192. PRODUCTION OF PHOENICINE ON SYNTHETIC MEDIA

1. *PENICILLIUM PHOENICEUM* VAN BEYMA
2. *PENICILLIUM RUBRUM* GRASBERGER-STOLL

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1. *PENICILLIUM PHOENICEUM* VAN BEYMA

PENICILLIUM PHOENICEUM, representative of Thom's classification of *Monoverticillata ramigena*, produces a red pigment, phenicine, which, being water-soluble, diffuses almost entirely into the medium. Its catalytic function in the respiration of living cells and certain of its properties were first described by Friedheim [1933]. The constitution, viz. 2:2'-dihydroxy-4:4'-ditoluquinone, was established by Posternak [1938].

Friedheim states that phenicine is produced by old cultures of the organism, grown on beerwort. On this medium the pigment does not appear until after the formation of the green conidia, but a further statement establishes the fact that phenicine is formed at the outset, when the mould is cultured on media rich in carbohydrates. *P. pheniceum*, which does not appear to have been previously grown on sugar media, was therefore inoculated on synthetic media containing 5% glucose, of which three were chosen, viz. acid Czapek-Dox, Raulin-Thom and Raulin-Dierckx, a neutral medium.

EXPERIMENTAL

A culture of this penicillium, obtained from the Centraalbureau voor Schimmelcultures, showed conidia formation in 3-4 days and pigment production in 4-5 days.

Nine litres of each of the three media were made up as follows:

Acid Czapek-Dox, pH 4.2. Glucose, 50 g.; NaNO_3 , 2.0 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; KH_2PO_4 , 1.0 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g.; distilled water to 1000 ml.

Raulin-Thom, pH 3.9. Glucose, 75 g.; tartaric acid, 4.0 g.; NH_4 tartrate, 4.0 g.; $(\text{NH}_4)_2\text{HPO}_4$, 0.6 g.; MgCO_3 , 0.4 g.; K_2CO_3 , 0.6 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.25 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.; distilled water to 1500 ml.

Raulin-Dierckx, pH 7.0, recommended for the extensive and varying pigment production which it induces among certain penicillia, was employed in a slightly modified form, glucose being used instead of the sucrose, specified by Biourge. Glucose, 46.6 g.; $(\text{NH}_4)_2\text{HPO}_4$, 0.4 g.; NH_4NO_3 , 2.66 g.; K_2CO_3 , 0.4 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.16 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g.; 66.7 ml. of a solution of 0.4 g. MgCO_3 and 0.71 g. tartaric acid in 100 ml. distilled water added and the volume adjusted to 1000 ml.

The media were distributed in 100 ml. amounts in 20 oz. flat bottles, which were plugged with cottonwool, sterilized by steaming on each of three consecutive days and inoculated with a spore suspension of *P. phoeniceum*, grown for 6–7 days on malt-agar slopes. The bottles were incubated in the dark at 20° for 18 days. Mycelium formation became apparent in 2–3 days, the entire surface of the liquid being overgrown in 5–6 days. At this stage, pigment appeared and was complete throughout by the 8th day, there being no formation of green conidia.

By the 10th day, the mycelium in all three cases had taken on a corrugated appearance. On the Czapek-Dox and Raulin-Thom media, the mycelial felts, upper and reverse sides, were white, while on the Raulin-Dierckx substrate, the felt was light brown, very thin and heavily perforated. Furthermore, this medium was golden yellow throughout almost the entire period of growth, while in both the other cases, the colour varied from shades of red to violet, allowance being made for the intensity of colour due to the increasing concentration of pigment.

pH determinations were made at intervals of 3 days, the mixed contents of four bottles being used for each reading. These results are contained in Table 1.

Table 1

Medium ... Days of incubation	Czapek-Dox		Raulin-Thom		Raulin-Dierckx	
	pH	Colour of substrate	pH	Colour of substrate	pH	Colour of substrate
0	4.2	—	3.9	—	7.0	—
3	4.6	—	4.1	—	6.1	—
6	6.7	Violet	4.9	Red	3.9	Red
9	5.1	Red	8.0	Violet	3.3	Orange
12	3.7	Red	6.4	Violet	1.8	Golden yellow
15	3.4	Red	5.4	Red	1.8	Golden yellow
18	3.8	Red	6.6	Violet	1.9	Golden yellow

In correlating these figures, it appears that phenicine has two colour changes, which may be roughly defined by reference to the pH values, yellow to red, pH 1.8–3.4, and red to violet, pH 5.4–6.4. In addition to the trend of the pH figures, the selection of 18 days as the optimum period of growth was supported by the fact that individual bottles, of 16, 17, 19 and 20 days' incubation showed slightly decreased yields of the pigment. There now remained 60 bottles of each medium.

The mycelium was strained off and washed with a small quantity of water, the washings being added to the main bulk of the filtrate. The mycelium, pressed and dried *in vacuo* at 55° was weighed and Soxhlet-extracted with no result of interest. An attempt to concentrate the filtrate *in vacuo* at 65° ended with the complete decomposition of the pigment.

