1965

Studies on amylases from Thermoactinomyces vulgaris

Mau-Jung Kuo
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd
Part of the Microbiology Commons

Recommended Citation
Kuo, Mau-Jung, "Studies on amylases from Thermoactinomyces vulgaris" (1965). Retrospective Theses and Dissertations. 3306.
https://lib.dr.iastate.edu/rtd/3306

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
KUO, Mau-Jung, 1933—
STUDIES ON AMYLASES FROM THERMO-
ACTINOMYCES VULGARIS.

Iowa State University of Science and Technology
Ph.D., 1965
Bacteriology

University Microfilms, Inc., Ann Arbor, Michigan
STUDIES ON AMYLASES FROM THERMOACTINOMYCES VULGARIS

by

Mau-Jung Kuo

A dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Bacteriology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa
1965
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>2</td>
</tr>
<tr>
<td><strong>PART I. ISOLATION, SCREENING, AND IDENTIFICATION OF AMYLASE-PRODUCING THERMOPHILIC ACTINOMYCETES</strong></td>
<td>6</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>7</td>
</tr>
<tr>
<td>ISOLATION</td>
<td>8</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>8</td>
</tr>
<tr>
<td>Results</td>
<td>9</td>
</tr>
<tr>
<td>PRELIMINARY SCREENING</td>
<td>12</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>12</td>
</tr>
<tr>
<td>Results</td>
<td>13</td>
</tr>
<tr>
<td>FINAL SELECTION OF THE ORGANISM</td>
<td>15</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>15</td>
</tr>
<tr>
<td>Temperature range for growth</td>
<td>15</td>
</tr>
<tr>
<td>Growth on synthetic medium</td>
<td>15</td>
</tr>
<tr>
<td>Standardization of the inoculum</td>
<td>15</td>
</tr>
<tr>
<td>Preparation of enzyme solution</td>
<td>16</td>
</tr>
<tr>
<td>Assay for amylase activity</td>
<td>16</td>
</tr>
<tr>
<td>Results</td>
<td>18</td>
</tr>
<tr>
<td>Temperature range for growth</td>
<td>18</td>
</tr>
<tr>
<td>Growth on synthetic medium</td>
<td>18</td>
</tr>
<tr>
<td>Standardization of the inoculum</td>
<td>18</td>
</tr>
<tr>
<td>Assay for amylase activity</td>
<td>18</td>
</tr>
</tbody>
</table>
IDENTIFICATION OF THE ORGANISM

Materials and Methods

Results
  Gelatin liquefaction
  Starch hydrolysis
  Cellulose decomposition
  Nitrate reduction
  Milk coagulation
  Growth in nutrient broth
  Growth on potato dextrose agar
  Growth on synthetic medium VII
  Growth on potato plug
  Growth on glucose asparagine agar
  Temperature range of growth
  Morphological characteristics

DISCUSSION

PART II. PRODUCTION OF AMYLASES

INTRODUCTION

INOCULUM

Materials and Methods
  Media for the production of spores and aerial mycelia
  Liquid medium for the build-up of inoculum
  Preparation of inoculum for the production medium

Results

Discussion

DETERMINATION OF OPTIMUM PHYSICAL CONDITIONS FOR AMYLASE ASSAY
Materials and Methods

Effect of pH on amylase activity 40
Effect of temperature on amylase activity 40
pH stability of the amylases 40
Temperature stability of the amylases 41

Results

Effect of pH on amylase activity 41
Effect of temperature on amylase activity 41
pH stability of the amylases 42
Temperature stability of the amylases 42

Discussion 51

SOME FACTORS AFFECTING THE PRODUCTION OF AMYLASES 53

Materials and Methods 53

Nitrogen sources 53
Carbon sources 54
Effects of C/N ratio 54
Determination of the optimum time of incubation 54
Effects of sequential addition of maltose and soluble starch to the medium 55
Effects of initial pH of the medium 55
Effects of different concentrations of calcium chloride 55
Effects of phosphate concentration 56

Results 56

Nitrogen sources 56
Carbon sources 56
Effects of C/N ratio 56
Determination of the optimum time of incubation 61
Effects of sequential addition of maltose and soluble starch to the medium 70
Effects of the initial pH of the medium 75
Effects of different concentrations of calcium chloride 80
Effects of different phosphate concentrations 81
Discussion 82

PART III. PURIFICATION AND SOME PROPERTIES OF THE AMYLASES OF T. VULGARIS 90

Introduction 91
Materials and Methods 91
Culture liquor 91
Amylase titration 91
Protein determination 92
Condensation of culture liquor 92
Protein precipitation with ammonium sulfate 92
Dialysis of enzyme solution 93
Removal of pigments 93
Treatment with organic solvents 93
Gel filtration 93
Amylase and protease assays for column chromatography 94
Ion exchange chromatography 94
Electrophoresis of the enzyme solution 96

Results 96
Condensation of culture liquor 96
Protein precipitation with ammonium sulfate 97
Dialysis of enzyme solution 97
Removal of pigments 97
Treatment with organic solvents 97
Gel filtration 104
Ion exchange chromatography 106
Electrophoresis of the enzyme preparation 122
Determination of optimum pH and temperature for amylase from peak A (Fig. 14) 123

Discussion 124

SUMMARY 137

LITERATURE CITED 139

ACKNOWLEDGMENTS 147
INTRODUCTION

Generally, thermophilic actinomycetes are amylase producers. They are found in soil, manure, peat, and other materials, growing together with mesophilic species. In most of these materials, competition for nutrients is keen. Yet through their thermophily, many species of this group of actinomycetes can be easily isolated and used for various studies.

In spite of the widespread distribution and ease of isolation of this group of organisms (Tendler and Burkholder, 1961; Kuster and Locci, 1963), no detailed studies have been made on amylases from any of these organisms. Most of the researches made so far have been related to antibiotic production. In view of these facts, it was thought to be of interest to study the amylases of thermophilic actinomycetes.

The present study was divided into three major parts, as follows:

1. Isolation and screening of amylase-producing, thermophilic actinomycetes from various sources. Selection of an isolate for use in further studies and identification of its taxonomic position.

2. Development of procedures favorable for maximum amylase yields.

3. Purification of amylase from the culture and studies on its properties.

It is evident that each objective listed above is itself an extensive topic and any results obtained in this research can only serve as a basis for further studies. It is hoped that in the future further developments in this field may grow out of this research.
REVIEW OF LITERATURE

Amylases are a group of enzymes capable of hydrolyzing such nutritive polysaccharides as starch and glycogen. They are distributed almost universally throughout the microbial, plant and animal kingdoms. Importance of starch and glycogen as major dietary carbon sources in nature has drawn intensive studies on this group of enzymes, and in recent years many amylases have been purified.

Most purified amylases from microbial sources so far belong to a group of enzymes known as $\alpha$-amylases. These enzymes have some characteristics in common; they are slightly acidic, water soluble, and contain one gram atom of calcium per mole of enzyme (Fischer and Stein, 1960). These enzymes generally have molecular weights around 50,000; however, the amylase from Bacillus stearothermophilus has an unusually low molecular weight of 15,600 (Manning and Campbell, 1961). The amylase from Bacillus subtilis in dimer form has a molecular weight of 100,000; it can be present in monomer form where the molecular weight is halved (Stein, 1957, Menzi, Stein, and Fischer, 1957). Stein and Fischer (1960) showed that this monomer-dimer transformation involved zinc, which served to bind two monomers to make a dimer.

Presence of calcium ion in the amylase molecule gives microbial $\alpha$-amylases resistance to denaturation by extreme pH, heat and chemical agents. The molecule also becomes resistant to the action of proteases (Stein and Fischer, 1958). Removal of calcium from amylases molecules results in liability of the enzyme to denaturation by heat, acid condition and urea, and attack by various proteases (Hagihara et al., 1956; Yamanaka and Higashi, 1957).
In the presence of alcohol, acetone, or ammonium sulfate, amylases are readily adsorbed to starch (Hagihara, 1958). This characteristic has been used extensively in the purification of these enzymes.

Alpha amylases have been considered to be endoamylases. The enzymes act randomly on the $\alpha-1\rightarrow4$ linkages, resulting in rapid diminution of viscosity and average molecular weight of the substrate. The primary products are oligosaccharides (dextrins) which are later broken down to yield maltose, some glucose, isomaltose, and branched-chain products of low molecular weight. The end products formed vary with the source of amylase, concentration of enzyme and substrate, and other factors. The $\alpha-1\rightarrow6$ linkage constituting the branching points of amylopectin and glycogen are not attacked by $\alpha$-amylases. The enzymes are so called because the reducing hemiacetal group liberated by their hydrolyses is in the $\alpha$ optical configuration.

Beta amylases, on the other hand, are exoamylases. They are capable of attacking the polysaccharides only from the non-reducing outer chain ends and do so in a regular manner, breaking every alternate bond to produce maltose. They are called $\beta$-amylases because the maltose produced is in the $\beta$-configuration. They are exclusively of vegetative and microbial origin. Gamma amylases, or glucamylases, are exoamylases of vegetative and microbial origin. Glucamylases break glucosidic bonds starting from the non-reducing end of amylose to produce $\alpha$-glucose and a shortened amylose chain. In both $\beta$ and $\gamma$ amylases, the action stops at the first branching point because these enzymes cannot split the $\alpha-1\rightarrow6$ bonds.

Some bacteria such as *Bacillus macerans* produce an amylase that acts on starch to produce Schardinger dextrins (Tilden and Hudson, 1942).
Cramer and Steinle (1955) showed that macerans amylase attacked the sixth glucosidic bond from the non-reducing end of the amylose chain, and produced a new glucosidic bond between carbon 1 of the sixth glucosyl unit and carbon 4 of the terminal glucosyl unit. According to Freudenburg and Cramer (1950) five distinct crystalline compounds may be obtained: the α, β, γ, s, and r dextrins. The α- and β- forms are obtained in far the greatest yield though over-all and individual yields vary according to the conditions of enzyme action (McClenahan, Tilden and Hudson, 1942). French and Rundle (1942) on the basis of X-ray data suggest that α-dextrin has a six-membered ring of glucose units and β-dextrin a seven-membered ring. The γ-dextrin has an eight-membered ring while s- and r-dextrins, known also as δ and ε dextrins, are addition products with higher alcohols.

There are not too many studies made on the amylases from the thermophilic microorganisms. Most of the studies made so far have been of bacterial origin. Thus Imsenecki, Solntzeva and Kuzyurina (1942) reported the isolation of amylase of Bacillus diastatieus at 60C, showing good liquefying activity at 100C and some saccharifying activity at 85C. Stark and Tetrault (1951) and Hartman, Wellerson, and Tetrault (1955) reported that a partially purified α-amylase from Bacillus stearothermophilus was active after 12hr. at 90C. Campbell (1954, 1955) purified and crystallized the α-amylase from the culture of Bacillus coagulaus. The optimum pH for the activity of this enzyme at 55C was found at 6.5-8.0. The enzyme obtained from the 55C preparation showed only 6-10% reduction in activity after 1 hr, whereas 35C preparations showed a 90-92% reduction in 1 hr.
Manning and Campbell (1961) isolated an α-amylase from the culture of Bacillus stearothermophilus. This pure enzyme was found to have the optimum temperature for activity at 55-70°C and optimum pH at 4.6-5.1. Calcium ion stimulated its activity. The enzyme in the native state existed as a semi-random or random-coiled well-hydrated molecule, with secondary structure due to the presence of disulfide bonds (Manning, Campbell, and Foster, 1961). Its amino acid composition and terminal group were also determined (Campbell and Manning, 1961; Campbell and Cleveland, 1961).

Studies on the amylases from actinomycete organisms, especially of thermophilic origin are relatively few. Hyslop and Sleeper (1964) reported purification of an α-amylase from the culture of Streptomyces albus, a mesophilic actinomycete. Some kinetic properties were reported. Fujii, Ookubo and Mikawajiri, (1963) reported isolation and crystallization of amylase produced by an Actinomyces species. Sakamoto and Shuzue (1958) studied the production of protease and amylase by representatives of the genus Streptomyces; 10 strains of Streptomyces were reported to utilize glucose, maltose, lactose, and sucrose as carbon sources for the production of protease and amylase. Gelatin, meat extract, peptone, wheat gluten and soybean casein were good nitrogen sources. Proteases and amylases were produced in large quantities when the C/N ratio was 5-10.

Though studies on the production and purification of amylases have been accumulated in great amount recently, yet no paper has been published on the amylase studies of the thermophilic actinomycetes. Many members of this group of organisms are known to hydrolyze starch, but no detailed study has ever been made on this subject yet.
PART I. ISOLATION, SCREENING, AND IDENTIFICATION OF AMYLASE-PRODUCING THERMOPHILIC ACTINOMYCETES
INTRODUCTION

In the studies of amylases from thermophilic actinomycetes, it was first necessary to isolate many strains from various sources and to examine these isolates for the quantity of amylases produced by each. Final selection of a strain for detailed investigation would then be possible. Since soil and composts are rich in thermophilic actinomycetes, these materials were used as the sources of isolates.
For the isolation of this group of microorganisms, advantage was taken of the fact that few mesophiles, which are causes of trouble during the isolation of certain actinomycetes at ordinary incubation temperature, grow at high temperature. The few colonies of Eubacteriales which develop at 55C have colonial characteristics different from those of actinomycetes, so those with features of actinomycetes may readily be selected for sub-culture.

Materials and Methods

Most of the soil samples listed in Table 1 were obtained from the Iowa State University Soil Testing Laboratory; however, Ringsted and Independence soil samples were collected directly from those localities in Iowa. Dung and compost samples were collected at various points in the locality of Ames, Iowa. Each sample was plated in the following manner: One gram air-dried soil or compost, which had been previously kept at 55C for two days to decrease the number of vegetative-cell contaminants, was suspended in 100 ml of sterile water and the dilution bottle was vigorously shaken for one minute to disperse the spores or mycelia. The bottle was kept stationary for a few minutes to allow the heavy material to sediment. Suitable serial dilutions were made in distilled water, and the dilutions were pipetted onto the surface of prepoured plates containing agar medium Ia of Tendler and Burkholder (1961). Plates were spread with a flamed and cooled glass hockey stick. Fifty plates were used for each sample in the isolation. After two days of incubation at 55C, the actinomycete colonies which had
developed on the plates were picked and streaked on another plate of medium Ia to which penicillin G had been added at a concentration sufficient to suppress the growth of gram positive bacteria. The isolates thus freed from contaminants were then subjected to the test for amylase production. This was performed by flooding the surface of the culture plate with Lugol's iodine solution. Formation of a colorless zone around the colonies indicated amylase production. Those actinomycetes that grew at 55°C and produced substantial zones of starch hydrolysis were saved for further investigation.

Results

The results are summarized in Table 1. It was noted that many isolates from the same source were very similar to one another in colony character and pigmentation. Only one isolate of apparently similar strains from the same sample was preserved in the collection. More colonies and fewer contaminants were obtained when samples were plated on partially dried plates than on freshly poured plates. For this reason, excess moisture on the surface of the solidified medium in the plate was removed by flaming the agar with a Bunsen burner. Uridil and Tetrault (1959) reported the desirable effect of partial desiccation of the culture medium on the isolation of thermophilic actinomycetes. A medium modified after that of Uridil and Tetrault (1959) by addition of 0.2% starch was used in some of the present isolation experiments and gave as satisfactory results as medium Ia.
The bacterial contaminants usually encountered in the experiments belonged to the genus *Bacillus*. Molds encountered were apparently of species of Ascomycetes.

Some media, such as AGS medium (El-Nakeeb and Lechevalier, 1963), modified Benedict's medium (Porter, Wilhelm, and Tresner, 1960), starch agar (0.5% starch) and glycerol asparagine agar II (Waksman, 1961), recommended for the isolation of aerobic actinomycetes, yielded less colonies than the two isolation media used routinely in the present study. Only a few isolates from medium Ia could grow on many of the afore-mentioned medium. Use of Czapek's nutrient agar resulted in too many contaminants that overgrew the organisms desired. Therefore, up to present, medium Ia (Tendler and Burkholder, 1961) and soy-typticase medium (Uridil and Tetrault, 1959) seemed to be the most favorable media for the isolation of thermophilic actinomycetes.

The thermophilic actinomycetes maintained on medium Ia did not produce blue or greyish-green pigments. This was demonstrated by the absence of such characteristic pigments on this medium by strains No. 58, 59, 60, and 61 of the collection, obtained from Purdue University and known to be good pigment producers.

