PREPARATION AND PROPERTIES OF THE AMYLASES PRODUCED BY BACILLUS MACERANS AND BACILLUS POLYMYXA[1]

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The history of the *Aerobacillus* group has been reviewed by Porter, McCleskey, and Levine (1937), who compared all the available "species" and concluded that there were really only two, *A. macerans* and *A. polymyxa*; of the five earlier recognized by Donker (1926), two, *A. violaris* and *A. amaracrylus*, were no longer available for study, and a third, *A. acetoethylicus* Northrop, could not be differentiated from *A. macerans*. Porter and his co-workers agreed with Donker that *A. asterosporus* was identical with *A. polymyxa*. They accepted Donker's description of the genus *Aerobacillus* which has priority over that of Pribram (1933) and includes spore-forming rods which grow aerobically and anaerobically and decompose a great number of carbohydrates, including starch and glycogen, with production of carbon dioxide and hydrogen. The *Aerobacillus* group has now been made a subgenus of the genus *Bacillus* (1939), hence the latter is the correct genus name.

Bacillus macerans and *B. polymyxa* are very similar morphologically and physiologically and have similar habitats, occurring in decaying vegetables and in soil and water. Porter, McCleskey, and Levine differentiated them in four ways: (1) by their behavior in the Voges-Proskauer test, in which *B. macerans* gave a negative and *B. polymyxa* a positive reaction, (2) by growth on media containing rhamnose and sorbitol, in which *B. macerans* produced acid and gas, while *B. polymyxa* did not, (3) by their optimal temperature range, which was higher for the *B. macerans* type, and (4) by serological reactions, *B. macerans* being a homogeneous antigenic type as distinct from the heterogeneous *B. polymyxa* type.

[1] Contribution from the Division of Chemistry, National Institute of Health, U. S. Public Health Service.

Both bacteria are recorded as starch-hydrolyzers (Bergey) but the striking action of *B. macerans* is its conversion of starch to nonreducing crystalline substances. These were described by the discoverer of *B. macerans* and are referred to for that reason as the Schardinger dextrins (Schardinger, 1905, 1908–9). They have been studied by a number of chemists in connection with investigations of the structure of starch (Pringsheim and Langhans, 1912; Pringsheim and Eissler, 1913; Karrer and Nägeli, 1921; Freudenberg and Jacobi, 1935; Freudenberg and Meyer-Delius, 1938). Schardinger also observed the formation of acetone by *B. macerans*, and a number of reports have been published regarding the practical production of acetone and the mechanism of its formation (Northrop, Ashe and Senior, 1919; Northrop, Ashe and Morgan, 1919; Arzberger, Peterson and Fred, 1920; Peterson, Fred and Verhulst, 1921; Fred, Peterson and Anderson, 1923; Speakman, 1925; Bakoṇyi, 1926; Zacherov, 1930). Kluyver and van Niel (1926) found that *B. polymyxa* produced butylene glycol instead of acetone.

The experiments reported here were the logical sequel to the finding that the formation of crystalline dextrins from starch by *B. macerans* is due to an enzyme separable from the bacteria (Tilden and Hudson, 1939). Studies of the optimal cultural conditions for production of the enzyme and of the optimal pH and temperature for enzyme activity were essential for effective use of the enzyme. The action of *B. polymyxa* on starch also proved to be due to an enzyme present in bacteria-free filtrates of cultures. The *B. polymyxa* amylase was quite different from the *B. macerans* enzyme, not only in its action on starch, which it converted to reducing substances, but also in the conditions of temperature and hydrogen ion concentration required for optimal activity. Because of convenience of preparation, simplicity of culture medium, and relative stability, the *B. polymyxa* enzyme may well prove to be a useful amylase.[2]

[2] Study of several other bacteria known to decompose starch (*Bacillus subtilis*, *B. vulgatus*, *B. niger*, *B. aterrimus*) revealed that they also elaborate amylases. These were of the *polymyxa* type, forming reducing substances but no crystalline dextrins.

ORIGIN OF CULTURES

We were fortunate in securing a large number of cultures for study: the Schardinger culture of *B. macerans*, Northrop's

