THE ISOLATION AND TENTATIVE IDENTIFICATION OF AMYLASE PRODUCING BACTERIA ON A SYNTHETIC BASAL MEDIUM

By

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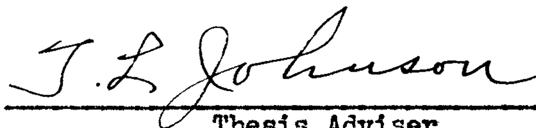

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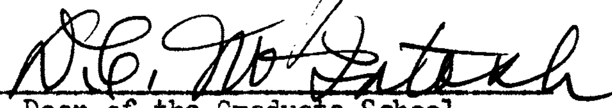

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TABLE OF CONTENTS

	Page
LIST OF TABLES	v
Chapter	
I. INTRODUCTION	1
II. LITERATURE REVIEW	3
III. EXPERIMENTAL	6
A. Sources and Methods of Isolation of Amylolytic Organisms	6
Methods and Results	7
Discussion	8
B. Purification of Cultures	9
Methods and Results	9
Discussion	11
C. Description and Tentative Identification of the Isolates	12
Methods and Results	12
Discussion of Methods	12
Discussion of Results	16
D. Action of Chemical and Physical Agents on the Production of Amylase as Indicated by the Iodine Test	22
Methods and Results	22
Discussion	24
IV. GENERAL DISCUSSION	28
V. SUMMARY	32
BIBLIOGRAPHY	34

LIST OF TABLES

Table Page

I. Number of Amylolytic Cultures from Various Sources 7

II. Number of Cultures Purified by Each Method 10

III. Physical Characteristics of the Isolates 17

IV. Physiological Characteristics 18

V. Effect of Sodium Chloride on Growth and Starch Breakdown 25

VI. Effect of Agitation on Growth and Amylolytic Activity 26

VII. Effect of pH on Growth and Amylolytic Activity 27

I. INTRODUCTION

The principle objective of this investigation was the isolation and identification of microorganisms that produce a significant amount of amylase when cultivated upon a synthetic basal medium utilizing starch as a source of carbon, and the effect of certain chemical and physical agents on amylase production.

In a survey of the literature only one reference was found that mentioned the use of a synthetic basal medium with the addition of starch, or a starchy substance, as a source of carbon. This reference material was by Tilden and Hudson (1942). However, these workers did not comment on the success or failure of their work utilizing the synthetic medium base. The desired result in this investigation was, that by the development of suitable media and cultural techniques, the isolation of organisms particularly adapted to hydrolyze starch.

In a synthetic basal medium all of the compounds are known and only the source of carbon, starch, is of an organic nature. Since these enzymes are of a proteinaceous nature (Sumner and Myrback, 1950), it seems that a much purer enzymatic extract could be obtained from an inorganic substrate than could be obtained from a substrate containing diverse proteins, which would be precipitated with the desired enzymes. The synthetic basal medium selected for use here was the one devised by Omeliansky (1904) for use with cellulose decomposers, with the substitution of starch for cellulose as a source of carbon (part III, A).

In an attempt to eliminate organisms not producing amylase, the sources of inocula were placed directly into synthetic basal starch medium, incubated, and treated with Lugol's iodine solution (1 Gm. iodine, 2 Gm. potassium iodide made up to 300 ml.) and the colonies showing a wide zone of hydrolysis, 5 mm. or better, were picked for future investigation.

These colonies were examined microscopically and many of the isolates appeared to be mixtures. Such mixed cultures have been reported in relation to cellulose decomposing organisms (Debrunfaut, 1847; Omeliansky, 1913). The isolates were purified and tentatively identified according to Bergy's Manual of Determinative Bacteriology and the Manual for Pure Culture Study of Bacteria.

Further investigations were performed on the effect of physical and chemical environmental changes on amylase production. The agents investigated were; pH, various chemical salts, and agitation of the culture.

II. LITERATURE REVIEW

From the first reports of the breakdown of starch containing materials by bacterial and fungal activities this action has been of industrial importance. The earliest records of the preparation of malt dates back to about 7000 B.C. with beer brewing becoming an established craft about 5000 B.C. (Lazar, 1938). Bacterial and mold amylases were undoubtedly used for centuries in Japan and China, but investigations into the nature of this action and the enzymes involved did not materialize until sometime after the work of Kirchhoff (1815) and Payen and Persoz (1833).

The earliest research with the breakdown of starch was in 1815 when Kirchhoff discovered "diastase" and in 1817 when Clement asserted that starch may be fermented without intermediate conversion to sugar. Payen and Persoz (1833) named the active principle of barley malt "diastase" and used the iodine staining property of starch to follow its action. These workers even effected a degree of concentration and purification of the enzyme by alcohol precipitation. Also working with malt diastase Marker (1878) postulated that there are two "diastatic ferments" in malt. This was the origin of the characterization of malt diastase as a mixture of alpha- and beta- diastase.

Probably the first record of the decomposition of starch by microorganisms came in 1819 with the work of deSaussure. He reported on the seemingly spontaneous decomposition of starch paste which had been left exposed to the atmosphere. Debrunfaut (1847) showed that the sugar, termed maltose by him, which he obtained from starch by the action of malt amylase,

and which in the course of time had come to be regarded as identical with glucose, was in fact different from this sugar, yielded by starch on hydrolysis with dilute acids. This work was expanded by O'Sullivan from 1872 to 1878 and he found that the maltose obtained from starch on hydrolysis with malt amylase was a disaccharide with about twice the rotatory power of glucose, but with a lesser reducing action toward Fehling's solution.

