Studies in the Fermentation of Cellulose.

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It is well known that the fermentation of cellulose matter in stagnant pools gives rise to an evolution of marsh gas. The same fermentation takes place during the anaerobic decomposition of sewage matter in the septic tank, and under certain conditions the quantity of gas evolved has been so considerable that it has been possible to make economic use of it.

It has thus been for a long time in the mind of one of us that if conditions could be so adjusted that the cellulose fermentation were developed to its utmost activity it might be possible to make economic use of large quantities of potential fuel in the form of waste paper and vegetable debris which at present have little or no value.

It is thus important to determine which forms of cellulose are most readily attacked by the organisms concerned in the fermentation, and what are the optimum conditions for the activity of the organisms.

The chief work hitherto done on the anaerobic fermentation of cellulose has been by Omelianski. (Compt. Rend. 1895, *121*, 653; 1897, *125*, 11 1; Chem. Centralbl. 1900, i, 918, from Arch. Sci. Biol. St. Petersb. *7*, 411; J. C. S. abstracts, 1902, ii, 468, from Central. Bact. Par. 1902, *8* ii, 193; 225; 257; 289; 321; 353; 385). Omelianski observed that such fermentation under unaerobic conditions is due to two different organisms, one of which produces marsh gas and the other hydrogen. According to him, the products of these fermentations were:—

Products.		% in marsh gas fermentation.		% in hydrogen fermentation.	
Fatty acids	...		49·9		66·9
Acetic acid	...	43·9		35·9	
Butyric acid	...	6·0		31·0	
Carbon dioxide	...		43·2		29·0
Methane ...	...		6·8		4·1

These results have been partially confirmed by Connick by using the same cellulose material and the same source of bacteria, (Am. Chem. Ab. 1917, 54, from Compt. Rend. Soc. Biol. *79*, 156).

Omelianski and other workers in this field have invariably used filter paper or cotton, both of which are normal celluloses. No mention has however been made in the literature on this subject of any attempts to employ oxy-or hemi-celluloses and consequently no information is available either as to their fermentability or the products of their fermentation.

The work described in the present paper was therefore undertaken in order to obtain information in the following directions:—

1. The character of raw material best suited for fermentation.
2. The conditions of optimum activity of the organisms.
3. The quantity and character of the products of fermentation.

Raw materials of fermentation. The general procedure followed in each experiment consisted in inoculating the necessary cellulose material with what may be called an emulsion of the necessary bacteria and allowing the action to continue over a certain period, the products being collected and analysed.

The "bacterial emulsion" was prepared as follows:—

A certain quantity of sludge was taken from the bottom of the septic tank, and some cellulose material such as banana stems and skins and filter papers were richly inoculated with it. When the cellulose was completely fermented more material was added and this process was continued till finally the rate of gas obtained per day was about 10 times the rate at the beginning. It was this sludge from the bottom of the fermentation vessel that was used in all the following experiments and for convenience called "Bacterial Emulsion." It gave on analysis 63·1% organic matter and 2·45 % nitrogen while the original sludge from the septic tank gave 65% organic matter and 2·35 nitrogen.

For the sake of comparative study the following substances were selected.

(i) Filter paper (Karl Schleicher and Schuell) representing ... "Normal celluloses"

(ii) News paper (Statesman, Calcutta), letter paper ("Silver Cross" A. Pirie & Sons), Brown paper. } representing "lignocelluloses"

(iii) Banana skins, Banana stems, Mahua waste, } representing "hemicelluloses"

The chief criterion to determine the degree of utility of any of the above mentioned substances is the intensity with which the fermentation proceeds, other conditions such as the type of bacteria, temperature and concentration remaining the same.

50 gms of each substance were placed in half Winchester bottles and 50 cc. of sludge from the bottom of the septic tank were added. The bottles were filled up with water to let the fermentation go on under anaerobic conditions.

Light was also excluded by wrapping the bottles in paper. Since only comparative values were required, the experiments were carried out at atmospheric temperature.

The quantities of gas evolved furnished an indirect measure of the resistance of different substances. They were collected over water and analysed in a Bone and Wheeler Apparatus.

It required about 4 days for the fermentation to begin. The average rates of gas generation after that time were :—

in the case of	filter paper	25 cc per day
,,	newspaper	42 ,, ,,
,,	letter paper	51 ,, ,,
,,	brown paper	8 ,, ,,
,,	banana skin	69 ,, ,,
,,	plantain stems	45 ,, ,,
,,	mahua waste	5 ,, ,,

A blank experiment was made to see that the sludge itself without any cellulose gave no gas.