Through this experiment, a collection of 118 isolates of amylolytic, thermophilic actinomycetes was built up; all other isolates were discarded. The collection did not contain an outstanding amylase producer. Many of the isolates showed almost the same extent of amylase activity with the crude iodine test employed in this experiment.
Table 1. Isolation of thermophilic actinomycetes from various sources

<table>
<thead>
<tr>
<th>Sources</th>
<th>Kind of material</th>
<th>No. of isolates</th>
<th>No. of amylase producers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISU Sheep Barn</td>
<td>Dung</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>ISU Dairy Farm 1</td>
<td>Compost</td>
<td>53</td>
<td>18</td>
</tr>
<tr>
<td>ISU Dairy Farm 3</td>
<td>Compost</td>
<td>61</td>
<td>20</td>
</tr>
<tr>
<td>ISU Swine Farm 3</td>
<td>Compost</td>
<td>50</td>
<td>11</td>
</tr>
<tr>
<td>ISU Swine Farm 4</td>
<td>Compost</td>
<td>34</td>
<td>4</td>
</tr>
<tr>
<td>ISU Swine Farm 5</td>
<td>Compost</td>
<td>63</td>
<td>15</td>
</tr>
<tr>
<td>ISU Swine Farm 6</td>
<td>Compost</td>
<td>31</td>
<td>5</td>
</tr>
<tr>
<td>Blairsburg 53062</td>
<td>Soil</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Boone 52995</td>
<td>Soil</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Boone 53429</td>
<td>Soil</td>
<td>56</td>
<td>1</td>
</tr>
<tr>
<td>Cambridge 53203</td>
<td>Soil</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td>Douds 52781</td>
<td>Soil</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Independence</td>
<td>Soil</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td>Jewell 53398</td>
<td>Soil</td>
<td>35</td>
<td>31</td>
</tr>
<tr>
<td>Nevada 53225</td>
<td>Soil</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>Penier 52890</td>
<td>Soil</td>
<td>93</td>
<td>8</td>
</tr>
<tr>
<td>Primghar 52860</td>
<td>Soil</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Ringsted</td>
<td>Soil</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>Ryan 53059</td>
<td>Soil</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Sac City 52864</td>
<td>Soil</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Sibley 53045</td>
<td>Soil</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>53325</td>
<td>Soil</td>
<td>70</td>
<td>12</td>
</tr>
</tbody>
</table>
PRELIMINARY SCREENING

With this collection of thermophilic actinomycetes at hand, it was necessary to select the better isolates for further studies on amylase production. Five criteria were established for further screening:

1. The organisms should be easy to maintain.
2. They should grow rapidly in the ordinary media.
3. They should be able to grow on the synthetic media.
4. They should be amylase producers.
5. They should have a wide temperature range for growth and be stable at these temperatures.

Materials and Methods

Tests for both 1 and 2 were performed using medium Ia. The incubation temperature was 55°C. A 5°C cold room was used for storage of the cultures. Those strains which grew rapidly at 55°C and which persisted at low temperature for more than three months without being subcultured to the new medium were selected for further studies. Those strains which satisfied the requirements 1 and 2 were then grown on media II and VII (Tendler and Burkholder, 1961); the latter is a synthetic medium and the former a complex medium.

For the amylase determination, a loopful of the spores from a slant culture on medium Ia, grown at 55°C for 3 days, was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of liquid medium VII (Tendler and Burkholder, 1961). The flasks were shaken at 120 r.p.m. on a rotary shaker (Model V, New Brunswick Scientific Co.) for 42 hr. at a temperature of
55°C. The culture was filtered through Whatman No. 5 filter paper and the final volume of the filtrate was brought back to the original 50 ml by the addition of distilled water. The Somogyi-Nelson procedure (Somogyi, 1938; Nelson, 1944) was used for the amylase titrations. This procedure measures the production of reducing substances from a starch substrate.

Those isolates which satisfied requirements 1 to 4 were subjected to a growth temperature study. Each isolate was inoculated onto slants of medium Ia, which were incubated at temperatures of 32, 37, 45, 55, 60, and 63°C. The slants were examined periodically for growth and sporulation.

Results

About four-fifths of the isolates in the collection satisfied requirements 1 and 2. Sixty isolates grew on medium VII. Only 11 isolates did not grow on medium II. One hundred and nine isolates produced amylases and 6 isolates did not grow in the medium; the remaining 3 isolates grew very slowly. Among the 109 amylases producers, 10 isolates were superior to all others in their amylase yields.

These ten isolates were checked for their growth characters and persistence at low temperature. All satisfied requirements 1 and 2. All grew on the synthetic medium VII, but two isolates grew slowly on it. When these 10 isolates were subjected to growth temperature experiments, all grew well at 45 and 55°C; only 3 grew at 63°C, one at 32°C and 6 at 37°C. Since requirements 1, 2, and 3 were satisfied by all of these isolates, those with high amylase yields, rapid mycelial growth and wide growth temperature range were chosen for further studies. These three isolates,
No. 5, 92, and 116, were found to possess the most desirable characteristics of all of the isolates studied.
FINAL SELECTION OF THE ORGANISM

By using these three isolates, No. 5, 92, and 116, comparisons were made for some physiological features so that final selection of one organism out of these three would be possible.

Materials and Methods

Temperature range for growth

The temperature range for growth was re-examined using spot inoculation of poured plates. Each petri plate contained 40 ml of medium Ia, the surface of which was dried by flaming before use. A loopful of spores was inoculated at the center of the plate. The inoculated plates were incubated at 32, 37, 45, 55, 60, and 63°C respectively for five days.

Growth on synthetic medium

Medium VII with and without 1.0% gelatin was used. The cultures were incubated at 55°C for five days. The amount of medium per plate and the method of inoculation were identical with those mentioned just above.

Standardization of the inoculum

For the quantitative comparison of amylase productivity of different isolates, it was necessary to standardize insofar as possible the amount and physiological state of the inoculum. Two methods were examined. In both methods, the organism was first grown in petri plates containing 40 ml of N-Z case medium (2% soluble starch, 0.5% N-Z case, 0.1% KH₂PO₄, 0.1% yeast extract, 0.05% MgSO₄, and 2% agar) for three days at 55°C. Aerial mycelia and spores were formed during the incubation. In method 1, 5
discs, cut out of the cultures with a sterile, 11 mm diameter cork borer, were transferred into 50 ml of the medium Ia broth in a 250 ml Erlenmeyer flask. These five discs contained, according to plate counts on Ia agar, approximately $10^7$ colony-forming units. In method II, Tanguay's procedure (1959) was used. The number of cells was adjusted by transferring a certain volume of the stock culture containing about $10^7$ cells into a flask containing liquid medium Ia. The cell suspensions prepared with this method were stored at -10°C before use.

**Preparation of enzyme solution**

An inoculated flask of Ia broth was shaken continuously on a shaker (120 r.p.m.) at 55°C for 24 hr. Five ml of the culture were then transferred into 50 ml of medium II or VII in a 250 ml Erlenmeyer flask. The flask was then shaken at 100 r.p.m. for 42 hr. at 55°C. The culture was then centrifuged to remove the inoculum discs as well as mycelia and the volume of the filtrate was brought back to 50 ml by the addition of distilled water. The pH of the culture filtrate was determined with a Beckman glass electrode pH meter.

**Assay for amylase activity**

In the first few experiments, the Somogyi-Nelson method was used and the amylase titer expressed in Somogyi units. In these experiments, for the convenience of the determination of amylase titer, a standard curve was first made using glucose (analytical grade, Fisher Scientific Co.). In later experiments, since studies were aimed mostly at the dextrinogenic characters of enzymes and also for the economy of time, a modified method
of Smith and Roe (1949), and Manning and Campbell (1961) was used. The method was as follows: A 1% solution of Lintner's soluble starch (Merck and Co.) was prepared in 1/15 M phosphate buffer, pH 5.9. To each of two test tubes of a set was added 5 ml of the buffered starch substrate, 3 ml of 1/15 M phosphate buffer (pH 5.9) and one ml of 0.5 M NaCl. The third tube of a set served as a reagent blank. The tubes were incubated in a 55°C water bath until equilibration of temperature was attained. To one tube containing starch was added 1 ml of suitably diluted culture filtrate. After 15 minutes of incubation, 2 ml of N HCl were added to the tube to stop the reaction. 1 ml of this culture filtrate was also added to the starch control tube and the reagent blank tube, respectively, and immediately 2 ml of N HCl were added to each. After thorough mixing, one ml of each of the 3 tubes was pipetted into separate 50 ml volumetric flasks containing 0.5 ml of N HCl and about 40 ml distilled water. Color was developed in each flask by the addition of 0.1 ml of an iodine solution (3.0% KI and 0.3% I₂). The contents of the flasks were brought to volume and thoroughly mixed. The resultant blue solutions were poured into colorimeter tubes and the absorbancies read at 620 mμ with a Bausch and Lomb Spectronic 20 Colorimeter. Enzyme activity was expressed by the equations:

\[
\frac{B-A}{B} \times (\text{mg of starch originally present}) = \text{mg of starch hydrolyzed}
\]

\[
\frac{B-A}{B} \times 100 = \text{per cent starch hydrolyzed}
\]

where B is the absorbancy of the starch control and A the absorbancy of the digest.
Results

**Temperature range for growth**

In all three isolates, no growth was observed at 32 and 63°C. Growth occurred from 37 to 60°C. All three strains appeared to have an optimum growth temperature of about 55°C.

**Growth on synthetic medium**

All three strains grew on the synthetic medium VII to some extent. Growth was promoted when 1% gelatin was added to the medium. Among the three cultures examined, No. 5 grew best, and No. 92 showed poorest growth.

**Standardization of the inoculum**

When method I was used, all of the isolates grew rapidly. However, when the plating-out method and mycelial dry weight measurements were compared, it was found that with plating-out method, deviations within the replicates of the same isolate were about ten to hundred fold while with mycelial dry weight measurements, they were about 5%. With method II, deviations either with the plating-out method or by mycelial dry weight measurement were less than those obtained with method I. This was true when the cell suspensions were freshly made. However, upon storage of the suspensions at -10°C the cultures decreased in viability, so less and less correlation was encountered between the results of plating-out and dry weight measurement.

**Assay for amylase activity**

The results are shown in Tables 2 and 3.
Table 2. Amylase production of three isolates of thermophilic actinomycetes in medium I\textsuperscript{a} Cultures were incubated at 55°C for 42 hr.

<table>
<thead>
<tr>
<th>Culture Number</th>
<th>5</th>
<th>92</th>
<th>116</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final pH</td>
<td>7.5</td>
<td>7.7</td>
<td>7.3</td>
</tr>
<tr>
<td>% Starch hydrolyzed\textsuperscript{b}</td>
<td>33.6</td>
<td>15.6</td>
<td>23.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Initial pH of the medium, 6.6 Glucose was replaced by 0.5% starch.

\textsuperscript{b}Each culture filtrate was diluted 5 fold with distilled water before assay.

Table 3. Amylase production of three isolates of thermophilic actinomycetes in medium VI

<table>
<thead>
<tr>
<th>Hours of incubation</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>%</td>
<td>pH</td>
<td>%</td>
</tr>
<tr>
<td>Culture No. 5</td>
<td>7.4</td>
<td>22.1</td>
<td>7.5</td>
<td>13.7</td>
</tr>
<tr>
<td>92</td>
<td>6.6</td>
<td>18.7</td>
<td>7.3</td>
<td>12.1</td>
</tr>
<tr>
<td>116</td>
<td>6.9</td>
<td>21.2</td>
<td>6.6</td>
<td>21.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Final pH of the culture Initial pH of all cultures, 6.9

\textsuperscript{b}Per cent starch hydrolyzed. Each culture filtrate was diluted 5 fold with distilled water before assay.
Data presented in these two tables indicated that isolates No. 5 and 116 were better amylase producers than No. 92, in media II and VI. Isolate No. 5 grew fastest of all three isolates and the pH of the medium changed more rapidly than with other isolates. Isolate No. 5 appeared to be the best strain of these three for further experiments because it had the following desirable features:

(a). Sporulated readily.

(b). Good amylase production.

(c). Easy maintenance of the culture.

(d). Fast and abundant mycelial growth.

(e). Grew on the synthetic medium.
IDENTIFICATION OF THE ORGANISM

Since isolate No. 5 had been selected as the organism to be used in further studies of the production and purification of amylases, it was desirable to identify its taxonomic position. According to Bergey's Manual of Determinative Bacteriology (1957), species of thermophilic actinomycetes are either classified in the genus Streptomyces or Thermoactinomyces. Some morphological and physiological examinations of the features of this isolate were, therefore, made to identify this organism.

Materials and Methods

The following media were used: nutrient gelatin (Manual of Microbiological Method, 1957), starch agar (2% soluble starch, 2% agar, 0.5% N-Z case, 0.5% KH$_2$PO$_4$, and 0.05% CaCl$_2$), cellulose medium (components identical with the starch agar except the soluble starch was replaced by 1.2% cellulose and the agar was omitted, nitrate broth (Manual of Microbiological Method, 1957), litmus milk (Manual of Microbiological Method, 1957), nutrient broth (Difco), synthetic agar medium VII (Tendler and Burkholder, 1961), potato plug and glucose asparagine agar. Unless otherwise mentioned, the culture was incubated at 55°C for 4-5 days. An 11 mm disc of the colony grown on medium Ia for 48 hr. was used to inoculate each tube of test medium.

Results

Gelatin liquefaction

Gelatin was completely liquified within 24 hr. after incubation. The
aerial mycelium of the colony was white and spores formed after 72 to 92 hr. of incubation.

**Starch hydrolysis**

Starch hydrolysis was observed on the area surrounding the colony. The enzymes produced were probably extracellular enzymes since the culture filtrate from 48 hr. broth cultures showed excellent amylase activity.

**Cellulose decomposition**

Cellulose was not used by the organism. There was slight growth of the organism on the cellulose-containing medium, but an equal amount of growth was obtained on the control basal medium. Therefore, the growth was ascribed to be because of non-cellulosic components in the medium.

**Nitrate reduction**

Nitrites were slowly produced from nitrate.

**Milk coagulation**

Coagulation and peptonization were observed within 24 hr.

**Growth in nutrient broth**

White to greyish white pellicles were formed on the surface of the medium.

**Growth on potato dextrose agar**

Abundant aerial mycelium was formed. Aerial mycelium was white to greyish white and powdery. Abundant spores were formed in 72 to 96 hr.
Growth on synthetic medium VII

Colonial development and sporulation were not as rapid as on potato dextrose agar. The aerial mycelium was white to yellowish brown and dark brown pigmentation was observed in the submerged hyphae.

Growth on potato plug

Abundant aerial mycelium and spores were formed. The aerial mycelium was white to light grey.

Growth on glucose asparagine agar

Abundant aerial mycelium was formed. The colony was light yellowish brown in color.

Temperature range of growth

As has already been discussed, no growth was observed at temperatures below 37°C and above 60°C.

Morphological characteristics

When the aerial hyphae and spores taken from the 72 hr. culture on potato dextrose agar were observed, spherical and ellipsoidal spores were borne singly at the ends of short branches. The size of the spores ranged from 0.4-1.0 μ in width and from 0.6-1.2 μ in length. The mean size of the spores was 0.6x0.7 μ. The sporophores were 0.5-1.5 μ in length. The width of the aerial hyphae was 0.4-0.8 μ.

The characteristics of isolate No. 5 were compared with descriptions of thermophilic actinomycetes given in Bergey's Manual (1957), Corbaz, Gregory, and Lacey (1963) and Erikson's papers (1953). The isolate closely resembled Thermoactinomyces vulgaris in morphological and physiological
characteristics. Some differences were observed between isolate No. 5 and published descriptions of *T. vulgaris* (Bergey's Manual, 1957). The isolate did not grow at temperature above 60°C and it also produced non-soluble yellow to brown pigments on synthetic media. The organism did not seem to fit into *T. thalophilus* nor *T. monosporus* since no growth was obtained at 37°C, the aerial mycelium was white to greyish white to yellow, and no soluble pigments were produced. In addition the isolate grew rapidly on the potato plug, while *T. thalophilus* and *T. monosporus* reportedly do not. The organism evidently was not a species of Streptomyces because its conidia were not formed in chains, as is characteristic of the members of this genus. The isolate, therefore, was assigned the species name, *T. vulgaris*.

To reaffirm its identity, the organism was sent to Lacey\(^a\) who confirmed the assigned epithet as being correct.

\(^a\)Lacey, Maureen E., Rothamsted Experimental Station, Harpenden, Hartfordsire, Great Britain. Identification of a strain of thermophilic actinomycetes sent by the author. Private communication. 1962.
DISCUSSION

There may be some questions about the criteria used to select an amylase-producing, thermophilic actinomycete for subsequent detailed study. These criteria were established with the purpose of obtaining, under defined experimental conditions, a few isolates which were suitable for further researches. Thus, isolate No. 5, which had been selected for detailed study, might not have been the most desirable amylase producer of the 118 isolates if another basal culture medium and other criteria had been used. For example, isolate No. 5 was later found to produce large quantities of proteases; the presence of these proteases was a great disadvantage during amylase purification.

Throughout the isolation of thermophilic actinomycetes from soil and compost, rather small numbers of colonies had been obtained with medium Ia. This medium was better than the rest of the media recommended by Tendler and Burkholder (1961), Erikson (1952), Corbaz, Gregory, and Lacey (1963), Kuster and Williams (1964), and El-NaKeeb and Lechevalier (1963). Medium Ia, however, seemed to be rather selective toward some thermophilic actinomycetes, since the colonies that developed on this medium were always similar to each other and none of the isolates produced green, grey, or orange pigmentation which are characteristic frequently reported for some thermophilic actinomycetes. The textures of the colonies obtained were very similar and none of the isolates produced any soluble pigments into the surrounding agar. However, some cultures obtained from other sources which produced green pigments in the original medium ceased to produce this pigment when transferred onto medium Ia agar, so it was still unknown
whether these similar colonies were actually closely related. Since the nutritional requirements of most of the thermophilic actinomycetes are unknown, it was not possible to estimate the selectivity of this medium.

During the isolation, no microscopic examination of each isolate was made, so it was not possible to state how many of the isolates belonged to the genus *Streptomyces* and how many to *Thermoactinomyces*.

Desiccation of the sample at 55°C seemed also to have exerted a selective effect on the isolation of the organisms. It may have been this rather than the medium Ta itself. When samples were held for two days at such a high temperature, vegetative cells of most microorganisms might have been destroyed. Only heat-resistant spores, chlamydospores, sclerotia, ascocarps and basidiocarps probably survived under this treatment. Addition of penicillin G to the medium helped to suppress the growth of *Bacillus species*. It also decreased the development of the number of thermophilic actinomycetes to the extent of about 15%. It was not known whether such treatment had eliminated some species of thermophilic actinomycetes.

