Butyric Acid and Butyl Alcohol Fermentation of Hemicellulose- and Starch-Rich Materials

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A group of bacteria, capable of fermenting the hemicelluloses and the starch of various plant materials, is isolated from the soil. The acids and alcohols produced in the fermentation of these materials consist principally of butyric acid and butyl alcohol. Wheat middlings give a good yield of fermentation products, while corn meal is not readily fermented. The addition of concentrated corn steep liquor exerts a markedly favorable effect on the fermentation processes, especially in the case of the corn meal, where a good yield of butyl alcohol is obtained. Other plant products and plant materials, such as molasses, alfalfa, and corncobs support good growth of the organisms, but the fermentation process is not particularly vigorous. Purified hemicelluloses are not fermented; in the process of separation and purification, they are rendered resistant to decomposition by these organisms.

IN THE study of decomposition of hemicelluloses by microörganisms (*14*), there was isolated from the soil a group of anaërobic bacteria, belonging to the *Clostridium butyricum* Prazmowski group, and capable of utilizing hemicellulose-rich materials; in this process some of the hemicelluloses and starch, as well as certain sugars, were converted into organic acids and alcohols. Several cultures belonging to this group of bacteria were isolated and cultivated; they were found to represent a number of strains which were similar in their morphological appearance but which varied considerably in their fermentation capacity and in their ability to produce acids and alcohols from carbohydrates. They were found to be closely related to the butyric acid bacteria, but were distinguished from the typical representatives of this group by the fact that they grew not only upon sugars and starch but also upon certain hemicelluloses.

A detailed review of the literature of the bacteria forming butyric acid and butyl alcohol is found in the work of McCoy, Fred, et al. (*8*). The investigations on the anaërobic decomposition of pectins, especially in connection with the anaërobic retting of flax, and of starches and cellulose have an important and direct bearing upon the problem under consideration. Beginning with the work of Winogradsky and Fribes (*19*) and Beijerinck (*1*) and ending with that of Carbone (*2*), Makrinov (*7*), and Ruschmann (*12*), an extensive literature has accumulated on the role of bacteria in the retting process. A number of organisms, all of which belong to the butyric acid bacteria, was described; they varied in the nature of the carbohydrates attacked and in the relative amount of butyric and other acids, as well as alcohols, produced in the fermentation process. These organisms could not attack cellulose; some, however, acted upon various hemicelluloses and starches. There is no doubt that certain organisms, probably related to this group, are also capable of fermenting true cellulose (*6*).

Fermentation of Wheat Middlings

The specific problem under consideration deals with the production of organic acids and alcohols by pure cultures of anaërobic bacteria isolated from soil, using as a substrate material rich in starch and in hemicellulose. The methods of isolation and cultivation of the organisms were those commonly employed in the study of anaërobic bacteria. Wheat middlings were employed as a basic medium for the growth of these organisms. The proximate analysis of this was as follows:

	%
Moisture	11.14
Total nitrogen	2.56
Ash	3.79
Starch	16.72
Hemicelluloses	34.66

The term "hemicellulose" is commonly employed in the literature in a very loose manner. It is used here to designate those carbohydrates which are readily hydrolyzed by hot dilute mineral acids (2 per cent hydrochloric) and converted into reducing sugars, with the exception of starch, glycogen, and inulin. Some of the hemicelluloses are pure polysaccharides, while others contain uronic acid groups, like the pectins, and can be spoken of as polyuronides (*9*).

The media used for the growth of the bacteria and for the production of the organic acids were prepared by placing definite 4 to 5 per cent quantities of middlings, about 1 per cent calcium carbonate, and tap water in a series of flasks of varying dimensions. The flasks were plugged with cotton, sterilized, and inoculated with portions of a 48-hour culture of the organisms. During the growth process there developed large quantities of various gases. In some of the experiments the gases were measured quantitatively and qualitatively. A flask containing about 6 liters of medium was found to produce well over 20 liters of gas, which consisted of about 45 per cent hydrogen, the rest being carbon dioxide and a small amount of methane. The different strains of the organisms differed, however, in the composition of the gases formed in the fermentation process.

The acids produced by these organisms were both volatile and nonvolatile. In the presence of calcium carbonate only small amounts of alcohol were found in the culture; however, in the absence of the carbonate, large quantities of alcohols were formed, while the acid accumulation was reduced, a phenomenon well known in the case of the butyl alcohol fermentation process.

