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Bacterial Alpha Amylase Paper Disc Tests on Starch Agar

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Placing and incubating a split barley seed on the surface of a starch-gelatin medium in a glass dish, Wijsman in 1889 demonstrated the presence of two starch-hydrolyzing enzymes diffusing with different velocities through the gel and yielding different end products (Van Klinkenberg, 1934). Since then, microbiologists have used the principle of this procedure to demonstrate the presence of amylase in cultures of microorganisms. Techniques used in the assay of antibiotics have been applied to the Wijsman method estimating quantitatively the potency of fungal, pancreatic and serum amylase (Lulla and Sreenivasaya, 1946; Sherwood, 1947; Sherwood, 1951; Goodman 1950; Tamagawa, 1951; Simonart and Yü Chow, 1951; Kotake, Koaze and Saito, 1952; Dingle, Reid and Solomons, 1953). The enzyme solution was placed into agar wells or into penassay cylinders or it was applied to paper discs. It was allowed to diffuse under controlled conditions of temperature and time. Upon flooding the surface with a diluted iodine solution, clear circles of hydrolysis became visible. Depending on the method, the concentration of the starch ranged from 0.1 to 3.6 per cent, that of the agar from 0.8 to 2.0 per cent, the amount of substrate per Petri plate from 5 to 25 ml., the temperature of incubation from 30 C to 45 C and the time of incubation from 12 to 24 hr. Also, the type of starch, presence or absence of disinfectants, and the method of applying the enzyme, as well as its amount and concentration, differed from investigator to investigator. However, regardless of the method, the diameter or the area of the circle of hydrolysis, within limits, was always related to the potency of the amylase. The use of the Wijsman method for the estimation of the potency of bacterial alpha amylase has not yet been reported.

It was the purpose of this investigation to study critically various treatments conceivably influencing the rate and the extent of the hydrolysis of soluble starch by a bacterial alpha-amylase preparation, so as to be able to make recommendations that will help toward the establishment of a standard experimental procedure to be used in testing the potency of bacterial alpha amylase.

GENERAL CONSIDERATIONS

Factors affecting the substrate, the enzyme solution, and the ensuing reaction were studied by (I) varying the concentrations of the starch and of the agar, the amount of substrate per Petri plate, and the type and concentration of different buffers, as well as the pH; (II) varying the concentration, the amount per paper disc, and the method of applying the enzyme solution; and (III) varying the time and the temperature of incubation, respectively.

Each of these factors was used at different levels and tested in different combinations. Since it was not expedient to vary all levels of all factors simultaneously, appropriate groupings were made of the factors for study so that the influence of the interactions that were considered important a priori could be studied. Studies were conducted for two different purposes: (1) to determine the optimum level to be used for the several factors in experiments determining the potency of the amylase and (2) to determine the relationship that exists between the various factors involved in such experiments.

The experimental design used to test the effects of various levels of each factor on the efficiency of the experiment and to determine the potency of the amylase was of the usual factorial type. Since every test for homogeneity of the variance (Snedecor, 1946) failed to disclose a lack of homogeneity of the variance observed within the various factorial levels, treatments yielding the largest zone diameters were considered to be the most sensitive as far as potency determination is concerned. This homogeneity of experimental error can probably be attributed to the restriction placed upon the range of a variable to those values that were ex-

perimentally feasible. Even with this restriction, however, cases did arise in which the most sensitive combination of levels of factors according to the maximum zone criterion is not to be recommended since the levels involved values on the periphery of experimental feasibility. For example, although agar concentrations below 1.0 per cent and starch concentrations below 0.2 per cent yielded the larger zone diameters, the use of semisolid agar and of starch concentrations too low to give zones with sharp, well defined edges is not to be recommended.

Ordinarily three samples of the enzyme powder were prepared and aliquots of each sample were placed on four paper discs per Petri plate, using four plates per sample. This resulted in 16 replications per sample, a total of 48 per lot. To determine the treatment or treatments yielding the largest diameter, the treatments were ordered by mean diameters and separated into statistically significant groups by the method of Tukey (1949). Since the error term in the procedure involves only the sample to sample variation, the use of more than one disc and more than one plate per sample did not increase the number of degrees of freedom in the error term, but simply increased the precision of the experiment in an inherent fashion.

In summarizing the results of each factorial experiment the ordered treatment means are given and the statistically significant (using the 5 per cent level of significance) groups of means are separated by horizontal lines.

