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The Relative Influence of Microorganisms and Plant Enzyms on Corn Silage Fermentation

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The Relative Influence of Microorganisms and Plant Enzyms on Corn Silage Fermentation

BY ALVIN R. LAMB

HISTORICAL

Ever since the fermentation of silage has been studied and discussed the question of the agent causing the fermentation has been in controversy. Some investigators have made the statement, based on evidence more or less incomplete, that microorganisms are solely responsible for the changes undergone by the ensiled forage. Other workers, who have based their conclusions on equally incomplete data, have held that in silage produced under proper conditions bacteria and yeasts do not figure to any appreciable extent, but that the plant cell itself is the cause of the chemical changes which take place in its constituents. Still other writers have sometimes taken sides on the subject without presenting any new data bearing on the problem.

Among the earliest workers on the chemistry and biology of silage formation were Burrill and Manns (3),[1] who found many species of bacteria in the silage, and stated that they were the cause of the chemical changes. Babcock and Russell (1) made silage in the presence of chloroform, ether, and benzene, obtaining in each case a change of color, some increase in acidity, and typical silage odor and flavor. These results and deductions made from other observations on the gases of the silo, the number of bacteria found, and on silage made from mature and immature corn led them to believe that bacteria were nonessential and that the cause of the fermentation was mainly the intramolecular changes which occur in protoplasm under anaerobic conditions when ordinary metabolic processes are suspended. Harding (8), working under their direction, found bacteria but no constant flora in silage. E. J. Russell (17) came to the conclusion that the primary and essential changes in silage fermentation were brought about by the plant cell and its enzyms and that the changes caused by bacteria were secondary and nonessential. Esten and Mason (6) found such large numbers of bacteria and yeasts in silage that they considered them the only important factor concerned. Hunter

[1]Reference is made by number to bibliography, pp. 330-331.

and Bushnell (10) recently found large numbers of the *Bacterium bulgaricus* group in silage and considered their activities very important. All the work mentioned above has been done with silage made from the corn plant (*Zea mays*), which is the chief silage crop in this country.

Investigators in plant physiology have found evidence of the evolution of carbon dioxid and the formation of alcohol under aseptic conditions in the tissues of many plants, including maize. The distinction as to whether this is due to the action of enzyms within the cells or to respiratory activities of the cell protoplasm is not made; but similar respiratory changes appear to be common to the majority of plants, especially in their seeds. It has been suggested that the respiration of plants under anaerobic conditions is identical with alcoholic fermentation. In many cases some of the alcohol is further oxidized or otherwise changed, but the ratio of carbon dioxid to alcohol is often found to be comparable to that of ordinary alcoholic fermentation by the zymase of yeast. Of course, anaerobic conditions obtain in the silo after the first few hours. Doroféjew (4) found that the respiration of injured leaves was accelerated. This may have some significance in connection with the chopping of corn before it is ensiled. Zaleski and Reinhard (27) and others have noticed similar effects in wounded vegetable tissues. With various seeds placed under anaerobic conditions Godlewski and Polzeniusz (7), Stoklasa et al. (20, 21), Minenkoff (13), and a number of others have found that alcohol and carbon dioxid are produced. Similar results have been obtained by others with other plant tissues than the seeds. Although Mazé and Perrier (12) have questioned the results of Stoklasa, it is still possible that such respiratory activities play a part in the formation of silage.

Certain enzyms have been shown to be present in corn grain. A proteoclastic enzym has been found by Vines (24) and likewise by Scheunert and Grimmer (18), who found an amylase also present. Sigmund (23) found a lipase in both the resting and germinating seeds of maize. Price (15) demonstrated the presence of a peroxidase, a catalase, a protease, an invertase, and a glucosidase in cornstalks. White (26) found proteoclastic and amyloclastic enzyms in maize seeds, which retained their activity in seeds 20 years old.

STATEMENT OF PROBLEM

A sharp differentiation between the activities of enzyms and microorganisms in a given medium is practically impossible. Both are susceptible to injury and destruction by heat and are more or less similarly subject to the inhibitive effect of our

common antiseptics. Moreover, some of the principal manifestations of bacterial activity are identical with those of enzym action, as, for example, the evolution of carbon dioxid, production of alcohol, rise in temperature of medium, and hydrolysis of protein. Antiseptics, if used in high enough concentration to inhibit all bacterial growth, seem also to exercise a deleterious effect on the plant tissues and their enzyms. Aseptic conditions can be maintained for respiration experiments on small amounts of plant tissue, but it would be very difficult to produce silage under such conditions. The problem attacked in the work at the Iowa Agricultural Experiment Station here reported was therefore to differentiate as accurately as possible between the results of the various activities of these two kinds of agents. A number of different experimental methods were employed in the effort to arrive at a distinction between them.