The analysis of the volatile acids was carried out according to the following procedure:

At the end of the incubation period the acids were liberated by the addition of sufficient 10 per cent H_3PO_4, and the medium was filtered through cotton. An aliquot portion of the culture was steam-distilled so as to remove the volatile acids; the distillate containing the organic acids was then neutralized and concentrated, thus removing the alcohols, ketones, and other volatile products present. The acids were again liberated and determined by fractional steam distillation, at constant volume, according to the Dyer method (*4*). However, this method was found to be not fully reliable when applied to known mixtures, and an attempt was therefore made to apply the Werkman (*17*)

procedure of separation of organic acids, by means of isopropyl ether, as well as the Duclaux (*3*) method as modified by Virtanen and Pulkki (*13*).

It was found in a preliminary experiment on the influence of the incubation period upon the production of volatile acids from wheat middlings that the maximum acid production was reached, at 28° to 30° C., in 11 days; after that, the acid content of the cultures began to diminish. Further investigations brought out the fact that the various strains of the organisms did not behave alike, some producing considerable quantities of acid in a shorter time than others.

In order to determine what constituents in the middlings are utilized by the organism, a proximate analysis (*16*) was made of the solids in the culture at the beginning and at the end of the decomposition period—namely, 11 days. The results presented in Table I show that the cellulose, lignin, and protein of the medium were not attacked at all or were utilized only to a very limited extent, the residual material having a much higher relative concentration of these complexes than the original medium. The starch had nearly all disappeared, while a part of the hemicelluloses (including the pentosans) and some of the fats were decomposed by the organism. The dried residue weighed just about half of the original dry material added to the culture. The loss in weight was almost completely accounted for by the disappearance of the starch, the hemicelluloses, and the fats. The organic nitrogen present in the middlings was sufficient to supply the needs of the organism. The addition of inorganic nitrogen, in the form of ammonium salts or nitrates, did not have any favorable effect upon the growth and acid production.

TABLE I. PROXIMATE CHEMICAL COMPOSITION OF WHEAT MIDDLINGS AND OF FERMENTED RESIDUE

	Total Control Culture	Fermented Residue: No $CaCO_3$ Total amount left	No $CaCO_3$ Decomposed	$CaCO_3$ Total amount left	$CaCO_3$ Decomposed
	Grams	*Grams*	*%*	*Grams*	*%*
Total dry material	380	178	53.16	202	...
Ether-soluble portion	14.9	8.7	41.61	8.0	46.31
Hemicellulose and starch[a]	150	46.3	69.13	43.8	70.80
Pentosan	73	45.9	37.12	51.1	30.00
Starch	95	4.4	95.37	4.2	95.58
Cellulose	30.4	25.6	15.79	26.9	11.51
Lignin	32.6	...	...	30.9	5.21
Protein	55.1	51.8	5.99	47.6	13.61

[a] Hemicellulose figure is calculated from the reducing sugars (× 0.9) produced on hydrolysis with 2 per cent HCl for 5 hours at 100° C.; the pentosan is calculated from the furfuraldehyde yield; the starch was determined by hydrolysis with an active diastase preparation.

The alcohol production by the organisms was determined in the following manner:

An aliquot portion of the steam distillate of the culture was titrated to phenolphthalein with 0.1 *N* potassium hydroxide. A larger aliquot was then carefully neutralized, using the titration figures, and the alcohols and other volatile materials present in the culture distilled over directly; an aliquot portion of this distillate containing less than 10 mg. of alcohol was analyzed by the Dupres method, as outlined by Northrop (*10*). The alcohol was oxidized by an excess of 0.2 *N* potassium dichromate, and the excess dichromate determined by titration with potassium iodide and sodium thiosulfate. This method also accounts for the aldehydes and other reducing substances which may be present in the distillate.

The nature of the alcohol was determined by adding sufficient dichromate to the alcoholic distillate, placing in pressure flasks, and heating in a steam bath for 45 minutes in order to oxidize the alcohol to the corresponding acid; the contents were made alkaline with potassium hydroxide, and most of the liquid was distilled over. The residue was acidified with sulfuric acid and slowly distilled. The distillate was found to contain butyric acid, the odor being so strong as to make a further chemical test unnecessary. Other alcohols beside butyl may have been produced, but only in negligible quantities.

The alcohol content of the culture was found to increase with an increase in the acid content and with the advance of the period of incubation, as shown in Table II. The addition of calcium carbonate modifies considerably the relative acid and alcohol production of the organisms.

