pyocyanine formation is easily affected by various circumstances independently of the bacterial growth, the choice of the medium was considerably difficult. It was observed that the strain supplied to the present experiment did not form pyocyanine in the organic medium prepared from natural materials such as bouillon, yeast extract, malt or kojic extract and some kinds of commercial preparation of peptone.

After various attempts the following medium has been chosen to be most favorable for pyocyanine formation : 3 g of glycerol, 1 g of peptone (Poly or

Mikuni peptone), 0.1 g of asparagine (replaced by aspartic or glutamic acid), 0.1 g of NH_4NO_3, 0.025 g of K_2HPO_4 and 0.025 g of $MgSO_47H_2O$ were dissolved in 100 ml of distilled water and pH was adjusted to 7.4.

Cicconi[12] reported that no pyocyanine formation in the medium containing yeast extract was based on the existence of the heat-stable inhibiting matter for pigment formation and that pigmentation was recovered by the addition of excess of alanine to the medium. On the other hand, it was observed that tap water could not be used for the purpose of the present experiment, since a decreasing effect on pyocyanine formation was brought probably on account of an inhibitory

Table 1. Effect of carbon sources on pyocyanine formation.

Carbon source	Assimilation	pH	Pyocyanine formation	pH
Glycerol	##	7.4	###	8.4
Glucose	##	6.5	—	4.5
Glucose (+$CaCO_3$)	##	7.2	###	8.0
Fructose	##	7.2	##	8.2
Sucrose	+	7.0	—	8.6
Maltose	trace	7.0	—	8.2
Lactose	trace	7.0	—	8.4
Xylose	+	4.8	—	5.8
Galactose	+	5.8	—	5.6
Mannose	+	5.3	—	5.0
Raffinose	##	6.8	—	8.0
Rhamnose	—	7.0	—	8.2
Sorbitol	+	6.8	—	8.4
Dulcitol	+	6.8	—	8.4
Mannitol	##	7.2	+	8.3
Gluconic acid	##	7.8	###	8.4
Lactic acid	##	8.0	##	8.4
Citric acid	—	7.2	—	8.2
α-Ketoglutaric acid	##	8.0	###	8.2
Fumaric acid	##	8.2	+	8.6
Succinic acid	##	8.2	+	8.4
Malic acid	##	8.0	+	8.6
Acetic acid	+	7.4	—	8.2
Plant oil (soya-bean)	##	7.4	##	8.4
Nil	—	7.0	—	8.4

Assimilation test was performed with the following synthetic medium: 2 % carbon source, 0.2 % NH_4NO_3, 0.05 % K_2HPO_4, 0.05 % $MgSO_47H_2O$ and 0.001 % $FeSO_47H_2O$, pH 7.0. Organic acids were used as natrium salts except gluconic acid which was a calcium salt. ##, ## and + represent the bacterial cell number of about 5×10^8, 2×10^8 and 10^8 in rough estimation. For pyocyanine formation test, the following medium was used: 2 % carbon source (organic acid was supplied as a calcium salt), 1 % peptone, 0.1 % NH_4NO_3, 0.1 % asparagine, 0.025 % $MgSO_47H_2O$ and 0.025 % K_2HPO_4, pH 7.4. ###, ##, ## and + show the amount of pyocyanine equivalent in color intensity to about 40, 30, 20 and 10 % $CuSO_45H_2O$ solutions, respectively. Incubation was carried out at 37° for 4 days in both tests.

action of metal ion.

As will be described later, however, the excess of any component of the medium would prevent the pigmentation, so that it may not always be reasonable to attribute the inhibition of pigmentation simply to the existence of inhibitory substance.

Carbon Source

Among various carbon sources, glycerol is considered to be most favorable for pyocyanine formation. When glucose (higher than 2%) was substituted for glycerol pyocyanine was not formed at all. However, when it was supplemented with $CaCO_3$, glucose was found to be the same desirable carbon source as glycerol (Table 1).

Disaccharides such as sucrose, maltose and lactose were hardly assimilable. Glycerol, glucose, fructose and such organic acid as gluconic, lactic or α-ketoglutaric acid were available for the present experiment. It was found that among various organic acids in the members of trycarboxylic acid cycle, only α-ketoglutaric acid was effective and that natrium salt of these organic acids revealed a decreasing effect on pyocyanine formation. It is, on the other hand, interesting to note that soybean oil can be used as a source of carbon for pyocyanine formation.