EXPERIMENTAL METHODS

Silage made in the laboratory in glass jars has been used in the greater part of this work. Experimental conditions can in this way be easily controlled, and comparisons are thus possible. The corn was chopped in a small silage cutter or was taken from the college farm silage cutter and packed as tightly as possible into cylindrical wide-mouth jars which were closed with rubber stoppers. Each was provided with an outlet tube for excess gases, which was closed with a pinchcock. Silage made in this manner is perfectly and normally preserved with characteristic appearance and aroma. Comparisons of chemical data between silage from the farm silos and from laboratory silos show no considerable difference. Of course, no two lots of silage are ever exactly alike chemically. The writer has previously made both corn silage and mixed silages in this manner (9, 11). Results obtained show no evidence that this laboratory silage is essentially different from silage made from similar material in an air-tight farm silo.

Some of the corn used in this work was grown to maturity in the greenhouse. Both greenhouse corn and field-grown corn were used, to prevent any possible abnormal results. The greenhouse corn was generally nearly as good in quality as the field-grown corn.

The analytical methods used are based upon the characteristic chemical changes which take place in silage fermentation. The formation of acids and alcohols, the evolution of carbon dioxid, the disappearance of simple sugars, and the degradation of protein, which are the principal chemical phenomena of the fermentation, have been measured by the methods described

below. Results from methods based on these chemical changes show, as the conventional estimations of crude protein, fiber, ether extract, etc., do not, the nature and extent of fermentation and the character of the silage, as nearly as chemical analysis can show. In each case comparisons were made with similar figures obtained on samples of the green corn from which the silage was made. In each case the chemical determinations were made upon the juice expressed from the silage in a Buchner press, under a pressure of 300 to 400 kgm. per square centimeter. This method of sampling facilitates the chemical examination, insures a well-mixed sample, and makes possible more comparable results.

The methods used are as follows:

Total acidity.—Ten c. c. of silage juice were diluted to about 500 c. c. with carbon dioxid free water, and titrated with decinormal barium hydroxid solution in the presence of phenolphthalein till a distinct pink appeared by reflected light against a white background.

Volatile acidity.—One hundred c. c. of juice were distilled in a current of carbon dioxid free steam. To hasten the liberation of volatile acids and alcohols, 100 gm. of sodium chlorid were added to the juice. About 600 c. c. of distillate were titrated with baryta water in the presence of phenolphthalein.

Alcohols (Distillation method).—The distillate from the volatile acid determination was neutralized with baryta water (solid phenolphthalein being added) and concentrated by repeated distillation with sodium chlorid (2). About 50 c. c. of alcohol solution were oxidized[1] in a pressure flask in a boiling water bath for 30 to 40 minutes, and the volatile acids then distilled off four or five times, with additions of carbon dioxid free water. The volatile acids were titrated and calculated as ethyl alcohol.

Alcohols (Aeration method).—In this method (5) a current of air was drawn thru the silage juice, which was saturated with ammonium sulphate, into concentrated sulphuric acid. The sulphuric acid-alcohol solution was then oxizided with the potassium dichromate solution, and distilled and calculated as in the previous method. Although other alchols are formed in silage in small amounts, all were calculated as ethyl alcohol.

Total sugars.—Fifty c. c. of juice were clarified with neutral lead acetate, the excess lead precipitated with anhydrous sodium carbonate, an aliquot allowed to stand 24 hours with hydrochloric acid, neutralized, and the total reducing sugars determined on an aliquot by either the Defren-O'Sullivan method or

[1]The oxidizing solution used was made up in the following proportions: 10 gm. of potassium dichromate ($K_2 Cr_2 O_7$), 20 gm. of sulphuric acid (H_2SO_4), 70 gm. of water.

the slightly modified volumetric method of Schoorl (19). In any given series the same method was used to insure comparable results.

Amino nitrogen.—The amino nitrogen was determined on the diluted juice with the Van Slyke apparatus (22). This determination shows the relative degree of hydrolysis of protein if used on the same or similar material at successive periods.

Ammonia nitrogen.—A 50-c. c. sample of the juice was distilled with magnesium oxid, according to the official fertilizer method.

Moisture.—Samples of about 100 gm. of silage were dried to constant weight, in most cases in a vacuum oven at 60° C.

SILAGE MADE UNDER ANTISEPTIC CONDITIONS

Corn silage made in the presence of ether and chloroform, as done by Babcock and Russell (1), is, of course, well preserved and evolves, after the antiseptic has been allowed to evaporate, an odor quite aromatic and characteristic of silage. The exact amount of antiseptic which should be added to inhibit bacterial growth without seriously impairing enzymic action is, however, very difficult to estimate. Moreover, Wagner (25) has found that certain bacteria may flourish in the presence of benzene, phloroglucinol, phenol, and phenolic derivatives. Some experimental silage was made, however, in the presence of chloroform, toluene, and cresol. Analytical data on these are shown in table I.

TABLE I—ANALYTICAL DATA ON ANTISEPTIC SILAGE

(Calculated on basis of 100 gm. of dry silage.)