MATERIALS AND METHODS

Materials

The same batch of materials was used throughout the entire investigation: soluble starch (according to Lintner),[1] granulated agar,[2] $Na_2HPO_4 \cdot 7H_2O$, KH_2PO_4 and $CaCl_2 \cdot 2H_2O$[3], alpha-amylase[4], filter paper discs[5] (cat. no. 740 E, 12.7 mm diameter), forced draft incubator[6] and pressed Pyrex Petri plates.

Preparing the Substrate

Agar was carefully weighed and added to one half of the total volume of Soerensen's phosphate buffer, $M/15$, pH 6.0. The remaining buffer solution was used to prepare a cold starch slurry and to rinse the beakers containing the starch and the agar, respectively. The starch agar medium, in a cotton-stoppered Erlenmeyer flask, was autoclaved at 15 lb pressure for 30 min. The medium was allowed to stand at room temperature for 15 min before being placed into a forced draft incuba-

[1] Merck and Co., Inc., Rahway, New Jersey.
[2] Baltimore Biological Laboratories, Baltimore, Maryland.
[3] Mallinckrodt Chemical Works, St. Louis, Missouri.
[4] Nutritional Biochemicals Corp., Cleveland, Ohio.
[5] Schleicher and Schuell Co., New York City, N. Y.
[6] Precision Scientific Co., Chicago, Illinois.

tor at 52 C for another 15 min. Using a 25 ml graduated glass cylinder, 20 ml of the medium were poured into sterile Petri plates. After the starch gel had hardened, the plates were stored in the refrigerator. Storing the substrate for at least two weeks had no detrimental effects.

It is not necessary to use aseptic technique, but contamination should be minimized. A sterile substrate can be prepared by pouring 20 ml of the melted medium into wide test tubes (25 × 150 mm), plugging the tubes with cotton, and autoclaving at 15 lb pressure for 30 min. The substrate is then poured into sterile Petri plates or it is stored in the refrigerator and melted again before use.

Preparing the Enzyme Solution

To make a one per cent amylase solution, 0.25 gm of enzyme powder was carefully weighed and placed into a dry test tube. Twenty-five ml of 0.0125 M calcium chloride solution were added from a volumetric pipette. The powder was stirred with a glass rod until completely dissolved. The solution was kept cold at all times.

Applying the Enzyme Solution

Before applying the amylase to the substrate, plates were removed from the refrigerator, allowed to reach room temperature, and were then randomized and labelled. Not more than two filter paper discs were placed on the surface of the starch agar gel at any one time and the enzyme solution was applied immediately. This procedure was necessary because of the tendency of the discs to absorb moisture rapidly from the medium. A 0.2 ml graduated pipette was used to apply accurately 0.07 ml of enzyme solution to each disc. The outside of the pipette was wiped dry with absorbent tissue paper before placing the tip to the center of the disc. Before the Petri plate was closed, the inside of the lid was wiped dry. Water of condensation dripping from the lid onto the medium caused a distortion of the zones of hydrolysis. After eight to ten plates had been treated, they were stored upright in a forced draft incubator at 52 C. It is advisable to stack them on empty plates to avoid excessive drying of the bottom plate. When discs are dipped into the enzyme solution, they are drained by touching the side of the test tube. Dipping the discs is a much more convenient procedure than applying a measured amount of enzyme. However, the reliability of this technique must be put to a vigorous test before it can be recommended.

Measuring the Zones of Hydrolysis

After a certain period of incubation, discs were removed from the surface of the medium and were discarded. The starch agar gel was then flooded with a dilute iodine solution and the excess was poured off.

Diameters of the colorless circle of hydrolysis (against a blue background) were quickly read to the nearest 0.2 mm on a Fisher-Lilly zone reader.[7] The reagent used to flood the substrate was a N/10 aqueous iodine-potassium iodide solution, using two parts to one part of water.

RESULTS

I. Varying the Conditions of the Substrate

Buffers. Three different buffer solutions of the same hydrogen-ion concentration, but each at two molarities,

TABLE 1. Effect of different buffers, and buffer concentrations, and hydrogen-ion concentrations of one buffer on the diameter of the zones of hydrolysis

| TREATMENT | TREAT-MENT NO. | STATISTICS OF 48 ZONES | | | ORDERED MEANS† | |
		Means*	Standard Deviation	Coefficient of Variation	Mean Diameter	Treatment No.
				%	mm	
Soerensen's M/15	1	24.80	0.265	1.07	24.82	2
pH 6.0 M/30	2	24.82	0.310	1.25	24.81	4
Universal M/15	3	24.79	0.260	1.05	24.80	1
pH 6.0 M/30	4	24.81	0.234	0.94	24.79	3
Citrate M/15	5	21.60	0.436	2.01	23.68	6
pH 6.0 M/30	6	23.68	0.262	1.10	21.60	5

* Least significant difference: 0.136 mm.

