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Biochemical Characteristics of Species of Penicillium Responsible for the Rot of Citrus Fruits

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Studies in the Biochemistry of Micro-organisms.

Part XVIII.—*Biochemical Characteristics of Species of* Penicillium *responsible for the rot of Citrus fruits.*

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Decaying oranges or other citrus fruits are almost invariably infected by one of two moulds, *viz.*, *P. digitatum* Saccardo (= *P. olivaceum* Wehmer), which causes the olive-coloured rot, and *P. italicum* Wehmer, which is responsible for the blue-green rot. It has been found in the course of work that each of these species has very definite biochemical characteristics which are described in this paper.

1. *P. digitatum* Saccardo (*P. olivaceum* Wehmer).

This species was first adequately described by Wehmer in "Beiträge zur Kenntnis einheimischer Pilze," Heft 2: "Untersuchungen über die Fäulnis der Früchte," Jena, 1895. Later, Thom (1910), in "Cultural Studies of Species of *Penicillium*," p. 31, renamed this species on the grounds of priority, *P. digitatum* Saccardo.

Physiologically it is distinguished from almost all other species of *Penicillium* by the fact that while it grows readily on organic media, it either refuses to grow or grows with difficulty on synthetic media containing nitrogen as sodium nitrate (Thom, p. 32).

In the course of work described in Part IV of this series on the carbon balance sheets of species of *Penicillium*, it was found that three strains of *P. digitatum* give unusually large amounts of carbon as "Carbon in H_2SO_4." The strains used were the following:—

(*a*) *P. digitatum*, Catalogue No. Ad. 52. This strain was purchased in 1925 from the Centraalbureau voor Schimmelcultures at Baarn under the name *P. olivaceum* Wehmer. It was sent for confirmation to Dr. Thom, who wrote, "Your No. 52, marked *P. olivaceum*, is correctly named as the olive-coloured rot of oranges, but the name should be changed to *P. digitatum* to comply with the rules."

(*b*) *P. digitatum*, Catalogue No. Ad. 81. This strain was isolated at Ardeer in 1926 from a mouldy orange. It gives a very poor growth on synthetic media containing sodium nitrate, and was identified by Dr. Thom.

(*c*) *P. digitatum*, Catalogue No. Ad. 102. This strain was purchased in 1927 and is the American Type Culture Collection No. 1113, isolated originally by Dr. Thom from a mouldy orange.

The carbon balance sheets prepared for these strains, which are given in Part IV,

Table III, p. 60, are all of the same type and are chiefly remarkable for the fact that each of the three strains tried gives rise to very considerable amounts of carbon in the sulphuric acid bubbler, indicating the production in fair amount by this species of some relatively volatile material. In addition, considerable amounts of "carbon in volatile neutral compounds" are produced, varying from about 20 per cent. with Ad. 52 to 30 per cent. with Ad. 102.

In order to investigate the nature of these products the following experiments were carried out: A quantity of the usual Czapek-Dox 5 per cent. glucose medium was made up and 350 c.c. of this placed in each of 60 1-litre conical flasks. These were plugged with cotton wool and sterilized by steaming on each of three consecutive days. After sterilization, 20 flasks were heavily sown with spores of Ad. 52, 20 were sown with Ad. 81, and 20 were sown with Ad. 102. Each flask was then fitted with a sterile rubber bung which carried two sterile glass tubes plugged with sterile cotton wool. One of these tubes reached almost to the surface of the liquid and was connected to a supply of sterile air. The other tube was cut short at the bung, and was connected to a bubbling tube which dipped beneath the surface of an absorbent solution contained in a boiling tube. In some cases this absorbent solution consisted of water, in others of normal sodium hydroxide, and in others of an aqueous solution of 2 : 4-dinitrophenylhydrazine hydrochloride. The whole of the flasks and fittings were incubated in the constant temperature room at 24° – 26° C. No growth was apparent in any flasks for at least a fortnight, but from that time onwards a slow growth started, and by the time the experiment terminated quite a fair growth had been obtained. Two flasks out of the 60 became infected and were replaced. Strains Ad. 52 and Ad. 102 grew rather better than strain Ad. 81.

All the flasks were sown on 25th February, 1929, and aeration was not commenced until 23rd March, 1929, from which time until the end of the incubation period about 250 c.c. of sterile air were passed through each flask each day for six days a week. At the end of the incubation period the contents of the flasks were filtered from the mycelium, each strain being dealt with separately. An average sample was taken for analysis, and a summary of the analytical results is given in Table I. The treatment of the bubblers, of the filtered metabolism solution A and of the mycelium B, are given later.

Table I.