Kind of silage	Total acidity (N/10)	Alcohol	NH_2—N
	C. c.	*Gm.*	*Gm.*
Typical green corn	34	0	0.052
Toluene silage (2 per cent)	31	Trace.	.057
Chloroform silage (5 per cent)	250	.....	.176
Cresol silage (0.5 per cent)	255	0.15	.199
Normal silage	675	.81	.256

The amount of toluene added seems to have been enough to stop practically all change. No bacterial growth was obtained from the chloroform silage. It seems likely that the jar of cresol silage contained a limited number of active organisms, as two forms were isolated from this silage, one of them an acid former, and no evidence of spore formation could be obtained with either of these organisms. Some of the results of the work with antiseptics have been introduced here to show the apparent impossibility of obtaining conclusive results, at least with silage, by using such methods alone. These data, however, may be of

some value when compared to other data shown on the following pages. Further experiments with varying amounts of antiseptics were not attempted, as it seemed likely that other methods would give more conclusive results.

OTHER EXPERIMENTAL SILAGE

An effort was made to learn the effect of bacteria and yeasts alone, by heating jars of chopped corn to destroy the enzyms, followed by inoculation with an infusion of normal silage, which should carry the normal mixed flora of silage. After inoculation the jars were in each case incubated at 28° to 30° C., with a control jar of normal silage made from the same sample of corn. The analytical data, which are interesting but not conclusive, are shown in table II.

TABLE II—SILAGE HEATED AND INOCULATED

(Data on 100 c. c. of juice)

Kind of silage	Total acidity (N/10)	Volatile acidity (N/10)	Alcohol	NH_2-N.
	C. c.	*C. c.*	*Gm.*	*Gm.*
Green corn	23	1.8	0	0.044
Normal silage (control)	375	118.0	.265	
Corn heated to 80° and inoculated	274	123.6	.345	.135
Green corn	43	.....	0	.111
Normal silage	318	109.6	.150	.214
Corn heated to 90° and inoculated	198	99.1	.244	.068
Green corn	19.5	1.2	0	.024
Normal silage	342.5	73.1	.297	.114
Corn heated to 85° and inoculated	301.5	58.4	.188	.035

The prominent part which may be played by yeasts in the fermentation of silage under certain conditions was demonstrated by adding to a jar of silage sufficient tartaric acid in solution to make 2 per cent of the weight of the silage. An acid mixture of this strength practically inhibits bacterial action and favors the development of yeasts. A comparison of the acidity of the silage with the amount of tartaric acid added showed that evidently no other acid had been formed, except some volatile acid, which might possibly have come from the oxidation of alcohol. The quantity of alcohol found in 100 c. c. of this silage juice was 1.746 gm., expressed as ethyl alcohol, while normal silage juice contain only from 0.20 to 0.45 gm.

RATE OF CHEMICAL CHANGES IN SILAGE FERMENTATION

A more conclusive method of differentiating between the activities of enzyms and of microorganisms was suggested by a paper by Rahn (16), who discussed the usefulness of curves

in the interpretation of microbial and bichemical processes. It is shown by Rahn that the curve which is obtained when the formation of products of fermentation or other biochemical process is plotted, taking as abscissæ the total time elapsed and as ordinates the total amounts of compounds produced, is in many cases indicative of the nature or cause of the change. If the change is caused by enzymic action and is, therefore, purely chemical, the active mass of the agent causing the change does not increase as the reaction progresses; and the decreasing concentration of the substance acted upon and the accumulation of end products tend to decrease the rate of change. Thus, the curve becomes convex toward the Y axis. The mass of enzym does not increase unless there are living cells present to elaborate more enzym. However, if organisms are present and are active, they multiply until the exhaustion of nutrients or the accumulation of end products retards and finally stops their increase. Until this time the rate of change increases with the number of organisms, and the resulting curve is convex toward the X axis. Then there is a point of inflection, after which the curve becomes similar to the enzymic curve. An examuie of a curve of this type is shown in fig. 1, a typical "fermentation curve". There is always a point of inflection, or change in direction of curvature in a bacterial curve, provided the data begin before the number of organisms has reached its maximum. These two kinds of curves are discussed at some length by Rahn in the article cited (16).

In natural or mixed fermentations, such as the formation of silage, it is possible that different processes taking place at the same time will destroy the natural form of the curve. Or if both chemical and biological factors are present and producing the same substance, e. g., alcohol, the nature of the curve might be variable, depending upon the relative "active mass" of the two agents. In the case of so large an inoculation that the bacteria do not multiply materially, a bacterial curve might possibly resemble the curve of an enzymatic process. However, the chemical composition of the material and the extent of the fermentation depend upon many variable factors, such as soil and meteorological conditions, the method and rate of filling the silo, and opportunity for inoculation. The silage resulting is thus a variable product, depending on these and other factors. Therefore it seems very unlikely that any considerable error in the interpretation of curves drawn from silage data would persist thru a number of entirely separate experiments. This is evident from the data given in the following pages.

