[CONTRIBUTION FROM THE NEW JERSEY AGRICULTURAL EXPERIMENT STATION]

Lactic Acid Production by Species of Rhizopus[1]

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Although the capacity of forming lactic acid is not widespread among the fungi, it seems to be definitely established that various species belonging to the genus *Rhizopus* are capable of producing this acid from a variety of carbohydrates, including glucose, maltose, sucrose, dextrin and starch. According to Saito,[2] inulin, lactose and sucrose cannot be used as a source of acid; Kanel[3] found, however, that the last carbohydrate is readily utilized. The presence of calcium carbonate is essential for the formation of appreciable quantities of the acid. Ammonium salts or compounds which produce ammonia, such as urea, are preferable to nitrates.

The nature and yield of the lactic acid vary considerably, depending largely upon the specific nature of the organism, including strain specificity, and upon the conditions of cultivation. Saito obtained only a low yield of the levo-rotatory form of the acid; Kanel, however, reported a yield, both from sugar and from starch, of 40% of the *d*-form; the period of incubation was twenty days. Ward, *et al.*,[4] also found that 32 to 62% of sugar was transformed into *d*-lactic acid in sixteen days.

Practically all the fungi capable of producing lactic acid from carbohydrates have been found among the *Mucorales*, chiefly in the genus *Rhizopus*. The only other exception so far reported was found to be a species of *Monilia*.[5] Saito employed in his investigations *Rh. chinensis*. According to Ehrlich,[6] *Rh. nigricans* and *R. tritici* are capable of bringing about the formation of lactic acid. Takahashi[7] used four different strains of *Mucor*. The organism employed by Kanel was *Rh. japonicus*, or a closely related form; Ward, *et al.*, worked with *Rh. oryzae* and *Rh. arrhizus*. When the incubation period is prolonged more than twenty days, the concentration of the lactic acid was shown[3] to be gradually reduced, and other acids, notably fumaric, are formed.

(1) Journal Series Paper of the Department of Soil Microbiology. N. J. Agr. Expt. Station.

(2) K. Saito, *Centrbl. Bakt.*, II, **29**, 289–290 (1911).

(3) E. Kanel, *Microbiologia*, **3**, 259–265 (1934).

(4) G. E. Ward, L. B. Lockwood, O. E. May and H. T. Herrick, THIS JOURNAL, **58**, 1286–1288 (1936).

(5) K. Miyaji, "Res. Bull. 10, Gifu Imp. Coll. Agr.," 1930.

(6) F. Ehrlich, *Ber.*, **52**, 63 (1919).

(7) T. Takahashi and T. Asai, *Centrbl. Bakt.*, II, **89**, 81–84 (1936).

Experimental

In the study of decomposition of plant materials in composts and in soil, a culture of *Rhizopus*, designated as MX, was isolated, which had the capacity of producing, from various carbohydrates large quantities of lactic acid. A comparison was later made of several strains of *Rhizopus* kept in the culture collection, and another organism, No. 36, was selected. The two organisms varied in cultural appearance, especially in the rapidity of spore formation and in the yields of lactic acid. MX was closely related to *Rh. nodosus* or *Rh. arrhizus*. The No. 36 was morphologically very similar to MX.

Several preliminary experiments, using glucose as a source of carbon, were carried out in order to determine the nature of the acid produced. It was soon found that, during the early stages of the growth of the organism, namely, between three and fifteen days, the acid is almost exclusively lactic acid. This was demonstrated by the precipitation of the calcium salt of the acid with alcohol and analysis of the salt. However, after fifteen to twenty days' growth, the lactic acid was transformed slowly to lower acids (fumaric?), as shown by the increase in the calcium content of the salt. It was felt, therefore, that when the determinations were made in cultures only ten to eighteen days old, the calculation of the lactic acid equivalent of the calcium in the salt did not involve any large error. This checked well with the organic matter content of the culture solution, which, after all the sugar had been consumed, was due almost entirely to the acid produced.

Once the nature of the acid was established, a series of experiments was carried out to determine the nutrient requirements of the organisms and especially the acid production from different carbohydrates, under different conditions of culture.

In a preliminary experiment it was found that during the growth of the organism MX in a 5% glucose solution, with ammonium sulfate as a source of nitrogen, the reaction was usually changed, within three days' growth at 28°, from *p*H 7.4 to 4.0 or 4.1. Little lactic acid was formed, unless calcium carbonate was added. When a 10% glucose solution was introduced into flasks containing the fully grown fungus pad, rapid acid production began, especially when the pad was obtained in a medium containing calcium carbonate.

The following culture medium was at first employed

Glucose	100 g.
NH_4NO_3	2.0 g.
K_2HPO_4	1.0 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
$FeCl_3$	0.02 g.
Tap water	1000 cc.