INFLUENCE OF CORN STEEP UPON FERMENTATION OF MIDDLINGS AND CORN MEAL

Corn steep liquor was found to exert a pronounced stimulating effect upon the fermentation process, as shown by the increased yield of volatile organic acids. The composition of this material was as follows:

	%
Moisture	49.16
Ash	8.76
Nitrogen	4.00

The pH of the steep was 4.5.

The influence of the corn steep upon the fermentation of middlings and corn meal is brought out in Table III. The addition of corn steep resulted in a marked increase in acid and alcohol production, both in the presence and absence of calcium carbonate.

TABLE III. INFLUENCE OF CORN STEEP ON FERMENTATION OF WHEAT MIDDLINGS[a]

Corn Steep	$CaCO_3$	Culture 5: Volatile acid		Culture 5: Alcohol		Culture 97: Volatile acid		Culture 97: Alcohol	
%		*Cc. 1 N*	*%*[b]	*Cc. 1 N*	*%*	*Cc. 1 N*	*%*	*Cc. 1 N*	*%*
0	0	7.74	1.51	395.71	16.29	9.03	1.77	94.60	3.48
0.5	0	11.70	2.29	348.03	14.32	11.61	2.27	47.24	1.94
1.0	0	17.20	3.37	407.1	16.75	21.07	4.12	45.28	1.86
2.0	0	18.06	3.53	427.66	17.60	15.48[c]	3.03	64.94[c]	2.67
0	+	37.84	7.40	23.62	0.97	57.62	11.28	29.52	1.21
0.5	+	44.72	8.75	52.62	2.17	65.36	12.79	42.30	1.74
1.0	+	40.85	7.99	59.01	2.43	68.80	13.46	32.47	1.34
2.0	+	51.17	10.01	58.03	2.39	69.66	13.63	29.52	1.21

[a] The method of sterilization of the media was modified according to the following procedure: 500 cc. of water plus the solid material were placed in flasks and sterilized for 1.5 hours at 15 pounds per square inch (1.05 kg. per sq. cm.) pressure; 400 cc. sterile water were then added, and the medium was sterilized an additional 30 minutes. The flasks were inoculated with 5 cc. of a 24-hour tube culture and incubated for 6 days.

[b] Percentage of dry middlings as butyric acid and butyl alcohol.

[c] Fermentation not complete.

The nonvolatile acid fraction was next investigated. A portion of the fermented medium was subjected to steam distillation; the residue left was then made into a plaster with anhydrous sodium sulfate and extracted for 72 hours with ether in a Soxhlet extractor. The nonvolatile portion gave a positive Uffleman test in both cases, indicating the presence of lactic acid or other α-hydroxy acids.

Among the volatile acids, butyric seems to be the only important acid produced in the fermentation process. Other

TABLE II. INFLUENCE OF CALCIUM CARBONATE ON FORMATION OF BUTYRIC ACID AND BUTYL ALCOHOL FROM MIDDLINGS

(800 cc. of medium used; 100 cc. of 40 hour old inoculum of same composition as flask to be inoculated; plain medium = 5 per cent wheat middlings, $CaCO_3$ medium = 5 per cent wheat middlings + 1 per cent $CaCO_3$)

Incubation Period	Nature of Product	Culture 5: Plain medium Titration[a]	Culture 5: Plain medium Percentage[b]	Culture 5: $CaCO_3$ medium Titration	Culture 5: $CaCO_3$ medium Percentage	Culture 97: Plain medium Titration	Culture 97: Plain medium Percentage	Culture 97: $CaCO_3$ medium Titration	Culture 97: $CaCO_3$ medium Percentage
Days									
1	Volatile acid	9.42	1.84	59.60	11.66	14.77	2.89	45.32	8.87
	Alcohol	202.51	8.33	137.32	5.65	121.99	5.02	131.38	5.41
3	Volatile acid	15.24	2.98	69.20	13.54	14.79	2.89	78.35	15.33
	Alcohol	203.79	8.39	195.99	8.07	95.62	3.94	92.55	3.81
5	Volatile acid	15.82	3.10	78.60	15.38	17.80	3.48	82.24	16.09
	Alcohol	274.14	11.28	188.87	7.77	186.70	7.68	116.63	4.80

[a] In terms of cubic centimeters of normal acid or alcohol.

[b] Percentage of dry middlings as butyric acid or butyl alcohol.

