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Studies on the cellulose-decomposing bacteria found parasitic in the alimentary canal in ruminants and other animals

I. On the isolation of the bacteria

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Very few studies[8, 13] have been made on the cellulose-decomposing bacteria found parasitic in the alimental canal in ruminants, while a number of studies[1–7, 9–12] made of those in soils. These bacteria, found in the intestine of horses and pigs and in the rabbit's coecum are believed to play an important role in supplying some nutrients to the hosts. Little or none, however, has been known about the strain or strains which predominate in these animals, nor about the effect on the nutrition of their hosts of the product yielded by their activity. In order to throw some light on these problems, a series of study have been undertaken. The present paper deals with a new method of isolation of this type of bacteria.

RESULTS

EXPERIMENT I

(*1*) *Examination of the routine methods of bacterial isolation.*

As shown in Table 1, liquified cellulose or filter paper is used in all these solid media, and cellulose or filter paper in all liquid media. In these methods, bacterial isolation is effected as in the manner shown in Fig. 1. The cellulose-decomposing bacteria are accumulated on the filter paper in the liquid medium A; bacteria thus accumulated are transferred to the solid medium B and left there till a clear zone is formed; colonies in the zone are transferred back to the liquid medium A, and put back again to a solid medium. The procedure is

Table 1.

Inventers	Ingredients			
Omeliansky	KH_2PO_4	1.0 g	NaCl	trace
	$MgSO_4$	0.5 g	Distilled water	1000 cc
	$(NH_4)_2SO_4$	0.5 g	Cellulose liquid	1000 cc
Dubos	$MgSO_4$	0.5 g	KCl	0.5 g
	$NaNO_3$	1.0 g	$FeSO_4$	trace
Mc. Bee	$(NH_4)_2SO_4$	0.5 g	$CaCO_3$	1.0 g
	KH_2PO_4	0.5 g	Agar	10.0 g
	$MgSO_4$	0.5 g	Cellulose liquid	1000 cc
	NaCl	trace	Cellulose	
Van Viterson	K_2HPO_4	1.0 g	Cellulose liquid	500 cc
	Cellulose	15.0 g	Distilled water	500 cc
	Peptone	5.0 g	$CaCl_2$	0.3 g
	$NaNH_4HPO_4 4H_2O$	1.0 g		

N. B. Bojanovsky used silicagel for the isolation.

repeated till a pure strain is obtained. It is difficult to isolate the pure strain from among the bacteria which are accumulated by repeated transfer from a liquid medium to a solid medium. Yamada[3] reported

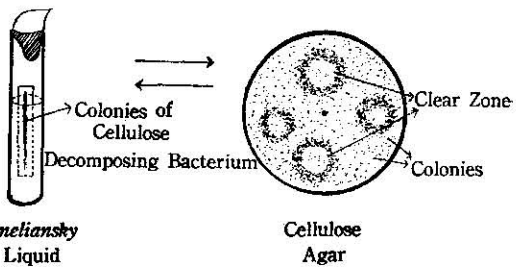

Fig. 1. Scheme showing the routine method of isolating cellulose-decomposing bacteria.

that he failed after two years of trial to obtain a pure culture of cellulose-decomposing bacteria by this method of accumulation. On the other hand, the formation of a clear zone on a solid medium requires a long period of time, depending the thickness of the agar, the pH of the medium and the proportion of the desired bacteria to the contaminating microbes. Moreover, since the clear zone itself is inhibited not only by pure strains but by contaminating strains due to the decomposition of cellulose, a difficulty always results in distinguishing between the genuine and the contaminating bacteria. It is worthy of note that notwithstanding this difficulty, Asai and Ueda[4] succeeded in isolating several strains of mesophilic cellulose-decomposing bacteria,

and Asai and Yamada[3] isolated thermophilic strains of the same bacteria by a modification of Hungate's method.

(2) *Author's method for isolation of bacteria.*

Examinations of the routine methods disclosed that contaminating bacteria were capable to grow on the saccharide products of the cellulose-decomposing bacteria and that, as cellulose being diffused throughout the agar in the form of gel, many forms of bacteria became naturally mixed up. The fact implies that the liquid cellulose is readily converted into sugar by the bacteria. Hence, a modification was made of adding strips of cellulose to the following medium.

