to form the amylases from some inactive combination or state.

A comparison of the influence of heavy water upon the plant amylases studied here and upon pancreatic amylase previously investigated[1] is of interest.

The activity measurements with all of these different amylases lead to the same conclusion: that even 99% heavy water has no appreciable effect upon the activities of these enzymes provided the hydrolysis of the starch is carried out under conditions which favor the action and minimize the inactivation of the amylase concerned. This finding assumes added significance when it is remembered that the different amylases were studied as highly purified products, in a condition which increases their sensitivity to their chemical environment.

The measurements of the stabilities of these amylases, on the other hand, give different results. With pancreatic amylase, there is decreased stability of the enzyme in the presence of heavy water,[1] while with the amylases of barley (β) and of malted barley (α and β) an increased stability of the enzyme in the presence of heavy water is observed. No attempt is made to explain these differences at this time.

Summary

Working with highly purified heavy water and with highly purified preparations of the amylases of barley (β) and of malted barley (α and β), it has been found that heavy water (99%) has no appreciable influence upon the hydrolysis of starch as catalyzed by any of these enzymes provided the conditions of the hydrolysis are such as to minimize the deterioration of the amylase and to favor its action. Similar results previously have been reported for highly purified pancreatic amylase.

Stability measurements show that inactivation of these plant amylases is much less rapid and less pronounced in highly purified heavy water than in similarly purified ordinary water.

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[CONTRIBUTION FROM NEW JERSEY AGRICULTURAL EXPERIMENT STATION]

The Production of Fumaric Acid by Molds Belonging to the Genus *Rhizopus*[1]

BY JACKSON W. FOSTER AND SELMAN A. WAKSMAN

Fumaric acid is one of the less commonly encountered products of mold metabolism. Aside from the important biochemical fact that this acid is unsaturated, it is of particular interest because, with only two exceptions, the organisms capable of producing this acid were always found to belong to the family *Mucoraceae*. The two exceptions are the isolation by Wehmer[2] of *Aspergillus fumaricus*, which gave almost 70% conversion of the sugar to fumaric acid, and the separation by Raistrick and Simonart,[3] of an unstated quantity of this acid from the culture medium of a *Penicillium*. However, it was later found[4] that upon continued cultivation Wehmer's organism lost the ability to produce fumaric acid.

Many species of *Rhizopus* have been found capable of producing varying quantities of fumaric acid, frequently accompanied by one or more other acids. Ehrlich,[5] using a culture designated as *Mucor stolonifer* (*Rhizopus nigricans*), first established, in 1911, this property for filamentous fungi. No neutralizing agent was added to the cultures, as a result of which low yields of acid were obtained. Ehrlich considered this acid to be an intermediate product in the breakdown of the carbohydrate by the fungus, rather than an end-product of metabolism. Takahashi, *et al.*,[6] found that *Rhiz. japonicus* gave a 16% yield of fumaric acid, accompanied by a small amount of lactic acid, whereas other species of *Rhizopus* produced smaller quantities of the fumaric acid; ethyl alcohol, formic, malic and acetic acids were also detected in the culture solutions. Takahashi suggested that the lactic acid originated from the fumaric acid by decarboxylation; gluconic acid was believed[7] to be an intermediate

(1) Journal Series Paper, N. J. Agricultural Experiment Station, Department of Soil Chemistry and Microbiology.

(2) C. Wehmer, *Ber.*, **51**, 1663 (1918).

(3) H. Raistrick and P. Simonart, *Biochem. J.*, **27**, 628 (1933).

(4) W. Thies, *Centrbl. Bakt.*, II, **82**, 321 (1930).

(5) F. Ehrlich, *Ber.*, **44**, 3737 (1911); **52**, 63 (1919).

(6) T. Takahashi, K. Sakaguchi and T. Asai, *Bull. Agr. Chem. Soc. (Japan)*, **2**, 5 (1926); **3**, 59 (1927).

(7) T. Takahashi and T. Asai, *Proc. Imp. Acad.* (Japan), **3**, 86 (1927).

product between glucose and fumaric acid. Gottschalk[8] suggested that fumaric acid is formed by *Rhizopus* through the pyruvic acid stage, according to Neuberg's fermentation scheme. However, neither Ehrlich and Bender[9] nor Butkewitsch and Federoff[10] could find any evidence for this assumption. The latter made a detailed study of the formation of fumaric acid by *Rhizopus nigricans*. In the presence of calcium carbonate a yield of 31.4% was obtained, and only 5% in the absence of the neutralizing agent. Mature fungus pellicles were found capable of converting sugar to fumaric acid even in the absence of nutrient minerals; under these conditions 20% yields were obtained in the presence of calcium carbonate and only 2% in its absence. The following scheme was proposed for the formation of fumaric acid: ethyl alcohol is first formed and is oxidized to acetic acid; the condensation of the latter takes place according to the Thunberg–Wieland reaction, giving succinic acid which is then dehydrogenated to fumaric acid.

Kanel[11] investigated the production of lactic and fumaric acids by two strains of *Rhizopus* in relation to the Eh of the medium. Lactic acid formation was associated with a low *r*H, whereas the accumulation of fumaric acid was favored by a high *r*H. More recently, Ward, *et al.*,[12] and Waksman and Foster[13] found small amounts of fumaric acid constantly associated with various species of *Rhizopus* which produced largely lactic acid. Bernhauer and Thole[14] have shown that the initially formed fumaric acid may be further transformed into malic acid by *Rhizopus;* when 0.005% zinc sulfate was added to a technical glucose medium the yield of fumarate was increased from 20.7 to 41.6%. These results were contrary to the findings of Waksman and Foster,[15] who reported that zinc favored growth of *Rhizopus* at the expense of the accumulation of fumaric acid. These authors[16] further described a case of association of fumaric acid production with sexuality in a strain of *Rhizopus nigricans*.