Nitrogen Source

The medium already described contained peptone, asparagine (aspartic or glutamic acid) and NH_4NO_3 as sources of nitrogen. Peptone and amino acid, of cource, play the role of carbon source for bacterial growth, but not at all for pyocyanine formation. As shown in Table 2, peptone is essential for pyocyanine formation whereas other nitrogen sources can, to some extent, be substituted for one another.

Table 2. Effect of nitrogen source.

Nitrogenous matters	(Glycerol)	Peptone	Asparagine	NH_4NO_3 (KNO_3)	$(NH_4)_2HPO_4$	Pyocyanine formed
Peptone	+	+	+	+	*	###
	−	+	+	+	*	−
	+	+	−	+	*	##
	+	+	+	−	*	##
Asparagine	+	−	+	+	*	−
	−	−	+	+	*	−
	+	−	+	−	*	−
	−	−	+	−	*	−
$NH_4NO_3(KNO_3)$	+	−	−	+	*	−
	(+)	(+)	(+)	(+)	*	(##)
$(NH_4)_2HPO_4$	+	+	+	*	+	−
	+	−	+	*	+	−

Concentration of components was the same as in Table 1 except the case of nitrogenous matters in the absence of peptone, which were tested at the concentration of 0.2 % as sole source of nitrogen. Incubation was performed at 37° for 4 days.

When NH_4NO_3, KNO_3 or $(NH_4)_2SO_4$ was singly used as a source of nitrogen, the bacterial growth was considerably poor and no pyocyanine was formed. Any pyocyanine formation was never observed with $(NH_4)_2HPO_4$ for the nitrogen source despite of the presence of peptone, and this fact, as will be illustrated later, was found to be due to its phosphate ion. In the case of urea used as sole source of nitrogen, the lag phase of bacterial growth was prolonged and pyocyanine was scarcely produced. Similarly, glutamic or aspartic acid did not support the formation of pyocyanine, while the bacterial growth was fairly satisfactory. In spite of the author's observation that when no peptone was supplied to the medium pyocyanine formation could not be expected at all, other investigators have reported that peptone in the medium could be substituted with several amino acids such as glycine, L-leucine or DL-alanine[13] and that alanine or tyrosine brought an increasing effect on pyocyanine formation.[14]

On the other hand, acid-hydrolysate of peptone was found by the author to be used as well as peptone for the purpose of pyocyanine formation. From these results, the effective constituent of peptone can be presumed to be amino acid, or otherwise to be another factor stimulating the formation of pyocyanine, since in the present experiment the same result could not always be obtained as the other worker's on the effect of amino acid. The above-mentioned facts also suggest that peptone-requirement for pyocyanine formation may be divided according to the varieties of bacterial strains.

Concentration of Carbon and Nitrogen Sources

In the present experiment using the growing cell of bacteria the concentration or the ratio of components in the medium cannot simply be discussed only about the pyocyanine formation, because the bacterial growth is also controlled by the proportion of individual component prior to the pigmentation.

On the other hand, only total nitrogen must not be argued on pyocyanine formation, since the form of nitrogen is brought up, for instance, two nitrogenous components of NH_4NO_3 and asparagine cannot be replaced only by NH_4NO_3 at an equal level of nitrogen as far as pyocyanine formation is concerned. However,

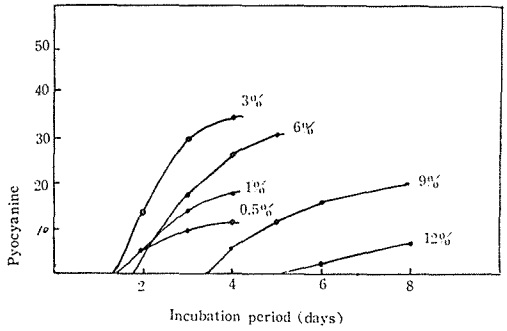

Fig. 1. Effect of concentration of carbon source.

