

Studies on *Bacillus thermofibrincolus* n. sp.

I. Description of the organism.

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This paper deals with a new organism which is thermophilic and capable of decomposing the cellulose.

Recently the microbial decomposition of cellulose has been studied rather extensively by different investigators and several new organisms have been isolated. The authors have been especially interested in the members of thermophilic group since they have the intimate relation to the process of composting. There are several investigations undertaken on the thermophilic bacteria especially in connection with the canning and dairy industry, viz. MORRISON and TANNER¹⁾, KRÖHN²⁾, ROBERTSON³⁾, FEIRER⁴⁾ and others. But the number of investigations on the thermophilic, cellulose fermenting organisms is somewhat limited, and some of them are reviewed here.

Review of Literature.

The bacterial decomposition of cellulose at high temperature was noted by MACFAYDEN and BLAXALL⁵⁾ in 1899, and later in 1912, PRINGSHEIM⁶⁾ investigated the fermentation products biochemically which was followed by KROULIK⁷⁾ who reported on the cultivation of impure cultures. In 1923 LANGWELL and LYMN⁸⁾ succeeded in isolation of a new strain of bacteria which is capable of fermenting

- 1) MORRISON, L. E., and TANNER, F. W., *Bot. Gaz.* 77, 171, 1924; TANNER, F. W., and MORRISON, L. E., *J. Bact.* 7, 343, 1922.
- 2) KRÖHN, V., *Ann. Acad. Sci., Finland*, ser. A, 21, 1, 1924.
- 3) ROBERTSON, A. H., *New York (Geneva) Agr. Exp. Station, Tech. Bull.* 130—131, 1927.
- 4) FEIRER, W. A., *Soil Science*, 23, 47, 1927.
- 5) MACFAYDEN, A. and F. R. BLAXALL, *Trans. Jenner Inst. of Preventive Medicine*, ser. 2, 182, 1899.
- 6) PRINGSHEIM, H., *Zeits. f. Phys. Chem.*, 78, 266, 1912; *Centbl. f. Bakt. Abt. II*, 38, 513.
- 7) KROULIK, A., *Centbl. f. Bakt., Abt. II*, 36, 339, 1912—13.
- 8) LANGWELL, H. and A. LYMN, *J. Soc. Chem. Indus.*, 42, 280, 1923.

the wood-pulp at 65° C. and produces acetic and lactic acid, alcohol, methane, hydrogen and carbon dioxide. The general characteristics of the organism grown on the glucose agar are given in Table I. It is especially noteworthy in this case that the organism grows well on the nutrient agar medium and does not lose its power of fermenting cellulose on its return to the cellulose medium. Mm. Khouvine¹⁾ isolated *Bac. cellulase dissolvens*, an anaerobic and grows best at 35—51° C. The organism produces a small amount of acetic and lactic acid, alcohol, CO₂ and H₂ gases, no flagellum; GRAM's negative, and no carbohydrates other than the cellulose is decomposed. NEUBERG and COHN²⁾, in 1923, isolated many strains of bacteria and as the intermediate products such as acetaldehyde, glucose and cellobiose were demonstrated. *Closteridium thermocellum* (n. sp.) was isolated by VILJOEN, FRED and PETERSON³⁾ in 1926, and has the characteristics which are given in Table I. The optimum temperature for the organism is 65° C; the peptone is used as the nitrogen source; 70—90% the spruce pulp is fermented and 50—55% acetic acid, 5—25% alcohol and a small amount of lactic acid, are produced besides CO₂ and H₂ gas, and the pigment is produced. In 1928, WOODMAN and STEWART⁴⁾ isolated an organism which is able to utilize an inorganic N such as ammonia sulfate but has no proteolytic action; 4—7 × 1.0 μ large and positive to GRAM's stain. Besides the cellulose, sucrose, fructose, glucose, maltose, arabinose, mannite, inuline, dextrose and glycerine are fermented with production of acid but no gas is produced. Again lactose, dulcitol and salicin are not fermented. In 1928, COOLHAAS⁵⁾ isolated an organism, *B. thermocellulolyticus* n. sp. of which the optimum temperature is 50—55° C.