K_2HPO_4—1.0 g	$CaCO_3$—2.0 g	$(N_4H)_2SO_4$—10 g	NaCl—trace
$MgSO_4$—0.3 g	Distilled water	—1000 cc	Agar—10.0 g

Both aerobic and anaerobic culture on this medium at 37°C resulted in a better growth of the bacteria as shown in Fig. 2.

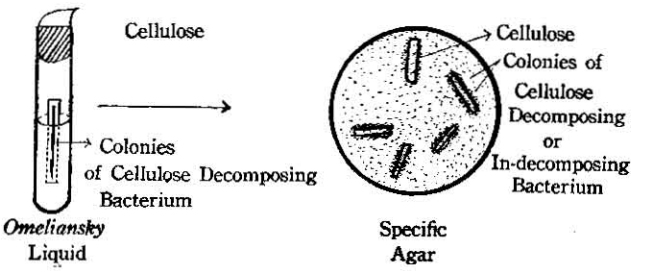

Fig. 2. Scheme showing the author's method in which the cellulose-decomposing bacteria are inoculated on the specific solid medium around each piece of the filter paper.

The isolation procedure finally adopted is as following. The bacteria are accumulated on a strip of filter paper in Omeliansky's medium; in two to three days, there are formed colonies of bacteria around each piece of the paper producing yellow or bluish pigments. A loopful amount of bacteria of each separate colony is tested on the filter paper in Omeliansky's liquid medium for identification of the microbes. It is soon observed that the paper is torn into pieces, suggesting that sample bacteria (both cellulose-decomposing and otherwise) were capable of growing in a medium composed of the aforesaid ingredients without filter paper and that the growth of any contaminating bacteria might be more or less inhibited due to a lack of carbon source supplied from decomposed cellulose. The bacteria thus grown are accordingly inoculated on the aforesaid solid medium with no piece of filter paper in it.

This inoculation was repeated further several times. It will be

observed after two weeks that bacteria in some colonies formed are cellulose-decomposing and those in other colonies are not. Thus, the isolation of the cellulose-decomposing bacteria can be readily carried out within a short period of time by growing them on this filter paper free medium. Ten strains of this form of microbes have so far been isolated from the rumen contents of 15 cows and are under the identification which will be reported later in more detail.

Comment of the experiment I

Most of strains isolated by the author's method are those of mesophilic methane-producing cellulose-decomposing bacteria and several of them are those of thermo-philic hydrogen-producing type. The difference in number between the former and the latter strains obtained may be ascribable to the scarcity of the materials, or may be due to a growth of the former strains to the latter. It is the author's opinion that the filter paper decomposing bacteria isolated by this method may be classified as cellulose-decomposing microbes, although the colonies of these microbes differs from those hitherto reported in that they are bluish or grey without the pigmentation and form tiny circumscribed granules. The strains isolated are mainly the genus *Pseudomonas* which is characterized by its colony being depressed in the agar medium. The colonies formed on the solid medium by bacteria, either cellulose-decomposing or otherwise, are all small in size due to a lack of cellulose as the carbon source. These strains may generally be taken as purified after being grown in diluted form by a plate culture. It is to be mentioned that the microbes thus isolated form colonies of the same type on the author's solid medium, and their cellulose-decomposing power is always been ascertained by a comparable decomposition of the filter paper.

Experiment II

An attempt to reduce the time required for isolation.

As previously reported, a new method described in Experiment I enabled the author to isolate pure strains of the mesophilic and the thermophilic cellulose-decomposing bacteria from the rumen (the former being more abundant than the latter). The time required for the isolation is given in Fig. 3.

Procedure--A. The accumulation of bacteria is followed by the loosening of the filter paper in 2 to 3 weeks (2 weeks in the case of the methane-producing group of the bacteria, 3 weeks in that of the oxygen-producing group).