TABLE 1

Source, cultural reactions, and enzyme activity of cultures of B. macerans

NO.	SOURCE	FERMENTATION OF RHAMNOSE*	FORMATION OF ACETYL METHYL CARBINOL (V.P. TEST†)	IODINE TEST FOR CRYSTALLINE DEXTRINS	UNITS OF ENZYME IN 1 ML. 3-WEEKS OLD CULTURE‡
843	Am. Type Cult. Coll. (1934 catalog)	+	−	+	1.0
277	N. R. Smith (Christensen's 8275)	+	−	+	3.0
278	N. R. Smith (Northrop's *acetoethylicus*)	+	−	+	2.0
573	M. Levine	+	−	+	2.0
577	M. Levine	+	−	+	2.0
583	M. Levine	+	−	+	3.0
585	M. Levine	+	−	+	0.5
588	M. Levine	+	−	+	3.0
18	Isolated from fractionally sterilized potato	+	−	+	2.0
2037	Isolated from fractionally sterilized potato	+	−	+	2.0
888	N. R. Smith (isolation)	+	−	+	3.0
646	N. R. Smith (*B. nigrificans* Cameron)	+	−	+	1.0
649	N. R. Smith (*B. nigrificans* Cameron)	+	−	+	0.5

* One per cent rhamnose agar slants containing Andrade indicator and made with a long butt. The slants were inoculated with a platinum needle, which was also used for stab inoculation of the butt.

† The medium was made with sodium chloride in place of phosphate (N. R. Smith, J. Bact., **39**, 757, 1940) and the test made with O'Meara reagent (Levine, Epstein, and Vaughn, Am. J. Pub. Health, **24**, 505–510, 1934).

‡ One unit is the amount of enzyme required to convert 30 mg. of starch in 30 minutes at 40° to the "brown-violet stage" (see text). The figure in each instance represents the maximal value obtained with a given strain.

B. acetoethylicus, and several cultures of *B. macerans* and *B. polymyxa* isolated from soil, water supplies, and decaying vegetables.[3] In the course of the present research, two cultures of

[3] Grateful acknowledgement is hereby made to Prof. Max Levine, of Iowa State College, and to Dr. N. R. Smith, of the Bureau of Plant Industry, U. S. Dept. of Agriculture, through whose courtesy most of the cultures were obtained.

B. macerans were isolated from potatoes. The data regarding sources, cultural reactions, and enzyme production of the various cultures are recorded in table 1 (*B. macerans* cultures) and table 2 (*B. polymyxa* cultures). The iodine test for crystalline dextrins (Tilden and Hudson, 1939) served as a convenient means of differentiation and was confirmed by the rhamnose and Voges-Proskauer tests and by serological reactions.

TABLE 2

Source, cultural reactions, and enzyme activity of cultures of B. polymyxa

NO.	SOURCE	FERMENTATION OF RHAMNOSE*	FORMATION OF ACETYL METHYL CARBINOL (V.P. TEST†)	IODINE TEST FOR CRYSTALLINE DEXTRINS	UNITS OF ENZYME IN 1 ML. 3 WEEKS OLD CULTURE‡
251	N. R. Smith (*B. asterosporus*)	−	+	−	3.0
252	N. R. Smith (isolation)	−	+	−	2.5
279	N. R. Smith (isolation)	−	+	−	4.0
280	N. R. Smith (isolation)	−	+	−	1.7
293	N. R. Smith (isolation)	−	+	−	0.8
297	N. R. Smith (isolation)	−	+	−	0.66
354	N. R. Smith (McCoy)	−	+	−	10.0
812	N. R. Smith (*asterosporus*)	−	+	−	1.0
813	N. R. Smith (*asterosporus*)	−	+	−	6.0

* See footnote *, table 1.

† See footnote †, table 1.

‡ One unit is the amount of enzyme required to convert 30 mg. of starch in 30 minutes at 40°C. to the disappearance of violet color with iodine (see text). The figure in each instance represents the maximal value obtained with a given strain.

Certain *B. macerans* cultures have proved to be consistently better enzyme producers than others, notably Nos. 583 and 588 from Levine, and Christensen's No. 8275. The Schardinger culture (No. 843, American Type Culture Collection) was one of the least active cultures biochemically. The use of the more active cultures in the production of crystalline dextrins doubtless explains why we (McClenahan, Tilden and Hudson in preparation) have obtained yields of 50–60 per cent as compared with the 25–30 per cent reported by Schardinger and others.

The *B. polymyxa* cultures showed a similar variation in their

ability to produce enzyme, No. 354 being outstanding in its activity.

CONDITIONS FOR DEVELOPMENT OF ENZYMES IN CULTURES

The presence of *B. macerans* amylase was first observed in cultures grown on potato, which was added in 10–20 gram portions to 100 ml. of calcium carbonate suspension. This medium was similar to the one used by Schardinger (1905, 1908–9). Addition of starch to the ordinary laboratory media (beef-infusion broth, peptone water) did not render them favorable for production of *B. macerans* enzyme, though the *B. polymyxa* enzyme formed abundantly on a simple starch peptone medium.