Glycerol was given at a desired concentration to the following basal medium : 1% peptone, 0.1% asparagine, 0.1% NH_4NO_3, 0.025% K_2HPO_4 and 0.025% $MgSO_47H_2O$, adjusted to pH 7.4. Incubation temperature was 37°C. Pyocyanine formed was indicated as a relative amount in comparison with standard solution of $CuSO_4$ shown in Table 1.

at any rate, it was a conspicuous phenomenon in the present experiment that pyocyanine formation was easily affected by the concentration of various components in the medium. As shown in Fig. 1, at a higher proportion of carbon source, pyocyanine formation is gradually delayed or decreased. On the contrary, at an increasing concentration of nitrogen source such as NH_4NO_3 the effect on pyocyanine formation is scarcely observed with the exception of peptone which is also utilized by the bacteria as a source of carbon. Smaller amounts of component necessary to add to the peptone medium do not support the formation of pyocyanine (Table 3).

Table 3. Effect of concentration of nitrogen sources.

Concentration of nitrogen source (%)		pH	Pyocyanine
Peptone	0.50	7.8	++
	1.00	8.4	+++
	2.00	8.8	++
	4.00	9.2	trace
	6.00	9.2	−
Asparagine	0.02	7.8	++
	0.05	8.0	++
	0.10	8.4	+++
	0.20	8.4	+++
	0.40	9.0	+++
NH_4NO_3	0.02	8.4	+
	0.05	8.2	++
	0.10	8.2	+++
	0.20	8.4	+++
	0.40	7.8	+++

Basal medium: 3% glycerol, 1% peptone, 0.1% asparagine, 0.1% NH_4NO_3 0.025% K_2HPO_4 and 0.025% $MgSO_47H_2O$, pH 7.4. Experiments were carried out with the medium containing requisite amount of one nitrogen source and the other materials of the same concentration as in the basal medium. Incubation temperature, period and representation of pyocyanine were the same as in Table 1.

As a general rule, the optimal concentration is peculiarly required in any case of components of the medium. As will be described later, not only in the case of carbon source but also of the other inorganic matter, the effect of higher concentration was shown on pyocyanine formation. These results may rather suggest a possibility of the relationship between the osmotic pressure of the medium and the enzyme action in the biosynthesis of pyocyanine.

Inorganic Matter

Five ions of K, PO_4, SO_4, Mg and Fe were reported to be essential for pyocyanine formation.[14] As was mentioned before, the strain supplied to this experiment could not form pyocyanine in the synthetic medium without peptone.

Therefore, the effect of individual component on pyocyanine formation had to be discussed in the presence of peptone. The five ions described above should be essential rather for the bacterial growth than for the pyocyanine formation.

Although the bacterial growth is fairly satisfactory in the presence of peptone, $MgSO_4$ must be supplied to the medium in order to expect the formation of pyocyanine, whereas the necessity of other inorganic matters is not pointed out (Table 4).

Table 4. Effect of inorganic matters.

Concentration (%, mg%*)	$MgSO_4$-$7H_2O$	$(NH_4)_2SO_4$	$MgCl_2$-$6H_2O$	K_2HPO_4	KCl	$(NH_4)_2HPO_4$	$FeSO_4$-$7H_2O$
0.010	++	(−)	(+)	+++	(+++)	(+++)	+++
0.025	+++	(−)	(++)	+++	(+++)	(++)	+++
0.050	+++	(−)	(++)	++	(++)	(++)	+++
0.100	+++	(−)	(++)	−	(++)	(−)	+++
Nil	−	(−)	(−)	++	(++)	(++)	+++

Basal medium was the same as in Table 3.

* Concentration of $FeSO_4 7H_2O$. During the test of one component, the concentration of other inorganic matters was fixed.

$(NH_4)_2SO_4$ and $MgCl_2$ were substituted for $MgSO_4$; KCl and $(NH_4)_2HPO_4$, for K_2HPO_4, respectively. Incubation was performed at 37° for 5 days. Pyocyanine formed was represented in the same way as in Table 1.

It is likely that the effect of $MgSO_4$ is ascribable to being supplemented by the addition of it to the peptone medium which is insufficient in content of Mg and SO_4 ions for pyocyanine formation, while these ions would not be deficient for bacterial growth. With PO_4 ion, a noticeable effect was found out: by increasing concentration of phosphate, pyocyanine formation is gradually decreased and at the concentration higher than 0.1% of K_2HPO_4, pyocyanine formation is completely stopped. However, in the literature, it is not so unusual for pyocyanine formation to point out the case in which phosphate is employed at higher concentration than 0.1%.[15,16,17]

Relation between Fluctuation of Sugar and Pyocyanine Formation

Fig. 2 shows the result of the experiment with the glucose medium containing $CaCO_3$. In general, pyocyanine formation was revealed after the incubaction of 48 hours when sugar was almost consumed. The same fluctuation of glycerol would be expected as in the case of glucose mentioned above, although the higher the concentration of glycerol, the more delayed appearance of pyocyanine as was shown before (Fig. 1). It is suggested from these results that pyocyanine formation will occur after the logalithmic phase of the bacterial growth.