Soerensen's pH 5.0	7	22.14	0.506	2.29	24.1	8
M/15 6.0	8	24.11	0.314	1.30	23.6	9
7.0	9	23.63	0.307	1.30	23.1	10
8.0	10	23.10	0.310	1.39	22.1	7

* Least significant difference: 0.329 mm.

(0.2 per cent starch, 1.0 per cent agar, 20 ml per plate; 1.0 per cent amylase, 0.07 ml per disc; 3 samples, 4 plates with 4 discs per treatment; 52 C for 6 hours.)

† The solid lines indicate that the differences between the treatments are significant, the broken lines indicate the differences between the treatments border on significance.

in a 3 x 2 x 2 factorial design, and one of these buffers at one molarity, but four different hydrogen-ion concentrations were compared. The results are shown in table 1.

As one can observe from the results, Soerensen's phosphate and Universal buffer (DiCarlo and Redfern, 1947), at either molarity, were equally useful. There was no significant difference between the average zone diameters in the presence of these buffers. However, citrate not only yielded significantly smaller zones, but decreased the zone diameters with increasing concentration of the buffer.

[7] Fisher Scientific Co., Pittsburgh, Pennsylvania.

pH. The hydrogen-ion concentration of the substrate had a profound effect on the reaction. The differences between the average zone diameters differed significantly from one pH to the next in the range from pH

TABLE 2. Effect of different starch and agar concentrations and of different amounts of substrate per plate on the diameter of the zones of hydrolysis

| STARCH CONC. | AGAR CONC. | AMOUNT/PLATE | TREATMENT NO. | MEAN ZONE DIAMETER* | ORDERED MEANS† | |
					Mean Diameter	Treatment No.
%	%	ml		mm	mm	
0.2	1.0	10	(1)	24.8	24.8	1
		20	(2)	24.6	24.6	2
	1.5	10	(3)	24.1	24.1	3
		20	(4)	23.8	23.8	4
	2.0	10	(5)	23.5	23.5	5
		20	(6)	23.2	23.2	6
1.0	1.0	10	(7)	22.7	22.7	7
		20	(8)	22.7	22.7	8
	1.5	10	(9)	22.1	22.1	9
		20	(10)	21.9	21.9	10
	2.0	10	(11)	21.5	21.5	11
		20	(12)	21.2	21.2	12

* Least significant difference: 0.402 mm.

0.2	1.0	20	(13)	24.25	24.59	14
		30	(14)	24.59	24.25	13
	2.0	20	(15)	23.31	23.31	15
		30	(16)	23.21	23.21	16
1.0	1.0	20	(17)	22.11	22.21	18
		30	(18)	22.21	22.11	17
	2.0	20	(19)	21.42	21.42	19
		30	(20)	21.32	21.32	20

* Least significant difference: 0.6355 mm.

(1.0 per cent amylase, 0.07 ml. per disc.; 3 samples, 4 plates with 4 discs per treatment; 52C for 6 hours).

† The solid lines indicate that the differences between the treatments are significant, the broken lines indicate the differences between the treatments border on significance.

5.0 to pH 8.0. The largest zones were obtained at pH 6.0, while pH 5.0 was particularly unfavorable.

Volume. Different volumes of substrate per plate using different combinations of starch and agar concentrations were tested in 3 x 2 x 2 and in 2 x 2 x 2 factorial experiments. The results are shown in table 2. The amount of the substrate per plate in either ex-

periment did not influence greatly the average diameter of the zones. However, every increase of 0.5 per cent of the concentration of the agar decreased significantly the zone diameters. The five-fold increase in the concentration of the starch had a drastic effect on the zones of hydrolysis.

