A STUDY OF CERTAIN FACTORS AFFECTING THE PRODUCTION AND ACTIVITY OF BACTERIAL AMYLASES PRODUCED BY ORGANISMS GROWN IN A SYNTHETIC MEDIA

By

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I INTRODUCTION

The purpose of this investigation was to make certain qualitative and quantitative studies of factors affecting the production and activity of Bacterial Amylases from 18 strains of organisms isolated by Hardwick in 19[illegible].

Hardwick isolated and tentatively identified 18 cultures of micro-organisms which evidenced amylolytic activity when grown upon a medium containing starch as the only organic constituent.

Preliminary tests using the media recommended by Hardwick[1] indicated that the turbidity of the media would interfere with testing procedures. A simpler, less turbid media was devised.[2] This media proved to be satisfactory in all respects.

This work includes; (1) A study of the effect of various concentrations of sodium chloride and potassium chloride upon the rate of growth of the organisms and their capacity to produce amylase; (2) Determination of the optimum pH and optimum temperature of amylolytic activity; (3) The quantitative measurement of amylolytic conversion of starch by the use of a modified photocolorimetric method.

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[1] Omelianski's Broth, with starch substituted for cellulose.

Sodium Ammonium Phosphate	1.5 gm.
Monopotassium phosphate	1.0 gm.
Magnesium Sulphate	0.2 gm.
Faultless Starch	2.0 gm.
Water to one liter	

II LITERATURE REVIEW

The literature of starch fermentations and starch chemistry dates to the beginnings of civilization. Brewing was one of the first sciences known to man. Records indicate that the preparation of malt was known as early as 7000 B. C.

Beer brewing had become an established industry among the Phoenecians and Egyptians by 5000 B. C. These ancients knew nothing of the conversion of starch to sugar by malt. They only knew that malt was necessary if they were to obtain the desired yield of alcohol. (Lazar, 1938).

The earliest scientific research concerning amylolysis occurred in 1815 when Kirchoff isolated the active ingredient of barley malt. In 1817, Clement asserted that the fermentation of starch without conversion to intermediate sugars was possible. In 1833, Payen and Persoz conducted classic research in concentrating the enzyme by precipitation with alcohol and following its activity through the use of the starch-Iodine color reaction. Marker (1878) theorized that there were two enzymes present in malt and from his work arose the realization of the existance of alpha- and beta-amylase.

The microbiology of starch was first investigated by deSaussure who in 1819 reported the seemingly spontaneous decomposition of a starch paste which had been left in the open. Debrunfaut (1847) proved that the sugar obtained from starch by the action of malt was not, as previously believed, glucose, but was instead a new sugar which he named maltose. O'Sullivan (1872-1878) expanded this work and proved that maltose was a disaccharide with nearly twice the rotatory power of glucose.

Atkinson (1881) studied the enzyme active on rice starch used in the preparation of a rice beer. This enzyme he called Koji Diastase.

Previous to 1890, a number of incidental notations on the fermentation of starch by micro-organisms had been made. The first organized investigation of the reduction of starch by micro-organisms was conducted in organisms toward starch. Takamine (1898) prepared an amylase of fungal origin which he named Taka-diastase. Muenter (1910) studied certain characteristics of Taka-diastase in detail.

Schardinger (1901-1911) cultivated Bacillus macerans on certain starch media and obtained from this organism an amylase which would reduce starch to crystallin or Schardinger dextrins. The Schardinger dextrins consist of two water soluble, non-reducing dextrins which can be easily detected. The two dextrins were named arbitrarily alpha- and beta-Schardinger dextrins.

Commercial preparations of bacterial amylases were first made in 1917 from Bacillus subtilis by Boiden and Effront. In the period 1917-1931, several detailed investigations upon the amylases of Bacillus subtilis and Bacillus mesentericus were made.

Among the most notable of the recent investigators into the production and properties of bacterial amylases are Tarr (1934), Tilden and Hudson (1939), Kerr (1942, 1943), Wilson, Schoch, and Hudson (1943), Balls and Schwimmer (1944), Peltier and Beckford (1945), Beckford, Kneen, and Lewis (1945), Schwimmer (1945), Beckford, Peltier, and Kneen (1946), and Kneen and Beckford (1946).

The media, experimental methods, and micro-organisms used by these recent workers differ widely. The media used ranged from a potato slurry (Tilden and Hudson) to a wheat bran mash (Beckford, Kneen, and Lewis). The micro-organisms employed included Bacillus subtilis,

Aspergillus oryzae, Bacillus polymyxa, Bacillus macerans, Bacillus graveolens, Bacillus mesentericus, Bacillus mycoides, and spores of Bacillus subtilis.