Effect of Oxygen

It was reported by some workers[18] that a certain strain of *Pseudomonas aeruginosa*, in the presence of nitrate, could grow under an intensive anaerobic condition and the leuce base of pyocyanine was produced anaerobically. The same result, at least concerning pigmentation, could not be obtained by the present author.

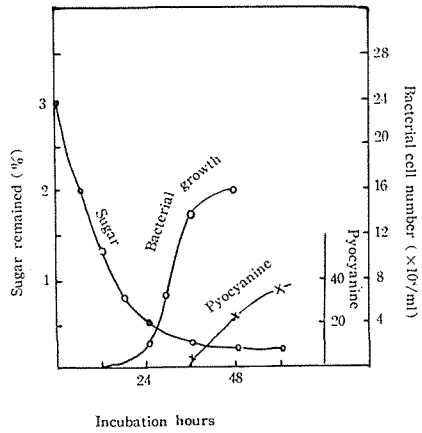

Fig. 2. Relation between the fluctuation of sugar and pyocyanine formation.

Medium : 3% glucose, 1% peptone, 0.1% NH_4NO_3, 0.1% asparagine, 0.025% K_2HPO_4, 0.025% $MgSO_47H_2O$ and 5% $CaCO_3$. Incubation temperature was 37°. The expression of pyocyanine was the same as in Table 1.

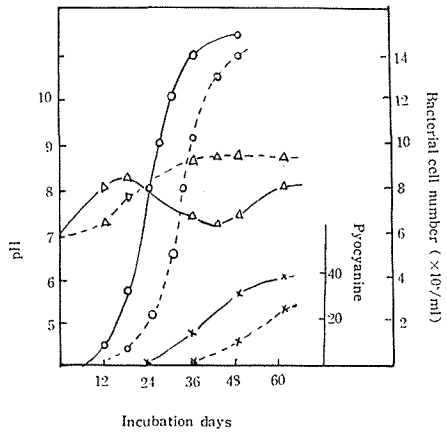

Fig. 3. Effect of aeration.

Medium : 3% glycerol, 1% peptone, 0.1% asparagine, 0.1% NH_4NO_3, 0.025% K_2HPO_4 and 0.025% $MgSO_47H_2O$, adjusted to pH 7.0. Incubation temperature was 37°. Aeration was performed at the capacity of about 500 ml of air per 200 ml of cultural solution per minute. (△…△…△), (○…○…○), (×…×…×) Express pH, bacterial growth and pyocyanine formation in static state ; (△—△—△), (○—○—○) and (×—×—×) do. under aeration, respectively.

Bacteria were usually incubated so as to be on spacious surface of the medium. Fig. 3 represents the results of the experiments on the effect of aeration. It will be seen in Fig. 3 that pyocyanine formation as well as bacterial growth is accelerated by the aeration.

Effect of pH

As was already described, no pyocyanine takes place in the glucose medium without $CaCO_3$. This fact signifies that pyocyanine formation is affected by pH of the medium (Table 5). The bacterial growth was allowed at the pH higher

Table 5. Effect of pH.

Carbon source	Nil		Ca-salt(+$CaCO_3$)		Na-salt	
	Pyocyanine	pH	Pyocyanine	pH	Pyocyanine	pH
Glycerol	###	8.2	*	*	*	*
Glucose**	##	7.8	*	*	*	*
Glucose	—	5.2	###	7.8	*	*
Lactate	*	*	##	7.8	##	9.6
α-Ketoglutarate	*	*	###	8.0	##	9.4
Fumarate	*	*	+	7.6	—	9.0

** 3% peptone medium.

Experimental methods were, on the whole, the same as in Table 1.

than 6, and for the pyocyanine formation optimal pH was observed to be in the range from 7.4 to 8.4. When final pH value of the medium was observed to be higher than about 9, the cultural solution became viscous and pyocyanine formation was obstructed. The reason why the natrium salt of organic acids causes the decrease of pyocyanine formation is considered to be due to the pH.