Among the first investigations of the microorganisms and enzymes involved was one publication by Atkinson (1881) on the diastase of "Koji," a fermented rice beverage in Japan. Following the first more or less incidental observations of Fitz (1877), Marcano (1882), Wortmann (1882), and Prillieux (1879), Fermi in 1890 undertook a more comprehensive study of the behavior of a number of well known bacteria toward starch. While working with the diastatic substances of fungal growth Takamine (1898) described the preparation of Taka-diastase. This fungal enzyme does not give the guaiacum reaction and the ratio of amyloclastic to saccharogenic activity is considerably higher than for malt amylase. Muenter (1910) noted that the action of Taka-diastase is injured by alcohol and ether, and that ammonium sulfate is the only salt useful for its precipitation. He also reported on the separation of the enzymes by dialysis, effects of small and large amounts of maltose, and the effects of temperature on the amylolytic activity of these enzymes.

Schardinger (1908, 1909, 1911) cultivated Bacillus macerans on starches and obtained from these starches "crystalline dextrins." These dextrins are a mixture of two water soluble, non-reducing dextrins which may be isolated with ease. These compounds are known as the alpha- and beta-Schardinger dextrins.

The discovery of bacterial amylases for commercial purposes is credited to the work of Boiden and Effront. These workers introduced the use of

Bacillus subtilis for the production of amylases in 1917. Considerable detailed investigations have been performed on the amylases of Bacillus subtilis and Bacillus mesentericus; these amylases have found wide application in industry. Effront (1923) contributed more work on the amylo-process and the industrial application of bacterial amylases.

More recent work with bacterial amylases has been by Kluyver (1931), Wallerstein (1939), Williams (1939), and Tilden and Hudson (1942). Also contributing greatly in this field are Kerr (1942, 1943, 1950), Balls and Schwimmer (1944), and Beckord, Peltier, and Kneen (1946). Other workers in many fields have contributed to this study but they are far too numerous to list here. A more detailed bibliography may be found in Walton's Survey of Starch Chemistry (1929), Enzymes and Their Role in Wheat Technology, edited by A. Anderson (1946), R. W. Kerr's Chemistry and Industry of Starch (1950), and Thaysen and Galloway The Microbiology of Starch and Sugars (1930).

III. EXPERIMENTAL

A. Sources and Methods of Isolation of Amylolytic Organisms

In search of bacteria possessing high amylolytic activity, many sources of rough inocula have been investigated. Bechamp, in 1886, found certain organisms in chalk which were capable of directly fermenting starch. In a report by Peltier and Beckord (1945) it was reported the best sources for obtaining these organisms were ropy bread, starches, flours, thin stillage, air, and manures. Of these sources, the ropy bread was the most fruitful and the manures least fruitful. The sources of rough inocula used here were silage from the bottom of an old silo, sputum, field soil, soil around grain elevator, dust inside the grainery, samples of rat feces at the grainery, air organisms, dried beans, and household starch.

In an attempt to isolate organisms different from those obtained by previous workers the medium used was that devised by Omeliansky (1904) for cellulose decomposers. Starch was substituted for the cellulose in a measured amount. This medium contains the nutrients necessary for growth of the strains of bacteria isolated and was prepared as follows: to a liter of distilled water is added, 1 gram ammonium sulfate, 1 gram dipotassium phosphate, .5 gram magnesium sulfate, 3 grams calcium carbonate, trace of sodium chloride, and 2 grams of starch; this solution was placed in containers (flasks or tubes) and autoclaved for 15 minutes at 15 pounds pressure. For the preparation of slants and plates 1.5 per cent Bacto-Agar was added. This medium proved more satisfactory for this purpose than did

Czapek's[1] or the medium described in the Manual for Pure Culture Study of Bacteria (Leaflet II_{50}-17), in that it provided more abundant growth than did the other two media.

Methods and Results

In order to obtain organisms evidencing amylolytic activity from the rough inocula, a random sample of the material was inoculated into 100 ml. of Omeliansky's broth and incubated for 48 hours at 37 degrees. These cultures were then streaked onto starch agar plates of the same medium.

After incubation at 37 degrees for 48 hours the plates were tested for starch hydrolysis with Lugol's solution (1 gram of iodine and 2 grams of potassium iodide made up to 300 ml. with distilled water). They were flooded with Lugol's solution and the liquid poured off after one minute. The colonies showing wide zones of hydrolysis, 5 m. m. or better, were picked and placed on starch agar slants. These cultures were retained for future use. The number of cultures obtained from each source is shown on Table I.

TABLE I

NUMBER OF AMYLOLYTIC CULTURES FROM VARIOUS SOURCES

Source	Silage	Sputum	Field Soil	Soil GE*	Dust GE*	Rat Feces	Media Contam**	Dried Beans	Starch
Number of Isolates	40	2	27	50	8	23	9	3	5

* Grain Elevator, Stillwater, Oklahoma

** Media Contamination

[1] S. A. Waksman, Principles of Soil Microbiology, p. 226.

