Studies on the cellulase produced by Aerobic Mesophilic bacteria
by Richard A Hammerstrom

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology
Montana State University

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Master of Science in Bacteriology

at

Montana State College

Approved:

Head, Major Department

Chairman, Examining Committee

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Bozeman, Montana
May, 1953

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ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr. R. H. McBee under whose guidance this program was conducted and to those of the staff at Montana State College who helped by word and deed throughout the course of this investigation.

ABSTRACT

A historical background regarding microbial cellulose decomposition is presented. Particular emphasis is placed on those studies relevant to enzyme preparation and examination. Four organisms of mesophilic, facultatively aerobic nature were used in the early portion of the work although most of the later work was conducted with two of these organisms. Methods of cultivation, types of media, and the growth characteristics of the organisms were investigated. Cellulolytic material was produced by growing the organisms in a liquid medium and concentrating the medium by evaporation. The presence of cellulase in these preparations was demonstrated by the formation of reducing substances after the addition of cellulose. Cellulase preparations were found to be most active at a pH of 5.5, and were stable toward drying and storage for long periods. They were not inactivated at temperatures up to 50 C. The cellulase was shown to be constitutive rather than adaptive since it was produced during growth on glucose or cellobiose as well as on cellulose. The end-product of the cellulase activity on cellulose was cellobiose. A cellobiase could not be demonstrated.

INTRODUCTION

Although a reasonable amount of work has been reported concerning cellulose degradation by microorganisms, review of the literature shows that even the basic considerations are not understood. The characteristics of the enzymes responsible for the breakdown of cellulose, their mode of action, and the intermediary products are not well known. The results obtained by workers in this field are not in agreement.

Cultures of known purity were not available for the study of cellulose decomposition until their isolation by McBeth and Scales (1913 .

Mitscherlich, in 1850, was probably the first worker to systematically study the decomposition of cellulose. He arrived at the conclusion that the decomposition of cellulose in manure piles was carried out by microorganisms. Although a variety of microorganisms was studied for the next 50 years, it was not until 1904 that Van Iterson concluded that the soil harbored aerobic bacteria which were capable of carrying out the decomposition process. This and other early work was conducted with either admittedly impure cultures or with cultures which were later proven to be impure. This work with impure cultures and the inherent difficulties involved in obtaining pure cultures led many workers to believe the cellulose could be decomposed only in a state of symbiosis (Enebo, 1948). It is easily understood how a great deal of confusion has arisen from these earlier studies conducted with impure cultures.

The aerobic phase of cellulose decomposition was further studied by Kellerman, et al., (1913), and McBeth and Scales (1913). Because of the inaccessibility of certain early papers, the following historical background

was obtained from Siu (1951). Omelianski voiced the criticism that the cleared zones on the cellulose-agar plates of McBeth and Scales were due to the dissolution of the carbonates contained in the medium rather than to the decomposition of the cellulose. In 1913 Löhnis and Lochhead, however, proved the zones were due, in part at least, to the decomposition of the cellulose. Hutchinson and Clayton in 1919, and in 1924 Waksman and Heukelekain demonstrated beyond a doubt that certain of Kellerman's organisms and several others were capable of clearing the cellulose from areas of cellulose-agar plates.

The principle contributions toward the understanding of the nature of the enzymic cellulose decomposition process were made from work conducted with species of Cytophaga, Vibrio, and certain of the fungi, principally the Aspergillus. In 1912 Pringsheim, using arresting techniques through heat and antiseptics such as toluene, acetone and iodoform appears to have been the first worker to report on the nature of the enzymes involved in cellulose decomposition. In 1923 Khouvine also worked with the cellulolytic enzymes and concluded on the basis of her inability to obtain cell-free enzyme mixtures that the process must be endoenzymic in nature. This opinion was supported by Meyer who made similar studies in 1943. The next available report was made by Fahraeus (1944), who, working with the Cytophaga, found that the process was apparently hydrolytic since glucose was formed from cellulose. Perlin, et al., (1947), working with mixed cultures of vibrios, found the products of cellulose decomposition to be mainly carbon dioxide, a pigment resembling riboflavin, a bacterial polysaccharide, and traces of acid. Carbon dioxide was found to be essential for cellulose

decomposition, and phosphorylation inhibitors prevented growth on cellulose. Levinson, Mandels, and Reese (1951) concluded that there are two distinct enzyme systems involved in cellulose decomposition. Working with fungi and Cytophaga, they determined that the enzyme, which they labeled C_X, degraded the cellulose into various soluble cellulose derivatives, mainly the sugar cellobiose, and the absorption through the cell membrane takes place, in part at least, at this level. A beta-glucosidase, which is produced only in trace amounts outside the cell, is involved in the further degradation of the soluble-celluloses into glucose. The presence of a cellobiase in the medium is not prerequisite to the utilization of the sugar cellobiose. Whitaker and George (1951), working with various wood-rotting fungi and studying the metabolic pattern of cellulose utilization, found that along with d-mannitol and ethanol, the end-products were mostly mycelium and carbon dioxide. No reference is found as to whether any or all of these metabolic processes are found to exist in the mesophilic, aerobic bacterial form of cellulose degradation. Very little work seems to have been done on this particular type of cellulose decomposition.