The authors isolated an organism which ferments the cellulose at 65° C. and will be reported on the following pages as to its morphology and some physiological characteristics indicating that it is a new species.

Experimental.

I. Culture medium:

For the isolation and cultivation of the organism, VILJOEN's medium of the following composition is used.

Sodium ammonium phosphate-NaNH ₄ HPO ₄ ·12H ₂ O	2.0 g.
Monobasic potassium phosphate-KH ₂ PO ₄	1.0
Magnesium sulfate-MgSO ₄ ·7H ₂ O	0.3

1) KHOUVINE, V., *Ann. de l'Inst. Pasteur*, 37, 711, 1923.

2) NEUBERG, C. and R. COHN, *Biochem. Zeitsch.*, 139, 527, 1923.

3) VILJOEN, J. A., FRED, E. B. and W. H. PETERSON, *J. Agr. Sci.*, 16, 1, 1916.

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

5) COOLHAAS, C., *Centbl. f. Bakt., Abt. II*, 76, 38, 1928.

Sodium chloride-NaCl.	0.1 g.
Peptone	5.0
Calcium carbonate-CaCO ₃	excess.
Tap water	1,000. cc.
Cellulose (filter paper)	15.0 g.
PH	7.34

100 cc. of the medium was placed in 300 cc. Erlenmeyer flask and sterilized, and was inoculated with about 5 grams of well rotted earth where an extremely rapid decomposition of fallen leaves has been observed annually; incubated at 65° C. The medium becomes turbid after 7—12 hours and gas bubbles are observed after 8—20 hours; the filter paper becomes yellow and begins to float on the surface or comes to 'head'; after vigorous fermentation, the filter paper loses its original form and the excess portion sinks to the bottom and becomes yellowish brown.

II. Isolation:

The isolation of the organism was carried out as follows: 5 cc. of 48 hours old culture in which vigorous fermentation is taking place, is transferred into a new culture medium and incubated at 65° C. The same procedure is repeated at every 48 hours until the culture attains purity under microscope. Then the culture was plated to ascertain its purity by the colonies produced. After a long consecutive cultivation, the organism weakens sometime which can be invigorated by adding a small amount of the compost extract which is prepared as follows: the dried compost and water is mixed in proportion 1 : 5 and autoclaved for one hour and half at 15 pounds pressure. The extract thus prepared is added to the weakened culture amounting to 5—10% the medium.

III. Morphological description:

The morphological description of the organism was carried out according by the Official Methode of the Society of American Bacteriologists, as will be noted below:

a) Vegetative cells:

The culture was grown in the cellulose medium at 65° C. for 24 hours and examined by staining with Indian ink; the form is long rod, $4.2 \times 0.5 \mu$ on average and some $3.0 - 7.5 \mu$, arranged singly or several cells in chain with somewhat rounded ends, (see Plate XXII.); the capsule is present and stained with WELCH's glacial acetic acid distinctly.

b) Sporangia:

Only a few sporangia are produced in a week-old culture in the cellulose medium at 65° C. but the numerous of them are produced on the starch agar medium. The sporangia are long rods, some of them measure $3.0 - 4.0 \times 0.5 \mu$ but the majority, $3.0 \times 0.5 \mu$, (see Plate XXII.); endspores present, examined by carbol-fuchsin stain alone and also counterstained with methylene blue, located

terminally and ellipsoidal in form; limit of size, $1.4-1.5 \times 0.9 \mu$ and the majority $1.5 \times 0.9 \mu$.

c) Motility:

Motile under the hanging drop, some grown in the cellulose medium at 65°C . for 24—48 hours and the others grown in broth and on the nutrient agar at 65°C . for 48 hours. The flagella were stained by GRAY's method¹⁾ and found to be peritrichous having 10—14 flagella. (see Plate XXII.)

d) Staining reactions:

Positive to GRAM's stain while 1—4 days old and becomes negative after seven days; stained better with carbol fuchsin, gentian violet than with LÖEFFLER's methylene blue, malachite green, saffranin and rosaniline.