Discussion

The data in Table I indicate that the most fertile sources for obtaining starch reducing organisms were soil around the grain elevator, silage, field soil, and rat feces. The poorer sources were sputum, dried beans, starch dust in grain elevator, and media contamination. This data indicate that materials high in starchy substances, or other carbohydrates, are better sources for the isolation of amylase producing organisms. It also indicates that environmental conditions influence the activities of these bacteria.

B. Purification of Cultures

More than 150 colonies of organisms were picked that indicated strong amylolytic activity. Microscopic examination showed that many (45) of these were mixed cultures. Others (Debrunfaut, 1847; Omeliansky, 1913) have reported similar results among the cellulose decomposers.

Methods and Results

The following methods were used to separate the mixed isolates:

1) Serial Dilution Method to extinction, using nutrient starch agar and the synthetic basal medium. The nutrient-starch medium consists of; .3 per cent beef extract, .5 per cent peptone, .2 per cent starch, 1.5 per cent agar. This medium and the synthetic basal medium were both prepared, placed in 15 ml. quantities in test tubes and sterilized.

The procedure followed was the same utilizing both media, the tubes of agar were melted and allowed to cool to about 43 degrees. They were then used as dilution blanks by adding to one tube a loopful of the culture and mixing thoroughly, two loopsful were transfered from the first tube into a second tube and this tube mixed thoroughly. This procedure was repeated until four dilutions had been made of the original loopful of culture.

After the dilutions were prepared the agar was poured into petri dishes and incubated at 37 degrees for 48 hours. After the incubation period these plates were treated with Logol's solution and those colonies showing a zone of hydrolysis of 5 mm. or better were placed on slants for use in the future.

2) Streak-Plate Method utilizing the synthetic basal-starch medium, synthetic basal-starch medium plus gential violet, synthetic basal-starch medium plus tyrothricin, and eosin-methylene blue agar.

The use of the gentian violet and the tyrothricin in the culture medium was recommended by the Manual for Pure Culture Study of Bacteria (Leaflet II-13). The concentration of the gentian violet in the medium was one part of the dye to one-hundred thousand parts of the medium. The selective bacteriostatic action of tyrothricin was obtained at a concentration of one part of the antibiotic to fifty-thousand parts of the medium.

The method of streaking the plates was the same in all instances. After the plates had been poured and the agar solidified they were each streaked with a broth culture. A loopful of the culture was placed on the agar and spread across one side of the plate two or three times, the loop was sterilized and streaked across the first streaks three or four times onto a second section of the plate. The loop was again sterilized and the second streaks were spread over the remaining portion of the agar surface. The plates were then incubated at 37 degrees for 24 to 48 hours. They were tested with iodine solution and colonies exhibiting large zones (5mm. or better) were picked.

TABLE II

NUMBER OF CULTURES PURIFIED
BY EACH METHOD

Method	Serial Dilution		Streak Plates			
Media	Syn. Basal Medium	Nutrient Starch	Syn. Basal Medium	Gentian Violet	Tyro-thricin	E M B
No. of cultures	12	12	25	13	7	5

Discussion

Of the two methods used for purifying these cultures it is apparent (note table II) that the streak plate method was more successful. Of the original 150 cultures only 74 were retained in pure culture. Of these 74 cultures, 50 of them were obtained from the streak plate method, and the remaining 24 were from the dilution plates. These 74 isolates were tested for their amylolytic abilities by placing them on synthetic starch media and testing them with iodine after incubation. From this group 15 cultures were discarded for the lack of starch breakdown. Of the remaining organisms 20 of them were selected for further study. These organisms were selected by the extent of the starch hydrolysis zone. Of these organisms some of them produced a very large zone (10 to 15 mm).

C. Description and Tentative Identification of the Isolates

The organisms isolated were identified with the aid of Bergey's Manual of Determinative Bacteriology (1948).

Methods and Results

The media and procedures utilized in these determinations were those recommended by the Manual for Pure Culture Study of Bacteria.[1] The tests performed include the gram stain reaction, presence of endospore, cell morphology, and motility (see table III); utilization of carbohydrates, nitrate reduction, gelatin liquefication, acetylmethylcarbinol production (Voges-Proskauer reaction), action on milk, and citrate utilization (see table IV).

Discussion of Methods

The examination of the isolates for cell morphology and spore formation was made on the same preparation. The cultures used were 36 hours old and stained with a dilute solution (1:100) of gentian violet as a simple stain. This stain presents a clear picture of the individual cell morphology and the presence and position of endospores. The gram stain technique was Hucker's Modification[2] performed on 18 hours cultures. The motility studies were performed in a hanging drop preparation. The medium for the motility studies was the synthetic basal starch medium in a broth preparation. The tubes were inoculated and incubated at 37 degrees from 12 to 18 hours before examination.

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[1] Manual for Pure Culture Study of Bacteria. Leaflet IV$_{46}$-6.

[2] Ibid., p. 8.

The fermentation of carbohydrates was carried out in a basal nutrient medium containing .5 per cent of the carbohydrate to be tested, with phenol red as an indicator. The carbohydrates were prepared by filtration[3], and added aseptically to the tubes of basal medium after they were sterilized. For the detection of gas production Durham fermentation vials were placed in the test tubes before sterilization.

After addition of the carbohydrates the tubes were inoculated with a uniform amount of a cell suspension of the organisms. This suspension was prepared by washing a 24 hour slant culture with sterile water. These tubes were incubated at 30 degrees and examined at 24 and 48 hours. The characteristics were the production of either an acid or alkaline reaction and the presence or absence of gas. In an acid reaction the indicator was yellow, if neutral the red color was unchanged, and if alkaline the medium became purple. Any gas produced by the isolates was detected by displacement of the medium in the Durham fermentation vials.