The methods of determining the properties of bacterial amylases vary with the purpose and whim of the investigator. There is no standardization of methods or values. Each worker devises his own method or modifies another method to suit his own purposes. Of necessity, each investigator, when measuring a particular property must devise an arbitrary unit of measure. This consistant inconsistancy renders evaluation of previous work difficult.

III EXPERIMENTAL

A. Normal Values of Growth and Amylase Production

Before an evaluation of the amylase production of these organisms could be made, it was first necessary to establish standards or normal values of quantitative and qualitative nature and devise methods whereby both the normal values and experimental results could be measured.

1. Measurement of Bacterial Numbers.

The plate count was used to determine bacterial numbers.

2. Colorimetric Determination of Starch Utilization.

One of the prerequisites for successful colorimetry is the use of a fluid which is not too dense to allow light rays to pass through. The media used by Hardwick was so turbid that it was impossible to use this media as a part of a colorimetric determination. It was necessary, therefore, to devise a media which contained no organic constituents except starch, which was relatively clear in character, and which would support the growth of the organisms used. A media meeting these requirements is described in the introduction.

For determining quantitatively the amount of starch remaining in the media, colorimetry was chosen because colorimetric methods are usually fast, relatively simple, and reasonably accurate. Colorimetry cannot be used if the color intensity and the concentration of the unknown are not proportional, or do not follow Beer's law. The transmittance of a solution containing light absorbing particles is dependent upon (1) the nature of the substance, (2) the wavelength of light, (3) the amount, or concentration, of light absorbing particles in the solution. The relation of these

factors was established by Beer. Beer's law may be expressed as follows: At a given wavelength of light $T = 10^{-klc}$, where T = transmittance, k is the constant characteristic of the substance, c is the concentration of light absorbing particles, and l is the depth of the solution through which the light passes. In the usual practice of colorimetry, k and l are constant and c is the variable factor.

Several methods are known to determine whether or not Beer's law is applicable to a given color reaction. The method of choice here was to plot the logarithm of the percent transmittance against the concentration of the starch solution. The actual procedure was as follows: Starch concentrations of 0.0005%, 0.001%, 0.0015%, 0.002%, 0.0025%, 0.003%, 0.0035%, and 0.004% were prepared. Ten ml. of each solution was pipetted into a standard colorimeter tube. To this was added 0.1 ml. of Gram's iodine.[1] The tube was then thoroughly mixed. A faint blue color was developed. The tubes were allowed to stand 10 minutes in order to allow the color to develop fully. A blank consisting of 10 ml. of water and 0.1 ml. of Gram's iodine was used to standardize the instrument. The tubes containing the various starch solutions were then placed in the colorimeter and the percent transmittance at 650 microns, wavelength, determined and recorded.

If the graph drawn from the data obtained by this procedure has a relatively straight line with a negative slope, then it may be assumed that Beer's law is applicable to the starch-Iodine color reaction.

Table I illustrates the graph obtained from this determination. It

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[1] Gram's Iodine

Iodine	1 gm.
Potassium Iodide	2 gm.
Water	300 ml.

may be seen that the graph does contain a relatively straight line between concentrations of 0.0005% and 0.003%. It will also be noticed that the line has a negative slope. At concentrations greater than 0.003%, Beer's law does not hold true.

Using the information thus gained, the following testing procedure was devised: 1.0 ml. of the culture fluid to be tested was placed in a 100 ml. volumetric flask. The flask was then filled to the mark with distilled water. The contents of the flask were then mixed thoroughly by agitation. Ten ml. of the dilute culture media was then transferred to a standard colorimeter tube. To this was added 0.1 ml. of Gram's iodine. The tube was then agitated until the fluid therein was well mixed. The tube was then set aside for 10 minutes to allow the color to develop fully. A blank was then prepared from culture fluid from which all the starch had been digested. The standard was prepared from uninoculated media which had been prepared simultaneously with the culture being tested. This uninoculated media contained 0.2% starch. It was then diluted to 1-100, giving a final concentration of 0.002%. This is the maximum amount of starch that could be present in one of the solutions being tested. This concentration is well within the range necessary for determinations with the photoelectric colorimeter using the concepts set forth by Beer.

Ten ml. of the diluted standard was then placed in a standard colorimeter tube and 0.1 ml. of Gram's iodine added. The color was allowed to develop for 10 minutes and then the densities of the standard and the unknown determined at a wavelength of 650 microns.

