HELLINGER, E. (1951). *J. gen. Microbiol.* 5, 633-639.

Requirements for Pyocyanine Production by *Pseudomonas aeruginosa* (Schroeter) Migula

By ESTHER HELLINGER

Daniel Sieff Research Institute, Rehovot, Israel

SUMMARY: Substances found to be essential for pyocyanine production by *Pseudomonas aeruginosa* were Mg, PO₄, SO₄ and NH₄ ions, together with a carbon source, preferably glucose, glycerol or ethanol. Ca, Na, K, Fe ions were not essential, nor was asparagine. Pyocyanine was formed in nutrient broth prepared with distilled water; the addition of chalk improved pigment production. Reliable and good yields were regularly obtained on an ordinary broth medium containing glucose (0.2%) and chalk.

Recommended media for stimulating pyocyanine production were tested. Turfitt's medium gave more reliable pyocyanine formation when the NH₄NO₃ was replaced by NH₄Cl and chalk was added. Increasing the MgSO₄ · 7H₂O content of this medium from 0.025% to 0.2% inhibited pyocyanine production.

Various amino-acids were tested. Glycine, L-tyrosine, DL-alanine, L-leucine, when added separately or in various combinations to a salt mixture+glucose, glycerol or ethanol permitted the production of pyocyanine. A mixture of glycine, L-leucine and DL-alanine (recommended by Burton, Campbell & Eagles, 1947) exerted a stimulating effect on pyocyanine production far superior to that obtained on any other medium tested.

The highest yield reached was 23.1 mg. pyocyanine/100 ml. of a medium consisting of: 0.4% DL-alanine; 0.8% L-leucine; 0.2% MgSO₄ · 7H₂O; 0.05% K₂HPO₄; 0.001% FeSO₄ · 7H₂O; 1% glycerol and chalk.

There is agreement in the literature that sulphate, phosphate and magnesium are essential for pyocyanine production by *Pseudomonas aeruginosa* (*Ps. pyocyanea*). Jamieson (1942) reported that, by chance using tap water of lake origin for his media, the majority of certain organisms he had previously considered to be *Achromobacter* spp. produced blue-green pigment; this would place them as *Pseudomonas* spp. Even media such as proteose-peptone when prepared with distilled water, failed to yield pigment, but with tap water did so. Jamieson found that best pigmentation was produced on Sullivan's medium: asparagine 1%, magnesium sulphate 0.2%; dipotassium phosphate 0.1%; distilled water. He thought this was due primarily to asparagine being a source of nitrogen more adequate for pigment formation than peptone. Burton, Campbell & Eagles (1948) found that K and Fe ions in addition to SO₄, PO₄, and Mg ions were necessary. They stated that Na, NH₄, Mn, Zn, AsO₄ or Cu could not be substituted in their defined medium (containing also 1% glycerol, 0.6% glycine, 0.6% leucine) for the above ions, and that NH₄, Mn, Zn, AsO₄ and Cu ions inhibited pyocyanine production. On the other hand Liot (1923) found that ammonium ion (as an organic salt) was necessary for pyocyanine formation. Young (1947) found that no pigment was produced in media containing over 1% glucose, the inhibition being attributed to the attainment of low pH values. Turfitt (1937) recommended the

following medium for good pigmentation: NH_4NO_3 , 0.1 %; K_2HPO_4 , 0.025 %; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025 %; ethanol, 0.1 %; distilled water.

A strain of *Ps. aeruginosa* isolated by the author from the River Yarkon, Israel, showed no pigment production under anaerobic conditions, but produced good pigmentation when grown aerobically on nutrient broth or nutrient agar prepared with distilled water. Because of this difference from Jamieson's findings, the requirements of our strain of *Ps. aeruginosa* for pyocyanine production were examined.