The analytical methods outlined above were used to obtain data on the chemical changes occurring during the early period

of silage fermentation, in order that curves such as just described might be plotted. The data obtained by Neidig (14) on this part of the fermentation process indicate the impossibility of obtaining regular curves and strictly comparable results by taking samples from a farm silo, owing to the variability in composition of the silage in different parts of the silo, and the necessity of the perfect exclusion of air thruout the process. Therefore, silage was made in the laboratory in small jars as mentioned above. The chopped corn was very thoroly mixed, and after a sample had been taken for the initial analysis, it was packed tightly into jars. The jars were kept under the same conditions, generally in an incubator at 28° to 32° C., following the average rise of temperature in the silo. One jar was opened each day and the juice pressed out and analyzed. This method should give as nearly correct results on the rate of change as it is possible to obtain, it being granted that silage formation in small jars is perfectly normal. The changes which were found were very similar to the changes observed by Neidig in the farm silos, except that the uniformity of the samples gave much more uniform and regular curves.

A series of nine jars of silage (series 1), using corn grown to maturity in the greenhouse, gave very interesting results. The corn was mixed and ensiled as described above. The determinations on the green corn and on each day's sample of silage juice were made, as usual, in a strictly comparable manner. The analytical data are given in table III.

The curves plotted from the data in table III are shown in figs. 1 to 4. The curves showing the disappearance of sugars and formation of acids are similar in shape and are typical of bacterial fermentation. The amino-nitrogen curve, which shows the rate of hydrolysis of protein, and the alcohol curve both show the abrupt rise at the beginning which characterizes

TABLE III—SERIES 1: FORMATION OF ACID AND ALCOHOL, DISAPPEARANCE OF SUGARS, AND INCREASE IN AMINO NITROGEN IN SILAGE.

(All data calculated to sample of 100 c c. of silage juice.)

Age of silage	Total acidity N/10 sol.	Alcohol	NH_2—N.	Total sugars	Total sugars which disappeared
Days	*C. c.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
0	27.5	0.002	0.069	5.299	0.000
1	47.5	.144	.083	4.934	.365
2	99.7	.065	.089	4.526	.773
3	166.2	.067	.098	2.774	2.525
4	190.0	.082	.089	2.323	2.976
5	233.7	.090	.106	2.078	3.221
6	253.6	.129	.108	1.742	3.557
7	278.3	.095	.109	1.867	3.432
8	226.1	.082	.109	2.203	3.096
12	228.0	.114	.109	2.270	3.029

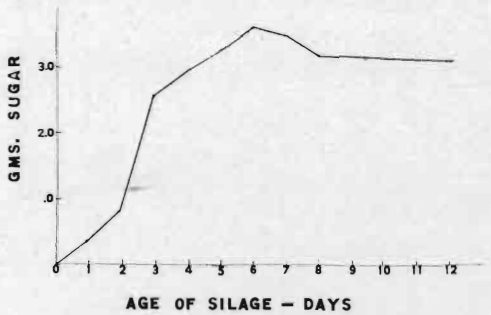

Fig. 1—Curve showing the disappearance of sugars in series 1.

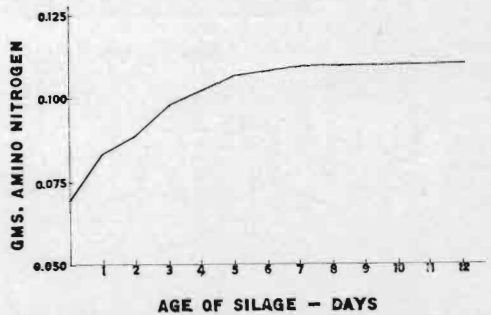

Fig. 3—Curve showing the increase in amino nitrogen (and the rate of hydrolysis of protein) in series 1.

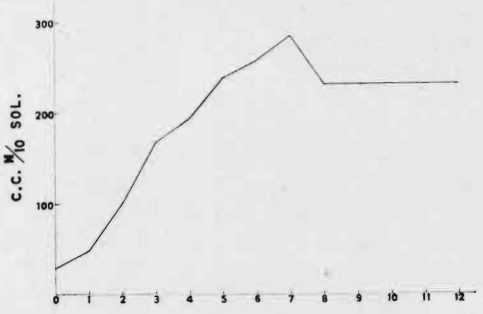

Fig. 2—Curve showing the development of acidity in series 1.

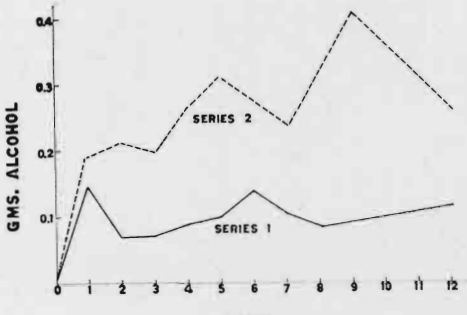

Fig. 4—Curves showing the formation of alcohol in series 1 and 2.

the enzmyic type of curve. However, between the second and third days during the same period in which there appears a marked increase in bacterial activity in the sugar and acid curves, there is also a second rise in NH_2-N curve, which is probable evidence of some proteoclastic action by bacteria.

