CVII. THE METABOLISM OF *ASPERGILLUS TAMARII* KITA. KOJIC ACID PRODUCTION

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THE ability of the organisms of the *Flavus-oryzae-tamarii* group of *Aspergilli* to produce kojic acid (5-hydroxy-2-hydroxymethyl- γ -pyrone) from various substrates has been widely reported. Birkinshaw *et al.* [1931] have suggested two possible mechanisms for the production of kojic acid from a pentose. The first assumes the breakdown of the substrate to acetaldehyde with a subsequent series of condensations to kojic acid. This is supported by the general ability of these organisms to produce ethyl alcohol. The other assumes that the carbon source is anabolized into a reserve material which is later hydrolysed and its units transformed to kojic acid.

The significance of phosphoric esters in metabolism leads us to consider their possible role in kojic acid production. Moreover, since these organisms have been shown to be ethyl alcohol producers it is of interest to determine whether kojic acid arises by way of ethyl alcohol or one of its intermediates such as acetaldehyde. Johnson [1934] and Walker [1936] have shown that alcohol production by species of *Aspergillus* could be inhibited anaerobically. Similar results have been observed with *Penicillia*. Under these conditions they found that citric acid production was not inhibited but rather increased, indicating that the mechanism of alcoholic fermentation was not involved. Nord *et al.* [1936] have shown that phosphorylation is not essential for alcohol production by species of *Fusaria*. Johnson *et al.* [1937] indicates that since the action of iodoacetate is on cozymase there should be no difference between the aerobic and anaerobic actions. In the same communication it was shown that acid production was actually increased in the presence of iodoacetate.

The present communication concerns the mechanism of kojic acid production by *A. tamarii*. Various substrates have been investigated, including mycelial substance; the production of aldehyde during fermentation has been followed by means of dimedon fixation and the effects of iodoacetate and fluoride determined.

EXPERIMENTAL

A culture of *A. tamarii* Kita (kindly supplied by Prof. H. Raistrick), from the culture collection of the London School of Hygiene and Tropical Medicine, was used in all of these studies. The culture was checked as to purity and showed characteristic morphology. On the usual Czapek-Dox medium the organism consistently produced kojic acid after 6 days' incubation at 20°. Transplants were made frequently on Czapek-Dox medium of the following composition: glucose, 50 g.; NaNO₃, 2 g.; KH₂PO₄, 1 g.; KCl, 0.5 g.; MgSO₄, 7H₂O, 0.5 g.; FeSO₄, 7H₂O, 0.01 g.; distilled water, 1000 ml.

The pH of this medium was 4.85 (quinhydrone electrode) after sterilization by steaming for 1 hr. on three successive days.

Availability of different carbon sources. Compounds which might be expected to arise in the course of kojic acid production from hexoses or those which showed peculiarity of structure such that they might offer suggestions as to essential linkages were selected for experiment. The media employed were made up in the usual manner with 5% of the carbon source to be studied substituted for the glucose. The media were distributed in 200 ml. wide-mouth bottles in 50 ml. quantities and sterilized in the usual way. The media were then sown with 1.0 ml. of a heavy 9-day-old spore suspension of the organism and incubated at 20°. The substrates employed were of the highest purity obtainable, Eastman Kodak (pure) Kahlbaum or Pfanstiehl.

The metabolism solutions were tested periodically for kojic acid production by the addition of a drop of 10% FeCl_3 to a few ml. of the culture solution which gives a deep red coloration with kojic acid in concentrations above 1 : 200,000.

Transfer technique of mould metabolism study. It was observed that growth on the various substrates was extremely poor and a special technique was therefore employed which consisted of growing the organisms on Czapek-Dox medium (containing glucose) for 9 days. At this time the growth was good and the FeCl_3 colour very strong. The medium was then washed away from the growth and the growth itself washed repeatedly with sterile Czapek-Dox basal salt solution until the washings gave no further FeCl_3 colour. At this point the new medium containing the carbon source to be studied was introduced aseptically and incubation continued at 20°.

