Studies on Bacillus thermofibrincolus.

III. Physiological studies (continued.)

By

Arao Itano and Satiyo Arakawa.

[July 29, 1930.]

The description[1] and physiological studies[2] of the organism were reported in the two previous reports respectively. This paper deals with the further physiological studies in two parts, namely Part I. The influence of some factors on the production of volatile acids and alcohol, such as culture method, temperature, oxygen, amount of peptone, amount of cellulose, various nitrogen sources: Part II. Nitrogen metabolism in culture medium.

Part I.

The influence of some factors on the production of volatile acids and alcohol:

As it was reported previously, the thermophilic cellulose fermenting bacteria in general produce formic, acetic, butyric, lactic acids and alcohol, and also hydrogen and methane gases as well as carbon dioxide. The production of these substances vary qualitatively as well as quantitatively by the species and the ecological conditions. For example, in case of Cl. thermocellum[3], it was reported to produce acetic, butyric and a trace of lactic acids, and alcohol, CO_2 and H_2 gases; and for the production of acetic acid, peptone and yeast water are said to be the good organic nitrogen source. At the sametime, the amount of acetic acid produced is influenced by such factors as, the amount of peptone supplied viz., without an addition of peptone, a slight production while 1% gives the maximum production; smaller the amount of cellulose supplied better the production of the acid; larger the culture flask is, more alcohol produced; period of incubation influences the process as follows, the maximum production of alcohol reaches on the third day while the acetic acid, on the third or fourth day; and the amount of alcohol decreases as the time passes.

1) Itano, A. and Arakawa, S., Berichte des Ōhara Institut etc., Bd. IV, Heft 2, 265, 1929.
2) ibid , Bd. IV, Heft 3, 357, 1930.
3) Viljoen, J. A., Fred, E. B. and Peterson, W. H., J. Agr. Sci., 16, 1, 1926.

The quantitative investigation of this nature is very rare and this investigation was undertaken to determine the amount of volatile acids and alcohol as a whole and the qualitative tests will be reported later.

Experimental.

Culture method:

20 cc. of medium[1] was placed in a large test-tube (20 × 188 mm) and two strips of filter paper (15 × 120 mm) weighing approximately 0.30 g. were dipped in the medium. 0.20 cc. of inoculum was used and the culture was incubated at 65 °C., in Freas incubator. In the course of incubation, the tube was shaken at various intervals so that the calcium carbonate in the medium was stirred up to neutralize the acid produced. This procedure was employed throughout the investigation unless stated otherwise.

Analytical method:

After a certain period, the original volume is made up and the content is centrifuged so that the calcium carbonate, unfermented portion of cellulose were thrown down and the clear portion of liquid was used for the analyses.

Determination of volatile acids:

The volatile acids were determined by the method given by FRED and his co-worker[2] which is carried out as follows; 50 cc. of the test solution is placed in a long-necked flask, and 20 g. of monosodium phosphate and 15 cc. of 85% phosphoric acid (MERCK) are added and enough distilled water is added to bring the content to a definite volume; then the distillation is carried out until no acid reaction is observed by the litmus paper (200—250 cc.) distillate is usually sufficient; the distillate is titrated with N/10 NaOH by using phenolphthalein as an indicator.

Determination of alcohol:

The alcohol was determined by the method described DOX and LAMB[3] which is as follows; 100 cc. of the culture medium is saturated with salt and distill 60—70 cc. and the distillate is treated with 10 g. of potassium bichromate and 20 g. of sulfuric acid and placed in the similar flask as in the case of volatile acid determination. Then two glass beads are added to prevent bumping and foaming, and distillation is carried out by keeping the content at the same volume. Thus the distillation is continued until the last portion of the distillate showed the acidity within 0.5 cc. of N/10 NaOH, using phenolphthalein as the

1) ITANO, A. and ARAKAWA, S., Loc. cit., Bd. IV, Heft 2, 266.
2) FRED, E. B. and other, J. Biological Chem., 39, 347, 1919.
3) DOX, A. W. and A. R. LAMB, J. Amer. Chem. 38, 2561, 1916.

indicator and the alcohol was calculated. The method was controlled by using chemically pure substance.