*Taxonomy of thermophilic actinomycetes is still in a state of flux.* Waksman and Corke (1953) established a new genus, *Thermoactinomyces*, to encompass some species which possessed the features of *Micromonospora* except for growth at 50-65°C. There was controversy over the dependability of this thermophily as a good taxonomic character at the generic level (Corbaz, Gregory, and Lacey, 1963; Erikson, 1953; Tendler and Burkholder, 1961). Küster (1963) recently revised the taxonomy of *Streptomyces*. According to his criteria, isolate No. 5 should be placed in the genus *Thermoactinomyces*. The isolate coincided very well with the descriptions of *T. vulgaris* given by Erikson (1953) and Kuster and Locci
(1963). Until the taxonomy of thermophilic actinomycetes undergoes further revision, it would be reasonable to designate this isolate as *T. vulgaris*. 
PART II. PRODUCTION OF AMYLASES
INTRODUCTION

After the selection of an organism for the studies of amylases, it was necessary to find some favorable conditions for its growth and amylase production. Since no paper had been published on the requirements of T. vulgaris for amylase production, it was required to sort out some factors relating to the production of the enzyme and set up recommended procedures to obtain amylases. Efforts were, therefore, concentrated on the development of inoculum and medium which would give high yields of amylases.
INOCULUM

It is known that in other groups of organisms there is a close relationship between the age of the spore used as the inoculum and the saecharification power of the final beer (Aikawa, Shomatsu and Takahara, 1965). In T. vulgaris no study had been made on this subject. Since cells from logarithmic phase of growth were generally used as inoculum, experiments were carried out first to find the growth pattern of T. vulgaris.

Materials and Methods

Media for the production of spores and aerial mycelia

Through preliminary experiments, two solid media were developed. These were as follows: Medium A (A agar) consisted of 2% soluble starch (Malinkrodt), 1% N-Z case (Sheffield), 0.1% yeast extract (Difco), 0.1% KH$_2$PO$_4$, 0.05% MgSO$_4$·2H$_2$O, and 2% agar (Difco). The pH of A agar was adjusted to 6.3 with N HCl prior to sterilization of the medium 15 min. at 15 p.s.i. in the autoclave. Medium B (B agar) had the following components: 2% soluble starch, 1% N-Z case, 0.1% yeast extract, 0.1% KH$_2$PO$_4$, 0.1% CaCl$_2$, and 2% agar. The pH of B agar was adjusted to 6.3 with N HCl before autoclaving.

Liquid medium for the build-up of inoculum

The medium (C broth) contained 2% soluble starch, 1% N-Z case, 0.1% yeast extract, 0.05% D-glucose, 0.1% KH$_2$PO$_4$, and 0.05% MgSO$_4$·2H$_2$O. C broth was dispensed into 250 ml Erlenmeyer flasks which were covered with cotton-cheesecloth closures. Each flask contained 50 ml of broth. The pH of the medium was adjusted to 6.5 with N HCl after autoclaving.
Preparation of inoculum for the production medium

Stock cultures of the organism were maintained on plates of medium Ia which were stored at 4°C. Cotton swabs previously immersed in sterile distilled water were used to remove the spores and aerial mycelia from the surfaces of the stock culture plates and these cells were then smeared uniformly on the surfaces of flamed plates, each containing 40 ml of either A agar or B agar. These plates were incubated at 55°C for two days. Only those plates on the surface of which thick and uniform aerial mycelia and spores had developed were selected as a source of inoculum for broth cultures. Discs containing uniform growth were cut from the culture with an 11 mm diameter cork borer. By random sampling from different plates, 5 discs were inoculated into each flask of C broth. These inoculated flasks were placed on a shaker and shaken at 120 r.p.m. for certain time intervals at 55°C. Growth of the organism was determined either by obtaining the dry weight of mycelium per flask or viable count. To obtain the mycelial weight of the culture, the culture was filtered through Whatman No. 5 filter paper. The mycelial mass was then dried at 85°C until the weight became constant. For viable counts, appropriate dilutions of the cultures were spread on the surface of nutrient agar (Difco). The plates were incubated at 55°C for 24 hr. and the number of colonies was counted. Determination of the final pH of the culture was made by reading the pH of the filtrates with a Beckman glass electrode pH meter (Model H 2).

Results

When the cells were grown either in A or B agars, aerial mycelia rapidly developed within 24 hr. after inoculation. Observed from the
bottom of the plate, the whole culture was yellow. The reverse of the culture gradually turned light brown during another 24 hr. of incubation. By this time aerial mycelia began to fragment and form spores and the surface of the entire colony began to assume a dry, cracked appearance. By swabbing the surface of the plate with cotton swabs, many cells and spores could be detached from the colonies. This was not possible when the culture was only 24 hr. old. Another 24 hr. incubation increased the powdery texture of the surface of the colonies. Large numbers of spores were formed. This was the most favorable time for use of the culture as inoculum because great numbers of pellets could be formed when an inoculum from this stage of growth was introduced into C broth. Prolonged incubation of the culture, however, was always accompanied by initiation of lysis of cells and development of plaques which made the culture unsuitable for inoculum.

Three types of lysis were noted on the agar-containing media. The first type of lysis was a complete clearing of the whole culture. In this type of lysis, the aerial mycelia which developed on the surface of the medium lysed rapidly and uniformly over the entire plate. Within 24 hr. after inoculation, no more aerial mycelia remained. Further incubation of the culture did not result in outgrowth and development of resistant strains. This type of lysis was most commonly met when an old culture on medium Ia was used as an inoculum, but also occurred sometimes when only a 48 hr. old culture was used as the inoculum. Occasionally, complete clearing of the entire plate might happen on the 48 hr. culture grown in either A or B agar. The second type of lysis was marked by lysis of only the aerial mycelia at the central portion of the colony; mycelia at the
exterior portions remained unaffected or slightly affected. In many cases pin-point aerial mycelia might remain at the very center of the colony surrounded by a circular clear zone of lysis which in turn was surrounded by aerial mycelia. This kind of lysis occurred only when colonies were isolated from one another. Lysis usually began when the cells were grown for two days or more. The third type of lysis was the formation of plaques on plates. The plaques began to show up after 24 to 36 hr. of incubation. The plaques were small, circular, and clear (0.1 ~ 0.8 mm in diameter) and isolated from one another; the whole culture was never completely lysed. The size and plaque characteristics resembled very much those caused by phages No. 575 and 800 of Agre's (1961).

Preliminary studies on the growth of the cultures when 48 and 72 hr. inocula were used showed that there were no remarkable differences either in mycelial weights or viable counts of resulting cultures. The 72 hr. inoculum had some disadvantages in that germination of cells was sometimes delayed and chances of lysis of the culture increased. Since the 48 hr. culture yielded as large an inoculum as the 72 hr. culture and lysis was less frequent, this incubation period is recommended. Before the discs were cut out of the plates, all of which showed signs of lysis were eliminated.

When colony discs from A agar were used to inoculate C broth, the results shown in Table 4 were obtained.

When colony discs from B agar were used to inoculate C broth, the results shown in Table 5 were obtained.
Table 4. Growth of *T. vulgaris* in C broth when cells from A agar were used as the inoculum

<table>
<thead>
<tr>
<th>Hours of Incubation</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
<th>36</th>
<th>48</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycelial wt. (mg)/flask</td>
<td>0</td>
<td>17.1</td>
<td>109.4</td>
<td>115.3</td>
<td>38.4</td>
<td>19.6</td>
<td>19.6</td>
<td>9.1</td>
<td>7.4</td>
</tr>
<tr>
<td>Final pH</td>
<td>6.7</td>
<td>6.6</td>
<td>6.3</td>
<td>6.4</td>
<td>6.7</td>
<td>7.7</td>
<td>8.1</td>
<td>6.9</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 5. Growth of *T. vulgaris* in C broth when cells from B agar were used as the inoculum

<table>
<thead>
<tr>
<th>Hours of Incubation</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
<th>36</th>
<th>48</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycelial wt. (mg)/flask</td>
<td>0</td>
<td>14.6</td>
<td>81.8</td>
<td>103.5</td>
<td>109.1</td>
<td>95.2</td>
<td>88.9</td>
<td>74.4</td>
<td>50.0</td>
</tr>
<tr>
<td>Log. viable count/ml</td>
<td>4.25</td>
<td>5.47</td>
<td>6.98</td>
<td>7.17</td>
<td>6.77</td>
<td>5.74</td>
<td>4.87</td>
<td>3.70</td>
<td>2.48</td>
</tr>
<tr>
<td>Final pH</td>
<td>6.7</td>
<td>6.5</td>
<td>6.1</td>
<td>5.9</td>
<td>6.3</td>
<td>6.9</td>
<td>7.1</td>
<td>7.7</td>
<td>8.1</td>
</tr>
</tbody>
</table>

The growth of the organism in C broth with an inoculum from the culture of either A or B agar was characterized by the formation of pellets which increased in size and number as the incubation period was extended. By the time the pH of the culture began to rise after initial drop, no individual pellets could be detected, but masses of dispersed submerged hyphae were seen suspended in the culture. Light yellowish brown pigments were formed at this stage of growth. White powdery to cottony aerial mycelia usually appeared on the wall of the flask slightly above the culture level. When the cultures were incubated further, the culture gradually
decreased in viscosity and the mycelia became more fragmented and well dispersed, accompanied by increased brown pigmentation of the culture and decreased mycelial mass. The latter two characteristics could be noticed with the unaided eye. The pH of the culture continued to rise. When the pH of the culture was approximately 8, lysis of the culture became so marked that few mycelia could be recovered from the culture.

**Discussion**

Results shown in Table 5 indicated that this organism had a rapid phase of growth in the first 24 hr. of incubation when a culture from B agar was used as the inoculum, the resultant growth phase occurred during the first 18 hr. of incubation. No lag phase could be detected in both treatments. This might have been because of the very similar nature of the components of the agar and broth media, or because the lag phase of the organism was shorter than 6 hr. and escaped detection. Difficulties in determining growth of the organism were first encountered by the reliability of using either viable counts or mycelial weight of the culture as the criterion. As can be seen from the data in Table 5, there was good correlation between the viable counts of the culture and mycelial mass. However, in view of the filamentous nature of growth of the organism, sole use of viable counts as an index of growth was discontinued because it was impossible to distinguish the colonies developed from one cell from those developed from a hypha or masses of hyphae. The numbers of colonies developed from the unit volume of culture were not necessarily proportional to the numbers of cells actually present in the cultures. Determination of the growth of the organism based on the mycelial dry
weight seemed more reliable than the viable count because dry weights measured growth on a protophasmic basis. With this method, however, it was impossible to estimate the original number of cells present in 5 culture discs from A and B agar. To determine the original concentration of cells in these 5 discs, the results obtained by the viable count were compared with those from plating out an aqueous suspension of the discs following homogenization in a semi-micro Waring Blender for 3 minutes. The homogenized sample gave about 20 times as many colonies as those of the unhomogenized sample. Still it was impossible to estimate the actual number of cells participating in the growth. Cells in these 5 discs were, therefore, estimated to be in the magnitude of $10^5$ to $10^7$. It was obvious that swabbing of the plates with cells from cultures grown on medium Ia would result in large variations of numbers of cells on each plate. Therefore, careful selection of plates with both uniform distribution of aerial mycelia and similar thickness of hyphae on the surface of the solid medium was necessary. This procedure, paired with random sampling of discs from many such plates, probably resulted in the use of rather uniform amounts of inoculum for each flask of the liquid medium, so these flasks would be suitable for comparisons of different treatments. Standardization of the amount of inoculum by preparing the cell suspensions in glycerol (Tanguay, 1959) was not applicable to the present study because the cells and spores of T. vulgaris did not retain a high level of viability upon storage. Furthermore, because of the peculiar nature of the organism, it was difficult to obtain uniform distribution of heavy cell suspensions, so use of this method was abandoned.

Lysis of the organism during the preparation and building-up of
inoculum was a great disadvantage for further studies of amylase production. Two of the three types of lysis described above seemed to be due to autolysis. Henssen (1957) described a rapid autolysis of the vegetative mycelium in the thermophilic actinomycetes sometimes before aerial mycelium was formed. Webley (1958) observed an increasing alkalization of the medium, rising to pH 9, after 8-10 days in *T. vulgaris*. He suggested the rapid autolysis, particularly of the aerial hyphae, was due to this strong alkalization. Addition of 0.1-0.2% yeast extract to the medium retarded to some extent the autolysis of the aerial mycelium at high temperatures. The phenomenon of autolyzed aerial mycelium in an early stage was observed at high temperatures; it did not occur when the cultures were kept at low temperature (4°C) and occurred to a small extent only at room temperature. This was confirmed in the inoculum experiments of the present study, and for the preservation of *T. vulgaris* cultures, a 4°C cold room was, therefore, chosen. On the other hand, Locci (1963) described the autoinhibition process in *T. vulgaris*. The third type of lysis, that of plaque formation on the agar media, strongly indicated the lysogeny of isolate No. 5. Since this strain was isolated from soil and actinophages occur abundantly in nature, particularly in manures and soils (Gilmour and Butala, 1951), it was quite possible that isolate No. 5 was susceptible to actinophage infection. Some attempts were made to obtain a culture free of phage by using UV irradiation and acridine dyes, but these were unsuccessful. More detailed studies on the lytic nature of this organism would be stimulating.

It was also interesting to note the different responses of the organism toward A and B agars. These two media were different only in the
presence of some inorganic salts such as calcium chloride and magnesium sulfate. The organism grew faster and produced less acids in C broth when the inoculum was produced in A agar than in B agar. Whether this was due to the presence of magnesium ion rather than calcium ion in medium A was unknown; however, proteolytic enzymes from some thermophilic *Streptomyces* species are stabilized by the addition of Ca$^{++}$ (Mizusawa, Ichishima and Yoshida, 1964). Since microbial amylases also are stabilized by Ca$^{++}$, it was not easy to determine whether addition of Ca$^{++}$ ion would favor amylase production as well as prevention of lysis. Since Na$^+$, K$^+$, Mg$^{++}$, and Fe$^{++}$ have also been reported to activate proteolytic enzymes, no conclusion could be made at this stage. Detailed studies on the metal metabolism of the organism in relation to enzyme production would be an interesting subject for future study.

It was decided from these experiments that the recommended procedure for preparing standardized primary and secondary inocula would be: (1) Growth of the organism on A agar for 48 hr., (2) use of 5 culture discs (11 mm diameter) cut from selected plates of the primary inoculum per 250 ml Erlenmeyer flask containing 50 ml of C broth of pH 6.3. The flasks of secondary inoculum are shaken at 120 r.p.m. on the rotary shaker at 55°C for 12 hr. If B agar and C broth are used, the secondary inoculum should be incubated an additional 3 to 6 hr.

A agar, which was developed when the purification studies were almost over, seemed superior to B agar, both in pH control and mycelial growth. Though A agar had been used less often than B agar, the A agar is recommended for further amylase studies because, as seen in a later section, more amylases could be obtained when this medium was used.
Before any studies could be made on the nutritional requirements of the organism for improved amylase yields, it was mandatory to find the optimum temperature and pH for amylase activity. When these conditions were known, an amylase unit could be defined, thus, quantitative comparisons of different treatments would be possible. It was evident that no purified amylase was available at this stage, so properly diluted filtrate of the culture liquor had to be used. This liquor would contain various sorts of enzymes related or unrelated to amylases as well as other impurities which might interfere with determination of amylase activity. More critical and quantitative experiments would be necessary as soon as any purified amylases could be obtained.

Materials and Methods

A medium with the following components was used for the production of amylases to be used in the determination of optimum pH and temperature for enzyme activity: 2% soluble starch, 0.5% N-Z case, 0.1% yeast extract, 0.1% KH₂PO₄, and 0.5% CaCl₂. The pH of the medium was adjusted to 6.3-6.4 with N HCl. After autoclaving, each 250 ml Erlenmeyer flask containing 50 ml of broth received 5 ml of secondary inoculum. The flasks were shaken at 100 r.p.m. for 48 hr. in a 55°C incubator. Mycelia were removed by centrifugation. The pH of the supernate was observed. An aliquot of this supernate was diluted properly and its amylase titer determined by the dextrinogenic method described in Part I of this thesis. If necessary, mycelial dry weights were determined using the method mentioned in the
previous section, Part II of this thesis. The above procedure, from the introduction of 5 ml of inoculum into the described medium to the determination of mycelial dry weight, was used as the standard procedure throughout the later experiments unless otherwise mentioned. Each treatment was prepared in triplicate and results given are the mean values of these triplicates.

**Effect of pH on amylase activity**

For the pH range of 5.3 to 8.0, 1/15 M phosphate buffer (Senya and Kondo, 1958) was used. For the pH range below 5.0, a 1/10 M acetate buffer satisfied the purpose. The whole procedure was identical with that described in Part I of this thesis, except the initial pH of the reaction mixture varied.

**Effect of temperature on amylase activity**

Water baths were used for different temperature treatments which ranged from 40 to 75°C. Phosphate buffer, 1/15 M, pH 5.9, was used in all treatments.

**pH stability of the amylases**

Acetate and phosphate buffers of different pHs were prepared in the same way as described two paragraphs previously. Properly diluted culture liquor was mixed with each buffer. All of the tubes containing enzymes were incubated at 60°C. At certain time intervals, samples were removed from these tubes and were pipetted into starch-buffer tubes as well as tubes of plain buffer. Both sets of tubes contained phosphate buffer of pH 5.9. The tubes were incubated at 60°C for 15 minutes before amylase titrations were made.
Temperature stability of the amylase

Tubes containing diluted culture liquor in phosphate buffer of pH 5.9 were placed in water baths maintained at 60, 70, 80, and 90°C. After appropriate time intervals, samples were taken from the amylase preparations and amylase assays were made at 60°C for 15 min. at pH 5.9.