The ability to reduce nitrates to nitrites has assumed importance as a differential character in the identification if some bacteria. To test for this ability the isolates were inoculated into Bacto-nitrate agar and Bacto-nitrate broth.[4] These tubes were incubated at 37 degrees for 48 hours. After incubation they were examined for gas as shown by foam in the broth or cracks in the agar. They were next tested for nitrite by placing a few drops of the sulfanilic acid[5] and the alpha-naphthylamine[6] reagents into

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[3] Ibid., Leaflet II_{50}-5.

[4] Difco Laboratories, Difco Manual, p. 134.

[5] Sulfanilic Acid, 8 gms; 5N Acetic Acid, 1000 ml.

[6] Alpha-naphthylamine, 5 gm; 5N Acetic Acid, 1000 ml.

each tube of broth and on each agar slant. If the nitrate has been reduced to nitrite a distinct pink or red color appears. A control of uninoculated media was tested at the same time.

The methyl red and Voges-Proskauer tests were both performed on the same culture. The medium for this test was placed in large tubes in 10 ml. quantities, sterilized, and inoculated with the isolates. They were incubated for 72 hours at 37 degrees and tested for the cleavage of glucose with the production of acid and acetylmethylcarbinol. Although the same medium was used for both of the reactions the tests were performed in different tubes. Controls were tested also.

The methyl red test was performed by adding a small amount of the methyl red reagent[7] to 5 ml. of the culture and observing the color reaction. A positive test was when the culture was sufficiently acid to turn the methyl red a distinct red; a yellow color with the indicator was regarded as a negative test.

The test for acetylmetlylcarbinol (Voges-Proskauer) was performed by adding 5 ml. of the culture to an equal volume of 10 per cent solution of potassium hydroxide and allowing it to stand overnight. A positive test was denoted by the production of an eosin color layer in the upper part of the tube.

The citrate test is dependent upon the ability of the bacteria to utilize sodium citrate as a sole source of carbon in a synthetic medium. The medium, consisting of sodium ammonium phosphate, potassium phosphate, magnesium sulfate, sodium citrate, and bromthymol blue was inoculated with the isolates and incubated at 37 degrees for 48 hours. A positive test was growth of the organism giving an alkaline reaction which was evidenced by

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[7] Manual for Pure Culture Study of Bacteria, Leaflet V_{47}-20.

deep blue color in the medium. A negative reaction was the lack of growth, resulting from the inability of the organism to utilize the citrate as a sole source of carbon.

The medium used for gelatin liquification was Bacto-nutrient gelatin as prepared by the Difco Laboratories.[8] It was nutrient broth to which had been added 12 per cent gelatin. This medium was used chiefly to detect the proteolytic properties of bacteria measured by their ability to liquify gelatin. In performing the test the nutrient gelatin was dissolved in water and distributed into test tubes then sterilized. Each tube was inoculated with one of the isolates and incubated at 37 degrees and examined daily over a period of two weeks. To test for liquefaction the gelatin tubes were placed in the refrigerator for 30 minutes. They were then examined to see if on cooling, the contents remained in a liquid state or solidified. A positive test is denoted by the gelatin remaining in a liquid condition.

The milk used as a culture medium here was that prepared by the Difco Laboratories (Bacto-Litmus Milk). This is dehydrated skim milk to which has been added .5 per cent litmus as an indicator. The media was suspended in water, placed in tubes, and sterilized. Each tube was then inoculated with one of the isolates, incubated at 37 degrees and readings taken at 24 and 36 hours. The reactions that were important was the production of either acid or alkaline, the peptonization or coagulation of the milk and reduction of the indicator. Litmus will present a red color in acid, purple in alkali, and will be colorless if reduced by the organism. If peptonization takes place there will be a definite serum layer on the milk; coagulation of the casein takes place in strong acidity with a definite curdled appearance.[9]

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[8] Difco Laboratories, Difco Manual, p. 26.

[9] Manual for Pure Culture Study of Bacteria, Leaflet V_{47}-21.

Discussion of Results

On an examination of the physical properties of the isolates (table III) was recognized that the majority of these organisms were gram-positive, spore-forming bacilli. Only isolates 35B and 37A were gram negative and did not form endospores. All 18 of these isolates were rod-shaped and showed motility. Isolate 57 presented a sluggish movement and its motility was recorded as doubtful.

In the physiological characterization of bacteria the cleavage of carbohydrates is of major importance. A list of the carbohydrates and the action of the isolates on them may be found in table IV. Good growth was obtained on all of the carbohydrates, with all of the isolates attacking arabinose with the production of acid. It was noted that none of the organisms produced a noticeable amount of gas on any of the carbohydrates.

Arabinose, dextrose, maltose, and sucrose were attacked by all 18 of the isolates with either an acid or alkaline reaction. The remaining carbohydrates, ranked from those most widely used to those utilized by a few isolates, are: galactose and trehalose (17); glycerol, inulin and xylose (16); lactose and raffinose (10); rhamnose and dextrins (5).