Determination of amount of cellulose fermented:

After a certain period, the culture medium was treated with acid alcohol so that calcium carbonate, cellular materials, pigment are dissolved; the mixture was filtered through a filter paper of which weight has been determined previously. The filter was washed with warm and cold water, alcohol and either in turn until no chlorine was found; and it was dried to the constant weight. The difference between the initial weight was taken as the amount of cellulose fermented.

Results.

1. Volumetric change of culture medium during the incubation:

Since the culture is incubated at 65°C., the evaporation of the medium has to be overcomed. VILJOEN and his co-workers[1] covered the mouth of the flask with tin foil to prevent the evaporation. In this test, a large size test-tube was left with the cotton plug only and the amount of evaporation was observed. The results are shown in Table I.

Table I.

Volumetric Change of Culture Medium.

Days.	Change of cc.	Loss cc.	Average per day. cc.
0.	100.0	—	—
1.	91.0	9.0	9.0
3.	76.0	24.0	8.0
7.	54.5	45.5	6.5

To prevent such a marked evaporation, the test-tube culture was placed inside of another larger test-tube which is provided with a piece of cotton and 20 cc. of water at the bottom, and the outside tube was stoppered with cork which has a fine hole. By this method, the evaporation was checked satisfactorily during 4—5 days incubation at 65°C.

2. Influence of culture method:

The different forms of filter paper were used, as fine pieces of strip and α-cellulose (89%) in Erlenmyer flasks or in test-tube were experimented as to the availability and the following results as shown in Table II were obtained.

1) VILJOEN and others, Loc. cit.

Table II.

Influence of Culture Method.

Treatment.	Days.	Cellulose fermented.			Volatile acid. (Acetic.)		Alcohol. (Ethyl.)	
		Initial.	Fermented.					
		g.	g.	%	mg.	%	mg.	%
Broken filter paper in flask.	8—12	3.00	1.80	60.00	593.13	32.95	165.80	9.21
Strip of filter paper hanged in flask.	6—7	3.47	2.73	78.90	637.63	23.36	84.70	3.10
α-cellulose in flask.	3—7	1.63	1.30	79.75	436.10	33.54	140.30	10.79
Strip of filter paper suspended in test-tube.	3—7	1.51	1 22	80.79	309.00	25.33	58.50	4.80

N. B. α-Cellulose used in this investigation was kindly supplied by Dr. S. KOMATSU of Kyoto University to whom our gratitude is due.

Table II indicates that the better fermentation was obtained by using the strip of filter paper suspended than the fine pieces which naturally settled to the bottom. More filter paper was fermented in the test-tube than that in the flask although the amount of products were the reverse.

3. Influence of temperature:

As to the influence of temperature on the growth of the organism, it was reported in the previous paper, and the influence on the products are reported in Table III.

Table III.

Influence of Temperature.

°C.	Days.	Cellulose fermented.		Volatile Acid. (Acetic.)		Alcohol. (Ethyl.)	
		g.	%	mg.	%	mg.	%
65	4	1.2906	85.52	319.50	24.76	124.43	9.64
50	10	0.8192	54.28	274.50	33.51	98.75	12.05

N. B. The initial amount of cellulose was 1.5092 g.

The activity of the organism was greatest at 65°C., and at 50°C., even after ten days, the amount of filter paper fermented was less than that of the other. However the total amount of products was greater at 50°C, which may be due to the fact that at 65°C some of the products are consumed by the organism as they are produced and again some may be lost on evaporation.

4. Influence of oxygen:

The influence of oxygen on the volatile acids and alcohol production was determined by 1) ordinary test-tube culture method; 2) double tube method; and 3) BUCHNER's anaerobic method. The following results were obtained as shown in Table IV.

Table IV.

Influence of Oxygen.

Culture method.	Amount of Cellulose fermented.		Volatile Acid. (Acetic)		Alcohol (Ethyl)	
	g.	%	mg.	%	mg.	%
Test-tube.	1.3298	88.08	282.00	21.21	171.82	12.90
Double-tubes.	1.3200	87.46	273.60	20.73	228.35	17.30
BUCHNER's.	1.1118	73.67	229.00	20.63	312.51	28.11

N. B. The initial amount of cellulose was 1.5092 g.

As it was reported previously, the organism belongs to the facultative anaerobic. But according to Table IV, the cellulose was fermented mostly under the aerobic condition although no appreciable difference was observed as to the volatile acid production. However, twice as much as alcohol was produced under anaerobic condition than under aerobic condition.