These averages are calculated from the readings of the volume of gas taken daily, extending over a period of 30 days.

It seemed probable that these differences in the activities could be traced back to the nature of the cellulose - *i. e.* whether normal, derived from lignified fibres or hemicellulose, or to the presence of foreign bodies that might have been already existing either in chemical combination in the raw vegetable tissues, or added substances such as sizing, and loading matter, invariably found in papers.

The degree of closeness with which fibres are bound to each other in paper can also influence the activity of its fermentation since the closer the fibres the less is the surface exposed for bacterial action. The volume of the air space in a sample can be used as a measure of the compactness of the fibres.

The influence of the nature of the cellulose on the activity was ascertained by employing pure celluloses of the three kinds - normal, the type derived from lignified fibres and hemicellulose prepared in a pure form using the chlorination process of Cross and Bevan (Cross and Bevan - Cellulose, 1916, p 95). Normal cellulose was prepared from cotton, cellulose of the second kind from megasse and hemicellulose was prepared from banana stem. Small amounts of these pure celluloses were exposed to the action of fermentation bacteria, taking care to prevent foreign infection. The fermentation was allowed to go on in an incubator at a temperature of 35°-2 to 35°-4 C. The times taken by these substances to decompose completely were noted and thus an idea of the resistances of the different kinds of cellulose to bacterial action was obtained.

TABLE. I.

Cellulose.	Time taken for fermentation.
Cotton	17 days
Megasse	15 ,,
Plantain stem	11 ,,

It can be seen from this that the normal celluloses are the most resistant, celluloses from lignified fibres next in order and the hemicelluloses are the least resistant. This behaviour of the three kinds of celluloses is also observed with chemical reagents such as alkalis and acids (Cross and Bevan, Cellulose. 1916, 78).

In order to test the influence of the presence of foreign bodies, jute, wood fibre and a small stem of gram, all in their crude state, were inoculated with the fermentation bacteria, There was no fermentaion at all, showing that cellulose is not attacked when it is in combination with substances like pectin, lignin &c. which are always present in raw vegetable tissues. It is observed only in the case of hemicelluloses that the fermentation proceeds irrespective of the raw condition of the tissues (banana skins and stems).

For the investigation of the third factor *viz.*, influence of sizing, loading and air space in the case of papers, samples from all the papers used were analysed by the methods given in Sindall in Paper Technology, Chapter X.

The character of the fibres present was identified microscopically by methods given in Herzberg (Papier Pruefung, 1916, p 94) as well as chapters 11 and 12 from Sindall. The following table gives the results of analysis, the comparative rate of evolution of gas being given in c. c. per day.

TABLE II.

Gas in c. c. per day.	Filter paper. 15	Newspaper. 42	Letter paper 31	Brown paper. 8
Chemical constituents.				
Moisture	5·6	7·9	5·2	7·8
Ash	1·4	10·0	4·0	2·4
Rosin	nil	2·9	2·0	nil
Starch	traces	nil	3 0	,,
Composition by volume.				
Fibre	36·3	44·4	39·1	46·9
Ash	33	3 3	1·09	1·6
Rosin		2.3	1·28	
Air space	67·3	50·0	57·2	51·5
Fibrous contituents.	Cotton.	About 80 % esparto and remainder mechanical wood pulp	65 % esparto remainder wood pulp with a little cotton.	85 % mechanical wood pulp and remainder raw jute.

A consideration of the analyses given in Table II and the gas volumes at once shows that celluloses from lignified fibres are more active than normal celluloses, and this is in agreement with what has been already observed with pure substances.

Loading matter and rosin do not seem to have much effect since newspaper which contains as much as 10% loading matter and 2·9% rosin, is quite active.

Starch, however, seems to retard the action in some way, as can be seen from the low activity of letter paper.

As regards the choice of cellulose material, therefore, the results of these experiments can be summarised as follows :—

(1) The resistances of different kinds of cellulose are of different magnitude, normal celluloses being the most resistant, cellulose from lignified fibres come next in order and hemicelluloses are the least resistant.

(2) The cellulose in the raw material should not be in a state of chemical combination. This however does not hold in the case of hemicellulose, as found in banana skins and stems.

As mechanical wood pulp can be looked upon as cellulose in a combined state, it is unsuitable for fermentation.

