A STUDY OF PIGMENT PRODUCTION
BY *PSEUDOMONAS*

by

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INTRODUCTION

The organisms of the genus Pseudomonas are important because of their ubiquitous nature, their ability to spoil foods, and their pathogenicity to plants and animals.

Being found in soil, fresh and sea water, decaying fish and other putrifying matter, sulfur springs, petroleum and its products, various plant organs, and in many types of small and large animals, indicates that bacteria belonging to the genus Pseudomonas are among the most widespread of microorganisms.

One of the outstanding characteristics of Pseudomonas is its ability to produce pigments of greenish hue. This pigment production by Pseudomonas is an important aid in the identification of the genus Pseudomonas (Bergey, 1948). Pigment production by these organisms also has a relationship to their antibiotic activity (Young, 1947).

This paper deals with the collection and identification of cultures of Pseudomonas and a study of some of the factors involved in the pigment production.

REVIEW OF LITERATURE

The original isolation of Pseudomonas as a pure culture was brought about because of its ability to produce a pigment. The blue or blue-green stains that sometimes appeared upon surgical dressings attracted attention of many observers long ago. Even before the cause of this phenomenon had been discovered, Fordos investigated the nature of the coloring substance and in 1860 extracted pyocyanine, a blue-green pigment, from surgical dressings. Since that time, studies of the pigments of Pseudomonas have appeared with regularity.

Pyocyanine Production by Pseudomonas

In 1882, Gessard proved that the blue-green pigment, pyocyanine, was produced by an organism which he was able to isolate in pure culture. He carefully described its morphological and physiological characteristics (Jordan, 1899).

Pyocyanine is formed by Pseudomonas pyocyanea which synthesizes this blue-green pigment when grown in suitable media. The blue or blue-green pigment is secreted from the bacterium into the surrounding medium from which it may readily be obtained in crystalline form (Oppenheimer and Stern, 1939). Much of the literature on pyocyanine is of a contradictory nature.

Requirements for the Production of the Pyocyanine Pigment. Gessard (1891), showed that glycerol peptone agar provided an admirable medium for pyocyanine formation. His medium consisted of 5 percent glycerol, 2 percent peptone, 3 percent agar, and distilled water.

Jordan (1899) stated that pigment production does not depend on the presence of phosphates or sulfates, and that ammonium salts of succinic, lactic, acetic, or citric acids could be used as the sole source of carbon and nitrogen.

Phosphorous, Mg^{++}, Nitrogen, and Carbon were essential for growth according to Robinson (1932), and he could not find a synthetic medium that would equal Bacto-peptone in the yield of pyocyanine by *Pseudomonas aeruginosa*. He recorded that Na^{+}, K^{+}, Ca^{++}, Cl^{-}, SO_4^{--}, and CO_3^{--} were essential.

Sandiford (1937) grew *Pseudomonas* organisms in peptone water, and all showed pigment production. In 1943, Seleen and Stark reported that the best formation of pyocyanine occurred in Gessard's glycerol peptone medium.

Di Maggia (1946) found that he could produce pigment in media lacking in Na^{++}, Mg^{++}, Ca^{++}, and K^{+}. He stated also that glucose or mannitol could be used as a sole source of carbon; but maltose, sucrose, or lactose would not serve as the sole source of carbon.

Young (1947) discovered that *Pseudomonas aeruginosa* could not produce pigments in culture media containing sufficient glucose (over 1 percent) to establish and maintain an acid reaction. In studying the required amino acids for production of pyocyanine by *Pseudomonas*, Burton, Eagles, and Campbell (1947) used Gessard's glycerol peptone medium as a comparison for maximum pigment. Burton and co-workers found that 0.4 percent alanine and 0.8 percent leucine were the 2 amino acids which the organism required for pigment production and that 0.4 percent glycine gave nearly as good results as alanine. Later, K^{+}, PO_4^{---}, SO_4^{--}, Fe^{+++}, and Mg^{++} were found essential to formation of the pigment (Burton, et al., 1948).

Inhibition of Pyocyanine Pigment Formation. Pyocyanine formation was inhibited by diphenylamine and Cu^{++} ions in concentrations as low as 1:50,000 according to Kharasch (1936), but he also found that pigment formation by *Pseudomonas Pyocyanea* was not inhibited by Fe^{+++}, glutathione, or alloxantin.

Certain peptones were noticed to exert an inhibitory effect on the production of pyocyanine by Di Maggia (1946). Young (1947) found that enriching the medium with veal infusion or blood inhibited the pigment production. Pyocyanine formation was inhibited in varying degrees by Co^{++}, NH_4^{+}, Mn^{++}, Zn^{++}, As^{++}, and Cu^{++} according to Burton, et al. (1948).

Effect of Temperature. Jordan (1899) stated that he obtained the best pigment production at room temperature and in the dark. Emry and Roberts (1914), Sandiford (1937), and Seleen and Stark (1943) observed that 37° C. was best for the formation of pyocyanine. Burton and co-workers (1947), however, used 30° C. for the incubation temperature.

Effect of pH. Burton, et al. (1947) adjusted their media to pH 7.2. Robinson (1932) states that pH range 6.0 to 9.0 is satisfactorily for aerobic growth, but pH 7.5 to 8.0 gave best pigment production. Young (1947) stated that the best production of pyocyanine was between pH 7.5 and 8.35, but her results could be questioned since she did not use the same medium throughout all tests.

Fluorescent Pigment Production by Pseudomonas

Gessard (1890), who reported early studies on pyocyanine, also investigated a second pigment of Pseudomonas, which gives a yellow-green fluorescence. He stated that a given strain of Bacterium pyocyaneus may, by suitable cultural methods, be induced to form a fluorescent pigment alone, pyocyanine alone, or the two pigments simultaneously. At that time only one species of the present genus Pseudomonas (Bacterium pyocyanus) had been described.