5. Influence of peptone:

The influence of various amount of peptone (0—2%) was tried, at 65°C and incubated for four days. The results are given in Table V.

Table V.

Influence of Peptone.

Amount of peptone.	Amount of cellulose fermented.		Volatile acid (acetic).	
%	g.	%	mg.	%
0.00	0.6860	45.45	78.00	11.52
0.25	1.2450	82.49	237.60	19.08
0.50	1.2760	84.55	345.60	27.08
1.00	0.3705	24.54	200.70	54.17
2.00	0.2357	15.62	129.60	54.99

N. B. The initial amount of cellulose was 1.5092 g.

Table V indicates that 0.5% peptone gave the best result and 1% peptone depressed the cellulose fermentation. Although the fermentation took place where no peptone was added, it may be due to a small amount of peptone which was brought in with the inoculum. Again the volatile acids increased in proportion with the amount of peptone.

6. Influence of cellulose:

The influence of different quantity of cellulose on the fermentation products was determined by varying the amount of cellulose, incubated for four days at 65°C. The results are given in Table VI.

Table VI.

Influence of Initial Concentration of Cellulose

Initial Amount of Cellulose.	Amount of Cellulose fermented.		Volatile Acid (Acetic).		Alcohol (Ethyl).	
%	g.	%	mg.	%	mg.	%
0.75	0.6694	88.71	190.50	28.46	38.09	5.54
1.50	1.1272	74.69	336.00	29.81	52.34	4.64
2.20	1.7418	76.94	420.80	24.16	183.68	10.55
3.75	1.8494	49.02	487.50	26.36	174.05	9.41
5.15	2.1012	40.55	457.50	21.77	152.03	7.24

As Table VI indicates the cellulose was fermented best when 0.75—2.20% cellulose was added and the production of volatile acids showed the similar tendency. But the larger quantity viz. 2.20—5.15% seemed to be better for the alcoholic production.

7. Influence of nitrogen source:

The quantitative tests as to the influence of nitrogen source were undertaken and reported on the preceding report. This gives the results of quanlitative investigation as shown in Table VII.

Table VII.

Influence of Nitrogen Source.

Nitrogen source.	Amount of Cellulose fermented.		Volatile acid (Acetic).		Alcohol (Ethyl).	
	g.	%	mg.	%	mg.	%
Meat extract.	0.7587	50.27	292.38	38.53	57.25	7.81
Egg albumin.	1.1385	75.44	319.08	28.02	67.15	5.90
Peptone.	1.3478	89.31	310.50	23.04	90.85	6.74
Casein.	1.1291	74.81	204.00	18.07	60.35	5.34
Ammonium sulfate.	0.8133	53.89	171.60	21.10	7.20	0.89

N. B. The initial amount of cellulose was 1.5092 g.

Table VII indicates that 89.31% cellulose was fermented where the peptone was supplied and 75%, for both egg albumin and casein; 38.53% volatile acids produced for meat extract; 28.02%, for egg albumin; while 7.81% alcohol was produced for meat extract and 6.74%, for peptone; and ammonium sulfate had no influence on the alcoholic production.

8. Influence of incubation period:

The influence of length of incubation was investigated with and without an addition of calcium carbonate to the medium, and the following results were obtained as given in Table VIII and also shown by Fig. I and II.

Table VIII.

Influence of Incubation Period.

No. of days.	Initial.		First.		Third.		Seventh.	
	$+CaCO_3$	$-CaCO_3$	$+CaCO_3$	$-CaCO_3$	$+CaCO_3$	$-CaCO_3$	$+CaCO_3$	$-CaCO_3$
Total acidity. cc.	6.0	10.5	8.0	10.8	4.0	18.0	3.8	22.5
P_H	7.63	6.98	7.34	7.00	7.36	6.33	7.34	6.09
Volatile acid (acetic) mg.	0.00 (0.00%)	0.00 (0.00%)	21.00 (26.32%)	19.50 (38.77%)	310.50 (32.02%)	61.80 (12.84%)	306.00 (23.00%)	102.00 (14.77%)
Alcohol (ethyl) mg.	0.00 (0.00%)	0.00 (0.00%)	2.50 (3.13%)	2.50 (4.97%)	59.25 (6.11%)	12.50 (2.60%)	25.00 (1.88%)	23.00 (3.33%)
Amn't of cellulose fermented g.	0.00 (0.00%)	0.00 (0.00%)	0.0798 (5.29%)	0.0503 (3.33%)	0.9698 (64.26%)	0.4812 (31.88%)	1.3306 (88.17%)	0.6906 (45.76%)

Figure I.