Nitrates were reduced to nitrites and citrate utilized as a source of carbon by all of the isolates. Gelatin was liquified in all cases except by isolates 28 and 36. The methyl red test was negative in all instances. The Voges-Proskauer reaction was positive in all cultures except isolates 9, 25B and 36. Of the eighteen isolates tested on litmus milk, fourteen of them produced an alkaline reaction and peptonized the milk. Of the four not producing this alkaline reaction (35B, 37A, 54, 66) only one (54) showed coagulation of the milk and litmus reduction, which was a typical

TABLE III

PHYSICAL CHARACTERISTICS
OF THE ISOLATES

ISOLATES	GRAM REACTION	ENDOSPORES**	CELL MORPHOLOGY	MOTILITY
2	+	ST	Rods	+
6	+	T	Rods	+
9	+	C	Rods	+
15	V*	C	Rods	+
16	+	T	Rods	+
21A	+	C	Rods	+
21B	+	T	Rods	+
25B	+	C	Rods	+
28	V*	ST	Rods	+
35B	-	-	Rods	+
36	+	T	Rods	+
37A	-	-	Rods	+
41	+	T	Rods	+
52	V*	ST	Rods	+
54	+	ST	Rods	+
57	+	C	Rods	+
66	+	ST	Rods	+
71	+	C	Rods	+

* Variable.

** C (central), T (terminal), ST (subterminal), - (absent).

TABLE IV

PHYSIOLOGICAL CHARACTERISTICS

Isolate	Lactose	Inulin	Trehalose	Glycerol	Galactose	Raffinose	Maltose	Rhamnose	Xylose	Arabinose	Sucrose	Dextrose	Dextrin	Nitrates	V.* P.*	Citrate	Gelatin	Milk #
2	-	+	+	+	-	-	+	-	+	+	+	+	-	+	+	+	+	+
6	-	+	+	-	+	A	A	-	-	+	+	+	-	+	+	+	+	+
9	A	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+
15	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
16	-	+	+	+	+	A	A	-	+	+	A	+	-	+	+	+	+	+
21A	-	+	+	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+
21B	+	A	+	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+
25B	A	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+
28	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+
35B	A	A	A	A	A	A	A	A	A	+	A	A	A	+	+	+	+	PR
36	A	A	A	A	A	A	A	+	+	+	+	A	A	+	-	+	-	+
37A	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	PR
41	-	-	-	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+
52	A	A	+	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+
54	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
57	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	CR
66	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+	+	+	R
71	-	-	+	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+

A(alkaline), +(acid), -(neutral) for carbohydrates
*Voges-Proskauer Reaction (acetylmethylcarbinol)
#+(alkaline peptonization), CR(coagulation-litmus reduction)
R(reduction of litmus), PR(peptonization and reduction of litmus)

acid reaction in milk.[10] The other three (35B, 37A, 66) demonstrated the reduction of litmus to a colorless state. Isolates 35B and 37A peptonized the milk while 66 showed only litmus reduction.

The characteristics in Table III and Table IV place these isolates in the Class Schizomycetes and the Order Eubacteriales. They were further classified to the Family Pseudomonadaceae (Isolates 35B and 37A) and the Family Bacillaceae (all other isolates).

The Order Eubacteriales and Families Pseudomonadaceae and Bacillaceae are described by Bergey's Manual, page 65, as:

Order I. Eubacteriales Buchanan.
(Jour. Bact., 2, 1917, 162)

Simple and undifferentiated cells which are either spherical or rod-shaped. The rods may be long or short, straight or curved or spiral. Some groups or species are non-motile, others show locomotation by means of flagella. Elongated cells devide by transverse fission and may remain attached to each other in charms. Spherical organisms devide either by parallel fission producing chains, or by fission alternating in two or three planes producing thus either tetrads or cubes of 8 and multiples of 8 cells. Many spherical cells form irregular masses in which the plane of division cannot be ascertained. Endospores occur in some species. Some species are chromogenic, but only in a few is the pigment photosynthetic (bacteriochlorophyll or other chlorophyll-like pigments).

Family II Pseudomonadaceae Winslow et. al.
(Jour. Bact., 2, 1917, 555)

Cells without endospores, elongate rods, or more or less spirally curved. One genus (Mycoplana) has branched cells. Usually motile by polar flagella which are either single or in small or large tufts. A few species are non-motile. Gram-negative (a few doubtful gram-positive tests are recorded in Pseudomonas). Grow well and fairly rapidly on the surface of ordinary culture media excepting Methanomonas and some vibrios that attack cellulose. They are preferably aerobic, only certain vibrios including Disulfovibrio being anaerobic. Either water or soil forms, or plant and animal pathogens.

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[10] Difco Laboratories, Difco Manual, p. 141.

Family XII Bacillaceae Fisher
(Jahrb. F. Wiss. Bot., 27, 1895, 139)

Rod-shaped cells, capable of producing spores, other with peritrichous flagella or non-motile; monotrichous flagellation has been reported but is doubtful. Endospores are cylindrical, ellipsoidal or spherical, and are located in the center of the cell, subterminally or terminally. Sporangia do not differ from the vegetative cells except when bulged by spores larger than the cell diameter. Such sporangia are spindle-shaped when spores are central, or wedge-or drumstick-shaped when spores are terminal. Usually Gram-positive. Pigment formation is rare. Aerobic, microaerobic, or anaerobic. Gelatin is frequently liquefied. Sugars are generally fermented, sometimes with the formation of visable gas. Some species are thermophilic, ie. will grow readily at 53° C. Mostly saprophytes, commonly found in soil. A few are animal, especially insect, parasites or pathogens.