METHODS AND MEDIA

Method of estimating pyocyanine. The pyocyanine was extracted from culture media and the colour intensity of extracts was compared with those of known concentrations of pure pyocyanine in chloroform with a photoelectric colorimeter (Lumetron Electrophotometer; scale standardized at 100 with pure chloroform, colour filter 620, light intensity +10). The logarithms of the Lumetron readings when plotted against pyocyanine concentration gave a straight line relationship.

Growth and extraction of pyocyanine. Twenty ml. quantities of medium in 50 ml. round, flat-bottom flasks were sterilized at 110° for 45 min. The pH after sterilizing was determined on separate 5 ml. quantities. Each flask was inoculated with one loopful of a young active culture and incubated at 37° . In the first experiments, one set of flasks was examined for pyocyanine on the 7th or 8th day, and a further set after a longer incubation period. In later experiments pyocyanine extractions were carried out on the 4th and 7th/8th day. In practically all cases pigment production decreased on prolonging the incubation beyond 4–5 days.

Table 1. *The composition of various media used to ascertain requirements of Ps. aeruginosa for pyocyanine production*

Constituent	Concentration (%)	Medium number															
		1	2	3	4	5	6	7	8	9	10	11	12	16	17		
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.02	—	+	+	+	—	+	+	+	+	+	—	+	—	+		
CaCl_2	0.01	+	—	+	+	+	+	+	+	+	+	—	—	—	—		
NaCl	0.1	+	+	—	+	+	+	+	+	+	+	—	—	—	—		
KH_2PO_4	0.1	+	+	+	—	+	+	+	—	+	+	+	+	—	—		
K_2HPO_4	0.1	—	—	—	—	—	—	—	—	—	—	+	+	+	+		
MgCl_2	0.02	—	—	—	—	+	—	—	—	—	—	—	—	—	—		
Na_3PO_4	0.2	—	—	—	—	—	—	—	+	—	—	—	—	—	—		
NaHCO_3	0.1	—	—	—	+	—	—	—	—	—	—	—	—	—	—		
FeCl_3	0.001	—	+	+	+	+	+	—	+	+	+	—	—	—	—		
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.001	+	—	—	—	—	—	—	—	—	—	—	—	—	—		
KNO_3	0.1	—	—	—	+	—	+	—	—	—	—	—	—	—	—		
NH_4NO_3	0.1	+	+	+	+	+	—	+	+	+	+	—	—	—	+		
Glycerol	2.0	+	+	+	+	+	+	+	+	—	+	+	+	+	+		
Asparagine	0.05	+	+	+	+	+	+	+	+	+	+	—	+	+	+		

+ = present in medium; — = absent from medium.

The individual cultures were extracted with 4–5 successive portions (5 ml.) of chloroform. Generally the chloroform extract was turbid; it was easily cleared by a brief centrifugation. To ensure perfect clarity for colorimetric measurements it was filtered through a filter paper wetted with chloroform, and the volume made up the original volume of the culture. When too concentrated the solution was suitably diluted.

Media. The composition of fourteen media is given in Table 1. In addition the two media recommended by Turfitt (1937) for stimulating the green fluorescent pigment of *Ps. fluorescens* (0.05 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.05 % K_2HPO_4 ; 0.3 % asparagine) and pyocyanine production by *Ps. pyocyanea* (0.025 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.025 % K_2HPO_4 ; 0.1 % NH_4NO_3 ; 0.1 % ethanol) were tested (media 18 and 19 respectively). Other media tested were: standard broth containing 0.3 % Difco meat extract + 0.5 % Bacto Proteose peptone made up with distilled water (No. 18); the same + 0.2 % glucose (No. 14); standard broth + 0.2 % glucose + chalk (No. 15).