Influence of Incubation Period on Production of Volatile Acids and Alcohol.

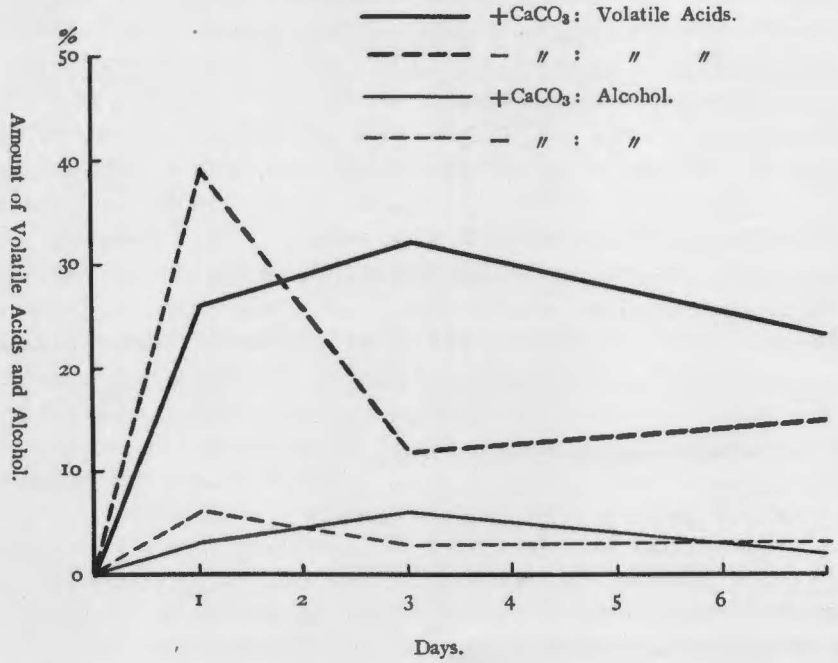

Figure II.

Influence of Incubation Period on Cellulose Fermentation.