A similar series of determinations (series 2) made two months later shows a somewhat different set of curves. Field-grown corn at the proper stage of maturity was chopped in the silage cutter, inoculated with material carrying the usual flora of the farm silage cutter, and ensiled as before. In this series total and volatile acid, alcohol, sugars, amino nitrogen, and ammonia nitrogen were determined. The analytical data are shown in table IV.

TABLE IV—SERIES 2: FORMATION OF TOTAL AND VOLATILE ACID AND ALCOHOL, AMINO NITROGEN, AMMONIA NITROGEN AND DISAPPEARANCE OF SUGARS IN SILAGE.

(Data on 100 c. c. of silage juice.)

Age of silage	Total acidity (N/10)	Volatile acidity (N/10)	Alcohol	NH_2-N.	NH_3-N.	Total sugars	Disappear of sugars
Days	*C. c.*	*C. c.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
0	17.3	1.4	0.000	0.021	0.006	3.139	0.000
1	97.0	42.1	.188	.053	.008	1.622	1.517
2	160.3	56.9	.210	.060	.010	1.008	2.131
3	205.4	65.2	.197	.066	.010	.648	2.491
4	235.2	68.4	.262	.095	.026	.235	2.904
5	250.6	68.6	.307	.098	.018	.158	2.981
7	254.4	70.6	.238	.097	.019	.019	3.120
9	263.0	71.0	.403	.104	.021	.019	
12	266.9	78.9	.256	.084	.019	.120	
15	274.6	75.8	.347	.112	.025	.161	
21	291.8	84.0	.354	.128	.027	.....	
30	296.6	79.5	.337	.149	.028	.....	

The form of the acid curves of this series (fig. 5), when compared to the usual form of acid curves, suggests the possibility of so large an inoculation with acid-forming bacteria that the maximum in numbers was reached during the first 24 hours. The curve showing the disappearance of sugars (fig. 6) has the same form. The curves showing the formation of alcohol (fig. 4) is of the enzymic form, like the corresponding curves in series 1. Irregularities in these two alcohol curves

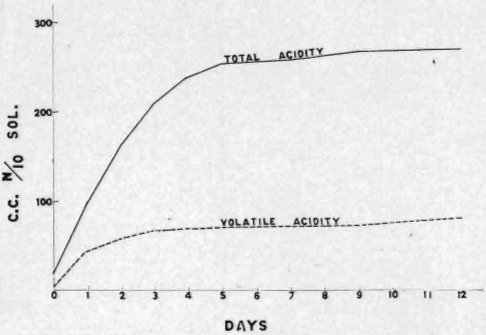

Fig. 5—Curves showing the development of acidity in series 2.

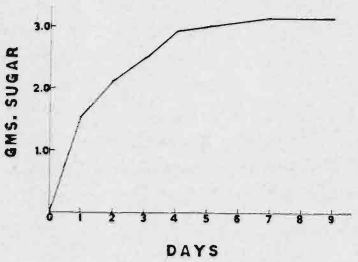

Fig. 6—Curve showing the disappearance of sugars in series 2.

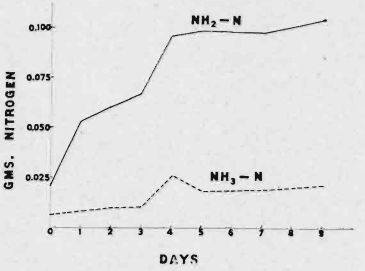

Fig. 7—Curves showing the increase in amino nitrogen and ammonia nitrogen in series 2.

suggest the possibility that each is the resultant of the formation of alcohol by one or more agents and its simultaneous oxidation by other agents. Or there might possibly be some variation between individual samples in the series. The amino-nitrogen curve (fig. 7) shows an evident enzymatic protein hydrolysis during the first three days. Between the third and fourth days, however, an abrupt rise takes place, which bears out the assumption made from series 1, viz, that bacteria figure in the hydrolysis of protein after the first two or three days. It is noteworthy that a similar rise takes place during the same period in the sugar, alcohol, and ammonia-nitrogen curves, indicating a general increase in the activity of microorganims at that time. As previously mentioned, just such a simultaneous rise was noticed in three of the curves in series 1 between the second and third days. This observation lends strength to the evidence in favor of enzymic action in these cases during the first two days.

Corn grown in the greenhouse was used for series 3. This corn made an excellent growth and was practically as good in quality as field-grown corn. The corn was chopped in the laboratory and ensiled as before, but with little opportunity for inoculation. Analytical data on this series are shown in table V. The first jar of silage was opened when only 12 hours old.

TABLE V—SERIES 3: FORMATION OF ACIDS AND ALCOHOLS AND DISAPPEARANCE OF SUGARS.

(Data on 100 c. c. of silage juice.)