Potatoes varied considerably in their suitability, chiefly with age. No appreciable amount of *B. macerans* enzyme developed on media made with new potatoes, but excellent enzyme solutions were secured from cultures grown on a medium prepared with potatoes which had been stored for a year or more but were free from rot. The different portions of the tuber were equally favorable, even the sprouts, and no differences could be detected among varieties:[4] Idaho, Irish Cobbler, Katahdin, Green Mountain, and Georgia Triumph. Sweet potatoes and carrots, which are similar to potatoes in their ash constituents and quantity of protein (Sherman, 1926; Winton and Winton, 1935) apparently furnished little or none of the substances needed by *B. macerans* for amylase production (table 3). The best culture medium found for this organism was, because of its constancy and ready availability, rolled oats. This medium could be used also for production of *B. polymyxa* amylase, especially if fortified with additional starch, but was less favorable than the peptone-starch medium. Table 4 records a comparative experiment on the cultural conditions for the production of the two enzymes.

The presence of one of the less soluble alkaline earth carbonates (calcium or strontium) was essential as a buffer. The buffer mechanism was studied in detail in the case of *B. macerans*,

[4] Through the kindness of Dr. F. J. Stevenson, of the U. S. Horticultural Station in Beltsville, Md., samples of potatoes were obtained representing different varieties and different ages (time after harvesting).

and the results are summarized in table 5. The best conditions were provided by a buffer which gave an initial pH of 6.8–7.0 and prevented a fall below 6.6. Barium carbonate, which is

TABLE 3

Conditions for development of B. macerans enzyme in cultures

INOCULUM	200 ML. MEDIUM BUFFERED WITH 2 PER CENT CALCIUM CARBONATE		pH*		MAXIMAL ENZYME VALUE§ UNITS IN 1 ML. CULTURE 3 WEEKS OLD
			Initial	Final	
		per cent			
2 ml. pooled cultures 25 days old	White† potato	10.....	7.6	7.2	2.0
		20.....	7.5	6.8	2.0
		40.....	7.4	6.6	2.0
	Sweet† potato	10.....	7.1	7.2	0.3
		20.....	7.0	7.2	0.1
		40.....	6.8	7.2	0.1
	Carrot†	10.....	7.1	7.2	0.2
		20.....	7.0	6.8	0.2
		40.....	6.8	6.6	0.2
	Rolled oats	2.....	7.4	7.2	1.7
		4.....	7.4	6.6	2.0
	Cornmeal, yellow‡	2.....	7.3	6.8	0.5
		4.....	7.3	6.3	0.5
	Cornmeal, white‡	2.....	7.4	6.4	1.0
		4.....	7.2	6.3	0.6
	Rolled oats buffered with M/5 phosphates	2.....	7.3	6.5	0.5
		4.....	7.3	6.5	0.5

* Determined electrometrically.

† Percentages given are on the basis of fresh (wet) tissue.

‡ The white cornmeal used was more finely ground than the yellow.

§ See footnote ‡, table 1 and table 2.

more soluble than calcium or strontium carbonate and gave a higher final pH, was less favorable. Magnesium carbonate (basic) inhibited enzyme formation altogether; the pH remained at about 8.0; growth was not inhibited. In order to maintain

TABLE 4

Comparison of conditions for development of enzymes of B. macerans and B. polymyxa

Maximal enzyme value (units in 1 ml.) of cultures on various media*

	CULTURE	2% STARCH 1% PEPTONE	10% FRESH POTATO†	5% ROLLED OATS		5% CORNMEAL	
				No added starch	+1% starch	No added starch	+1% starch
B. polymyxa	251	1.3	1.5	2.0	2.0	1.5	2.5
	252	2.5	1.5	1.5	2.0	1.0	1.5
	354	10.0	0.6	3.0	6.0	3.0	6.0
B. macerans	277	No appreciable enzyme formed	1.5	3.0	2.0	1.0	0.75
	583		0.5	3.0	3.0	1.0	1.0
	588		1.5	1.5	1.0	1.0	1.0
	18		0.75	1.5	1.0	1.0	1.0
	2037		0.25	2.0	2.0	0.4	0.25

* See footnote ‡, table 1.

† Percentage calculated on the basis of fresh (wet) tissue.

TABLE 5

*Effect of buffer substances on development of enzyme in cultures of B. macerans**

25 GM. OF FRESH POTATO IN 250 ML. OF	pH†		MAXIMAL ENZYME VALUE‡ UNITS IN 1 ML.
	Initial	Final	
Water	6.1	5.6	0.1
0.5% calcium carbonate	6.7	5.8	0.5
1.0% calcium carbonate	6.8	6.2	1.5
2.0% calcium carbonate	7.0	6.6	3.0
4.0% calcium carbonate	7.0	6.8	2.0
2.0% strontium carbonate	7.0	6.6	3.0
2.0% barium carbonate	7.0	6.8	1.5
2.0% magnesium carbonate (basic)	8.5	8.5	None
M/5 phosphate buffer	7.0	6.3	0.66
M/5 phosphate buffer	7.3	6.9	0.66
M/1 phosphate buffer	7.0	6.6	None
M/1 phosphate buffer	7.3	6.9	None

* The inoculum in this experiment was 1 ml. of a 24-day-old culture of *B. macerans* No. 18, grown on potato medium, buffered with calcium carbonate.