	Strain Ad. 52.	Strain Ad. 81.	Strain Ad. 102.
Incubation period in days	79	93	76
	per cent.	per cent.	per cent.
Glucose by polarimeter	0·650	1·186	0·985
Glucose by Wood-Ost method	0·645	1·098	0·972

Treatment of bubblers.—The absorbent bubblers mentioned on p. 356 were treated as follows :—

(*a*) The contents of the bubblers containing water were united with solution A and worked up with this solution.

(*b*) The contents of the bubblers containing normal sodium hydroxide were tested for volatile acids, but practically none were found.

(*c*) The contents of the bubblers containing 2 : 4-dinitrophenylhydrazine remained clear throughout the experiment and indicated the absence of aldehydes or ketones.

Treatment of Solution A.—The filtered metabolism solution from each of the three strains had a very pleasant ethereal smell. The treatment of the solution was the same with each of the three strains. The solution, to which was added the aqueous absorbent solution described above, was distilled at ordinary pressure from a large bolt-head flask fitted with a long YOUNG's fractionating column. Two fractions were collected, fraction 1 boiling at 70° C. to 98° C. and fraction 2 at 98° C. to 99° C. These fractions were now further fractionated, again using a YOUNG's column. The following three fractions were obtained in each case :—

(*a*) Boiling point, 70° to 73° C. (mainly 71° C.).
(*b*) Boiling point, 78° to 81° C. (mainly 79·5° C.).
(*c*) Boiling point, 88° to 98° C. (gradual rise).

The behaviour on distillation of the metabolism solution from each of the three strains was exactly similar, the weights of distillate obtained being somewhat different as is shown in Table II.

TABLE II.

	Strain Ad. 52.	Strain Ad. 81.	Strain Ad. 102.
Weight in gm. of fraction (*a*), B. Pt. 70°–73° C.	6·03	3·07	4·40
Weight in gm. of fraction (*b*), B. Pt. 78°–81° C.	22·66	8·08	12·93
Weight in gm. of fraction (*c*), B. Pt. 88°–98° C.	17·23	4·20	33·80

Treatment of fraction (*a*).—Fraction (*a*) was shown to consist principally of *ethyl acetate* by the following means :—The whole of fraction (*a*) from strain Ad. 52 was shaken with about its own volume of calcium chloride solution (50 gm. $CaCl_2$ and 50 gm. water), separated and dried overnight over solid calcium chloride. It was distilled and gave two fractions, one weighing 1·25 gm. and boiling at 72°–74° C., while the other weighed 2·87 gm. and boiled at 75° C. (boiling point of ethyl acetate = 77° C.).

0·964 gm. of the fraction boiling at 75° C. was hydrolysed by boiling with an excess of N/1 NaOH for one hour under reflux. The excess of sodium hydroxide was titrated with N/1 nitric acid to aqueous phenolphthalein. 9·25 c.c. of N/1 sodium hydroxide were used, corresponding to an equivalent of 104 (theoretical for ethyl acetate = 88). The neutralised solution was now distilled. The distillate gave a positive iodoform reaction at 60° C. (not in the cold) and a carbon content on wet combustion corresponding to 0·517 gm. of ethyl alcohol (if the fraction boiling at 75° C. were pure ethyl acetate this amount would be 0·504 gm.). The distillation residue was evaporated to small bulk, filtered, silver nitrate added and the precipitated silver salt recrystallised from boiling water. It proved to be silver acetate. 0·2120 gm. gave 0·1363 gm. of silver on ignition, corresponding to 64·3 per cent. silver (theoretical for CH_3COOAg = 64·8 per cent.).

Hence the fraction boiling at 75° C. consists of ethyl acetate containing small amounts of ethyl alcohol, which is known to be difficult to remove entirely from ethyl acetate. The corresponding fraction (*a*) from strains Ad. 81 and Ad. 102 gave exactly similar results.

Treatment of fraction (*b*).—Fraction (*b*) was shown to consist principally of ethyl alcohol by the following means :—A portion of fraction (*b*) from strain Ad. 52 was treated with *p*-nitrobenzoyl chloride and the resultant *p*-nitrobenzoate recrystallised. It crystallised in plates and melted at 55·5°–56·5° C., and its melting point was unchanged on admixture with a sample of ethyl *p*-nitrobenzoate.

The corresponding fractions (*b*) from strains Ad. 81 and Ad. 102 gave exactly similar results.

Treatment of fraction (*c*).—Fraction (*c*) from each of the three strains was shown to consist principally of ethyl alcohol which was identified as the *p*-nitrobenzoate, while the higher boiling fraction consisted of water.