TABLE IV. EFFECT OF CORN STEEP ON FERMENTATION OF CORN MEAL[a]

(900 cc. of medium containing 4.5 per cent corn meal; inoculum, 5 cc. of a 24-hour culture)

INCUBATION PERIOD	CULTURE	NATURE OF PRODUCT	CORN MEAL ALONE		CORN MEAL + $CaCO_3$		CORN MEAL + CORN STEEP		CORN MEAL + CORN STEEP + $CaCO_3$	
Days			*Cc. 1 N*	%[b]	*Cc. 1 N*	%	*Cc. 1 N*	%	*Cc. 1 N*	%
3	5	Volatile acid	4.30	0.94	22.79	4.96	1.72	0.37	69.23	15.05
		Alcohol	3.14	0.14	3.14	0.14	183.92	8.41	111.40	5.09
	97	Volatile acid	2.41	0.52	16.34	3.55	4.73	1.03	54.18	11.78
		Alcohol	1.50	0.07	1.50	0.07	39.30	1.80	18.30	8.37
6	5	Volatile acid	6.88	1.50	38.27	8.32	1.72	0.37	79.12	17.20
		Alcohol	8.40	0.38	5.26	0.24	161.86	7.40	183.92	8.41
	97	Volatile acid	3.01	0.65	32.51	7.07	5.59	1.22	75.68	16.46
		Alcohol	1.50	0.07	3.60	0.16	73.94	3.38	44.54	2.04
10	5	Volatile acid	6.45	1.40	44.55	9.69	3.01	0.65	64.93	13.93
		Alcohol	9.50	0.43	15.14	0.69	277.46	12.69	151.34	6.92
	97	Volatile acid	3.44	0.75	42.14	9.16	5.16	1.12	85.14	18.51
		Alcohol	2.54	0.12	9.90	0.45	92.22	4.22	41.40	1.89
30	5	Volatile acid	9.37	2.04	64.93	14.12	5.50	1.20	72.93	15.86
		Alcohol	18.30	0.84	81.30	3.72	253.50	11.59	234.60	10.73
	97	Volatile acid	3.70	0.80	68.80	14.96	6.02	1.31	83.42	18.14
		Alcohol	5.54	0.25	96.72	4.42	138.64	6.34	107.12	4.90

[a] Corn steep = 0.5%; $CaCO_3$ = 1.0%.
[b] Percentage of dry corn meal as butyric acid and butyl alcohol.

acids were present but only in small concentration. This was further confirmed by an analysis of the volatile acids produced in the fermentation, using the procedure of Orla-Jensen (*11*) whereby the potassium salt of the acid is precipitated fractionally by silver nitrate and the silver content of the various fractions determined. The following results were obtained:

FRACTION	Ag IN SALT Culture 6	Culture 7	FRACTION	Ag IN SALT Culture 6	Culture 7
	%	%		%	%
1	55.1	55.5	5	55.3	55.6
2	55.1	55.6	6	55.4	56.1
3	55.4	55.3	7	55.3	55.7
4	55.5	55.6	8	55.5	54.2

The theoretical silver content of silver butyrate is 55.38 per cent. Butyric, therefore, appears to be the only volatile acid produced by the two organisms.

When, instead of middlings, corn meal mash was used, very little fermentation took place. In order to determine whether this was due entirely to the inability of the organisms studied to attack the starch in the corn or to a lack of certain nutrients, the effect of corn steep upon the fermentation of corn mash was studied. The medium consisted of 900 cc. of water and 4.5 per cent corn meal, some of the flasks receiving previous to sterilization 0.5 per cent corn steep, some 1 per cent calcium carbonate, and some both. The flasks were inoculated with 5-cc. portions of 24-hour cultures of two organisms (5 and 97) and placed in the incubator. At the end of definite periods of incubation, the cultures were analyzed for volatile acid and for alcohol.

The results presented in Table IV show that culture 5 is capable of producing large amounts of acid and alcohol from corn meal when corn steep is added to the medium, with and without calcium carbonate. However, the corn steep depressed acid production by culture 5 in a medium lacking a neutralizing agent, while culture 97 was stimulated. Since the corn steep was distinctly acid in reaction, this difference in behavior of the two organisms may have been due to a greater sensitivity of culture 5 to the acid conditions thus created. The differences may also have been due to the fact that culture 5 possesses a high alcohol-producing capacity, indicating the existence in this organism of a more efficient mechanism for the immediate reduction of the acids to alcohols, thus preventing their accumulation. In the presence of calcium carbonate, more active fermentation of the corn meal took place, as evidenced by the concentration of the products formed. These results further emphasize the variability of the different strains in their fermentation capacity: culture 97 produced less acid at the start of the fermentation than culture 5, but in the end overtook it. In alcohol production, however, culture 97 was always inferior to culture 5. There was a sharp increase in alcohol production in the 30 day old culture 97 (without the corn steep but with calcium carbonate) which seems to be abnormally high. No satisfactory explanation can be suggested for this.