IV. Cultural characteristics:

a. Colonies on the nutrient agar; the colonies appeared after 15 hours at 65°C ., medium, surface growth, circular, smooth surface with entire edge; finely granulated internal structure; irregular deep colonies, punctiform and filamentous.

b. Liquid culture:

1) Cellulose medium; 24 hours old culture in the cellulose medium at 65°C ., becomes strongly turbid and forms grayish white membrane and produce a peculiar odor resembling to that of acetic acid; abundant flocculent sediment. The gas bubbles are produced 7—12 hours after inoculation, and the filter paper becomes yellow after 24 hours and begins to be broken up after 36 hours, and the broken cellulose is pushed up and forms 'head'. (see Plate XXIII.)

2) Nutrient broth; grayish white membrane is formed after 24 hours at 65°C .; strongly turbid and a small amount of sediment which is viscid on agitation is formed; strong odor is noted.

3) Glucose broth; 24 hours old at 65°C ., slightly grayish white membrane is formed; no gas; odor, present; abundant grayish white sediment which is viscid on agitation is formed as the culture becomes old.

4) Agar stroke; 24 hours old at 65°C ., moderate growth of echinulated form, smooth surface with flat elevation; optically translucent, glistening and grayish white; bad odor; butyrous; no color is present at 24 hours but becomes yellowish brown later; condensed water becomes turbid. The similar growth was observed on the cellulose medium.

5) Gelatin medium;

Since no growth of the organism takes place at 20°C ., the following special procedure was adapted to ascertain its liquefaction. After the gelatin medium was inoculated, it was placed in an incubator at 65°C . for 24 hours, and subsequently immersed in the cold water. The control tube was treated similarly. On cooling under the running water, the control tube solidified but the

1) GRAY, P. H. H. J. Bact. 12, 273, 1926.

inoculated tube remained liquid. From this test, it seems to indicate that the gelatin was liquefied by the organism.

6) Potato medium; 24 hours old at 65° C., grayish white with moist glistening growth took place but no change in color of potato; the liquid in tube became turbid.

V. Physiological Description :

The S. A. B. methods were employed and found to be as follows :

1) Production of indol ;

In both the cellulose and nutrient broth after 2—3 days, the positive tests were obtained by both SALKOWSKI's and GORÉ's methods.

2) Production of hydrogen sulfide :

Testing with the lead acetate paper in both the cellulose and nutrient broth, a marked production of hydrogen sulfide was noted after 24 hours growth.

3) Catalase reaction :

A marked catalase reaction is noted as it was reported in a separate paper¹⁾ published in 1928.

4) Production of acetyl methyle carbinol :

The Voges-Proskauer reaction was applied to both cultures, the cellulose and glucose broth media, grown 1 or 3 days at 65° C. and 37° C. respectively, and was found that the production of acetyl methyl carbinol was negative. As the control, *B. sutitis* and *B. mycoides* were grown in glucose broth, for 3 days at 30° C., and the test was applied by which a marked positive results were obtained, so that the accuracy of the test was ascertained.

(5) Reaction in milk :

Brom-cresol-purple was used as an indicator, and the culture was grown at 65° C. A slight acid production took place after three days and coagulated; a marked production of acid on the third and wheyed; the peptonization was noted on the tenth day. The litmus milk was reduced after six hours and red-dened on the fifth days. The reduction of methylene blue milk began on the fourth hour.

6) Nitrate reduction :

Nitrite was formed in the nitrate broth, nitrate nutrient agar and nitrate cellulose medium, after 24 hours at 65° C., but no gas was produced even after five days. The test was carried out by Griess' reaction.

7) Hydrolysis of starch :

The clear zone was produced round the colonies on the starch agar plate after 24 hours growth at 65° C. The test was carried out by the iodine method as usual.

8) Fermentation of carbohydrates :

Besides the cellulose, hemicellulose, starch, raffinose, salicin, sucrose, lac-

1) ITANO, A. and S. ARAKAWA, *Berichte d. Ohara etc.* III, 505, 1928.

tose, maltose, mannose, galactose, fructose, glucose, xylose and arabinose are fermented.

9) Oxygen relation :

According to the BUCHNER's method as applied here, the organism is facultative in regard to the oxygen requirement.