† Determined electrometrically.

‡ See footnote ‡, table 1.

the optimal pH range with acetate or phosphate buffers in media prepared with oatmeal, cornmeal, or potato, it was necessary to

use a concentration of M/5 to M/1, which was apparently unfavorable to enzyme production by *B. macerans*.

The development of *B. macerans* enzyme was most rapid at 37.5° to 40°. It formed slowly at 30°, but none was detected in cultures kept at 20°, even after many weeks. Temperatures above 42° were definitely inhibitory to enzyme formation and growth. The *B. polymyxa* amylase developed abundantly at room temperature (20–25°); its formation was not inhibited, however, at 37.5°, and this temperature proved satisfactory for stock cultures of both organisms.

The period of active growth and fermentation precedes that of maximal enzyme formation, which is not attained until after gas production has practically ceased. The enzymes are presumably liberated as the aged bacterial cells autolyze. The period required for maximal enzyme development averages three to four weeks, but occasionally a high value will be reached in 10–14 days. Probably none of the media used contain optimal amounts of the substances which favor enzyme production, since the enzyme content tends to be less after the cultures have been grown for many generations on the same medium; it is possible to improve the yield, then, by transferring oatmeal cultures to potato slants for a generation or two, or *vice versa*. An enzyme value of 1 unit per milliliter has been obtained on a simple mineral-starch medium[5] when each liter of medium received an inoculation of 5 ml. from an oatmeal culture of one of the more active *B. macerans* cultures (277, 888); even this relatively small amount of inoculum carried over sufficient of the essential substances from the oatmeal to permit one generation of growth and enzyme formation.

Once formed, the enzymes are remarkably stable. Even at incubator temperature, the enzyme value of a pure *B. macerans* culture may remain unchanged for many days, and at 5° it is stable for at least a year, irrespective of whether or not it has been separated from the bacteria. Contamination with other bacteria, as would be expected, reduces the enzyme value, and grossly contaminated cultures may contain no enzyme at all.

[5] One per cent soluble starch containing 0.1 per cent NaCl, 0.1 per cent $MgSO_4$, 0.2 per cent $(NH_4)_2SO_4$ and 1 per cent $CaCO_3$.

PROPERTIES OF THE ENZYMES

For determining the optimal range of temperature and pH for activity of the enzymes, solutions purified by acetone precipitation were used; the *B. polymyxa* enzyme was precipitated by

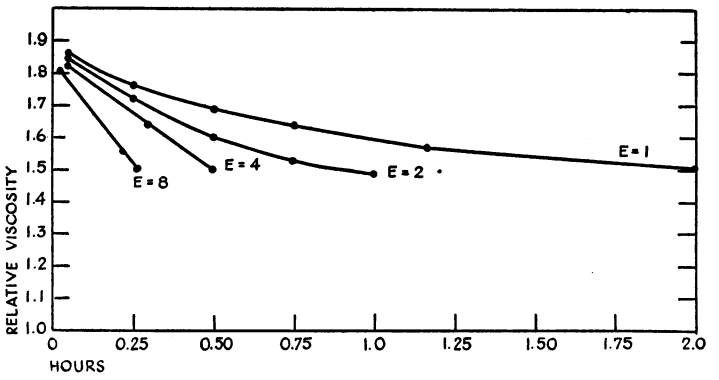

CHART 1. EFFECT OF INCREASING QUANTITIES OF THE B. MACERANS ENZYME (*E* ON RATE OF FALL IN VISCOSITY OF 3 PER CENT STARCH SOLUTION

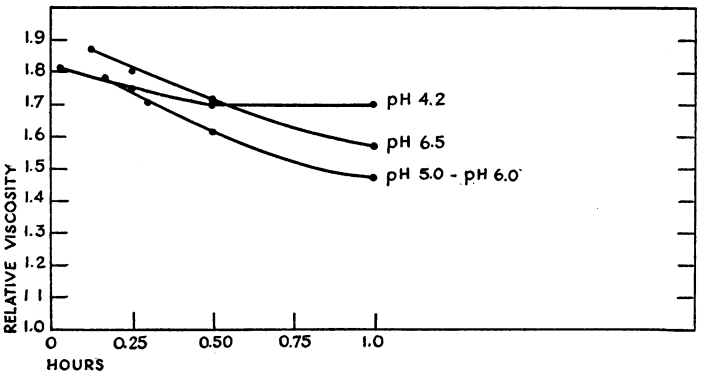

CHART 2. INFLUENCE OF pH ON THE VELOCITY OF THE REACTION BROUGHT ABOUT BY THE B. MACERANS ENZYME

acetone under conditions similar to those used for the *B. macerans* enzyme (Tilden, Adams and Hudson, in press).