Since disagreement on nearly every phase studied with regards to the aerobic, mesophilic bacterial type of cellulose decomposition can be found in the literature, it was with the intent of securing basic information regarding this subject that the following study was made. The work was planned in such a manner that it could be carried on as a continuation of the earlier studies made by Nishio (1952) and would supply the basic information necessary for more precise studies on the cellulolytic enzymes of the mesophilic, aerobic bacteria. This basic part of the work involved

(1) re-examination of cultural techniques, (2) the effects of various carbohydrates and cultural conditions on cellulase production and (3) the preparation and examination of the cellulase-complex and the products of cellulose decomposition.

MATERIALS AND METHODS

Cultures

The bacterial cultures used in these experiments were isolated from soils neighboring Montana State College by Nishio (1952) and were designated by her as 11A, 11C, 2BF, and 2BR. They are mesophilic, facultatively aerobic bacteria, and although attempts were made by Nishio to classify the organisms, nothing definite was determined other than that the organisms were members of the Eubacteriales. No further attempts were made during this study to establish a more limiting classification. The general nature of the organisms is such that optimal growth is obtained in the pH range 7.0-8.0 at a temperature of 35-40 C. Small amounts of beef extract, peptone, or yeast extract were found to be equally suitable in respect to obtaining increased growth and cellulolytic activity. The organisms are apparently of such a nature that they can digest with equal vigor, cuprammonium cellulose, prepared according to Kellerman and McBeth (1912), ball-milled cellulose, prepared according to Hungate (1950), or cellulose precipitated from sulfuric acid solution prepared by the method of Scales (1916).

It was found early in the experiments that the morphology of the organisms and the gram staining reaction, were not reliable criteria upon which to base cultural purity. Both characteristics appeared to depend upon medium composition, age of the culture, consistency of technique and other unrecognized factors. Consequently, cultural purity was based upon repeated subculturing from stock cultures into petri dishes containing cellulose-agar medium and the selection of cellulolytic colonies for

further subculturing. It was found that contaminating forms, which inevitably appear in plating methods, could be readily distinguished, and could be eliminated through careful picking of colonies.

Cultural techniques

Culture media. Stock cultures were carried in brain liver heart (semi-solid) medium where they remained viable for at least 3 months at room temperature. Screw-capped tubes were used to prevent evaporation. Although no closely observed or controlled tests to determine how long the organisms would remain viable on brain liver heart medium were carried out, it was found that the organisms were extremely hardy in respect to temperature variations and desiccation when cultured in this fashion.

Demonstration of cellulolytic activity of the organisms by the plate method was made with McBeth and Scales (1913) cellulose-agar medium with the addition of 0.1 per cent yeast extract. Although originally offered as an enrichment medium for the isolation of cellulolytic soil organisms, it was found to be suitable for routine cultivation. The medium consisted of:

0.1 per cent K_2HPO_4
0.1 per cent $MgSO_4 \cdot 7H_2O$
0.1 per cent NaCl
0.2 per cent $(NH_4)_2SO_4$
0.2 per cent $CaCO_3$
0.5 per cent cellulose
1.0 per cent agar
and tap water.

For those cultures which were liquid, a medium suggested by R. E. Hungate, of Washington State College, for the isolation of organisms from the gut of the termite was used with success. This salt solution is composed of equal parts of:

Solution A:

1.5 per cent $Na_2HPO \cdot 12H_2O$

Solution B:

1.2 per cent NaCl
0.3 per cent KH_2PO_4
0.09 per cent $(NH_4)_2SO_4$
0.04 per cent $MgSO_4 \cdot 7H_2O$
0.03 per cent $CaCl_2$

To this was added an equal volume of a solution containing 0.2 per cent yeast extract and 0.1-0.5 per cent carbohydrate in tap water. The final pH of this medium was about 7.0 without further adjustment. This medium was used in all cases where the cultures were grown for the preparation of enzyme materials. It was found that, although growth did not appear to be as rapid as when the McBeth and Scales medium was used as a liquid medium, the results were more favorable. This was due to the fact that in the absence of the calcium carbonate the disappearance of the cellulose was more easily seen.