INFLUENCE OF TYPE OF INOCULUM

A study was next made of the influence of nature of inoculum for bringing about the final fermentation. Flasks containing 900 cc. of water and 5 per cent wheat middlings were inoculated with 5 cc. of three different inocula, incubated for 5 days, and the acid and alcohol determined. The inocula were prepared as follows:

1. The plain inoculum consisted of a 24-hour culture of vegetative cells.
2. In the soil inoculum the organism was grown in a medium consisting of 100-gram portions of Sassafras sandy soil plus 2 grams of wheat middlings for 30 days; some of the soil was then used to inoculate wheat-middling culture tubes; after 24 hours of incubation these were employed for the inoculation of the large flasks.
3. In the spore inocula, 2 to 4 week old cultures were heated at 80° C. for 10 minutes to destroy the vegetative cells and the weak spores; the culture thus heated was used for inoculation purposes.

Comparatively little difference was obtained between the three different inocula; the plain vegetative cells gave somewhat lower yields of acid and alcohol. The inoculum of spores gave a higher yield, probably due to the fact that in the process of pasteurization the weak and less desirable cells were eliminated. An inoculum of this type usually shows a longer lag phase, because of the time required for the germination of the spores, but results in a more vigorous fermentation as has been pointed out by Weyer and Rettger (*18*). The soil inoculum gave results falling between the other two types; this represents a modified form of spore inoculation, since, after 30 days of growth in the soil culture, practically all of the organisms were largely in the spore state. Inoculation of a culture tube with the soil and subsequent incubation results in a fresh and vigorous vegetative culture, which can be used to inoculate the large flasks. A combination of the soil culture and pasteurization is known to be made use of in a commercial production of butyl alcohol (*5*).

FERMENTATION OF VARIOUS PLANT MATERIALS

In a search for an inexpensive plant material which might serve as a substrate for the growth of hemicellulose- and starch-fermenting organisms, the fermentation of blackstrap molasses and of alfalfa was attempted. The molasses employed had the following composition:

	%
Total nitrogen	0.78
Reducing sugars	17.25
Total sugars	49.58

It was difficult to obtain a preliminary fermentation of this material in the inoculum, which was prepared for the enrichment of the culture. The fermentation of the molasses, as shown in Table V, did not prove to be of any decided advantage over the wheat middlings, and its use was therefore discontinued.

TABLE V. FERMENTATION OF BLACKSTRAP MOLASSES

(6 per cent molasses + 1 per cent $CaCO_3$; 100 cc. actively fermenting inoculum used for 800 cc. of media)

Incubation Period	Volatile Acid Produced			
	Culture 5		Culture 97	
Days	*Cc. 1 N*	*%[a]*	*Cc. 1 N*	*%*
7	56.01	9.13	85.17	13.89
11	61.63	10.05	99.35	16.20

[a] Percentage of molasses as butyric acid.

The dried alfalfa contained 7.86 per cent hemicellulose, 12.79 per cent reducing sugar (calculated as glucose) in the hot and cold water extracts, 2.1 per cent total nitrogen, and 13.4 per cent ash. A medium was prepared containing, per flask, 74.4 grams of dry alfalfa and 800 cc. of water. After 8 days of incubation the inoculated flasks were analyzed for volatile acidity and alcohol. The results given in Table VI show that the alfalfa is fermented by both organisms, but not to a very large extent. A fair amount of alcohol was produced, however, under all conditions.

TABLE VI. FERMENTATION OF ALFALFA

(800 cc. of medium, 74.4 grams dry alfalfa; corn steep, 0.5 per cent, and $CaCO_3$ 1 per cent when used; 8-day incubation)

Composition of Medium	Culture 5				Culture 97			
	Volatile acid		Alcohol		Volatile acid		Alcohol	
	Cc. 1 N	*%[a]*	*Cc. 1 N*	*%*	*Cc. 1 N*	*%*	*Cc. 1 N*	*%*
Alfalfa	4.73	0.56	47.88	1.19	7.74	0.92	30.16	0.75
Alfalfa + $CaCO_3$	30.10	3.56	30.16	0.75	27.95	3.31	26.32	0.66
Alfalfa + corn steep	5.16	0.61	71.74	1.79	11.61	1.37	41.72	1.04
Alfalfa + corn steep + $CaCO_3$	25.80	3.05	28.62	0.71	26.66	3.16	20.92	0.52

[a] Percentage of dry alfalfa as butyric acid or as butyl alcohol.

