A study of some biochemical and physiological properties of an extreme thermophile
by Jorj Terry Ulrich

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Microbiology
Montana State University

Abstract:
A gram-negative nonsporeforming extreme thermophilic bacterium was isolated from an alkaline hot spring in Yellowstone National Park. The results of physiological and cultural studies indicate the organism to be optimally adapted to alkaline pH (7.5 - 7.8) and to extreme temperatures (70 C). Studies of the morphology of the organism using light and electron microscopy indicate a lack of spores and flagella and reveal nothing unique to the architecture which would explain the ability to survive high temperatures. The organism was found to be a strict aerobe, heterotrophic for its carbon needs. Respiration studies done at 50, 60, and 70 C showed optimum respiratory activity at 70 C. Spectrophotometric analysis of cells grown at these temperatures revealed uniform concentrations of cytochromes a, a3, b, and c, indicating that a decrease of oxygen tension with an increase in temperature was not the rate limiting factor for the aerobic respiration of these organisms. A thermostable β-galactosidase (E.C. 3.2.1.23 β-D-galactoside galactohydrolase) was found to be inducible in this organism. The induction parameters and the repression of β-galactosidase synthesis by glucose indicate the presence of a lactose operon similar to that found in mesophilic organisms.

The enzyme was purified 78-fold and the optimum temperature and pH were determined to be 80 C and pH 5.0, respectively. The enzyme was activated by both manganese and ferrous iron, indicating a metal ion dependency of the catalytic site. Sulfhydryl activation and thermal stabilization indicate the thermophilic β-galactosidase is a sulf-hydryl enzyme. Kinetic determinations at 80 C established a Km of 2.0 x 10”^M for the chromogenic substrate o-nitrophenyl-p-D-galacto-pyranoside (ONPG) and a Ki of 7.5 x 10^-3 for lactose. The Arrhenius energy of activation was calculated to be 13.7 Kcal/mole. A molecular weight of 5.7 x 10^5 daltons was estimated by elution from Sepharose 4B. Pronase digestion, alkaline hydrolysis, and charcoal adsorption did not affect the thermostability of the purified enzyme, indicating the molecule was not stabilized by extrinsic factors.

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ABSTRACT

A gram-negative nonsporeforming extreme thermophilic bacterium was isolated from an alkaline hot spring in Yellowstone National Park. The results of physiological and cultural studies indicate the organism to be optimally adapted to alkaline pH (7.5 - 7.8) and to extreme temperatures (70 C). Studies of the morphology of the organism using light and electron microscopy indicate a lack of spores and flagella and reveal nothing unique to the architecture which would explain the ability to survive high temperatures. The organism was found to be a strict aerobe, heterotrophic for its carbon needs. Respiration studies done at 50, 60, and 70 C showed optimum respiratory activity at 70 C. Spectrophotometric analysis of cells grown at these temperatures revealed uniform concentrations of cytochromes a, a_3, b, and c, indicating that a decrease of oxygen tension with an increase in temperature was not the rate limiting factor for the aerobic respiration of these organisms. A thermostable β-galactosidase (E.C. 3.2.1.23 β-D-galactoside galactohydrolase) was found to be inducible in this organism. The induction parameters and the repression of β-galactosidase synthesis by glucose indicate the presence of a lactose operon similar to that found in mesophilic organisms. The enzyme was purified 78-fold and the optimum temperature and pH were determined to be 80 C and pH 5.0, respectively. The enzyme was activated by both manganese and ferrous iron, indicating a metal ion dependency of the catalytic site. Sulfhydryl activation and thermal stabilization indicate the thermophilic β-galactosidase is a sulfhydryl enzyme. Kinetic determinations at 80 C established a K_m of 2.0×10^{-3}M for the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) and a K_i of 7.5×10^{-3}M for lactose. The Arrhenius energy of activation was calculated to be 13.7 Kcal/mole. A molecular weight of 5.7×10^5 daltons was estimated by elution from Sepharose 4B. Pronase digestion, alkaline hydrolysis, and charcoal adsorption did not affect the thermostability of the purified enzyme, indicating the molecule was not stabilized by extrinsic factors.

INTRODUCTION

> "Well may we affirm, that every part of the world is habitable! Whether lakes of brine, or...warm mineral springs--the wide expanse and depths of the ocean--the upper regions of the atmosphere, and even the surface of perpetual snow--all support organic beings."
> (Charles Darwin, Voyage of H.M.S. Beagle, Ch. IV)

These observations voiced by Charles Darwin in 1833 have been well established through biological investigations. Microorganisms have been successfully cultured from many environmental extremes, including those of temperature, redox potential (Eh), pH, salinity, hydrostatic pressure, nutrient concentration, and electromagnetic and ionizing radiations. A general review of these factors has been given by Brock (10). He states that there are three ways in which an organism can adapt to an extreme environment: (i) it can develop a mechanism for excluding the factor, (ii) it can develop a mechanism for detoxifying the factor, or (iii) it can learn to live with the factor. It is the last possibility that this thesis will deal with in considering some physiological and biochemical aspects of an extreme thermophilic bacterium.

The Biochemical Approach to Thermophily

Many theories have been promulgated in attempts to explain the nature of life at high temperatures. The data collected in support of these theories has been obtained primarily by the isolation and

study of various cellular components.

