FUNGAL GROWTH IN SYRUP OF TOLU

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A sample of Syrup of Tolu showing fungal growth had a distinct odour resembling toluene and yielded colonies of a species of *Penicillium* on subculture. The isolated organisms were grown on media containing sub-inhibitory concentrations of either benzoic or cinnamic acids as sole carbon source, and in the presence of cinnamic acid the toluene-like odour was apparent. Cultures of *P. nigricans* and of five recently isolated species or strains within species of *Penicillium* behaved similarly. Attempts to characterise the product with the toluene-like odour failed because of the presence of interfering substances. Samples of tolu syrups prepared with different sucrose contents, and adjusted to different pH values, were inoculated with the isolated fungus: preparations adjusted to pH 4·0–4·2 appeared to support growth irrespective of sucrose concentrations within limits of 50–67 per cent w/w, provided the inoculum was large.

Syrup of Tolu B.P. is a preparation in which spoilage due to microbial growth would appear to be unlikely. High sucrose concentrations are considered to be inimical to yeast and mould growth and the extracted benzoic acid which this preparation contains is a recognised fungistat and bacteriostat.

Experimental and Results

An amber 80 fl. oz. bottle of Syrup of Tolu of commercial origin was found to contain a submerged fungal growth and to have a pronounced odour which resembled toluene. The bottle closed with a bakelite cap fitted with a cork wad was opened after storage for several months at room temperature. There was no evidence of extensive production of carbon dioxide and the syrup was pH 3·96. Portions were plated on malt agar and Czapek-Dox agar and incubated at 24°. Colonies of only one type developed and these showed microscopical characters typical of the genus *Penicillium*.

It appeared that the substance of toluene-like odour in the infected syrup was produced by the fungus from one or both of the constituent aromatic acids: benzoic or cinnamic acids, or their esters. The possible utilisation of these acids raised the question of whether, as the sole nutritional source of carbon, they would support growth of the fungus. These points were investigated by the following fungistatic evaluation of the acids. A series of solutions containing graded concentrations of benzoic or cinnamic acids in Czapek-Dox medium at pH 5·0 were prepared. The media contained either no added sugar or 5 per cent of glucose or sucrose. The media were distributed in 10 ml. volumes in sterile, capped test tubes and were inoculated with spore suspensions, which were prepared by weighing small quantities of air-dry fungal spores,

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adding sufficient sterile water to provide 0·2 mg. spores per ml., and shaking vigorously to disperse the spores. An even distribution resulted if the suspensions were stored for two hours at room temperature, with intermittent shaking, before use. The tubes of media were inoculated with three drops of suspension delivered from the Cook and Yousef[1] pipette, so that the inoculum comprised about 10 μg. of spores. The

TABLE I

GROWTH OF *Penicillium* SPECIES IN MEDIA CONTAINING BENZOIC ACID IN VARYING CONCENTRATIONS AT pH 5·0

Concentration (per cent w/v)		2·0	1·0	0·5	0·25	0·10	0·05	0·005	Minimal fungistatic concn
Medium	Test organism								
Basal	T	—	—	+s	+++	+++	++	+	1·0
	N	—	+	+++	+++	+++	++	+	2·0
	A	—	—	—	—	+++	++	+	0·25
	B	—	—	+	+++	++	++	+	1·0
	C	—	+s	+	++	+++	++	+	2·0
	D	—	—	++	+++	+++	+++	+	1·0
	E	—	—	+	++	+++	++	+	1·0
With 5 per cent glucose	T	—	—	+s	++	+++	+++	+++	1·0
	N	—	+s	+++	+++	+++	+++	+++	2·0
	A	—	—	—	++	+++	+++	+++	0·5
	B	—	—	+	++	+++	+++	+++	1·0
	C	—	+s	++	+++	+++	+++	+++	2·0
	D	—	—	++	+++	+++	+++	+++	1·0
	E	—	—	+s	++	+++	+++	+++	1·0
With 5 per cent sucrose	T	—	—	+s	++	+++	+++	+++	1·0
	N	—	—	+	++	+++	+++	+++	1·0
	A	—	—	+	++	+++	+++	+++	1·0
	B	—	—	+s	++	+++	+++	+++	1·0
	C	—	—	++	+++	+++	+++	+++	1·0
	D	—	—	+	++	+++	+++	+++	1·0
	E	—	—	+	++	+++	+++	+++	1·0

The strains referred to in the table are T: isolated from the original infected syrup; N: *Penicillium nigricans*; A, B, C, D, E: freshly isolated *Penicillium* species.