The isolates 35B and 37A were placed in the Family Pseudomonadaceae on the following characteristics: they are rod-shaped, Gram-negative, motile, aerobic organisms that grow well on ordinary culture media and produce no endospores. These isolates (35B and 37A) were further classified into the Genus Pseudomonas on the characteristics of absence of pigment, gelatin liquefaction, no visable gas from carbohydrates, and litmus reduction in milk with an acid reaction.

The Gram-positive organisms were classed as belonging to the Family Bacillaceae. These isolates (2, 6, 9, 16, 21A, 21B, 25B, 36, 54, 57, 66, and 71) besides being Gram-positive are rod-shaped organisms which are motile, aerobic, and produce endospores. The fermentation of carbohydrates without gas production and their aerobic nature placed them in the Genus Bacillus.

These isolates were further classed into collective groups. They were: (1) Bacillus subtilis group (2, 6, 16, 21A, 21B, 54, 57, 66, and 71). These isolates were placed in this group because of their action on carbohydrates, gelatin, nitrates, xylose, arabinose, and the production of acetylmethylcarbinol. (2) Bacillus megatherium group (9, 25B, and 36). The

characteristics of these organisms are very similar to the B. subtilis group. The main difference is that acetylmethylcarbinol is not produced.

Four of the isolates (15, 28, 41, and 52) were gram-variable. Their overall characteristics were those of the genus Bacillus. These organisms were placed in this genus and further identification was not attempted.

D. Action of Chemical and Physical Agents on the Production of Amylase as Indicated by the Iodine Test

In an attempt to accelerate growth and consequently, or, independently, increase the production of amylase, a variety of chemical and physical agents were tested to determine their effect.

The agencies employed here were as follows: (1) Chemical salts in varying concentrations. (2) Agitation of broth cultures during incubation. (3) Variation of pH of the substrate.

Methods and Results

1. Chemical salts in varying concentrations. The salts used were potassium chloride, potassium metabisulfite, sodium acetate, sodium sulfite, and sodium chloride. These salts were added to the synthetic basal starch medium[1] in the final concentrations of 0.1, 0.01, 0.001, and 0.0001 per cent. These media and a synthetic basal medium control, containing none of the above salts, were placed in large test tubes, sterilized, and inoculated. For the inoculation an agar slant was flooded with sterile water and the cells suspended. These cells were washed twice by using sterile water and a centrifuge. Inoculation was made by pipette, placing each culture into a tube of each concentration of the five salts tested and the control medium. These tubes were then incubated at 30 degrees for 36 hours and observations made for starch breakdown and any effect on growth.

Starch breakdown was tested by removing 1 ml. of the broth culture with a pipette, placing it into a Kahn test tube (7.5 cm. x 1 cm.), and adding

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[1] The sodium chloride was excluded for this exercise.

2 drops of Lugol's iodine solution. Growth was measured by turbidometric estimations of the increase in the denisty of the broth.

The concentrations of potassium chloride, potassium metabisulfite, sodium acetate, and sodium sulfite applied here did not produce an appreciable effect on either the growth of the hydrolytic activities of the isolates. However, the use of sodium chloride produced more favorable results in this type of action as is shown on Table V.

2. <u>Agitation of the cultures during incubation</u>. Each organism was grown on a starch agar slant and a washed cell suspension prepared as an inoculum. The medium employed was the synthetic basal starch medium and was used as a broth. This medium was placed into 250 ml. Erlenmeyer flasks in 100 ml. quantities, each flask was pipette inoculated with a washed cell suspension and placed on a rotary shaking machine.[2] The incubation temperature was 30 degrees and each culture was examined for growth and starch hydrolysis (iodine method) at 24 and 48 hours. An identical set of flasks were incubated under the same conditions, except for the agitation, as a control set. The results may be found in Table VI.

3. <u>Variation of the pH of the substrate</u>. The growth of the isolates and their amylolytic properties were tested at three different pH levels. These levels were 5.5, 7.0, and 8.5. The medium, the preparation of the inoculum, and method of inoculation was the same as that used with the salts.

The pH was adjusted by the use of 0.1 N hydrochloric acid and 0.1 N sodium hydroxide. The pH was determined by the use of a Beckman pH meter.[3] After sterilization a check was made on the various pH levels to determine any changes developing during the sterilization process. None were detected.

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[2] New Brunswick Scientific Co., New Brunswick, N. J.

[3] Beckman Glass Electrode pH Meter, Model H-2.

After incubation, 30 degrees for 36 hours, these tubes were tested by the iodine method (part 1 this section) and the results listed in Table VII.

Discussion

The stimulation of bacterial growth for enzyme production through the addition of salts has been relatively univestigated. Walton (1928) presented very few references on this subject prior to 1928. Kerr (1950), and Sumners and Myrback (1950), mentioned the use of electrolytes to produce an accelerator or depressor effect on amylase activity. However, this effect was on the action of the enzyme and not on its production by the microorganisms. The primary interest here was the influence on growth of amylase producing organisms and not on activity of the enzyme.

Potassium chloride, potassium metabisulfite, sodium acetate, and sodium sulfite were tested with negligible and inconclusive results. Sodium chloride showed a progressive increase in both growth and enzyme activity, i.e. increased concentration of sodium chloride produced an increase in growth and starch breakdown.