(3) The sizing and loading material affect the activity only to a slight extent.

It is obvious that some rapid means of determining the suitability of cellulose materials of fermentation other than complete analysis is desirable and the action of Schweitzer's reagent naturally suggested itself for this purpose.

The best recipe for making this reagent is given by de Toni (cf. Tunmaun: Pflanzen Mikrochemie 1913, s 546).

A series of experiments was carried out to determine whether any relation existed between the resistance of the cellulose to this reagent and to the fermentation bacteria. These results are summarised in the following tables :—

TABLE III.

	Time taken by Schweitzer's reagent to dissolve the paper.	Time taken by fermentation bacteria for same.	REMARKS.
Filter paper	... 1 minute	18 days	
Newspaper	... 6 minutes	15 days	
Letter paper	... 8 ,,	24 ,,	
Brown paper	... 13 ,,	38 ,,	
*Banana skins ,, stems			* The substances never dissolved completely, so it was not possible to see when the actual disintegration took place.

The insolubic residues of the substances after the action of the Schweitzer's reagent were compared with the corresponding residues in the fermentation bottles.

Table IV.

		Residue after the action of Schweitzer's reagent.	Residue after the action of bacteria.
1.	Filter paper ...	No residue left.	Dissolved in 11 months, no residue left.
*2.	Newspaper ...	No appreciable residue left.	25 % residue left after 15 months, in the form of small yellowish bits.
*3.	Letter paper ...	No appreciable residue left.	50 % residue left after 15 months.
4.	Brown paper ...	No appreciable residue left.	The paper being composed principally of raw fibres, could show very little activity and only a small fraction was fermented in 15 months.
5.	Banana stems	Everything dissolved except epidermis.	No residue left after 3½ months, except the epidermis.
6.	Banana skins		
7.	Mahua waste ...	Large quantity of residue remained permanently.	Very little could be fermented even after 15 months.

In the light of these experiments it can be concluded that the Schweitzer's reagent has a reaction on the different kinds of cellulose roughly parallel to the reaction due to the bacteria, and can be used at any rate to differentiate between fermentable and non fermentable material.

2. *The conditions of activity of the organisms:*

Omelianski showed that two organisms took part in the fermentation. B. Methanicus evolving methane, andB. Hydrogenii evolving hydrogen.

These were separated by somewhat tedious methods of subculture

Attempts were therefore made during the present research to grow the bacteria on solid media similar to that used by Kellerman and Mcbeth (Am. Ch. Abs. 1912, 3279, from Central Bact. Parasitenkunde II Abs. 34,484—494).

(* In the fermentation of newspaper and letter paper, it is possible that the surface of the material during the long period of 15 months has got covered with a film of some poisonous product, perhaps due to the presence of sizing and loading matter, thus preventing further bacterial action. This is also confirmed to some extent by the observation that when the paper was washed with water and freshly inoculated, the fermentation was re-established and continued for some time. It could not, however, attain the initial vigour.

The composition of the medium used in the present researches was as follows :—

Potassium phosphate	1	gm
Magnesium sulphate	0-5	,,
$(NH_4)_2 SO_4$	traces.	
Sodium chloride	1	gm
Water	100	cc.
Agar	2	gm

To this solution, 2 per cent of cellulose (filter paper pulp) and 2 per cent starch were added to make up the respective media.

Plate cultures were made on these media; the Petri dishes were kept in a dessicator containing alkaline pyrogallate to ensure anaerobic conditions. The dessicator was then exhausted to about 30 mm. pressure in order to avoid the formation of a very high pressure due to the gases which would be evolved by the action of the bacteria upon cellulose. The dessicator was opened every day to observe the growth and exhausted again before keeping it in the incubator. The method however was not successful, there being no growth of the Omelianski bacteria.

Slope cultures were also tried without success. An observation was made in this connection that although the bacteria could not grow on the solid medium, they could do so in the water given out by the medium as could be seen from the evolution of gas.

The method of Omelianski thus appears the one most likely to be successful in separating and isolating the organism. From the practical point of view it was therefore decided to be more important to determine the effect of environment on the naturally accumulating bacterial emulsion than to spend time in isolating a pure culture which would not be likely to be capable of economic use in practice.

(a) In the first place the effects were examined of the addition of the following chemical substances on the activity of the mixed fermentation *viz* : acid, alkali, metals, salts.