A similar experiment was carried through using as a source of carbohydrate ground corncobs, in concentration of 5.87 per cent. The results, reported in Table VII, show that a fair amount of volatile acidity is produced in the fermentation of untreated corncobs; however, the alcohol production was almost negligible. Culture 97 gave a much lower fermentation of the corncobs than culture 5.

TABLE VII. FERMENTATION OF CORNCOBS

(800 cc. of medium containing 5.87 per cent ground corncobs, 0.5 per cent corn steep, 1 per cent $CaCO_3$; 8-day incubation)

Composition of Medium	Culture 5				Culture 97			
	Volatile acid		Alcohol		Volatile acid		Alcohol	
	Cc. 1 N	*%[a]*	*Cc. 1 N*	*%*	*Cc. 1 N*	*%*	*Cc. 1 N*	*%*
Corn cobs	4.73	0.89	1.68	0.07	4.30	0.81	1.68	0.07
Corn cobs + $CaCO_3$	19.52	3.66	1.68	0.07	11.18	2.10	1.68	0.07
Corn cobs + steep	7.74	1.45	7.84	0.31	6.45	1.21	1.68	0.07
Corn cobs + steep + $CaCO_3$	35.69	6.69	6.30	0.25	27.09	5.08	3.22	0.13

[a] Percentage of dry corn cobs as butyric acid or as butyl alcohol.

In order to determine the particular constituents of the corncobs that have undergone fermentation, liter flasks containing 45.53 grams of the dry material in 800 cc. of solution were inoculated with 5 cc. of a 24-hour culture on middlings. All the flasks had 4-gram portions of corn steep added to them (0.5 per cent concentration), and one set of the flasks received an additional 8 grams of calcium carbonate. After 11 days of incubation the flasks, to which no calcium carbonate was added, were removed and analyzed. The flasks containing the calcium carbonate were incubated for 20 days; these flasks inoculated with culture 97 failed to ferment for more than 1 or 2 days after inoculation, while the flasks inoculated with culture 5 were producing gas actively until the very end of the incubation period, thus confirming the previous observations of the greater activity of this strain.

The results presented in Table VIII show definitely that the hemicelluloses of the corncobs are attacked by the anaërobic bacteria, especially by culture 5, in the presence of calcium carbonate. The starch, which is present in the corncobs only in very small amounts, was also attacked, but did not seem to play an important role in supplying a source of energy to the organisms. The hemicelluloses of the corncobs are made up primarily of pentosans, particularly xylan. The decomposition of the pentosan was especially pronounced in the presence of calcium carbonate. The pentosan content in the decomposed residues was found to be greater than the hemicellulose obtained by the method of hydrolysis with dilute acid. This discrepancy is due to the fact that in the distillation of the pentosans with 12 per cent hydrochloric acid, not only the pentosans, but also the uronic acid complexes which do not give very much reducing sugar on hydrolysis with 2 per cent hydrochloric acid, are converted to furfuraldehyde.

TABLE VIII. COMPOSITION OF CORNCOBS AT BEGINNING AND END OF FERMENTATION BY CULTURES 5 AND 97

(800 cc. of medium, 0.5 per cent corn steep in all flasks, 1 per cent $CaCO_3$, where used; no $CaCO_3$, 11-day incubation; $CaCO_3$, 20-day incubation)

	Control	Culture 5		Culture 97
		No $CaCO_3$	$CaCO_3$	No $CaCO_3$
	Grams	*Grams*	*Grams*	*Grams*
Total residual dry material	45.530	41.500	39.200	41.800
Total solids in filtrate	2.032[a]	4.510	14.351	4.030
Ash in filtrate	0.349[a]	0.762	9.002	0.710
Hemicelluloses	16.432	14.025	9.967	14.446
Pentosans	15.990	14.865	10.451	15.401
Starch	0.850	0.402	0.476	0.658
Nitrogen	0.151[b]	0.241	0.187	0.202
Ash in residue	0.633	0.212	2.234	0.213
Volatile acid produced, cc. 1 *N*[c]		8.64	46.78	8.64
Alcohol produced, cc. 1 *N*[d]		3.40	9.00	4.10

[a] From corn steep.
[b] Nitrogen in corn steep = 0.160 gram.
[c] The corresponding amounts (in per cent) of dry corncobs fermented, as butyric acid, are 1.67, 9.05, and 1.67.
[d] The corresponding amounts (in per cent) of dry corncobs fermented, as butyl alcohol, are 0.14, 0.37, and 0.17.