Gaughran (33) proposed the concept that the composition of cellular lipids may set the limits of the growth temperatures for microorganisms. He suggested that cells cannot grow at temperatures below the solidification point of their lipids. He reported a decrease in lipid content concomitant with an increase in saturated fatty acids as the growth temperature of a mesophile was raised above its optimum. In contrast, the lipids of a thermophile remained relatively constant in both quantity and the degree of saturation. Marr and Ingraham (58) showed that the mesophile, Escherichia coli, reflected a progressive increase in saturated fatty acids and a corresponding decrease in unsaturated fatty acids as the temperature of growth was increased. However, they also found that temperature was not unique in determining the fatty acid composition, since altering the nutrition independently of temperature also resulted in major changes in fatty acid composition. Recently, Bauman and Simmons (7) have examined the fatty acids and polar lipids extracted from bacterial masses found in two Yellowstone National Park hot springs. Both populations had a series of C_{14} to C_{20} straight-chain acids with a maximum at C_{18}, and a series of saturated isoacids with a maximum at C_{17} in one case and C_{19} in the other. They concluded that the high maxima of these series may be attributed to their extreme thermophilic environment, but that

otherwise the distribution is unremarkable for organisms from such an extreme environment. In a study of thermophilic Bacillus species, Daron (20) reported that fatty acids having 16 or 17 carbon atoms accounted for over 30% of the fatty acid content of the cells. The proportion of unsaturated fatty acids varied inversely with the growth temperature and was never greater than 14%. In contrast, in a study of a mesophilic Bacillus species, fatty acids with 15 carbon atoms predominated and unsaturated fatty acids constituted 14% to 20% of the total fatty acids (44). Although the lipid basis for thermophily has not been supported by decisive experimental evidence, further study seems warranted in regard to the lipo-protein nature of the membranes of thermophilic organisms.

A second area of intense study has been the protein synthesizing machinery of thermophiles. Saunders and Campbell (74) isolated the mRNA of the obligate thermophile, Bacillus stearothermophilus, after a 30 second pulse of ^{32}P. The base composition was determined and the guanine plus cytosine (G + C) ratio of the mRNA agreed well with the G + C ratio of the deoxyribonucleic acid (DNA) isolated from the same strain. Recognizing that the base composition of mRNA is a reflection of the base composition of DNA, it was tempting to consider that the DNA from thermophiles should have a higher percentage of G + C, hence more triple bonding and more stability, than mesophiles. Marmur and

Doty (57) have tabulated the G + C ratios of the DNA of various mesophilic organisms as well as for B. stearothermophilus. The mesophilic G + C ratios range from 29% to 74%, while B. stearothermophilus has a G + C ratio of 44%. Brock and Freeze (12) reported a G + C ratio of 65% for Thermus aquaticus, a thermophile which grows optimally at 72 C. The temperatures necessary to achieve thermal melt (T_m) of the DNA of these thermophilic organisms studied is far in excess of their respective maximum growth temperatures.

The nucleotide composition of tRNA from B. stearothermophilus stain B (54) is very similar to that reported for E. coli (26).

Thermal stability of ribosomal RNA and ribosomes has been determined by thermal melting profiles. Ribosomes isolated from B. stearothermophilus undergo thermal denaturation at temperatures markedly higher than those obtained from E. coli (31, 74). Recently, Zeikus et al (85) have reported the ribosomes of T. aquaticus to have an even higher degree of thermostability than B. stearothermophilus. Saunders and Campbell (74) studied the ribosomal RNA of B. stearothermophilus to determine if the greater stability of its ribosomes was due to the presence of an unusual ribosomal RNA species. No significant differences were found between the ribosomal RNA species present in B. stearothermophilus and E. coli, as reflected by the nearly identical thermal melting profiles of the 16S and 23S ribosomal

RNA fractions from both organisms. It was postulated that the more stable ribosomes of the thermophile might reflect a difference in ribosomal proteins. However, a comparison of the amino acid composition of B. stearothermophilus ribosomes with that reported by Spahr (80) for E. coli ribosomes, again revealed no significant differences (74). Pace and Campbell (67) found that the thermal stability of ribosomes of 19 organisms, ranging from the psychrophile, Vibrio marinus, to the thermophile, B. stearothermophilus, correlated positively with maximal growth temperatures. In attempts to explain this positive correlation, the base compositions of the ribosomal RNA species of the 19 organisms were determined. The guanine-cytosine content increased and the adenine-uracil content decreased with increasing growth temperature. It was also shown in this study that no direct correlation existed between the guanine-cytosine content of ribosomal RNA and its respective DNA.

A subcellular protein-synthesizing system prepared from B. stearothermophilus was extensively studied by Friedman and Weinstein (32). Incorporation of ^{14}C-phenylalanine, lysine, and proline occurred across a temperature range of 30 to 70 C, with an optimum between 55 and 60 C, when directed by native mRNA molecules. Parallel studies with the E. coli system showed that endogenous incorporation of ^{14}C-phenylalanine at 65 C was only about 10% of that observed at 37 C.

However, when poly-uracil was used as a messenger, incorporation was greater at 37 C than at 65 C in the thermophilic system. The significant incorporation occurring at 37 C was unexpected, because B. stearothermophilus did not grow at 37 C in the growth medium utilized in these studies. They concluded that the lesion which prevented growth at 37 C did not involve the components of the protein synthetic apparatus of this organism.

Attempts to explain the molecular mechanism of thermophily has led to abundant research with thermostable enzymes. An apparent major breakthrough came when Manning, et al (55, 56) crystallized and characterized the extracellular enzyme, α-amylase, of B. stearothermophilus. Physicochemical characterization of this enzyme indicated that it was a small protein with a molecular weight of 15,600 deltons. Structurally it was unusual with a high proline content and a virtual absence of α-helix as determined by optical rotatory dispersion. The thermostability of α-amylase was attributed to a randomly coiled structure having the characteristics of a denatured protein. Recently, α-amylase from B. stearothermophilus has been the subject of further study (69). Attempts to crystallize the enzyme by the method of Manning et al (55) yielded crystals which were identical in appearance with those reported earlier to be α-amylase, but were in fact not the enzyme. Pfuller and Elliot (69) found the molecular weight of the enzyme to be 53,000 daltons and dependent on Ca^{++} for stability. Amino

acid analysis revealed a low proline content. The authors concluded that the α-amylase in these studies did not have a random coiled structure, but resembled the many other known α-amylases.

Contrasting the work of Manning et al (55) is the study of a thermostable protease from B. thermoproteolyticus (65). Physicochemical studies on this enzyme indicated that thermostability resulted from a rigid structure. The abundance of amino acids having hydrophobic side chains and the high content of tyrosine were suggested as factors contributing to this rigidity.