The agitation of the culture flasks on the rotary shaker presented encouraging results. In all cases the agitated cultures evidenced more growth and starch breakdown than did identical flasks incubated without agitation. Evidenced here, as was demonstrated with the sodium chloride, was the direct relation between starch breakdown and increase in growth. A further study of the effects of varying salt concentration and the effect of agitation on growth and amylase production would yield results which might be of practical importance.

On examination of Table VII it was found that these isolates were tolerant to the acidic and basic conditions tested. Those providing better

TABLE V

EFFECT OF SODIUM CHLORIDE ON GROWTH AND STARCH BREAKDOWN[4]

Organism	Concentration of NaCl*				Control**
	1	2	3	4	
15	+++	+++	+++	+++	++
16	+++	+++	+++	+++	++
2	+++	+++	+++	+++	+
57	+++	+++	+++	+++	+
71	+++	+++	+++	+++	+
6	++	++	+++	+++	+
21A	++	++	+++	+++	+
25B	++	++	+++	+++	+
52	++	++	+++	+++	+
66	++	++	+++	+++	+
9	+	++	+++	+++	+
21B	+	+	+++	+++	+
41	+	+	++	+++	+
35B	++	++	++	++	+
37A	++	++	++	++	+
36	+	++	++	++	+
54	+	++	++	++	+
28	+	+	++	++	+

*(1) 0.0001%, (2) 0.001%, (3) 0.01%, (4) 0.1%.
**Control contained no sodium chloride.

[4] Increased growth shows increased starch hydrolysis and vice versa.

TABLE VI

EFFECT OF AGITATION ON
GROWTH AND AMYLOLYTIC ACTIVITY

Isolate	Growth				Hydrolysis			
	1*		2**		1*		2**	
	24	48	24	48	24	48	24	48
16	++	+++	+	++	+	+	+	+
35B	+	+++	+	++	+	+	+	+
57	+	+++	+	++	+	+	+	+
2	++	+++	-	++	+	+	+	+
28	++	+++	-	++	+	+	-	+
9	++	+++	-	+	+	+	-	+
25B	+	+++	-	+	+	+	-	+
66	+	+++	-	+	+	+	-	+
6	+	++	-	+	+	+	-	+
57A	+	++	-	+	+	+	-	+
52	+	++	-	+	+	+	-	+
54	+	++	-	+	+	+	-	+
15	-	++	-	+	-	+	-	-
21B	-	++	-	+	-	+	-	+
21A	-	+	-	+	-	+	-	-
36	-	+	-	+	-	+	-	-
41	-	+	-	±	-	+	-	+
71	-	+	-	±	-	+	-	-

*Agitation

**No Agitation

TABLE VII

EFFECT OF pH ON GROWTH
AND AMYLOLYTIC ACTIVITY

pH	Isolate Number																	
	21A	25B	2	21B	6	41	66	9	28	35B	37A	54	57	52	16	71	36	15
5.5	+++	+++	+++	++	++	+	++	±	+	+	+	+	+	+	±	+	+	-
7.0	++	++	++	++	+	+	+	+	+	+	+	+	+	+	+	±	±	+
8.5	++	++	+	++	+	++	+	++	+	+	±	±	±	-	±	±	±	±

growth and more pronounced amylolytic activity at pH 5.5 were: isolates 2, 6, 21A, 25B, 36, 66, and 71. Of these organisms all were classified in the B. subtilis group except 25B and 36 which were placed in the B. megatherium group.

Isolates 15, 16, 37A, 52, 54, and 57 seemed to favor the pH 7.0. Representatives here of the B. subtilis group are 16, 54, and 57; the gram-variable group, 15 and 52; and the Family Pseudomonadaceae was 37A.

Two of the isolates grew better at pH 8.5. Of these isolate 41 was gram-variable and isolate 9 belongs to the B. megatherium group. A few of the organisms were not limited by the pH ranges used here. These were isolates 21B of the B. subtilis group, 28 of the gram-variable group, and isolate 35B of the Family Pseudomonadaceae.

The data in Table VII shows the effect of pH on growth and the ability of these organisms to hydrolyze starch. It can be seen that the majority of these organisms favor acidic conditions. However, there are a few which favor the pH 7.0 and two that show much better growth in the alkaline range. This information suggests that the optimum pH for growth and enzyme activity varies with the individual isolate.

IV. GENERAL DISCUSSION

In view of the data in this manuscript, as to the distribution of amylolytic organisms, it may be reasoned that meterials having starchy characteristics, or high carbohydrate content, are good sources of these organisms. This reasoning is supported by reports of Sjoberg (1921) and Peltier and Beckord (1945).

The purification of the isolates presented a problem. As has been reported with the cellulose decomposers (Groenewege, 1921; Omeliansky, 1913) some of the starch organisms exist in a very close relationship with each other. The nature of this relationship has been reported as symbiotic by most workers, but this statement is controversial. This association may well be commenselism, parasitism, or symbiosis.

The methods applied to purify these isolates are commonly employed to isolate colonies of different organisms foun in mixed cultures. These methods were: (1) the serial dilution method; (2) the streak plate method.

The serial dilution method for the isolation of bacteria is one of the oldest methods known to bacteriologists. The principle of its action is, that by use of a series of dilution tubes, and diluting a sample of the stock culture from one tube to another, eventually, individual cells will form distinct colonies. The data on Table II indicate that this method was inferior to the streak plate method as employed here.