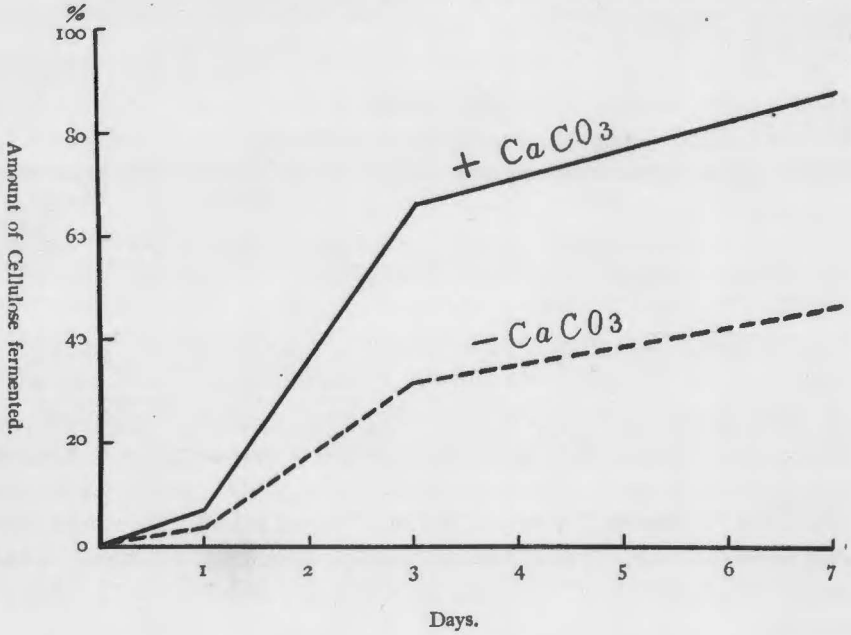

Table VIII and Fig. I indicate that on the first day, approximately 20 mg. of volatile acids were produced in both media, with or without calcium carbonate, which correspond to 26.32% cellulose fermented in presence of calcium carbonate and 38.77%, without calcium carbonate. On the third day, the total volatile acids increased, and 32.02% and 12.84% produced with and without calcium carbonate respectively. On the seventh day, the total volatile acids decreased to 23% and 14.77% with and without calcium carbonate respectively. In case of the cellulose fermentation, with calcium carbonate, 5.3% was fermented on the first day; 64.3%, on the third day and increased rapidly on the seventh day to 88.2% while without calcium carbonate, 3.3%, on the first day; 31.9%, on the third and 45.8%, on the seventh day. That is, with calcium carbonate, twice as much as cellulose was fermented than that of without it. This indicates that, without the presence of calcium carbonate, the accumulation of acid and subsequent increase of hydrogen ion concentration interferes with the activity of the organism. In fact, without calcium carbonate, P_H 6.6 was reached while it remained P_H 7.3 in the other. Considering these results, the rate of activity of the organism could be followed by determining the P_H value of the medium as noted by SANBORN[1].

1) SANBORN, J. R., J. Bact. 12, 1, 1926.

Part II.

Nitrogen metabolism in culture medium:

The nitrogen metabolism of bacteria in culture medium in general has been investigated quite extensively owing to its significance attaches to the subject. But no information is available on the thermophilic bacteria in regard to the proteolysis and its approximate index so far as we are aware. This paper deals with the production of amino- and ammonia-nitrogen in presence or absence of cellulose in the culture medium at 65°C. together with some such factors as total phosphate, amount of cellulose decomposed, P_H value and number of bacteria.

Regarding the approximate index of proteolysis, DE BORD[1] investigated with Bact. coli, Ps. pyocyanea, B. subtilis, C. botulinum and Cl. sporogenes in peptone medium with glucose, and found that the production of amino-nitrogen in such medium serves as an approximate index of proteolysis but ammonia can not be taken as such. Thus he obtained the results which substantiate the contention of SEARS[2]; KENDAL and BLY[3]; and which differ from those obtained by WAKSMAN[4], BERMAN and RETTGER[5]. For these reasons, it is very interesting to ascertain as to the nature of nitrogen metabolism of B. thermofibrincolus.

Experimental.

The same medium[6] was used and B. thermofibrincolus was cultured in a large test-tube at 65°C. and the amount of inoculum used was 1%. The methods of determination employed are as follows:

1. Ammonia-nitrogen;—by the combined method of HARPER[7] and FOLIN's modified[8].
2. Amino-nitrogen;—VAN SLYKE's method[9] was used after the ammonia was driven out.
3. Total phosphoric acid;—by Uranium acetate method[10].
4. Cellulose;—as described previously.
5. P_H value;—by the quinhydrone method.
6. Number of bacteria;—by BREED and DREW[11].

---

1) DE BORD, G. G., J. Bact., 8, 7, 1923.
2) SEARS, H. J., J. Infect. Dis., 19, 105, 1916.
3) KENDAL, A. I. and BLY, R. S., Ibid 30, 239, 1922.
4) WAKSMAN, S. A., J. Bact. 5, 1, 1920.
5) BERMAN, N. and L. F. RETTGER, J. Bact. 3, 389, 1918.
6) ITANO, A. and ARAKAWA, S., Loc. cit.
7) HARPER, H. J., Soil Science, 18, 409, 1924.
8) PETERSON, W. H. and co-workers, J. Bact. 15, 168, 1928.
9) VAN SLYKE, D. D., J. Biol. Chem., 9. 1911.
10) HAWK, P. B., Pract. Physiol. Chem., 1923.
11) BREED, R. S. and DREW, J. D., New York Agric. Exp. Sta. Tech., Bull. 49, 1916.

Results.

The results obtained are given collectively in Table IX and also shown in Fig. III and IV.

Table IX.

Fate of Amino- and Ammonia-Nitrogen.