Results

Effect of pH on amylase activity

The results are shown in Fig. 1. Amylase activity was not detected at pH 4.0. Slight activity was recognized when the pH was maintained at 4.4. Maximum enzyme activity was shown to be within the pH range of 5.6-6.5. A pH of 5.9 was optimum. Considerable amylase activity still could be detected at pH 8.

There was good coincidence of the amylase titers at pH 5.6 and 5.9 where the two buffer systems overlapped. This supported the validity of selecting a buffer system of pH 5.9 as the optimum condition for the enzyme activity. In the subsequent titration of amylases, therefore, this pH was used in all experiments.

Effect of temperature on amylase activity

The optimum temperature for amylase activity in the culture filtrate was found to be around 60°C, as seen in Fig. 2. In later experiments 60°C was selected as the temperature for the incubation of tubes in amylase assay.
**pH stability of the amylases**

The results are shown in Fig. 3. As expected, the amylase activity was very stable at pH 5.9. The enzymes were readily inactivated in alkaline media. With prolonged incubation, they were inactivated faster in the buffer of pH 6.5 than those adjusted to 5.6. Visual observation of the tubes containing enzymes showed rapid denaturation of proteins first in the pH 7.4 buffer; an increase in turbidity of the tubes could be observed within half an hour of incubation. The proteins soon precipitated. In the buffer systems of pH 4.6 and 7.0, flocculent particles appeared throughout the solution within an hr.; a fluffy precipitate formed during the next hour of incubation. Lesser quantities of a similar type of precipitate were formed at pH values of 6.5 and 5.9 after 3½ hr. of incubation. In the buffer of pH 5.6, protein precipitation was slowest and least pronounced.

The nature of these precipitates was not studied, but they probably consisted of protein at least in part, including denatured amylases.

**Temperature stability of the amylases**

As is shown in Fig. 4, the amylases were more stable at 60°C than at higher temperatures. No amylase activity could be detected in the tubes incubated at temperatures higher than 70°C for more than one hr. At these high temperatures, denaturation of the proteins was visible when the time of incubation was prolonged for more than half an hour.
Fig. 1. The effect of pH on amylase activity. Crude culture supernate was used as the enzyme source.
The graph shows the relative amylase activity (%) as a function of pH. The activity is measured in two buffer systems: acetate buffer and phosphate buffer. The pH range is from 3.5 to 8.0. The graph indicates that the amylase activity is highest in the range of pH 5.0 to 6.0.
Fig. 2. The effect of temperature on amylase activity. Crude culture supernate was used as the enzyme source.
Fig. 3. The pH stability of the amylases of *T. vulgaris*
Amylase activity at pH 5.9 (0 hr.) was taken as 100%
and the rest of the data converted to relative values
Crude culture supernate was used as the enzyme source
Fig. 4. The temperature stability of the amylases of *T. vulgaris*. Crude culture supernate was used as the enzyme source.
Discussion

When any amylase assays are made with the culture filtrate of the organism, it has to be realized that the results only reflect the net activity of all amylases present. The optimum pH and temperature determined for the amylase activities, therefore, do not reveal the properties of any individual amylase but rather exhibit the features of the pooled amylases in the filtrate. In spite of these reservations, it was necessary to define the amylase unit to be used in later experiments based on the best data on hand. An amylase unit was defined as the amount of enzyme which hydrolyses one mg of starch per minute at 60°C at pH 5.9. This unit was just one-tenth value of the amylase unit proposed by Manning and Campbell (1961). The reason of setting up this new unit was because the culture filtrate did not contain sufficient amylase activity to be expressed with their unit. The reason of using dextrinogenic method in the amylase assay was based on the priority given to α-amylase in the system.

When close examination was made of the data presented in Fig. 3 and Fig. 4, it could be noted that even under optimum conditions for the amylase activity, there were marked decreases with time in the amylase titers. Although the complex nature of the culture filtrate would not permit any simple interpretation of these results, it might be an interesting speculation to consider the proteases present in the filtrate as one of many factors involved in inactivation of amylases. The presence of proteinases had been demonstrated by the rapid gelatin liquefaction by the organism as mentioned in Part I, as well as the casein hydrolysis observed when aliquots of culture filtrate were added to casein solution. Viewed from the
substrate specificity and thermostability, proteases from some other species of Actinomycetes have broad spectrum and are resistant to high temperature (Krassilnikov, 1938; Krassilnikov and Koreniako, 1938; Nomoto, Narahashi, and Murakani, 1959 a, b; Miyasawa, Ichishima, and Yoshida, 1964). If this is the case with T. vulgaris proteases, separation of these enzymes from the amylases should be of primary concern in later purification studies of amylases.
SOME FACTORS AFFECTING THE PRODUCTION OF AMYLASES

After procedures for the preparation of inoculum and amylase assay had been standardized, factors influencing amylase production were investigated. Since no report had been published on amylase production by thermophilic actinomycetes and many factors might affect the yield of enzymes produced, a few of the major variables were selected for study. Major emphasis was given to the nutritional requirements of the organism in relation to its amylase production.

Materials and Methods

Nitrogen sources

In this series of experiments, various nitrogen sources were tested for their efficiency in supporting amylase production.

The basal medium contained 2% starch, 0.1% yeast extract, 0.1% KH₂PO₄, 0.05% CaCl₂, and 0.05% D-glucose. The following nitrogen sources were used: N-Z case (Sheffield Co.), Trypticase (B.B.L.), polypeptone (B.B.L.), bactopeptone (Difco), thiotone (B.B.L.), phytone (B.B.L.), potassium nitrate, ammonium sulfate, and ammonium nitrate (all analytical grade). The organic nitrogen sources were added to the basal medium at the 1% level, while the inorganic compounds were used at concentrations equivalent to the nitrogen contents of the organic sources, assuming that the nitrogen content of the organic sources was 16%. A standard procedure was followed, as described in Part II of this thesis.
Carbon sources

The components of the basal medium in this series of experiments were: 1% N-Z case, 0.1% yeast extract, 0.1% $\text{KH}_2\text{PO}_4$, 0.05% CaCl$_2$, and 0.05% D-glucose. The following carbon sources were examined at the 2% level: glucose, mannose, galactose, fructose, sucrose, maltose, mannitol, sorbitol, lactose, cellobiose, inulin, cellulose, and soluble starch (Mallinkrodt Co.). All of these carbohydrates were analytical grade. Each carbohydrate was dissolved in distilled water, was sterilized by filtration through a Seitz filter, and was added to sterile basal medium. Basal medium devoid of added carbohydrate was used as a control. The pH of each medium was adjusted to 6.4 with either NHCl or NaOH before the media were inoculated.

Effects of C/N ratio

The basal medium was the same as described in the previous section except the level of N-Z case was varied. Levels of starch (1.0, 1.5, 2.0, and 2.5%) were added to the basal medium in appropriate combinations so each carbohydrate and each nitrogen level was tested against each other. The initial pH of all of the media was adjusted to 6.3-6.5.

Determination of the optimum time of incubation

The basal medium was the same as that used in the previous section. Either soluble starch or maltose was used as the carbon source at the 2% level, along with 1.0% N-Z case. The cultures were shaken for either 36 or 48 hr. In the former case, a 12 hr. culture of C broth inoculated with colony discs from A agar served as the secondary inoculum, while in the latter case an 18 hr. culture of C broth inoculated with discs from B agar
was used.

**Effects of sequential addition of maltose and soluble starch to the medium**

Since maltose and soluble starch had different effects on the cultures, experiments were designed to discover the effects of addition of either maltose or starch to the cultures after certain periods of incubation. The starting medium was the basal described previously with the addition of 1.0% N-Z case and either maltose or starch. Addition of carbohydrates to the cultures was made 12, 24, or 36 hr. after inoculation. The culture was harvested 36, 48, 60, or 72 hr. after inoculation. Culture discs from B agar were used to inoculate C broth and after 18 hr. of growth, the latter culture was used as inoculum to inoculate all the media used in this experiment.

**Effects of initial pH of the medium**

Two per cent soluble starch and 1.0% N-Z case were added to the basal medium mentioned previously. The pH of the medium was adjusted, after sterilization, by using N NaOH and N HCl. An 18 hr. culture in C broth was used as the inoculum.

**Effects of different concentrations of calcium chloride**

The effect of calcium chloride on amylase production was studied using the basal medium described previously containing 2% soluble starch and 1% N-Z case. Calcium chloride was added at levels of 0.0, 0.05, 0.1, 0.2, and 0.3%, respectively. As inoculum, 18 hr. culture in C broth was used.
Effects of phosphate concentration

The components of the liquid medium and method of inoculation were identical with those mentioned just above, except that 0.05% calcium chloride and different levels of $\text{KH}_2\text{PO}_4$ (0.0, 0.1, 0.2, and 0.3%) were used. The initial pH of the medium was adjusted to 6.4 with $\text{N NaOH}$. An 18 hr. culture in C broth was used as the secondary inoculum.

Results

Nitrogen sources

The results are shown in Table 6. The three inorganic nitrogen sources examined were inadequate for substantial amylase production. Mycelial growth was also very poor in these media. On the other hand, the complex nitrogen sources supported good mycelial growth and amylase production. N-Z case and trypticase were the best of the six nitrogen sources examined for support of amylase production. Though no quantitative determination of mycelial growth was made in this experiment, visual observation of each culture indicated good mycelial growth in all the culture containing complex nitrogen sources except those containing thiotone. There seemed to be good co-relations among the amylase titer, pH change, and mycelial growth of the culture.

Carbon sources

The data given in Table 7 shows that both maltose and soluble starch were good sources for amylase production. The remaining carbohydrates did not induce substantial amylase production. Abundant mycelial growth was observed in the media containing fructose, mannitol, maltose, or soluble
Table 6. Effects of various nitrogen sources on the production of amylases by *T. vulgaris* No. 5 grown for 48 hr. at 55°C

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>36 Hours</th>
<th>48 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final pH</td>
<td>Amylase units</td>
</tr>
<tr>
<td>Bactopeptone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N-Z case</td>
<td>6.7</td>
<td>16.37</td>
</tr>
<tr>
<td>Phytone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Polypeptone</td>
<td>6.7</td>
<td>14.42</td>
</tr>
<tr>
<td>Thiotone</td>
<td>5.7</td>
<td>4.82</td>
</tr>
<tr>
<td>Trypticase</td>
<td>6.9</td>
<td>12.64</td>
</tr>
<tr>
<td>KNO₃</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>None</td>
<td>6.0</td>
<td>0.77</td>
</tr>
</tbody>
</table>

*An 18 hr. culture in C broth was used as the inoculum.*

Starch. The final pHs in these cultures were between 5.5-7.0. The pH of the cultures containing other carbohydrates were high and mycelial yields were less than those recorded for fructose, mannitol, maltose, or soluble starch. Mycelial growth, as well as slight amylase production, was observed in the control medium; this positive effect was ascribed to the presence of some nutrients in the yeast extract added.

It could not be concluded from the data in Table 7 that those carbohydrates which did not facilitate amylase production in 36 hr. cultures would also be poor amylase inducers if the cultures were grown for periods
Table 7. Effects of various carbohydrates on the production of amylases by T. vulgaris No. 5 grown for 36 hr. at 55°C

<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>Final pH</th>
<th>Mycelial weight per flask (mg)</th>
<th>Amylase units</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>5.6</td>
<td>162.7</td>
<td>3.92</td>
</tr>
<tr>
<td>D-galactose</td>
<td>7.8</td>
<td>113.6</td>
<td>1.17</td>
</tr>
<tr>
<td>D-mannose</td>
<td>7.9</td>
<td>117.7</td>
<td>1.94</td>
</tr>
<tr>
<td>D-fructose</td>
<td>5.9</td>
<td>239.7</td>
<td>1.94</td>
</tr>
<tr>
<td>Lactose</td>
<td>8.1</td>
<td>121.3</td>
<td>3.92</td>
</tr>
<tr>
<td>Maltose</td>
<td>6.4</td>
<td>260.2</td>
<td>37.65</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8.2</td>
<td>149.3</td>
<td>2.31</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>7.8</td>
<td>112.5</td>
<td>0.00</td>
</tr>
<tr>
<td>Mannitol</td>
<td>5.6</td>
<td>262.2</td>
<td>1.88</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>8.2</td>
<td>138.0</td>
<td>2.07</td>
</tr>
<tr>
<td>Inulin</td>
<td>8.0</td>
<td>132.4</td>
<td>1.54</td>
</tr>
<tr>
<td>Soluble Starch</td>
<td>6.9</td>
<td>196.4</td>
<td>53.67</td>
</tr>
<tr>
<td>None</td>
<td>8.1</td>
<td>104.2</td>
<td>2.75</td>
</tr>
</tbody>
</table>

A 12 hr. culture in C broth was used as the inoculum. Initial pH of the medium, 64

of time sufficiently short so that the pH would remain near neutrality. Through previous experiments, a final pH of 7.5 or less was found to be essential for the production of high amylase yields. In a subsequent experiment, therefore, cultures were incubated for only 24 hr. so pH changes would not be as drastic as in the 36 hr. cultures. The results showed that
none of the monosaccharides was a good inducer of amylase production, even though the pHs of the cultures mostly remained between 5.5 to 7.5. Maltose and soluble starch again were found to be good carbon sources for mycelial growth and amylase production. Nevertheless, the enzyme yields were less in the 24 hr. than in the 36 hr. cultures. The same trend was also noticed with other carbohydrates listed in Table 7.

Glucose and fructose were unique among the monosaccharides in that the pHs of the cultures remained below 7.0, even after 48 hr. incubation. Increases in the pH of culture containing either of these sugars were slow, even when rather rapid increases in mycelial weights were observed. This suggested that inclusion of either of these sugars in the medium might help to facilitate mycelial growth as well as pH control of the medium.

Effects of C/N ratio

From the above experiments it was learned that both maltose and soluble starch were good carbon sources for amylase production. Soluble starch was a better amylase inducer than was maltose, though pH control of cultures containing starch was more difficult. In this experiment, starch and N-Z case were chosen to find the optimum C/N ratio for amylase production. The results are shown in Table 8. Statistical analysis showed that levels of both starch and N-Z case had significant effects on amylase production. There was effect of interaction between starch and N-Z case on the yield. These effects were significant at the 1% level, as seen in Table 9.
Table 8. Effects of C/N ratio on the production of amylases from *T. vulgaris* No. 5 grown at 55°C for 48 hr.\(^a\)

<table>
<thead>
<tr>
<th>Starch (%)</th>
<th>N-Z Case (%)</th>
<th>Final pH</th>
<th>Amylase units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.5</td>
<td>7.3</td>
<td>5.02</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>8.4</td>
<td>5.87</td>
</tr>
<tr>
<td>1.0</td>
<td>1.5</td>
<td>8.6</td>
<td>3.90</td>
</tr>
<tr>
<td>1.5</td>
<td>0.5</td>
<td>7.0</td>
<td>19.70</td>
</tr>
<tr>
<td>1.5</td>
<td>1.0</td>
<td>8.3</td>
<td>5.80</td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
<td>8.5</td>
<td>5.12</td>
</tr>
<tr>
<td>2.0</td>
<td>0.5</td>
<td>5.9</td>
<td>23.35</td>
</tr>
<tr>
<td>2.0</td>
<td>1.0</td>
<td>7.3</td>
<td>47.23</td>
</tr>
<tr>
<td>2.0</td>
<td>1.5</td>
<td>8.0</td>
<td>24.18</td>
</tr>
<tr>
<td>2.5</td>
<td>0.5</td>
<td>7.7</td>
<td>43.07</td>
</tr>
<tr>
<td>2.5</td>
<td>1.0</td>
<td>7.8</td>
<td>41.35</td>
</tr>
<tr>
<td>2.5</td>
<td>1.5</td>
<td>7.5</td>
<td>36.18</td>
</tr>
</tbody>
</table>

\(^a\)An 18 hr. culture in C broth was used as the inoculum. Initial pH of the medium, 6.4
Table 9. Analysis of variance of the effect of starch versus N-Z case at different levels. The data were transformed to log (X + 1) for analysis.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>23</td>
<td>5.6962</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>11</td>
<td>5.2812</td>
<td>0.4801</td>
<td>13.8757**</td>
</tr>
<tr>
<td>Starch</td>
<td>3</td>
<td>0.8747</td>
<td>0.2916</td>
<td>8.4278**</td>
</tr>
<tr>
<td>N-Z case</td>
<td>2</td>
<td>1.3723</td>
<td>0.6862</td>
<td>19.8324**</td>
</tr>
<tr>
<td>Starch x N-Z case</td>
<td>6</td>
<td>3.0342</td>
<td>0.5057</td>
<td>14.6156**</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>0.4150</td>
<td>0.0346</td>
<td></td>
</tr>
</tbody>
</table>

**Significant at the 1% level

The above results indicated that best amylase yields could be obtained when the C/N ratio was 2 with the amount of starch at the 2% level. When starch was added at the 1% level, even at a C/N ratio of 2, the amylase yield was low. Increase of the N-Z case level not only facilitated the pH increase of the culture but also lysis of mycelia.

**Determination of the optimum time of incubation**

The results are shown in Fig. 5, 6, 7, and 8. All of these figures except Fig. 6 indicated that the highest amylase yield could be obtained when the pH of the culture reached near neutrality. Further increases of the pH above 7.5 resulted in sharply reduced yields. Based upon mycelial growth, amylase production was at its peak when the culture reached the maximum phase of growth. When maltose was compared with soluble starch, it
Fig. 5. The amylase titer and pH of *T. vulgaris* cultures at different times of incubation. The carbon source was 2% soluble starch. An 18 hr. culture in C broth was used as the inoculum.
Fig. 6. The amylase titer and pH of *T. vulgaris* cultures at different time of incubation. The carbon source was 2% maltose. An 18 hr. culture from C broth was used as the inoculum.
UNITS OF AMYLASE ACTIVITY PER ml.