Effect of Temperature

In the range from 15 to 40°, incubation was carried out. At 25°, the bacterial growth was, to some extent, delayed and the pigment primary formed changed to brownish blue before pyocyanine formation was fully performed. When the bacteria incubated at 37° for 2 days were removed to the incubator of 40°, pyocyanine formation was stopped and pigmentation was no longer recovered even when they were returned to the former state. At room temperature (about 15 to 18°) bacterial growth attained to a desirable state after a long time but pyocyanine was hardly formed. Optimal temperature was found to be 37° for pyocyanine formation as well as bacterial growth (Fig. 4).

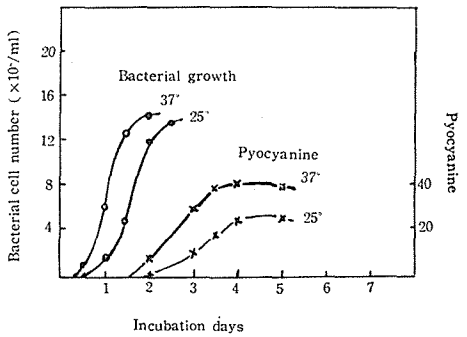

Fig. 4. Effect of incubation temperature and time.

Medium was the same as in Fig. 3 except pH 7.4. (×—×—×) Pyocyanine formation, (○—○—○) bacterial growth. Pyocyanine was expressed by the same way as in Table 1.

Variability of the Bacterial Strain

In the present study the author has experienced that the strain supplied was

susceptible to the bacterial mutation. Socolov[19] reported that the bacteria incubated in the desiccator filled with CO_2 lost the ability to form pigment and that the strain thus treated could not show the recovery of pigmentation and simultaneously lost biochemical characters such as gelatin-liquefaction or milk-coagulation. Similarly, it was presented by the other worker that the bacteria incubated on egg albumin at 57° for 5 minutes decreased the ability to form pyocyanine[20]. The author has observed that from one strain isolated at the start of the present experiment the following varieties were derived, spontaneously : changes in form of bacterial colony, decrease or increase in the capacity for the formation of pyocyanine and other antibiotic substance, appearrance of the assimilability of a certain organic acid (*e.g.* citric acid which is usually unable to be attacked by the living cell), *etc.*

Incubation Period

The optimal period for pyocyanine formation was found to be controlled by the concentration of components of the medium besides the incubation temperature, hence the conditions can not simply be ascertained. At higher concentration of glycerol, pyocyanine formation was considerably delayed or decreased as in the case of the experiment at lower temperature. The highest amount of pyocyanine was detected at the period of 3 to 4 days under a suitable cultural condition, after when the pyocyanine primary formed was observed to be gradually diminished owing to its oxidation (Fig. 4).

On the Viscidity of Cultural Solution

The aged cultured solution, in general, became viscous like egg white, especially in such a medium getting very alkaline as in the case of peptone in high concentration or of natrium salt of organic acids.

Dantz *et al.*[21] have noted the production of gelatin-like substance by the present bacteria and suggested this substance to be a kind of carbohydrates. The author has observed that when the medium became viscous, pyocyanine was hardly produced probably on account of the autolysis of the bacterial cells depending on very alkalinity of the cultured solution, and that the viscous solution was coagulated by the addition of formaldehyde or $HgCl_2$, suggesting the majority of these substances to be rather a kind of proteins.

SUMMARY

With a newly isolated strain of *Pseudomonas aeruginosa* the following experimental results on pyocyanine formation were obtained.

1. Pyocyanine could not be formed in the organic medium prepared from natural materials such as bouillon, yeast extract, malt or kojic extract, some kinds of commercial peptone, *etc.*

2. The excess of components in the medium prevented pyocyanine formation, especially it was noted that at the concentration higher than 0.1 % of K_2HPO_4, pyocyanine was not formed at all.

3. No pyocyaine formation in glucose medium was found to be due to the

effect of pH of the medium, so that glucose can also be a favorable source of carbon for pyocyanine formation as good as glycerol, by the addition of $CaCO_3$.

4. It is considered that peptone is an essential material not only for bacterial growth but also for pyocyanine formation as a source of amino acid or an existence of the substance promoting pigmentation.

5. Pyocyanine formation was observed to begin after the logarithmic phase of the bacterial growth.

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