Procedure—B. The germs thus accumulated are then transferred to a solid medium and left there till a colony is formed; the transplantation is repeated several times till a pure strain is obtained; in most cases it takes 1 to 3 weeks.

Procedure C. The cellulose-decomposing power of the germs can be checked in 1 to 2 weeks.

In short, for the pure isolation by the present method are required 30 to 50 days in contrast to the other methods by which are required one to several months (in some cases to a year or two). Though it is apparent that the bacterial isolation can be achieved within rather a short time-period, an attempt was made to make every step of culture as short as possible. The period required for (A) and (C) (Fig. 3) is needed for the cultivation in Omeliansky's liquid medium and so can be shortened by the promotion of the bacterial growth, whereas that spent for (B) is indispensable for the aquisition of genuine strains and can not be cut short. Hence, several stimuli suppliments which are known as promoting the cellulose-decomposing activity of the bacteria were added to the medium. The result obtained is given in Table 2.

Table 2.

	Order of decomposing stimulus added power.	Days required for starting decomposition of cellulose	
		Strain I	Strain II
1	Boiled extract of barley seeds	8.	6.
2	Boiled extract of clover stems and leaves	7.	7.
3	Sodium citrate	8.	8.
4	*Azotobacter chroococcum*	11.	11.
5	*Micrococcus*	12.	12.
6	Sodium-sulphate	12.	12.
7	Control	12.	12.

N. B. (1) The germs were determined as growing when the filter began to split.
(2 *Micrococcus*, a genus showing its cellulose-decomposing activity when the Clostridium in the stomach is isolated.
(3) *Azotobacter*—loopful, *Micrococcus*—loopful.

The boiled extracts of barley seeds and of the stems and leaves of the clover, each prepared by boiling a 10 g aliquot in 100 cc of water for 30 minutes is added to the medium in the ratio of 1 : 20 (by volume). The other stimuli supplements are added at a level of the

medium. The decomposition of cellulose is promoted in the medium containing one of the stimuli than in the control. It is clearly shown in Table 2 that the boiled extracts of these clover and barley seed, sodium citrate, and *Azotobactor* are particularly effective as decomposition-promotors. The decomposition-promoting effect of each stimulus varied with its amounts added, as shown in Table 3.

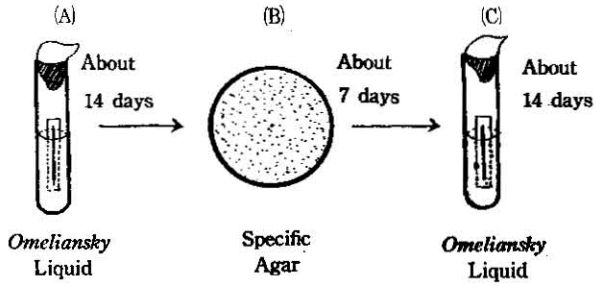

Fig. 3. Diagrammatic representation of the days needed to isolate the cellulose-decomposing bacteria by using the specific agar.

Table 3.

Order of decomposing power	Stimuli added to the medium	Stimulus medium ratio by volume	Number of days for the cellulose to split up	
			no. 3.	no. 4.
1	The boiled extracts of clover leaf stalks	1/20	8	8
		1/10	8	10
2	Sodium-citrate	1/10	8	8
		1/5	14	8
3	*Azotobacter chroococcum*	1 loop	16	15
		5 loops	18	17
4	Control		18	18

Generally speaking, the boiled extracts of clover leaves and that of barley seeds contain a non-nitrogenous substance as their main ingredients, and a lesser amount of amide. The effect of the soluble non-nitrogenous substance may be presumably due to acceleration of citric acid cycle by citric acid formation from non-nitrogenous substances, and that of *Azotobacter* is ascribable to the action of the zooglea. In a word, the decomposition promoting effects of these stimuli may presumably be attributed to the presence of carbohydrate ingredients available as energy sources. This assumption may not be incorrect, because the medium is purposely free from carbon source. Accordingly, several saccharides and nitrogenous matters for efficacy as stimuli suppliments are tested. The results are presented in Table 4.

Table 4.