(8) A. Gottschalk, *Z. physiol. Chem.*, **152**, 136 (1926); **172**, 314 (1927); **182**, 311 (1929).

(9) F. Ehrlich, F. and I. Bender, *Z. physiol. Chem.*, **170**, 118; **172**, 317 (1927).

(10) Wl. Butkewitsch and M. W. Federoff, *Biochem. Z.*, **206**, 440; **207**, 302 (1929); **219**, 87, 103 (1930).

(11) E. Kanel, *Microbiologia*, **4**, 636 (1935).

(12) G. E. Ward, L. B. Lockwood, O. E. May and H. T. Herrick, THIS JOURNAL, **58**, 1286 (1936); *J. Agr. Research*, **53**, 849 (1936).

(13) S. A. Waksman and J. W. Foster, *ibid.*, to be published (1938).

(14) K. Bernhauer and H. Thole, *Biochem. Z.*, **287**, 167 (1936).

(15) S. A. Waksman and J. W. Foster, *Compt. rend.*, **207**, 483 (1938).

(16) J. W. Foster and S. A. Waksman, *Science*, **88**, in press (1938).

Experimental

In an attempt to study the metabolism of fumaric acid producing fungi, a survey first was made of the ability of various genera and species of organisms belonging to the *Mucorales*, to produce this acid. The following number of strains in each genus were tested:[17] *Mucor* 11, *Rhizopus* 10, *Cunninghamella* 6, *Absidia* 6, *Circinella* 4, *Phycomyces* 2, *Syncephalastrum* 1 and *Mortierella* 1.

The following synthetic medium was used in the preliminary survey

Technical glucose	5.0%
$(NH_4)_2SO_4$	0.2%
$MgSO_4 \cdot 7H_2O$	.05%
K_2HPO_4	.05%
$Fe_2(SO_4)_3$	.001%
$ZnSO_4 \cdot 7H_2O$	.0005%

Two-hundred ml. portions of the medium were placed in 500-ml. Erlenmeyer flasks, sterilized for one hour in flowing steam on two successive days and inoculated with spores. In some cases duplicate cultures were used, in others quadruplicates. The cultures were incubated at 28°. After two to three days' growth, 5-g. portions of sterile calcium carbonate were added to the flasks and incubation continued for ten to seventeen days longer. The flasks were shaken in rotary fashion by hand several times daily to neutralize the acids formed, care being taken not to injure the pellicle.

Periodic observations upon the nature of the growth of the different fungi during this experiment indicated four distinct types: (1) abundant surface pellicle formation with high aerial mycelium; (2) abundant surface pellicle with very low aerial mycelium; (3) sub-surface growth, floating just submerged with little or no aerial mycelium; (4) submerged or bottom spongy growth, usually rather scant.

For chemical analyses, the culture liquid was filtered off, the fungus growth was pressed and washed a few times with water, and the washings added to the filtrate. The total filtrate was made up to volume for analysis. Alcoholic odors were quite pronounced in practically all the cultures. Aliquot portions were analyzed for sugar (modified Bertrand method), ammonia-nitrogen (magnesium oxide distillation), total soluble material and total soluble ash. Because of the survey nature of this experiment and because of the large number of cultures handled, it was believed that the total soluble ash, after accounting for the calcium sulfate produced due to the consumption of ammonia by the organism, was a sufficiently reliable index of the total acid produced. The presence of fumaric acid was determined by the formation of insoluble mercurous fumarate in 5% nitric acid.[18] Without going into a de-

(17) Some of these cultures were obtained from our own stock collection, some were freshly isolated from soils and composts. The authors wish to express their indebtedness to Dr. Charles Thom, of the Bureau of Plant Industry of the U. S. D. A., to Dr. A. F. Blakeslee of the Carnegie Institute of Experimental Biology and Dr. Elizabeth Clark of the N. J. Agricultural Experiment Station, for supplying a number of cultures.

(18) A. Ölander, *Z. physik. Chem.*, **A144**, 49 (1929).

tailed discussion of the data obtained, it is sufficient to state that, of the 41 cultures tested, nine forms, comprising 6 *Rhizopus*, 1 *Mucor*, 1 *Cunninghamella* and 1 *Circinella* were found to be capable of producing fumaric acid, under the conditions of the particular experiment. It is clear, therefore, that the ability to form fumaric acid is not a property characteristic of all strains of *Rhizopus*, and further that other genera belonging to the *Mucorales* possess this ability.

Qualitative tests for lactic acid also were made; ether extracts were made of 10-cc. portions of the cultures and tested by means of the thiophene reagent. Some quantitative analyses were made also, using the method of Friedmann and Graeser.[19] These tests indicated that all but two of the *Rhizopus* cultures produced considerable amounts of lactic acid in addition to the fumaric acid. Inasmuch as these two strains were also found to be decidedly superior in their capacity of producing fumaric acid, they were selected for further study; they will be designated as *Rhizopus nigricans* nos. 35 and 45.

A remarkable peculiarity in the behavior of no. 35 during this preliminary experiment focused interest upon this organism. Of the four replicates in this experiment, three produced 13–15% fumaric acid which was determined by Ölander's[18] modification of the Hahn and Haarmann method, whereas the fourth replicate did not show even a trace of fumaric acid. Much consideration was given to this anomalous behavior and, as will be shown later, it was found to be due to the specific concentration of carbohydrate in relation to available nitrogen. The conditions during this experiment were close to the critical point at which fumaric acid may or may not be produced.

