CLXXXVI. ALCOHOLIC FERMENTATION BY *ASPERGILLUS FLAVUS*, BREFELD.

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A. flavus and *A. oryzae* present a number of at least superficial resemblances, and occur in nature under similar conditions. Thom and Church [1926] state that though the typical *A. flavus* may be distinguished from the typical *A. oryzae* with great contrast, yet, in a whole series of the naturally occurring organisms, the separateness of these two groups breaks down.

The latter mould has been shown by Yabuta [1912, 1916, 1924] to produce kojic acid from carbohydrates. The accidental discovery that a yellow-green *Aspergillus* belonging to the *A. oryzae* group produced crystals of calcium kojate when grown on cane-sugar solution in presence of chalk, led me to try if *A. flavus* also gave this compound.

I found no kojic acid produced by a typical strain of *A. flavus*, but the experiment led to the unlooked-for result of considerable alcohol production by this mould. Under the same conditions *A. oryzae* produced no alcohol.

A perusal of the literature fails to reveal any undoubted capacity among the *Aspergilli* of fermenting sugar to alcohol, when allowed to grow on a liquid with exposure to air, such as obtains in an ordinary flask culture. Small quantities are formed when the mycelium of certain strains is deliberately submerged in the sugary liquid, or when the air supply is otherwise deliberately withheld.

Pasteur [1876] experimented on the alcoholic capacities of *Penicillium* and *Aspergillus glaucus* (he later had some doubts as to the identity of this "*A. glaucus*"), and reached the conclusion that these moulds when deprived of free oxygen, as by submerging the germinating spores or the mycelium, would decompose sugar after the manner of yeast; but in full growth, with plenty of air, *Aspergillus* would not yield alcohol.

Wehmer [1910] gives a good account of the question of alcohol production by *Aspergillus*, and concludes that the possibility of some strains producing alcohol in more than mere traces cannot be altogether brushed aside, but the matter needs further investigation.

Waksman [1922] gives a bibliographic review up to 1922 of the enzymes of micro-organisms. The conclusion that the anaerobic respiration of *A. niger*, when the mycelium is submerged, takes the nature of alcoholic fermentation, is supported by Kostytschew's experiment in which a three day-old mycelium,

submerged by glass beads and deprived of further air, produced alcohol and carbon dioxide in practically the usual yeast ratio. Zymase is not specifically attributed to other *Aspergilli* in this review.

Thom and Church [1926], in a summary of the enzymic and fermentative activities of the *Aspergilli*, recall that, in the case of the saké fermentation, the earliest workers attributed the alcohol production to *A. oryzae*, but that yeasts were subsequently proved to produce the alcohol.

Oppenheimer [1926] finds that *Aspergillus* can be induced to give anaerobic alcoholic fermentation, with certain precautions, but that aerobically absolutely no alcoholic fermentation results. He upholds Wehmer in questioning Elfing's statement of the production of 4.2 % alcohol by "*P. glaucum*." Oppenheimer states too that anaerobically the mould fungi are extremely sensitive to acids, and that one way of overcoming this is by keeping the solution neutral with chalk.

A. flavus does not appear to have received special consideration in this connection.

The very definite production of ethyl alcohol by *A. flavus* in the experiments about to be described, under conditions which would ordinarily be termed aerobic, is not necessarily at variance with the findings in the literature cited above. The conditions were exactly the same as those which suffice for such typically aerobic changes as the oxidation of glucose to gluconic acid and citric acid by *A. niger*. Indeed *A. flavus* itself produces some citric acid in these conditions. Whatever differences there are in the access of the mycelial cells of the two fungi to the air are intrinsic to the respective habits of growth of the two species. As noticed below, the mycelial felt of *A. flavus*, under these conditions, is rather remarkable for its apparent thickness and open texture; and since its upper surface remains practically only just above water throughout its growth, a certain degree of anaerobiosis must be enjoyed by the cells of its lower layers. Thus, if it is right to connect the alcohol production with this feature of the mould's growth, we have merely another instance of the essentially anaerobic nature of the alcoholic fermentation.

EXPERIMENTAL.

The culture used throughout was the type culture of *A. flavus* from the National Collection of Type Cultures at the Lister Institute.

The medium selected was based on Currie's formula quoted below: a medium originally devised for optimum citric acid yields with *A. niger* [Currie, 1917].