The calculation for the amount of starch present is as follows:

$$\text{Concentration of unknown} = \frac{\text{Density of unknown}}{\text{Density of standard}} \times 0.2$$

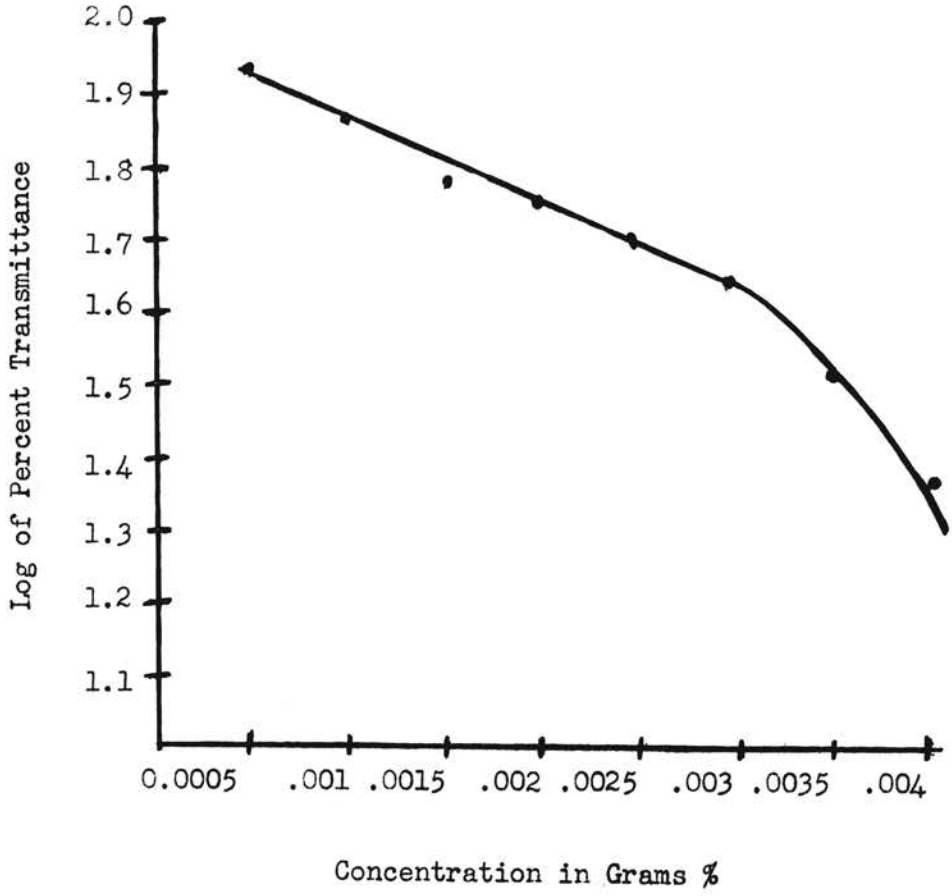
Calculation of the dilution is unnecessary since the standard and

the unknown are diluted equally. The answer is in percent.

The instrument used in these determinations was a Lumitron Colorimeter, Model 400-a.[1]

Table I

Graph to show that Beer's law is applicable to Starch-iodine color Reaction.



[1]Manufactured by the Photovolt Corporation, New York, New York.

3. Normal Values of Growth and Amylase Production.

a. In order to determine the effects of certain physical and chemical factors upon the growth and amylase production of these organisms, it was first necessary to establish normal values and ranges by making plate counts from the culture flasks daily for 4 days and every other day thereafter for a total of 8 days. From this procedure, the rate of growth could be determined. Table II illustrates the results of this work.

b. Using the same cultures employed in determining the rate of growth, the rate of starch conversion was also studied. The determination of starch was made by the photocolorimetric method hereinbefore described. The starch determinations were made daily for 4 days and every other day thereafter for a total of 8 days. The result of this study is given in Table III. The figures represent the amount of starch remaining in the culture flasks.

c. Normal pH Values.

At the conclusion of the incubation period, the pH of each culture flask was determined by the use of a Beckman Model H-2 Glass electrode pH Meter.[1] The incubation period consists of the time required to reduce all the starch in the media. Table IV indicated the pH of the culture fluid after all starch had been reduced.

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[1]National Technical Laboratories, South Pasadena, California.

TABLE II

Growth of Organisms in Synthetic Media as Determined by Plate Count.

Org. No.	Number of Organisms Per Milliliter Period of Incubation at 37°C. 1 Day	2 Days	3 Days	4 Days	6 Days	8 Days
2	30,000	200,000	200,000	6,000,000	7,500,000	4,000,000
6	33,000	350,000	300,000	5,000,000	6,000,000	4,000,000
9	29,000	200,000	300,000	4,000,000	5,000,000	3,000,000
15	30,000	150,000	200,000	4,000,000	5,000,000	4,000,000
16	45,000	300,000	300,000	5,000,000	5,000,000	4,000,000
21-A	31,000	200,000	300,000	4,000,000	5,000,000	4,000,000
21-B	20,000	430,000	400,000	6,000,000	6,000,000	6,000,000
25-B	10,000	100,000	200,000	4,000,000	5,000,000	3,000,000
28	10,000	230,000	200,000	6,000,000	8,000,000	7,000,000
35-B	10,000	640,000	300,000	400,000	3,000,000	6,000,000
36	less than 10,000	300,000	300,000	300,000	2,000,000	6,000,000
37-A	10,000	200,000	300,000	4,000,000	6,000,000	5,000,000
41	20,000	300,000	200,000	6,000,000	6,000,000	4,000,000
52	21,000	400,000	300,000	5,000,000	6,000,000	4,000,000
54	30,000	200,000	300,000	6,000,000	6,000,000	5,000,000
57	31,000	200,000	300,000	7,000,000	8,000,000	6,000,000
66	10,000	400,000	500,000	6,000,000	7,000,000	6,000,000
71	30,000	300,000	400,000	8,000,000	8,000,000	5,000,000
C.	0	0	0	0	0	0