Days.	Initial.		First.		Third.		Fourth.	
Treatment.	+ Cellulose.	− Cellulose.	+ Cellulose.	− Cellulose.	+ Cellulose.	− Cellulose.	+ Cellulose.	− Cellulose.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
NH_3-N.	16.22	16.22	19.46	19.60	14.35	18.63	16.15	17.11
NH_2-N.	10.08	10.08	10.50	13.74	17.68	17.57	21.34	17.67
Total P.	130.20	130.20	130.20	130.20	79.05	120.90	79.05	120.90
Cellulose decomposed.	0.0 (0.0%)	—	14.00 (0.01%)	—	532.75 (37.8%)	—	688.50 (48.9%)	—
P_H	7.67	7.67	7.43	7.37	7.40	7.91	7.38	8.03
No. of bacteria.	86,400 (4.9365)	86,400 (4.9365)	2,400,000 (6.3802)	480,000 (5.6812)	5,760,000 (6.7604)	336,000 (5.5263)	5,280,000 (6.7226)	672,000 (5.8274)

N. B. The figures indicate the amount in 100 cc. except the bacterial number which is given per 1 cc. The number in the parenthesis is the logarithm of the bacterial number.

Figure III.

Fate of Amino- and Ammonia-Nitrogen, and Quantity of Cellulose fermented in Culture Medium.

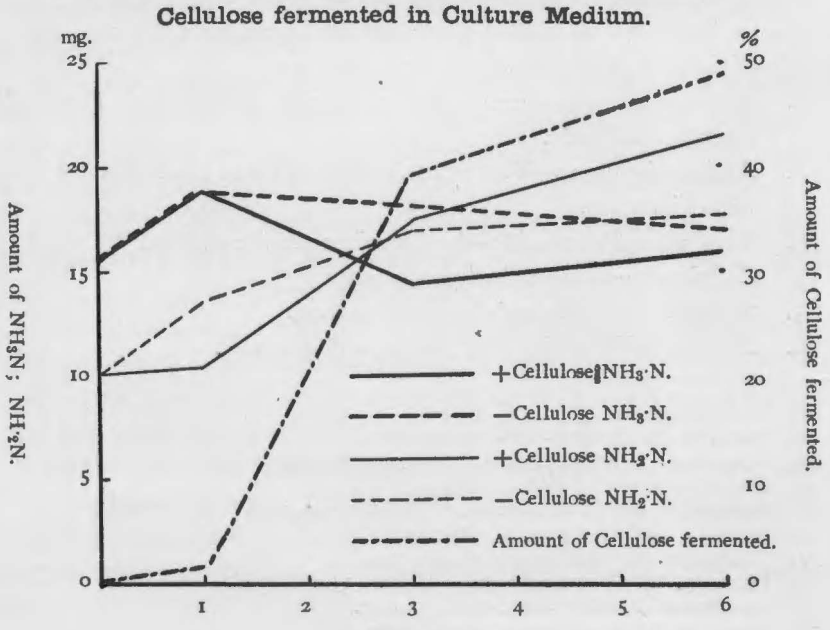

Figure IV.

Variation of Bacterial Number in Culture Medium.

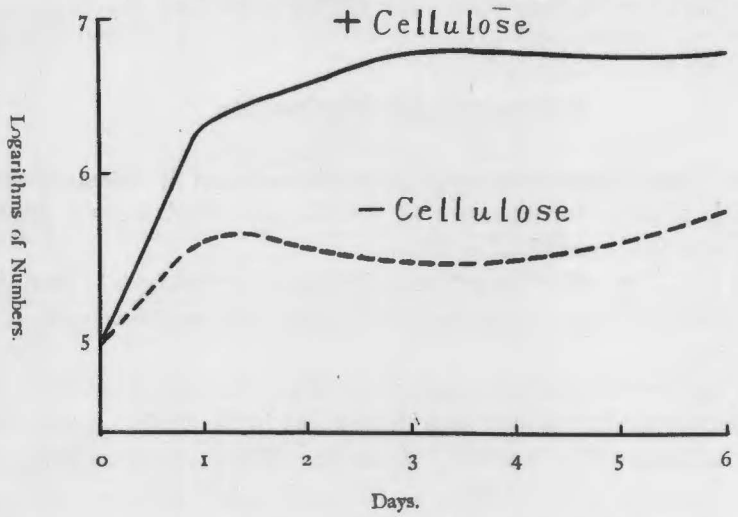

Table IX and Fig. III indicate that in the medium with cellulose added, ammonia increased to 3.22 mg. on the first day and on the third day when the vigorous cellulose fermentation took place, the decrease of 5.11 mg. was observed; and slight increase on the sixth day which indicates plainly that for the fermentation of cellulose so much of available nitrogen is consumed; and also a large amount of phosphoric acid was consumed. On the other hand, in the culture without cellulose, the similar result was obtained, on the first day, as to ammonia which gradually diminished later and the comsumption of phosphoric acid is not marked. Regarding the amino-nitrogen, where the cellulose was present, practically no change took place on the first day. After the second day, it increased in parallel with the amount of cellulose fermented.