Both α-amylase and protease are small extracellular enzymes and may not be representative of enzymes in the intracellular environment, where structural elements in the cell could perhaps contribute to thermostability. Studies of a thermostable intracellular enzyme have been made possible by the isolation and crystallization of a glyceraldehyde 3-phosphate dehydrogenase from B. stearothermophilus (2, 3). The molecular weight (163,500 daltons) was similar to other glyceraldehyde 3-phosphate dehydrogenases. Optical rotatory dispersion studies suggested a tightly structured molecule. Singleton, et al (78) have compared the amino acid content of the enzyme to counterparts from other sources (rabbit, lobster, and pig muscle). The compositions of the enzymes proved to be quite similar and the thermophilic enzyme did not exhibit an increased number of hydrophobic

residues. A recent examination of the subunit structure of glyceraldehyde 3-phosphate dehydrogenase from B. stearothermophilus has revealed no information concerning the thermostable property of the enzyme (5).

Freeze and Brock (30) have recently presented data on the purification and properties of the thermostable fructose 1,6-diphosphate aldolase of T. aquaticus. They found a molecular weight of 140,000 daltons, which was in agreement with the aldolases of Anacystis nidulans and B. stearothermophilus. A comparison of T. aquaticus enzyme with that of B. stearothermophilus was of interest, in that Thompson, et al, (81) showed that the B. stearothermophilus enzyme was inactivated rapidly at 75 C, whereas the T. aquaticus enzyme was quite stable even to 97 C. Both enzymes were activated by cysteine and had similar catalytic properties. It was concluded that the active site of the T. aquaticus enzyme was conserved during evolution, but because of its great heat stability, it is likely that other portions of enzyme are markedly altered.

Howard and Becker (39) have recently purified (approximately 1000-fold) the TPN^+ dependent isocitrate dehydrogenase from B. stearothermophilus. In a detailed analysis they compared the physicochemical properties of this enzyme to those of pig heart isocitrate dehydrogenase and those of the enzyme from the mesophile Azotobacter

vinelandii. The molecular weight of the thermophilic enzyme was 92,600 daltons as compared to molecular weights of 80,000 daltons and 60,000 daltons from A. vinelandii and pig heart, respectively. In general the catalytic properties of the three enzymes were similar. Amino acid analysis of the thermophilic enzyme showed neither an elevated average hydrophobicity nor a large number of tryptophan or tyrosine residues. The low half-cystine content of the thermophilic isocitrate dehydrogenase was in keeping with the values reported for aldolase and amylase from B. stearothermophilus. They concluded that a high degree of disulfide crosslinking was not necessary for thermal stability.

At the present time, the consensus among investigators is that thermostable proteins are inherently stable and not subject to extrinsic stabilizing factors (30, 39, 78). Ameluxen and Lins (4) have recently completed a comparative study on the thermostability of enzymes from B. stearothermophilus and a mesophile, B. cereus. Of the eleven enzymes examined, the thermophilic species were, in general, much more stable than the mesophilic species. However, the pyruvate kinase and the glutamic-oxaloacetic transaminase from the thermophile had thermostability characteristics quite close to those from the mesophile. The authors indicated that the possibility exists that not all thermophilic enzymes have intrinsic molecular thermostability,

and that such enzymes might well depend on the rate of synthesis as a controlling mechanism. An additional study, combining lysates of the mesophilic and thermophilic systems, indicated thermostability could not be conferred to the mesophilic protein. Although the absence of extrinsic stabilizing factors is suggested, the authors point out that stabilizing factors, if present, may have the capacity to bind only to the thermophilic protein.

Although the efforts of these aforementioned investigators on the thermostable macromolecules of thermophiles have been fruitful, they have not established the nature of their thermal tolerance.

The Physiological Approach to Thermophily

A contrast to the rigid biochemical approach to life at high temperatures is the physiological approach, directed at an examination of the total life processes at elevated temperatures.

In an early review on thermophily, Allen (1) put forth the hypothesis that growth of thermophiles at high temperatures depends on rapid synthetic events to replace the heat-damaged proteins. Supporting evidence for this concept was reported by Bubela and Holdsworth (13).. They found that B. stearothermophilus had a significantly faster rate of protein and ribonucleic acid turnover than did E. coli. This report (13) is at variance with the recent findings of Epstein and Grossowicz (27), who determined that a similar rate of

protein breakdown occurred in resting cells of a thermophile and E. coli. The latter investigators argue that although only breakdown of protein was determined, it can be safely assumed that similar results would be obtained if protein turnover, i.e., breakdown and resynthesis, had been measured. They concluded that their results are in accordance with the concept of increased thermal stability of the cellular proteins from thermophiles.

A useful way of expressing quantitatively the effect of temperature on microbial activity is in the form of the temperature coefficient or Q_{10} values. Theoretically then, an organism should be 16 times as active at 70 C as it is at 30 C, hence life processes in a thermophile should be quite rapid. A strain of B. stearothermophilus with an optimum temperature of growth at 65 C has been calculated to have a generation time of 11 minutes (64) as compared to that of E. coli with an optimum temperature of 40 C and a generation time of 21 minutes (41). Brock (11) has recently summarized the data on the growth rates of various bacteria (both mesophiles and thermophiles) at their optimum temperatures. An Arrhenius curve was plotted as the logarithm of the generations per hour versus the reciprocal of the optimum growth temperatures (absolute) of these organisms. This composite curve was compared to an Arrhenius curve for the growth of E. coli at various temperatures at and below its optimum. As the

composite curve was much shallower than the curve for E. coli, it was concluded that thermophiles do not grow as fast at their optima as has been predicted.

Another use of the Arrhenius equation has been to compare the rate of the total biological process (growth) with a change in temperature. Epstein and Grossowicz calculated the Arrhenius constant for a prototrophic thermophilic bacillus to be 15,000 and 13,500 cal/mole when grown in minimal and rich media, respectively (28). As the authors point out, these values are essentially similar to those given by others for mesophiles and psychrophiles (37, 76), indicating that life processes are indeed no faster at higher temperatures.