Requirements for the Production of the Fluorescent Pigment. Gessard (1892) concluded that fluorescense could be produced only if the medium contained decomposed lecithin. Lepierre (1895) found that fluorescence depended upon meat extractives, such as xanthine and creatinine, plus soluble albuminoids.

Jordan (1899) noticed that in a broth of asparagine, phosphate, and sulfate these organisms produced excellent fluorescent pigmentation and concluded that both phosphorous and sulfur were essential. The following compounds, arranged according to their relative influence, were found to stimulate the production of fluorescence: Asparagine, succinic acid, lactic acid, citric acid, tartaric acid, uric acid, acetic acid, oxalic acid, and formic acid. Jordan also warned, however, that if the concentrations of the chemicals were too great the pigment production would be hampered.

Later, in 1907, Benecke reported that the essentials for the production of the fluorescent pigment were Mg^{++}, PO_4^{---}, SO_4^{--}, and a very small amount of K^+, together with suitable sources of Carbon and Nitrogen (Turfitt, 1936).

Tanner (1918) thoroughly reviewed the literature up to that time and found that fluorescence was produced in gelatin, Uschinsky's medium, Frankel's medium, and Sullivan's medium. The latter medium contained only $MgSO_4$, asparagin, and dipotassium phosphate.

Later Georgia and Poe (1931) reopened the subject and named at least ten investigators who had reported the essential requirements for Pseudomonas fluorescence, and noted very little agreement as to requirement. They gave several reasons for the conflicting results and suggested that Mg^{++}, PO_4^{---}, and SO_4^{--} were all necessary for the fluorescent pigment formation. They proposed the following medium $MgSO_4$ (0.5g.), K_2HPO_4 (0.5g.), asparagine (3.0g.), and distilled water (1000 ml.). The following year they (Georgia and Poe, 1932) suggested that peptones varied in their ability to aid in fluorescent pigment formation simply because the peptones varied greatly in their composition, some lacking PO_4^{---}, Mg^{++}, or some other essential constituent for pigment production. They, as did Jordan, cautioned that too concentrated media will not satisfactorily support formation of fluorescence, even though the necessary constituents are present. For example, they stated a 0.5 percent peptone broth is more satisfactory than a 3 percent broth.

Turfitt (1936) agreed with Georgia and Poe that Mg^{++} and PO_4^{---} would produce growth but no fluorescent pigmentation, so Turfitt used their medium and obtained good yields of fluorescent pigment. In addition, Turfitt also observed that a trace of some heavy metal salt would cause complete inhibition of fluorescence, although growth proceeded. He advised, therefore to use only distilled water in preparing the culture medium.

Effect of Temperature. Jordan (1899) stated that best fluorescence was produced at room temperature. Georgia and Poe (1932) did not state the temperature used. Turfitt incubated his *Pseudomonas* at 25° C. for the formation of the green fluorescence, and according to Seleen and Stark (1943) the best temperature was between 20° and 30° C.

Effect of pH. Very few of the workers mentioned the pH of their medium, Jordan (1899) said the presence of acid conceals the color. Georgia and Poe (1932) determined that their cultures produced the best fluorescence around pH 6.8 to 7.3.

Reasons for the Conflicting Statements in the Literature

A short discussion seems pertinent because of the many contradictory statements found in the literature concerning *Pseudomonas*. The loss of pigment formation has been noticed since the early history of *Pseudomonas*. Charrin and Phisalix reported in 1892 that cultures of *Bacterium pyocyaneus* persisted in losing their ability to produce pigment (Turfitt, 1936). Varying results in experiments could be explained, therefore, by the fact that one worker might have had a strain of Pseudomonas which retained its ability to produce pigment, while another investigator might have had a variable or weak pigment producer.

Variations in the strains, themselves, could alter findings and, as an illustration of this, Baerthleim (1918) obtained six variants from one single strain of Bacterium pyocyaneus. These strains showed differences in colony formation, in size and shape of the rods, in the presence or absence of pigments, and in the different kinds of pigments produced.

The environmental conditions and how they were controlled should also be considered. The purity of the chemicals, glassware, water, etc.; the temperature of incubation; the pH of the medium; and the size tube or flask in which the Pseudomonas were grown, are all important conditions which could explain contradictory statements.

To add to the confusion, there have been reports of several other pigments in addition to the two main ones pyocyanine and the yellow-green fluorescent pigment which certain strains or species of Pseudomonas produce. For an example, Nakhimovshaya (1948) announced a new Pseudomonas species, Pseudomonas aurantiaca, which not only produces a green pigment, but also an orange-yellow pigment.

It is not difficult, therefore, to understand why there are contradictions in the literature and why classifying Pseudomonas by pigment production and other characteristics is not an easy task.

Turfitt (1936, 1937) made two very good observations on this subject. He noted, "In common with most other bacterial characteristics, pigmentation has, from time to time, been considered a variable cultural factor. But, certain specified media make the chromogenic character remarkably constant; and further, the organisms are closely related both morphologically and culturally.

EXPERIMENTAL METHODS AND RESULTS

Collection, Sources, and Preliminary Identification as to Genus

Fifty Pseudomonas cultures were collected so a representative study could be made. The organisms were obtained from different sources and Table 1 presents the original number of the culture, the letter designation assigned to those used in further studies, and the source from which each organism was obtained.

The collection included only cultures which formed pigment of some type on nutrient agar plus 1 percent glycerol. The cultures were placed in nutrient broth tubes and, after good growth appeared, were streaked on nutrient agar plates for re-isolation and purity studies. Well isolated colonies were picked from these plates after 48 hours incubation at 35° C. The picked colonies were placed on nutrient agar slants, and smears were made for morphological studies.

Gram stains were made on all 50 of the organisms and their size, shape, and arrangements were noted. All were gram negative; size ranged from 0.60μ to 1.2μ by 1.0μ to 2.0μ; the average size was 0.7μ by 1.5μ. Occurrence was singly, in pairs, or in irregular masses.