The usual method of measuring the velocity of the reaction brought about by saccharogenic amylases, by determination of the reducing substances formed, was found to be applicable to the *B. polymyxa* decomposition. A quantitative technique for

measuring the rate of the *B. macerans* action was found in the fall in viscosity of the starch solution.

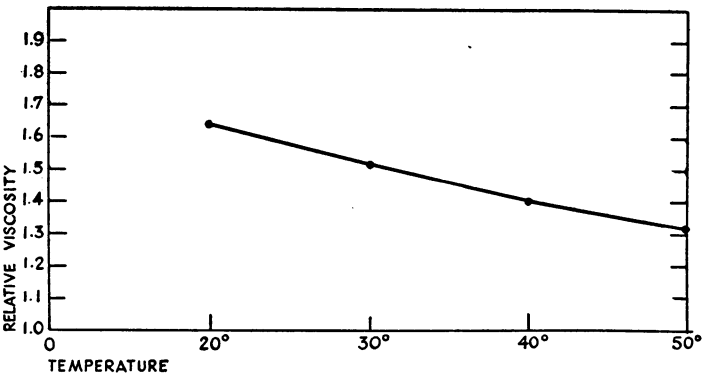

CHART 3. INFLUENCE OF TEMPERATURE ON THE VELOCITY OF THE REACTION BROUGHT ABOUT BY THE B. MACERANS ENZYME

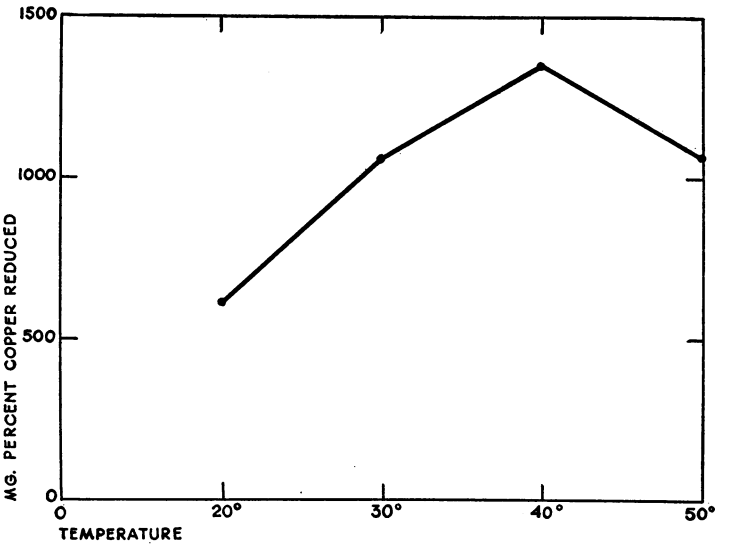

CHART 4. INFLUENCE OF TEMPERATURE ON THE VELOCITY OF THE REACTION BROUGHT ABOUT BY THE B. POLYMYXA ENZYME

As shown in chart 1, the relationship between the quantity of *B. macerans* enzyme used and the time required to produce a

given fall in viscosity follows the rule of Arrhenius for enzyme action ($QT = K$). Measurements made at 40° of the fall in viscosity at different hydrogen ion concentrations (chart 2) showed the reaction velocity to be at a maximum between pH 5.0 and 6.0, with slight decrease in activity at pH 6.5 and complete loss of activity at pH 4.2. The enzyme is relatively stable to heating, as shown by the linear form of the curve constructed by plotting the relative viscosity reached in a given time at 20°, 30° 40°, and 50°; there was a slight deviation between 40° and 50°