TABLE III

Reduction of Starch by Organisms Grown in an Inorganic Media

Org. No.	Period of Incubation 1 Day	2 Days	3 Days	4 Days	6 Days	8 Days
2	0.2%	0.19%	0.19%	0.0%	---	---
6	0.2%	0.18%	0.18%	0.0%	---	---
9	0.2%	0.19%	0.19%	0.0%	---	---
15	0.2%	0.19%	0.18%	0.0%	---	---
16	0.19%	0.17%	0.17%	0.0%	---	---
21-A	0.2%	0.19%	0.19%	0.0%	---	---
21-B	0.2%	0.19%	0.18%	0.0%	---	---
25-B	0.2%	0.2%	0.19%	0.0%	---	---
28	0.2%	0.2%	0.19%	0.0%	---	---
35-B	0.2%	0.18%	0.18%	0.0%	---	---
36	0.2%	0.2%	0.19%	0.10%	0.05%	0.0%
37-A	0.2%	0.2%	0.19%	0.10%	0.08%	0.0%
41	0.2%	0.19%	0.18%	0.0%	---	---
52	0.2%	0.18%	0.17%	0.0%	---	---
54	0.2%	0.18%	0.16%	0.0%	---	---
57	0.2%	0.19%	0.17%	0.0%	---	---
66	0.2%	0.18%	0.17%	0.0%	---	---
71	0.19%	0.18%	0.16%	0.0%	---	---
C.	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%

These figures represent the amount of starch remaining in flasks.

TABLE IV

pH of Culture Fluid at Time That All Starch Had Been Reduced

No.	pH
2	5.4
6	5.9
9	6.0
15	6.3
16	5.9
21-A	6.1
21-B	5.9
25-B	6.4
28	5.6
35-B	6.4
36	6.5
37-A	6.6
41	5.8
52	5.7
54	5.5
57	5.6
66	5.3
71	5.4
C.	6.8

B. Effect of Salts in Low Concentration

Winslow and Hotchkiss (1922), and Sherman and Holm (1922) conducted extensive research concerning the effects of many chlorine salts upon the growth and activity of certain bacteria. Among the salts tested by these workers were KCl and NaCl. These workers found that these salts in low concentrations stimulated the rate of growth of the organisms tested. In addition, salts in low concentration increase the pH range over which the organisms could survive.

1. Procedure

The basal inorganic media was prepared as previously described. To this basic formula was added sufficient NaCl or KCl to yield a culture fluid containing the following concentrations of the particular salt in question: 0.0001%; 0.001%; 0.01%; 0.1%; 0.2%; 0.25%; 0.5%; 1.0%; and 2.0%.

The media containing these various concentrations of salt was dispensed

in 100 ml. portions in 250 ml. Erlenmeyer flasks and autoclaved for 15 minutes at 15 lbs. pressure. When cool, the flasks were inoculated with the various organisms and incubated at 37° for a total of 8 days.

Plate counts were made daily for 4 days and every other day for a total of 8 days. The same schedule was followed in making starch determinations with the exception that starch determinations were discontinued when results indicated that no starch remained in the culture media.

2. Results.

a. NaCl

Of the 9 concentrations of sodium chloride tested, concentrations less than 0.1% showed no increase or decrease in the activity of the organisms. At a concentration of 0.1%, some increase in the activity of the organisms was noted. At a concentration of 0.2%, the organisms appeared to be further stimulated. The maximum stimulatory action was noted at a concentration of 0.25% NaCl. At concentrations above 0.25%, a progressive inhibition in metabolic activity was noted. Table V indicates the rate of growth as determined by the plate count of organisms grown in a basal media containing 0.25% NaCl.