Age of silage	Total acidity (N/10)	Volatile acidity (N/10)	Alcohol	Total sugars	Disappearance of sugars
Days	*C. c.*	*C. c.*	*Gm.*	*Gm.*	*Gm.*
0	14.0	2.7	0.001	3.850	0
½	25.0	2.8	.079	4.292	— .442
1	36.5	2.9	.123	4.060	— .210
2	39.0	3.0	.272	3.428	+ .422
4	41.5	3.1	.312	2.236	1.614
7	63.5	5.0	.414	1.660	2.190

The increase in the amount of sugars during the first day is interesting, and very probably due to the presence in the corn grain of amylase, which in this case produced sugar from higher carbohydrates faster than the sugar was used. The development of acids is remarbaly small, due perhaps to the slight opportunity for inoculation with acid-forming organisms. It is unfortunate that a larger number of jars was not filled in this experiment so that the later progress of acid formation could have been followed. Since there is no possibility in this series that the formation of acetic acid complicates the question of alcohol formation, some interesting observations are possible. The alcohol curve (fig. 8) after beginning like an enzymic curve takes another abrupt rise between the first and second days, probably when the yeasts become more active. The concomitant production of carbon dioxid in the silage of this series was also measured. (The data are given on page 327.) The carbon dioxid curve (fig. 13) shows the same trend thruout as the alcohol curve, including the rise just mentioned between the first and second days. The carbon dioxid: alcohol ratios show a general increase as the fermentation progresses, as follows:

Age of silage (days)...	½	1	2	4	7
Ratio.....	1:0.51	1:0.61	1:0.86	1:0.79	1:0.97

This increase might be either because the yeasts are taking an increasingly greater part in the fermentation and the ratio therefore approaches the ratio of the ordinary alcoholic fermentation, or because carbon dioxid from other reactions is included in the amount evolved during the first few days. Of course, the above is the result of but a single experiment on this point.

In the next series (series 4) the corn used was from the same greenhouse plot as the preceding. The use of the same tools, etc., a week later, gave opportunity for a much larger inoculation with acid-forming bacteria. This is evidenced by the data shown below. In this series bacteriological counts of some of the samples were made.[1] The technic used was as follows: The sample was ground in a sterile mortar for 15 minutes, 50 gm. weighed out, placed in a liter flask with 500 c. c. of sterile water and shaken 100 times. One c. c. was plated in various dilutions in a yeast-extract agar and incubated for 48 hours at 37° C. Data on this series are given in table VI.

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[1]Acknowledgment is gratefully made to Dr. R. E. Buchanan for the use of laboratory facilities and media for making these counts.

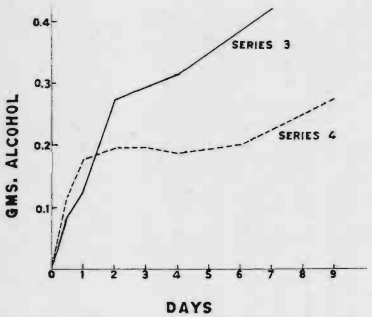

Fig. 8—Curve showing the formation of alcohol in series 3 and 4.

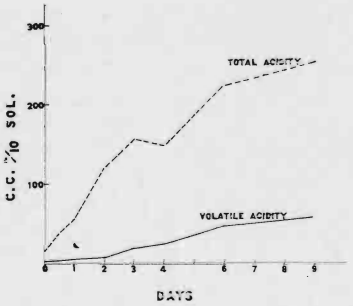

Fig. 9—Curves showing the development of acidity in series 4.

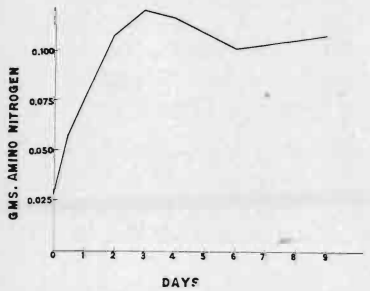

Fig. 10—Curve showing the increase in amino nitrogen in series 4.

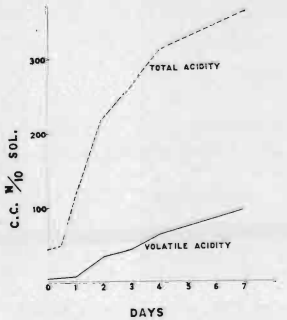

Fig. 11—Curves showing the development of acidity in series 5.

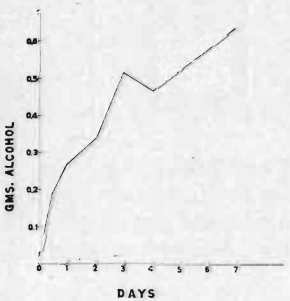

Fig. 12—Curve showing the formation of alcohol in series 5.

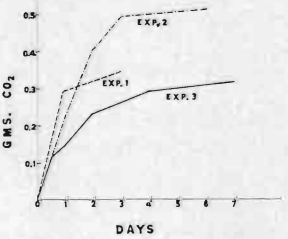

Fig. 13—Curves showing the rate of evolution of carbon dioxide. (Curves 2 and 3 are coincident during the first one-half day.)