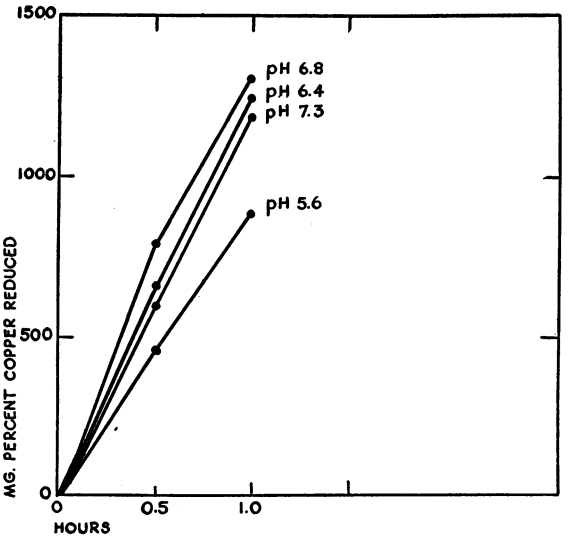

CHART 5. INFLUENCE OF pH ON THE VELOCITY OF THE REACTION BROUGHT ABOUT BY THE B. POLYMYXA ENZYME

hence some inactivation occurred in 1 hour at 50° (chart 3). Data obtained by means of the iodine test at 60°, 70°, and 80° showed no marked falling off in activity in 15 minutes until after the temperature passed 70°.

The *B. polymyxa* enzyme is less stable to heat. Comparison of the rate of formation of reducing substances in 1 hour at 20°, 30°, 40°, and 50° showed the curve flattening out between 30° and 40°, while at 50° the amounts of reducing substances formed were no greater than at 30° (chart 4). The optimal pH for the *B. polymyxa* enzyme at 40° was 6.8 (chart 5).

EXPERIMENTAL METHODS

Preparation of starch solution. Suspensions of soluble starch (Takamine) were made in 4 per cent concentration. The required amount (calculated as anhydrous) was placed in a volumetric flask, the flask half filled with distilled water, and rotated in a boiling water bath until the solution became clear. After the surface had been covered with boiling distilled water, the solution was placed immediately in a pressure cooker, already hot, and heated for 1 hour at 125°. A flask of distilled water was autoclaved at the same time, so that the solution, which was about 8 per cent when autoclaved, could be diluted with hot sterile water immediately after removal from the autoclave. After the solution was cool, sterile distilled water was added to the mark and the well-mixed solution distributed under aseptic conditions into lots of about 25 ml., the containers being closed tightly with sterile corks. Sterile solutions of starch were found to keep for many days without perceptible change if not opened; since no two starch solutions have quite the same viscosity, it was necessary to make a given series of experiments with the same lot of starch. Dilution to 3 per cent was made at the time of use with buffer and enzyme solutions. If it was desired to prepare crystalline dextrins, the addition of sterile enzyme was made before the volume was made to the mark.

Iodine test for crystalline dextrins. One milliliter of 3 per cent starch solution was incubated at 40° with 0.5 ml. clear supernate or filtrate from a culture grown for 2–4 weeks on suitable medium. At intervals 3 drops of the digest were transferred to a spot plate and a drop of N/10 iodine solution added. The microscopic appearances shown in the illustrations are typical of those seen when a loopful of the mixture was transferred to a microscope slide and examined after evaporation. The appearance of small blue dots (fig. 1), which the high power showed to be hexagonal crystals, characterized the first stage, just as the blue color began to change to violet. These increased in number and size as the reaction progressed. Later, long needles gradually spread out from the center until they covered the area of the drop and were noticeable without microscopic examination (fig. 2). This stage,

when the color was a brown violet, while not indicative of completion of the reaction, served as a suitable end point with which to compare different cultures of enzyme content and to determine the activity of purified enzyme preparations. One unit of enzyme is defined as that quantity which will convert 1 ml. of 3 per cent starch (30 mg.) to this point in 30 minutes at 40° at the optimal pH.[6] The brown violet stage very soon gives way to a brown stage (fig. 3), which lasts for many hours without further change.

When the material to be examined contained only traces of enzyme, several hours were required to convert 30 mg. of starch. In such instances it was found convenient to dilute the starch to 0.5 per cent and the iodine solution to N/60. The crystals were not abundant but were definitely demonstrable.

Crystalline dextrins were also demonstrable by the iodine test in oatmeal or potato cultures 8 to 12 hours old, if the inoculum was 0.5 ml., or 24 to 48 hours if the cultures were seeded by loop.

In the case of *B. polymyxa*, the disappearance of all violet color with iodine served as a suitable end point for comparing cultures for amylase content.

Viscosity measurements. Ostwald viscometers which permitted the passage of 8 ml. of water in about 75 seconds at 40° were set up in a glass water bath with accurate temperature regulation. The 4 per cent starch solution, prepared as described, was brought to 40° and sufficient phosphate buffer solution added so that the final volume (after addition of the enzyme) would have a phosphate concentration of M/10. The enzyme solution, previously brought to the same temperature, was then added, and the time of mixing recorded. Immediately 8 ml. were transferred to a viscometer and readings made at 15 minute intervals until the relative viscosity reached about 1.5.