Flagella staining was attempted on 15 of the organisms. Two different staining techniques were used. Both the modified Bailey's method and Casares-Gil's Flagella stain demonstrated the presence of flagella (Committee on Bacteriological Technic,

Table 1. The sources of the *Pseudomonas* organisms.

No. of organism	Later designation	Source
1		Brock weter
2		Well water
3		Feces (horse)
4		Brook water
5		Milk
6		Water
7	A	Urine
8		Cream
9	B	Cream
10	C	Well water
11	D	Well water
12	E	Sedimentation pond in Louisiana
13	F	A spring in Yellowstone Park
14	G	Salt water from a deep oil well
15	H	Ear abscess
16	I	A *Pseudomonas* stock culture
17		A *Pseudomonas* stock culture
18	J	Spoiled egg
19		" "
20	L	" "
21		" "
22	M	" "
23		" "
24	N	" "
25		Sinclair's finished kerosene
26		Phillips' distillate sample
27	O	A slanted separatory pit
28	P	A Sinclair crude oil sample
29	Q	A Sinclair crude oil sample
30		A supply spring in Yellowstone Park
31	R	Sedimentation pond in Texas
32		A culture marked *Pseudomonas olevorans*
33	S	A culture marked *Pseudomonas fermentons*
34		A sample from a cracked pressure distillate tank
35	T	Sulfur springs in Yellowstone Park
36	U	Roach tracks (contamination)
37	V	Oleomargarine sample
38		" "
39	W	" "
40	X	" "
41		" "
42	Y	" "

Table 1. (concl.)

No. of organism	Later designation	Source
43	Z	Oleomargarine sample
44	Aa	" "
45		" "
46	Bb	" "
47	Cc	" "
48	Dd	" "
49	K	Ear abscess
50		Milk

1946).

The following media were inoculated with the 50 organisms and used as an aid in classification.

- Gelatin plates
- Gelatin tubes
- Starch plates
- Milk plates
- Potato slants
- Indole broth
- KNO_3 broth
- Litmus milk
- Fermentation tubes (Durham)
    - Glucose
    - Sucrose
    - Maltose
    - Lactose
    - Mannitol

All inoculations were made from slants 48 hours old, and the temperature of incubation was 35° C., except for the gelatin tubes, which were incubated at 20° C. Results of the inoculations are given in Table 2. Some of the organisms did not show typical Pseudomonas reactions, and others of the collection were apparently losing the ability of pigment production. Thus, 30 organisms were selected from the 50 and designated with a letter.

Table 2. Characteristics of 50 Pseudomonas organisms.

Organism	Morphological[1]				Cultural[1]			Biochemical[1]										
								Acid formation										
	Motile	Spores	Flagella	Gram stain	Density	Form	Potato slant	Dex.	Suc.	Lac.	Man.	Mal.	Gel. liquef.	Nitro. reduct.	Indole form.	Litmus milk	Starch hydrol.	Casein digest.
1	+	-	+	-	*	Rod	B	+	-	-	-	-	+	-	-	AP	-	+
2	+	-	+	-	*	Rod	B	+	+	-	-	-	+	-	-	AP	-	+
3	+	-	+	-	*	Rod	LB	+	-	-	-	-	+	-	-	A	+	-
4	+	-	+	-	*	Rod	LB	+	-	-	-	-	+	-	-	AP	-	+
5	+	-	+	-	*	Rod	LB	+	-	-	+	+	-	+	-	ARd	-	-
6	+	-	+	-	*	Rod	LB	+	-	+	+	+	-	+	+	AcRd	-	-
7 (A)	+	-	+	-	*	Rod	B	+	-	-	-	-	+	-	-	AP	-	+
8	+	-	+	-	*	Rod	RB	+	-	-	-	-	+	-	-	AP	+	+
9 (B)	+	-	+	-	*	Rod	B	+	-	-	-	-	+	-	-	P	-	+
10 (C)	+	-	+	-	*	Rod	B	+	-	-	-	-	+	-	-	APRd	-	+
11 (D)	+	-	+	-	*	Rod	B	+	-	-	-	-	+	-	-	P	-	-
12 (E)	+	-	+	-	*	Rod	LB	+	-	-	-	-	+	-	-	AP	-	+
13 (F)	+	-	+	-	*	Rod	B	+	-	-	-	-	+	-	-	P	-	+
14 (G)	+	-	+	-	*	Rod	B	+	-	-	-	-	+	-	-	PRd	+	+
15 (H)	+	-	+	-	*	Rod	RB	+	-	-	-	-	+	-	-	AP	-	+
16 (I)	+	-		-	*	Rod	B	+	-	-	-	-	+	-	-	P	-	-
17	+	-		-	*	Rod	B	+	-	-	-	-	+	+	-	AP	-	+
18 (J)	+	-		-	*	Rod	LB	+	-	-	-	-	+	-	-	P	-	+
19 (K)	+	-		-	*	Rod	B	+	-	-	-	-	+	-	-	P	-	+
20 (L)	+	-		-	*	Rod	RB	+	-	-	-	-	+	-	-	P	-	+
21	+	-		-	*	Rod	RB	+	-	-	-	-	+	-	-	P	-	-
22 (M)	+	-		-	*	Rod	B	+	-	-	-	-	+	-	-	P	-	+
23	+	-		-	*	Rod	Rd	+	-	-	-	-	+	-	-	Rd	-	+
24 (N)	+	-		-	*	Rod	B	+	-	-	-	-	+	-	-	P	-	+
25	+	-		-	*	Rod	LB	+	-	-	-	-	+	-	-	Ac	-	+
26	+	-		-	*	Rod	LB	+	-	-	-	-	-	-	-	P	+	-
27 (O)	+	-		-	*	Rod	RB	+	-	-	-	-	+	-	-	AP	-	+
28 (P)	+	-		-	*	Rod	RB	+	-	-	-	-	+	-	-	AP	-	+

Table 2. (concl.)