Activated charcoal adsorbs phenicine from the medium made slightly alkaline, but the product, recovered by solvent extraction, was completely unsatisfactory. The acidified substrate was therefore extracted with CHCl_3 , which solution was then re-extracted with dilute aqueous NaHCO_3 . This process was repeated several times [cf. Friedheim, 1933].

The final CHCl_3 solution, dried over anhydrous Na_2SO_4 , was evaporated *in vacuo* and the phenicine recovered as microscopic yellow prisms. This, after a few further recrystallizations from pure benzene, yielded a mixture of prisms and prismatic needles, which when heated rapidly melted at 231–232° (decomp.), first becoming dark in colour and finally black. (Found: C, 61.72, 61.24; H, 3.68, 3.72 %; mol. wt. 267, 271. $\text{C}_{14}\text{H}_{10}\text{O}_6$ requires C, 61.31; H, 3.65 %; mol. wt. 274.)

For purposes of comparison of the metabolism of the organism on the three media, the dry mycelium and pigment yields, calculated in g. per litre of initial substrate are shown in Table 2.

Table 2

Medium	Mycelium g.	Pigment g.
Raulin-Thom	17.1	0.082
Czapek-Dox	13.2	0.510
Raulin-Dierckx	10.8	0.261

A further batch of bottles was inoculated and allowed to grow over a period of 7 weeks. The final pH of the medium (Czapek-Dox) was 8.1 and the mycelium and phenicine yields per litre were 13.4 and 0.204 g. respectively. From this, it appears that, lacking other sources of carbon, *P. phenicium* will utilize phenicine.

The concentration of NaNO_3 in the Czapek-Dox medium was found to have a direct influence on the production of mycelium and pigment. Accordingly, individual batches of 2 l. of the solution were made up, containing concentrations of NaNO_3 ranging from zero to 4 g./l. by 0.5 g. increments. Sterilized and inoculated as before, these were allowed to grow for 18 days and then treated as already described. The figures obtained are incorporated in Table 3.

Table 3

Conc. of NaNO_3 g./l.	Mycelium g./l.	Phenicine g./l.
0.0	No growth	—
0.5	5.1	0.174
1.0	8.9	0.345
1.5	11.5	0.540
2.0	12.8	0.504
2.5	12.2	0.320
3.0	10.4	0.202
3.5	10.2	0.148
4.0	9.5	0.057

Therefore, while pigmentation is practically entirely inhibited on a Czapek-Dox medium, containing 4.0 g. NaNO_3 /l., the optimum yield is obtained on a solution, containing 1.5 g./l.

2. *PENICILLIUM RUBRUM* GRASBERGER-STOLL

Penicillium rubrum, included in Thom's classification of the "Biverticillata symmetrica", produces, on acid Czapek-Dox synthetic medium, two pigments [Posternak, 1939], the first, yellow, diffusible and only slightly affected by changes in pH and, in older cultures, a compound which proves to be phenicine. The organism was therefore grown on the three synthetic media, acid Czapek-Dox, Raulin-Thom and neutral Raulin-Dierckx, for purposes of comparing the general metabolism and on Czapek-Dox solution containing varying concentrations of NaNO_3 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ for the influence of nitrogen and magnesium concentrations (cf. Part I).

EXPERIMENTAL

The culture of *P. rubrum* was obtained from the Centraalbureau voor Schimmelcultures. The yellow mycelium of the 2nd day, on malt agar, gave way to green conidia on the 3rd while pigmentation was apparent in 3–4 days. Later a plentiful separation of loose spores commences.

Nine litres of each of the three media were made up as described in Part I and distributed in 100 ml. amounts in 20 oz. flat bottles, which were plugged with cottonwool, sterilized by steaming on each of three consecutive days and inoculated with spore suspension of *P. rubrum*, grown for 4–5 days on malt-agar. The bottles, incubated in the dark at 20°, were allowed to grow for 18 days.

The following details were noted during growth: On the Czapek-Dox medium, mycelium formation became apparent in 2 days with a white overgrowth of the liquid in 3 days. The smooth light green felt of the 4th, changed to a heavily sporing darker green on the 5th day, there being no further change for the period of growth. The reverse, first yellow, altered to light brown and then gradually darkened in shade. The medium, first yellow, was faintly red on the 4th day and intense red on the 9th, there being no further change.

On the Raulin-Thom medium, the mycelium was slightly corrugated and yellow in colour on the 3rd day, changing to greenish yellow on the 4th and deepening to grey-green with blue-grey patches on the 12th. The reverse, first yellow, was brown on the 9th with gradual darkening in shade. Yellow in 4 days, the substrate altered through orange to orange-red on the 6th and to deep red on the 8th day.