RESULTS

In the first series of experiments (Table 2) growth occurred in all media except that containing no phosphate (No. 4). The defined media Nos. 1 to 10, when lacking Mg, SO_4 , PO_4 , NH_4 or glycerol, failed to produce pyocyanine. Ca,

Table 2. *Effect of composition of medium on growth, pH and pyocyanine production by Ps. aeruginosa grown at 37°*

Medium		pH values				Pyocyanine mg./100 ml. medium	
		Initial Series		Final Series			
		No.	Growth	1	2	1	2
1	moderate	6.4	6.4	7.3	8.1	None	None
2	good	6.3	6.4	6.1	6.4	2.45	0.35
3	good	6.4	6.4	6.2	6.3	2.24	0.28
4	none	6.7	6.4	6.7	7.6	None	None
5	good	6.3	6.3	7.5	7.0	Traces	None
6	good	6.4	6.5	6.8	7.5	None	None
7	good	6.3	6.4	6.3	6.2	1.87	0.1
8	good	7.8	6.9	7.4	6.8	1.79	0.1
9	good	6.3	6.9	6.5	7.3	None	None
10	good	6.3	6.4	6.1	5.7	1.72	0.15
11	moderate	6.6	—	6.5	—	None	.
12	moderate	6.5	—	4.9	—	None	.
13	very good	6.5	6.9	8.8	8.7	0.4	{ 0.18
					8.7		{ 1.37+
14	very good	6.4	6.7	7.3	7.4	1.62	0.29
15	very good	7.4	7.4	8.6	8.7	5.62	{ 12.63
					8.7		{ 9.38+
16	fair	7.7	—	6.5	—	None	.
17	good	7.5	—	4.9	—	0.15	.
18	good	7.7	7.0	7.8	8.5	None	None
19	good	7.8	7.0	7.2	4.2	1.42	None

* = Series 1 tested on 7th day; ** = Series 2 tested on 8th day; + = chalk added to medium.

Na, K, Fe ions and asparagine were not essential for pigment formation. Substitution of KNO_3 for NH_4NO_3 (No. 6) did not stimulate pyocyanine formation, indicating that the ammonium ion was required and not nitrate ion. In medium No. 12 (a defined medium containing Mg, SO_4 , PO_4 , asparagine and glycerol, but no NH_4 ion) pigment was produced, confirming the need for ammonium nitrogen. Turfitt's medium for *Ps. fluorescens* lacking in glycerol and ammonium salts but containing the essential ions Mg, SO_4 , PO_4 , with asparagine as nitrogen source (No. 18), also did not yield pyocyanine. In Turfitt's medium for *Ps. aeruginosa* containing Mg, SO_4 , PO_4 , and NH_4 ions, and ethanol instead of glycerol (No. 19), pyocyanine was formed but only after four days. Medium No. 17 which contained the substances which appeared to be essential for pigment formation (Mg, SO_4 , PO_4 , NH_4 ions and glycerol) gave at first a faint greenish pigmentation, which turned pink and became reddish by the fourth day, presumably due to the low pH reached; the acid salt of pyocyanine is port-wine coloured. On making alkaline a little pyocyanine was extractable from these cultures.

The best production of pyocyanine occurred on standard broth containing glucose (0.2%) + chalk (Medium 15): yields as high as 12.6 mg. pyocyanine/100 ml. medium were obtained. On unsupplemented standard broth pigmentation was erratic; some pyocyanine was formed when 0.2% glucose (without chalk) was added.

Ammonia, which appeared to be essential for pigment formation, is liberated by the organism from the peptone in standard broth. Turfitt's relatively simple medium (No. 19) contains NH_4NO_3 , but this medium was unreliable for pyocyanine production; fluorescein, which is also produced, was at times the sole pigment present. Trials were made substituting NH_4Cl for the NH_4NO_3 in this medium and doubling the ethanol content (i.e. to 0.2%); the pH of all media was adjusted to 7.4. In one medium of this series, ethanol was replaced by glycerol, and asparagine used instead of ammonium salt; growth was good, the pH of the medium rose to 8, fluorescein but no pyocyanine was formed. Pyocyanine formation was erratic on Turfitt's medium both with and without chalk. With NH_4Cl growth was fair to moderate, the pH of the culture medium always fell to 4.4–5.2, but no pyocyanine was formed; with the addition of chalk, the pH rose to 8.1 and pyocyanine was regularly formed although in variable amounts, the highest being 3.3 mg./100ml. medium. In general NH_4Cl with chalk was more reliable for pyocyanine formation and gave higher yields than NH_4NO_3 . These chemically defined media were not as good for pyocyanine production as standard broth containing glucose and chalk.