Organism	Morphological[1]				Cultural[1]			Biochemical[1]										
								Acid formation										
	Motil	Spore	Fla-gella	Gram stain	Den-sity	Form	Potato slant	Dex.	Suc.	Lac.	Man.	Mal.	Gel. lique.	Nitro reduc.	Indole forma.	Litmus milk	Starch hydro.	Casein diges
29 (Q)	+	-		-	*	Rod	RB	+	-	-	-	-	+	-	-	AP	+	+
30	+	-		-	*	Rod	B	+	-	+	-	-	-	-	-	P	+	-
31 (R)	+	-		-	*	Rod	LRB	+	-	-	-	-	+	-	-	P	-	+
32	+	-		-	*	Rod	B	+	-	-	-	-	-	+	-	Rd	-	-
33 (S)	+	-		-	*	Rod	BR	+	-	-	-	-	+	-	-	P	-	+
34	+	-		-	0	Rod	B	+	+	+	-	-	-	-	-	Rd	+	+
35 (T)	+	-		-	*	Rod	LB	+	-	-	-	-	-	-	-	AP	-	+
36 (U)	+	-		-	*	Rod	B	+	-	+	-	-	-	-	-	P	-	+
37 (V)	+	-		-	*	Rod	LB	+	-	-	-	-	+	-	-	P	-	+
38	+	-		-	*	Rod	LB	-	-	-	-	-	+	-	-	AcP	+	-
39 (W)	+	-		-	*	Rod	LB	+	-	-	-	-	+	-	-	P	-	+
40 (X)	+	-		-	*	Rod	LB	+	-	-	-	-	+	-	-	P	-	+
41	+	-		-	*	Rod	LRB	+	-	-	-	-	-	-	-	AcP	+	-
42 (Y)	+	-		-	*	Rod	LB	+	-	-	-	-	+	-	-	P	-	+
43 (Z)	+	-		-	*	Rod	LB	+	-	-	-	-	+	-	-	P	-	+
44 (Aa)	+	-		-	*	Rod	LB	+	-	-	-	-	+	-	-	P	-	+
45	+	-		-	*	Rod	B	+	+	+	+	-	+	-	-	Rd	-	+
46 (Bb)	+	-		-	*	Rod	LB	+	-	-	-	-	+	-	-	P	-	+
47 (Cc)	+	-		-	*	Rod	LB	+	-	-	-	-	+	-	-	P	-	+
48 (Dd)	+	-		-	*	Rod	B	+	-	-	-	-	+	-	-	P	-	+
49 (K)	+	-		-	*	Rod	B	+	-	-	-	-	+	-	-	AP	-	+
50	+	-		-	0	Rod	RB	+	-	+	-	-	-	+	-	AcP	+	+

[1] * density of the plate colony was translucent
L Light tan growth
B brown growth
R reddish growth
A alkaline reaction showed in litmus milk
Ac acid reaction showed in litmus milk
P peptonization showed in litmus milk

The cultural characteristics (note Table 2) agreed with many of the respective Pseudomonas species as described by Bergey's 6th. Edition (1948). Those strains selected for further study could be tentatively classified as Pseudomonas aeruginosa or Pseudomonas jaegeri mainly on the fact that these organisms grew well at 37° C. and produced pigments of greenish hue.

Factors Influencing Pigment Production

The 30 organisms were typed as to producing only pyocyanine I, only fluorescence II, or the production of both pigments III. To test for pyocyanine, a modified Gessard's glycerol peptone broth was used. This medium also inhibited any fluorescence. To obtain only the fluorescent pigment, Georgia and Poe's broth was used. Ingredients of both media are presented in the Appendix. Glycerol peptone broth tubes were inoculated from 24-hour slant cultures of glycerol peptone agar. Georgia and Poe's broth tubes were inoculated from 24-hour slant cultures of Georgia and Poe's agar. Incubation temperature was 30° C. Results of this experiment are given in Table 3. It was always necessary to shake the Gessard's glycerol peptone broth tubes vigorously. This shaking changed any of the leuco-pyocyanine to the blue-green pigment.

Effect of Temperature. Tubes of nutrient, glycerol peptone, and Georgia-Poe broth were inoculated with 24-hour cultures from their respective agar slant medium. The temperatures of incubation were 5°, 20°, 37°, and 43° C. Growth at 30° C. had previously been reported. The original pH of the media was not

Table 3. Pigment production in media after 8 days at 30° C. and the type number given to each organism.

Organism	Type[1]	Medium[2]	
		glycerol peptone	Georgia Poe
A	I	#	-
B	II	-	+
C	III	####	+++
D	III	####	++
E	III	#	+
F	III	#####	++
G	III	#####	++
H	III	##	+++
I	II	#	+++
J	II	-	++++
K	III	###	++
L	III	#	++++
M	II	-	+++
N	III	###	+
O	I	#####	-
P	II	-	+
Q	III	##	+++
R	II	-	+++
S	I	###	-
T	II	-	++++
U	II	-	++
V	II	-	+++
W	II	-	++++
X	II	-	++
Y	II	-	+++
Z	II	-	++
Aa	II	-	++++
Bb	II	-	++++
Cc	II	-	++++
Dd	II	-	++++

[1] I the organism produces only pyocyanine
II the organism produces only fluorescence
III the organism produces both pigments

[2] # a fair amount of pyocyanine produced
the maximum amount of pyocyanine produced
+ a fair amount of fluorescence produced
++++ a maximum amount of fluorescence produced
- no pigment

changed. Readings were made after 24 hours, 48 hours, and 5 days. The results of the inoculations are given in Table 4.

At 5° C. no pigment was formed in any of the media; but growth was checked in the nutrient broth tubes. Seven tubes showed definite signs of growth (A, B, G, M, N, P, and Z); 9 tubes showed questionable or slight growth (C, D, F, N, L, U, V, X, and Y); the remainder tubes showed no sign of growth after 2 weeks. 43° C. incubation tubes all showed growth (a few were questionable); pigment occurred in a few tubes of glycerol peptone medium; no pigment was observed in Georgia-Poe's medium.