Celluloses. Since preliminary experimentation showed not apparent variation when cellulose of different preparations were used, ball-milled cellulose (Hungate, 1950) was used during most of the later work because of the comparative ease with which it could be prepared.

Moist-chamber incubation. Since the incubation time necessary to produce zones of clearing in cellulose-agar plates sometimes extended to several weeks, it was found necessary to store the petri dishes in moist chambers such as coffee cans or other tightly-sealing cans containing clumps of water-soaked cotton. Sterilization between uses is recommended to reduce contamination of the plates with molds.

Methods of enzyme preparation

Quantities of cultures varying from 20 ml to 5 gallons were prepared with the idea of demonstrating the presence of cellulase or other enzymes in the cultural fluid after the disappearance of the cellulose or other substrates. In the first attempts toward demonstrating the presence of cellulolytic materials in the cultures, the results were not at all consistent, since on some occasions the activity could be demonstrated quite readily and on other occasions only after considerable incubation, or not at all. It was found that the concentration of the raw culture was an important factor. Several methods were used to concentrate the mixtures. In some cases evaporation to dryness at 50-55 C in an oven and subsequent rehydration to a reduced volume with distilled water was found to be satisfactory. Although quantitative tests were not conducted, it would appear that the dehydration had little or no effect upon the activity of the cellulolytic complex. In another attempt to prepare large volumes of enzyme concentrate, a vacuum distillation apparatus was employed satisfactorily. The vacuum obtained from a water aspirator was sufficient to maintain boiling at a temperature of 35-45 C.

To prevent overgrowth of contaminating microorganisms, all cultures which were to be tested for the presence of cellulolytic enzymes were treated with toluene. Although other materials could have been used to maintain sterility without the impairment of enzyme activity, it was found that toluene gave satisfactory results and it was used for this purpose throughout the experimentation. After the required incubation period, the cultures could be treated with toluene, placed in a rubber-

stoppered bottle and would remain sterile for long periods of time. If, however, the vessel was opened frequently, or allowed to remain open, care had to be taken to add more toluene periodically. Additional toluene was added after vacuum distillation since the toluene boiled away.

The difficulties encountered during the preparation of large volumes of cultures for the preparation of enzyme mixtures were quite numerous, with maintenance of sterility with a constant bubbling of air through the culture being the most difficult. Five-gallon carboys were employed with an air supply which filtered through an erlenmeyer flask filled with water as finely dispersed bubbles and then was further filtered through a tube packed with sterile cotton.

A rather large supply of moist, sterile air could be obtained in this fashion. Although it is perhaps not necessary, the medium was added at intervals rather than inoculating the entire amount at one time. The purpose of this method was to allow for a buildup of cellulolytic activity, but it also presented another opportunity for contamination. Perhaps the whole amount could be added at once and thereby reduce the possibility of airborne contamination encountered with multiple-opening of the carboys.

Methods of enzyme-effect analysis

Since the products of the enzymic hydrolysis of cellulose are known to contain the reducing sugars glucose and cellobiose, the following methods of testing for their formation from cellulose were established and used routinely.

Five ml portions of toluene-treated samples were placed in standard test tubes with 1 ml of approximately 5 per cent ball-milled cellulose.

The samples were immediately tested to insure the initial absence of reducing substances using Benedict's qualitative reagent in a 2:1 ratio of Benedict's solution to the sample and heating in a boiling water bath for 5 minutes. The formation of a green, yellow, or reddish color with Benedict's reagent was taken as an indication of the presence of reducing substances in the sample. After incubation for 1-6 days at 37 C, a positive test for reducing sugars indicated the action of the enzyme complex. No attempt to measure reducing substances quantitatively was made using this method, but experience permitted a crude evaluation to be made from one day to the next or from one sample to the next.

Where samples containing an objectionable amount of extraneous material were encountered, centrifugation prior to testing produced better results. Fehling's test was compared with Benedict's solution, but was found to be less sensitive. Benedict's solution was found to give recognizable reactions with as little as 0.01 per cent reducing substance in the form of glucose.

Analysis for glucose in the presence of cellobiose was done manometrically using a commercial glucose-oxidase catalase mixture, Deoxygenase, a product of Takamine Laboratories, Inc., and was obtained through the generosity of Dr. Leo Kline, Western Regional Research Laboratories, Albany, California. The glucose-oxidase was freshly prepared for each determination. A 16 mg per ml concentration was used in a sodium acetate buffer solution with a pH of 5.5. In relation to the specificity of the reaction achieved with glucose-oxidase, Keilin and Hartree (1948) reported that in relation to a glucose value of 100, the value of cellobiose is

0.06. Although the values achieved in this experiment were higher than that, i.e. 4.1 and 4.4, the reaction was judged as satisfactory for the qualitative determination of sugars formed by the action of cellulolytic enzyme preparations. Also the cellobiose used in the experiment might have contained small amounts of glucose. Mann (1946) and Blakley (1951) reported the successful use of glucose-oxidase preparations for the qualitative-quantitative determinations of sugars in biological systems.