(b) Other more purely physical conditions were studied either specifically, or incidentally in connection with experiments already cited. Such conditions were :

(i) The relative concentration of the inoculant and cellulose material.

(ii) The state of division of the latter.

(iii) The temperature of fermentation.

(iv) The effect of removal of products of fermentation.

(a) *The effect of certain added chemical substances on the fermentation.*

The following is the general method followed in these experiments. Equal sizes of strips of filter paper (5 cm × 1 cm) were put in bacteriological test tubes, the necessary amounts of solutions containing the chemical substance under investigation, were added and the whole sterilised. They were then inoculated with equal amounts (5 cc) of bacterial emulsion and kept in the incubator. The days taken by the paper strips to dissolve were noted in each case and were looked upon as inversely proportional to the activity of the bacteria.

(i) *Alkalinity or acidity.*

Hydrochloric acid and sodium hydroxide were used in these experiments. The activities observed are given side by side with the concentration in

TABLE V.

Percentage of acid alkali.	Days required for disappearance of paper.	Remarks.
− 1·5	15 days	
− 1·4	13 "	
− 1·3	13 "	
− 1·2	17 "	
− 1·1	17 "	
− 1·0	16 "	
− 0·9	15 "	
− 0·8	14 "	
− 0·7	13 "	
− 0·6	15 "	
− 0·5	15 "	
− 0·4	15 "	
− 0·3	13 "	
− 0·2	12 "	
− 0·1	12 "	
neutral	13 "	
+ 0·1	16 "	
+ 0·2	16 "	

TABLE V.—*Continued.*

Percentage of acid or alkali.	Days required for disappearance of paper.	Remarks.
+ 0·3	15 days	
+ 0·4	15 ,,	
+ 0·5	16 ,,	
+ 0·6	16 ,,	
+ 0·7	15 ,,	
+ 0·8	17 ,,	
+ 0·9	17 ,,	
+ 1·0	15 ,,	
+ 1·1	15 ,,	
+ 1·2	17 ,,	
+ 1·3	17 ,,	
+ 1·4 + 1·5	Not dissolved in 20 days.	Poisoning action appreciable

The concentrations are expressed as percentages and the positive and negative signs denote acidity and alkalinity respectively.

It can be seen from the above table that while alkalinity of 1·5% has no great influence on the fermentation, acidity of same strength has a decided retarding action.

(ii) *Metals.*

Bright foils (5 cm × 1 cm) of the following metals were kept in contact with the paper strips and the fermentation observed in the same manner as before. The results are given in

TABLE VI.

Metal.	No. of days required for disappearance of paper.	Remarks.
Blank	9 days	
Lead	10 ,,	
Copper		Poisoned. No action.
Tin	10 ,,	
Iron	11 ,,	
Aluminium	12 ,,	
Zinc		do

These experiments show that the presence of copper and zinc in metallic form is very injurious to the fermentation and hence these metals should be avoided in the construction of fermentation vessels.

(iii) *Effects of different salts.*

Concentrations of 0·01, 0·1 and 1 part per 100,000 of the positive radicals were used in these experiments.

The salts employed for this purpose were :—

Sulphates, copper, ferrous ammonium, zinc, magnesium ammonium and potassium.

Phosphates, sodium and potassium.

Acetates, 0·25 per cent solutions of following acetates were tried in the same manner—copper, zinc, ammonium and sodium.

In the case of lead acetate, the concentrations used were 0·01, 0·1 and 1 part per 100,000.

In the case of calcium acetate concentration ranging from 0·25 to 5·0% were tried. The following table shows the concentrations and the days taken for the disappearance of paper in each case.

TABLE VII.

Radical under expt.	Salt used.	Concentration of radical parts per 100,000.	Days required for disappearance of paper.	Remarks.
Blank.			9 days	
Cu.	$CuSO_4$	·01 part of Cu	14 ,,	
		·1 ,,	12 ,,	
		1 ,,	14 ,,	
Fe.	Ferrous	·01 part of Fe	11 ,,	Both Fe & NH_3 act.
	ammonium	·1 ,,	11 ,,	
	sulphate.	1 ,,	13 ,,	
Zn.	$Zn\ SO_4$	·01 part of Zn	15 ,,	
		·1 ,,	15 ,,	
		1 ,,	19 ,,	
Mg.	$Mg\ SO_4$	·01 part of Mg	13 ,,	
		·1 ,,	13 ,,	
		1 ,,	16 ,,	