In measuring the hemicellulose, starch, and pentosan content of the corncobs, separate samples of the material were used for each determination. The hemicellulose fraction included the starch as well. The ash content of the fresh material plus that of the corn steep added to the culture checked well with that found in the residue plus the filtrate, in the case of the flasks free from calcium carbonate. However, the flask receiving calcium carbonate gave, for some unexplainable reason, an abnormally high ash content.

The proximate analysis of the fresh corncobs was as follows:

	%		%
Total hemicellulose[a]	36.09	Total nitrogen	0.33
Pentosan	35.12	Ash	1.39
Starch	1.87		

[a] Calculated from sugar produced on hydrolysis with 2 per cent hydrochloric acid. The low nitrogen and ash content of the corncobs are probably responsible for the marked stimulating effect of the corn steep upon the fermentation of this material, as shown in Table VII.

INFLUENCE OF NITROGEN SOURCE UPON THE FERMENTATION PROCESS

A more detailed study was then made of the nitrogen and energy requirements of the organisms bringing about the fermentation. Four different media were used; these were placed, in 200-cc. quantities, in 300-cc. Florence flasks. The media were inoculated with 5-cc. portions of a 24-hour wheat-middling culture, incubated for 7 days, and analyzed for volatile acid, alcohol, ammonia nitrogen, and residual glucose. The different media had the following composition:

Composition	Medium 1	Medium 2	Medium 3	Medium 4
	%	%	%	%
Glucose	4.0	4.0	..	..
$(NH_4)_2HPO_4$	0.25	..	..	..
Corn steep	0.25	0.25	0.25	..
Casein	..	1.0	2.0	2.0
K_2HPO_4	..	..	..	0.25
$MgSO_4$	..	..	..	0.10

Distilled water was employed in the case of the first three media, while tap water was used in the last medium. The casein was brought into solution by sodium hydroxide, and the solution adjusted back to the neutral point by dilute hydrochloric acid. All the media were adjusted to pH 7.0; the addition of the corn steep to the casein solution resulted in the formation of a precipitate of the casein, owing to the acidity of the corn steep. The flasks were sterilized in flowing steam for 30 minutes on 3 consecutive days.

TABLE IX. NITROGEN AND CARBOHYDRATE UTILIZATION OF CULTURES 5 AND 97

(200 cc. of medium, 7-day incubation)

MEDIUM	CULTURE	VOLATILE ACID	ALCOHOL	GLUCOSE LEFT	NH_3 NITROGEN
		Cc. 1 N	*Cc. 1 N*	*Grams*	*Mg.*
1	5	3.10	9.59	6.28	77.78
	97	2.24	4.07	6.62	78.69
	Control	..	...	7.74	85.11
2[a]	5	2.15	19.42	5.72	1.23
	97	3.23	10.35	6.57	1.83
	Control	..	...	7.74	4.89
3[b]	5	..	2.73	..	16.67
	97	..	2.82	..	18.50
	Control	..	...	..	18.50
4[b]	5	..	3.48	..	17.28
	97	..	2.92	..	16.06
	Control	..	...	..	18.50

[a] 260.6 mg. of nitrogen added as casein.
[b] 521.2 mg. of nitrogen added as casein.

Culture 97 produced in medium 1 a sluggish fermentation, while culture 5 produced a considerable amount of gas in media 1 and 2 about 4 hours after inoculation. Eight hours after inoculation, culture 5 produced in medium 2 a spongy mass floating on the surface of the liquid, and buoyed up partly by the gas produced; culture 97 brought this about several hours later. This was due to the precipitation of the casein by the acid formed by the organisms. The flasks containing media 3 and 4 gave some evidence of gas production about 8 hours after inoculation; this ceased, however, in a very short time. An examination of the results given in Table IX reveals the fact that the fermentation obtained in all four media was not very vigorous. It is evident, however, that the organisms prefer casein as a source of nitrogen to the ammonium salt.

The results obtained by the use of media 3 and 4 show that the casein cannot be used as a sole source of energy by the organisms. This was further confirmed by the failure to obtain liquefaction by either organism of gelatin stabs incubated under anaërobic conditions in a MacIntosh and Fildes jar. A slight amount of gas was evolved after inoculation; this is probably due to the fermentation of the small amount of wheat middlings carried over with the inoculum; this can also account for the small amount of alcohol produced. The ammonia nitrogen in the casein media inoculated with the organisms was lower than that of the corresponding controls, showing that, as fast as the ammonia was formed in the degradation of the casein, it was used as a source of nitrogen by the organism, in the presence of available energy.