Table VI indicated the rate at which the starch in the media was hydrolysed by organisms grown in a media containing 0.25% NaCl. The increase in amylolytic activity is coincident with the increase in the rate of growth. Maximum amylolytic activity was coincident with maximum reproductive activity.

b. KCl

Of the 9 concentrations of KCl tested, 0.1% did show a slight acceleratory effect. This affect was evident in the rate of growth, but

TABLE V

Bacterial Numbers in Media Containing 0.25% NaCl

Org. No.	Period of Incubation					
	1 Day	2 Days	3 Days	4 Days	6 Days	8 Days
2	$3x10^3$	$7x10^4$	$7x10^5$	$7x10^5$	$5x10^5$	$2x10^5$
6	$3x10^3$	$6x10^4$	$7x10^5$	$7x10^5$	$6x10^5$	$2x10^5$
9	$4x10^3$	$7x10^4$	$6x10^5$	$6x10^5$	$6x10^5$	$3x10^5$
15	$4x10^3$	$7x10^4$	$7x10^5$	$6x10^5$	$5x10^5$	$4x10^5$
16	$2x10^3$	$6x10^4$	$7x10^5$	$7x10^5$	$5x10^5$	$2x10^3$
21-A	$5x10^3$	$5x10^4$	$6x10^5$	$5x10^5$	$5x10^5$	$3x10^5$
21-B	$4x10^3$	$6x10^4$	$6x10^5$	$6x10^5$	$6x10^5$	$2x10^5$
25-B	$4x10^3$	$8x10^4$	$7x10^5$	$6x10^5$	$6x10^5$	$4x10^5$
28	$3x10^3$	$8x10^4$	$8x10^5$	$6x10^5$	$5x10^5$	$2x10^5$
35-B	$4x10^3$	$5x10^4$	$5x10^5$	$7x10^5$	$7x10^5$	$5x10^5$
36	$2x10^3$	$2x10^4$	$6x10^4$	$2x10^5$	$3x10^5$	$5x10^5$
37-A	$3x10^3$	$3x10^4$	$5x10^4$	$3x10^5$	$3x10^5$	$3x10^5$
41	$5x10^3$	$7x10^4$	$6x10^5$	$6x10^5$	$6x10^5$	$4x10^5$
52	$4x10^3$	$6x10^4$	$7x10^5$	$7x10^5$	$6x10^5$	$3x10^5$
54	$3x10^3$	$8x10^4$	$6x10^5$	$7x10^5$	$5x10^5$	$1x10^5$
57	$3x10^3$	$6x10^4$	$7x10^5$	$7x10^5$	$5x10^5$	$5x10^5$
66	$4x10^3$	$8x10^4$	$7x10^5$	$6x10^5$	$5x10^5$	$2x10^5$
71	$3x10^3$	$8x10^4$	$8x10^5$	$7x10^5$	$6x10^5$	$6x10^5$
C.	0	0	0	0	0	0

TABLE VI

Amount of Starch Remaining in Culture Flask

No.	Period of Incubation					
	1 Day	2 Days	3 Days	4 Days	6 Days	8 Days
2	0.19%	0.16%	0.0%	---	---	---
6	0.2%	0.17%	0.04%	0.0%	---	---
9	0.2%	0.17%	0.0%	---	---	---
15	0.2%	0.18%	0.0%	---	---	---
16	0.2%	0.16%	0.0%	---	---	---
21-A	0.19%	0.17%	0.0%	---	---	---
21-B	0.2%	0.17%	0.0%	---	---	---
25-B	0.2%	0.15%	0.04%	0.0%	---	---
28	0.19%	0.16%	0.0%	---	---	---
35-B	0.2%	0.19%	0.11%	0.0%	---	---
36	0.2%	0.19%	0.13%	0.0%	---	---
37-A	0.2%	0.19%	0.10%	0.0%	---	---
41	0.2%	0.17%	0.0%	---	---	---
52	0.2%	0.18%	0.0%	---	---	---
54	0.19%	0.16%	0.0%	---	---	---
57	0.2%	0.17%	0.0%	---	---	---
66	0.2%	0.16%	0.0%	---	---	---
71	0.19%	0.15%	0.0%	---	---	---
C.	0.2%	0.2%	0.2%			

Culture fluid containing 0.25% NaCl.