It was necessary to filter the enzyme solutions, buffer solutions, and starch solution through hardened filter paper before use;

[6] The pH of the starch-enzyme mixtures was found to be about 6.0, i.e., within the optimal range, and a tenth molar concentration of phosphate buffer was required to change the pH appreciably. Hence the addition of buffer was unnecessary, except for determination of the limits of the optimal pH range, and it was also undesirable, since the formation of phosphate crystals was a source of confusion in testing solutions with iodine.

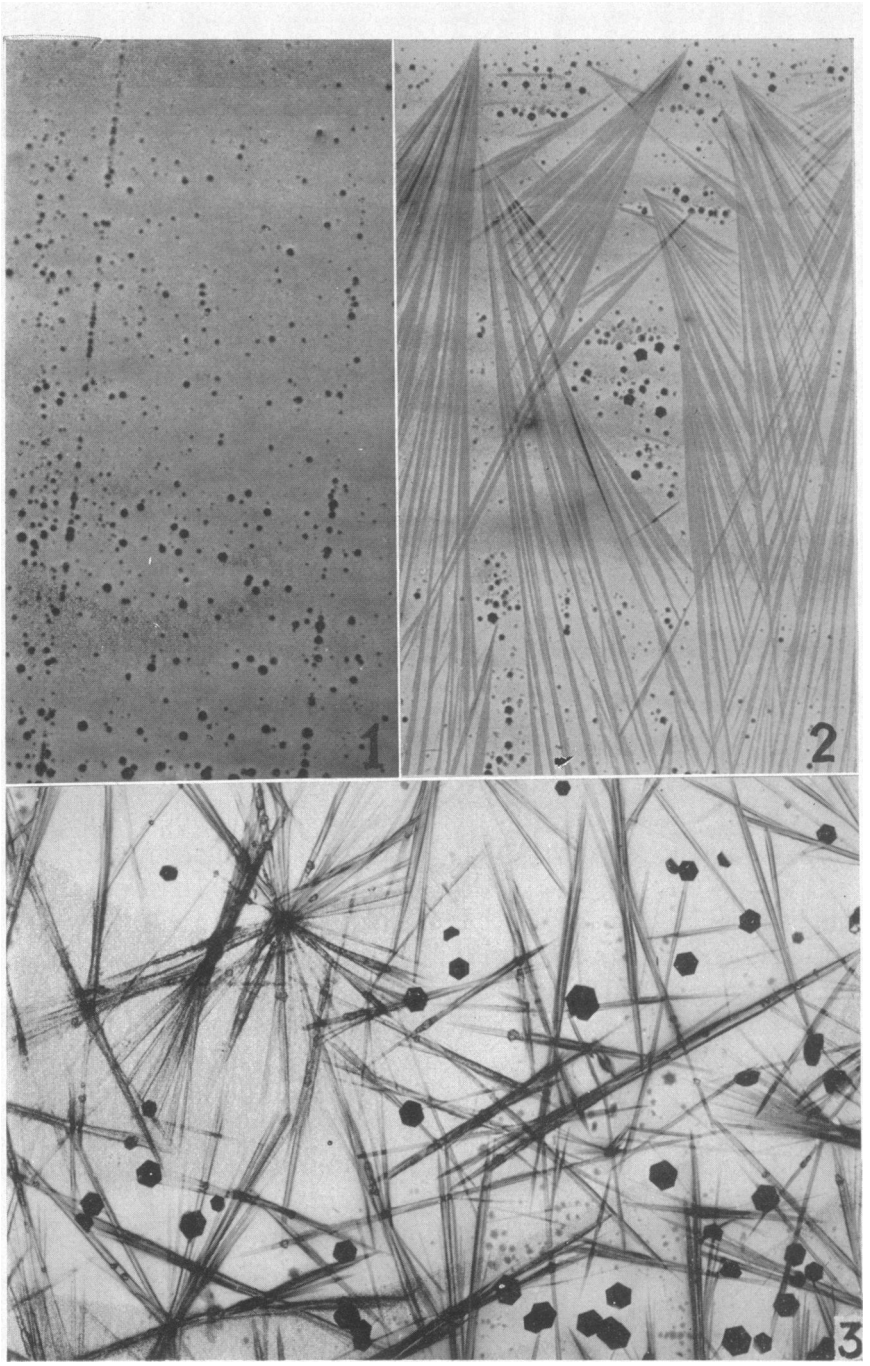

FIG. 1. IODINE TEST FOR CRYSTALLINE DEXTRINS

Microscopic appearance typical of the early stage of the decomposition of starch by the *B. macerans* enzyme. Magnification × 305.

FIG. 2. IODINE TEST FOR CRYSTALLINE DEXTRINS

Microscopic appearance typical of the "brown violet stage" of the decomposition of starch by the *B. macerans* enzyme. Magnification × 305.