— no growth visible;
+s very slight mycelial growth;
\+ submerged mycelial growth;
++ heavier growth with prescence of spores, covering part of the surface of the medium;
+++ heavy sporing growth forming a complete layer at the surface of the medium and with production of pigment in the medium.

tubes were incubated at 24° for seven days, after which they were examined for evidence of growth and detectable toluene-like odour. Each experiment was in duplicate for both acids in three media and for seven different organisms: that isolated from the infected syrup, a verified strain of *P. nigricans*, and five recently isolated species, or strains within species, of *Penicillium*.

The results are shown in Tables I and II. Growth of all the test organisms was inhibited by either benzoic or cinnamic acids when present in sufficient concentration. In the absence of a sugar in the medium, growth was supported by sub-inhibitory concentrations of either of the acids, but the toluene-like odour was detected only when cinnamic acid was used. That the amount of growth increased as the concentrations of the acids were increased in sugar-free media was taken as evidence of the utilisation of the acids. In contrast, the amount of growth in media

containing sucrose or glucose was independent of aromatic acid concentration except when the inhibitory concentration was approached. The organism isolated from the infected syrup had a tolerance to benzoic acid approximately equal to the other organisms studied, but the tolerance to cinnamic acid was decidedly lower than that of the other species examined. It appears that the presence of sucrose or glucose, at the

TABLE II

GROWTH OF *Penicillium* SPECIES IN MEDIA CONTAINING CINNAMIC ACID IN VARYING CONCENTRATIONS AT pH 5·0

Concentration of cinnamic acid (per cent w/v)		1·0	0·6	0·4	0·3	0·2	0·1	0·05	0·03	0·005	Minimal fungistatic concn. (per cent)
Medium	Test organism										
Basal	T	—	—	—	—	O+	o++	o+++	+	+s	0·3
	N	—	O+s	O+	O+	O++	o+++	++	+	+s	1·0
	A	—	—	—	O+	o++	o+++	++	+	+s	0·4
	B	—	—	O+s	O+	O++	o++	++	+	+s	0·6
	C	—	—	O+	O+	O+	o++	++	+	+s	0·6
	D	—	—	O+s	O+	O++	o+++	++	+	+s	0·6
	E	—	O+s	O+	O+	O+	o+++	++	+	+	1·0
With 5 per cent glucose	T	—	—	—	O+	O+	o++	+++	+++	+++	0·4
	N	—	O+s	O+	O+	O+	o++	o+++	+++	+++	1·0
	A	—	O+s	O+	O+	O+	+++	+++	+++	+++	1·0
	B	—	O+s	O+	O+	O+	+++	+++	+++	+++	1·0
	C	—	O+	O+	O+	O+	o+++	+++	+++	+++	1·0
	D	—	O+	O+	O+	o++	o++	+++	+++	+++	1·0
	E	—	O+	O+	O+	o++	o+++	+++	+++	+++	1·0
With 5 per cent sucrose	T	—	—	—	O+	O+	o++	+++	+++	+++	0·4
	N	—	O+s	O+	O+	o+	o++	+++	+++	+++	1·0
	A	—	O+s	O+	O+	O+	+++	+++	+++	+++	1·0
	B	—	O+s	O+	O+	O+	o+++	+++	+++	+++	1·0
	C	—	—	O+s	O+s	O+	o++	+++	+++	+++	0·6
	D	—	O+s	O+	O+	O+	o++	+++	+++	+++	1·0
	E	—	—	O+	O+	o++	o++	+++	+++	+++	0·6

The strains used are denoted by symbols, a key to which appears below Table I.

O strong smell of toluene-like substance;
o just detectable toluene-like odour;
— no visible growth;
+s very slight mycelial growth;
+ submerged mycelial growth;
++ heavier growth with presence of spores, the growth covering part of the surface of the medium;
+++ heavy sporing growth forming a complete layer at the surface of the medium and with the production of pigment in the medium.

concentrations which were employed, raises the tolerance of *Penicillium* species to cinnamic acid, but has a doubtful influence on resistance to benzoic acid.