In order to illustrate the balance of carbon and nitrogen in the growth of the organism, the results of a typical experiment are reported here. Three hundred-cc. portions of the medium were placed in 1000-cc. Erlenmeyer flasks, sterilized by means of flowing steam, on three consecutive days, inoculated with the spores of the fungus, and incubated at 28°. After forty-eight hours of undisturbed growth, 5-g. portions of sterile calcium carbonate were added to the flasks. Some of these were shaken daily by hand, for one or two minutes, and some were left undisturbed. Analyses were made after five and nine days.

The results (Table I) show that *Rhizopus* consumed the ammonium ion in preference to the nitrate ion, and that the above medium contained a considerable excess of nitrogen above the nutrient requirements. The cell synthesis was not very extensive, since only about 13% of the glucose consumed was utilized to cover this requirement.[8] The preference of the organism for the ammonium ion is also brought out in another experiment. Three media were prepared containing glucose, minerals and calcium carbonate in the above proportions. Different forms of nitrogen were used, namely, ammonium sulfate, 5 g. per liter, ammonium nitrate, 2.5 g. and ammonium phosphate 2.5 g. per liter. The cultures were incubated for ten and seventeen days at 28° (Table II). All the three salts were utilized readily as sources of nitrogen, the sulfate being somewhat more efficient than the others.

TABLE I

GLUCOSE DECOMPOSITION BY *Rhizopus* MX

On basis of 1 liter of medium[a]

Incubation, days	Culture shaken	Glucose, g. Left	Glucose, g. Consumed	Nitrogen left, mg. NH_3–N	Nitrogen left, mg. NO_3–N	Dry weight of pellicle, g.	Nitrogen in pellicle, mg.
5	+	19.1	65.1	75.0	310	8.45	217.3
5	−	28.0	56.2	110.0	330	6.96	208.0
9	+	..	..	..	...	9.74[b]	310.0

[a] Control contained 84.2 g. of pure glucose. [b] Ash in pellicle, 11.6%.

TABLE II

INFLUENCE OF NITROGEN SOURCE UPON ACID PRODUCTION BY *Rhizopus*

Nitrogen source	Incubation, days	*Rhizopus*	Glucose consumed, g.	Acid produced, g.[a]	Pellicle Dry weight, g.	Pellicle N, %
$(NH_4)_2SO_4$	10	No. 36	46.2	26.1	...	..
$(NH_4)_2SO_4$	10	MX	39.7	18.3	...	..
$(NH_4)_2SO_4$	17	No. 36	83.3	53.7	6.1	3.04
$(NH_4)_2SO_4$	17	MX	83.5	46.7	8.9	3.23
NH_4NO_3	10	No. 36	36.2	11.2	...	..
NH_4NO_3	10	MX	40.7	16.8	...	..
NH_4NO_3	17	No. 36	70.0	25.8	6.3	4.41
NH_4NO_3	17	MX	80.3	37.1	7.7	3.48
$(NH_4)_2HPO_4$	17	No. 36	62.4	29.9	...	..
$(NH_4)_2HPO_4$	17	MX	71.1	33.6	...	..

[a] Calculated from calcium equivalent.

In order to bring about the proper neutralization of the acid, it was sufficient to shake the flasks once or twice daily, for two or three minutes by hand, so as to bring the calcium carbonate, which was usually added in excess, in intimate contact with the acid. The results presented in Table III show that proper stirring of the culture, after a pellicle has been formed, which usually required forty-eight hours, is essential for maximum acid production.

TABLE III

INFLUENCE OF SHAKING UPON GLUCOSE DECOMPOSITION BY *Rhizopus* MX[a]

Treatment of culture	Glucose, g. Left	Glucose, g. Decomposed	Acid, produced, g.	Pellicle Dry weight, g.	Pellicle Ash, %	Pellicle Nitrogen, %
Unshaken	28.6	55.6	20.0	4.45	9.2	3.16
Shaken	10.9	73.3	30.9	..	...	..

[a] Thirteen days' incubation.

The medium was now modified, by reducing the concentration of the ammonium sulfate to 1.25 g. and the dipotassium phosphate to 0.5 g. per liter. The results of an experiment on prolonged incubation show (Table IV) that the most efficient production of the acid took place in eighteen days, at which time over 60% of the glucose was converted into acid; after twenty-eight days, the conversion was only 43%. When the acid was recovered by crystallization and alcohol precipitation, it was found to increase in calcium content with the age of the culture; the calcium oxide content of the younger cultures was 27.5% or close to the theoretical requirement for lactic acid, while in the older cultures it increased to 33 and even 35%. These results tend to confirm those of other investigators concerning the gradual transformation, in the older cultures of *Rhizopus*, of some of the lactic acid into lower acids.

TABLE IV

INFLUENCE OF PROLONGED INCUBATION ON ACID PRODUCTION BY *Rhizopus* FROM GLUCOSE

Organism	Incubation, days	Glucose consumed, g.	Acid produced, g.
MX	18	50.5	31.9
MX	25	75.2	..
MX	28	81.4	34.8
No. 36	18	36.8	22.3
No. 36	25	58.5	..