Treatment of Mycelium B.—The mycelium from each of the three strains, after being drained from the metabolism solution, was thoroughly extracted with boiling water, the extract from each strain being kept separate. In each case there separated from the cooled mycelium extract a white, flocculent, non-crystalline solid. A second extraction of the mycelium with boiling water gave a second crop of the same material, but the amount obtained by a third extraction was negligible. The yields of material obtained were as follows :—

	Gm.
Strain Ad. 52—Crops 1 and 2 together	4·81
Strain Ad. 81—Crop 1	2·14
Strain Ad. 81—Crop 2	2·37
Strain Ad. 102—Crop 1	1·97
Strain Ad. 102—Crop 2	2·00

Properties of product from mycelium extract.

The white, flocculent, non-crystalline solid separating from the hot aqueous mycelium extract was shown to be the same material from each of the three strains and to consist in each case of a complex carbohydrate.

This carbohydrate is quite white in colour if quickly filtered and quickly dried with alcohol and ether. It gives no colour with iodine and is almost insoluble in cold water. It dissolves moderately well in boiling water, from which it separates almost completely on cooling as a white flocculent solid. The material prepared from strain Ad. 81 had the following properties :—

(1) *Optical rotation.*—The material is strongly dextro-rotatory. Because of its insolubility in cold water the optical rotation was determined in a jacketed tube at 90° C. 0·495 gm. dissolved in 200 c.c. of hot water gave $[\alpha]^{90}_{\text{Hg. green}} = +299°$ and $[\alpha]^{90}_{\text{Hg. yellow}} = +270°$. The material from strain Ad. 52 gave corresponding values of $+292°$ and $+261°$, while that from strain Ad. 102 gave $+292°$ and $+266°$.

(2) *Hydrolysis by boiling dilute acid.*—0·100 gm. of the material from strain Ad. 81 was heated under reflux with 10 c.c. of N/1 H_2SO_4 for three and a half hours, cooled, 10 c.c. of N/1 NaOH added and made up to 25 c.c. with water. The optical rotation of this solution calculating as glucose corresponded to a concentration of 0·400 per cent. ($\equiv$ 0·100 gm. glucose), while glucose estimated by the Wood-Ost method gave 0·392 per cent. ($\equiv$ 0·098 gm. glucose).

(3) *Products of hydrolysis.*—0·500 gm. of the material from strain Ad. 81 was hydrolysed for three hours under reflux with 10 c.c. of N/1 H_2SO_4 and 10 c.c. of water. At the end of the hydrolysis period the mixture was titrated to phenolphthalein with N/1 NaOH, of which 10·02 c.c. were required. Hence, it is obvious that no acidic bodies are formed by hydrolysis.

The neutralised hydrolysis mixture was made up to 50 c.c. and its glucose content estimated (*a*) by polarimeter = 1·009 per cent., (*b*) by Wood-Ost = 1·006 per cent., corresponding to yields of 0·505 gm. and 0·503 gm. of glucose from 0·500 gm. of carbohydrate hydrolysed.

It was definitely proved that glucose is the only carbohydrate formed on hydrolysis by preparing the phenylosazone from the hydrolysis mixture. The whole phenylosazone was filtered off and dried. Its melting point was 203° C. 0·1 gm. dissolved in 2 c.c. of pyridine plus 3 c.c. of ethyl alcohol gave a rotation in a 2 cm. tube of —0·29°, corresponding to —1·45° for a 10 cm. tube (Neuberg gives —1·50° for pure glucosazone).

(4) *Effect of Enzymes.*—0·2 gm. samples of carbohydrate from each of the three strains were incubated at 25° C. with 0·2 gm. of Pangestin (commercial diastase) in 20 c.c. of water in the presence of toluene. There was no hydrolysis even after one week's incubation.

(5) *Analysis by combustion.*—A sample of the carbohydrate from Ad. 81, dried to constant weight *in vacuo* at 50° C. over P_2O_5, was analysed by SCHOELLER, Berlin, with the following results :—

TABLE III.

Weight of Substance analysed.	Weight of CO_2.	Weight of H_2O.	Percentage Carbon.	Percentage Hydrogen.
mgm.	mgm.	mgm.	per cent.	per cent.
4·855	7·800	2·83	43·82	6·52
4·481	7·165	2·58	43·76	6·47
Theoretical for $C_6H_{10}O_5$	—	—	44·42	6·22

2. *P. italicum* WEHMER.

This species was first described by WEHMER (*loc. cit.* p. 68), and is further described by THOM (*loc. cit.* p. 29). WEHMER showed that the blue-green rot of citrus fruits is a different species from the similarly coloured apple rot. THOM (*loc. cit.* p. 31) says "Pure cultures of *P. italicum* WEHMER can always be secured by finding decaying oranges in the market which have the blue-green areas of rot just beginning to appear upon them." During the course of other work it was observed that a reputed culture of *P. italicum* gave a characteristic colour reaction with ferric chloride. This appeared of sufficient interest to warrant the extension of the test to other cultures of *P. italicum* obtained from different sources and it has been found that the tests to be described are given by all genuine cultures of *P. italicum* which were used.