Where qualitative sugar determinations were conducted by the use of the osazone test, the method of Hassid and McCready (1942) was followed. The identification of the sugars was based on the observation of crystalline osazones and their solubility in hot solutions. Although the reaction is not always an absolute test of the identity of a sugar, as a number of sugars yield the same osazone, the reaction is specific enough to differentiate between cellobiose and glucose.

EXPERIMENTAL RESULTS

Re-examination of Cultural techniques and methods

Although the four cultures: 11A, 11C, 2BF, and 2BR had demonstrated the ability to decompose cellulose previous to this experimentation, the preliminary work consisted of subculturing the organisms on McBeth and Scales cellulose-agar medium in attempt to establish culture purity and to become familiar with cultural techniques. It quickly became evident that the organisms were polymorphic and gram variable.

The varieties of forms which were present after growth on Hungate's basal medium with various carbohydrates added are shown in Table I. It can be seen that with such variations in form and staining reaction these characteristics could not be depended upon as criteria of culture purity. Since, by definition, the organisms were mesophilic, aerobic and cellulolytic, it was concluded that the only sound criteria must be based upon these characteristics with the added consideration that the organisms must also be capable of utilizing cellobiose or glucose as had been demonstrated by Nishio (1952). Therefore, cultural purity was established on the basis of the organisms forming only one colonial type when grown on cellobiose- or glucose-containing medium and their subsequent growth with cleared zones on cellulose-agar medium.

When it was considered that the cultures were pure, experimentation was begun to determine the nature of the enzymes involved in the cellulolytic process by growing large volumes of the bacterial cultures. After an unsuccessful attempt to cultivate the organisms in a fluid medium consisting of the basal portion of McBeth and Scales medium, Hungate's basal-salts medium with 0.1 per cent yeast extract and 0.1 per cent sugar was

Table I

Morphological and staining reaction variations after growth for three days in carbohydrate-containing media.

Organism	Carbohydrate	Gram Reaction	Morphology
11A	Glucose	variable	Rods in chains and single; cocci in chains.
11A	Cellobiose	"	Rods of varying size.
11A	Ball-milled cellulose	"	Diplococci
11A	Scales cellulose	"	Very short rods
11C	Glucose	"	Short rods; chains
11C	Cellobiose	"	Short rods
11C	Ball-milled cellulose	"	Short, thick rods
11C	Scales cellulose	"	Long, filamentous rods
2BF	Glucose	"	Fat, short rods
2BF	Cellobiose	"	Extreme variation
2BF	Ball-milled cellulose	"	Long, filamentous rods
2BF	Scales cellulose	"	Short rods
2BR	Glucose	"	Short to medium rods
2BR	Cellobiose	"	Short rods
2BR	Ball-milled cellulose	"	Short to filamentous rods
2BR	Scales cellulose	"	Long, very thin rods

used successfully. Following the initial inoculation of 5 ml, additional medium was added after 6, 10, and 12 days' incubation to allow for any increase in cellulolytic activity that might take place. The final volume was kept at 20 ml, which in 150 ml erlenmeyer flasks would allow for a large surface of the medium to be in contact with the air. The activity of the cultures was stopped by the addition of 2 ml of toluene after a total incubation period of 15 days. At that time there was no visible evidence of fibrous cellulose in the medium. Five ml portions of the cultures were transferred to test tubes and 1.0 ml of approximately 5 per cent Scales cellulose were added. The mixture was tested with Benedict's solution for the presence of reducing substances at the beginning of the experiment and at intervals of approximately 24 hours thereafter. The test was negative at the beginning of the experiment, but after 5 days at 37C positive reactions were obtained showing that reducing substances had been formed. Thus, cellulase activity could be demonstrated by this method.