TABLE VII

Bacterial Numbers in Media Containing 0.1% KCL

	Period of Incubation					
No.	1 Day	2 Days	3 Days	4 Days	6 Days	8 Days
2	$2x10^3$	$1x10^4$	$9x10^4$	$1x10^5$	$3x10^5$	$4x10^5$
6	$3x10^3$	$1x10^4$	$9x10^4$	$2x10^5$	$3x10^5$	$3x10^5$
9	$2x10^3$	$1x10^4$	$8x10^4$	$2x10^5$	$3x10^5$	$2x10^5$
15	$4x10^3$	$2x10^4$	$5x10^4$	$2x10^5$	$4x10^5$	$2x10^5$
16	$1x10^3$	$1x10^4$	$6x10^4$	$1x10^5$	$3x10^5$	$4x10^5$
21-A	$2x10^3$	$3x10^4$	$5x10^4$	$2x10^5$	$3x10^5$	$2x10^5$
21-B	$2x10^3$	$2x10^4$	$6x10^4$	$2x10^5$	$3x10^5$	$3x10^5$
25-B	$3x10^3$	$2x10^4$	$7x10^4$	$2x10^5$	$3x10^5$	$3x10^5$
28	$4x10^3$	$3x10^4$	$9x10^4$	$5x10^5$	$6x10^5$	$4x10^5$
35-B	$2x10^3$	$1x10^4$	$3x10^4$	$2x10^5$	$3x10^5$	$2x10^5$
36	$2x10^3$	$1x10^4$	$4x10^4$	$1x10^5$	$1x10^5$	$2x10^5$
37-A	$2x10^3$	$2x10^4$	$3x10^4$	$1x10^5$	$1x10^5$	$3x10^5$
41	$2x10^3$	$3x10^4$	$8x10^4$	$2x10^5$	$3x10^5$	$3x10^5$
52	$4x10^3$	$3x10^4$	$8x10^4$	$2x10^5$	$3x10^5$	$2x10^5$
54	$2x10^3$	$2x10^4$	$1x10^5$	$3x10^5$	$3x10^5$	$2x10^5$
57	$3x10^3$	$3x10^4$	$9x10^4$	$3x10^5$	$3x10^5$	$3x10^5$
66	$2x10^3$	$2x10^4$	$8x10^4$	$2x10^5$	$3x10^5$	$2x10^5$
71	$4x10^3$	$2x10^4$	$8x10^4$	$4x10^5$	$3x10^5$	$2x10^5$
C	0	0	0	0	0	0

Number of organisms per cc.

TABLE VIII

Amount of Starch Remaining in Culture Flasks
Media Containing 0.1% KCl

	Period of Incubation					
No.	1 Day	2 Days	3 Days	4 Days	6 Days	8 Days
2	0.2%	0.17%	0.11%	0.0%	---	---
6	0.2%	0.18%	0.11%	0.0%	---	---
9	0.2%	0.17%	0.12%	0.0%	---	---
15	0.2%	0.17%	0.11%	0.0%	---	---
16	0.2%	0.16%	0.13%	0.0%	---	---
21-A	0.2%	0.18%	0.11%	0.0%	---	---
21-B	0.2%	0.17%	0.11%	0.0%	---	---
25-B	0.2%	0.18%	0.12%	0.0%	---	---
28	0.19%	0.17%	0.10%	0.0%	---	---
35-B	0.2%	0.17%	0.11%	0.0%	---	---
36	0.2%	0.19%	0.13%	0.05%	0.0%	---
37-A	0.2%	0.19%	0.15%	0.06%	0.0%	---
41	0.2%	0.18%	0.11%	0.0%	---	---
52	0.2%	0.17%	0.12%	0.0%	---	---
54	0.2%	0.16%	0.11%	0.0%	---	---
57	0.2%	0.17%	0.14%	0.0%	---	---
66	0.2%	0.17%	0.13%	0.0%	---	---
71	0.19%	0.17%	0.11%	0.0%	---	---
C	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%

was not pronounced in the rate of starch reduction. In no case was the acceleration of activity by KCl equal to the acceleration of activity by NaCl. The lower concentrations of KCl had no effect whatever. However, at concentrations above 0.1%, the organisms were inhibited in a direct ratio to the concentration. Table VII gives the results of plate counts on a media containing 0.1% KCl. Table VIII gives the rate of starch reduction in this media.

C. Optimum pH and Optimum Temperature of the Enzyme Filtrate.

In order to determine certain facts concerning the amylases produced by these organisms, it was necessary to separate the effects of the organisms' metabolism from the effects of the enzyme activity. The simplest way to do this was to filter the culture fluid through a Seitz filter and use the resultant filtrate as an amylase solution. It was found by preliminary tests that a filtrate evidencing maximum activity could be obtained from a culture which had been stimulated by 0.25% NaCl and incubated for 60 hours.

1. Optimum pH.

a. Procedure.

The sterile filtrate described above was adjusted to the pH being tested with the aid of a pH-Meter. A solution containing 0.01% starch was simultaneously adjusted to the same pH. 10 ml. of the 0.01% starch solution was placed in a 250 ml. Erlenmeyer flask. To this was added 50 ml. of the filtrate. No attempt was made to maintain sterility. At 5 minute intervals 0.1 ml. was withdrawn and placed on a depression spot plate. To this was added 1 drop of Gram's iodine. Starch reduction was considered complete when the typical blue color of the starch-iodine complex no longer appeared. The time required for the complete reduction

of starch was considered a measure of the potency of the enzyme.