HOURS OF INCUBATION

2% MALTOSE

pH OF THE CULTURE
Fig. 7. The amylase titer and pH of *T. vulgaris* cultures at different time of incubation. The carbon source was 2% soluble starch. A 12 hr. culture from C broth was used as the inoculum.
UNITS OF AMYLASE ACTIVITY PER ML.

MYCELIAL DRY WEIGHT PER 50 ML. CULTURE, MG.

HOURS OF INCUBATION

AMYLASE

PH

MYCELIAL WT.

50 100 150 200 250

0 50 100 150 200

0 12 24 36 48 60 72

6 7 8 9 10 11 12 13

6 7 8 9 10 11 12 13
Fig. 8. The amylase titer and pH of *T. vulgaris* cultures at different time of incubation. The carbon source was 2% maltose. A 12 hr. culture from C broth was used as the inoculum.
was noticed that maltose tended to retard the pH rise of the culture. Differences were noted in growth, pH changes, and amylase production when inocula of different ages were used. When 12 hr. cultures were used as the inoculum, the growth pattern as well as amylase production was very similar between maltose and starch supplemented cultures. Optimum amylase yields were obtained in the maltose containing medium when the culture was incubated for 24 to 36 hr., while in the starch-containing medium the optimum amylase yields were obtained after incubation for 36 hr. Prolonged incubation brought sharper drops in the yields in the starch-containing medium than in the maltose-containing medium. When an 18 hr. culture was used as the inoculum, there was an initial lowering of the pH of the culture in the starch medium. The pH then started to increase. Optimum amylase yields were obtained only after 48 hr. of incubation. Prolonged incubation caused sharp increases of the pH to 8 and decreased in the enzyme yields. In the maltose-containing medium inoculated with an 18 hr. culture, the pH increased during the first 6 hr. of incubation, sharply dropped back to pH 6, then did not change substantially with prolonged incubation. The amylase yield also increased gradually with time of incubation. In conclusion, the 12 hr. culture was found to be a better source of inoculum than the 18 hr. culture, maltose was a better carbon source than starch for initial growth and pH control of the culture, and starch was better than maltose as an inducer of amylase production if the pH of the culture could be controlled.

Effects of sequential addition of maltose and soluble starch to the medium

The previous experiments showed that the pH of the culture might be
controlled by use of maltose, yet pointed out the value of starch as a better enzyme inducer. Various combinations of these two carbohydrates were examined, therefore, regarding their effects on culture pH and amylase production. The results are shown in Tables 10a and 10b.

Table 10a. Effects of sequential addition of carbohydrates on amylase production^a

<table>
<thead>
<tr>
<th>Culture designation</th>
<th>Carbohydrate originally present</th>
<th>Carbohydrate added later</th>
<th>Time of addition (hr.)</th>
<th>Total hr. of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>1% starch</td>
<td>1% starch</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td>1B</td>
<td>1% starch</td>
<td>1% starch</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>1C</td>
<td>1% starch</td>
<td>1% starch</td>
<td>12</td>
<td>48</td>
</tr>
<tr>
<td>1D</td>
<td>1% starch</td>
<td>1% starch</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>1E</td>
<td>1% starch</td>
<td>1% starch</td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>1F</td>
<td>1% starch</td>
<td>1% starch</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td>2A</td>
<td>1% starch</td>
<td>1% maltose</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td>2B</td>
<td>1% starch</td>
<td>1% maltose</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>2C</td>
<td>1% starch</td>
<td>1% maltose</td>
<td>12</td>
<td>48</td>
</tr>
<tr>
<td>2D</td>
<td>1% starch</td>
<td>1% maltose</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>2E</td>
<td>1% starch</td>
<td>1% maltose</td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>2F</td>
<td>1% starch</td>
<td>1% maltose</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td>3A</td>
<td>1% maltose</td>
<td>1% starch</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td>3B</td>
<td>1% maltose</td>
<td>1% starch</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>3C</td>
<td>1% maltose</td>
<td>1% starch</td>
<td>12</td>
<td>48</td>
</tr>
</tbody>
</table>

^aAn 18 hr. culture was used as the inoculum.
Table 10a. (Continued)

<table>
<thead>
<tr>
<th>Culture designation</th>
<th>Carbohydrate originally present</th>
<th>Carbohydrate added later</th>
<th>Time of addition (hr.)</th>
<th>Total hr. of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D</td>
<td>1% maltose</td>
<td>1% starch</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>3E</td>
<td>1% maltose</td>
<td>1% starch</td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>3F</td>
<td>1% maltose</td>
<td>1% starch</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td>4A</td>
<td>1% maltose + 1% starch</td>
<td>-</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td>4B</td>
<td>1% maltose + 1% starch</td>
<td>-</td>
<td>-</td>
<td>48</td>
</tr>
<tr>
<td>4C</td>
<td>1% maltose + 1% starch</td>
<td>-</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>5A</td>
<td>2% starch</td>
<td>1% starch</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td>5B</td>
<td>2% starch</td>
<td>1% starch</td>
<td>36</td>
<td>60</td>
</tr>
<tr>
<td>5C</td>
<td>2% starch</td>
<td>1% starch</td>
<td>24</td>
<td>72</td>
</tr>
<tr>
<td>5D</td>
<td>2% starch</td>
<td>1% starch</td>
<td>36</td>
<td>72</td>
</tr>
<tr>
<td>6A</td>
<td>2% starch</td>
<td>1% maltose</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td>6B</td>
<td>2% starch</td>
<td>1% maltose</td>
<td>36</td>
<td>60</td>
</tr>
<tr>
<td>6C</td>
<td>2% starch</td>
<td>1% maltose</td>
<td>24</td>
<td>72</td>
</tr>
<tr>
<td>6D</td>
<td>2% starch</td>
<td>1% maltose</td>
<td>36</td>
<td>72</td>
</tr>
<tr>
<td>6E</td>
<td>2% starch + 1% maltose</td>
<td>-</td>
<td>-</td>
<td>72</td>
</tr>
<tr>
<td>7A</td>
<td>2% maltose</td>
<td>1% starch</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td>7B</td>
<td>2% maltose</td>
<td>1% starch</td>
<td>36</td>
<td>60</td>
</tr>
<tr>
<td>7C</td>
<td>2% maltose</td>
<td>1% starch</td>
<td>24</td>
<td>72</td>
</tr>
<tr>
<td>7D</td>
<td>2% maltose</td>
<td>1% starch</td>
<td>36</td>
<td>72</td>
</tr>
<tr>
<td>7E</td>
<td>2% maltose + 1% starch</td>
<td>-</td>
<td>-</td>
<td>60</td>
</tr>
</tbody>
</table>
Table 10b. Effects of sequential addition of carbohydrates on amylase production

<table>
<thead>
<tr>
<th>Culture designation</th>
<th>Final pH</th>
<th>Amylase units</th>
<th>Culture designation</th>
<th>Final pH</th>
<th>Amylase units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>6.6</td>
<td>12.49</td>
<td>4A</td>
<td>5.8</td>
<td>8.08</td>
</tr>
<tr>
<td>1B</td>
<td>6.7</td>
<td>13.58</td>
<td>4B</td>
<td>6.0</td>
<td>9.48</td>
</tr>
<tr>
<td>1C</td>
<td>8.0</td>
<td>1.51</td>
<td>4C</td>
<td>7.6</td>
<td>7.79</td>
</tr>
<tr>
<td>1D</td>
<td>8.0</td>
<td>2.46</td>
<td>5A</td>
<td>5.9</td>
<td>5.78</td>
</tr>
<tr>
<td>1E</td>
<td>8.0</td>
<td>0.74</td>
<td>5B</td>
<td>5.9</td>
<td>8.63</td>
</tr>
<tr>
<td>1F</td>
<td>7.7</td>
<td>3.35</td>
<td>5C</td>
<td>5.9</td>
<td>7.27</td>
</tr>
<tr>
<td>2A</td>
<td>5.7</td>
<td>7.24</td>
<td>5D</td>
<td>5.8</td>
<td>4.54</td>
</tr>
<tr>
<td>2B</td>
<td>6.6</td>
<td>20.49</td>
<td>6A</td>
<td>5.8</td>
<td>4.30</td>
</tr>
<tr>
<td>2C</td>
<td>7.9</td>
<td>2.41</td>
<td>6B</td>
<td>5.8</td>
<td>4.71</td>
</tr>
<tr>
<td>2D</td>
<td>7.9</td>
<td>17.01</td>
<td>6C</td>
<td>5.8</td>
<td>4.50</td>
</tr>
<tr>
<td>2E</td>
<td>8.0</td>
<td>11.08</td>
<td>6D</td>
<td>5.9</td>
<td>4.29</td>
</tr>
<tr>
<td>2F</td>
<td>8.0</td>
<td>10.30</td>
<td>6E</td>
<td>5.8</td>
<td>3.37</td>
</tr>
<tr>
<td>3A</td>
<td>6.0</td>
<td>7.17</td>
<td>7A</td>
<td>5.8</td>
<td>3.59</td>
</tr>
<tr>
<td>3B</td>
<td>5.9</td>
<td>7.02</td>
<td>7B</td>
<td>5.9</td>
<td>3.13</td>
</tr>
<tr>
<td>3C</td>
<td>8.0</td>
<td>11.52</td>
<td>7C</td>
<td>5.8</td>
<td>4.28</td>
</tr>
<tr>
<td>3D</td>
<td>7.3</td>
<td>32.15</td>
<td>7D</td>
<td>5.9</td>
<td>3.95</td>
</tr>
<tr>
<td>3E</td>
<td>8.1</td>
<td>4.02</td>
<td>7E</td>
<td>5.8</td>
<td>3.56</td>
</tr>
<tr>
<td>3F</td>
<td>8.0</td>
<td>3.98</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Some of the comparisons were examined statistically. Treatment 3D resulted in the greatest amylase yields. This treatment resulted in significantly greater amylase production than treatments 2B (significant at the 5% level) and 2D (significant at the 1% level). Thus, the best procedure of those examined was to include 1% maltose in the medium initially, add 1% starch after incubation for 24 hr., and harvest the culture after total incubation for 48 hr.

Since it was interesting to know what was happening during incubation in treatment 3D, an experiment was carried out to find the growth and amylase production pattern of the organism. The results are given in Fig. 9. It can be seen that during the initial growth phase there was a decrease of the pH of the culture and gradual increase of amylase titer. The pH of the medium kept decreasing for about 12 hr. following addition of the starch, then rose rapidly to near neutrality in another 12 hr. Further incubation resulted in increase of pH of the culture beyond the optimum condition for amylase yield. Amylase production, therefore, was found to increase rapidly following the administration of 1% soluble starch and within a total incubation period of 48 hr. the yield reached the maximum. At this time the pH of the culture usually lay between 6.7-7.5.

Use of a 12 hr. inoculum resulted in a slightly different growth pattern, as shown in Fig. 10. Growth of the cells was rapid. In the initial 12 hr. of growth, mycelial weights increased nearly 7 fold. Maximum mycelial weight was obtained between 24 and 36 hr. of incubation. Further incubation resulted in the lysis of cells. Here, a initial drop in the pH of the culture was not detected. Instead, the pH increased gradually for 36 hr. then increased rapidly to near 8; further incubation resulted in a
subsequent decrease in the pH down to 7.5. The amylase titer increased rapidly between 24 and 36 hr. reaching a maximum after 36 hr. of incubation. Sharp decreases in the titer were observed from 36 to 48 hr., and it was reduced to a negligible level after 60 hr. of incubation.

It was evident, from these two experiments, that inocula from cultures of different age could result in different patterns of physiology of the organism in relation to its amylase production. By comparing these two experiments, it would not be too difficult to choose the 12 hr. culture as the source of inoculum because use of this inoculum would result in higher amylase yields and shortening of the incubation time.

Effects of the initial pH of the medium

The results are shown in Table 11. It was evident from these data that for 48 hr. culture, the best initial pH of the medium for growth was around 6.0. No mycelial growth was found in the medium whose pH was beyond the pH range of 5.1-8.2. The best amylase yield was found with an initial pH of 6.4. Mycelial growth was almost negligible in the culture with an initial pH of 9.1. When a 12 hr. culture was used as an inoculum and the cultures were incubated for 36 hr., the optimum initial pH of the medium for maximum amylase production was still found to be around 6.5, as seen in Table 12. An initial pH of around 6.0 seemed to be less favorable for amylase production, although final mycelial weights were larger when this pH was used than when an initial pH of 6.5 was used, suggesting that autolysis might have been greater at the higher pH. When the initial pH of the medium was higher than 7, growth of the organism was very rapid but was followed by rapid lysis of the culture.
Fig. 9. The amylase titer and pH of *T. vulgaris* cultures at different time of incubation. One per cent of maltose was added to the medium and 24 hr. later, 1% soluble starch was supplemented. An 18 hr. culture in C broth was used as the inoculum.
77

- AMYLASE
- pH

1% MALTOSE
1% SOLUBLE STARCH

UNITS OF AMYLASE ACTIVITY PER ml

PH OF THE CULTURE

HOURS OF INCUBATION
Fig. 10. The amylase titer and pH of *T. vulgaris* cultures at different time of incubation. One per cent maltose was added to the medium and 24 hr. later, 1% soluble starch was supplemented. A 12 hr. culture in C broth was used as the inoculum.
Table 11. Effects of the initial pH of the medium on the production of amylases from *T. vulgaris* No. 5 grown at 55C for 48 hr.\textsuperscript{a}

<table>
<thead>
<tr>
<th>pH</th>
<th>Initial</th>
<th>Final</th>
<th>Mycelial weight per flask (mg)</th>
<th>Amylase units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.1</td>
<td>4.0</td>
<td>12.3</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>4.6</td>
<td>12.5</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>5.1</td>
<td>5.1</td>
<td>15.1</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>5.6</td>
<td>5.5</td>
<td>43.8</td>
<td>5.89</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>6.6</td>
<td>191.4</td>
<td>18.43</td>
</tr>
<tr>
<td></td>
<td>6.4</td>
<td>7.3</td>
<td>185.2</td>
<td>28.14</td>
</tr>
<tr>
<td></td>
<td>6.9</td>
<td>8.0</td>
<td>169.8</td>
<td>9.04</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>8.3</td>
<td>130.2</td>
<td>6.11</td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>8.3</td>
<td>108.0</td>
<td>2.75</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
<td>8.4</td>
<td>53.1</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>8.4</td>
<td>18.5</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>9.1</td>
<td>8.3</td>
<td>15.3</td>
<td>0.00</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Two per cent starch was used as the carbohydrate source. As inoculum, an 18 hr. culture in C broth was used. Mycelial weight in the control flask was 12.43 mg.

**Effects of different concentrations of calcium chloride**

The results showed that there was no significant difference in mycelial weights and amylase titers between the four treatments. Without the addition of calcium chloride to the medium, however, the titer and pH change of the cultures fluctuated greatly. When 0.3% calcium chloride was used, there was a slight decrease in mycelial weight as well as amylase
Table 12. Effects of the initial pH of the medium on the production of amylase from *T. vulgaris* No. 5 grown at 55°C for 36 hr.\(^a\)

<table>
<thead>
<tr>
<th>pH</th>
<th>Mycelial weight per flask (mg)</th>
<th>Amylase units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>5.8</td>
<td>71.37</td>
</tr>
<tr>
<td>5.9</td>
<td>6.7</td>
<td>231.04</td>
</tr>
<tr>
<td>6.5</td>
<td>7.2</td>
<td>211.40</td>
</tr>
<tr>
<td>7.1</td>
<td>7.6</td>
<td>142.24</td>
</tr>
<tr>
<td>7.5</td>
<td>8.1</td>
<td>100.91</td>
</tr>
<tr>
<td>7.9</td>
<td>8.0</td>
<td>56.66</td>
</tr>
</tbody>
</table>

\(^{a}\)The medium contained 2% soluble starch. A 12 hr. culture was used as an inoculum. Mycelial weight in the control flask was 11.85 mg.

The decrease, when statistically tested, was not significant at the 5% level.

**Effects of different phosphate concentrations**

The results are shown in Table 13. Best amylase production could be obtained when 0.1% KH\(_2\)PO\(_4\) was used; however, there was no significant statistical differences at the 1% level between the amylase titers obtained when 0.1 and 0.2% phosphate were used. More drastic pH change was seen in the control culture than in the cultures supplied with phosphate. Addition of 0.3% phosphate to the medium increased the buffering capacity of the medium, but decreased the amylase yields, though more mycelium was produced at this higher phosphate concentration.
Table 13. Effects of different phosphate concentrations on the production of amylases from *T. vulgaris* No. 5 grown at 55°C for 48 hr.\(^{a}\)

<table>
<thead>
<tr>
<th>% phosphate used</th>
<th>Final pH</th>
<th>Amylase units</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.8</td>
<td>10.51</td>
</tr>
<tr>
<td>0.1</td>
<td>7.2</td>
<td>27.44</td>
</tr>
<tr>
<td>0.2</td>
<td>6.8</td>
<td>26.38</td>
</tr>
<tr>
<td>0.3</td>
<td>6.5</td>
<td>14.76</td>
</tr>
</tbody>
</table>

\(^{a}\)An 18 hr. culture was used as the inoculum.