Adaptation of microorganisms to growth over different ranges has shown, with the exception of one group of organisms, that the temperature range for most microorganisms growing under normal environmental or laboratory conditions is not readily altered (29). The exception to this generalization has been reported with certain mesophilic bacteria which can often acquire the ability to grow at temperatures above their normal maximum for growth, and therefore become essentially thermophilic. Kluyver and Baars (45) were among the first to report evidence for adaptation when they showed that strains of the mesophile, Desulfovibrio desulfuricans, can acquire the thermophilic habit after culturing at high temperatures. Allen (1) reported that mesophilic

strains of B. subtilis, B. cereus, B. megaterium, and B. circulans consistently yielded thermophilic variants.

Due to the advent of the technique for studying genetic transformation in bacilli, it became possible to investigate the transfer of genetic information that determines thermophily. McDonald and Matney (61) showed that when mesophilic strains of B. subtilis (which are unable to grow at 55 C) were grown in the presence of DNA extracted from thermophilic strains of this bacterium, the mesophiles were transformed at the rate of about one organism in 10^4 for the ability to grow at 55 C. They concluded that the change to a thermophilic habit by these mesophilic bacteria involved a relatively small change in the metabolism of the organism.

Although the acquisition of new genetic information to explain the adaptation of an organism to a higher temperature is appealing, the following indicates the basis for this change may be physiological. Bausam and Matney (8) have shown that facultative bacilli growing in the mesophilic range require a brief period of adaptation at intermediate temperatures before gaining the capacity to initiate growth at thermophilic temperatures. Recently, Dowben and Weidenmuller (22) have reported that a genetically marked mesophilic bacterium, B. subtilis, could be induced to grow at temperatures as high as 72 C by slow adaptation. This adaptation was concomitant with an increase

in total cell protein. The ability of the organism for continued growth at high temperatures was, however, quickly lost upon transfer to the lower temperature.

In a theoretical paper, Orgel (66) has pointed out that adaptation to environmental conditions in which many enzymes are partially inactivated is not likely to occur by the emergence of mutant strains, as the required multiplicity of mutations would take too long. He states, on the other hand, adaptation to difficult environmental conditions may occur by relaxation of repression of enzyme synthesis if the change in the environment is gradual. This process of adaptation would then be accompanied by synthesis of increased amounts of many enzymes. He concludes that this physiological mechanism of adaptation may permit survival and growth of the organism for a sufficient number of generations to allow the emergence, after multiple mutations, of a strain particularly suited for that environment.

Thus the physiological approach to thermophily has shown that life processes at high temperatures appear to be no faster than those at mesophilic temperatures and that thermophiles could represent an adaptation from mesophilic ancestry.

Statement of Research Problem

The purpose of the research reported in the following pages was to combine the tools of biochemistry and physiology in a study of an

extreme thermophilic bacterium. The aspects of the study include the following: (i) a morphological and cultural examination of the bacterium, (ii) a study of the respiratory mechanism of this organism, based on the knowledge that the solubility of oxygen decreases as the temperature increases, (iii) a study to determine if thermophiles express similar genetic and biochemical control mechanisms as seen in mesophilic systems, and (iv) a purification and characterization of a thermostable enzyme from this organism to provide information as to the nature of its thermostability, as well as a comparison to its mesophilic counterpart.

MATERIALS AND METHODS

Isolation of the organism: The organism used in these studies was isolated from an alkaline thermal spring located in the Gibbon Meadow area of Yellowstone National Park. Samples of spring water containing the filamentous bacterial mats were placed in Nalgene bottles and transported to the laboratory.

Culture media: Initial isolations were cultured in 0.5% nutrient broth (Difco) at 70 C. Later, routine studies employed a medium containing 0.2% tryptone (Difco) plus 0.2% yeast extract (Difco) in a basal salts solution containing: 3.0×10^{-4}M $(NH_4)_2SO_4$, 1×10^{-3}M KCl, 4×10^{-3}M NaCl, 2.6×10^{-3}M $Na_2HPO_4 \cdot 7H_2O$, 5×10^{-5}M $Ca(NO_3)_2 \cdot H_2O$, 4×10^{-4}M $MgSO_4 \cdot 7H_2O$, and 0.5 ml Hutner's trace element solution (40) per liter. The pH was adjusted to 7.7 with NaOH. This medium will be referred to hereafter as the standard growth medium. Although initial isolates appeared to be pure cultures, plates containing the standard growth medium in 3.0% agar were streaked, wrapped in Saran Wrap (Dow Chemical Co.) to prevent drying, and incubated, inverted, just above the water surface in a covered water bath. Single, isolated colonies were picked for subculturing.

Nutritional requirements of the organism were studied following a scheme as outlined by Seaman (75). A chemically defined medium was devised containing 150 µg per ml of each of the following amino acids: cystine, isoleucine, glutamate, aspartate, proline, and serine in the

basal salts solution. Filter sterilized solutions of the growth factors biotin, B_{12}, lipoic acid, and paraaminobenzoic acid (PABA) were added to a final concentration of 0.3µg per ml. This medium will be referred to hereafter as the chemically defined medium.

Incubation conditions: A Blue M (MW-110A) Constant Temperature water bath, with a self-leveling reservoir, was used for incubation of unshaken tube cultures. The water bath was fitted with a plexiglass lid, which had holes drilled through it large enough to accommodate 16 mm screw capped culture tubes. The tubes were prevented from slipping through the holes by means of a rubber ring fastened just below the caps. For growth studies, a covered Blue M (MSB-1122A-1) Constant temperature shaking water bath was used. Agitation was maintained at 100 strokes per minute. Growth was measured at 640 nm in 500-ml Bellco side arm flasks containing 80 ml of medium by use of a Bausch and Lomb Spectronic 20 colorimeter. Dry weights were determined as outlined by Lichstein and Oginsky (49).

For large batch cultures a New Brunswich Micro-Ferm (MF-214) table top fermentor was used. A constant (70 C) temperature was achieved by a Heto-ultrathermostat (Birkerod, Denmark) circulating hot water pump attached to the heating coils of the fermentor. Airflow was regulated at 3 liters per minute and agitation at 250 rpm.