TABLE 3. *Comparison of 5 ml and 30 ml of substrate per plate*

STATISTICS	5 ML/PLATE	30 ML/PLATE
No. of Zones	44	47
Mean Zone Diameter (mm)	22.40	22.00
Standard Deviation (mm)	0.424	0.381
Coefficient of Variation—(per cent)	1.89	1.73
Student's t value	4.73	

(1.0 per cent starch, 1.0 per cent agar; 1.0 per cent amylase, 0.07 ml per disc; 6 samples, 6 plates with 2 discs per treatment; 52C for 6 hours.)

TABLE 4. *Effect of increasing starch concentrations, each at two agar concentrations, on the diameter of the zones of hydrolysis*

STARCH CONCENTRATION	AGAR CONCENTRATION	TREATMENT NO.	MEAN ZONE DIAMETER*	ORDERED MEANS† Mean Diameter	ORDERED MEANS† Treatment No.
%	%		mm	mm	
0.2	1.0	1	24.8	24.8	1†
	2.0	2	23.2	23.8	3
0.4	1.0	3	23.8	23.2	2
	2.0	4	22.4	23.1	5‡
0.6	1.0	5	23.1	22.8	7
	2.0	6	21.8	22.4	4
0.8	1.0	7	22.1	22.1	9
	2.0	8	21.3	22.1	6
1.0	1.0	9	22.1	21.3	8
	2.0	10	20.9	20.9	10

* Least significant difference: 0.302 mm.
† The solid lines indicate that the differences between the treatments are significant, the broken lines indicate the differences between the treatments border on significance.

(1.0 per cent amylase, 0.07 ml per disc; 3 samples, 6 plates with 4 discs each per treatment; 52C for 6 hours.)

The slight, but highly significant, effect of the volume of substrate was shown in an experiment in which 5 ml and 30 ml per plate were compared, with a minimum of 44 replications. Results are shown in table 3.

Larger zone diameters were obtained with 5 ml of substrate per plate; the difference produced by the two amounts was significant at the 1 per cent point. The

Student's t value on the experiment, with 44 replications at 5 ml and 47 replications at 30 ml, was 4.73.

Starch and agar concentrations. The effect of increasing starch concentrations upon the diameter of the zones of hydrolysis was tested by comparing five levels of starch concentration, each at two levels of agar concentration, in a 5 x 2 factorial experiment. The results are shown in table 4.

At any one agar concentration, increasing the starch concentration by 0.2 per cent decreased significantly the average zone diameter of the zones of hydrolysis. Also, at any one starch concentration, doubling the agar concentration had the same effect. The largest zone diameters were obtained with 0.2 per cent starch and 1 per cent agar. This was also observed in the experiments described in table 2.

In another experiment, 0.2, 0.1 and 0.05 per cent starch, each at 1 per cent agar, were compared. The average zone diameters increased significantly with decreasing starch concentrations. However, at starch con-

TABLE 5. *Comparison of average zone diameters obtained in repeated experiments*

(16 replications per sample)

STARCH CONCENTRATION	FIRST EXPERIMENT Sample No. 1	2	3	SECOND EXPERIMENT Sample No. 1	2	3	MEAN ZONE DIAMETER 96 ZONES
%							mm
0.2	24.50	24.88	24.86	24.71	24.79	24.90	24.77
0.4	23.63	23.64	23.75	23.79	23.95	23.85	23.77
0.6	22.87	23.09	23.49	23.10	22.91	23.24	23.22
0.8	22.50	22.72	23.08	22.71	22.82	22.80	22.77
1.0	21.78	22.04	22.55	21.86	22.09	22.01	22.06

centrations below 0.2 per cent the contrast between the colorless zone and the blue background became less distinct and the edges turned fuzzy. Since the usefulness of the method is governed by the ease and accuracy with which the zone diameters can be measured, 0.2 per cent starch remained the best choice.

The high degree of reproducibility of the method is shown by the following test. The experiment testing increasing concentrations of starch from 0.2 to 1.0 per cent and 1 per cent agar, shown in table 4, was repeated two weeks later with a freshly prepared substrate and enzyme solution. The results of the first and of the second experiment are shown in table 5.

When the logarithms of the starch concentrations between 0.2 and 0.8 per cent were plotted against the average diameter of 96 zones of hydrolysis, a straight line was obtained. The zones produced in the presence of 1.0 per cent starch were too small to lie on this line.

When the results with 2 per cent agar were plotted in the same manner, points were generally close to a straight line, but not on it. Apparently, 1 per cent agar

facilitated a better relationship between substrate and enzyme.