Discussion

Through preliminary experiments it was learned that *T. vulgaris* No. 5 grew faster and produced more amylases when it was grown in complex than in synthetic media. Growth of this organism in the synthetic agar Medium VII (Tendler and Burkholder, 1961), as shown in the previous section, suggested the possibility of growing it in the medium with an inorganic salt as the nitrogen source. Scarcity of mycelial growth and low amylase titer in media containing either nitrate or ammonium nitrogen indicated that this organism has fairly complex growth requirements. Further studies on the mineral, amino acid and vitamin requirements of the organism might shed some light on what substances are required for or to stimulate growth. N-Z case was the best nitrogen source of those examined. This substance supported massive mycelial growth and early mycelial development.

There were correlations between amylase titer and pH of the culture. During the experiments on nitrogen sources it was always noted through
visual observation that the originally compact and discrete masses of hyphae in the culture would gradually disintegrate and become sparse, indicating lysis of the culture. With the initiation of autolysis, the pH of the culture increased. When the pH reached the range of 6.6-7.5, generally the highest amylase titers were attained. Depending on the sources of nitrogen used, it was possible to harvest the culture in this pH range. Trypticase, for example, might be just as good a nitrogen source as N-Z case for amylase production if the culture could be harvested at a pH around 7. This substance usually caused rapid growth of the organism as well as lysis of the culture. Some other of the organic nitrogen sources examined were not so readily available to the organism, thus growth as well as enzyme production progressed slowly.

From the practical standpoint, it was found to be inadvisable to incubate the culture too long at high temperature. Water was lost rapidly from the culture and frequent supplement of water would increase the chance of contamination and might bring about some unexpected effects on the physiology of the culture. Also, the tendency of this organism to lyse prevented prolonged incubation of the culture. Pin-point areas of lysis sometimes appeared on A agar and B agar. These areas of lysis resembled very closely one of the two types of plaques described by Agre (1961), caused by Micromonospora vulgaris phages. Since 55C was reported by Agre (1962) to be optimum for phage multiplication, it was not advisable to incubate the culture too long lest the lysogenic organism, if it was, should in the course of growth start to lyse and release intracellular proteinases which would complicate the situation.
The remarkable nature of maltose and starch as the amylase inducers has long been known in many other microorganisms. Tonomura et al. (1961), for example, reported that carbohydrates such as maltose, isomaltose, starch, and glycogen were excellent \( \alpha \)-amylase inducers in *Aspergillus oryzae*. Tanabe and Ohyama (1955) pointed out the inhibitory effect of glucose on \( \alpha \)-amylase formation. Experiments with *T. vulgaris* showed close similarity of this organism to *A. oryzae* and other microorganisms in respect to inducibility of amylases to the carbohydrates used. Glucose could facilitate early development of mycelia, but like fructose and mannitol, the pH increase in the culture was slow. Increase of the pH was sharp in cultures grown in starch and certain other mono- and disaccharides. This suggested that the metabolic pattern of the organism might have utilized different metabolic pathways when different carbohydrates were metabolized. It was learned from the experiments that the organism produced acids in the early stages of growth and later, with the aging of the culture, released alkaline substances that increased the pH of the medium. It would be interesting to learn of the fate of starch and maltose within the cells of *T. vulgaris*, since it responded differently to these two carbohydrates.

The data in Table 7 also showed that there was no direct correlation between mycelial growth and amylase production. Fructose and mannitol both supported good mycelial growth but did not facilitate amylase production. Soluble starch and maltose both supported good mycelial growth and amylase production. From the practical viewpoint, it was more economical to use soluble starch than maltose, so the former was used more often in many experiments.
Studies on the effects of alteration of the C/N ratio on amylase production showed that this organism utilized soluble starch very rapidly. This was demonstrated by the rapid decolorization of the culture in response to the addition of iodine only after 6 hr. of incubation, even when 2% of starch was originally present in the medium. Increase of starch in the medium tended to bring about acid formation in the culture and increase of N-Z case brought the pH of the culture to the alkaline side. The organism developed massive mycelial mass with increased quantities of starch. Lysis of the culture was facilitated with increased amount of N-Z case, consequently, the amylase titer decreased. According to Chaloupka (1956, 1958), cultures of Streptomyces cultivated under different conditions secreted more proteases in an environment with a low concentration of nitrogen than in media rich in nitrogen. A decrease in the concentration of sugars in the medium brought about a decrease in the secretion of proteases. The results with T. vulgaris did not agree with the above findings. Though protease determinations were not made, it was speculated that increased concentration of N-Z case in the medium accelerated the pH increase of the culture with subsequent lysis of cells; thus resulted in increase of the pH of the culture.

As shown in Fig. 7 and 8, maximum amylase yield was found to take place when the mycelial mass began to lyse, as indicated by the slight decrease of mycelial weight of the culture at the time of harvest. There was a shift of the optimum time of incubation for amylase production when cultures of different ages were used as inocula. Cells from these two sources must be in different physiological conditions, as seen in the patterns of pH changes and amylase production of the cultures. Cells from the 18 hr.
culture were probably near the stationary phase because cultures inoculated with these cells showed a marked phase of adjustment, delayed mycelial growth, and delayed amylase production. When 12 hr. cultures were used as the inoculum, culture response to either maltose or soluble starch was very similar. No marked phase of adjustment could be detected. Mycelial growth was rapid and the amylase titer reached its maximum earlier. This different response of cells to maltose and soluble starch suggested the possibility of different rates of incorporation of these carbohydrates into the cells as well as different metabolic patterns followed. Cells from the 12 hr. culture must be physiologically younger than those from 18 hr. culture; this might have enabled cells from the former culture to utilize different carbohydrates without undergoing any marked adjustment. The real basis of the differential utilization of maltose and starch by this organism under such environmental condition needs further study.

From Table 10b it was evident that 3% carbohydrate was not appropriate for amylase production, regardless of when the carbohydrate was added. Abundant masses of mycelia were developed in such cultures, but amylase titers and pH of the culture were low. When 1% of either maltose or starch was used and another carbohydrate was added to the culture after 12 hr. of incubation, and if harvest was made at 36 hr., the cultures still did not undergo marked lysis. When the incubation was extended to 48 hr. or more, lysis could be observed, accompanied by low amylase yield, which indicated that rapid lysis occurred between 36 and 48 hr. When the carbohydrate supplement was made 24 hr. after the inoculation of a culture, a total of 48 hr. incubation seemed most favorable; prolonged incubation promoted further lysis of the culture which would result in lowering of the
amylase yields. It is not clear why addition of 1% carbohydrate, especially soluble starch, 24 hr. after the addition of the first 1% carbohydrate to the medium could result in higher amylase yield than when 2% carbohydrate was used from the beginning. Suzuki and Tanabe (1962) observed similar effects in *Aspergillus oryzae*. They found (1962b) high amylase yields could be secured if the carbohydrate was supplied to the culture when the reducing sugars in the cells were in the decline. If this was the case with *T. vulgaris*, then it would be encouraging to study the fate of these intracellular reducing sugars.

Generally, actinomycetes grow rapidly in alkaline media, and this held true in *T. vulgaris*. Rapid growth, however, did not result in high mycelial yield, for when the organism was grown in the medium of a pH higher than 7, rapid mycelial growth was always followed by early lysis of the culture. Low amylase yield and high protease activities were observed under these conditions. Also, it was noted that incubation of cells in media of initial pH 7.0 or 7.4, sometimes resulted in no growth at all or sudden clearing of the culture. This might have been due to actinophages present in lysogenic cells. As noted in *Streptomyces* species (Rautenstein, 1957), many lysogenic species easily liberate phage when grown as submerged culture for 48 hr. on a shaker. Since the plate culture of *T. vulgaris* No. 5 indicated its lysogenic nature, incubation of the cells at high pH might not only have accelerated autolysis but production and release of phages from the cells. When the cells were incubated in a medium of pH near 6.5, lysis sometimes took place, but with less frequency. The organism did not grow at a pH below 5; this was the lower pH limit of this
organism for growth. In contrast, an upper pH limit was not determined. These pH studies indicated that the initial pH of the medium should be adjusted between 6.2-6.5 for high amylase yield.

Addition of calcium chloride to the medium was originally aimed at the stabilization of amylases in the culture. Results with different levels of this salt showed that the calcium ions originally present in other components of the medium, particularly in yeast extract, would suffice. However, since wide fluctuations of the final pH of the cultures were observed in media unsupplemented with calcium chloride, a minimum of 0.05% was added. Proteases from some thermophilic Streptomyces species also require calcium ion for thermostabilization (Miyasawa, Ichishima, and Yoshida, 1964). Whether this is the same with T. vulgaris proteases is still unknown.

The studies with phosphate levels were done to determine the maximum phosphate concentration for high amylase titer. Together with yeast extract and proteins in the medium, phosphates were expected to exert buffering action. The results (Table 13) showed that addition of 0.3% phosphate buffered the medium well, but resulted in low yields of amylases. Whether this was due to toxic effects of phosphate or potassium, or strong buffer action which deterred increase of the pH of the culture to neutrality, was unknown. However, for the production of amylases, it was sufficient to use 0.1% phosphate.

From the above experiments, some knowledge was obtained of optimum conditions for good amylase production from T. vulgaris No. 5. The recommended procedure would be as follows:

(1) Growth of the organism on A agar for 48 hr.
(2) Five 11 mm diameter culture discs, cut out of different plates at random, are introduced into 50 ml of C broth (pH 6.4) in a 250 ml Erlenmeyer flask and the flask shaken at 55°C for 12 hr. at 120 r.p.m.

(3) The entire contents of one flask is used to inoculate 500 ml of a medium containing 1% maltose, 1% N-Z case, 0.1% yeast extract, 0.1% KH₂PO₄, 0.05% D-glucose, and 0.05% calcium chloride in a 2.5 l Fernbach flask. The pH of the flask is adjusted to 6.4 after autoclave-sterilization, and the flask is shaken at 55°C for 24 hr. at 100 r.p.m. One per cent soluble starch is added, and incubation is continued for another 12 to 16 hr. The culture is then harvested.
PART III. PURIFICATION AND SOME PROPERTIES OF THE AMYLASES OF \textit{T. VULGARIS}
Introduction

There are many publications on the purification of amylases from various sources; these procedures also might be useful in the purification of *T. vulgaris* amylases. However, viewed from the different sources and nature of the organism as well as enzyme system itself, it seemed necessary to examine modifications of various established procedures to determine conditions for purification and separation of the *T. vulgaris* amylases. Manning and Campbell (1961) published a procedure for the purification of *Bacillus stearothermophilus* amylase. In the present studies, a modification of their procedure was developed. Furthermore, other techniques were utilized.

**Materials and Methods**

In setting up steps for the purification of amylase, emphasis was placed on the increase of purity and reduction of amylase loss that would occur during treatments.

**Culture liquor**

Culture liquor was prepared in the manner described in the last section of Part II of this thesis. This was used as the starting material for the initial step of purification.

**Amylase titration**

The same dextrinogenic method as described in the previous section of this thesis was followed.
Protein determination

The Folin-Ciocalteau procedure modified by Lowry et al. (1951) was employed.

Condensation of culture liquor

The original culture in six 2.51 flasks was pooled; cells and agar discs were removed by passage of the broth culture through a Sharples centrifuge. The pH and volume of the supernate were measured and the pH adjusted to 6.5 with 2 N acetic acid. This culture supernate was then distributed about equally in 6 pyrex utility dishes (22x33 cm). The dishes were placed on the laboratory bench under 6 infrared lamps. Evaporation and cooling were facilitated with a 20 inch floor fan; the temperature of the liquor never exceeded 34°C. When the supernate was condensed to about 1/6 of its original volume, the liquor was pooled. Distilled water was used to dissolve the brown, sticky material adhering to the walls of the dishes and the wash solutions were added to the pooled, condensed liquor. The volume of the liquor was brought to 500 ml with distilled water, then the pH was adjusted to 6.1 with 2 N acetic acid.

Protein precipitation with ammonium sulfate

Powdered ammonium sulfate (reagent grade) was used in the initial protein precipitation. It was added to the condensed liquor at room temperature. The liquor was constantly stirred during the addition of the salt; stirring was continued for another 15 minutes after the salt was completely dissolved into the liquor. The liquor was then kept at 1°C over-night to allow protein precipitation to reach completion. Since this precipitation had to be carried out at low temperature, sodium sulfate was not tried.
Dialysis of enzyme solution

The ammonium sulfate-treated liquor was filtered through a Celite Analytical filter aid (Johns-Manville Products, L-665-A). The brown, sticky material on the filter cake was removed and dissolved in 100 ml of 0.2 M calcium acetate of pH 6.0. The solution was poured into dialysis tubing and dialyzed against 10 l of 0.01 M calcium acetate of pH 5.8 at 1C. To facilitate dialysis, the calcium acetate solution was stirred continuously. The dialysis solution was changed at least once. Dialysis was continued until no trace of sulfate ion could be detected in the solution.

Removal of pigments

The dialyzed enzyme solution was filtered through 5 gms of acid-treated, thoroughly washed, active carbon (Klearit, Jennision-Wright Co.) to remove the pigments. Treatment of the active carbon was according to the method recommended by Babbar, Powar, and Jogannathan (1962).

Treatment with organic solvents

Acetone (commercial grade), ethanol (95%), n-propanol (98%), and t-butanol (analytical grade) were examined for the efficiency in precipitation of T. vulgaris amylases. The solvents were stored at -10C before use. After mixing with the enzyme solution, the mixture was kept at -10C for gradual precipitation of proteins.

Gel filtration

Sephadex G-75 (Pharmacia, Uppsala, Sweden) was first allowed to swell in distilled water for 48 hr. at 4C. The swollen material was then filled into two glass tubes, each measuring 2 x 200 cm. These two columns were
then linked with 0.8 mm diameter polyethylene tubing and placed parallel to each other, with one column slightly elevated above the other. A Beckman solution metering pump (Model 746) was used to pump the effluent from the elevated column to the other one. Before use, the columns were equilibrated with 0.01 M calcium acetate of pH 5.8 through a 1 l separatory funnel attached to the top of the elevated column as a buffer reservoir. An LKS Radi-Rac fraction collector was used to collect the effluents.

**Amylase and protease assays for column chromatography**

For this purpose, antibiotic assay filter paper discs (Schleicher and Schuell Co., No. 740-E) were used. After saturation with the effluent of a fraction contained in a collection tube, the disc was placed on the surface of either an amylase assay agar plate (Hudman et al., 1957) or a protease assay agar plate (Lewis et al., 1957). Each plate contained 20 ml of substrate-containing agar. Four discs, spaced equidistantly, were placed on each plate. The plates were then incubated at 45°C. The incubation period was not kept constant since it was necessary only to compare enzyme level in different fraction of the same run. After incubation, 0.3% iodine solution was poured onto the amylase assay plates to define the zones of starch hydrolysis. The protease assay plates contained a milk-agar-buffer mixture; the zones of casein hydrolysis were discernible without further treatment. The diameters of the zones of starch or casein hydrolysis were determined with a Fisher-Lilly antibiotic zone reader.

**Ion exchange chromatography**

Preliminary studies were conducted to select suitable ion exchangers.
Approximately 3 gm each of Dowex 50 W-X8, Dowex 1-X4 (Baker Chemical Co.), Cellex-CM, and Cellex D (Bio-Rad Laboratories) were soaked in distilled water. Before use, Cellex-CM and Dowex 50 W-X8 were converted to the HR form with 2 N acetic acid. These two cation exchangers were then washed with 0.1 M acetate buffer of pH 5.0. After excess buffer was drained off, 20 ml of culture filtrate, pH 6.0, was added to each ion exchanger contained in a plastic centrifuge tube and the culture filtrate and exchanger were mixed thoroughly. The anion exchangers were examined similarly; 20 ml of culture filtrate, pH 6.0, was added to each tube containing 3 gm of the resin and the contents of each tube was mixed well. The tubes were then centrifuged and the supernate tested for amylase and protease activities.

In later studies, chromatography with Dowex 50 W-X8 and Cellex-CM was attempted. When Dowex 50 W-X8 was used, a 9 x 500 mm, glass stoppered burette was filled with 3 gm of material, the resin was converted into the HR form, as described above, then the column was adjusted to the desired pH with a suitable buffer. Five ml of enzyme preparation were added to the column and the column was developed. Other experimental conditions are described in detail in each figure. When Cellex-CM was used, 6 gm were soaked in distilled water, the material was packed into a 20 x 250 mm glass column (Fisher and Porter Co.), and was converted to the HR form with 2 N acetic acid. After adjustment of the pH of the column to the desired pH, 5 ml of the enzyme solution were added to the column. Other details of the experimental conditions can be found in the pertinent figures.
Electrophoresis of the enzyme solution

A Shandon Universal Electrophoretic apparatus (Colab Laboratories, Inc.) was used to develop the samples (5 μl) on 25 x 160 mm cellulose acetate strips (Scheicher & Shuell Co. Type 2500). Three different buffers of ionic strength 0.1 were prepared as follows: (1) Acetate buffer of pH 4.0; 20 ml of 2 M sodium acetate, 33.7 ml of 3.5 M acetic acid, 32 ml of 5 M NaCl, distilled water added to a volume of 2 l. (2) Phosphate buffer of pH 6.5; 16.6 ml of 0.5 M Na₂HPO₄, 3.7 ml of 4 M NaH₂PO₄, 32 ml of 5 M NaCl, distilled water added to a volume of 2 l. (3) Barbital buffer of pH 8.5; 80 ml of 0.5 M sodium diethylbarbiturate, 5.3 ml of 2 M HCl, distilled water was added to a final volume of 2 l.

For protein stain, bromphenol blue (BPB) was used according to the method recommended by Nakamura, Ohmura, and Kobayashi (1958). For the detection of amylase activity, each strip, after electrophoresis, was placed directly between two starch-impregnated, filter paper strips (35 x 180 mm, Whatman No. 2 filter paper). These strips were placed between two glass plates and were pressed tightly with screw-type clamps. The whole assembly was then incubated at 55°C for 4 hours. Amylase was located by spraying the filter paper with 0.3% iodine solution.

