STUDIES IN THE BIOCHEMISTRY OF MICROORGANISMS*

Part VII.—Terrein and Kojic Acid, Metabolic Products of Aspergillus stellatus Curzi

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Aspergillus stellatus Curzi, is shown to produce terrein (I) and kojic acid (II). Conditions favourable for the production of metabolites (I) and (II) are described.

During screening of fungi from spoiled bananas, besides many others, an interesting aerial contaminant belonging to the genus Aspergillus was isolated which was later on identified[1] as *Aspergillus stellatus* Curzi (*Rend. Acad. Nazl. Lincei*, **19,** 426–428 (1934). This species is also described under the *Aspergillus nidulans* group as *Aspergillus variecolor* (Berk. Br.)[2] Although *A. stellatus* was first described by Curzi in 1934 (*loc. cit*), no work on its metabolic products has so far been reported in the literature. There was, however, one report by Gupta and Viswanathan[3] in which they have claimed to have isolated antituberculous substance variecolin from *A. variecolor*. These workers, however, have not given any physical constants for variecolin. Our attempt to repeat their work was also unsuccessful. This has evinced interest in us to undertake these investigations.

When *A. stellatus* was grown on fully synthetic medium (Czapeck Dox) containing 5% glucose as the sole source of carbon and $NaNO_3$ as source of nitrogen and incubated at 24°C for 17 days, extraction of the broth with ethyl acetate afforded an oily product ν_{max} 3333 (—OH); 1720 (C=O) cm^{-1}. This oily ketol, however, could not be crystallized, nor could a solid derivative be obtained.

Since no crystalline metabolite could be obtained, it was considered worthwhile to modify the medium. During the course of work on *Curvularia siddiqui* in our laboratories, the use of synthetic medium enriched with carrot extract was found to have an effect on the formation of metabolic products, though its specific role was not understood.[4–6] Taking advantage of this experience, *A. stellatus* was grown on synthetic medium enriched with carrot extract but again no crystalline product was obtained. Next we thought of modifying the culture medium by changing the carbon and nitrogen sources (see Table 1).

Thus when *A. stellatus* was grown on synthetic medium enriched with carrot extract and having molasses as carbon source no solid metabolite was formed. Similar results were obtained using molasses as carbon source and diammonium tartrate as nitrogen source. The oily products obtained in the different experiments have the same R_f values. All of them show positive $FeCl_3$ test. However, when *A. stellatus* was grown on synthetic medium enriched with carrot extract and having diammonium tartrate as nitrogen source and incubated at 24°C for 17 days, it was interesting to note that in this case extraction of the broth with ethyl acetate afforded a crystalline

TABLE 1

Medium	Carbon source	Nitrogen source	Metabolite
Czapeck Dox medium	Glucose	$NaNO_3$	Oily viscous product
Czapeck Dox medium enriched with carrot extract	Glucose	$NaNO_3$	Oily viscous product
,,	Molasses	$NaNO_3$	Oily viscous product
,,	Molasses	DAT	Oily viscous product
,,	Glucose	DAT	Terrein (m. p. 127°C)

DAT = Diammonium tartrate

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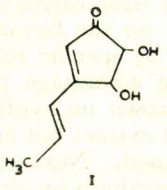
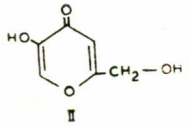
product, m.p. 127°C. The UV spectrum of this compound shows absorption at λ_{max} 275 mμ (ε26000) which is characteristic of congugated dienone. In the IR spectrum it shows peaks at ν_{max} 3620 and 3420 cm^{-1} (–OH), 1700, 1640 and 1575 cm^{-1} (—C(=O)—C=C—C=C—). The proton magnetic resonance (PMR) of this compound shows a doublet at τ 8.28 which can be ascribed to a methyl group attached to vinyl proton bearing carbon (H—C(—CH_3)=C—) two partially resolved doublet at τ 5.90 and 5.2 representing protons attached to hydroxyl bearing carbons and peaks at τ 4.01, 3.47, 3.2 which may be ascribed to vinyl protons. The compound analyses for $C_8H_{10}O_3$. In the mass spectrum, the molecular ion peak *m/e* comes at 154. The analytical and spectral data of this compound fits for structure I which is terrein.



I

Terrein is a mold metabolite first isolated by Raistrick[7] from *Aspergillus terreus* and its constitution was elegantly established by Barton and his co-workers.[8] Terrein was also reported to have been isolated by Grove[9] from *P. raistrickii* and also by a group of Japanese workers[10] from *A. fischeri*

In the foregoing experiments, the flasks containing culture medium and inocculant were incubated by allowing them to stand at 24°C for 17 days. Experiments were, however, also conducted by shaking the flasks during incubation. Thus it was observed when *A. stellatus* was grown on synthetic medium enriched with carrot extract and having glucose and $NaNO_3$ as sources of carbon and nitrogen, respectively, and incubated by shaking for 21 days, extraction of the broth with ethyl acetate furnished a crystalline product, m.p. 151°C. It gives positive $FeCl_3$ test, ν_{max} 3226 (—OH) and 1667 cm^{-1} (C=O). The compound analyses for $C_6H_6O_4$. In the mass spectrum the molecular ion peak *m/e* comes at 142. This compound was identified as kojic acid (II).