TABLE VII.—*Continued.*

Radical under expt.	Salt used.	Concentration of radical parts per 100,000.	Days required for disappearance of paper.	Remarks
K.	K_2SO_4	·01 part of K	11 days	
		·1 „	9 „	
		1 „	16 „	
NH_4.	NH_4Cl	·01 part of NH_4	12 „	
		·1 „	14 „	
		1 „	21 „	
PO_4.	Na_2HPO_4	·01 part of PO_4	14 „	
		·1 „	13 „	
		1 „	12 „	
	Na_2HPO_4	·25 % solution.	8 „	
	K_2HPO_4	„	8 „	
	Cu acetate	„		No action.
	Zn „	„		„
	NH_4 „	„	5 „	
	Na „	„	12 „	
	Calcium acetate.	„	13 „	
		·5%	16 „	
		1	16 „	
		1·5	16 „	
		2	16 „	
		2·5	16 „	
		3	17 „	
		3·5		Very feeble action.
		4		
		4·5		
		5		
Pb	Lead acetate.	·01 part of Pb	15 „	
		·1 „		No action.
		1		

The above experiments show that zinc has a retarding action when present in even such small quantities as 1 part per

100,000. Ammonia and copper also seem to have a deleterious effect.

A phosphate appears more and more advantageous as its relative quantity is increased. Sodium and potassium phosphates show an equal action.

As regards the acetates ·25% of copper and zinc acetates are sufficient to inhibit the action of the bacteria. Lead acetate in such small concentrations as ·1 part per 100,000 acts as poison.

Calcium acetate in quantities more than 3% is also harmful.

(b) *The effect of physical conditions on the fermentation.*

(i) *Relative concentration of inoculant and cellulose material.*

(1) In the following experiments different quantities of cellulose material were taken and inoculated with the bacterial emulsion. The third column in the following table gives the ratios—material to inoculant.

TABLE VIII.

Cellulose material. a.	Inoculant. b.	Ratio of a : b	Average rate of gas.	Remarks.
500 gms.	100 cc.	5 : 1	50	Stopped after 300 cc.
10	20	½ : 1	37	Did not stop.
10	100	1/10 : 1	81	do.

The experiments show that the fermentation cannot be accelerated by employing a large mass of material with a small amount of inoculant.

By gradually building up the bacterial emulsion it was found possible to obtain a daily volume of combustible gas equal to 80% of the volume of the space occupied by the fermenting material.

(2) *The state of division.*

Small pieces of paper (about 8 sq. cm.) as also of the banana skins and stems were used. It was often noted in these experiments that the sludge remained at the bottom and the paper or banana skin or stem pieces would float to the top of the liquid, being carried up with the gas formed in the fermentation.

To ascertain whether this would affect the fermentation, drops from the sludge and supernatant liquid were examined under a high power microscope. It was observed that a greater number of rod-like bacteria were present in the sludge than in the supernatant liquid. Fermentations were also carried out using the same quantities of filter paper and the sludge, but with pieces (about 8 sq. cm.) in one experiment and pulp in the other. The rates of gases in these experiments were observed and compared.

TABLE VIII*a*.

Quantities used : 5 gms. of filter paper plus 50 cc bacterial emulsion.

Date.	Gas by using pulp.	Gas by using pieces.	Remarks.
8th September.	10 cc.	10 cc.	
9th ,,	30 ,,	30 ,,	
19th ,,	95 ,,	95 ,,	
21st ,,	120 ,,	120 ,,	
22nd ,,	130 ,,	130 ,,	At this stage the the pieces were carried up to the top
25th ,,	160 ,,	150 ,,	and the action stopped.
22nd October	285 ,,	130 ,,	

In this experiment, though some of the pulp was soon floated to the top, it also carried some of the sludge with it and so the action could very well go on. But in the case of pieces the fermentation was stopped as soon as they floated to the top. One can conclude therefore that the cellulose material to be used for the fermentation must be in the form of pulp.

(3) *Temperature.*

It has already been mentioned that Omelianski found 35°C to be the optimum temperature for the fermentation bacteria. It is however important to know whether the action would go on with reasonable activity even at the ordinary temperature (20°—25°C).

In order to get some information about this point two fermentations were carried on side by side, one in an incubator keeping a temperature of about 30°C and the other outside at the room temperature (20°—25°). The gases evolved were recorded and compared.

TABLE IX.