Microscopy: A Nikon (S-KE) microscope was used for all microscopic observations. Photo-micrography was performed with an ME-35 camera attachment with Kodak Panatomic-X film developed with Microdal X, diluted 1:3. Gram stains were used routinely and flagella stains were performed by the method of Blenden and Goldberg (9).

For electron microscope examination, cells were grown in the standard growth medium, washed three times in 0.05M sodium phosphate buffer, pH 7.0. Equal amounts of cell suspension and 2.5% glutaraldehyde in sodium phosphate buffer were mixed in BEEM capsules and allowed to stand at room temperature for one hour. The cells were pelleted by centrifugation, the supernatant removed and pelleted cells were resuspended in 1% osmic acid and allowed to fix for 1.5 hrs at 4 C. The cells were again pelleted and washed three times in phosphate buffer. The cells were dehydrated in steps with increasing concentrations of acetone (20%, 70%, 100%), allowing 15 min incubation with each change. The cells were centrifuged once again and the supernatant liquid discarded. The pellet was covered with 100% propylene oxide and allowed to stand at room temperature for 15 minutes. The propylene oxide was decanted and the pellet was covered with infiltrating solution (50% propylene oxide plus 50% Araldite epoxy resin) for one hour at room temperature. The infiltrating solution was decanted and the pellet was suspended in 100% Araldite (Ciba) and

placed in a 60 C hot air oven until polymerization occurred. Thin sections were cut on a Reichert Om U2 ultramicrotome and stained with a saturated aqueous uranyl acetate solution followed by lead citrate, as described by Reynolds (70). Shadow casting of unfixed samples was accomplished by the use of palladium (shadow angle 3:1) in a Varian shadow casting apparatus. All specimens were observed with a Zeiss EM 9A electron microscope.

Dipicolinic Acid Assay: Dipicolinic acid (DPA) content of whole cells was assayed by the method of Janssen and Lund (43). Cells were grown either in the standard growth medium or on soil extract agar plates. Approximately 10 mg of harvested cells were washed three times in distilled water and resuspended in 5 ml of distilled water. In addition, an ammonium sulfate preparation of the cell free preparation containing 20 mg per ml of protein (by Lowry's determination) was diluted to 5 ml with distilled water. These samples were autoclaved for 15 min at 15 lb per in^2. After cooling the suspensions were acidified with 0.1 ml of 1N HCl and allowed to stand at room temperature for 30 minutes. After centrifugation at 10,000 x g for 10 min, a clear supernatant was obtained. One ml of freshly prepared reagent consisting of 1% $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 1% ascorbic acid in 0.5M acetate buffer at pH 5.5, was added to 4 ml of liquid supernatant. The color density was measured within one hour at 440 nm using

a Baush and Lomb Spectronic 20 colorimeter, and compared to a standard containing 100 µg of DPA.

Respiration studies: Respirometric studies were carried out at various temperatures using a Gilson Differential Respirometer. The cells were grown to mid-log phase in the standard growth medium at the temperatures that were used for oxygen uptake experiments (50, 60, and 70 C). The cells were then washed in sodium phosphate buffer (0.05M, pH 7.0) and resuspended in the complete defined medium. This suspension of cells was added to respirometer flasks in 3.0 ml volumes. A 20% solution of KOH was added to the center wells. After a 30 min equilibration period, oxygen uptake was recorded every 5 min for 30 min before and after the addition of various exogenous substrates from the side arm (final concentration of each substrate was 0.2%). An external auxiliary circulating hot water pump was used to achieve the 60 C and 70 C temperatures in the respirometer water bath. Temperatures in excess of 70 C were not attempted for technical reasons.

Cells for cytochrome analysis were grown in the standard growth medium at 50, 60, and 70 C, in liter quantities to mid-log. After harvesting by centrifugation, the cells were washed and resuspended in phosphate buffer. The oxidized vs. dithionite reduced difference spectra (15) were determined at room temperature using a Hitachi

Perkin-Elmer (M 356) spectrophotometer. The cytochrome concentrations were calculated with the appropriate extinction coefficients.

$$\underline{a} \left\{ {}^{604\text{-}630}_{\text{redox}} = 27, \text{ and } \underline{a}_3 \left\{ {}^{445\text{-}460}_{\text{redox}} = 164 \quad (82); \right.\right.$$

$$\underline{b} \left\{ {}^{563\text{-}575}_{\text{redox}} = 22, \text{ and } \underline{c} \left\{ {}^{550\text{-}540}_{\text{redox}} = 19 \quad (83). \right.\right.$$

Induction of β-galactosidase: Isolates, T2 and T6 were found to be inducible for β-galactosidase. For optimal induction, filter sterilized lactose was added to the standard growth medium at a concentration of 3×10^{-2}M. Strain T2 was used exclusively throughout the remainder of these studies.

A strain of E. coli K_{12} obtained from the Department of Microbiology at Montana State University, was included during assays for β-galactosidase as a comparative control. E. coli cultures were routinely grown at 37 C, pH 7.2, in 0.8% yeast extract, 0.8% tryptone in the basal salts solution previously described. For β-galactosidase induction of E. coli cultures, filter sterilized lactose was added at a concentration of 3×10^{-2}M.

Buffer solutions: Induced cells were washed, suspended, and assayed in one of the following buffer solutions: 0.05M sodium phosphate (pH 3.5 to 8.5), 0.05M sodium citrate (pH 3.5 to 8.5), 0.05M TRIS

(hydroxymethyl) aminomethane (pH 7.0), or 0.05M sodium phosphate plus potassium phosphate (pH 7.0). The standard buffer used throughout this work contained 0.05M sodium phosphate at pH 7.0 (buffer A).

Preparation of induced whole-cell suspensions for assay: To follow induction, aliquots of cells were removed from the culture at timed intervals, immediately chilled in an ice bath, centrifuged, and resuspended in one of the above buffers. Five ml volumes of these suspensions were treated with 0.5 ml of toluene-acetone (2:1), and incubated for 5 min, with agitation, at 25 C. The treated suspensions were immediately assayed for enzyme as described below.