Effect of cultural conditions on cellulase production. Because of the long incubation period necessary to produce cellulase-containing material from cultures grown on cellulose and the inevitable residual material encountered that made it impossible to obtain clear solutions of cellulase-containing material, the cultures were grown on cellobiose and glucose to determine if a more rapid cellulase production could be achieved. It was believed that, if cellulase were a constitutive enzyme it would be formed under these conditions. An experiment similar to the previous one was set up. Each organism was inoculated into Hungate's basal medium to which had been added glucose, cellobiose, ball-milled cellulose, and Scales cellulose

respectively. Additional medium was added at intervals and the incubation period was terminated by the addition of toluene when the cellulose had disappeared from the flasks to which it was added. Toluene and Scales cellulose were again added and the test for cellulase activity started. Although the time interval necessary to produce detectable amounts of reducing substances varied for the different substrates, all the cultures became positive within 6 days. Similar portions to which ball-milled cellulose was added after toluene treatment gave similar results. Apparently the enzyme-complex is equally adapted to action upon either type of cellulose. This experiment demonstrated that the cellulolytic enzyme-complex was constitutive in nature rather than adaptive. Since these results are contrary to the generally accepted belief (Siu, 1951) this experiment was repeated 3 times with similar results on each occasion. Although cellulase produced by growth on cellobiose and glucose has not been used for further work in these studies, it appears to be a valuable tool for future investigation.

Preparation and examination of cellulase

Mass cultures for enzyme production. In view of the previous results which showed that the enzyme activity could be demonstrated readily, and that concentration of the enzyme-complex was an important factor controlling the measurements of its activity, large volumes of cultures became necessary so that concentrated solutions could be obtained. Five-gallon carboys to which 2 liters of Hungate's medium containing ball-milled cellulose had been added were sterilized and inoculated with cultures 11A and 11C and incubated at 35 C. Large cultures of 2BF and 2BR were not

prepared. The carboys were shaken frequently and more medium was added when it was noted that the visible cellulose had nearly disappeared. The decomposition became more rapid after the first several days and larger amounts of medium could be added at each interval. When the carboys became approximately one-half full, the decomposition became less rapid and appeared to cease altogether. Attempts to stimulate further decomposition by the addition of more yeast extract failed. Although previous trials had shown that aeration did not markedly speed up decomposition, compressed-air was bubbled through the carboys. The air was rendered sterile by passage through a sterile, cotton-packed tube approximately 50 cm in length. A manifold was arranged to provide air for the several flasks. The decomposition did not proceed noticeably faster, and after a number of days, it became evident that the cellulolytic process was not progressing, therefore the action was stopped by the addition of toluene. Tests were conducted to determine the presence of cellulolytic-enzyme activity. The activity was found to be very slight.

It was noted that a precipitate appeared when the cultures were allowed to stand. Previous experiments had indicated that in centrifuged samples the precipitate possessed slightly greater cellulolytic activity than the supernatant. This led to an investigation of the cellulolytic activity of the various portions of these mass cultures. The gravity-precipitated portions of the cultures showed a markedly greater activity than the supernatant fluid.

By careful siphoning the supernatant fluid was removed from the carboys into a vacuum distilling apparatus and concentrated to approximately

one-tenth its original volume. The concentrated portion showed an increased cellulolytic activity. Both the precipitated and the condensed portions were placed in tubes and frozen for future work. Earlier findings had shown that freezing did not destroy the cellulolytic activity of the preparations.

pH. Since previous work on the effect of pH on the activity of the enzyme complex was studied only by the formation of reducing substances as indicated by the Benedict's test, it was thought that, in view of the variations shown, a more precise determination using more accurate methods might be of value. To do this, precipitated material from the mass-culturing of 11A, which had previously shown a high degree of cellulolytic activity, was arranged in 5 ml portions and adjusted, in increments of 0.5 to pH values ranging from 4.5 to 8.0 with a Beckman pH meter. Ball-milled cellulose and toluene were added and the cultures placed in a 37 C incubator. Benedict's tests showed the samples to be free or reducing substances at the beginning of the test period. Daily tests were made to determine the amount of reducing substances present, and to readjust the pH where necessary. After 4 days the Benedict's tests showed the samples to contain rather large amounts of reducing substances. The samples were centrifuged and 0.1 ml of each removed from the supernatant for testing. A Folin-Wu test for reducing sugars was performed on the samples. Figure 1 represents pH values plotted against color intensity recorded as mg glucose equivalents. The results show an optimal cellulase activity at a pH of 5.5 which agrees with the results reported by Siu (1951) on the cellulases of some of the fungi, the snail and some other cellulolytic bacteria.