Among the cultures used in the preliminary experiment, there were present two strains of *Rhizopus nigricans*, each comprising a pair of races designated as the (+) or female and the (−) or male races. A marked difference in the production of fumaric acid was found in the case of one pair. A further study was made of this phenomenon, in order to determine specifically what physiological dissimilarity could be attributed to the mere difference in sexuality of two organisms morphologically and genetically identical. Of particular interest was the fact that one of the active forms selected for further study, namely, no. 35, comprised one of the members of one of the two pairs, being a (+) or female race. The four cultures were inoculated into glucose–mineral medium of the above composition and calcium carbonate added after three days. Incubation took place at 28°, for fifteen days. In addition to the usual determinations, calcium in solution was measured by evaporating and igniting an aliquot. Following double precipitation as the oxalate the Ca^{++} was calculated from the potassium permanganate titration.

Table I reveals the fact that, in the case of no. 35 and no. 36, a most striking dissimilarity exists in the physiological activities of the male and female members of this pair of organisms as manifested by their metabolic products. This was already apparent during the course of growth where gross differences in the type and quantity of the fungus pad were discernible.[20] Number 35 was again found to produce fumaric acid vigorously, this behavior being remarkably different from that of its sexual mate. The latter failed to give a trace of fumaric acid even though it had metabolized even more carbohydrate than the former. These phenomena indicate a qualitative difference in the enzyme mechanisms of the two strains of this organism. Satina and Blakeslee[21] could correlate the sexuality of a large number of the *Mucorales* with certain biochemical reactions, such as reducing powers of cell extracts toward potassium permanganate and tellurium salts, Manoilov's reaction, catalase content, etc. These reactions differed quantitatively and not qualitatively as in the case of fumaric acid production.

TABLE I

GROWTH AND ACID PRODUCTION BY MALE AND FEMALE RACES OF RHIZOPUS NIGRICANS

Mg. per 200 ml. of culture solution

Culture, no.	35 (+)	36 (−)	37 (+)	38 (−)
Glucose consumed	6472	6622	8580	7520
Total soluble material	5896	2367	1362	2250
Soluble ash	1531	519	607	660
NH_3-N left	1.3	41.4	23.0	2.5
NH_3-N consumed	80.7	40.6	59.0	79.5
Fumaric acid produced	2059	None	None	None
Conversion,[a] %	31.8	..	..	..
Total Ca^{++} in soln.	837.5	107.7	170.6	201.7
Ca^{++} equiv. of NH_4^+ consumed	115.4	58.1	84.4	113.7
Ca^{++} due to organic acids	722.1	49.6	86.2	88.0
Fumaric acid equiv., calcd. from Ca^{++}	2094	..	..	..
% of total acids accountable as fumaric	98.3	..	..	..
Lactic acid	0	0	0	0

[a] Fumaric acid produced/glucose consumed.

Further examination of the data presented in Table I emphasizes again the fundamental physiological dissimilarity, especially with reference to efficiency of utilization of the carbohydrate substrate. Both strains consumed practically the same amount of glucose. Number 35, however, despite the fact that it had left a large part of the glucose molecule as fumaric acid (31.8% conversion), synthesized more than twice as much cell substance as no. 36; the latter left a bare fraction of the consumed sugar in the form of acids in the medium. The amount of growth may be judged from the ammonia consumed or may be calculated from the total nitrogen in the growth assuming a 5% nitrogen value for *Rhizopus* cell substance, as shown elsewhere.[13] The weight of the fungus pellicle was not obtainable directly, since considerable calcium carbonate was adhering to and was caught within the

(19) T. E. Friedmann and J. B. Graeser, *J. Biol. Chem.*, **100**, 291 (1933).

(20) No. 35 produced a normal, heavy, abundant, deep-gray surface pellicle. No. 36 formed a slimy, white, spore-free, floating, subsurface growth, with limited aerial mycelium. Microscopic examination showed that no. 35 produced typical long slender branching hyphae. No. 36 was composed of a spongy mass of short, thick stunted hyphae; yeast-like budding projections were abundant and true branching rare; few sporangia could be found; the protoplasm appeared to be densely granular; this description is characteristic of the submerged type of growth frequently observed among the *Mucorales*.

(21) S. Satina and A. F. Blakeslee, *Proc. Natl. Acad. Sci.*, **12**, 191, 197 (1926); **13**, 115 (1927); **14**, 308 (1928).

TABLE II

EFFECT OF ZINC AND GLUCOSE CONCENTRATION UPON GROWTH AND ACID PRODUCTION BY MALE AND FEMALE RACES OF RHIZOPUS NIGRICANS

Culture, no.	35 (+)				36 (−)			
Glucose concn., %	2.5		10.0		2.5		10.0	
Zinc present	−	+	−	+	−	+	−	+
Glucose consumed, mg.	2369	4752	4530	9795	863	4257	2235	10553
Fumaric acid produced, mg.	891	474	1040	2214	None	None	None	None
Conversion,[a] %	37.6	10.0	22.9	22.6	...	...	...	...
Nitrogen consumed, mg.	23.7	79.2	32.6	79.2	13.1	58.8	19.4	71.4
Total Ca in soln., mg.	337	264	462	934	42	92	87	177
Calcium due to organic acids, mg.	303	150	415	821	23	8	60	75
Calcium as fumaric acid, %	98.8	109	86.2	93.0	...	...	...	...

[a] See footnote in Table I.

mycelial network. The marked quantitative differences in the cell synthesis were obvious from the analysis of the pellicles:

	No. 35 mg.	No. 36 mg.
Total nitrogen consumed	69.1	28.9
Weight of pellicle calculated	1382	578

By calculating the efficiency of carbon utilization[22] by these two races of *Rhizopus*, no. 35 was found to convert 24.0% of the carbon of the sugar used into cell substance, whereas no. 36 was much less efficient, converting only 9.8%. The latter either oxidized the unaccountable carbon to carbon dioxide or left it as non-acidic metabolic products, largely ethyl alcohol. This alternative is the more likely, since the culture had a strong alcoholic odor; it is well known that alcohol formation is characteristic of the submerged type of growth of *Rhizopus*. A much smaller amount of carbon was unaccounted for in the no. 35 culture, which had only a faint alcoholic odor.