Preparation of cell-free extracts: Large batch cultures (10 l) grown for 12 hrs in the standard growth medium plus 3 x 10^{-2}M lactose were harvested using a Sorvall Continuous Flow System (KSB) attached to a Sorvall RC2-B (Refrigerated) Centrifuge. The temperature was controlled at 4 C and the speed at 25,000 x g. The cell paste (45-50g wet weight per 10 l) was washed three times and resuspended in the standard buffer to a concentration of 250 mg (wet weight cells) per ml. The cells were then ruptured with a Sorvall Ribi cell fractionator at 10 C using a pressure of 30,000 psi. Cell debris was removed by centrifugation at 40,000 x g for one hour at 4 C. The supernatant liquid of the cell-free extract was diluted with buffer and assayed

as described below.

Assay of β-galactosidase: The chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) was used to measure enzyme activity (48). Solutions of 5×10^{-3}M ONPG were prepared in the above buffers. To a 1.0 ml toluene-treated cell suspension or cell-free extract was added 4.0 ml of ONPG solution. The mixture was incubated for 10 min at 70 C, unless otherwise indicated. The reaction was terminated by holding the tube containing the reaction mixture in an ice bath for 2 min, followed by the addition of 5.0 ml of 0.5M Na_2CO_3. For routine assays no attention was given to a warm-up period, since all samples received the same incubation conditions. In the case of the whole cell assay, cells were removed from the assay mixture by centrifugation at 10,000 x g for 5 min. Cell-free assay mixtures remained clear and required no centrifugation. The absorbancy of the supernatant liquid was measured at 420 nm using a Beckman Model B spectrophotometer and converted to molar concentrations of o-nitrophenol (ONP) by multiplying by the appropriate molar extinction ($1/E_{cm}^{nM} = 240$ $nmole^{-1}cm^{-1}$) as determined from an ONP standard curve. Non-induced cells were used as appropriate control blanks for the whole cell assays, and tubes containing all reagents, except enzyme, were used as controls in the cell-free assay. ONPG thermal breakdown at 70 C for 30 min was negligible, but increased rapidly at temperatures above

80 C. Assays for E. coli β-galactosidase were conducted at 25 C in buffer A.

The specific activity of β-galactosidase for the whole-cell assay is expressed as nmoles of ONP liberated from ONPG per milligram of cell dry weight per minute. Dry weights were determined from a standard curve relating cell dry weight to the optical density (OD) of a cell suspension at 640 nm. The specific activity of the cell-free β-galactosidase is expressed as nmoles of ONP liberated from ONPG per milligram per ml protein per minute. Protein determinations were performed by the method of Lowry (52) and compared to a standard containing 100 µg of purified bovine serum albumen.

One unit of enzyme is equivalent to 1.0 nmole of ONP liberated from ONPG per minute, at 70 C and pH 7.0.

The differential rate of β-galactosidase induction, P, was obtained from the slope of a plot of specific activity against time.

Procedure for establishing optimal assay conditions: To determine the temperature of optimum activity of the enzyme, a 52-fold purified preparation containing 10 µg protein per ml was assayed at various temperatures. In addition, the same procedure was carried out using an enzyme preparation at 5 µg protein per ml and containing 5×10^{-3}M cysteine. A blank containing all reactants except enzyme was used at each temperature to correct for the thermal hydrolysis of ONPG.

Heat inactivation of the enzyme was followed by preincubating the 52-fold preparation in sodium phosphate buffer (pH 7.0), containing 10 µg protein per ml, at various temperatures for timed intervals. The samples were placed in an ice bath after removal from the water bath and then assayed for residual enzyme activity at 70 C. Another sample of enzyme was placed in pH 5.0 sodium phosphate buffer and pre-incubated at 70 C and then assayed at 70 C for residual activity.

The optimum pH for activity was evaluated by dialyzing the enzyme preparation against distilled water for 24 hours to remove the buffer. One ml portions of the enzyme preparation were then placed into a series of tubes. Solutions containing 5×10^{-3} M ONPG in either sodium phosphate or sodium citrate buffer were adjusted over a pH range of 3.5 to 8.5. Solutions of ONPG at the appropriate pH were added to the enzyme and incubated at 70 C.

A suspension of 12 hr lactose-induced cells was also evaluated for optimum pH and optimum temperature for activity. To establish an optimum pH for activity, cells were suspended in either sodium phosphate or sodium citrate buffer adjusted over a pH range of 3.5 to 8.5. Solutions of ONPG at the appropriate pH were added to 1.0 ml volumes of the cell suspensions and incubated at 70 C. To determine the optimum temperature for activity, 1.0 ml volumes of cells in sodium phosphate buffer (pH 7.0) were assayed at various temperatures.

Temperatures over 96 C were achieved by use of an oil bath.

Procedure for enzyme induction studies: Cells were grown to the early logarithmic phase of growth, in the standard growth medium. The cell suspension was then distributed into 5 flasks and 3 x 10^{-3}M concentrations of filter sterilized lactose, galactose, melibiose, and glucose and a 4 x 10^{-3}M concentration of isopropyl-β-D-thiogalactoside (IPTG) were added separately to the flasks. After timed intervals samples of cells were removed from each flask and the OD determined.

The samples were then cooled in an ice bath, washed and re-suspended in buffer A. After all samples had been collected, the cells were toluene-treated and assayed at 70 C. A non-supplemented culture was followed to determine basal levels of enzyme.

The induction response at 50, 60, 70, 75, and 80 C was followed using galactose or lactose as the inducer substrate.

Determination of enzyme specificity: Cultures of lactose, melibiose, and galactose induced cells were checked for enzyme specificity by assaying the cells with alpha and beta linked ONPG, and alpha and beta linked para-nitrophenyl-D-galactopyranoside (PNPG).

Procedure for catabolite repression studies: A culture was grown to the early logarithmic phase of growth in the standard growth medium plus 3 x 10^{-2}M glucose, to a second 3 x 10^{-2}M pyruvate, to a third

3 x 10^{-2}M glucose plus 7.5 x 10^{-3}M adenosine-3'5' cyclic mono phosphate (cAMP), to a fourth 3 x 10^{-2}M glycerol. Samples were removed from the cultures at timed intervals, the OD recorded and the sample was washed and resuspended in buffer and chilled. After all samples had been collected, the cells were toluene-treated and assayed at 70 C.