Kojic acid production was followed as in the first experiment and after 50 days' incubation the metabolism solutions were made acid and extracted with ether. The following compounds gave no kojic acid and gave growth only in instances marked (+): sodium formate, acetate (+), propionate, oxalate, citrate, tartrate (+), fumarate (+), lactate (+), succinate (+), pyruvate (++), asparaginate (+), malonate, malate ($\pm$), glycollate; glycine (+), alanine (++), cysteine (+), ethylene, propylene and trimethylene glycols, propylene oxide, pentaerythritol, *D*-inositol (cf. Tamiya [1932] and Katagira & Kitahara [1929] who obtained kojic acid from *A. oryzae*), *L*-ascorbic acid, furfuraldehyde, Czapek salts, distilled water.

Using the special transfer technique the following compounds gave slow and slight production of kojic acid: *D*-adonitol, dulcitol, mannitol, sorbitol, galactose and xylose. More rapid production was observed with glycerol, fructose, gluconolactone, mannose, β -glucose, glucose and sucrose, respectively, sucrose being the most effective; neither erythritol nor α -methyl glucoside gave any kojic acid.

The results indicate that, as has been shown for the members of the *Flavus-oryzae* group, of the large group of carbon compounds investigated only glycerol, the pentoses, pentahydric alcohols, hexoses, hexahydric alcohols and disaccharides served as sources of carbon for *A. tamaraii* in the production of kojic acid.

Cellular material as a substrate for kojic acid production. To determine whether some reserve material was built up by the cell and subsequently broken down to kojic acid several experiments using mycelia of *A. tamaraii* obtained from growth on hexoses, glycerol, erythritol, xylose and sucrose as the source of carbon in the Czapek-Dox medium were carried out.

The mycelia were washed free from kojic acid, dried and ground to a fine powder. Czapek-Dox medium in which the glucose was replaced by 5% of dried mould preparation was made up and sterilized in the usual manner; 9-day growths were washed thoroughly, transferred to the mould media and incubated at 20°. Even after 90 days' incubation kojic acid could not be

detected which seems to indicate that the mycelium of *A. tamarii* does not contain a reserve polysaccharide or cellular constituent which can give rise to kojic acid after prolonged incubation under the conditions of this experiment.

Inhibition of phosphorylation and organic phosphate breakdown by fluoride and iodoacetate. A series of experiments was undertaken to determine whether the production of kojic acid depends upon an initial phosphorylation of substrate molecules with subsequent breakdown or rearrangement to give rise to kojic acid. The general method employed by Meyerhof & Kiessling [1935] using sodium fluoride and sodium iodoacetate was used in these experiments. The concentrations of inhibitor employed were *M*/300, *M*/400 and *M*/500. These concentrations were selected since Walker [1936] has reported that *M*/500 iodoacetate prevents the production of ethyl alcohol by *A. niger*. Experiments with *A. tamarii* also showed no ethyl alcohol production when iodoacetate was used in these same concentrations.

A series of 2 oz. wide-mouth bottles each containing 25 ml. of Czapek-Dox glucose medium was made up and divided into six groups containing respectively, *M*/300, *M*/400 and *M*/500 sodium fluoride; *M*/300, *M*/400 and *M*/500 sodium iodoacetate. The media were sterilized as usual, the pH determined by quinhydrone electrode, adjusted to 4.85 and inoculation was made with 1.0 ml. of a fresh suspension of spores of a 9-day-old culture of the organism. Incubation was at 20°. At intervals, duplicate, random bottles were examined. Analyses were carried out to determine (1) dry weight of the mycelial growth, (2) residual glucose (by polarimeter), (3) pH (by quinhydrone electrode), (4) titratable acidity and (5) kojic acid production as determined by the iodine absorption method suggested by Birkinshaw & Raistrick [1931]. At the same time 350 ml. portions of the Czapek-Dox medium containing sodium iodoacetate or sodium fluoride (*M*/300) were made up in 1 l. conical flasks. These were sown after sterilization as usual and incubated at room temperature (20–22°) for 27 days. At the end of this period the flasks were removed and the kojic acid produced was isolated according to Birkinshaw *et al.* [1931]. The material was identified by crystal structure, m.p. and mixed m.p. with an authentic sample.

By carrying out determinations at frequent intervals over 30 days the following general observations were made on the effects of fluoride and iodoacetate on the general metabolism of *A. tamarii*. There is no significant effect on the pH or the titratable acidity changes during the course of metabolism with the concentrations employed, except in the case of *M*/300 iodoacetate which inhibited growth during the first 10 days. The final pH and titratable acidity after 30 days were practically the same as in the control.