The mycelial felt on the Raulin-Dierckx medium was corrugated and yellow on the 4th day and yellow-green on the 5th, deepening on continued growth. The yellow reverse changed slowly to a final brown. First faintly yellow, the medium deepened to golden yellow, without further change.

pH determinations were made at intervals of 3 days, the mixed contents of 4 bottles being used for one reading. In the case of the Czapek-Dox and Raulin-Thom media, the increase in acidity was too slow to obtain clearly defined values but, on the 18th day, the respective reactions were pH 2.9 and 2.6. Both these values are included in the pH range, 1.8–3.4, in which the change from yellow to red occurs in solutions of phoenicine (cf. Part I). The reaction of the Raulin-Dierckx solution altered from pH 7.0 to 1.9 in 6 days and showed an upward trend to the final reaction of pH 2.3.

After 18 days' growth, the mycelium was strained off, washed with a small quantity of water and, when pressed, dried *in vacuo*. In filtering, no precautions were taken to retain the loose spores. The acidified filtrate and washings were extracted with CHCl_3 and dilute NaHCO_3 solution (cf. Part I). The yellow diffusible pigment, mentioned by Posternak, is not present in quantity and, being less soluble in NaHCO_3 than is phoenicine, is partly retained by the CHCl_3 during extraction while the remainder separates out as a film on the walls of the vessel when the NaHCO_3 extracts are acidified. As the phoenicine, obtained on recrystallizing from benzene, melts sharply at 231–232°, it appears that this yellow pigment is completely lost in the extraction. The yields of phoenicine and dry mycelium for the three media are calculated per initial litre of substrate (Table 4).

Table 4

Medium	Mycelium g.	Phoenicine g.
Czapek-Dox	8.9	1.457
Raulin-Thom	12.4	1.370
Raulin-Dierckx	12.3	0.142

It was found that the age of the inoculating culture tended to diminish the yields; a 79-day old culture on malt agar when used for the inoculating spore suspension gave (Table 5):

Table 5

Medium	Mycelium g.	Phoenicine g.
Czapek-Dox	9.8	1.281
Raulin-Thom	11.5	1.060
Raulin-Dierckx	12.0	0.124

A further batch of 4 l. of Czapek-Dox medium was inoculated from a 6-day old culture and incubated as before for 24 days. Four bottles were taken at intervals of 3 days and the contents extracted, the yields of phoenicine being given in Table 6:

Table 6

Days of incubation	Phoenicine g./l.	Days of incubation	Phoenicine g./l.
0	—	15	1.043
3	—	18	1.403
6	0.150	21	1.260
9	0.590	24	1.183
12	0.703		

From these results, it seems that, as for *P. phoeniceum* (cf. Part I), 18 days are the optimum period of growth for *P. rubrum*, and further that this organism will also utilize phoenicine as a source of carbon.

In dealing with the influence of nitrogen concentration, individual batches of 2 l. of Czapek-Dox solution, containing amounts of NaNO_3 , ranging from zero to 4 g./l. by 0.5 g. increments, were inoculated from 7-day old cultures and incubated for 18 days. Table 7 gives the production of phoenicine and dry mycelium per litre.

Table 7

NaNO_3 g./l.	Mycelium g.	Phoenicine g.
0	—	—
0.5	2.8	0.400
1.0	4.9	0.645
1.5	6.1	0.851
2.0	7.5	1.327
2.5	10.8	1.307
3.0	11.3	1.890
3.5	11.8	0.772
4.0	11.7	0.633

Two litre amounts of Czapek-Dox medium, containing 0, 0.1, 0.2, 0.3, 0.4 and 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ /l. were inoculated from 6-day old cultures and grown, under the usual conditions. The resultant values for mycelium and pigment production are contained in Table 8:

Table 8

Conc. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ g./l.	Mycelium g./l.	Phoenicine g./l.
0	2.1	0.220
0.1	8.3	1.560
0.2	9.4	1.568
0.3	10.5	1.848
0.4	8.7	1.436
0.5	9.2	1.360

Analysis of Tables 7 and 8 shows that the optimum concentrations of NaNO_3 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ are 3.0 and 0.3 g./l. respectively. A solution of the following composition was then made up and inoculated in the usual manner: glucose, 50 g.; NaNO_3 , 3.0 g.; KCl , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g.; K_2HPO_4 , 1.0 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water to 1000 ml.

After 18 days, the dry mycelium and phenicine yields per litre of initial substrate, obtained as already described were: mycelium, 11.4 g.; phenicine, 2.0 g. This result is of particular interest as the new yield shows a mean 45 % increase over those already obtained.

SUMMARY

P. phoeniceum produces phenicine more readily on synthetic media than on beerwort and in the case of one medium, acid Czapek-Dox, yields the pigment in greater quantity, viz. 51 mg./100 ml. as compared with 44 mg. on a beerwort substrate [Friedheim, 1938].

On Czapek-Dox and Raulin-Thom media, *P. rubrum* produces nearly equal quantities of phenicine, quantities which are $2\frac{1}{2}$ –3 times greater than the optimum yield on Czapek-Dox medium of *P. phoeniceum*. The organism is similarly, but not identically, influenced by pH and nitrogen concentration and gives a greatly increased yield of the pigment, when grown on a specially modified medium.

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