Results

Condensation of culture liquor

When the culture supernate was condensed in the utility dishes, its pH usually dropped 0.2 to 0.3 units by the end of the operation. During condensation, about 8% of the amylase activity was lost, as shown in Table 16.
**Protein precipitation with ammonium sulfate**

When ammonium sulfate was added, the pH of the condensed culture supernate was decreased to 5.3-5.6 depending on the amount of salt added. The optimum concentration for the maximum yield of amylase in the precipitate was found to be 50% of its saturation at 25°C, as shown in Fig. 11. Some of the brown pigments remained in solution, and were eliminated when the material was filtered through Celite.

**Dialysis of enzyme solution**

When the protein solution from the precipitation with 50% ammonium sulfate was dialyzed, some brown pigments could be seen diffusing out of the dialysis tube into the surrounding solution. After completion of dialysis and removal of the calcium sulfate which formed, the amylase activity was checked against the original, undialyzed solution; about 5% of the amylase activity was lost during this step of the purification procedure.

**Removal of pigments**

When the dialyzed enzyme solution was filtered through active carbon, and the filtrate was compared with unfiltered solution for enzyme activity, it was found that about 12% of the amylase activity present in the unfiltered solution was lost. Brown pigments were completely removed from the solution. About 15% protease activity was removed by the same treatment.

**Treatment with organic solvents**

The results are shown in Table 14. Acetone, ethanol, and t-butanol were all effective for the precipitation of amylases; n-propanol was less effective at the concentration examined. When more detailed studies were
Fig. 11. Effect of ammonium sulfate concentration on the precipitation of *T. vulgaris* amylases
Fig. 12. Effect of acetone on the precipitation of the amylases of *T. vulgaris*. Precipitation was carried out at -10°C.
Fig. 13. Effect of ethanol on the precipitation of the amylases of *T. vulgaris*. Precipitation was carried out at -10°C.
Graph showing the relationship between ethanol/enzyme solution (V/V) and relative amylase activity (%). The x-axis represents the ethanol/enzyme solution (V/V) ranging from 1.0 to 3.0, and the y-axis represents the relative amylase activity (%) ranging from 0 to 100.
Table 14. Recovery of amylase activity after precipitation with different solvents

<table>
<thead>
<tr>
<th>Precipitating agent</th>
<th>Enzyme to solvent ratio (v/v)</th>
<th>Units of amylase per ml</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>1:1</td>
<td>150</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>387</td>
<td>47</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1:1</td>
<td>213</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>374</td>
<td>45</td>
</tr>
<tr>
<td>n-propanol</td>
<td>1:1</td>
<td>255</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>154</td>
<td>19</td>
</tr>
<tr>
<td>t-butanol</td>
<td>1:1</td>
<td>165</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>375</td>
<td>46</td>
</tr>
</tbody>
</table>

made with acetone and ethanol, it was found that with acetone, the best volume ratio was one volume of enzyme solution to two volumes of acetone, whereas in ethanol, the ratio was one to 2.5, as shown in Fig. 12 and 13. Statistical analyses showed there was no significant difference between the treatments acetone 1:2 (volume:volume) and ethanol 1:2.5.

**Gel filtration**

The second acetone precipitate was dissolved in 50 ml of 0.01 M calcium acetate, pH 5.6, and 10 ml of the enzyme solution were added to equilibrated columns of Sephadex. The columns were developed by adding 0.01 M calcium acetate solution of pH 5.8 at the rate of 1.4 ml per minute, giving the results shown in Fig. 14. At least 3 to 4 amylase species were present
in the acetone precipitate. Proteases were eluted in only one peak. Amylase fractions in peak A and/or B were completely free from proteases. Amylase activity in peaks C and D was contaminated with proteases. Peak B, which is relatively inconspicuous in Fig. 14, might be just an artifact; however, this "shoulder" to peak A was obtained in several subsequent determinations.

When 20 ml of an undialyzed enzyme solution obtained from a single precipitation with ammonium sulfate was added to the columns, the results shown in Fig. 15 were obtained. Peak A was greatly diminished in size, whereas peak C was increased markedly in size. However, peak D and the protease peak were not substantially different in size when preparations from different stages in the purification procedure were used as the starting material. Peak B, which was relatively inconspicuous in Fig. 14, was pronounced. When effluents from tubes 62 to 72, shown in Fig. 15, were pooled and precipitated with acetone twice, as had been done in the experiment shown in Fig. 14, the results presented in Fig. 16 were obtained. Only two peaks, corresponding to peaks A and C, appeared. Peak C was diminished considerably in size, while peak A had increased immensely.

When an enzyme preparation obtained from the second acetone precipitation was applied to the column in which Sephadex G-50 or G-100 had been used, little separation of amylase peaks was obtained and the amylase peaks mostly overlapped the protease peak. The column size used in these experiments was 20 x 500 mm. Similar but slightly better enzyme separation was obtained when Sephadex G-75 was used. When a column of 20 x 2000 mm was used, better separation of amylase from protease as well as differentiation
of amylase peaks was observed when using Sephadex G-75, but not when either G-50 or G-100 was used.

**Ion exchange chromatography**

Four ion exchangers were compared qualitatively for their capacity to adsorb enzymes and it was found that, under the experimental conditions used, both Cellex-CM and Dowex 50 W-X8 adsorbed amylases and proteases, as shown in Table 15. Pigments were not adsorbed to any of the ion exchangers.

Table 15. Effect of different ion exchangers on the adsorption of enzymes and pigments

<table>
<thead>
<tr>
<th>Adsorption</th>
<th>Amylases</th>
<th>Proteases</th>
<th>Pigments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellex-CM</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cellex-D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dowex 50 W-X8</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Dowex 1-X4</td>
<td>±</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

An enzyme preparation from the second acetone precipitation was dissolved in 50 ml of 0.01 M calcium acetate, pH 5.8, and 25 ml of the enzyme solution were added to a column in which Cellex-CM had been filled in HR form. The column was washed with calcium acetate solution of pH 6.1 (20 mg of calcium acetate per 1 distilled water). Only pigments were eluted from
Fig. 14. Separation of amylases and proteases on Sephadex G-75 packed into two columns, each measuring 2 x 200 cm. The columns were equilibrated with 0.01 M calcium acetate of pH 5.8 before use. Ten ml of the enzyme solution, obtained from dissolving the enzyme precipitate from the second acetone treatment in 50 ml of 0.01 M calcium acetate (pH 5.6), were applied to the column. The column was eluted with 0.01 M calcium acetate of pH 5.8.
14 ML. OF EFFLUENT PER TUBE
PLATES WERE INCUBATED AT 45°C FOR 18 HOURS

- AMYLASE
- PROTEASE

DIAMETER OF ZONE OF HYDROLYSIS, MM

TUBE NUMBER
Fig. 15. Gel filtration of amylases and proteases on Sephadex G-75. The columns were prepared and eluted in the manner described in Fig. 14. Twenty ml of an undialyzed enzyme preparation obtained from a single precipitation with ammonium sulfate was applied to the columns.
16 ml of effluent per tube
Plates were incubated at 45°C for 9 hours

- □ AMYLASE
- ○ PROTEASE

Diameter of zone of hydrolysis, mm
Fig. 16. Gel filtration of amylases on Sephadex G-75
The columns were prepared and eluted in the manner described in Fig. 14. An amylase preparation from peak C (tubes 62 to 72) of Fig. 14 was applied to the columns.
13.5 ml. OF EFFLUENT PER TUBE
PLATES WERE INCUBATED AT 45°C FOR 9 HRS.
NO PROTEASE ACTIVITY DETECTED
the column. No amylase activity could be detected. Since amylase might have remained adsorbed to the column, the column was washed thoroughly with 500 ml of 0.067 M calcium acetate, pH 7.2. Protease activity was detected in some tubes, but no amylase activity could be found. The column was then washed with 0.5 M calcium acetate, pH 6.7, but still no amylase activity could be found. From these experiments the conclusion was reached that amylase was firmly adsorbed onto the column or was destroyed.

When 5 ml of enzyme solution made from an acetone precipitate were added to the Cellex-CM column, and the column was washed with 500 ml of distilled water, then with 500 ml of 0.3 M acetic acid, no amylase or protease activity could be detected in any effluent, though pigments were eluted. When the Cellex-CM column was buffered to pH 3.8 with 0.1 M acetic acid and after the addition of 5 ml enzyme solution (pH 5.0) the column was washed continuously with 150 ml of 1/15 M phosphate buffer of pH 5.9, and 300 ml of 1/15 M phosphate buffer of pH 8.8, no amylase activity was found in any effluent. Protease activity was detected in fractions obtained from washing the column with phosphate buffer of pH 8.8 and this activity was found in the effluents whose pH ranged from 4.1 to 8.2. Pigments were eluted in the first 6 tubes where the pH of the effluent was around 3. The results are shown in Fig. 17. If an enzyme preparation was added to a Cellex-CM column that had previously been buffered to pH 3.7 with 0.1 M acetate and then the column was washed with 100 ml of 0.1 M acetate buffer, pH 3.7, then with 150 ml of 0.2 M calcium acetate, pH 5.5, and finally with 150 ml of 0.2 M calcium acetate, pH 7.3, slight amylase activity could be detected in the fractions of pH 5.2 to 5.3 which came from washing the column with calcium acetate of pH 5.5. Protease activity was found in the fractions with
pH ranging from 4.0 to 7.3. Also, a fraction with pH 3.7 (first tube) showed strong protease activity. Thus, all attempts to isolate the amylases of *T. vulgaris* using Cellex-CM columns were unsuccessful.

In experiments using a Dowex 50 W-X8 column, enzyme solution from the second acetone precipitation was added to the column in which the resin had been converted to the HR form. One hundred ml of distilled water were first used to wash the column and each tube received 10 ml of the effluent. This was followed by washing with 100 ml of 0.2 M calcium acetate, pH 5.0 and finally with 100 ml of 0.2 M calcium acetate, pH 4.5. Amylase activity was detected in tubes 14 to 24, as shown in Fig. 18, where the pH of the effluents fluctuated between 5.2 to 5.6. Two peaks were present. Proteases were eluted in at least 3 peaks; one at pH 2.1, one between pH 3.3 and 5.6, and one between pH 4.5 and 5.1. Thus, the amylases and proteases were not separable under these conditions. When the resin was converted to HR form with 0.1 M acetic acid, pH 3.0 and 5 ml of enzyme solution, pH 6.0, was added and 100 ml of 0.1 M acetate buffer of pH 3.2, 150 ml of 0.1 M acetate buffer of pH 4.1, 150 ml of 0.1 M acetate buffer of pH 5.0, and 150 ml of 0.1 M sodium acetate of pH 7.4 were used in sequence described above to wash the column, an amylase peak was found in fractions where the pH of the effluent was from 5.0 to 7.2. At least 5 protease peaks were detected (Fig. 19) corresponding to pH of the effluent at 3.0 or less, pH 3.7 to 4.0, pH 4.1 to 4.8, 4.8 to 7.3, and above 7.3. An amylase peak overlapped with one of the protease peaks, as shown in Fig. 19. Though the results seemed to be promising for the separation of some proteases, it was still difficult to separate amylases from proteases with this method. If the resin was first buffered with 0.1 M acetate of pH 4.7 and 5 ml of the
Fig. 17. Ion exchange chromatography on a Cellex-CM column of an enzyme preparation from the second acetone precipitate. The column was buffered with 1/10 M of acetate, pH 3.8, before use. After the addition of 5 ml of enzyme solution, pH 5.0, 150 ml of 1/15 M phosphate buffer (pH 5.9) and 300 ml of 1/15 M phosphate buffer (pH 8.8) were used to elute the column.
8.5 ml. of effluent per tube
plates were incubated
at 45°C for 12 hours

- - AMYLASE
- - PROTEASE

Diameter of zone of hydrolysis, mm.

Tube number
enzyme solution (pH 4.7) were added, followed by washing the column with 100 ml of pH 4.7, 150 ml of pH 5.3, and 150 ml of pH 5.9, all of 0.1 M acetate buffer, the amylase peak showed up in the first tube of 4.6. Three protease peaks were present; the first peak at the pH range of 4.5 to 4.6, the second peak at pH 4.6 to 5.2, and the third peak at pH 5.6 to 5.7. Amylase activity was slight. When the resin was used in NaR form and buffered to pH 4.0 with 0.1 M acetate buffer and later 5 ml of the enzyme solution of pH 4.3 were added, followed by washing the column first with 100 ml of 0.1 M acetate buffer, pH 4.0, 100 ml of 0.1 M acetate buffer, pH 5.0, then with 100 ml of 1/15 M phosphate buffer of pH 5.9 and finally with 100 ml of 1/15 M phosphate buffer, pH 8.0, two amylase peaks were observed; the first one being associated with effluents of pH 4.0 to 4.3 and the second one with effluents of pH 4.3 to 5.8. Pigments were associated with the first peak. Four protease peaks were found; the first peak at pH 4.3 to 4.0, the second at pH 4.0 to 4.3, the third at pH 4.3 to 5.8, and the last at pH 5.8 to 7.5. No separation of amylases from proteases could be made.

Thus use of Dowex 50 W-X8 for the separation of the amylase from the protease of *T. vulgaris* was unsuccessful. However, it was learned that from these experiments there were at least 5 proteases present in the enzyme solution from the second acetone precipitation. One was eluted at low pH of 2 to 3, three at the pH range of 4 to 7, and one at pH 7 or higher. These proteases were separable from one another.

One experiment was carried out with Cellex-D, a DEAE cellulose. Eight gm of Cellex-D, previously allowed to swell in distilled water, was filled into a 20 x 250 mm glass column. Before use, calcium acetate of pH 6.1 (20 mg calcium acetate per 1 distilled water) was used to buffer the column.
Fig. 18. Ion exchange chromatography of the enzyme preparation on a Dowex 50 W-X8 column. The resin was converted to HR form before the addition of the enzymes. After addition of the enzymes, the column was eluted with 0.2 M calcium acetate as follows: 100 ml of pH 5.8, 100 ml of pH 5.0, and 100 ml of pH 4.5.
10 ml. EFFLUENT PER TUBE
PLATES WERE INCUBATED
AT 45°C FOR 12 HRS.

- AMYLASE
- PROTEASE
- pH

DIAMETER OF ZONE OF HYDROLYSIS, mm.

pH

TUBE NUMBER
Fig. 19. Ion exchange chromatography of the enzyme preparation on a Dowex 50 W-X8 column. The resin was first converted to HR form. Five ml of the enzyme solution (pH 6.0) were applied to the column. The column was then eluted with 0.1 M acetate as follows: 100 ml of pH 3.2, 150 ml of pH 4.1, 150 ml of pH 5.0, and 150 ml of pH 7.4.
10 ml. EFFLUENT PER TUBE
PLATES WERE INCUBATED AT 45°C FOR 18 HRS.

- AMYLASE
- PROTEASE
- pH

Diameter of Zone of Hydrolysis, mm.

pH

Tube Number
The same buffer was used to wash the column after the introduction of 5 ml of enzyme solution (pH 6.0) from the second acetone precipitation. No amylase activity was detected in the effluents. Only proteases were detected, and three peaks were found. No further experiment was carried out with this ion exchanger.

Besides ion exchange chromatography described above, a starch-Celite column, as recommended by Schwimmer and Balls (1948), was examined as a possible means to isolate the amylases of T. vulgaris. The flow rate was slow and no amylase activity could be detected, so no further studies were made with this kind of column.

**Electrophoresis of the enzyme preparation**

Since experiments with gel filtration gave good separation of amylases from proteases, as shown in Fig. 14, enzyme solutions from peaks A and C were examined electrophoretically. Also, as a control, an enzyme preparation from the second acetone precipitation was used.

A sample from tube 95 of peak A (Fig. 14) was run in the barbital buffer of pH 8.5 for 18 1/2 hr. with an initial voltage of 290 volts and a constant current of 2 mamp per strip. An amylase band was seen on the anode side, about 10 cm away from the origin. No other protein was detected on the strip. Three or four amylases bands were discernible on the control strip, and many bands could be detected when the strip was stained to detect proteins. A sample from tube 95 of peak A (Fig. 14) was run at pH 6.5. The experiment was run at constant current of 2 mamp per strip for 10 hr. with an initial voltage of 290. One very faint amylase band was observed 1 cm away from the origin toward the anode. No other protein bands
were detected. Two to three protein bands were present on the control strip, with two amylase bands on the anode side. When the experiment was in a buffer of pH 4.0 at 2 mamp per strip for 5 hours with an initial voltage of 150, very faint amylase activity was observed very near to the origin, yet still on the anode side. The band was very weak and very difficult to identify as a band, even with the protein stain. The control sample yielded one or two inconspicuous, diffuse bands of amylase activity; there were 4 to 5 protein bands on the strip. Electrophoresis with effluents from tubes 87 and 116 yielded results almost identical to those reported in this paragraph.

**Determination of optimum pH and temperature for amylase from peak A (Fig. 14)**

Since fractions from peak A contained only one amylase by gel filtration and zone electrophoresis, the effluent in tube 95 was suitably diluted and used in the determination of its optimum pH and temperature for activity. A universal buffer of ionic strength 0.1 (Senya and Kondo, 1958) was used in these experiments. The experiments were carried out in the manner described in Part II of this thesis. The results are presented in Fig. 20, 21, and 22. It can be seen that the optimum pH and temperature for the amylase activity in peak A were nearly identical to those in which crude culture filtrate was used as an amylase source. The enzyme was stable at 60°C; only 15% of its activity was lost during 16 hr. of incubation. At temperature above 70°C, 86% or more of the activity was lost within half an hour and no activity could be detected in 1 or 2 hr. This suggested that the amylase in peak A might be present in the second acetone precipitation and in the original culture liquor in greatest quantities of all amylases.
Another explanation is that all amylases in the crude liquor have the same optimum temperature and optimum pH range for activities. The first hypothesis seems probable because when the effluents from tubes 87 to 118 were pooled and total amylase units present were determined, it gave 3670 units which was equivalent to 67% of amylases contained in the original 10 ml of enzyme solution.