An attempt was made to isolate and identify the substance of toluene-like odour. One litre volumes of Czapek-Dox basal medium containing 0·2 per cent w/v cinnamic acid at pH 5·0 were inoculated with spores obtained from subculture of the original infected syrup, and the media were kept aerated and agitated by a stream of filtered, compressed air which was saturated with water vapour at the incubation temperature of 24°. The air-stream passing from the media was passed through a cold trap surrounded with ice. After incubation for five days, the condensate

in the cold trap was found to possess a strong odour resembling toluene and it appeared to be an aqueous solution of the metabolic product. Examined spectrophotometrically, the solution was found to show a maximal optical density at 242 mμ, compared with which a saturated aqueous solution of toluene showed peaks of absorption at 262 and 268 mμ. When equal portions of the condensate were treated with an equal volume of either 0·05N HCl or 0·05N NaOH, a difference in optical density was found between the acid and alkaline solutions, the maximal difference occurring at 257 mμ. It was considered that the true λ_{max} of the toluene-like substance was obscured by other metabolic products whose ultra-violet absorption was affected by change in pH.

Possible conditions for growth of *Penicillium* species in Syrup of Tolu were investigated as follows. Samples of the syrup were prepared to contain varying sucrose concentrations: 50, 55, 60, 62, 63, 64, 65, and 67 per cent w/w, in addition to a sample containing the Pharmacopoeial concentration of 66 per cent w/w. The pH of the preparations lay within the range of 2·8–2·9, compared with the value of 3·96 for the sample of infected syrup. A second series of samples were prepared in which the above sucrose concentrations were maintained, but the pH adjusted to 4·0–4·2 by the addition of sodium hydroxide. A 10 ml. portion of each of the samples prepared was placed in each of two sterile, stoppered test tubes, thus giving two series of tubes containing all of the samples which were prepared. One series was infected with one drop of the original infected syrup, and the other series was inoculated with approximately 1 mg. of air-dry spores obtained by subculture of the infected syrup. After incubation for three months at 24°, it was found that none of the samples infected with the smaller inoculum showed evidence of growth. Of the samples adjusted to pH 4·0–4·2 and subjected to a heavy inoculum of spores, a number showed well-defined growth and had developed the toluene-like odour. Growth occurred in this series of samples at sucrose concentrations of 55, 60, 62, 64, 65 and 67 per cent w/w, so that there appeared to be no relation between sucrose content, over the concentration range examined, and suitability for growth at the pH studied. None of the heavily inoculated syrups of pH 2·8–2·9 showed growth.

Discussion and Conclusions

It seems that benzoic or cinnamic acids can be used by several species of *Penicillium* as a sole nutritional source of carbon, provided the concentration of the acid lies below inhibitory levels. Similar behaviour was found with seven species, or strains within species, or *Penicillium*, these being morphologically or culturally distinct, and it is therefore possible that the capacity to utilise these acids is widespread in this genus. Growth in near-inhibitory concentrations of cinnamic acid was accompanied by the production of a substance of toluene-like odour. Toluene would appear to be a reasonable product of breakdown of cinnamic acid by a rupture at the unsaturated linkage, leaving a 2C compound, for example acetate, which could be metabolised.

From the attempts to establish the conditions required for growth of species of *Penicillium* in Syrup of Tolu, it is suggested that:

(i) variation in sucrose concentration within the limits of 55–67 per cent w/w had no effect in permitting growth;

(ii) growth would occur in samples adjusted to a less acid reaction (pH 4·0–4·2); and

(iii) growth in less acid samples took place only when the inoculum was large (1 mg. spores), but not when the small inoculum (0·016 ml. of original infected syrup) was used.

Dependence of microbial growth on the pH of solutions of aromatic acids is in accordance with the findings of Hoffman, Schweitzer and Dalby[2,3], Rahn and Conn[4], and Goshorn and Degering[5] that inhibition of fungi, yeasts and bacteria respectively by benzoic acid and certain of its derivatives is largely dependent upon the concentration of undissociated acid, the benzoate ion having a much lower inhibitory activity. It will be observed that growth did not take place in all samples adjusted to a less acid reaction and heavily inoculated with spores, from which it appears that factors other than those which have been considered here may operate to decide initiation of fungal growth.

It is concluded that fungal growth can occur in Syrup of Tolu when the concentration of aromatic acids is deficient, when the reaction of the product is less acid than that expected, and when the product is exposed to very large fungal inocula.

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