COMPOSITION OF THE GASES PRODUCED IN FERMENTATION

As to the composition of the gases produced in the anaërobic fermentation, reference may be made to the results of a typical experiment reported in Table X. The medium used in this experiment consisted of 37.5 grams of wheat middlings, 5.0 grams corn steep, and 7.5 grams calcium carbonate in 1000 cc. of water. At various intervals samples of the gas liberated during the fermentation were removed and analyzed. The results show that during the early stages of growth the gas consisted largely of hydrogen and carbon dioxide. With advancing growth the concentration of the hydrogen diminished and that of carbon dioxide increased, owing no doubt partly to the liberation of some carbon dioxide from the carbonate by interaction with the acids produced. There was very little methane or carbon monoxide at any time during the fermentation process.

TABLE X. COMPOSITION OF GASES LIBERATED IN FERMENTATION OF WHEAT MIDDLINGS AT DIFFERENT STAGES OF GROWTH

(Gas concentration, in per cent)

GAS	AGE OF CULTURE			
	20 hrs.	26 hrs.	43 hrs.	67 hrs.
CO_2	56.3	60.3	68.2	78.8
O_2	0.9	0.6	0.8	0.8
CO	0	0	0.1	0.2
CH_4	0.7	0	0.5	0.2
H_2	41.2	38.7	27.7	18.0

DISCUSSION

The results of the above investigations, as well as others not reported here, prove beyond any doubt that the organisms under consideration are able to bring about the decomposition under anaërobic conditions or the fermentation of a variety of carbohydrates, including sugars (glucose, lactose), starch, hemicelluloses (mannan, galactan), but not of cellulose or of lignin. The most important point to be established by these investigations is the ability of certain anaërobic bacteria to utilize various hemicelluloses and produce acids and alcohols. It was essential to use plant materials rich in one or more specific hemicellulose, as shown for the pentosans in the experiment on the fermentation of corncobs. When Irish moss was used as the substrate, culture 5 decomposed within a few days 84.3 mg. out of 531.4 mg. of the galactan added to the culture. When salep root was used, the culture decomposed the mannan actively, producing an abundant quantity of gas. It is of interest to recall here that culture 5 was isolated from soil by the use of a mannan medium, and 97 by the use of a xylan medium.

When freshly isolated, the bacteria were much more active in bringing about the fermentation of purified hemicelluloses, as shown elsewhere (*14*, *15*). Continued cultivation in the laboratory, in pure culture, resulted in a marked deterioration of the fermentation capacity, especially when purified hemicelluloses were used as sources of energy. No attempt was made to study this phenomenon in detail. There is no doubt, however, that the vigor of the strains can be reëstablished by proper methods of cultivation and selection.

SUMMARY

The acid- and alcohol-forming capacity of a series of anaërobic bacteria isolated from soil and capable of fermenting hemicelluloses and starch was studied. Two organisms were selected for most of the investigations reported here. Both forms produced large amounts of butyric acid and smaller concentrations of nonvolatile acid, containing some lactic. One of the cultures produced a typical butyl alcohol fermentation. Corn meal was not fermented readily, but the addition of corn steep had a markedly favorable effect upon the process of growth and fermentation. Wheat middlings were found to give the best medium for both growth and fermentation; the addition of corn meal to this medium did not exert any favorable effect, while the addition of corn steep did.

The addition of calcium carbonate to the medium resulted in the suppression of alcohol production and in an increase in volatile acidity.

Molasses, alfalfa, corncobs, and certain other plant products can be used as a medium for the growth of these organisms, but the extent of fermentation was found to be parallel to the amount of available carbohydrate.

Although the organisms were able to utilize pentosans in the form of corncobs, mannans in the form of salep root, and

galactan in the form of Irish moss, very little growth was produced on the purified hemicelluloses.

Casein and ammonium salts can be utilized as sources of nitrogen, the former being preferred.

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The Alchymists

by

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The original of this, No. 33 in the Berolzheimer Series of Alchemical and Historical Reproductions, is 23 by 32 inches in size. It is now in New York and is owned by The Ehrich Galleries who purchased it in Germany, and by whose coöperation we have been supplied with the photographic negative from which our half-tone cut was made. This is the eighth Teniers painting in the series.