Since the concentration of the starch solution was 0.01% and the amount used was 10 ml., we can express the amylase activity as the time required for 50 ml. of the enzyme filtrate to reduce 0.001 gm. of starch.

The temperature at which these reactions took place is a factor in their activity. Consequently, the reaction flasks were incubated at a temperature of 37° C. in a water bath.

The pH levels tested were 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0.

b. Results.

All the filtrates except 36 and 37-A evidenced greatest amylolytic activity at a pH range of 5.5 to 6.0. Filtrates from organisms 36 and 37-A showed greatest activity at a pH of 6.5. Table IX illustrates the results of this part of the thesis study.

2. Optimum Temperature.

a. Procedure.

50 ml. of the enzyme filtrate prepared as described herinbefore was placed in a 100 ml. Erlenmeyer flask containing 10 ml. of a 0.01% starch solution. The contents of the flask were mixed well and placed in a water bath, the temperature of which had been previously adjusted to the desired degree. At 5 minute intervals, 0.1 ml. of the mixture in the flask was removed and placed on a depression spot plate. To this was added 1 drop of Gram's iodine. If a blue color resulted, starch was still present in the media. The time required to reduce the starch in the mixture was taken as an indication of the potency of the amylase.

The temperatures tested were 37°, 47°, 57°, 67°, and 77° Centigrade.

The pH of all solutions was adjusted to 5.5.

b. Results.

The results are listed in Table X.

TABLE IX

Time in Minutes Required for 50 ml. of Enzyme Filtrate to Reduce 0.001 gm. of Starch at Various pH Levels

	pH								
No.	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
2	40	35	30	15	15	20	35	45	60+
6	35	30	30	10	10	15	35	45	60+
9	35	35	30	10	10	15	40	45	60+
15	35	35	30	15	15	20	45	50	60+
16	40	35	30	10	10	15	30	45	60+
21-A	35	30	30	15	15	15	35	45	60+
21-B	35	30	30	15	10	20	40	50	60+
25-B	40	35	30	15	15	20	40	50	60+
28	30	25	25	5	5	15	25	50	60+
35-B	60+	35	35	20	15	20	25	45	60+
36	60+	60+	60+	45	35	25	30	55	60+
37-A	60+	60+	60+	45	30	25	35	60+	60+
41	30	30	30	15	15	25	35	45	60+
52	35	30	25	15	15	30	45	55	60+
54	35	30	25	10	15	25	35	50	60+
57	40	30	25	10	10	25	40	50	60+
66	35	30	25	10	15	30	40	55	60+
71	35	30	25	10	15	25	40	55	60+

TABLE X

Time Required for 50 ml. Enzyme Filtrate to Reduce 0.001 gm. of Starch at Various Temperatures. (In Minutes)

	Temperature Being Tested				
No.	37° C.	47° C.	57° C.	67° C.	77° C.
2	15	15	10	20	60+
6	15	10	10	20	60+
9	10	10	5	15	60+
15	15	10	5	10	45
16	10	10	5	15	60+
21-A	15	15	10	20	60+
21-B	15	10	5	15	60+
25-B	15	15	10	30	60+
28	5	5	5	20	60+
35-B	20	20	15	15	60+
36	45	30	45	60+	60+
37-A	60+	20	50	60+	60+
41	15	15	15	30	60+
52	15	10	5	40	60+
54	10	10	10	20	60+
57	15	10	5	25	60+
66	10	10	10	20	60+
71	10	5	5	15	55

IV DISCUSSION

The properties of amylases produced by micro-organisms grown on a synthetic media using starch as the only organic constituent have not been extensively investigated. Most commercial preparations of bacterial amylases are prepared from a potato slurry or from a mash consisting of some starchy product and suitable phosphate buffers.

The bacteria which produce the greatest yield of amylase are of the Bacillus group. The organisms of this group are quite hardy and are possessed of an enzyme system that enables them to utilize a great variety of organic substances as a source of carbon and energy. From this study we may see that the organisms enployed here are capable of utilizing starch as their sole source of carbon and energy.

Most amylases, with the exception of malt, are activated by Chlorine ions. This phenomenon was mainfested in the acceleration of starch hydrolysis when a suitable concentration of sodium chloride was added to the culture media. In this case, a suitable concentration was 0.25%. By comparing Tables II and III with Tables V and VI, the more rapid rate of starch reduction and the increased rate of growth of the organisms is apparent. The time required for complete starch hydrolysis was shortened from 4 days to 3 days. This increase in amylolytic activity was accompanied by a sudden increase in bacterial numbers.

KCl was not as effective as an accelerator as NaCl was. KCl did display some ability to speed the growth of these organisms, but never to a degree comparable to the acceleratory effect of NaCl. KCl manifested an inhibitory effect at much lower concentrations than did NaCl. A

comparison of Tables II and III with Tables VII and VIII will point out that some increase in metabolic activity did result from the addition of KCl, but that this increase is not of great consequence.