FIG. 3. IODINE TEST FOR CRYSTALLINE DEXTRINS

Microscopic appearance characteristic of the "brown stage" of the decomposition of starch by the *B. macerans* enzyme. Magnification × 305.

the presence of an occasional fiber was apt to clog the fine capillary and spoil the experiment.

The electrometric method was used for determinations of pH.

Measurements of reducing value of B. polymyxa digests were made by the Shaffer-Hartmann macro technique (Shaffer and Hartmann, 1920–21) in a 300 ml. Erlenmeyer flask with a rubber stopper into which a piece of capillary tubing was inserted. With this precaution oxidation of the precipitated cuprous oxide was prevented, and duplicate titrations checked within 0.05 ml.

The desired pH was obtained by the addition of phosphate buffer solutions sufficient to give a final concentration of tenth molar.

Isolation of crystalline dextrins. The digests were prepared under aseptic conditions; before making the starch solution to volume, sterile enzyme was added in the amount needed for the conversion of the desired amount of starch in a given time as determined by the iodine test. At the end of the digestion the small amount of light fluffy sediment remaining was removed by filtration, the filtrate placed in a wide-mouth flask, and 5 per cent trichloroethylene by volume added. The mixture was cooled in ice and stirred mechanically 2 to 3 hours. A heavy white precipitate formed, which, after a day or two in the refrigerator, settled in a compact mass. The supernate was withdrawn, concentrated *in vacuo* to one-fifth its volume, and stirred with trichloroethylene. The trichloroethylene precipitation was carried out in two stages because of the tendency of the concentrated filtrate to form a jelly-like mass when treated with the solvent. The combined precipitates were filtered by gravity in the refrigerator and washed with a little ice-cold distilled water. After 24 hours the precipitate was transferred from the paper to a flask, 10 ml. of boiling water was added for each gram of the moist precipitate, and the mixture was kept in a boiling water bath under a hood and shaken frequently. The precipitate went into solution as the trichloroethylene evaporated. The solution was filtered hot through a layer of decolorizing carbon. Beta dextrin crystallized promptly. After a day or two in the cold room (5°)

the crystals were filtered off and the solution concentrated to about one-fifth volume, when an additional crop of beta dextrin was obtained. The mother liquor was finally concentrated to a syrup, from which the alpha dextrin was precipitated by the addition of alcohol at refrigerator temperature in portions at intervals of a day or two until the solution became about 70 per cent alcohol. Beta dextrin was recrystallized from a 25 per cent solution in boiling water; alpha dextrin from a 30 per cent solution in hot 70 per cent alcohol.

The iodine test again proved useful in differentiating the beta from the alpha dextrin. The brown precipitate produced by iodine in solutions of beta dextrin was found to consist exclusively of long yellow needles and orange-brown prisms as was observed long ago by Schardinger (1908–9). These were uniform and were obtained with constancy in recrystallized beta dextrin of constant rotation. Solutions of the residual dextrin, which is presumed to be Schardinger's alpha dextrin, yielded a green solution in the iodine test, as Schardinger noted; on evaporation, needles and prisms which appeared green were always found mixed with blue hexagons. Further study of the residual crystalline dextrin is obviously desirable.

SUMMARY

Study of a considerable number of cultures of *Bacillus macerans* having the characters of the species as established by Porter, McCleskey and Levine showed that all developed the unique amylase capable of converting starch to the non-reducing crystalline (Schardinger) dextrins. The amount of enzyme produced varied from culture to culture, a few cultures being outstanding in their enzyme activity. Cultures of *Bacillus polymyxa* showed similar quantitative variation in the elaboration of an amylase of a type similar to the amylases already known. Optimal cultural conditions have been established for the production of these two enzymes, and the iodine test for crystalline dextrins has been shown to be a simple and reliable one for distinguishing these two bacteria.

The amylases of *Bacillus macerans* and *Bacillus polymyxa*

have characteristic properties. The *B. macerans* enzyme is relatively stable to heating, no marked inactivation occurring in 1 hour at 50°. The *B. polymyxa* amylase is inactivated to a considerable extent by exposure to 50° for the same length of time. The optimal pH was determined at 40°, both enzymes being active and relatively stable at this temperature. The *B. macerans* amylase showed its greatest activity at pH 5.0 to 6.0, the *B. polymyxa* at pH 6.8.

The preparation of the characteristic amylases of these two bacteria is a relatively simple and inexpensive procedure, and the conditions established for maximal enzyme production and starch hydrolysis provide a basis for their possible commercial usefulness. The *B. macerans* enzyme, in particular, seems to have many theoretical applications to carbohydrate chemistry which merit consideration.

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