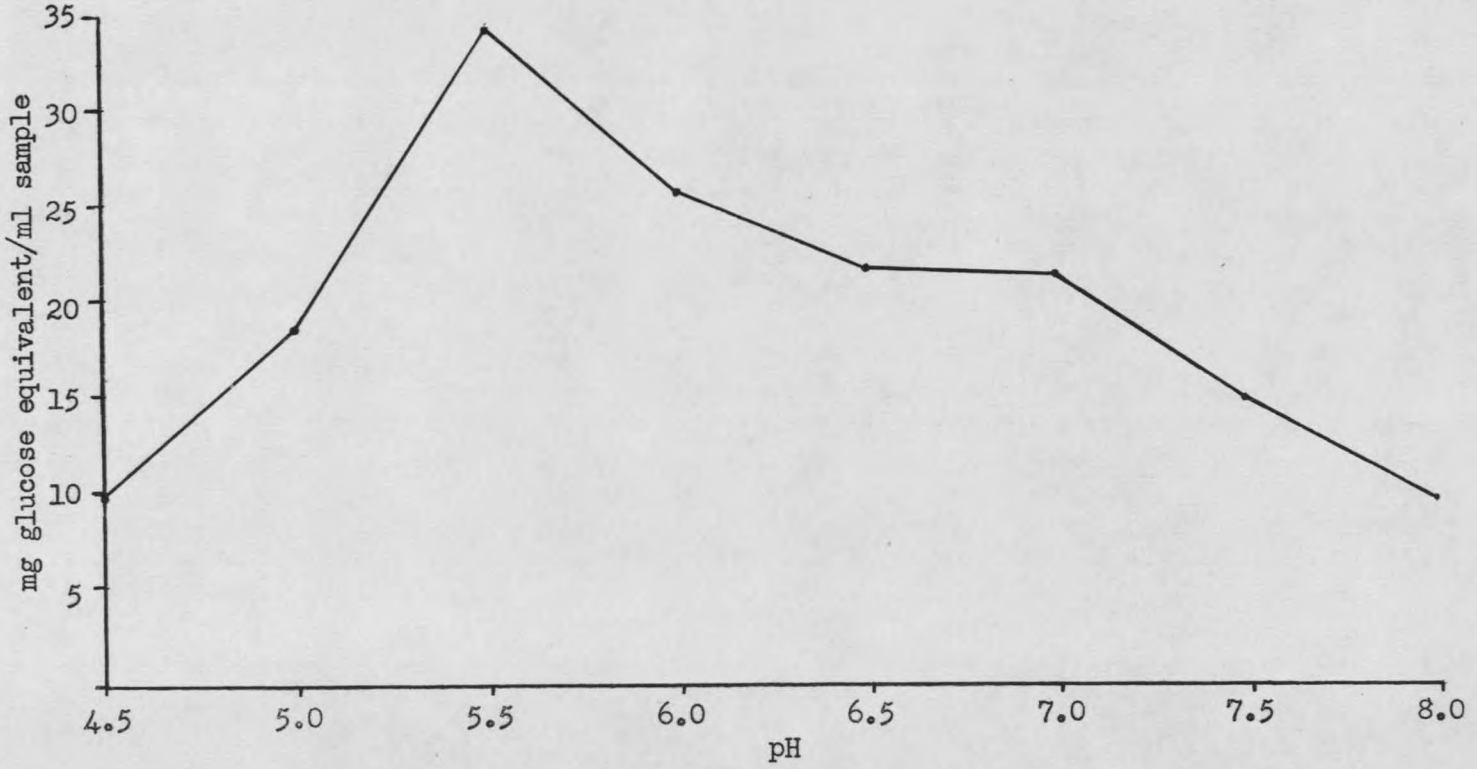

figure 1. The effect of pH on cellulase activity

Temperature effects on cellulase complex. Although the effects of various temperatures were not studied precisely, it became necessary in the course of this investigation to determine how the enzyme-containing mixtures could be stored for future work.

Cellulase-containing samples were obtained from the mass culture of 11A. Five ml samples to which were added one ml of toluene, were stored at temperatures of -20, 5, 25, 45, 50, and 68 C. The frozen sample was "quick" frozen in a deep-freeze unit and allowed to thaw slowly to room temperature. At the end of a 3 day storage period, one ml of approximately 5 per cent ball-milled cellulose was added to each sample and the samples were incubated at 37 C. Daily tests for reducing substances were performed using Benedict's solution. All the samples except the one stored at 68 C contained a large quantity of reducing substances after four days incubation. It appears that the cellulase is stable at ordinary temperatures, but is inactivated at temperatures much above 50 C.

Desiccation effects on cellulase complex. In the earlier attempts at concentrating the cellulolytic complex, 5 ml of each culture were placed in evaporating dishes and allowed to completely dry at 50-55 C. Samples of this material, rehydrated after nearly a year of storage at room temperature, showed considerable activity. Apparently the cellulolytic complex will remain active for long periods of time when stored in this fashion. Samples which were desiccated at 68 C failed to show activity within several hours after the desiccation was complete.