Tables I and II show that in the presence of *M*/300 iodoacetate growth is completely inhibited during the early stages of incubation but that when growth is established metabolism is very active and in 8 days the treated mould consumes as much glucose as the control does in 20 days. No significant effect on glucose metabolism is noted with lower concentrations of iodoacetate. During the early stages of growth sodium fluoride appears to exercise no effect on glucose breakdown. From the 15th day onwards the control utilizes only very little glucose, whereas the fluoride-treated growths continue to metabolize the glucose at a rapid rate.

Sodium fluoride appears to exercise no effect on growth as indicated by the dry weight of mycelium produced. *M*/300 and *M*/400 iodoacetate inhibit the establishing of a growth but do not appear to affect the growth once it is established. *M*/500 iodoacetate actually appears to accelerate growth slightly.

Table I. *Growth and glucose dissimilation of A. tamarii Kita grown on Czapek-fluoride and Czapek-iodoacetate media*

Incubation days	Control		Sodium fluoride (glucose %)			Sodium iodoacetate (mycelium g./flask)		
	Glucose %	Mycelium g./flask	M/300	M/400	M/500	M/300	M/400	M/500
4	4.40	0.059	4.40	4.20	4.32	0.000	0.046	0.051
6	3.68	0.109	3.86	3.68	3.50	0.000	0.055	0.110
9	2.52	0.158	2.68	2.00	2.52	0.000	0.116	0.203
12	1.77	0.195	2.24	1.52	1.77	0.061	0.168	0.213
15	1.54	0.199	1.23	1.07	1.25	0.158	0.200	0.205
20	1.42	0.195	0.71	0.71	0.84	0.164	0.192	0.198
25	1.31	0.198	0.65	0.65	0.77	0.182	0.187	0.217
30	—	0.201	0.35	0.40	0.57	0.185	0.198	0.225

Table II. *Kojic acid production by A. tamarii on Czapek-fluoride and Czapek-iodoacetate media*

Incubation days	Control	Sodium fluoride (mg./flask)			Sodium iodoacetate (mg./flask)		
		M/300	M/400	M/500	M/300	M/400	M/500
9	8.5	28.2	70.5	27.3	—	4.7	18.8
12	29.9	23.4	84.0	87.0	Trace	41.7	31.3
15	58.4	81.0	104.0	92.5	102.2	115.0	52.0
20	54.0	95.3	106.5	87.5	171.0	120.0	69.8
25	—	95.3	118.0	121.0	156.5	115.0	67.5
30	75.4	84.0	104.0	102.0	138.0	117.0	57.6

From Table II it appears that, in spite of the rather slight effects of the inhibitors on the general metabolism, iodoacetate and fluoride do increase kojic acid production.

Using the transfer technique, mycelia grown in 1 l. flasks when transferred to media containing *M*/100 inhibitor were still able to produce kojic acid. The kojic acid was isolated and identified.

Kojic acid production in phosphate-free media. To determine whether phosphate is necessary for kojic acid production an experiment was carried out in which 9-day growths from 1 l. flasks containing 350 ml. Czapek-Dox glucose medium were washed thoroughly with phosphate-free salt solution and then transferred to Czapek-Dox glucose medium which contained no phosphate. It was notable that the sugar was metabolized somewhat more rapidly, and kojic acid production was more rapid but not increased as indicated in Table III.

Table III. *Glucose decomposition and kojic acid production by A. tamarii in Czapek-Dox and Czapek-Dox (P-free) media*

Incubation days	Czapek-Dox (P-free)		Czapek-Dox	
	Residual glucose %	Kojic acid mg./ml.	Residual glucose %	Kojic acid mg./ml.
0	5.00	—	5.00	—
3	3.24	3.28	2.86	1.76
6	1.14	9.68	1.14	7.16
10	0.00	9.42	0.76	8.84
13	0.00	8.40	0.57	8.84
15	0.00	8.40	0.00	9.68
20	0.00	7.41	0.00	8.41

That the more rapid production was not due to change in the *pH* of the medium was determined by transfer to phosphate medium containing K_2HPO_4 at the *pH* of the phosphate-free medium. The production of kojic acid was not accelerated.