The results were put on a comparative basis as tabulated below. This was done by adding all the positive (+) signs for each medium at different temperatures. From Table 4 and the tabulation below, 30° C. appears to be the best temperature for the production of the fluorescent pigment; the results for pyocyanine show little difference between 30° and 37° C.

Temperature of incubation	Medium		
	glycerol peptone	Georgia-Poe	nutrient broth
20°	14	46	8
30°	42	70	13
37°	38	45	11

Effect of pH. As noted before, not much work had been done as to the effect of pH on pigment production of Pseudomonas; therefore, the media were made as usual and the original pH was measured on the glass electrode instrument.

Table 4. Effect of temperature on pigment production.

Temperature	Medium	Organism with their type number[1]														
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
		I	II	III	III	III	III	III	III	II	II	III	III	II	III	I
5°	Glyc. pep.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Georgia-Poe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nut. broth	*	*	?*	?*	-	?*	*	?*	-	-	-	?*	*	*	-
20°	Glyc. pep.	-	-	-	2+	-	+	+	-	-	-	+	2+	-	3+	-
	Georgia-Poe	-	+	+	+	-	2+	2+	2+	2+	2+	2+	3+	+	+	-
	Nut. broth	-	-	-	2+	-	-	+	-	-	-	2+	2+	-	+	-
30°	Glyc. pep.	+	-	4+	4+	+	5+	5+	2+	-	-	3+	+	-	3+	5+
	Georgia-Poe	-	+	3+	2+	+	2+	2+	3+	3+	4+	2+	4+	3+	+	-
	Nut. broth	-	-	2+	-	+	2+	-	-	-	-	-	2+	-	3+	-
37°	Glyc. pep.	+	-	2+	3+	-	3+	5+	+	-	-	2+	4+	-	3+	2+
	Georgia-Poe	-	-	3+	2+	-	2+	2+	+	+	2+	2+	3+	2+	2+	-
	Nut. broth	-	-	-	-	-	-	-	-	-	-	-	4+	-	4+	-
43°	Glyc. pep.	-	-	-	-	-	-	3+	-	-	-	+	3+	-	-	-
	Georgia-Poe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nut. broth	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Table 4. (concl.)

Temperature	Medium	Organism with their type number[1]														
		P	Q	R	S	T	U	V	W	X	Y	Z	Aa	Bb	Cc	Dd
		II	III	III	I	II	III	II	II	II	II	II	II	II	II	II
5°	Glyc. pep.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Georgia-Poe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nut. broth	*	-	-	-	-	?*	?*	-	?*	?*	*	-	-	-	-
20°	Glyc. pep.	-	-	-	3+	-	+	-	-	-	-	-	-	-	-	-
	Georgia-Poe	2+	2+	+	+	2+	+	2+	2+	2+	3+	3+	3+	3+	2+	2+
	Nut. broth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30°	Glyc. pep.	-	2+	-	3+	-	-	-	-	-	-	-	-	-	-	-
	Georgia-Poe	-	3+	3+	+	4+	2+	3+	4+	2+	3+	2+	4+	4+	4+	4+
	Nut. broth	-	-	-	3+	-	-	-	-	-	-	-	-	-	-	-
37°	Glyc. pep.	-	+	-	3+	-	4+	-	-	-	-	-	-	-	-	-
	Georgia-Poe	-	2+	2+	+	2+	+	+	2+	2+	2+	2+	+	2+	2+	2+
	Nut. broth	-	+	-	3+	-	4+	-	-	-	-	-	-	-	-	-
43°	Glyc. pep.	-	-	-	2+	-	-	-	-	-	-	-	-	-	-	-
	Georgia-Poe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Nut. broth	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

[1] * growth
?* questionable growth
+ pigment production by *Pseudomonas*
- no pigment production (at 5° C. it means no growth)

The original pH for glycerol peptone was close to pH 6.5; for Georgia-Poe medium, pH 7.0; and for nutrient broth, pH 7.5. Each medium was adjusted to pH 6.5, 7.0, 7.5, 8.0, and 8.5. Glycerol peptone and Georgia-Poe's medium were adjusted with H_2SO_4 or KOH, and nutrient broth with HCl or NaOH depending on whether an acid or an alkali was needed. All reactions were checked on the glass electrode.

Inoculations were performed as in the temperature experiment and the tubes were incubated at 30° C. 24 hour, and 4 and 5 day readings were made. The results of the inoculations are given in Table 5. The comparative results were made as in the temperature experiment and tabulated below.

pH	Medium		
	glycerol peptone	Georgia Poe	nutrient broth
6.5	(original) 40	(original) 53	12
7.0	39	70	14
7.5	39	68	(original) 13
8.0	42	53	16
8.5	43	41	16

Pyocyanine production seems to be favored by alkaline media between pH 8.0 to 8.5; but, the totals were so close that the difference between pH 6.5 and 8.5 is very small. The original pH of Georgia and Poe medium seems to be the best for fluorescence; that is, a pH around neutral or 7.0. The pigment produced in the nutrient broth probably was mainly pyocyanine as the pigment was extracted with chloroform; although no attempts were made to determine the relative amounts of the two pigments produced in this medium.