It is of no small interest to note that the acid formed by no. 35 was practically pure fumaric acid, which amounted to 98.3% of the total acidity as expressed by the Ca^{++} in solution. The other acids produced in small amounts by these organisms remain as yet unidentified although lactic acid could not be detected in the ether extracts of the culture. Volatile acids were present only in traces in all four cultures, less than 2 cc. of 0.1 *N* sodium hydroxide being required to neutralize the total volatile acidity in each. The male (no. 38) and female (no. 37) races of the other strain of *Rhizopus nigricans* exhibited no such dissimilarity of physiological behavior, as brought out in Table I. Thus it appears that the distinctive physiological specificity of races nos. 35–36 is not necessarily widespread among species of *Rhizopus*.

It has been shown elsewhere[15] that the presence of certain metallic ions markedly modifies the physiology of *Rhizopus*. Since the effect of carbohydrate–nitrogen ratio of the medium is also very pronounced, it was thought that possibly during this and other experiments on the female (+) and male (−) races, the inability of the latter to produce fumaric acid could be ascribed to the interrelation of the influences of the metallic elements and glucose concentration. In order to test this point, the two organisms were inoculated in duplicate into a series of flasks of the basic medium (200 ml.) containing 2.5 and 10% C. P. anhydrous glucose; some received zinc at the rate of 1.2 p. p. m. (10 mg. $ZnSO_4 \cdot 7H_2O$ per liter of medium) and others were left without zinc. All flasks had 2.8 p. p. m. iron (10 mg. ferric sulfate per liter). Calcium carbonate was added on the third day and the cultures were incubated for eleven days. At the time of analysis, no. 35 had produced heavy pellicles on the zinc medium and much thinner pellicles on the zinc-free medium. No. 36 produced good pellicles only on the zinc-containing medium.

The cultures were boiled vigorously for several minutes before filtration and analysis, in order to ensure complete neutralization of the acids by the calcium carbonate.

The results presented in Table II show that the male and female races of the particular strain of *Rhizopus nigricans* are distinctly different in the nature of their growth, regardless of sugar concentration and the presence of zinc. Under no conditions could fumaric acid be found in culture no. 36, while no. 35 produced this acid in abundance. No. 36 is a very feeble acid producer, as shown by the amount of calcium in solution.

In order to characterize chemically the acid formed by no. 35, the residual culture liquid was acidified and extracted with ether. Analyses made on the air-dry crystalline material in the extract were compared with a sample of known fumaric acid, with the following results:

Test	Unknown acid	Known fumaric acid
Melting point, °C.	275	284
(Unsatn.)$KMnO_4$ in cold	Decolorized immediately	
Neut. equiv.	60.5	58.0
$HgNO_3$ in 5% HNO_3	Hg precipitate	Hg precipitate

A study was now made of the factors governing the production of fumaric acid by the specific organisms, using the two selected strains of *Rhizopus* (nos. 35 and 45). Sixty 200-ml. portions of 2.5% glucose medium containing the above nutrient salts were placed in 500-ml. Erlenmeyer flasks and sterilized, as shown above. Two flasks were set aside as controls and a set of 28 flasks each inoculated with spore suspensions of no. 35 and no. 45. Sterile 3-g. portions of calcium carbonate were added after two or three days of incubation. Six flasks of each organism were removed at intervals of six, nine, thirteen and eighteen days. Two of each of the six cultures were analyzed immediately. In the remaining four flasks the liquid was

(22) Carbon content of cell substance grown in absence of calcium carbonate was found by analysis to be 45%.

TABLE III

INFLUENCE OF OXYGEN TENSION ON FUMARIC ACID PRODUCTION BY PELLICLES OF RHIZOPUS

Culture no.	Age of pellicle, days	Glucose consumed,[a] g.			Fumaric acid produced, g.			Conversion, %		
		Stat.[b]	Anaer.	Aer.	Stat.	Anaer.	Aer.	Stat.	Anaer.	Aer.
35	6	3.708	2.820	4.048	1.720	0	0.420	46.4	..	10.4
	9	2.748	1.636	3.542	1.370	0	1.420	49.8	..	40.1
	13	3.696	1.684	3.362	0.880	0	0.070	23.9	..	2.1
	18	3.408	1.052	2.548	.800	0	.415	23.5	..	16.3
45	6	4.388	3.792	4.388	2.220	0.370	1.000	50.6	9.7	22.8
	9	4.206	2.528	3.924	1.380	.410	1.600	32.6	16.2	41.0
	13	3.664	3.514	3.822	2.020	.700	1.970	55.1	20.0	51.5
	18	1.100	1.764	1.076	0.495	.185	0.423	45.0	10.5	39.3

[a] 150 ml. of 3.3% glucose used as substrate. [b] Stat. = stationary, Anaer. = anaerobic, Aer. = aerated cultures.

poured off aseptically, the fungus pellicles washed twice with sterile distilled water, and 150 cc. of 3.3% glucose solution as well as 5 g. of sterile calcium carbonate added. Two of these four pellicle cultures were incubated stationary, one was saturated with hydrogen to keep anaerobic, and the remaining culture aerated vigorously through the liquid for the period of incubation, usually two to five days. The cultures were then filtered, the pellicles squeezed and washed twice with water. The combined filtrate was analyzed for sugar, ammonia, ash and fumaric acid. Surprisingly enough, the growing cultures did not show any fumaric acid at any stage of development, and contained, in general, very little acid. This finds a ready explanation in the results presented in Table VI, where it is shown, that, under conditions of relatively low carbohydrate and high nitrogen nutrition, as prevailed in this experiment, acid formation is greatly repressed during the growth of the organisms; further data dealing with this question will be published elsewhere. However, the pellicles obtained in this experiment and treated as described above possessed the power of producing fumaric acid, as shown by the copious yields of this acid when the pellicles were allowed to act on glucose solutions in the absence of nitrogen and minerals, as shown in Table III. Thus, even though these organisms may grow and synthesize abundant cell substance without forming a trace of fumaric acid, they still possess the enzyme mechanism for producing this acid.