10) Thermal death point :

The test was carried out in an oil bath using a glass tube, 7 × 200 mm. and the glass wall of 1 mm. thick. One cc. of 48 hours old culture in the cellulose medium, PH 7.0, was sealed in the glass tube and heated. The organism was killed after 8 minutes heating at 130—132° C. Approximately 16 millions of the organism were present in one cc. of the culture used.

VI. Comparative study of the thermophilic cellulose fermenting bacteria.

The comparative study of some of the thermophilic cellulose fermenting bacteria which are closely related to each other was undertaken and the results are noted in Table I :

Table I.
Comparative Study of Some Thermophilic Cellulose
Fermenting Bacteria.

Authors.	LANGWELL & LYMN	ITANO & ARAKAWA	VILJOEN, FRED & PETERSON
Name of organism.	—	Bacillus thermofibrincolus.	Closteridium thermocellum.
Size of rods, μ .	4.0 × 0.4	4.2 × 0.5	5.0 × 0.4
Size of spore, μ .	—	1.5 × 0.9	0.9 × 0.6
Flagella.	absent	peritricus	peritricus
Gram's stain.	—	+	—
Nutrient broth	grow.	grow.	grow.
Glucose broth.	gas.	no gas, acid, light membrane, abundant viscid sediment.	gas and acid, pellicle, sediment.
Nutrient agar. stroke	glistening, moist, butyrous.	moist, translucent, butyrous.	moist, glistening, butyrous.
Agar colony.	surface & bottom small.	ditto.	ditto. (starch agar.)
Potato.	yellow, moist, potato browned.	gray, white, moist, potato no change.	yellow, potato browned.
Milk	acid coagulation in 5 days, no digestion, reduce litmus.	acid, coagulation in 3 days, wheyed, gas, reduce litmus.	slight acid coagulation in 3 days, wheyed, gas.
Indol	—	positive.	negative.
Catalase.	—	positive.	positive

Table I. (continued.)

Authors	LANGWELL & LYMN.	ITANO & ARAKAWA.	VILJOEN, FRED & PETARSON.
Methyl adetyle carbinol.	—	negative.	negative.
Best N source.	—	albumin.*	peptone.
Fermentation of Carbohydrates	starch. hemicellulose, glucose, xylose.	Cellulose, starch, hemicellulose, raffinose, glucose, lactose, maltose, mannose, galactose, fructose, sucrose, xyrose, arabinose, salicine.	ditto.

* The action on the albumin will be reported in the next paper.

As Table I indicates, the organism resembles closely to *Cl. thermocellum* more than the other. However the close examination reveals that the said organism differs from *Cl. thermocellum* in regard to two, three important points, and some other biochemical differences will be reported, in detail, in the following paper. Again it is obvious to state that this organism differs from *B. celulosae dissolvens*¹⁾ and also from that which was reported by WOODMAN and STEWART²⁾. Accordingly the authors consider that the organism described herein as a new species and propose the name, *Bacillus thermofibrincolus* n. sp.

Summary and Conclusions.

The authors isolated an organism which decomposes the cellulose vigorously at 65° C., and described its morphological and cultural characteristics. As the results of the investigation, the authors consider it as a new species and propose the name, *Bacillus thermofibrincolus* n. sp.

Further the biochemical description such as fermentation products will be reported in the following paper.

1) KHOUVINE, Y., *Ann. de l'inst. Pasteur*, 37, 711, 1923.

2) WOODMAN, H. E. and J. STEWART, *J. Agr. Science*, 18, 713, 1928.

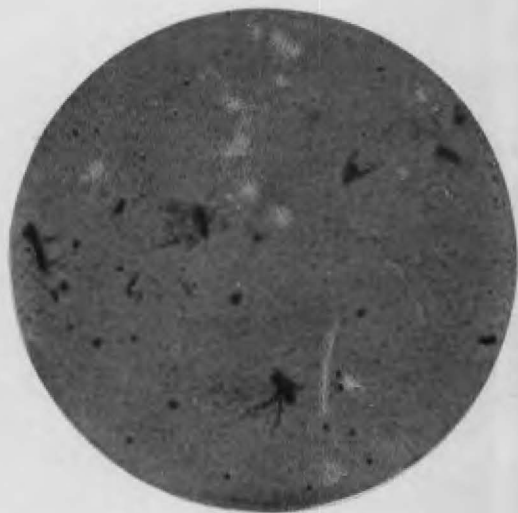
PLATE XXII.