Table 5. Effect of pH on pigment production.

pH	Medium	Organism with their type number[1]														
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
		I	II	III	III	II	III	III	III	II	II	III	III	II	III	I
6.5	Glyc. pep.	+	-	-	4+	-	5+	5+	2+	+	-	3+	+	-	3+	5+
	Georgia-Poe	-	+	3+	2+	-	+	2+	-	-	-	-	+	-	3+	-
	Nut. broth	-	-	-	+	+	2+	2+	+	3+	4+	2+	2+	3+	+	-
7.0	Glyc. pep.	+	-	4+	4+	-	4+	5+	+	-	-	2+	3+	-	3+	+
	Georgia-Poe	-	+	3+	2+	+	2+	2+	3+	2+	4+	2+	4+	3+	+	-
	Nut. broth	-	-	-	2+	-	-	3+	-	-	-	-	2+	-	2+	-
7.5	Glyc. pep.	2+	-	4+	4+	-	4+	4+	+	-	-	3+	2+	-	3+	2+
	Georgia-Poe	-	+	2+	2+	+	2+	3+	2+	3+	5+	3+	4+	4+	2+	-
	Nut. broth	-	-	-	2+	-	+	2+	-	-	-	-	2+	-	3+	-
8.0	Glyc. pep.	+	-	4+	5+	-	5+	4+	-	-	-	3+	2+	-	4+	2+
	Georgia-Poe	-	-	+	2+	-	2+	2+	+	3+	4+	2+	3+	3+	2+	-
	Nut. broth	-	-	-	3+	-	+	3+	-	-	-	-	2+	-	3+	-
8.5	Glyc. pep.	+	-	4+	5+	-	5+	5+	+	-	-	+	4+	-	4+	2+
	Georgia-Poe	-	-	+	2+	-	1+	+	+	+	3+	2+	2+	4+	+	-
	Nut. broth	-	-	-	3+	-	+	3+	-	-	-	-	2+	-	3+	-

Table 5. (concl.)

pH	Medium	Organism with their type number[1]														
		P	Q	R	S	T	U	V	W	X	Y	Z	Aa	Bb	Cc	Dd
		II	III	II	I	II	III	II	II	II	II	II	II	II	II	II
	Glyc. pep.	-	2+	-	3+	-	-	-	-	-	-	-	-	-	-	-
6.5	Georgia-Poe	+	+	2+	-	3+	2+	3+	3+	2+	2+	+	3+	3+	2+	+
	Nut. broth	-	-	-	3+	-	-	-	-	-	-	-	-	-	-	-
	Glyc. pep.	-	+	-	5+	-	4+	-	-	-	-	-	-	-	-	-
7.0	Georgia-Poe	+	3+	3+	-	4+	2+	3+	4+	2+	3+	2+	4+	4+	4+	4+
	Nut. broth	-	-	-	4+	-	-	-	-	-	-	-	-	-	-	-
	Glyc. pep.	-	+	-	5+	-	4+	-	-	-	-	-	-	-	-	-
7.5	Georgia-Poe	+	2+	4+	-	3+	2+	3+	3+	3+	3+	2+	2+	2+	3+	2+
	Nut. broth	-	-	-	3+	-	-	-	-	-	-	-	-	-	-	-
	Glyc. pep.	-	+	-	5+	-	4+	-	-	-	-	-	-	-	-	-
8.0	Georgia-Poe	+	+	3+	+	3+	+	2+	2+	2+	2+	2+	2+	2+	2+	2+
	Nut. broth	-	-	-	4+	-	-	-	-	-	-	-	-	-	-	-
	Glyc. pep.	-	2+	+	5+	-	4+	-	-	-	-	-	-	-	-	-
8.5	Georgia-Poe	-	+	+	+	+	2+	2+	3+	2+	2+	2+	2+	2+	+	2+
	Nut. broth	-	-	-	5+	-	-	-	-	-	-	-	-	-	-	-

[1] + pigment production by *Pseudomonas*
- no pigment production

Carbon Sources Necessary for Pigment Production

In testing simple carbon sources for their ability to stimulate the production of pigments by Pseudomonas, the following experiment was made.

The basic medium without any carbon source was Ashby's salt solution (ingredients in Appendix), NH_4SO_4, and distilled water. One percent of dextrose, sucrose, lactose, maltose, mannitol, xylose, glycerol, sodium acetate, and asparagin were the carbon compounds used. The pH of these 1 percent "broth tubes" ranged from pH 7.0 to 7.9. The tubes were innoculated from 24-hour nutrient agar slant cultures. Incubation temperature was 30° C.

Results of this experiment are given in Table 6. On the tenth day 1 ml of chloroform was added to each tube and shaken to detect the presence of pyocyanine. Sodium acetate broth showed the strongest pyocyanine reaction by the chloroform test while slight amounts were observed in dextrose, mannitol, and asparagin broth tubes. The yellow-green fluorescent pigment appeared in the asparagine medium in the greatest amount. Only slight pigment was produced in any of the media except the sodium acetate and asparagine medium, and it should be noted also that no pigment was visible in the xylose, lactose, maltose and sucrose broth tubes.

Table 6. Pigment production by *Pseudomonas* species in various carbon sources.

Carbon compound	Organism with their type number[1]														
	A I	B II	C III	D III	E II	F III	G III	H III	I II	J II	K III	L III	M II	N III	O I
Dextrose	-	-	±	-	-	-	-	-	-	-	-	-	±	-	-
Glycerol	-	±	-	-	-	-	-	-	-	-	±	±	-	±	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	±	±	-	±	-	±	±	±	±	±	±	±	±
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium acetate	1+	-	2+	1+	-	3+	±	1+	1+	+	2+	2+	3+	3+	±
Asparagine	1+	1+	4+	3+	1+	4+	3+	3+	3+	3+	3+	3+	4+	3+	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 6. (concl.)