II. *Varying the Conditions of the Enzyme Solution*

Amount. The effect of varying the amount of enzyme solution applied to each filter paper disc was tested by comparing four different amounts under the usual experimental conditions. The results are shown in table 6.

Although the largest zones were obtained with the largest amount of enzyme solution applied to the disc, some of the enzyme solution overflowed from the disc to the substrate. Consequently, 0.07 ml per disc was

TABLE 6. *Effect of varying the amount of the enzyme solution applied to each disc on the diameter of the zones of hydrolysis*

AMOUNT OF AMYLASE/DISC	TREATMENT NO.	STATISTICS OF 20 ZONES			ORDERED MEANS†	
		Mean Zone Diameter*	Standard Deviation	C.V.	Mean Diameter	Treatment No.
ml		*mm*	*mm*	%	*mm*	
0.05	1	24.22	0.242	1.00	25.54	4
0.06	2	24.66	0.386	1.57	25.01	3
0.07	3	25.01	0.246	0.98	24.66	2
0.08	4	25.54	0.323	1.26	24.22	1

(0.2 per cent starch, 1.0 per cent agar, pH 6.0, 20 ml per plate; 1.0 per cent amylase; 2 samples, 10 plates with 2 discs each per treatment; 52 C for 6 hours).

* Least significant difference: 0.432 mm.

† The solid lines indicate that the differences between the treatments are significant, the broken lines indicate the differences between the treatments border on significance.

TABLE 7. *Comparison of two methods of applying the enzyme solution to the paper disc*

METHOD OF APPLICATION	MEAN ZONE DIAMETER	STANDARD DEVIATION	COEFFICIENT OF VARIATION
	mm	*mm*	%
0.07 ml	27.06	0.344	1.26
Dipping	29.01	0.292	1.00

(0.2 per cent starch, 1.0 per cent agar, pH 6.0, 20 ml per plate; 1.0 per cent amylase; 1 sample, 13 plates with 4 discs each per treatment; 52 C for 6 hours.)

used routinely and is recommended for use. There was no significant difference between the zone diameters produced by 0.06 and 0.07 ml per disc; an additional advantage of using 0.07 ml.

When the logarithms of the amount of the enzyme solution per disc were plotted against the average zone diameters a straight line was obtained between 0.05 and 0.07 ml. The average zone diameters produced by 0.08 ml per disc were too large to lie on this line. Obviously, 0.08 ml is too much liquid to be held by the disc.

Dipping and applying. Dipping the paper disc into the enzyme solution is a more convenient method than applying a measured amount to the disc. Consequently,

both methods of applying the enzyme solution were compared under the usual experimental conditions. The results are shown in table 7.

TABLE 8. *Effect of increasing the concentration of the amylase solution on the diameter of the zones of hydrolysis*

(Diameter in mm)

AMYLASE CONCENTRATION	AMYLASE POWDER SAMPLE NO.		DIFFERENCE BETWEEN SAMPLES	MEAN DIAMETER OF POOLED SAMPLES	
	1	2		d	d²
%					
4.0	29.75	29.32	0.43	29.54	872.6
2.0	28.47	28.85	0.38	28.66	821.4
1.0	27.87	27.87	0.00	27.87	776.6
0.5	27.02	26.77	0.25	26.90	723.6
0.25	26.12	25.97	0.15	26.05	678.6
0.12	24.72	24.70	0.02	24.71	610.6
0.06	23.92	23.72	0.20	23.82	567.4
0.03	23.07	22.40	0.67	22.74	517.1