A large quantity of the calcium salt of the acid was now collected by concentrating the medium, crystallizing, dissolving the salt in hot water and reprecipitating with alcohol. The calcium salt was then acidified with phosphoric acid, extracted with ether and the zinc salt prepared. Both salts gave, by chemical analysis and rotation, the dextrorotatory form of the lactic acid.

When starch was substituted for glucose as a source of energy, active lactic acid production was also found to take place. Potato starch was used in the form of a paste, 50 g. per liter. The raw starch contained 86% of pure starch, by chemical analysis. Ammonium sulfate, as a source of nitrogen, and the above minerals were added to the medium. Five hundred-cc. portions were placed in 1000-cc. flasks and sterilized in flowing steam. The flasks were inoculated and incubated in an undisturbed condition for forty-eight hours at 28°; an excess of calcium carbonate was then added; the flasks were shaken twice daily. After seventeen days' incubation, the starch completely disappeared and 26.4 g. of lactic acid, calculated from the calcium salt, was produced per liter of medium. This is equivalent to a yield of over 60%.

(8) On the basis of carbon content, this would be equivalent to over 15%.

The results of two other experiments, using culture MX, show (Table V) that the starch is at first transformed into reducing sugar, and the latter is gradually changed to lactic acid. The yield of the acid was as high with starch as a source of energy as with glucose, namely, 74.2% in the first experiment, and 50% in the second. Considerable sugar was left in the cultures in the last experiment, even after fourteen days.

TABLE V

PRODUCTION OF ACID BY *Rhizopus* FROM STARCH

On basis of 50 g. of raw potato starch[a]

Experiment 1			Experiment 2		
Period of incubation, days	Sugar as glucose, g.	Acid produced, g.	Period of incubation, days	Sugar as glucose, g.	Acid produced, g.
4	19.3	9.0	5[b]	23.2	8.4
6[b]	10.8	21.5	9	14.5	17.1
8	1.8	27.6	14	6.5	21.3
11	0.6	31.9			

[a] Equivalent to 43 g. of dry starch. [b] Starch completely disappeared.

A more detailed study was now made of the utilization of starch as a source of carbon for the production of lactic acid (Table VI). In the absence of calcium carbonate, no lactic acid accumulated; a large part of the starch was converted, however, into sugar, and remained as such; the growth of the organism was considerably less, and some of the starch remained unhydrolyzed even after eleven days. In the presence of calcium carbonate (1 g. per 2 g. of starch), the starch was also converted to sugar, but the latter was transformed rapidly to lactic acid. Nearly 60% of the starch was changed to the acid within eight days of incubation, and over 65%, in eleven days. After that period, the acid was gradually destroyed and became transformed to lower acids as shown by the increasing ash content. The nature of these acids was not determined.

TABLE VI

TRANSFORMATION OF STARCH TO SUGAR AND TO LACTIC ACID BY *Rhizopus*

On basis of 50 g. of raw potato starch[a]

	No $CaCO_3$		$CaCO_3$ present		
Incubation, days	Sugar as glucose, g.	Acid produced, g.	Sugar as glucose, g.	Acid produced, g.	CaO content of solution, g.
4	0.67	Trace	0.75	5.22	1.80
6	13.80	Trace	14.18	22.20	7.66
8	14.28	0	3.21	25.57	9.22
11	14.40	0	0	28.08	10.20
18	...	...	0	16.50	7.54

[a] Equivalent to 43 g. of dry starch.

A study of the influence of temperature on the lactic acid production revealed the fact that 28–37° was the optimum, the rate of the process being reduced rapidly above and below that temperature. A decrease in lactic acid production was accompanied, in starch media, to a certain point, by an increase in sugar accumulation, emphasizing the fact that two distinct enzyme mechanisms are responsible for the two processes.

Inulin was also utilized as a source of carbon by the organism, at a much slower rate, however, than either glucose or starch. Raw artichoke juice or cooked artichoke extract were used as sources of this carbohydrate. When the inulin was first hydrolyzed by means of a dilute mineral acid, lactic acid production took place more rapidly. No sugar accumulated in the inulin medium. These facts point to limited formation of the enzyme inulase by species of *Rhizopus*.

Summary

Two species of *Rhizopus*, isolated from composts of decomposing organic matter and from soil, were found capable of producing lactic acid from glucose, starch and inulin. As much as 60 to 70% of the first two carbohydrates was converted to the *d*-lactic acid in ten to eighteen days at 28°.

The starch was at first converted to sugar, and the latter gradually changed to lactic acid. When conditions were not favorable to the formation of the acid, as a lack of neutralizing agent or at an unfavorable temperature, the sugar accumulated.

Inulin was only slowly converted to lactic acid, due to insufficient production of inulase by the organisms.

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