Average value (from 30 readings) of gas at 30°C. per day.	Average value of gas from fermentation at room temperature.	Ratio of gas formation at 30° to same at 20°—25°.
13 cc.	8·4	1·5

It appears therefore that the fermentation proceeds $1\frac{1}{2}$ times quicker at 30° than at ordinary temperature (20°-25°C).

(4) *Removal of products.*

It is to be expected that the removal of the liquid from the fermentation bottle would maintain a concentration of products below a certain limit, thus promoting the fermentation. The following experiments were therefore tried. (i) Changing 1/10th of the total volume of liquid in the fermentation bottle by addition of fresh water every day and (ii) changing $\frac{1}{4}$th of the total volume every day. The rates of gas formation were noted in each case. The results are entered in

TABLE X.

Quantities used: 25 gms. of filter paper plus 200 cc bacterial emulsion. Volume of fermentation jar—2 litres.

(In apparatus A 500 cc of distilled water were daily put by means of a tap funnel, a corresponding amount of water in the apparatus being consequently thrust out. In apparatus B, only 100 cc of water were daily put.)

Dates.	Days from start.	Total water replaced in A.	Total water replaced in B.	Gas in A.	Gas in B.	Ratio of gas in A & B.
22nd Aug.						Expt. started.
		cc.	cc.	cc.		
26th	4	2000	400	390	110	3·4
30th	8	4000	800	800	230	3·5
4th Sep.	12	6000	1200	1100	340	3·5
8th	16	8000	1600	1450	465	3·1

The experiment shows that by changing the water from the fermentation bottle, the products are carried away and thus the fermentation goes on about 3 times more quickly.

The results obtained from these experiments as regards the favourable conditions for efficient fermentation indicate that :—

(i) The speed of the fermentation can be maximum only with a certain ratio of the cellulose material and the inoculant.

(ii) The material should be present in a fine state of division.

(iii) The fermentation is one and a half times quicker at 30° than at ordinary temperature (20°—25°C).

(iv) The removal of products increases the efficiency of the fermentation.

3. *The quantity and character of the products of fermentation.*

(1) *Analysis of gaseous products.*

The gases given out in the fermentation were collected over water and analysed. They were as follows :—

TABLE XI.

Material fermented.		CH_4 %	H %	CO_2 %	Calorific value per cubic foot (calculated)*
Filter paper	...	81·9	14·5	3·5	508·4
Newspaper	...	88·8	4·4	6·8	532·8
Letter paper	...	78·6	12·6	8·7	485·8
Banana skins	...	80·6	3·1	7·4	533·6
Banana stems	...	87·0	5·1	7·5	525·8

The average calorific value of the above gases, 516·8 is 1·45 times that of the coal gas which is only 356 according to Davis (loc. cit.)

As the gases have ~~have~~ been collected over water, the quantity of carbon dioxide in it will depend on the amount of water. The above figures show what sort of gas can be expected in the presence of a fairly large excess of water.

The above analyses show that hemicelluloses give a higher proportion of methane than normal celluloses, the latter give a higher proportion of hydrogen.

(2) *Analysis of soluble end products.*

These consist mainly of organic acids present in the form of very dilute solutions, so that distillation methods of

* These values were calculated from the composition of the gas. Marsh gas was taken to have a calorific value of 592 units and hydrogen 163 (Davis: Chemical Engineering Vol. 1, p. 326) This unit is defined as the number of calories per cubic foot of gas, the gas being measured at 0°C and 30 inches mercury pressure

analysis had to be tried, since identification by means of ethyl esters seemed out of the question. The Duclaux method (Duclaux: Traité de Microbiologie, 1900, ii, 385; see also Upsom, Plum and Scott, J. Am. Ch. S. *39*, 731; Lamb ibid *39*, 746; Gillespie and Walters ibid *39*, 2027) was therefore the only one which could be used for the identification of volatile acids. One of the imperfections of this method is that a slight quantity of a foreign substance, though non-volatile, in the distilling flask is sufficient to upset the distillation values. This disadvantage has been overcome by the important modification of Dyer (J. Bio. Chem. *28*, 445) in which the distillation is carried out with steam, the volume of liquid in the distilling flask being always kept constant.

In the present experiments, the quantity of acid was so little that the steam distillation method could not be used. The quantity of acetic acid that would come over in 100 cc. distillate by ordinary distillation according to the Duclaux method would require a litre and a half of distillate by Dyer's modification (see values given on page 452 in Dyer's paper loc. cit.). So that unless one had a sufficient quantity of acid, Dyer's method could not be used.