(1)



(2)

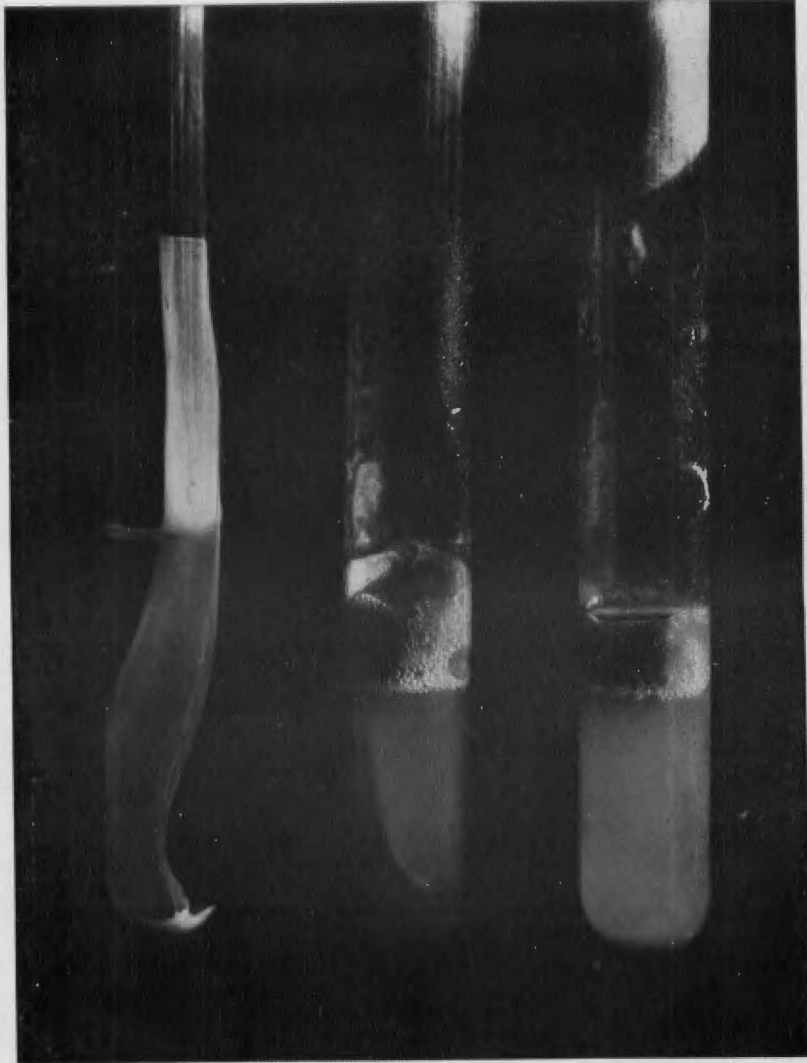


(3)

PLATE XXII.

- 1) *Bac. thermofibrincolus* n. sp. : multiplying cells in the cellulose medium, 24 hours old at 65° C.; Indian ink stain; $\times 1,500$.
- 2) *Bac. thermofibrincolus* n. sp. : spores and sporangia, 24 hours old at 65°, on the starch agar slant; Indian ink stain; $\times 1,500$.
- 3) *Bac. thermofibrincolus* n. sp. : flagella stain, 24 hours old at 65° C., in the cellulose medium; stained by GRAY's method; $\times 675$.
(Photo. ARAKAWA.)

PLATE XXIII.



(1)

(2)

(3)

PLATE XXIII.

Fermentation of cellulose by *Bac. thermofibrincolus* n. sp. :

(1) Control, filter paper in the cellulose medium.

(2) 48 hours after inoculation, at 65° C.

(3) 72 " " " " " "

(Photo, ARAKAWA.)