II

Kojic acid is a mold metabolite first isolated by Saito[11] from *Aspergillus oryzae* and subsequently by a variety of microorganisms.[12] The identity of this compound was confirmed by comparing the R_f values and by taking the mixed m.p. with that of an authentic sample of the material (m.p. 151°C.) In the above experiment 960 ml of the broth (from 4 flasks) were extracted with ethyl acetate and the yield of kojic acid was 1.6 g. Our observation that 'shaking' is an important factor in the above experiment is compatible with the known facts that kojic acid is produced in an aerobic process and for that purpose "the oxygen requirements of the mold had to be satisfied by bubbling oxygen through the medium or by constant agitation".[12]

Experimental

All melting points are corrected. UV absorption spectra were determined with a Beckman spectrophotometer model D.K. 2 in 95% ethanol. IR spectra were determined with a Beckman IR 5. PMR spectra were recorded on Varian A60 using tetramethylsilane as internal reference.

Organism.—During screening of spoiled bananas, a strain of *Aspergillus* was isolated, which was identified by C.M.I. as *Aspergillus stellatus* Curzi and was catalogued under No. (I.M.I. 112543).

Aspergillus stellatus was first inoculated on ordinary Czapeck Dox medium in test tubes and incubated at 24°C for 9 days. This 9-day old culture was then used to inoculate flasks containing the culture medium.

Preparation of Carrot Extract.—Raw carrots were peeled off to remove roots and skin, washed and cut into small pieces. 200 g of these carrots were boiled in 700 ml of distilled water. After filtration the volume of the extract was made up to 1 l. with distilled water.

Terrein

Cutural Conditions.—The medium used was composed of glucose 50 g; K_2HPO_4 1.0 g; KCl 0.5 g; $MgSO_4.7H_2O$ 0.5 g; $FeSO_4.7H_2O$ 0.01 g and aqueous carrot extract 1 l.

In a typical batch, 5 (1-l.) conical flasks were taken, each containing 340 ml of the above media and autoclaved at 10 lb pressure for 20 min. 75.6 g per litre of diammonium tartrate solution was sterilized and 10 ml of it was added asceptically to each of the flasks. This precaution was taken to prevent discoloration of the media which occurs when diammonium tartrate is sterilized along with the medium.

These flasks containing culture medium (pH 4.5) were inoculated with 9-day old tube culture of *A. stellatus* and incubated at 24°C for 17 days.

During incubation, broth (25 ml) was drawn asceptically, first after a week and then every third day for pH and optical rotation measurements. The data is recorded in Table 2.

TABLE 2.

Days of incubation	pH	Angle of rotation	Glucose%
1st	4.5	1.05	5.00
8th	4.9	0.72	3.40
11th	6.2	0.23	1.05
14th	6.4	0.18	0.85
17th	7.2	0.00	0.00

Isolation of Terrein.—After 17 days the mycelia was removed by filtration (suction) and the borth (1475 ml) was extracted with ethyl acetate. The organic layer was dried (anhydrous sodium sulphate) and solvent removed to give terrein as a gummy product which congeals to solid on standing (1.91 g). Crystallization from ethyl acetate afforded colourless, needles (1.7 g) m.p. 127°C mixed m.p. with authentic sample (m.p. 127°C) undepressed. $[\alpha]_D^{30}+136$ (*c* 1 in water). Found: C, 62.30, H, 6.62; O (by difference), 31.08%. Terrein $C_{10}H_8O_3$ requires: C, 62.32; H, 6.54; O,31.14%. UV absorption bands at λ_{max} 275 mμ (ε26000). IR absorption bands at λ_{max} 3620, 1700, 1640, 1575 cm^{-1}. The PMR spectrum shows peaks at τ 8.28 ($-CH_3$), τ 5.90, 5.22 (protons attached to hydroxyl bearing carbon), τ4.01, 3.47, 3.2 (vinyl protons).

Kojic Acid

Cultural Conditions.—The medium used was composed of glucose 50 g; $NaNO_3$ 3 g; K_2HPO_4 1 g; KCl 0.5 g; $MgSO_4.7H_2O$ 0.5 g; $FeSO_4.7H_2O$ 0.01 g and aqueous carrot extract 1 l.

In a typical batch, four (1-l.) conical flasks, each containing 350 ml of the above media (pH 4.5), were taken inocculated with 9-day old tube culture of *A. stellatus* and incubated at 24°C on shaker for 21 days. During incubation, broth (25 ml) was drawn periodically for pH and optical rotation measurements. The data is given in Table 3.

TABLE 3.

Days of incubation	pH	Angle of rotation	Glucose%
1st	4.5	1.05	5
6th	5.5	0.75	3.5
9th	5.9	0.58	2.7
12th	6.4	0.34	1.6
16th	7.0	0.20	0.95
21st	7.4	0.00	0.0

Isolation of Kojic Acid.—After 21 days, the mycelia was filtered off (suction) and the broth 960 ml was extracted first with ethyl acetate and then with ether. The filtrate was saturated with sodium chloride and again extracted. The combined extracts were dried over anhydrous sodium sulphate. Removal of the solvent by distillation afforded Kojic acid which crystallises from ethyl acetate as colourless prisms (1.6 g) m.p. 151°C. Mixed m.p. with authentic sample (m.p. 151°C) undepressed. ν_{max} 3226 (–OH), and 1667 cm^{-1} (C=O). Found: C, 50.72; H, 4.48; O (by difference), 44.8%. Kojic acid $C_6H_6O_4$ requires: C, 50.70; H, 4.22; O,45.49%.

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