A suggestion of a difference in the physiological activity between the two organisms is to be found in a comparison of rates of nitrogen consumption. No. 45 seemed to be considerably more vigorous in this respect. The low residual ash points to little or no acid formation; the major part of the ash left was due to the unused minerals in the medium.

The pellicle cultures prepared at different stages of growth of these two organisms yielded several important facts: they produce fumaric acid abundantly and vigorously in the complete absence of available nitrogen and minerals. Under these conditions, no growth of the organism takes place; the cell substance merely respires the glucose in the culture. As much as 40–50% of the sugar consumed accumulates as calcium fumarate. Compared with the activity of the organisms during actual growth, on the basis of sugar consumed, the pellicles are extremely vigorous; it required eighteen days to consume slightly more than 4 g. of glucose during the growth period and only three to six days by the action of the pellicles.

A very significant difference in the physiology of the two organisms was brought out clearly by their peculiar behavior in an atmosphere of hydrogen: no. 35 did not form even a trace of fumaric acid in four tests, whereas no. 45 produced in all cases a considerable amount of acid, equivalent to as much as 30–40% of that produced under aerobic conditions. This experiment was performed three times, using a commercial hydrogen atmosphere, with essentially the same results. However, when anaerobiosis was obtained by means of carbon dioxide, no fumaric acid was formed by either no. 35 or no. 45 pellicles. A third distinguishing feature is to be deduced from the influence of age of the pellicle upon its fumaric acid producing capacity. This factor exerted no apparent effect upon the vigor of no. 45, but with increasing age a marked debility was induced in no. 35.

Finally, the effect of aeration testifies decisively to the dissimilarity in the physiology of these two fumaric acid producing organisms. In all cases, aeration through the culture liquid was definitely deleterious to the fumaric acid mechanism of no. 35, as compared with stationary cultures, whereas the effect upon no. 45 was practically negative. It is quite possible that the acid was formed by no. 35 and, in the presence of an excess of oxygen, was further oxidized. This assumption, as well, points to differences in the metabolism of the two organisms.

In order to obtain further suggestions as to the quantitative nature of the acid produced by the pellicles, some of the stationary cultures were analyzed for the calcium in solution (Table IV). Fumaric acid was formed by no. 45 almost exclusively; in the case of no. 35, this acid amounted to only about 55% of the total acids formed. In other words, no. 35 produced a large amount of other as yet unidentified acids, under the above experimental conditions. Under anaerobic conditions, some acid formation

TABLE IV

CONVERSION OF GLUCOSE BY PELLICLE CULTURES OF RHIZOPUS

Mg. per 150 ml. of culture

Organism no.	35	45
Glucose consumed	4388	4388
Fumaric acid found	1190	2210
% conversion	27.1	50.4
Total Ca^{++} as CaO in soln.	1053	1083
Fumaric acid equiv. of CaO in soln.	2180	2241
CaO per g. glucose consumed	240	247
% of total acid as fumaric	54.6	98.6

by no. 35 occurred as brought out by the small amount of soluble calcium, due to acids other than fumaric. The anaerobic cultures had a strong alcoholic odor, indicating that some of the glucose was converted into this neutral compound.

It is to be recalled that many of the *Mucoraceae* are strongly fermentative organisms, even under aerobic conditions, and especially under limited oxygen tensions. This appears to be true of the fumaric acid organisms. Ether extracts of 10-ml. portions of the acidified culture gave in all cases negative thiophene tests for lactic acid.

The results of this experiment point to the fact that there is justification for considering "strain specificity" even when a single process such as fumaric acid formation is under consideration. These results offer a clue to the discrepancies obtained by Gottschalk,[8] on the one hand, and Ehrlich and Bender[9] and Butkewitsch and Federoff[10] on the other.

The calcium salts in the pellicle cultures of no. 45 were crystallized from the liquid by concentrating, filtering, washing and recrystallizing. An equivalent amount of concentrated hydrochloric acid was then added and the relatively insoluble fumaric acid crystallized out upon concentration; it was recrystallized once more from water and dried. Another portion of the residual pellicle culture liquid was acidified and extracted with ether. The crystalline material was recrystallized from hot water and dried. The analysis of the acid obtained from both the ether and water extracts proved the identity of the acid with that of fumaric.

	Ether extract	Water-soluble acid	Known fumaric acid (twice recrystallized)
Melting point, °C.	287	288	289
Mixed melting point, °C.	289	...	...
Neut. equiv.	56.8	58.8	58.1
$KMnO_4$ unsatn. test	Decolorized in cold		
M. p. of *p*-nitrobenzyl ester	Prepared from ether and water mixed lots = 149°, uncorr.		

Culture no. 45, a neutral strain, was selected for detailed investigation of the factors influencing fumaric acid formation. The choice was made because of the rapid and abundant growth, the vigor in producing fumaric acid, and the consistently high yields of this acid to the almost complete exclusion of other acids.