Carbon compound	Organism with their type number[1]														
	P	Q	R	S	T	U	V	W	X	Y	Z	Aa	Bb	Cc	Dd
	II	III	III	I	II	III	II	II	II	II	II	II	II	II	II
Dextrose	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	*	-	-	-	-	-	-	-	-	-	*	-
Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	*	-	*	-	*	-	-	*	*	-	-	*	*	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sodium acetate	*	-	-	3+	-	3+	-	-	-	*	-	+	*	-	-
Asparagine	2+	2+	1+	3+	3+	3+	3+	3+	4+	3+	3+	3+	3+	3+	3+
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1] + pigment production by *Pseudomonas*
- no pigment production
* slight pigment production

Nitrogen Sources Necessary for Pigment Production

This experiment was carried out to find the effect of simple nitrogen sources on the production of pigment. The twelve compounds tested and their formulas are listed below. The basic medium was Ashby's Salts solution, 1 percent glycerol, and 0.1 percent of the nitrogenous compound being studied.

para-amino benzoic acid --- $NH_2C_6H_4COOH$
l-lysine ------------------ $NH_2(CH_2)_4CH(NH_2)COOH$
l-histidine --------------- $C_3H_3N_2CH_2CH(NH_2)COOH$
l-arginine ---------------- $NH_2C(:NH)NH(CH_2)_3CH(NH_2)COOH$
nicotinic acid ------------ $C_5H_4(COOH)N$
l-tyrosine ---------------- $HOC_6H_4CH_2CH(NH_2)COOH$
l-cysteine ---------------- $SCH_2CH(NH_2)COOH$
dl-ornithine -------------- $CH_2(NH_2)(CH)CH(NH_2)COOH$
l-aspartic acid ----------- $COOHCH_2CH(NH_2)COOH$
l-tryptophane ------------- $C_6H_4NHCH:CH_2CH(NH_2)COOH$
l-asparagine -------------- $NH_2COCH_2CH(NH_2)COOH$
riboflavin ---------------- $C_{17}H_{20}N_4O_6$

The solutions all tested pH 7 or above. The tubes were inoculated from 24-hour nutrient agar slant cultures. Incubation temperature was 30° C. Results of this experiment are given in Table 7.

Presence of pyocyanine was tested by shaking the tube with 1 ml of chloroform on the 6th day of incubation. The media containing the following compounds gave a positive test for pyocyanine, and they are listed in order of decreasing stimulating ability: Tyrosine, histidine, asparagine, aspartic acid, arginine, tryptophane, lysine, ornithine, cystine, and riboflavin. No pigment was produced in the presence of either para-amino benzoic acid or nicotinic acid.

Table 7. Pigment production by <u>Pseudomonas</u> species in various nitrogen sources.

Nitrogenous compound	Organism with their type number[1]														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
	I	II	III	III	II	III	III	III	II	II	III	III	II	III	I
Cystine	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-
Histidine	2+	+	2+	2+	-	2+	2+	3+	2+	2+	1+	3+	2+	3+	+
Asparagine	2+	1+	4+	2+	+	3+	1+	3+	2+	2+	2+	3+	1+	2+	-
Ornithine	+	2+	+	-	-	-	-	1+	-	1+	-	+	-	1+	-
p-amino benzoic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspartic acid	2+	2+	2+	1+	+	1+	1+	3+	3+	2+	2+	3+	2+	2+	+
Arginine	3+	2+	3+	+	-	3+	2+	3+	2+	2+	2+	3+	1+	3+	-
Lysine	3+	2+	-	-	-	-	-	+	-	-	+	1+	-	2+	-
Tryptophane	+	2+	1+	+	-	1+	+	+	+	1+	2+	1+	+	2+	+
Nicotinic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine	3+	+	2+	+	1+	3+	3+	2+	2+	2+	3+	3+	2+	3+	-
Riboflavin	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-

Table 7. (concl.)

Nitrogenous compound	Organism with their type number[1]														
	P II	Q III	R III	S I	T II	U III	V II	W II	X II	Y II	Z II	Aa III	Bb II	Cc II	Dd II
Cystine	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
Histidine	-	2+	-	3+	-	2+	2+	+	2+	2+	1+	2+	3+	2+	2+
Asparagine	1+	1+	-	3+	-	2+	2+	1+	3+	2+	2+	2+	2+	3+	2+
Ornithine	-	+	-	3+	2+	+	-	+	+	+	2+	1+	-	+	1+
p-amino benzoic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspartic acid	+	+	-	1+	+	1+	1+	2+	2+	2+	1+	1+	1+	1+	+
Arginine	-	1+	+	2+	-	+	2+	2+	1+	3+	2+	3+	2+	3+	3+
Lysine	-	2+	+	+	+	-	-	2+	-	+	-	+	+	+	+
Tryptophane	-	-	-	1+	2+	+	+	+	1+	1+	1+	1+	+	1+	+
Nicotinic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine	+	+	1+	3+	+	+	+	+	+	2+	-	1+	+	1+	1+
Riboflavin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1] + pigment production by *Pseudomonas*
\- no pigment production

DISCUSSION

Attempts to relate the source of the organism to the type of pigment produced did not yield any definite correlations. That is, the Pseudomonas cultures which produced only pyocyanine (Type I) came from urine, a slanted separatory pit, and from a stock culture of unknown origin; the organisms producing only fluorescence (Type II) came from cream, a stock culture of unknown origin, spoiled eggs, an oil sample, sedimentation pond, sulfur springs, and oleomargarine; and the Pseudomonas organisms producing both pigments were isolated from well water, spring water, salt water in deep oil well, ear abscess, spoiled eggs, and oil sample. The only pigment produced by the Pseudomonas organisms from oleomargarine samples in this study was the fluorescent type of pigment.

In studying the effect of temperature on the Pseudomonas organisms it was found that at 5° C. no pigment was produced; at 20° C. both pigments were noticeable, but more fluorescent pigment was obtained that pyocyanine; at 30° C. the best fluorescence was produced; at 37° C. the pyocyanine about equaled the pyocyanine produced at 30° C.; and at 43° C. no fluorescent pigment was produced and only noticeable amounts of pyocyanine were obtained. Room temperature or 30° C. was used by Jordan (1899) and Burton, et al. (1947); and 37° C. was the temperature employed by Emry and Roberts (1914), Sandiford (1937), and Seleen and Stark (1943) for the production of pyocyanine. Thus, the results, showing that either 30° or 37° C. produced the maximum pyocyanine pigment, substantiate previous work as to the best temperature for the production of pyocyanine. It was also observed that 30° C. was the best temperature for fluorescence; this result agreed with past workers.