The streak plate method was a means of procuring isolated colonies of these organisms. By repeated streaking onto a plate of medium well isolated colonies were obtained the use of gentian violet, tyrothricin, and

eosin-methylene blue agar, as streak plates, was an attempt to devise a means for the selective isolation of these organisms.

The bacteriostatic action of the gentian violet has been known for many years. Its usefulness is well recognized and recommended by the Manual of Pure Culture Study of Bacteria. Here was a method for obtaining the organisms belonging to the gram-negative group (35B and 37A) by inhibiting the gram-positive organisms.

The use of tyrothricin was designed to obtain any gram-positive, spore-forming bacilli present. This antibiotic, produced by the B. brevis group, is primarily effective against the gram-negative organisms and non-spore forming gram-positive organisms. This method was rather successful in obtaining the spore-forming bacilli.

The eosin-methylene blue agar is primarily a differential medium for the identification of organisms of the coli-aerogenes group. It is a test for gram-negative intestinal and soil bacteria which typically ferment lactose and sucrose. Only five isolates were obtained here, but they were from mixed cultures which previous attempts failed to separate.

By consulting Table II the conclusion may be drawn that, of the methods employed here, the streak plate method was the most effective for the purification of these mixed cultures. The media employed here played an important part in the disruption of the close relationship of some of these isolates. The use of gentian violet, tyrothricin, and eosin-methylene blue agar made it possible to break this association in many cases. There were a few cultures in which none of the methods employed were sufficient to break this association between these isolates.

The physical and physiological characteristics (Tables III and IV) were invaluable in the tentative identification of the isolates. A consideration of the physical characteristics immediately places these organisms into

three major groups. These are: (1) the gram-positive group, (2) the gram-negative group, and (3) the gram-variable group.

The observation of endospores in all the gram-positive and gram-variable organisms, and all of them being aerobic, motile rods, they were collectively placed into the Family Bacillaceae. The absence of endospores, in the gram-negative group, and the demonstration of motility aided in classifying these organisms in the Family Pseudomonadaceae. Further classification depends on the biological activities of the organism.

The more important physiological characteristics are: carbohydrate utilization without gas production, citrate utilization, gelatin liquefaction, action on milk, the formation of acetylmethylcarbinol, and nitrate reduction. These features aided immensely in placing the organisms in their proper genera.

The effects of salts, pH, and agitation on the growth and amylase activity of the isolates may be found in part D of the experimental section.

An overall function was observed in relation to sodium chloride and agitation. Both of these factors, applied separately here, produced a marked increase in bacterial growth and starch breakdown. This was an interesting observation and warrants further investigation. The sodium chloride may have influenced the activity of the amylase itself, causing increased breakdown of the starch with more energy becoming available to the bacteria. Such an "activation" of enzyme is known to occur with salivary amylase in the presence of the chloride ion.[1]

There was increased growth and enzyme activity noticed during agitation. Whether the increase was due to aeration, continual separation of the bacterial cells, or increased contact between the enzyme and substrate

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[1] Hawk, P. B., et. al., Practical Physiological Chemistry, p. 310.

surface is unknown. It was recognized that many devices for aeration of cultures in industry[2] and the laboratory[3] exist. Therefore, this influence may be due to the increased availability of oxygen.

The variation in the pH of the medium showed a wide range of results. The majority of the isolates grew better at pH 5.5, a few at pH 7.0, and only two at pH 8.5. Three of the isolates presented the same results at all three pH levels. All groups of the isolates—gram-positive, gram-variable, and gram-negative—were represented in all three pH levels. These results suggest that the optimum pH level for growth and enzyme production varies with the individual species and is not constant in any one group of isolates.

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[2] Prescott, S. C., and C. G. Dunn, p. 91.

[3] Beckord, L. D., G. L. Peltier, and E. Kneen, 1946.

V. SUMMARY

This thesis problem was the isolation and tentative identification of amylolytic bacteria on a synthetic basal medium, and determinations of the effect of various chemical and physical agents on the growth of the bacteria and the production and activity of the enzyme.

Of the various sources used for inocula it was found that the best sources for isolating this type of bacteria were soil around the grain elevator, silage, field soil, and rat feces from the grain mill. It was observed that in many cases the isolates were existing as a mixed culture under a very close relationship. For further investigation and identification these mixed cultures were purified. Various methods and media were used.

The organisms were isolated and identified by methods described in the *Manual for Pure Culture Study of Bacteria* and according to *Bergey's Manual of Determinative Bacteriology*. It was found that the majority of these organisms were of the Family Bacillaceae with only two belonging to the Family Pseudomonadaceae.

The chemical and physical agents tested were: (1) The effect of various concentrations of salts in the medium on the growth of the bacteria and their amylolytic activity; (2) The effect of agitation of the cultures on a rotary shaking machine during incubation as measured by starch hydrolysis and growth; (3) The effect of varying the pH levels on the growth and amylolytic activity of the isolates. The salts used gave negligible results, except for the sodium chloride. This salt demonstrated increased growth and enzyme

activity in direct relation to the increase in concentration. The amount of increase varied with the individual isolates. The agitation gave accelerated growth and starch hydrolysis with nearly every isolate. The different pH levels demonstrated variation in tolerance and optimum pH levels.

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