Butkewitsch and Federoff[10] investigated the fumaric acid formation by mature *Rhizopus* pellicles in the absence of all minerals and nitrogen with and without neutralizing agents. In the presence of calcium carbonate, yields of 20% were obtained and only 2% in its absence. The pellicles of no. 45 produced, in the presence of calcium carbonate, far greater amounts of fumaric acid than are usually reported, namely, up to 50% of the sugar consumed. These yields have been obtained consistently from both zinc-containing and zinc-free pellicles. Butkewitsch and Federoff used no zinc in their cultures, and this, plus the fact that they used extremely long incubation periods, justifies the assumption that the growths that they have obtained were comparable to the depressed and relatively limited growths of the zinc-free cultures in these experiments. It was of interest to determine whether zinc pellicles possessed the capacity of accumulating fumaric acid without a neutralizing agent. This experiment was carried out on a relatively large scale, in order to assure that the activity of the fungus is a property characteristic of a large mass of cell substance, rather than of a small mass of growth which conceivably might not respond typically with the accumulation of free acid. Seven-hundred ml. portions of the usual 5% glucose-salt medium with 1.2 p. p. m. zinc were distributed in eight 3-liter Fernbach flasks and sterilized. *Rhizopus* no. 45 was used as the inoculum, calcium carbonate was added after three days, and incubation was continued at 28° for nine days. At this time a luxuriant, thick vegetative pellicle with abundant aerial mycelium each covering an area of approximately 350 sq. cm. was present in the flasks. These were drained free of the nutrient medium and washed several times with sterile distilled water until the last trace of calcium carbonate was removed; fresh 700-ml. portions of sterile carbohydrate solution were now introduced, as follows: flasks 1 and 2, 5% glucose; 3 and 4, 10%; 5 and 6, 15%; 7 and 8, 2% starch. Seventy-five gram portions of sterile calcium carbonate were added to flasks 2, 4, 6 and 8, whereas 1, 3, 5 and 7 were left without any neutralizing agent.

Acid formation took place vigorously, as could be seen by the highly refractive streams descending from the bottom of the pellicle when the flasks were held up to the light. The flasks were shaken frequently during the course of the experiment, to neutralize the acid. On the fifth day, calcium fumarate began to crystallize out in flask 4; on the following day, the bottom of flask 3 was covered with numerous beautifully formed needle-shaped crystals. These crystals were removed and recrystallized from ether. The m. p. was 290°; potassium permanganate was decolorized in the cold. The crystals proved to be free fumaric acid which was formed in such abundance that it saturated the culture and crystallized out. After six days, 75-cc. samples of the liquid were withdrawn aseptically from each flask, filtered and analyzed. The *p*H of the calcium carbonate-free cultures was measured potentiometrically using a glass electrode. After seven days of incubation, all the liquid in the flasks was drained off, the pellicles washed, and replaced a second time with 700-cc. portions of 5% glucose with and without calcium carbonate as above. These were then incubated for nine days further and analyzed. The results are reported in Table V.

This experiment proves conclusively that *Rhizopus* pellicles can produce free fumaric acid in large amounts in the complete absence of a neutralizing agent. The free acid accumulated as such in the medium and eventually attained concentrations sufficient to crystallize out in the culture as free acid. This accumulation of acid lowered the *p*H to 2.4, at which point the crystallization occurred. In the other flasks, the *p*H values (2.5–2.6) suggest that the concentration was such that crystallization would have occurred shortly thereafter.

The ability of the organism to produce fumaric acid at this high hydrogen ion concentration is noteworthy, for it is much below the range of activity of most living systems. Special mention should be made of the fact that, even under such adverse conditions, the efficiency of acid formation in the flasks containing 10% glucose was not

TABLE V

FUMARIC ACID PRODUCTION BY RHIZOPUS PELLICLES, IN PRESENCE AND ABSENCE OF $CaCO_3$

Replacement of carbohydrate	Glucose consumed, g. First		Glucose consumed, g. Second		Fumaric acid produced, g. First		Fumaric acid produced, g. Second		Conversion, % First		Conversion, % Second	
$CaCO_3$ present	+	−	+	−	+	−	+	−	+	−	+	−
Glucose 5%, 5%	34.1	21.5	25.9	10.0	12.7	7.3	7.1	1.9	37	34	28	17
Glucose 10%, 5%	35.8	30.2	26.6	8.9	8.5[a]	6.7	6.8[b]	2.8	24[a]	22	25[b]	32
Glucose 15%, 5%	61.8	32.3	26.2	13.7	11.6[a]	6.3	8.3[b]	3.6	19[a]	20	32[b]	26
Starch 2%, glucose 5%	..[c]	..	21.6	8.4	6.9	4.8	4.1	1.3	...[c]	..	19	15

[a] Does not include considerable Ca fumarate caked on pellicle. [b] Includes crystals from 1st replacement; see footnote[a]. [c] All starch disappeared. Glucose left: $-CaCO_3$, 1.490 g.; $+CaCO_3$, 0.770 g. Approx. conversion: $-CaCO_3$, 30%; $+CaCO_3$, 40%.

TABLE VI

FUMARIC ACID FORMATION BY RHIZOPUS UNDER DIFFERENT CONDITIONS OF NITROGEN NUTRITION[a]

Concn. of N source, % $(NH_4)_2SO_4$	Concn. of N source, % Urea	Glucose consumed, g. $(NH_4)_2SO_4$	Glucose consumed, g. Urea	Fumaric acid produced g. $(NH_4)_2SO_4$	Fumaric acid produced g. Urea	Conversion, % $(NH_4)_2SO_4$	Conversion, % Urea	Nitrogen consumed, mg. $(NH_4)_2SO_4$	Nitrogen consumed, mg. Urea	Pellicle, g. Urea
0.01	0.005	14.90	13.59	3.32	1.29	22.5	9.5	72	57	1.67
.02	.01	14.16	14.85	1.98	79	13.9	5.3	147	117	2.44
.03	.015	13.35	15.03	2.10	54	15.8	3.6	116	136	2.48

[a] 200-ml. portions of medium; glucose control = 17.2 g.

much below that in the corresponding cultures with calcium carbonate, namely, 22.2% as against 23.9%; the efficiency of acid production was also practically the same in the 5% glucose flasks. It is to be emphasized further that the organism produced considerable acid even during the second replacement of the sugar using the same pellicle. The organism thus continued to function as an enzyme system through several sugar replacements and its capacity to produce fumaric acid was not exhausted. As was to be expected, the cultures containing calcium carbonate utilized the glucose at a higher rate but with no greater efficiency.