To determine whether phosphorylation is essential for kojic acid production from glycerol and from xylose experiments similar to those carried out with glucose were undertaken. It was found that kojic acid was produced on both glycerol-phosphate-free and xylose-phosphate-free media. There was, however, no apparent difference in the rate of kojic acid production or in the quantity produced as was shown to be the case in kojic acid production on glucose-phosphate-free medium.

Production of kojic acid in presence of aldehyde-fixing agents. In order to fix acetaldehyde that might be formed in the course of metabolism by the organisms they were grown in the presence of dimedon (dimethyldihydroresorcinol) [Neuberg & Reinfurth, 1920], or sodium sulphite. Controls were tested by the Rimini reaction and found to contain no acetaldehyde. Media were prepared as in the experiments to determine the effects of fluoride or iodoacetate. The dimedon was added aseptically (10 g./600 ml. medium) and the bottles sown with a heavy suspension of spores. Incubation was at 20°.

At the same time 1 l. flasks containing 350 ml. portions of the Czapek-dimedon medium were sown and incubated. The metabolism solution was worked up after 27 days' incubation, the kojic acid isolated and identified. No aldomedon could be isolated.

The results (Table IV) indicate that kojic acid production is not inhibited by dimedon and that metabolism in the presence of this fixing agent followed an apparently normal course. Growth was somewhat stimulated.

Table IV. *Kojic acid production by A. tamarii in presence of dimedon*

Incubation days	Growth g./flask	<i>pH</i>	Titratable acidity ml. 0.1 <i>N</i> NaOH/10 ml.	Kojic acid mg./flask
0	—	4.80	3.35	—
6	0.067	5.05	4.01	Trace
12	0.220	5.95	8.72	58.4
25	0.231	6.15	11.52	114.0

Another experiment using sodium sulphite as the fixing agent was carried out. The transfer technique was employed using growths from 1 l. flask cultures which were transferred to Czapek-Dox medium containing Na_2SO_3 (0.02 *M*). Incubation was at 20°. Owing to the alkaline reaction of the medium metabolism was considerably diminished and kojic acid production was slow but definitely positive.

Availability of different sugars for kojic acid production. A group of sugars consisting of sucrose, glucose, fructose, galactose and α -methyl glucoside were used as the source of carbon in Czapek-Dox medium; 9-day growths from 1 l. flask cultures were transferred to media containing the above sugars.

The results of the experiment are summarized in Table V. The slow and poor production from galactose is striking. Sucrose appears the best source of carbon for kojic acid production.

Table V. *Kojic acid production by A. tamarii Kita from various sugars*

Incubation days	mg. Kojic acid/ml.			
	Glucose	Sucrose	Fructose	Galactose
3	1.76	2.30	0.71	—
6	7.16	5.87	0.43	—
10	8.84	10.00	3.56	0.142
13	8.84	10.90	8.13	0.93
15	9.68	11.75	6.28	1.42
20	8.41	9.68	7.70	1.71

CONCLUSIONS

From a study of the growth and metabolism of *A. tamarii* Kita in the presence of fluoride and iodoacetate, and from a study of kojic acid production by transfer cultures of the organism on phosphate-free media it has been shown that the production of this metabolic product from glucose, xylose and glycerol probably does not take place by way of phosphoric esters. Moreover, the dissimilation of glucose in a phosphate-free medium gives rise to undiminished and more rapid production of kojic acid. In the xylose and glycerol media, as well, no changes in acid production could be observed.

The appearance of kojic acid does not seem to involve a preliminary breakdown to acetaldehyde with subsequent resynthesis since the addition of fixing agents such as dimedon or sulphite did not prevent kojic acid production and no fixation products could be isolated.

Under the conditions of these experiments the organism does not appear to be able to produce kojic acid from mould substance produced on a variety of substrates. This seems to indicate that the acid does not arise by way of the breakdown of previously synthesized storage products such as mould polysaccharides.

The organism can utilize a variety of sugars in kojic acid production. Sucrose is most readily assimilated with glucose only slightly less readily so. Galactose and fructose are less readily available, the former being only very slowly attacked.

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