The tests, which are described later, were given by all the following strains of *P. italicum* WEHMER :—

(*a*) Catalogue No. Ad. 17. Isolated at Ardeer in 1925 from a mouldy lemon.

(*b*) Catalogue No. Ad. 84. Isolated at Ardeer in 1926 from a mouldy orange.

(*c*) Catalogue No. Ad. 85. Isolated at Ardeer in 1926 from a mouldy orange.

(*d*) Catalogue No. Ad. 86. Isolated at Ardeer in 1926 from a mouldy orange. Cultures Ad. 84, Ad. 85 and Ad. 86 have all been identified by Dr. THOM as strains of *P. italicum.*

(*e*) Fourteen other strains of *P. italicum* isolated from mouldy oranges and lemons obtained from markets in different parts of Great Britain, in order to ensure differences in strains.

Single spore cultures of each of the above 18 strains of *P. italicum* were prepared and used for inoculating tubes, each containing 10 c.c. of the modified CZAPEK-DOX glucose medium containing twenty times the amount of ferrous sulphate given in Part I, p. 7.

The medium was sterilized by steaming for half an hour on each of three consecutive days. After inoculation the tubes were incubated in the dark at 24° C. At intervals of 6 days, 15 days, 20 days and 40 days, one tube of each culture was tested, and while it was found that not all cultures gave a positive result in 6 days or 15 days, each of the 18 strains tried gave a definite positive result after 40 days' incubation.

The test was carried out as follows :—The metabolism solution, without sterilizing, was poured from the mycelium and filtered through a small filter paper. The filtrate was then divided into two equal parts which were treated as follows :—

(*a*) To one-half was added a single drop of 10 per cent. ferric chloride solution in water. A transient green colour is produced when the ferric chloride comes in contact with the solution, but this colour disappears on shaking. Further addition of ferric chloride solution drop by drop, until a slight excess has been added, gives rise to a permanent, beautiful, emerald-green colour which varies in intensity with different strains, some of the strains giving a very intense reaction. If this green solution is now diluted to such an extent that the green colour is only just obvious, and two or three drops of a freshly prepared aqueous solution of potassium ferricyanide are now added, a deep blue colour is immediately obtained indicating the reduction of the ferric chloride to the ferrous state. In our opinion this is due to the oxidation by the ferric chloride of some metabolic product of *P. italicum*, with the formation of (*a*) ferrous chloride which now reacts with potassium ferricyanide to give the Prussian blue colour, and (*b*) an oxidation product which now gives a deep emerald-green colour with ferric chloride.

(*b*) To the other half of the filtered metabolism solution was now added, drop by drop, a filtered saturated solution of bleaching powder, with shaking between the additions. In every case a copious white precipitate was formed and a purple colour was produced, varying in intensity from a pale amethyst to a permanganate colour. With subsequent addition of excess of bleaching powder solution this purple colour disappears, leaving a colourless solution. It was noticed that the intensity of the ferric chloride reaction runs parallel with the intensity of the purple colour given with bleaching powder, and it is proposed to attempt the isolation and chemical investigation of the product which is responsible for the above colour reactions.

We are of the opinion that these colour reactions are not only characteristic of, but specific for *P. italicum* Wehmer, since out of a large collection of different species of different genera, *P. italicum* Wehmer is the only mould in our possession which gives the colour reactions above described. The reaction given by *P. italicum* with ferric chloride might be confused with the somewhat similar reaction given by *Citromyces* species and described in Part XI. There are two very definite differences. In the first place the *Citromyces* colour is developed *immediately* with ferric chloride, while the colour with *P. italicum* requires *an excess* of ferric chloride. Further, *Citromyces* species do not give the characteristic purple colour with bleaching powder, which as described above is given by *P. italicum*.

Summary.

The biochemical characteristics are described of two species of *Penicillium* responsible for the rot of citrus fruits, *viz.*, *P. digitatum* SACCARDO (*P. olivaceum* WEHMER) and *P. italicum* WEHMER.

P. digitatum SACCARDO is unique amongst species in our collection in the fact that it produces from glucose considerable amounts of ethyl acetate. In addition it also produces ethyl alcohol and a new polysaccharide which gives rise to glucose on hydrolysis.

P. italicum WEHMER produces from glucose a new metabolic product which is characterized by its colour reactions with ferric chloride and bleaching powder. These colour reactions are characteristic of and diagnostic for *P. italicum* WEHMER.

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