The carbon–nitrogen ratio in the nutrient medium proved to be of vital importance for the fumaric acid producing capacity of the organism. This was conclusively shown on comparing the cultures containing varying carbohydrate concentrations and a constant nitrogen source. The following experiment was performed in order to throw further light upon this problem. Two sources of nitrogen, namely, ammonium sulfate and urea, were added to a 10% glucose-mineral medium plus zinc, in concentrations of 1, 2, 3, g. per liter of the first and 0.5, 1.0, 1.5 g. per liter of the second. *Rhizopus* 45 was used for inoculation; incubation took place for nine days. Only the ammonium sulfate set received calcium carbonate. The pellicles from this set were drained, washed as usual, and reserved for further treatment. The urea pellicles were washed twice, squeezed and dried overnight at 80°, then for two hours at 100° and weighed. The data presented in Table VI serve to emphasize the fact that a high carbon:nitrogen ratio in the medium is most favorable for fumaric acid production by *Rhizopus*. Progressive nitrogen enrichment induced corresponding diminution in fumaric acid production, both in regard to absolute yield and efficiency of conversion. Urea as a source of nitrogen has an adverse influence on acid formation as compared with ammonium sulfate.

The 22.5% conversion of glucose to fumaric acid in the presence of 0.01% ammonium sulfate is about the highest yield obtained during the growth of the organism in a zinc-containing culture. Despite the low supply of nitrogen, the organism consumed the largest amount of glucose. This possibly may be explained by the fact that the organism assimilated rapidly all the available nitrogen, since only 1.6 mg. of nitrogen was left in the culture, and then commenced to function as a pellicle which would respire the glucose at a much higher rate.

Steinberg[23] reported that nitrate nitrogen was available to *Asp. niger* only if molybdenum was present in the medium. *Rhizopus* 45 was tested for nitrate utilization under these conditions but in no case was nitrate found to be an available source of nitrogen for this organism.

The ammonium sulfate pellicles were treated with 150 cc. of a 10% glucose solution plus calcium carbonate, and incubated at 28°. In three days, a copious crystallization of calcium fumarate occurred in the 2 g. per liter pellicles and in the 1 g. per liter pellicles. In five days, both 3 g. per liter pellicles gave crystals. The tenacity with which the crystals of calcium fumarate clung to the pellicles and encrusted them obviated any attempt at an accurate quantitative determination of the fumaric acid without destroying the pellicle. It was decided, therefore, instead of removing the crystals that had deposited on the bottom of the flasks, to remove only the liquid from the flasks, leaving all the crystals. After one washing with distilled water, 150 cc. more of 10% glucose + calcium carbonate was added, and in like fashion the process repeated a third time. By this time so much crystalline calcium fumarate had accumulated in the flasks that the whole culture presented a solid mass of crystals. This was particularly true of the 1 g. per liter pellicles. The sugar consumed during the first two intervals is given here. The third replacement was not analyzed. The 2 g. per liter pellicles were obviously the most vigorous.

Nitrogen content of original culture, g./liter	Sugar consumed, g. 1st period	Sugar consumed, g. 2nd period
1	11.2	8.1
2	12.7	12.3
3	11.3	10.7

(23) R. A. Steinberg, *J. Agr. Research*, **55**, 891 (1938).

This experiment again shows that *Rhizopus* pellicles can continue to function as enzyme systems producing fumaric acid for a long time after being deprived of nitrogen and minerals; also that an accumulation of the end-product of this reaction is not particularly harmful to the process.

Since small amounts of trace elements exert such a profound influence on the capacity of the organism to yield fumaric acid during growth, it appeared quite possible that the presence of various elements in the glucose replacement solutions might affect the process in some way or other. Large size pellicles were obtained in 3-liter Fernbach flasks as before. In order to secure extra large pellicles, the nitrogen content of the 10% glucose–zinc medium was raised to 3 g. of ammonium sulfate per liter. After nine days, pellicle cultures were prepared as usual containing 750 cc. of 10% glucose and calcium carbonate, with the following treatments

1, nothing	4, 1 g. K_2HPO_4
2, 1 g. $MgSO_4 \cdot 7H_2O$[24]	5, 1 g. $Fe_2(SO_4)_3$
3, 10 g. $MgSO_4 \cdot 7H_2O$[24]	6, 1 g. $ZnSO_4 \cdot 7H_2O$

In three days, abundant crystal formation was obtained in flasks 1, 4, 5, 6; in four days in no. 2; in five days no. 3. After seven days of incubation, the cultures were boiled until all the calcium fumarate was dissolved, and analyzed. The results presented in Table VII indicate that $MgSO_4 \cdot 7H_2O$ (10 mg./liter) and dipotassium phosphate, in concentrations used, were decidedly unfavorable to acid formation by pellicles. Although the ferric sulfate was somewhat repressive to glucose utilization, it was not unfavorable to the efficiency of conversion. Dipotassium phosphate, on the other hand, gave a high glucose utilization, as might be expected from its role in the primary phosphorylation in glucose dissimilation but the transformation process was diverted to other products at the expense of the fumaric acid.