TABLE VI—SERIES 4: FORMATION OF ACIDS AND ALCOHOL, AMINO NITROGEN, DISAPPEARANCE OF SUGARS, AND BACTERIAL COUNTS IN SILAGE.

(Data all on 100 c. c. of juice, except bacterial counts.)

Age of silage	Total acidity (N/10)	Volatile acidity (N/10)	Alcohol	NH_2-N.	Total sugars	Disappearance of sugars	Bacteria per gram of silage
Days	*C. c.*	*C. c.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	
0	15.5	2.7	0.001	0.028	6.662	0	
½	39.0	3.5	.109	.058	7.036	— .374	9,900,000
1	55.5	4.6	.176	.076	7.594	— .932	
2	121.0	7.1	.192	.108	5.030	(+)1.632	106,500,000
3	156.0	18.9	.193	.120	3.764	2.898	119,000,000
4	148.5	24.5	.182	.116	4.636	2.026	38,800,000
6	223.5	47.8	.197	.101	2.324	4.338	
9	254.0	58.2	.268	.107	2.190	4.472	106,000,000

It should be noted that the preliminary increase in sugars occurs as it did in series 3. The comparatively small bacterial count in the 4-day-old silage is reflected in the total acid, amino nitrogen, and sugar columns. The alcohol curve (fig. 8) shows the same enzymic form as before. The acid curves (fig. 9) show the usual bacterial form. The amino-nitrogen curve (fig. 10) is of the enzymic form as before. The curves showing the disappearance of sugar in series 3 and 4, tho not reproduced here, approach the bacterial form.

A final lot of silage (series 5) was made from corn grown in the fields. This corn lay in the field or on wagons 15 to 20 hours after cutting. It was taken from the farm silage cutter, mixed, and ensiled as before. The data are given in table VII.

TABLE VII—SERIES 5: FORMATION OF ACIDS AND ALCOHOL IN SILAGE.

(Data on 100 c. c. of juice.)

Age of silage	Total acidity (N/10)	Volatile acidity (N/10)	Alcohol
Days	*C. c.*	*C. c.*	*Gm.*
0	45.0	6.0	0.022
½	47.5	6.5	.190
1	123.5	7.5	.269
2	218.5	36.8	.342
3	263.0	45.3	.515
4	312.5	65.2	.465
7	362.5	96.8	.633

The initial acid and alcohol content are rather high, as the corn stood so long after cutting. The acid curves (fig. 11) show the usual bacterial form and the alcohol curve (fig. 12) the usual enzymic form, with the later rise presumably due to yeasts.

The data obtained from these five series of experimental silage will be discussed later.

EVOLUTION OF CARBON DIOXID

The amount of carbon dioxid evolved by silage, a constant and characteristic phenomenon of the process, was measured by absorption in caustic potash solution. The silage was packed into cylindrical specimen jars with wide mouths which were fitted with specially made rubber stoppers. A ⅛-inch galvanized-iron pipe was led to the bottom of each jar and the corn was tightly packed around it. This pipe was closed at the top with a rubber tube and a pinchcock. An outlet tube at the top of each jar was connected to an absorption train. The gas was forced thru the train by its own pressure, which was always greatest during the first day, gradually decreasing thereafter. At the end of each period the iron inlet tube of one of the jars was connected to a soda-lime tube and a current of air was drawn thru the absorption train for 30 minutes to remove the carbon dioxid remaining in the jar. The silage could then be removed for analysis. The data from the three experiments are given in table VIII.

TABLE VIII—EVOLUTION OF CARBON DIOXID IN SILAGE.

(All data calculated to sample of 100 gm. of silage.)

Age of silage	Evolution of carbon dioxid		
	Experiment 1 (Gm. CO_2)	Experiment 2 (Gm. CO_2)	Exp'm't 3* (Gm. CO_2)
Days			
½			0.115
1	0.288	0.228	.148
2	(†)	.404	.226
3	.341	.490	
4	(†)	(†)	.292
5	(†)	(†)	
6		.508	
7			.315

*These data are from series 3, p. 323.

The curves plotted from these data are shown in figure 13. All are of the enzymic form, and check with the observations of the writer on all the silage he has made, viz, that the evolution of gas is always greatest during the first day or two, and nearly ceases after about four days. In most cases the rate of evolution is evidently kept up after the first day or two by contributions from bacteria and yeasts. In experiment 3 the curve shows a change of direction and a distinct rise during the second day, coincident, as remarked above, with a similar rise in the alcohol curve in the same experiment (cf. series 3).