Duclaux's method consists in distilling 110 cc. of the unknown mixture of acids, the distillates being collected in 10 cc fractions. The acidity in each of these fractions is determined by titration with standard alkali and is expressed as percentage of total acid present in the distilling flask or as percentage of the total acid distilled over in 100 cc. distillate. These percentages or distillation numbers are constant and fixed for any particular volatile fatty acid. If only one acid is present, it can at once be known by comparison with the standard distillation numbers given by Duclaux. If two acids are present, the distillation numbers will depend upon the relative amounts of the two acids. The amounts of the two acids can be found out by the simple rule of three. With three acids the case is more complex, but if they are known acids their percentages can be calculated. A very efficient method for these calculations is given by Gillespie and Walters.

Pure acetic acid and pure butyric acid were first tried to see that the technique of the experiment was right. The distillation figures were calculated as percentages of the total acid distilling over in 100 cc. These figures correspond with those of Gillespie and Walters within reasonable limits.

TABLE XII. Acetic Value.

Successive 10 cc. fractions.	% acid distilled over. Mean of two experiments.	Differences.	Gillespie and Walters figures.	Difference.
10 cc.	7·4	7·4	8·0	8·0
20	15·3	7·9	16·3	8·3
30	23·5	7·2	24·6	8·3
40	32·3	8·8	33·5	8·9
50	41·7	9·4	42·7	9·2
60	51·6	9·9	52·1	9·4
70	62·2	10·6	62·4	10·3
80	73·5	11·3	73·4	11·0
90	86·0	12·5	85·7	12·3
100	100·0	14·0	100·0	14·3

The deviations in the figures in columns 3 and 5 may be due to impurity in the acetic acid, which could be detected by potassium chromate and nitric acid.

TABLE XIII. *n*-Butyric acid.

10 cc.	17·1	17·1	16·7	16·7
20	32·5	15·4	31·9	15·2
30	46·4	13·9	46·7	13·2
40	58·0	12·2	57·9	12·2
50	69·1	10·5	68·8	10·9
60	78·1	9·0	77·7	9·1
70	85·8	7·7	85·8	7·9
80	92·0	6·2	92·1	6·3
90	96·6	4·6	96·8	4·7
100	100·0	3·4	100·0	3·2

The liquid products in these experiments were obtained by fermenting two kilos of plantain stem pieces (moisture 83·6%) in a 40 litre drum by adding 2·5 litres of sludge. After fermentation the clear supernatant liquid 30 litres was neutralised with caustic soda so that no volatile acid should evaporate while boiling. The liquid was boiled down to about 4 litres in a steam pan and then concentrated on a water bath to about 700 cc. The volume of this solution was made up to 1000 cc.

250 cc of this solution were treated with enough sulphuric acid to liberate the fatty acids, and distilled. The distillate was redistilled to obtain the volatile fatty acids without

impurity. The final distilate was 175 cc. Out of this 110 cc. were taken and distilled according to Duclaux's method. The distillates were collected in 10 cc fractions which were then titrated with N/50 caustic soda solution.

TABLE XIV.

Fractions. 100 cc.	cc of N/50 NaOH.	% of acid distilled over.	Total distillate.	% of acid distilled over.	Gillespie and Walters figures for pure acetic acid.
1	1·65	8·0	10	8·6	8·0
2	1·75	8·4	20	16·4	16·3
3	1·7	8·2	30	24·6	24·6
4	1·8	8·7	40	33·3	33·5
5	1·9	9·2	50	42·5	42·7
6	1·9	9·2	60	51·7	52·1
7	2·0	9·7	70	61·4	52·4
8	2·3	11·1	80	72·5	73·4
9	2·6	12·5	90	85·0	85·7
10	3·1	15·0	100	100·0	100·0
	20·7				

A consideration of the figures in the last two columns shows that the percentage values obtained in the present distillation are almost identical with the values for acetic acid. It is therefore possible that either only acetic acid is present or a mixture of acids giving the above distillation ratios as a whole which is however somewhat improbable. Hence no conclusion can be drawn without making a quantitative examination of the distillate.

The first 10 cc fraction should be expected to contain a greater proportion of a higher acid if any present, and the last fraction should contain a greater proportion of the lower acid (cf Duclaux).