L-Tyrosine, L-tryptophan, L-cystine and glycine were added separately and in various combinations to 0.2% glucose, 2% glycerol or 0.2% ethanol; the salts present were: 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.025% K_2HPO_4 ; 1% NH_4NO_3 or NH_4Cl . The media were adjusted to pH 7.4 and cultures incubated for four days at 37°. All cultures showed good growth, the final pH varying from 7.6 to 8.7. Pyocyanine was produced only in the media containing tyrosine or glycine. Yields from glycine media were distinctly better than from tyrosine

media; the presence of chalk increased these yields, although the effect was apparently not due to control of pH. There were no striking differences between media containing either glucose, ethanol or glycerol. A comparison of pyocyanine yields from media containing 0.2 % glycine + ammonium salts, with those from media lacking glycine, gave results somewhat in favour of the latter.

The two media of Burton, Eagles & Campbell (1947) and recommended by them for maximum production of pyocyanine were compared with the standard broth containing glucose and chalk. One of these media contained

Table 3. *Effect on pyocyanine production of L-leucine, with glycine or DL-alanine in a glycerol + salt mixture,* as compared with standard broth + glucose*

Final pH Pyocyanine (mg./ 100 ml. medium)	0.4 % glycine + 0.8 % L-leucine		0.4 % DL-alanine + 0.8 % L-leucine		Standard broth + 0.2 % glucose	
	—CaCO ₃	+CaCO ₃	—CaCO ₃	+CaCO ₃	—CaCO ₃	+CaCO ₃
	8.0	7.7	8.1	7.9	8.6	8.7
	14.5	19.5	19.5	23.1	7.3	9.15

* 1 % glycerol; 0.04 % K₂HPO₄; 0.2 % MgSO₄ · 7H₂O; 0.001 % FeSO₄ · 7H₂O.

0.4 % glycine, the second 0.4 % DL-alanine; both were at pH 7.4 and contained also 0.8 % L-leucine, 1 % glycerol and a salt mixture (MgSO₄ · 7H₂O, 0.2 %; K₂HPO₄, 0.04 %; FeSO₄ · 7H₂O, 0.001 %). Pyocyanine extractions were carried out after four days at 37°. The results (Table 3) with both media of Burton *et al.* were striking; they gave yields of pyocyanine far superior to the supplemented standard broth. The addition of chalk to the media of Burton *et al.* tended further to increase the pyocyanine yield.

Table 4. *Pyocyanine production on various media*

(The growth was very good in all media.)

No.	Medium	CaCO ₃	Final pH value	Pyocyanine (mg./100 ml. medium)
1	MgSO ₄ · 7H ₂ O 0.2 %	—	4.6	(trace)
	K ₂ HPO ₄ 0.04 %			
	NH ₄ Cl 0.1 %			
	Ethanol 0.2 %			
2	MgSO ₄ · 7H ₂ O 0.2 %	—	8.3	6.85
	K ₂ HPO ₄ 0.04 %			
	Glycine 0.4 %			
	L-Leucine 0.8 %			
	Ethanol 0.2 %			
3	As No. 2 +	—	8.6	5.46
	FeSO ₄ · 7H ₂ O (0.001 %)	+	8.4	5.73
4	As No. 3 but with glycerol (1 %) re- placing ethanol	—	6.9	10.44
		+	6.9	10.44
5	Standard broth +	—	8.6	3.58
	glucose (0.2 %)	+	8.4	7.19

In further tests with the media of Burton *et al.* it was found (Table 4) (a) that although comparatively good yields of pyocyanine were obtained with ethanol, glycerol was better; (b) ferrous sulphate did not appear to exert an influence on pyocyanine formation; (c) increasing the concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ from 0.025 % to 0.2 % inhibited pyocyanine formation in the given medium.