(0.2 per cent starch, 1.0 per cent agar, pH 6.0, 20 ml per plate; 0.07 ml amylase per disc; 2 samples each; 2 plates with 4 discs each per treatment; 52 C for 8 hours.
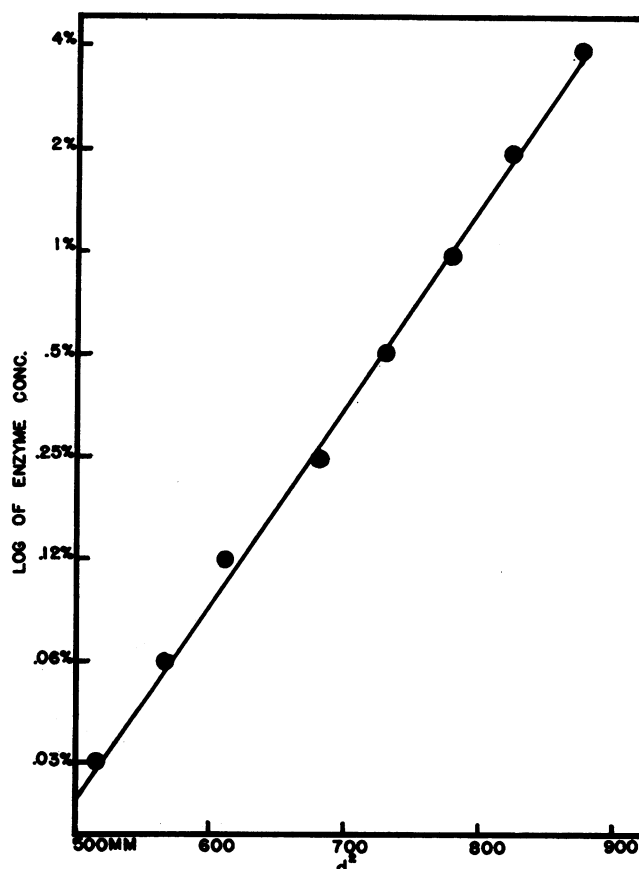

FIG. 1. The effect of amylase concentration on the size of the zone of hydrolysis. Each point represents the average of 16 zones.

Dipping the disc into the enzyme solution resulted in zones which were significantly larger than those obtained from applying 0.07 ml to each disc. Apparently, dipped paper discs absorb more than 0.07 or even

0.08 ml of enzyme solution. This has the advantage of enabling the investigator to lower the concentration of the enzyme solution below the point where measurable

TABLE 9. *Effect of different temperatures and periods of incubation on the diameter of the zones of hydrolysis*

(Diameter* in mm)

PERIOD OF INCUBATION HOURS	TEMPERATURE OF INCUBATION			
	41	51	61	71
3	19.59	20.02	20.88	20.96
6	23.28	24.32	25.44	22.40
9	26.36	28.04	28.66	24.51
12	28.99	30.97	31.22	26.26
24	38.11	41.21	38.32	29.65

(0.2 per cent starch, 1.0 per cent agar, pH 6.0, 20 ml per plate; 1.0 per cent amylase, 0.07 ml per disc; 2 samples, 4 plates with 4 discs each per treatment).

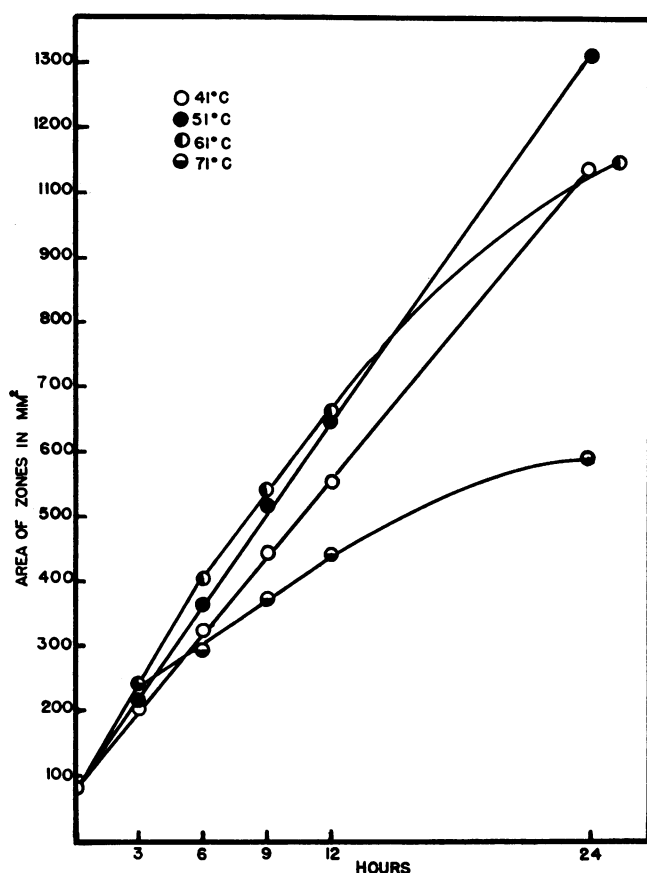* Least significant difference : 0.348 mm.

FIG. 2. The effect of temperature and the length of incubation on the zone of hydrolysis. Each point represents the average of 32 zones.

zones are still being obtained with 0.07 ml of enzyme solution per disc.