An additional study was performed to observe the effect of lowering the glucose concentration used to repress enzyme synthesis. The procedure was the same as described above, except that the glucose concentration used was 5 x 10^{-3}M.

Enzyme purification procedures: Cell-free extracts of 12 hr lactose-induced cultures were centrifuged for one hour at 40,000 x g to remove cell debris. The supernatant liquid was dialyzed overnight against buffer A. The preparation was then precipitated with two volumes of cold (4 C) acetone. The resulting precipitate was collected by a 10 min centrifugation at 15,000 x g and resuspended in buffer A. The insoluble material was removed by centrifugation and the supernatant liquid was again precipitated by the addition of solid ammonium sulfate. Enzyme activity was precipitated with 22.5-35% ammonium sulfate saturation at 23 C. The ammonium sulfate precipitate was removed by centrifugation, resuspended in buffer A and dialyzed against the same for 24 hours.

Following dialysis the precipitate was concentrated against solid polyethylene glycol to a volume of 2.0 ml and layered on a 2.5 x 35 cm column containing Sephadex G200. The sample was eluted with buffer A and collected in 3 ml aliquots by use of a Buchler Fractomette (M-3-4300) automatic fraction collector. Flow rate was 15 ml per hour. The void volume (V_o) was determined by the use of Blue Dextran 2000. Enzyme activity of the eluent was determined by an ONPG assay of an aliquot (0.2 ml) from each tube. The presence of protein was determined by an A_{280} scan of the eluent using a Beckman DU spectrophotometer.

The pooled peak fraction from the Sephadex G200 elution was concentrated by dialysis against solid polyethylene glycol to a volume of 3.0 ml and layered on a 2.5 x 35 cm column containing DEAE-Sephadex A50. Flow rate was 20 ml per hour. The sample was eluted with a linear gradient of 0.0M to 0.5M NaCl in the standard buffer. Enzyme activity and protein determinations were accomplished as discussed above.

Kinetic observations: To follow the kinetics of hydrolysis of ONPG varying concentrations of ONPG were placed in a series of tubes and allowed to pre-warm to the assay temperature being considered. To start the reaction, pre-warmed enzyme was added to the tubes. The reaction was terminated by holding the tubes in an ice bath for 5 min

followed by the addition of Na_2CO_3. The hydrolysis of ONPG was determined colorimetrically at A_{420}.

Michaelis-Menten (K_m) Determination: The K_m values were determined by plotting the kinetic data by the method of Lineweaver and Burke (50) in which the reciprocal of the Michaelis-Menten equation is written as $\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{(S)}$.

Inhibitor studies using lactose as a competitive inhibitor of ONPG were evaluated using the Dixon (21) plot. In this procedure the concentration of the ONPG was constant and the inhibitor concentration was varied.

The Arrhenius activation energy was calculated from the slope of the straight line plot of the logarithm of the initial activity (velocity constant) versus the reciprocal of the absolute temperature.

Molecular weight determination: The enzyme and several known molecular weight standards were eluted from a Sepharose 4B column and the elution volumes (V_e) were plotted semilogarithmically. The standards used were: catalase (2.4×10^5 daltons), E. coli β-galactosidase (5.4×10^5 daltons), and the monomer and dimer forms of urease (5×10^5 and 10^6 daltons, respectively). The peak fractions of these proteins were determined by a A_{280} scan. In addition, catalase was identified by testing the fractions with 0.5% hydrogen peroxide, urease by the

titration method of Gorin, et al, (36), and both E. coli β-galactosidase and the thermophilic β-galactosidase by the ONPG assay. In order to separate the activities of the two β-galactosidases, a 0.5 ml amount of eluent was taken from each tube across the fraction and assayed at 25 C for E. coli β-galactosidase. A second series of 0.5 ml amounts was taken from the eluted fraction and placed in a water bath for 5 min at 70 C. The tubes were removed and cooled in an ice bath, the substrate (ONPG) added and assayed at 70 C. To determine if all of the E. coli β-galactosidase was destroyed after 5 min at 70 C, a sample of enzyme, containing 10-fold more protein than was in the peak fraction of the eluent from Sepharose 4B, was treated for 5 min at 70 C. All E. coli enzyme activity was lost after this treatment.

Disc gel electrophoresis: Disc gel electrophoresis was performed in 5.0% polyacrylamide gels with 0.05M phosphate buffer, pH 7.2. Approximately 50 µg of sample in 20% sucrose was subjected to 8.0 ma of direct current for 3-4 hours. After electrophoresis the enzyme was detected by staining the protein with Coomassie brilliant blue. In addition the enzyme activity of unstained tubes could be detected by incubating a gel in the ONPG solution and observing the yellow chromophore. Gels developed by each technique were scanned on a Joyce Chromoscan Densitometer.

RESULTS

Isolation and initial observations: Samples of water containing the microbial mats transported from the hot spring to the laboratory and subsequently inoculated into tubes containing 0.2% tryptone and 0.2% yeast extract, required 24-36 hours incubation at 70 C, for the appearance of visible turbidity. If, however, the microbial mats were placed directly into the prepared standard medium, kept warm in a thermos during transport to the laboratory and incubated at 70 C, visible turbidity was evident within 8-10 hours. Growth in unshaken tubes appeared first as a pellicle at the surface. As the density increased, the bacterial mass became yellow in color and began to diffuse throughout the tubes, with the heavier clumps sinking to the bottom. Microscopic examination of this growth revealed gram negative filaments ranging in length from 8 μm to an estimated 150 μm and having a width of 0.4 μm to 0.6 μm (Fig. 1 upper left).