Sucrose	125-150 g.	} per 1000 cc.
NH ₄ NO ₃	2.0-2.5 g.	
KH ₂ PO ₄	0.75-1.0 g.	
MgSO ₄ ·7H ₂ O	0.20-0.25 g.	
HCl to p _H 3.4-3.5 (5-4 cc. N/5)		

Small conical flasks, filled to a depth of about half an inch with the above medium, were used, and a little chalk, autoclaved separately, was added to each. This procedure is necessary to avoid undesirable changes in the medium. Fernbach [1916] states that otherwise partial and even complete precipitation of phosphates may occur at the temperature of sterilisation.

After inoculation from mature subcultures of *A. flavus*, the flasks were incubated at 28° for 10–12 days.

The cultures early acquired a rather heavy, somewhat fruity alcoholic smell, and bubbles of gas were disengaged from the chalk. The growth of the mycelium was rapid and in most cases it remained free from spores. The mycelium, though not dense, was unusually thick, reaching 3 mm. in 10 days. Ordinary pressure from the fingers expressed a large volume of liquor from the mould; microscopical examination of a cross-section showed an open network of hyphae. This mode of growth is doubtless important in relation to alcohol production.

The liquor from the cultures, augmented with the liquor expressed from the moulds, was filtered and distilled. The distillate gave the iodoform and dichromate reactions. After redistillation, saturation with potassium carbonate, and separation, a considerable fraction distilled between 78° and 79° and gave the tests for ethyl alcohol.

The fermented liquor also contained a considerable quantity of calcium salts in solution. Part of this is accounted for by calcium citrate. This was precipitated on boiling, and the presence of the acid confirmed by Denigès's test. Liquors from cultures which spored contained more citrate than those which did not spore. The latter, however, invariably contained in solution much calcium in the form of compounds precipitable by strong alcohol. Further work on the calcium salts in solution is in progress.

A series of cultures was also studied to obtain some idea of the rate of production of alcohol by *A. flavus* and of the quantity of alcohol produced. The solution from the cultures was distilled and the distillate tested for alcohol by the colorimetric method of Bertrand and Thomas [1920]; the total reducing substances and residual cane-sugar were estimated by the decoloration method, before and after hydrolysis with HCl. All the cane-sugar appeared to be inverted by the eighth day.

The data are summarised in the following table.

Differences with regard to sporulation appear to account for the major irregularities in the results, whilst in any such series of cultures, where ideas of continuous changes can only be got from examination of different individuals, some irregularities are to be expected.

Table I.

Culture	Age in days	Wt. of wet mould (g.)	Wt. of dry mould (g.)	Sugar con- sumed (g.)	Alcohol pro- duced (g.)	g. alc. per 100 cc. liquor	g. alc. per 100 g. sugar consumed	Condition as regards sporu- lation
1	2½	8.0	0.20	1.55	0.11	0.21	7.1	No spores
2	3½	9.2	0.35	2.35	0.18	0.32	7.6	"
3	4½	9.6	0.38	3.35	0.62	1.19	18.5	"
4	5½	10.7	0.41	3.75	0.90	1.71	24.0	"
5	6½	10.7	0.72	5.31	1.00	1.96	18.8	"
6	6½	—	1.20	5.95	0.62	1.26	10.4	Sporing
7	7½	12.5	0.50	5.25	1.25	2.43	23.8	No spores
8	8½	9.9	1.50	6.73	0.38	0.81	5.6	Sporing
9	9½	14.0	1.00	6.85	1.00	2.02	14.6	No spores
10	9½	12.9	1.00	—	1.17	—	—	"
11	10½	10.3	1.80	6.85	0.25	0.53	3.6	Sporing
12	12½	15.7	0.87	7.49	1.30	2.82	17.3	No spores
13	13½	14.3	0.77	7.33	1.30	2.76	17.7	"
14	22½	—	0.97	7.80	0.50	1.11	6.40	"
15	22½	12.3	2.19	7.85	0.002	0.004	0.03	Sporing
16	23½	10.0	1.60	7.85	0.00	0.00	0.00	"

SUMMARY.

A. flavus was not found to produce kojic acid under conditions in which *A. oryzae* is known to give it.

In presence of chalk, *A. flavus*, without any precautions to exclude air from the cultures, produces considerable quantities of ethyl alcohol.

In a series of cultures the alcohol amounted to 2-3 % in the fermented liquor, and represented 15-25 %, by weight, of the sugar consumed.

Under the same conditions, *A. oryzae* was not found to produce any alcohol.

During the fermentation with *A. flavus* considerable quantities of chalk are dissolved, some calcium citrate is formed and some soluble calcium salts precipitable by alcohol.

When the mould forms spores, the yield of alcohol is much reduced and the quantity of citrate increased.

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