Amylase concentration. The effect of increasing the concentration of the amylase solution on the diameter of the zones of hydrolysis was tested under the usual experimental conditions, incubating the plates for 8

hr at 52 C, instead of the usual six hr. A four per cent amylase solution was prepared in 0.0125 M calcium chloride solution and was diluted with this diluent to give 2.0, 1.0, 0.5, 0.25, 0.12, 0.06 and 0.03 per cent enzyme powder. The results are shown in table 8.

When the logarithms of the concentration of the amylase were plotted against the square of the average zone diameter of pooled samples a straight line was obtained, shown in figure 1. When the results of each sample of amylase were plotted separately, using the

TABLE 10. *Ordered means of zone diameters and their differences*

ORDERED MEANS†	TREATMENT	
	Hrs.	Temperature C
41.21	24	51
38.32	24	61
38.11	24	41
31.22	12	61
30.97	12	51
29.65	24	71
28.99	12	41
28.66	9	61
28.04	9	51
26.36	9	41
26.26	12	71
25.44	6	61
24.51	9	71
24.32	6	51
23.28	6	41
22.40	6	71
20.96	3	71
20.88	3	61
20.02	3	51
19.59	3	41

† The solid lines indicate that the differences between the treatments are significant, the broken lines indicate the differences between the treatments border on significance.

diameter instead of its square, straight lines were obtained between 0.03 and 0.12 per cent and between 0.25 and 1.0 per cent amylase with one lot, and between 0.03 and 2.0 per cent with the other sample of enzyme powder. Apparently, the number of replications used was too small to give more precise results. The close relationship between enzyme concentration and zone diameter, despite a more than 100-fold increase in enzyme concentration, is being utilized at present to develop an assay for the quantitative estimation of the potency of bacterial amylases. It is of interest to note that the differences between the average zone diame-

ters of each sample between 0.06 and 1.0 per cent amylase did not exceed 0.25 mm.

III. *Varying the Temperature and Period of Incubation*

The effect of the temperature and of the period of incubation on the size of the zones of hydrolysis was studied in an experiment utilizing four temperatures and five periods of incubation. At each time interval, four plates were randomly removed from the incubator. The experiment lasted ten days because the same incubator was used. The substrate was the same throughout, but the enzyme solution was freshly prepared at each temperature. At 41 C and 51 C, the edges of the zone of hydrolysis were well-defined. At 61 C after six hr of incubation, the edges turned fuzzy. At 71 C this occurred after three hr. Since after 24 hr of incubation many zones exceeded the measurable range of the zone reader, the diameters were merely approximated. The results are shown in table 9 and graphically in figure 2. The ordered means are shown in table 10.

Heat inactivation of the enzyme solution became noticeable at 61 C and 71 C. This explains why in three instances among the ordered means a difference in temperature of 10 and 20 degrees did not produce significantly different zone diameters at differing periods of incubation.

The data of table 9 are presented merely to show the effects of temperature and time on the rate and the extent of the dextrinization. They are not meant to be used as recommendations. Although the largest zones of hydrolysis were obtained after 24 hr of incubation, heat inactivation and poorly defined edges of zones prohibit the use of this period of incubation. Since shorter periods of incubation are always desirable in enzyme studies, depending on the temperature of incubation, periods up to twelve hr are suggested.

DISCUSSION

The starch-agar, filter paper disc method for measuring the potency of amylase preparations is apparently a sensitive method which can be controlled. Small changes in the pH of the substrate, the concentration of the starch, of the agar and of the enzyme solution caused statistically significant changes in the diameter of the zone of hydrolysis. Obviously, the ingredients of the substrate and of the enzyme solution must be prepared with care. The amount of enzyme solution applied with a pipette to each disc also must be measured carefully. On the contrary, the volume of substrate per plate between 10 and 30 ml and the use of Soerensen's phosphate or universal buffer at m/15 or m/30 had no significant effects on the zone diameters. These observations indicated the need for careful manipulation of the materials. In this investigation, under a variety of conditions affecting the substrate, enzyme and reaction, the coefficients of variation for mean diameters ranged from 0.94 to 2.29 per cent, being generally closer to 1 than 2 per cent.

Time and temperature of incubation are critical factors which must be carefully observed. Heat inactivation of the enzyme during incubation should be avoided. These conditions differ from one enzyme preparation to the next and must be experimentally determined. Therefore, no recommendations can be made concerning the optimum length and temperature of incubation. Generally, plates should not be incubated more than 12 hours.