Products of cellulose hydrolysis

Manometric sugar identifications. Since the degradation of the cellulose

is believed to follow, in part at least, a hydrolytic pathway with the formation of cellobiose and, in some cases, glucose. An experiment was conducted to determine to which level of degradation the cellulolytic complex under study carried the substrate. To do this it became necessary to formulate a method by which cellobiose could be distinguished from glucose, since the commonly-used methods (Folin-Wu, Fehling's, Benedict's) are tests for reducing substances and cellobiose and glucose are both reducing sugars. This differentiation was accomplished by using an enzyme, glucose-oxidase, which attacks glucose and not cellobiose. The high degree of specificity of this enzyme makes it adaptable for determining the presence of glucose in a mixture of sugars.

A Warburg manometric analysis was performed using cultural preparations which were known to contain reducing substances formed by the cellulolytic process. Duplicate samples of glucose (2 mg per ml), culture containing formed reducing substances, and cultural material not allowed to act upon cellulose were run. One ml of each was placed into a Warburg vessel, along with 1.0 ml of sodium acetate buffer and, 0.5 ml of a glucose-oxidase preparation (Desoxygenase) in a concentration of 16 mg per ml was added to each vessel sidearm. Air was used as an atmosphere over the materials. After equilibration the substrate in the sidearms was dumped and manometric readings were made until equilibrium was again reached. The glucose-oxidase oxidizes glucose to gluconic acid with the uptake of 62.2 microliters of oxygen per mg of glucose. The results of this experiment are presented in Table II. Since the results showed no increase in glucose content of the culture after cellulase activity, it appeared that

Table II

Amount of glucose formed by cellulase-complex activity on cellulose

Substrate	Micro-liters O_2 consumed	Per cent efficiency	mg glucose
Glucose.....	111.6	89	2.0
	109.7	89	2.0
Culture plus reducing material.....	19.5	-	0.35
	14.3	-	0.25
Culture not allowed to act on cellulose.....	22.1	-	0.39
	26.4	-	0.45

the reducing substance formed by the action of the enzyme in this experiment was not glucose.

Manometric determinations, in order to identify the sugars formed at each pH level, were also made using the samples which were adjusted to pH values 5.5 and 7.0 and used to determine the effect of pH on cellulase activity. The samples were centrifuged and two 0.1 portions were removed from each sample. To one 0.1 ml portion at each pH value was added 0.3 ml of 3 N HCl. The acidified portion was heated in boiling water for 45 minutes to hydrolyze any cellobiose in the sample to glucose. After hydrolysis the acid was neutralized to the red range of phenol red with sodium hydroxide, and all four samples were diluted to a volume of 2 ml with sodium acetate buffer pH 5.5. As a comparison, glucose determinations were run in duplicate in conjunction with the sample determinations. To the main part of each of 4 Warburg vessels was added 1.0 ml of sample and 1.0 ml sodium acetate buffer solution. 0.5 ml of a glucose-oxidase (16 mg per ml) in buffer solution was added to each sidearm. The atmosphere was air and the temperature of the water bath was 37 C. After equilibration the sidearms were dumped and recordings made until equilibrium had again been reached. It is evident (Table III) that any significant difference in the amount of glucose in the hydrolyzed and un-hydrolyzed samples would be caused by the hydrolytic process, and an increase in glucose content during hydrolysis would indicate that the reducing substance formed as a result of the cellulolytic process was probably cellobiose. The results indicate that cellobiose was the sugar which accumulated in the samples.

Table III

Effect, as glucose formed, of acid hydrolysis on reducing substance formed by cellulase activity

Substrate	Micro-liters O_2 consumed	Per cent efficiency	mg glucose
Run 1			
Glucose control.....	59	94	1.0
	57	92	1.0
Hydrolyzed samples from pH 5.5	9	-	0.16
	15	-	0.22
Hydrolyzed samples from pH 7.0.....	10	-	0.16
	5	-	0.10
Run 2			
Glucose control.....	41	86	-
	64	88	-
Non-hydrolyzed sample pH 5.5	4	-	0.06
	1	-	0.02
Non-hydrolyzed sample pH 7.0	6	-	0.10
	6	-	0.10

Osazone preparations. In conjunction with the manometric qualitative sugar determinations it was thought that further identification of the products of cellulase activity could possibly be obtained through the use of the osazone test. Portions of the same preparations used for manometric determinations were used in this experiment. One ml of culture was mixed with 0.15 gm sodium acetate and 0.1 gm phenylhydrazine hydrochloride and heated in boiling water for 30 minutes. Microscopic examination of the resultant material failed to show glucosazone crystals in any case. Crystals similar to cellobiosazone were observed. The crystals dissolved when the tubes were again placed in boiling water, a property of cellobiosazone. Glucosazone is not redissolved by heating to the temperature of boiling water. Thus it appears that in verification of previous results the product formed by the cellulolytic process is cellobiose.