From these results, it seems that in case of B. thermofibrincolus it behaves similarly as those organisms which were investigated by DE BORD in presence of glucose and the amino-nitrogen can be taken as an approximate index of proteolysis. Also it is clear that B. thermofibrincolus has proteolytic enzyme while such thermophilic cellulose fermenting bacteria as described by WOODMAN and STEWART[1] do not contain the proteolytic enzyme. Again the presence of proteolytic enzyme in B. thermofibrincolus has a great significance in connection with the fermentation of compost. Where no cellulose was added, the amino-nitrogen increased gradually up to the third day but no further change took place after that which may be due to lack of energy supply.

Further as to the change of bacterial number, it is shown in Fig. II which indicates that with the cellulose added, the number increased five times on

1) WOODMAN, H. E. and STEWART, J., J. Agr. Sci. 18, 713, 1928.

the first day, and seventeen times, on the third day; eight times, on the sixth day comparing to that which no cellulose was added, and no marked increase observed in the latter anytime which may be due to the lack of energy supply.

Summary and Conclusions.

Part I. The influence of ecological conditions upon B. thermofibrincolus was undertaken as to the quantity of volatile acids and alcohol produced. The results may be summarized as follows:

1. At 65°C., 45.5% evaporation took place in seven days in case of the test-tube culture but it was found that the double tube method prevents it to a great extent.

2. In the test-tube culture, more cellulose was fermented but less volatile acids and alcohol were produced than those found in the flask culture. When 1.5—3.0% cellulose was supplied, about 60—88% was fermented within 3—7 days; and 24—33% volatile acids (as acetic acid) and 3—12% alcohol (as ethyl) were produced relative to the fermented cellulose.

3. The optimum temperature was found to be 65°C., and at 50°C., it took twice as much time as that required at 65°C., and even then only 63% cellulose was fermented in comparison with that of 65°C., although the percentage of the amount of products was rather high.

4. Culturing by BUCHNER's anaerobic method, less amount of cellulose was fermented than that under aerobic condition, either ordinary or double tube method, but double quantity of alcohol and the equal amount of volatile alcohol were produced.

5. As to the influence of peptone, 0.5% concentration was the best and as a whole, less the amount of peptone was better for the cellulose fermentation. But the production of volatile acids was in proportion to the amount of peptone.

6. The best fermentation was obtained when 0.75—2.20% cellulose was added although it seemed to produce more alcohol as more cellulose was supplied.

7. The influence of nitrogen source, seems to be in the following order: Peptone>egg albumin>casein>ammonium sulfate>meat extract, for the cellulose fermentation; meat extract>egg albumin>peptone>casein>ammonium sulfate, for the production of volatile acids; meat extract>peptone>egg albumin>casein>ammonium sulfate for alcoholic production. It was especially noted that the organic nitrogen was essential for the alcoholic production.

8. As to the influence of incubation period, the test was carried out with and without an addition of calcium carbonate to the cellulose medium. With the presence of calcium carbonate, at the end of third day, more than one half

and on the seventh day, about 88% cellulose was fermented and the production of volatile acids and alcohol was the greatest on the seventh day also. On the other hand, without the calcium carbonate, the activity was decreased by one half and it was due to the increase of hydrogen ion concentration which seems to serve as an index of physiological activity of the organism.

Part II. The nitrogen metabolism of B. thermofibrincolus was investigated, and the results are summarized as follows:

1. B. thermofibrincolus has proteolytic enzyme and the production of amino-nitrogen can be taken as an approximate index of proteolysis as in the case with those bacteria investigated by DE BORD.

2. The amino-nitrogen production is governed by the source of energy supplied.