From the data obtained, the following recommendations can be made for the use of a sensitive starch-agar, filter-paper disc method: 0.2 per cent soluble starch, 1 per cent agar prepared in Soerensen's phosphate buffer, m/15, pH 6.0 using 20 to 25 ml per plate; amylase preparation (0.03 to 4.0 per cent w/v) in 0.0125 m calcium chloride solution, applying 0.07 ml to each disc (12.7 mm diameter); incubating the plates at 40 or 50 C up to 12 hr.

The inhibitory effect of the citrate buffer on the amylase was unexpected and is being investigated further.

Increasing the concentration of the enzyme preparation 100-fold did not influence appreciably the straight line relation that existed between the logarithms of the concentration and the resulting zone diameters. Consequently, it will be possible to develop an assay procedure for measuring the potency of bacterial amylase solutions. This is being done in our laboratories at present.

SUMMARY

Important steps of a paper-disc, starch-agar diffusion method for estimating the potency of bacterial amylases were investigated critically in experiments which were statistically designed and analyzed.

Slight changes in the concentration of the starch, of the agar, of the amylase, in the pH of the substrate and in the amount of the enzyme per disc caused statistically significant changes in the diameter of the zones of hydrolysis.

Large changes in the amount of the substrate per plate, ranging from 10 ml to 30 ml, and in the use of Soerensen's phosphate or of a universal buffer, each at m/15 or m/30 concentration, had no effect on the zone diameters.

Period and temperature of incubation were critical factors and must be determined experimentally for each type of amylase. For the bacterial amylase under test, 41 C and 51 C up to 24 hr were satisfactory. At higher temperatures heat inactivation of the enzyme occurred rapidly.

Over a certain range, the amount of enzyme per disc,

the concentration of the starch and the concentration of the amylase, respectively, were linearly related to the diameter of the zone of hydrolysis.

The method is rapid, convenient and highly accurate, the coefficient of variation being approximately one per cent, and is being used to develop a standard assay procedure for bacterial amylases.

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The Pectolytic Activity of Molds Isolated from Black Raspberries[1]

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The use of the Howard mold count in the quality grading of food products had its inception in 1911 when it was first used in connection with tomato products to indicate the efficiency of trimming, sorting and manufacturing procedures (Howard, 1911). Needham and Fellers (1925) applied the Howard method to strawberries and blackberries and this method has since been applied to other berry fruits including raspberries. Mold counts are currently regarded by some workers as synonymous with the extent of decomposition and mushiness.

Rendle (1933) who was concerned with "mushiness" of raspberries during their transportation to canning factories, made no mention of molds. He showed that the pectic constituents cementing the cell walls of this fruit are subject to more rapid changes than occur in most fruits during ripening and that these changes take place even more rapidly after the fruit is picked.

Byssochlamys fulva was found responsible for the breakdown of pectinous material in raspberries and complete disintegration of the fruit (Olliver and Smith, 1933). The degree of softening was unrelated to the amount of visible mycelia.

Botrytis cinerea, which is known to attack berry fruits, required a pH of 6.5 on potato decoction medium

for optimum pectolytic enzyme production (Fernando, 1937). When conditions were made acid little enzyme activity was noted despite good growth of the mold. It is concluded that relatively little is known as to the conditions under which fungi produce pectolytic enzymes (Phaff, 1946).

Recently, producers and processors of black raspberries in Michigan have been concerned, especially during humid seasons, with government seizure and rerouting of their produce executed on the basis of the Howard mold count. This matter was investigated by Fabian *et al.* (1951) who showed that no tissue breakdown of the black raspberries was noted even when the mold count as determined by the Howard method was 80 per cent. Beneke (1950a) isolated and identified the molds present in 1719 druplets of black raspberries. From this large number of druplets he isolated *Alternaria* sp. in 641 instances, *Cladosporium* sp. in 262 instances, *Pullularia* sp. 81 times, *Fusarium* sp. 66 times, *Botrytis* sp. 65 times, *Penicillium* sp. 59 times, *Rhizopus* sp. 25 times, *Trichoderma* sp. 21 times and eight other genera such as *Aspergillus* sp. and *Oospora* sp. in lesser numbers. The first two genera, *Alternaria* and *Cladosporium*, accounted for approximately 70 per cent of the total number of molds isolated from black raspberries. The present study was concerned with

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