The effect of reaction of the medium on the pigment production by Pseudomonas was more noticeable on the fluorescent pigment than on the pyocyanine. That is, the range of pH from 6.5 to 8.5 did not noticeably change the amount of pyocyanine produced. Burton, et al. (1947) thought pH 7.5 to 8.0 (or 8.35) was better pyocyanine production. The data presented in Table 5 indicates that pyocyanine production is not extremely sensitive to change in reaction. The reactions reported by other workers as optimum fall within the range where good pyocyanine formation was obtained.

Although fluorescence was produced by the Pseudomonas organisms in the pH range of 6.5 to 8.5, the best production of fluorescent pigment was obtained around pH 7.0 to 7.5, which agrees with Georgia and Poe's result of pH 6.8 to 7.3 (1932).

The data obtained from the experiment concerning carbon sources necessary for the stimulation of pigment formation show that sodium acetate and asparagine (added to a simple salts medium with NH_4SO_4) stimulate good fluorescent and pyocyanine pigment formation. There was only slight pigment produced from mannitol, glycerol, or dextrose. According to Young (1947) the acid formed the sugar breakdown could prevent the formation of any pigment; but the 30 organisms tested did not form acid from mannitol, lactose, maltose, and sucrose.

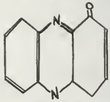
The Pseudomonas organisms which produced only fluorescent pigment (Type II) showed almost 5 times more pigment in the presence of asparagine than in sodium acetate media; there was not much difference in the amount of pyocyanine pigment (Type I) produced in the two media. As might be expected, Type III, which formed both pigments, gave a greater total pigment production in the asparagine medium.

Di Maggia (1946) found that glucose or mannitol could serve as the sole source of carbon for pyocyanine production, while maltose, sucrose, or lactose could not. If faint or slight pigment formation is considered as positive pigment production, the data presented in Table 6 compares favorably with Di Maggia results.

Lepierre (1895) stated that he thought creatinine or xanthine had to be present for the production of fluorescence. The results obtained on fluorescent pigment production by Pseudomonas agree with the data recorded by the majority of previous workers; for, Jordan in 1899 noticed that asparagine as the sole source of carbon produced excellent fluorescent pigmentation. Georgia and Poe (1931) included asparagine as sole source of carbon and nitrogen in their medium and Turfitt (1936) could find no compound superior to asparagine for the production of the fluorescent pigment.

The study made with the nitrogenous compounds revealed that many of them could stimulate the Pseudomonas to produce one or both of the pigments. The data in Table 7 show asparagine to be the best single addition to the simple Ashby's medium for the production of fluorescent pigment. Arginine, aspartic acid, and histidine media showed good fluorescence; tyrosine and tryptophane showed fair fluorescent pigment. Lysine and ornithine gave only slight pigmentation.

The greatest amount of pyocyanine produced was in the tyrosine medium (Table 7). Pyocyanine has the structural formula of



, Oppenheimer and Stern (1939). The cyclic structure of the tyrosine molecule probably leads to easy synthesis of the pigment.

The present study has shown a number of factors which influence the production of pyocyanine and fluorescent pigments by Pseudomonas. As noted before, the importance placed on pigment formation in the present classification, according to Bergey (1948), indicates that further knowledge of this phenomenon is desirable. Particular attention should be given to the variability of strains grown on different media as well as the relative amounts of the two pigments produced by different strains of the organisms, at the various stages of their growth cycle. Such information would probably facilitate the identification and classification of members of the genus Pseudomonas.

SUMMARY

1. Little correlation could be noted between the source of the organisms and the type of pigments produced.

2. Pseudomonas organisms were found which produced only pyocyanine or the fluorescent pigment; some of the strains produced both of the pigments.

3. It was found that fluorescence production by Pseudomonas cultures was favored by a medium of pH near neutrality (pH 7.0 to 7.5) and an incubation temperature of 30° C.

4. Favorable conditions for pyocyanine production were observed over a wider range of incubation temperature and reaction of the media than existed for the fluorescent pigment formation. A slightly alkaline reaction and temperatures between 30° and 37° C. were best for the production of pyocyanine.

5. Asparagine served best of all substances tested as the sole source of carbon and nitrogen for the production of the fluorescent pigment by Pseudomonas.

6. Of the following compounds, cystine, histidine, asparagine, ornithine, para-amino benzoic acid, aspartic acid, arginine, lysine, tryptophane, nicotinic acid, tyrosin, and riboflavin, tyrosine proved to be the best nitrogen source for the production of pyocyanine by Pseudomonas.

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APPENDIX

Culture Media

Gessard's Glycerol Peptone Broth

This medium was modified by the addition of salts in the concentrations recommended by Burton, et al. (1948).

Glycerol	50 ml. (5%)
Bacto-Peptone	20 grams (2%)
K_2HPO_4	0.4 grams (0.04%)
$MgSO_4 \cdot 7\ H_2O$	20 grams (2%)
Fe_2SO_4	0.01 grams (0.001%)
Distilled water	950 ml.

It should be noted that if agar is to be added to this broth medium for a solid medium, 3 percent agar is necessary.

Georgia and Poe's Broth

This medium was not modified and the asparagine was the sole source of carbon and nitrogen as suggested and used by Georgia and Poe (1931).

Asparagine	3.0 grams (0.3%)
K_2HPO_4	0.5 grams (0.05%)
$MgSO_4 \cdot 7H_2O$	0.5 grams (0.05%)
Distilled water	100.0 ml.

To make this broth into a solid medium only 2 percent agar is needed.

Ashby's Salt Solution

This salt solution was modification of Ashby's original salts medium for the growth of <u>Azotobacter</u> used by Farris and Gainey (1944). Each ingredient should be dissolved in water before the next is added.

K_2HPO_4	1.8 grams
KH_2PO_4	0.7 gram
NaCl	0.2 gram
$MgSO_4 \cdot 7H_2O$	0.2 gram
$CaCl_2$	0.02 gram
$CaCO_3$	0.02 gram
$FeCl_3$	Trace
MoO_3	3.0 ppm
Distilled water	1000.0 ml.