The first and the last fractions after titration were evaporated down to 2 cc with a drop of dilute sulphuric acid to neutralise the colour due to phenolphthalein used in the titration. They were then tested qualitatively for acids by Dyer's orientation tests (Dyer J. Bio. Chem. *28*, 445). The tests depend upon the relative solubilities of iron and copper salts of the fatty acids in water and amyl alcohol. *Only acetic acid could be detected in* both the samples.

The following tests for formic acid were also applied to the sample from the last fraction (1) ammoniacal silver nitrate (2) mercuric chloride (3) potassium chlorate and nitric acid. No formic acid could be detected.

It seemed then that by the fermentation of plantain stem which contained hemicellulose, acetic acid was the only acid formed. But this cannot be definitely stated unless some more experiments are performed and uniform results obtained.

The investigation of products in dilute solution is a somewhat tedious process and large quantities of material need to be handled. It suggests itself however as a possible method for differentiating various typical celluloses and obtaining some insight into their constitution.

(iii) *Intermediate products.*

In the consideration of the products from the fermentation, no mention has been made up to now of the existence and recovery of any intermediate products. Experiments were made on the lines suggested by Pringsheim's work on the endoenzymes of the cellulose fermentation bacteria (Zeits. Physio. Chem. 1912, 78, 266) where he interrupted the bacterial action with the use of antiseptics, thus allowing the endoenzymes to act. Pringsheim recommended the use of iodoform. But experiments were made here with (1) toluene (2) chloroform (3) iodoform (4) thymol water. The intermediate products according to Pringsheim were cellobiose and dextrose, both reducing sugars; but none of these could be detected in these experiments in spite of the fact that the antiseptic had effectively checked the fermentation.

Summary of Results.

It has been shown that :—

1. Hemicelluloses are the best material to use for the fermentation. Waste papers can also be used with certain limitations.
2. Schweitzer's reagent can be employed as a rapid test in the selection of a suitable material for fermentation.
3. Small quantities of lead, copper and zinc salts or the presence of copper and zinc foils, stop the fermentation.
4. Acidity of the medium beyond one per cent has a retarding influence on the fermentation.

5. The rate of fermentation is at a maximum (a) with a certain ratio of cellulose material and the inoculant, (b) at a temperature near the optimum (35°C), (c) if the products are effectively removed from time to time.
6. By gradually building up the bacterial emulsion it is possible to obtain a daily volume of combustible gas equal to 80% of the volume of the space occupied by the fermenting material.
7. The gaseous products from the fermentation, if collected over water, contain 85% of methane and are therefore of high calorific value.
8. Hemicelluloses give only acetic acid as the soluble product. Butyric acid has not been detected.

General Conclusions.

Production of power gas.

It is evident from the above that under certain conditions it is possible to obtain power gas in economic quantities from the numerous waste cellulose materials available.

The gas will be very useful as a source of power in places like the Western Coast of India which has no coal fields of its own and where importation of coal is bound to be very costly. On the Western Coast especially there are ample waste cellulose materials such as plantain skins and stems and other vegetable refuse. These materials fortunately contain hemicelluloses which give the best results in the fermentation. The temperature on the Western Coast also is sufficiently high and is therefore another favourable condition. These considerations, then, show that the fermentation of cellulose might prove a useful process of production of a power gas for industrial purposes.

It is also possible that the fermentation might be employed as a method of utilization of cellulose residues from various industries using plant products as their raw material. Special investigations are however necessary for each residue of this sort, before the fermentation process can be applied successfully.

Recovery of acetic acid.

Hitherto the only large scale application of the cellulose fermentation for the production of power gas has been in connection with septic tanks *e. g.* at Matunga, (See James, Drainage

Problems of the East, chapter 8, G. J. Fowler, Bacteriological and Enzyme Chemistry, p. 284). Recovery of acetic acid from the effluent of the septic tank while quite possible would be unsatisfactory.

On the other hand if the cellulose fermentation tank were supplied with a dilute solution of ammonium phosphate instead of sewage, the effluent could be mixed with alcohol and passed over an acetifier and in this way the acetic acid in the effluent recovered along with the acid produced from the alcohol.

Thermophilic bacteria.

It appears from the work of Macfadyan (J. Path. Inst. 1894) and Pringsheim (Zeits. Physio. Chem. 1912, 78, 268) that the thermophilic bacteria are capable of fermenting cellulose more rapidly than Omelianski's bacteria. An investigation of the favourable conditions for their action would therefore be very desirable from the point of view of obtaining large quantities of power gas from the fermentation of waste cellulose material.

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APRIL 1920.