Discussion

Aside from obtaining an enzyme as pure as possible, it was important to prevent its loss during purification. In this respect, when the original culture supernate was first condensed in room temperature, pH adjustment was necessary. Although the nature of all components present in the supernate was not clear, it was known from previous experiments that a rise of pH of the culture above neutrality generally resulted in rapid loss of amylase activity. It was also learned that incubation of culture supernate at pH below 5 would cause coagulation of proteins accompanied by decreased amylase recovery. The pH of the supernate was, therefore, adjusted to 6.5. The decrease of approximately 8% of activity that occurred during concentration might have been diminished if condensation was done at a lower temperature. However, equipment was not available for rapid concentration of large volumes at low temperature.

Elimination of *T. vulgaris* cells from the culture might not be proper if maximum yield was expected. *Aspergillus oryzae* is known to produce both extra- and endocellular amylases (Suzuki and Tanabe, 1962b; Tonomura, Futai, and Tanabe, 1963; Tonomura et al., 1963). This was also the case with *Bacillus marcerans* (DePinto and Campbell, 1964). *T. vulgaris* was not
The amylase from peak A (tube 95) of Fig. 14 was used as the enzyme source.
RELATIVE AMYLASE ACTIVITY, %

PH

4.0

4.5

5.0

5.5

6.0

6.5

7.0

7.5

126
Fig. 21. The effect of temperature on the activity of the amylase from peak A (tube 95) of Fig. 14
Fig. 22. The temperature stability of the amylase from peak A (tube 95) of Fig. 14
examined for the presence of endocellular amylase, but the presence of cell-bound amylases in other microorganisms indicates that such a study would be of interest. One or more of the amylases examined in the present study might have been of endocellular origin because, at the time of harvest, some cells had already undergone lysis.

Three or four peaks of starch-hydrolyzing activity revealed through gel filtration (Fig. 14) indicated presence of at least 3 or 4 amylases in the enzyme solution from the second acetone precipitation. According to the theory of molecular sieving of gel filtration, the amylase fraction in peak D should have the largest molecular size of all amylases present in the solution and the amylase in peak A the smallest. Since the amylase in peak D was contaminated with proteases, more refined techniques would be necessary to separate it from these proteases. Peak C, on the other hand, was overlapped only partially with the protease peak, and it would probably not be too difficult to separate this amylase fraction completely from the proteases in the future. Use of the recycling chromatographic technique, first developed by Porath and Bennich (1962), might result in adequate separation of all amylases and proteases and other enzymes present in the solution.

Use of long columns in gel filtration had some disadvantages, such as difficulties in packing the columns evenly, in removal of air bubbles trapped in the columns, in washing the columns, and in controlling the flow rate, as listed by Chersi (1964), and Tiselius, Porath, and Albertsson (1963). Gel filtration, however, proved to be an effective technique in the enzyme purification. Loss of enzyme was almost negligible when proper buffer was used.
It was interesting to note that the amylase in peak C (Fig. 15) could be converted into another type of amylase, represented by peak A. Electrophoresis with effluent from tube 96 of Fig. 16 showed that the amylase in this fraction was identical with that present in tube 95 of Fig. 14. Electrophoresis with effluent from tube 66 of Fig. 15 gave one prominent amylase. However, occasionally 2, and sometimes 3, amylase bands appeared. These extra bands were generally diffuse and not conspicuous; one seemed to be identical to the band shown by the enzyme present in tube 95 of Fig. 14. When the fractions from tubes 66 and 95 of Fig. 14 were used to compare the action pattern of amylase in each fraction, it was found that the amylase in each fraction gave almost identical results, suggesting the amylases in these two fractions might be isozymes.\(^a\) This interconversion of isozymes has been described by other workers, such as Jolly and Mason (1965), who found that the tyrosinase in mushroom existed in multiple forms. They reported that interconversion of these isozymes could be obtained through changes in pH, ionic strength, and protein concentration of the system. In *Bacillus subtilis*, detailed studies on the association and dissociation of \(\alpha\)-amylases also have been made (Isemura, Kakiuchi, and Eto, 1960; Isemura and Kakiuchi, 1962; Kakiuchi, Imanishi, and Isemura, 1964; Kakiuchi, Hamaguchi, and Isemura, 1965). Interconversion of polymer and monomer forms of amylases in this organism was also induced by changes in the system described above. They reported the existence of an equilibrium

---

\(^a\) Robyt, John F., Dept. of Biochem. & Biophysics, Iowa State University, Ames, Iowa. Data from the studies of action pattern of these two fractions of amylases. Private communication. 1965.
among these isozymes (Kakiuchi, Hamaguchi, and Isemura, 1965). The similar monomer dimer transformation was also reported in \( \alpha \)-chymotrypsin (Massey, Harrington, and Hartley, 1956; Tinoco, 1957). Judging from these examples, it was thought that appearance of 1 to 3 amylase bands from the sample in tube 66 of Fig. 15 on the strip might have been due to dissociation of polymer into dimer or monomer forms. From this viewpoint, it was possible that even in tube 95 of Fig. 14 interconversion might take place. Though electrophoresis experiments usually gave only one protein and amylase band in these strips in 3 different pH buffers, the technique might not be sufficiently sensitive to conclude definitely that there was always only one amylase present in that fraction. The amylase band either on starch-impregnated paper or the stained strip was sometimes diffuse; there were some isolated bands, but they were so faint in color that they could not be definitely attributed to the presence of amylase activity. Therefore, in future studies, more sensitive methods would be helpful. In addition, studies utilizing the ultracentrifuge would be of interest.

As to the application of ion exchange chromatography to the isolation of amylases, all attempts made so far were unsuccessful. Yet the success of other investigators in use of ion exchange chromatography for the separation of amylases from other microorganisms indicates that this technique might be applied to the separation of the amylases of \( T. \) vulgaris if appropriate condition can be found. Use of DEAE cellulose with the addition of diisopropylfluorophosphate, as recommended by Stein and Fischer (1961), might be useful and indeed Mhatre separated the amylases from the proteases
of *T. vulgaris* using this method. However, the electrophoresis pattern obtained by Mhatre did not show any separation of the different amylases present in the preparation; only one amylase band was detected. Though the chromatographic procedures used in the present study did not result in isolation of the amylases from the enzyme preparation, the presence of at least 5 proteases was detected. In the future, if detailed studies on the proteases were to be carried out, this ion exchange chromatography would be of much use.

The amylase in tube 95 was rather unstable at high temperature (Fig. 22). The enzyme had optimum temperature at 60°C but was rapidly destroyed about 70°C. This thermolability was not because of lack of calcium ion, which is required for stability by some α-amylases. Calcium was detected in the fraction in the solution, so it must be present in sufficient quantity for binding with the enzyme. Therefore, this thermolability may be due to the peculiar nature of the enzyme protein itself.

Through the above experiments, the recommended procedure for the purification of amylases from *T. vulgaris* was set as follows:

1. Centrifugation of the pooled culture liquor to remove cells and agar discs
2. Condensation of the culture supernate
   The pH is adjusted to 6.5 with 2 N acetic acid, then the supernate is condensed to 1/6 of its original volume.

---

*Mhatre, N. S., Enzymology Research Laboratory, Miles Chemical Co., Elkhart, Indiana. Data from analyses made with the enzyme preparation sent by the author. Private communication. 1965.*
(3) Precipitation of proteins with ammonium sulfate

Before the additional of the salt, the pH of the liquor is adjusted to 6.1 with 2 N acetic acid. Ammonium sulfate is added to 50% saturation (25°C), with constant stirring, at room temperature; after complete solution of the salt, stirring is continued for another 15 minutes. The solution is kept at 1°C overnight for complete precipitation of the proteins.

(4) Dialysis. After filtration through Celite, the brown, sticky substance is dissolved in 100 ml of 0.2 M calcium acetate, pH 6.0. The solution is dialyzed against 0.01 M calcium acetate (pH 5.8) at 1°C until the dialysis solution is free of sulfate ion.

(5) Removal of pigments

The dialyzed solution is filtered through 5 gm of acid-treated active carbon.

(6) First acetone precipitation

The pH of the enzyme is adjusted to 5.8 with 1 N acetic acid. Two volumes of commercial acetone (-10°C) are added to one volume of enzyme solution. The mixture is allowed to stand at -10°C for 3 to 5 hr. The precipitate is dissolved in 100 ml of 0.01 M calcium acetate, pH 6.0.

(7) Second acetone precipitation

Acetone precipitation is repeated as given in the previous step, except that the solution is kept at -10°C for 18 hr.
Specific activities and yields obtained during a typical purification procedure, as described above, are given in Table 16.

Table 16. Typical results obtained during the purification of *T. vulgaris* amylase

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total units</th>
<th>Specific units/mg act. pro.</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear filtrate</td>
<td>2650</td>
<td>82300</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td>Condensed filtrate</td>
<td>500</td>
<td>75640</td>
<td>2.2</td>
<td>92</td>
</tr>
<tr>
<td>Dialyzed and decolorized solution</td>
<td>165</td>
<td>60120</td>
<td>163.4</td>
<td>73</td>
</tr>
<tr>
<td>First acetone precipitation</td>
<td>100</td>
<td>38830</td>
<td>762.0</td>
<td>47</td>
</tr>
<tr>
<td>Second acetone precipitation</td>
<td>50</td>
<td>27350</td>
<td>1763.0</td>
<td>33</td>
</tr>
</tbody>
</table>

^An 18 hr. culture was used as the inoculum.

^An amylase unit was defined as that amount of amylase which hydrolyzes 1 mg of starch per min. at 60°C at pH 5.9.

It is evident from the data in Table 16 that the yield was low; less than 20 mg of material could be expected from 3 l of original culture liquor.

(8) Gel filtration

The enzyme precipitate from the second acetone treatment is dissolved in 50 ml of 0.01 M calcium acetate, pH 5.6. The enzyme solution is run through Sephadex G-75 columns prepared in the manner described previously. Effluents from peak A are pooled and concentrated.

Through the above procedure, amylase can be purified. Many attempts
were made to crystallize the amylase but without any success. This was probably because of the low concentration of enzyme protein present in the preparation. If this was the case, it would be necessary to increase the yield of amylases originally present in the culture.
SUMMARY

1. In the search of amylase-producing, thermophilic actinomycetes from soil and manure, a strain of *Thermoactinomyces vulgaris* was selected for use. Some of its morphological and physiological characteristics were studied.

2. In the preparation of inoculum to be used in the production of amylases from the organism, two solid media, A and B, and a liquid medium (C broth) were developed. The optimum period of incubation for the growth of this organism on either A or B agar was 48 hr. When the cultures from these two media were used to inoculate the C broth, 12 hr. incubation in the liquid medium was optimum for the development of culture for inoculum if cells from A agar were used, while 18 hr. was appropriate for cells from B agar. A 12 hr. inoculum was superior to that from an 13 hr. culture for the production of amylases.

3. For amylase production, N-Z case was a satisfactory nitrogen source. Generally, organic nitrogen sources were superior to inorganic nitrogen sources. Among the carbohydrates examined, maltose and soluble starch were amylase inducers. Fructose and mannitol supported profuse mycelial growth but not amylase production.

4. High amylase yields were obtained when 1% N-Z case was used with 2% of soluble starch or maltose, 0.1% yeast extract, 0.1% KH₂PO₄, 0.05% calcium chloride, and 0.05% D-glucose. The initial pH of the medium should be adjusted to 6.3-6.5.

5. Amylase production could be enhanced by using 1% maltose and 1% N-Z case as the initial concentration of sugar and nitrogen in the initial
medium, supplementing further with 1% soluble starch after incubation for 24 hr.

6. The pH of the culture could be used as an indicator for time of harvest. The optimum time for harvest was when the pH reached approximately 7.2-7.5. Maximum amylase yields were usually obtained at the point when lysis of the cells was just starting.

7. A procedure for the growth of the culture as well as amylase production was described.

3. A procedure for the purification of amylases was described.

9. There were at least 3 amylases present in the culture. One of these amylases was found to be chromatographically and electrophoretically pure.

10. This amylase had an optimum pH around 6.0 and optimum temperature of 60°C for maximum activity. It was rather unstable at temperatures above 70°C.

11. Transformation of one amylase species into another was observed between two amylases present in the enzyme preparation. These two amylases had nearly identical action patterns.

12. There were at least 5 proteases in the culture liquor. These enzymes were not separable with gel filtration but could be separated with ion exchange chromatography.
LITERATURE CITED

Agre, N. S.

Aikawa, T., Oote, S., and Takahara, Y.

Babbar, I. J., Power, V. K., and Jagannathan, V.

Breed, R. S., Murray, E. G. D., and Smith, N. R.

Campbell, L. L.

Campbell, L. L.

Campbell, L. L. and Cleveland, P. D.

Campbell, L. L. and Manning, G. B.

Chaloupka, J.

Chaloupka, J.

Chersi, A.
Corbaz, R., Gregory, P. H., and Lacey, M. E.

Cramer, F. and Steinle, D.

DePinto, J. A. and Campbell, L. L.

El-NaKeeb, M. A. and Lechevalier, H. A.

Erikson, D.

Erikson, D.

Fischer, E. H. and Stein, E. A.

French, D. and Rundle, R. G.

Freudenburg, K. and Cramer, F.

Fujii, N., Ookubo, M., and Mikawajiri, H.

Gilmour, C. M. and Butala, D.

Hagihara, B.

Hagihara, B., Nakayama, T., Matsubara, H., and Okunuki, K.

Henssen, A.


Hyslop, P. and Sleeper, B. P.

Imsenecki, A. A., Solntzewa, L. I., and Kuzyurina, L. A.

Isemura, T. and Kakiuchi, K.

Isemura, T., Kakiuchi, K., and Eto, H.

Jolly, R. L. and Mason, H. S.

Kakiuchi, K., Hamaguchi, K., and Isemura, T.


Krassilnikov, N. A.
Krassilnikov, N. A. and Koreniako, A. I.
1938 The phenomenon of autolysis in Actinomycetales. II. Influence of environmental conditions upon autolysis of actinomycetes and proactinomycetes. Mikrobiologiya (Abstract) 7: 829-837.

Küster, E.

Küster, E. and Locci, R.

Küster, E. and Williams, S. T.


Locci, R.

Lowry, O. H., Rosebrough, N. J., Fair, A. L., and Randall, R. J.

Manning, G. B. and Campbell, L. L.

Manning, G. B., Campbell, L. L., and Foster, R. F.

Massey, V., Harrington, W. F., and Hartley, E. A.

McClenahan, W. S., Tilden, E. B., and Hudson, C. S.
1942 A study of the products obtained from starch by the action of the amylase of Bacillus macerans. J. Am. Chem. Soc. 64: 2139-2144.
Menzi, R., Stein, E. A., and Fischer, E. H.  

Mizusawa, K., Ichishima, E., and Yoshida, F.  

Nakamure, M., Ohmura, K., and Kobayashi, N.  

Nelson, N.  

Nomoto, M. and Narahashi, Y.  

Nomoto, M. and Narahashi, Y.  

Porath, J. and Bennich, H.  


Rautenstein, J. I.  

Sakamoto, S. and Shuzue, K.  

Schwimmer, S. and Balls, A. K.  
Senya, K. and Kondo, Y.

Simpson, F. J. and McCoy, E.

Smith, S. W. and Roe, J. H.


Somogyi, M.

Starke, E. and Tetrault, P. A.
1951 Isolation of bacterial, cell-free, starch saccharifying enzymes from the medium at 70°C. J. Bacteriol. 62: 247-249.

Stein, E. A.

Stein, E. A. and Fischer, E. H.

Stein, E. A. and Fischer, E. H.

Stein, E. A. and Fischer, E. H.

Suzuki, H. and Tanabe, O.

Tanabe, O. and Ohyama, J.
Tanguay, A. E.

Tendler, M. D. and Burkholder, P. R.

Tilden, E. B. and Hudson, C. S.

Tinoco, I., Jr.

Tiselius, A., Porath, J., and Albertssen, P. A.

Tonomura, K., Futai, F., and Tanabe, O.

Tonomura, K., Iwana, K., Futai, F., and Tanabe, O.

Tonomura, K., Suzuki, H., Nakamura, H., Kuraya, K., and Tanabe, O.

Uridil, J. E. and Tetrault, P. A.

Waksman, S. A.

Waksman, S. A. and Corke, C. T.

Webley, D. M.
Yamanaka, T., Higashi, T., Horio, T., and Okunuki, K.  
1957 Denaturation and inactivation of enzyme proteins. VIII.  
Thermodynamic aspects of the denaturation of crystalline  
ACKNOWLEDGMENTS

The author wishes to express his deep appreciation to Dr. Paul A. Hartman for the opportunity to work in his laboratory, for his encouragement, and help throughout the author's stay at Iowa State and in research.

The author also wishes to acknowledge the members of Dr. Hartman's research group for their help during the author's research and discussion developed during work. He also likes to express his appreciation to all the professors in his department for many valuable suggestions. Thanks are also extended to the members of the author's committee and the fellow graduate students in the department.

This work was supported by an assistantship from the Iowa Agricultural and Home Economics Experimental Station, Ames, Iowa, and the author wishes to thank the University for the financial assistance.