RISE IN TEMPERATURE

Another characteristic phenomenon of silage fermentation, but one very much misunderstood in the early days of silage making, is the rise in temperature of the silage. Temperatures

as high as 130° F. have been observed in the silo at or near the surface of the silage. This excessive heating is due to activity of microorganisms greatly accelerated by the presence of atmospheric oxygen, and occurs whenever silage is uncovered and left exposed to the air for a time. The temperature deep in the silo, however, protected from the air and sufficiently removed from the conduction of heat from the surface of the silage, is rarely higher than 80° F. It is rather unsatisfactory to attempt to obtain curves characteristic of bacterial or enzymic fermentation from the rise in temperature of the medium, on account of the number of somewhat extraneous factors involved. The outside temperature is always a factor, and the rise in temperature of the silage might easily affect the rate of chemical reactions or of bacterial growth thus increasing the rate of temperature rise and perhaps changing the nature of the curve. Data which have been obtained from the farm silos[1] and from very carefully insulated laboratory silos suggest that the greater part of the heat developed is due to microbial action. It is considered unnecessary to reproduce these data here as similar data have been published (14). However, one table is subjoined (table IX) showing the rise in temperature at the surface of the silage in one of the farm silos. An iron pipe was forced down into the silage for about 4 feet, and a thermometer, immersed in a test tube full of water, lowered into the pipe so that the bulb was 2 feet below the surfac of the silage. The top of the pipe was closed except when the thermometer was pulled up for reading. These data when plotted give a smooth and typical bacterial fermentation curve. Of course, this does not exclude the possibility of some heat prdouction by enzym action.

TABLE IX—RATE OF HEATING AT SURFACE OF SILAGE IN BRICK SILO.

Date	Time	Age of silage	Temperature	Outside temperature
		Days	°F.	F°.
Sept. 19	4 p. m.	0	71.2	61
Do	12 mid.	.....	75.4	
Sept. 20	9 a. m.	.....	82.0	67
Do	12 m.	.....	85.6	
Do	6 p. m.	1	100.8	
Sept. 21	9 a. m.	.....	113.4	58
Do	12 m.	.....	115.3	
Do	4 p. m.	2	117.6	
Sept. 22	9 a. m.	.....	122.2	53
Do	6 p. m.	3	123.2	
Sept. 23	12 m.	4	125.6	52
Sept. 24	12 m.	5	127.2	61
Sept. 25	12 m.	6	128.8	62

[1]With the cooperation of the Agricultural Engineering section of the Iowa station.

DISCUSSION AND CONCLUSIONS

It appears that neither microorganisms nor plant enzyms are alone responsible for the changes which take place in corn silage fermentation. The curves in these pages show that acid production is mainly if not entirely a phenomenon of bacterial activity in the silage. The results from the other experimental silage described above also suggest that the greater part of the acid is produced by microorganisms.

The curves showing the disappearance of sugars are, like the acid curves, generally of the bacterial fermentation type. Altho some of the sugar is undoubtedly changed by enzym action, the greater amount seems to be metabolized by bacteria and yeasts.

The formation of alcohol, however, is evidently a phenomenon primarily of the respiratory or enzymatic activity of the plant cells. This is suggested by investigators in plant physiology, who have often found zymase in plants, and is corroborated by the nature of the alcohol curves shown. The curves suggest a later production of alcohol by yeasts, and results from the other experimental silage support this idea. As stated above, Esten and Mason (6) found large numbers of yeasts in corn silage. Both factors, therefore, probably have a share in the alcohol production.

A similar statement holds good for the hydrolysis of protein as indicated by the amino-nitrogen content of the silage. Proteoclastic enzyms are present in corn grain, and the curves show evidence of their activity. Both the later rise in the amino-nitrogen curves noted above and the results from silage in which the enzyms were destroyed show some proteoclastic activity by microorganisms also. It is noteworthy that E. J. Russell (17) found end products of protein hydrolysis in corn silage made in the presence of toluene, which hydrolysis he ascribed to enzym action.

The evolution of carbon dioxid must be dne largely to enzym action. The curves shown all agree on that point, even when the first period was only 12 hours. Evidence that yeasts produce a part of the carbon dioxid after the first day has also been pointed out.

The rise in temperature of the silage is not great except at the surface, where the material is in contact with air. Microorganisms seem to be responsible for most of the heating, but the partial influence of enzyms is not excluded.

SUMMARY

The question of the respective causal relationship of microorganisms and plant cell enzyms to the fermentation of corn silage has long been in controversy. It is difficult to differen-

tiate between the activities of these two kinds of agents. Work with antiseptics both by earlier investigators and by the writer is not conclusive. Experimental silage, other than antiseptic silage, has been made, with results of some value; but the most conclusive evidence is obtained by the determination of the rate of change in various phenomena of the fermentation under normal conditions. Curves plotted from these data show that bacteria are mainly responsible for acid production and the concomitant disappearance of sugars. Alcohol is formed first by plant enzyms and later by yeasts. Protein is hydrolyzed first by enzyms and later by microorganisms. Carbon dioxid evolution seems to be very largely due to respiratory or enzymic activities, but yeasts probably have a share in its production after the first day or two. Microorganisms are probably largely responsible for the heating of the silage. Both kinds of factors are always present during silage fermentation and the process is due to the activities of both in the absence of air.

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