DISCUSSION

Although experimentation has of necessity been of a general nature up to this point in the study, certain information concerning the morphology, cultural characteristics, and properties of the cellulase-complex as well as the products formed by its action have been obtained.

The complex morphological situation encountered in these organisms would tend to support the concept of Nishio (1952) that the organisms were of the genus Corynebacterium since polymorphism is encountered in members of this group. It would be difficult to say at this time just what factor or factors control the morphological state or staining reaction of the organisms.

Although the method of pure culture study utilized in this work, i.e. growth of only one colonial type, all members of which cleared zones on cellulose-agar plates subsequent to growth on cellobiose- or glucose-containing medium, leaves much to be desired, it was the only reasonable method available at the time the investigation was made.

The cellulase appears to be exoenzymic since it decomposes cellulose at some distance from the colony. Although there has been controversy over this subject, this seems to offer the simplest explanation and no good evidence to the contrary has been offered.

Apparently the types of cellulose offered for decomposition play no great part in the cellulolytic process since the results were similar on all types tested. This situation is not surprising in view of the many states in which cellulose is encountered in nature.

The production of cellulase was not found to be dependent upon the presence of cellulose, but rather was also produced when the organisms were grown on glucose and cellobiose, the two most probable intermediates in cellulose hydrolysis. Cellulase has been thought to be an adaptive enzyme in those organisms which are not obligately cellulolytic, however it is difficult to understand how a material of such low solubility and high molecular weight as cellulose could influence the production of cellulolytic enzymes within the cell. In view of the results obtained in this investigation, the cellulase appears to be constitutive and is elaborated in larger quantities in response to the hydrolytic products of cellulose. The results establish the fact that cellulose is not necessary for the production of cellulase.

The cellulase produced by these organisms is a very stable enzyme, since it will withstand temperatures up to 50 C, complete drying, and storage at room temperature for long periods of time. These are common characteristics of other hydrolytic enzymes and make the cellulase a convenient enzyme for study. Although the optimal pH for its activity was found to be 5.5, this is not the pH for optimal growth of these organisms. This situation has been found to exist with the cellulases elaborated by other cellulolytic organisms.

Although it has been possible to demonstrate the action of a cellulase which hydrolyzes cellulose to cellobiose, a cellobiase which hydrolyzes cellobiose to glucose has not been found. This failure may be due to an inactivation of the cellobiase by the methods used, but this does not seem likely in light of the known stability of emulsin and other

beta-glucosidases. The organisms may utilize cellobiose directly without a conversion to glucose per se, as has been demonstrated by Hungate (1944) with Clostridium cellobioparus. It may be that the cellobiose is endocellular and is inactivated by the disintegration of the cells by toluene. However, one would not expect this inactivation to be immediate or complete upon cellular destruction. The utilization of cellobiose without a demonstrable cellobiase leaves the utilization mechanism in doubt. Several possibilities might be concerned with this step: (1) the mechanism could be phosphorolytic with the formation of non-reducing substances, or (2) the mechanism could have formed glucose which was immediately degraded to some non-reducing substance. A similar situation has been found to exist in the case of the thermophilic bacteria (McBee, 1948) which can attack cellobiose but not glucose. (3) There may be other unrecognized methods of utilization.

SUMMARY

Four mesophilic, facultatively aerobic bacteria of the order Eubacteriales were studied in respect to cultural methods, pure culture techniques, preparation of cellulolytic material, and examination of the cellulolytic process. The organisms demonstrated extreme polymorphism and variation in staining reaction. A method of pure cultural study involving growth on cellulose agar medium is presented.

Types of cellulose apparently have little or no effect upon the production of cellulase since its action could be demonstrated independently of living cells after growth on any of the types tested. The cellulase-complex is apparently constitutive and not adaptive in nature since cellulase activity could also be demonstrated in cultures grown on cellobiose and glucose.

The influence of pH on cellulolytic activity was studied and found to have a considerable effect, with the greatest activity being exerted at pH 5.5.

The effect of various temperatures on the cellulase complex is discussed. Acceptable storage temperatures include -20, 5, and 25 C. A few days at 68 C inactivated the cellulase complex. Desiccation, accomplished at 50-55 C did not inactivate the cellulase, but desiccation at 68 C did.

Methods are discussed for the production of large volumes of enzyme-containing material. Concentration can be accomplished successfully either by vacuum distillation or by evaporation at controlled temperatures.

Several aspects of the bacterial cellulolytic process and the utilization of cellobiose without a demonstrable cellobiase are presented. These mechanisms may be phosphorolytic or unrecognized in nature.

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