By studying Tables II, V, and VII, some information concerning the rate of growth and the behavior of the organisms in relation to starch reduction can be gained. Evidently, the number of organisms present in the cultures after 1 day's incubation is largely dependent upon the magnitude of the inoculum. By the second day of incubation, the bacterial numbers stabilize. If the media contains no accelerating agents, the bacterial numbers remain about the same on the third day of incubation. On the fourth day, or third if some accelerator is present, bacterial numbers increase tremendously. This increase is accompanied by the reduction of all starch in the media. By the sixth day of incubation, bacterial numbers reach their zenith and by the eighth day, some reduction in the size of the bacterial population is evident.

From the assembled data the following statements may be made:

(1) Maximum reproductive activity occurs during the fourth day of incubation unless sodium chloride (0.25%) or potassium chloride (0.1%) is added, in which case maximum reproductive activity occurs on the third day of incubation.

(2) Maximum reproductive activity and maximum amylolytic activity are coincident.

(3) Sodium chloride in a concentration of 0.25% increases the rate of reproduction and the rate of starch reduction.

(4) Potassium chloride in a concentration of 0.1% manifests some accelerator action, but not to a degree equal to 0.25% NaCl.

The optimum pH for the enzyme filtrates is evidently between 5.5 and 6.0. Table IX indicates that acid conditions are more favorable to

amylolytic activity than are alkaline conditions. DiCarlo and Redfern (1947) conducted extensive research concerning the properties of bacterial alpha-amylases, and their results concerning the optimum pH coincide quite closely with the results listed in Table IX. At a pH over 7.5 very little amylase activity is noted. However, at pH levels as low as 4.0, some enzyme activity is demonstrated.

By comparing the data in Table IV with the data in Table IX, it can be seen that the optimum pH for the enzyme filtrate corresponds with the pH of the media at the time of greatest amylolytic activity and reproductive activity.

The optimum temperature for filtrates containing amylases from these organisms was found to be near 57° C. As the temperature increased, enzyme activity increased until at 57° C. maximum activity was demonstrated. A further increase in temperature was not accompanied by an increase in amylase activity, but was accompanied by a drastic decrease in the activity of the enzyme. This phenomenon of increased enzyme activity when the temperature is increased is characteristic of most enzymes. According to Kleiner (1948) the activity of an enzyme is increased 2 to 4 times by each 10° C. temperature rise until the optimum temperature is reached. Above this optimum temperature, the rate decreases and at a certain point, the enzyme is destroyed.

The results relative to optimum temperature differ somewhat with the results obtained by DiCarlo and Redfern, but not drastically so. DiCarlo and Redfern indicate that the maximum amylase activity occurred at a temperature of 50° C. at a pH of 5.5.

Throughout the course of this experimentation, two organisms were conspicuous by their lack of conformity. These organisms are #36 and

#37-A. The exceptional behavior of these two organisms was to be expected, however, as they are the only organisms in the group which were Gram negative. All others were Gram positive aerobic sporeformers which Hardwick (1952) had placed in the ~~Genus~~ Family Bacillaceae. Organisms #36 and #37-A were placed in the ~~Genus~~ FAMILY Pseudomonadaceae by Hardwick. The amylases produced by these two organisms at no time equaled the potency of the remaining 16 organisms. These two organisms did not thrive on the synthetic media.

V SUMMARY

This thesis problem was the study of certain factors affecting the production and activity of Bacterial amylases produced by organisms grown in synthetic media.

In order to determine the amount of starch utilized by these organisms, a photocolorimetric method of starch determination was employed. This photocolorimetric determination was quick, easy, and sufficiently accurate for this purpose.

The organisms used in this study were 18 bacterial isolates described by Hardwick in 1952. These isolates evidenced amylolytic activity when grown on a synthetic media. The media described by Hardwick proved unsatisfactory for this study. Consequently a more suitable media was devised.

It was found that sodium chloride in a concentration of 0.25% stimulated the rate of growth and the amylolytic activity of these organisms.

Potassium chloride in a concentration of 0.1% had some stimulatory effect upon the organisms' metabolism, but this effect never equaled the effect of 0.25% NaCl.

The optimum pH for the activity of filtrates containing the amylases produced by these various organisms was found to be between 5.5 and 6.0.

The optimum temperature for the filtrates made from these organisms was near 57° C. There was a progressive increase in enzyme activity as

the temperature increased. Above this optimum, enzyme activity decreased and finally became nonexistant.

Organisms 36 and 37-A did not behave in a manner similar to the rest of the organisms. In general, they were not as active. These two organisms are gram negative. The rest are Gram positive.

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