Order of decomposing power	Stimulus supplements added at level of %		Days required for decomposing cellulose	Stage in decompos.
1	Xylose	0.05 %	6	⫴
2	Lactose	0.05 %	7	⫴
3	Dextrine	0.05 %	9	⫴
4	Glucose	0.05 %	10	⫴
5	*Azotobacter*	1 loop	11	⫴
6	Dulcit	0.05 %	12	⫴
7	Mannit	0.05 %	13	±
8	Urea	0.05 %	13	±
9	Salicine	0.05 %	13	±
10	Maltose	0.05 %	14	+
11	Saccharose	0.05 %	15	+
12	Sodium nitrite	0.05 %	15	±
13	Potacium nitrite	0.05 %	15	±
14	Control		21	+

⫴ Completely decomposed ⫴ Torn into small strips

⫴ Nearly so + So in parts ± Torn into halves.

When xylose, lactose and glucose were added to the medium in concentration of 0.05% and 0.1%, the result as shown in Table 5 was obtained.

Table 5.

Order of decomposing power	Stimulus added and the adding ratio	Days required for the cellulose decomposing	Opt. conc.
1	Xylose	5 4	0.05 % 0.1 %
2	Lactose	5 5	0.05 % 0.1 %
3	Detrine	8 7	0.05 % 0.1 %

The effects of sugars are more pronounced than those amides, as shown in Table 6. The promoting effects on cellulose-decomposing power due to the presence of these sugars and of the boiled extracts of barley seeds and of the leaves and stems of the clover are also shown in Table 6 for comparison.

Table 6.

Order of decomposing power	Stimulus-medium	Time required for the cellulose-decomposing (Day)
1	Arabinose (Monosaccharide)	5
2	Xylose (Monosaccharide)	6
3	Saccharose (Oligosaccharide)	6
4	Galactose (Monosaccharide)	6
5	Mannose (Monosaccharide)	6
6	Glucose (Monosaccharide)	7
7	Lactose (Oligosaccharide)	7
8	Boiled extracts of barely seeds	8
9	Dextrine (Polysaccharide)	8
10	Clover-leaf's boiled extracts	9
11	Raffinose (Trisaccharide)	9
12	Mannit (Monosaccharide)	9
13	*Azotobacter*	9
14	Dulcit (Monosaccharide)	9
15	Urea	12
16	Inuline (Polysaccharide)	12
17	Maltose (Oligosaccharide)	12
18	Control	15

Discussion of the experiment II

It is noteworthy that the boiled extracts of barley seeds and clovers and the body contents of the *Azotobacter* was inferior to certain forms of pentose and hexose and that among the saccharides used, pentose is the best promotor of the decomposition of cellulose.

Résumé

In order to obtain a pure culture of the cellulose-decomposing bacteria, an agar medium has been devised which is composed of K_2HPO_4 (10 g), $CaCO_3$ (2.0 g), $MgSO_4$ (0.5 g) $(NH_4)_2SO_4$ (1.0 g), NaCl (trace), distilled water (1000 cc), and agar (1.0 g). Although cellulose is not included in this medium, the cellulose-decomposing bacteria and the other bacteria actually form colonies. In dilute method a pure culture is obtained. When the cellulose-decomposing bacteria which are obtained by this method were inocultated, no decrease is noticed in their activity. The mesophilic cellulose-decomposing bacteria and also the thermophilic cellulose-decomposing bacteria can be cultured on this agar plate, but the latter is rarely seen. A further investigation will

be needed to account for the difference of abundance between these two types.

It has been found in the experiment I that by using the specific agar after culturing in Omeliansky's medium, a pure culture of the cellulose-decomposing bacteria was obtained in a comparatively short time. Shortening of the time of isolation is realized by adopting specific agar, but not by the applying Omeliansky's medium. It takes ordinarilly about 30–50 days to obtain a pure culture, but if time of culture on Omeliansky's medium can be shortened, the isolation will be more readily made. It is to be noted in this respect that carbohydrate is a good stimulus to cellulose decomposition. Especially pentose group and some of hexose group are found excellent.

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