TABLE VII

EFFECT OF MINERALS ON FUMARIC ACID PRODUCTION BY RHIZOPUS PELLICLES[a]

Mineral treatment	Glucose consumed, g.	Fumaric acid produced, g.	Conversion, %
None	60.51	26.11	43.2
$MgSO_4 \cdot 7H_2O$, 1 g.	63.37	26.09	41.1
$MgSO_4 \cdot 7H_2O$, 10 g.	58.00	20.06	34.6
K_2HPO_4, 1 g.	65.52	19.39	29.6
$Fe_2(SO_4)_3$, 1 g.	52.08	22.92	44.0
$ZnSO_4 \cdot 7H_2O$, 1 g.	64.35	28.74	44.7

[a] 10% sugar solution used.

Discussion

The evidence presented here as well as by other investigators points to the importance of "strain specificity" of cultures of *Rhizopus* for acid production. Not only do the various morphologically identical strains of *Rhizopus nigricans* differ strikingly in their acid forming capacities but also the male and female races of even the same strain exhibit decided differences. However, this was not characteristic of even a significant number of sexual pairs. It is not impossible that sexual differences in this group of fungi may eventually be correlated with other as yet unstudied physiological processes. Some may produce none or very little acid, forming largely alcohol, others may produce lactic acid predominantly, others fumaric acid; still some may form mixtures of the two as well as of succinic, malic, oxalic[25] and other acids.

The concept that different fungus cultures of a given morphological entity possess the same or even similar physiological properties is not supported by these investigations. This is already well known in the case of *Asp. niger*. Moreover, there are suggestions that the mode of formation of even a specific process such as fumaric acid production may differ among fungi. Any attempt to offer an explanation for the wide diversity of physiological types of a morphological entity necessarily must await further accumulation of experimental facts. One may be tempted, however, to speculate upon the possibility of physiological evolution within the stable morphological group. Many organisms of the *Rhizopus* group are almost strictly fermentative in their metabolism and the possession by some of the oxidative phase of the respiratory process such as occurs in fumaric acid production represents a much higher level of efficiency of energy utilization. The presence of the C_4 dicarboxylic acids in cultures of these fungi suggests possible relation to the respiratory scheme which Szent-Gyorgi[26] has shown for animal cells. It is possible that the enzyme equilibrium supposed to exist between these acids functioning as a chain of hydrogen carriers is unbalanced in the case of these fungi with the resultant preponderance of fumaric acid. Accumulation of fumarate by *Rhizopus nigricans* 45, even up to saturation of the culture solution, does not repress continued acid formation.

The experiment reported in Table III emphasizes the fact that a property characteristic of a specific organism may not manifest itself even under usual conditions of culture and hence may mislead one into making erroneous conclusions and interpretations.

(24) The magnesium sulfate flasks were of special interest in view of the recent report by Butkewitsch and Trofimova [*C. R. Acad. Sci. U.S.S.R.*, **17**, 221 (1937); *C. A.*, **32**, 2559 (1938)] that the presence of this salt in pellicle cultures of *Asp. niger* greatly stimulates citric acid formation.

(25) Wl. Butkewitsch, *Biochem. Z.*, **182**, 99 (1927).

(26) A. von Szent-Gyorgi, "Perspectives in Biochemistry," Cambridge Univ. Press, 1937, p. 165.

The cell substance of *Rhizopus* still possesses a vigorous capacity for acid formation despite the fact that this acid may not have been produced during active growth. A point of interest is the fact that the pellicle retains for some time its function as an enzyme system continuing to produce acid from as many as three or four successive replacements with fresh sugar solutions.

Extensive experimentation has been undertaken dealing with the use of submerged growths of *Rhizopus* and all the evidence points definitely to possible industrial utilization of the fermentation processes for fumaric acid production. The fungus can be grown rapidly and abundantly in large containers corresponding to tank conditions with use of rapid aeration and adequate agitation in the presence of calcium carbonate. The rate of growth under these conditions is greatly accelerated (twenty-four to forty-eight hours as compared to five to seven days for stationary surface growth) and the yields of acid obtained thus far give much promise of a practical significance. The advantages of a deep-tank mold fermentation process over the shallow pan process are obvious. An added feature of interest is the fact that the original nutrient culture solution may be drained off through the false bottom used for aeration leaving the mass of fungus mycelium remaining in the container which then is recharged with pure sugar solution and calcium carbonate; the fermentation of this replaced sugar by the preformed mycelium commences immediately at a high rate, resulting in considerable economy of time. The fungus mycelium still possesses a high fermentative capacity through several sugar replacements although with gradually diminishing vigor. One disadvantage encountered is the crystallization and solidification of the culture solution due to the limited solubility of calcium fumarate. This may be obviated by use of lower concentrations. The use of pure sugar solutions results in the preparation of a product of a high degree of purity and the relative insolubility of fumaric acid permits liberation of the free acid direct from the culture solution without separation first as the calcium salt, thereby facilitating greatly the process of recovery.

Summary

The production of fumaric acid by fungi is largely limited to species of *Rhizopus*. However, not all organisms of this genus are able to produce this acid. Certain species found among other genera of *Mucoraceae*, namely, *Mucor*, *Cunninghamella* and *Circinella*, were also found capable of producing fumaric acid.

In the case of one strain of *Rhizopus nigricans*, the female race was characterized by the ability to form fumaric acid whereas its sexual mate did not possess this capacity.

One strain of *Rhizopus nigricans* was found to be particularly characterized by rapid and abundant growth, and high yields of fumaric acid, which amounted to 40–50% of the carbohydrate consumed. This was true especially of the action of mature pellicles acting upon carbohydrate in the complete absence of minerals and nitrogen. The rate of acid production was greater in the presence of calcium carbonate than in its absence. The pellicles continued to convert sugar to fumaric acid through several replacements of sugar solutions.

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