DISCUSSION

The results obtained with our strain of *Ps. aeruginosa* do not agree with Jamieson's (1942) finding that tap water was essential for pyocyanine formation on nutrient broth; pyocyanine was obtained in nutrient broth cultures prepared with distilled water, but the addition of calcium carbonate did enhance its formation. Wrede & Strack (1924) found that media containing asparagine + glucose as carbon source gave good pyocyanine yields, and recognized the importance of maintaining the organism in a medium containing peptone (1 %) to preserve its ability to give good yields of pigment. In the present experiments asparagine was not essential for pyocyanine formation; when it was added to a medium containing glycerol, MgSO_4 and K_2HPO_4 no pyocyanine was formed, yet on replacing asparagine with an ammonium salt pyocyanine was formed. Peptone is favourable for the maintenance of the organism's ability to give good yields, but a peptone medium did not ensure high pyocyanine yields unless the medium also contained glucose and chalk. We obtained pyocyanine in nutrient media + 2 % glucose, whereas Young (1947) found no pigment in media containing over 1 % glucose.

The present findings do not agree with those of Burton *et al.* (1948) that K and Fe are essential ions for pyocyanine formation. The media used without added iron contained less than 0.02 p.p.m. iron and yet pyocyanine was formed.

Ammonium ion appeared essential for pigment formation and we do not agree with Liot (1923) who claimed that pyocyanine is formed only in the presence of an organic ammonium salt. Ammonium salts as sole nitrogen source in media containing 0.025 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025 % K_2HPO_4 , with glucose, glycerol or ethanol gave somewhat better yields than when glycine was added to the medium, whereas in media containing 0.2 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.04 % K_2HPO_4 pyocyanine production was inhibited. Turfitt's medium for pyocyanine formation was found to be unreliable, but by replacing ammonium nitrate with ammonium chloride and chalk the pigment was regularly formed.

The media devised by Burton *et al.* (1948) were superior in supporting pyocyanine production to all the other media tested with a particular strain of *Ps. aeruginosa* in the present work.

I wish to express my gratitude to my laboratory assistant Mr Haim Gruener for his valuable help. I also thank my colleague Dr Frieda Goldschmidt for kindly testing the media for contaminating iron.

REFERENCES

- BURTON, M. O., EAGLES, B. A. & CAMPBELL, J. J. R. (1947). The amino-acid requirements for pyocyanine production. *Canad. J. Res., C* 25, 121.
- BURTON, M. O., CAMPBELL, J. J. R. & EAGLES, B. A. (1948). The mineral requirements for pyocyanine production. *Canad. J. Res., C* 26, 15.
- JAMIESON, M. C. (1942). Requisites for recognition of the blue-green *Pseudomonas*. *Sci. Agric.* 22, 401.
- LIOT, A. (1923). Culture du bacille pyocyanique sur milieux chimiquement définis. *Ann. Inst. Pasteur*, 37, 234.
- TURFITT, G. E. (1937). Bacteriological and biochemical relationships in the *pyocyanus-fluorescens* group. II. Investigations on the green fluorescent pigment. *Biochem. J.* 31, 212.
- WREDE, F. & STRACK, E. (1924). Über das Pyocyanin, den blauen Farbstoff des *Bacillus Pyocyaneus*. I. *Hoppe-Seyl. Z.* 140, 1.
- YOUNG, C. (1947). Pigment production and antibiotic activity in cultures of *Pseudomonas aeruginosa*. *J. Bact.* 54, 109.

(Received 18 July 1950)