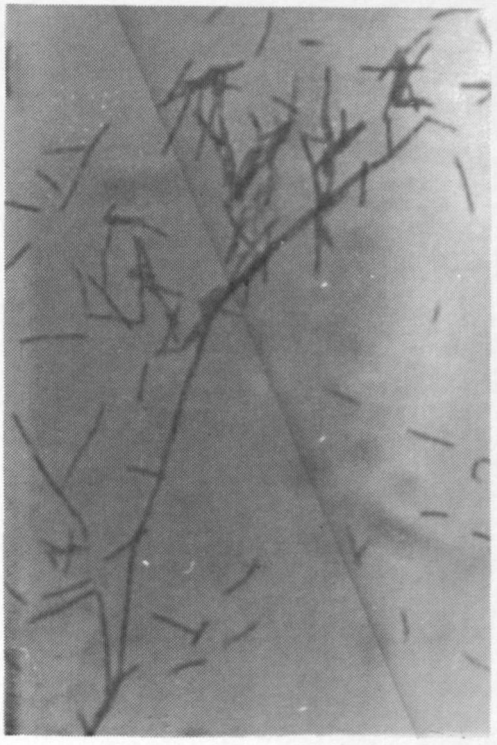
Pure cultures were obtained by streaking these enrichments on agar plates. Dense, spreading, yellow-pigmented colonies were observed after 24-36 hours incubation at 70 C. Isolates were stored either by lyophilization of broth cultures or by freezing the broth culture directly. The cultures were viable after storage under either condition for up to at least six months.

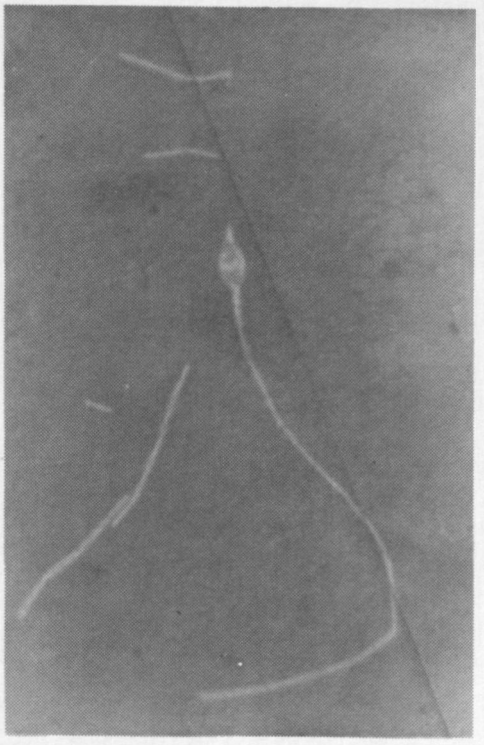
Morphology: Microscopic examination of unshaken cultures consistently

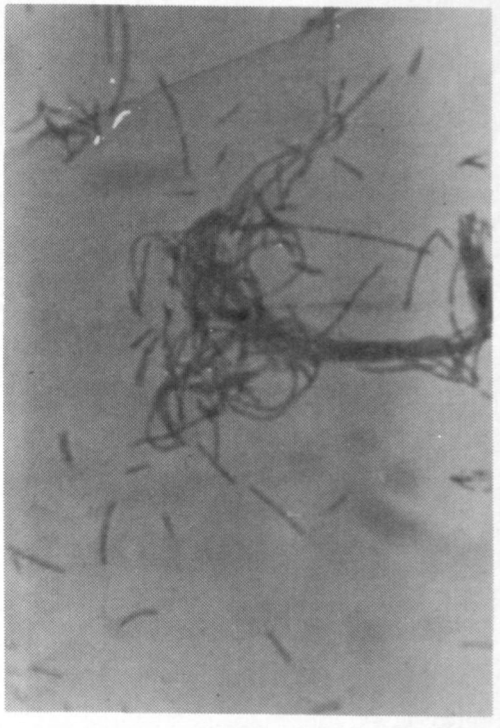
showed gram negative filaments of varying lengths. Aerated or shaken cultures produced cells of a more uniform length (6-8 μm). On occasion cells were seen which had a swollen area along the filament (Fig. 1-upper right). Microscope slides immersed in the growth medium allowed cells to adhere to the glass. Microscopic examination of these slides showed aggregations of the cells, either as linear arrays (Fig. 1-lower left) or as rosettes (Fig. 1-lower right). Flagella stains were negative, while control strains with B. pumilus displayed prominent flagella. To verify the absence of spores, dipicolinic acid (DPA) assays were done on cells from a variety of growth conditions. In all instances the assays were negative, while a sporulated culture of B. stearothermophilus gave an assay of 5 μg DPA per 20 mg wet weight of spore-cell suspension.

A shadow casted specimen (Fig. 2) observed under the electron microscope also indicated the lack of flagella in this organism. There appeared to be a great deal of amorphous material associated with the surface of the organism. This material may represent a slime or secretion which provides the organism with the ability to aggregate and to form bacterial mats in its natural hot spring environment.

There appears to be nothing unusual about the ultrastructure of the organism as shown by thin-sectioning (Fig. 3). The cell







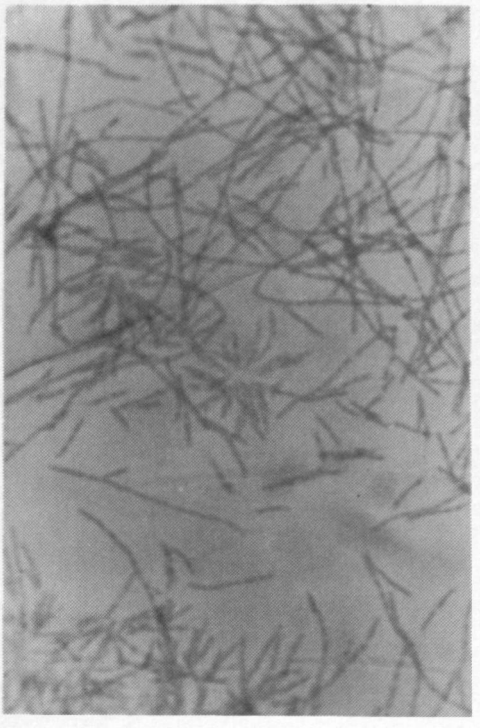


Figure 2 Electron micrograph of shadow cast organism.

Magnification: 48,000x.

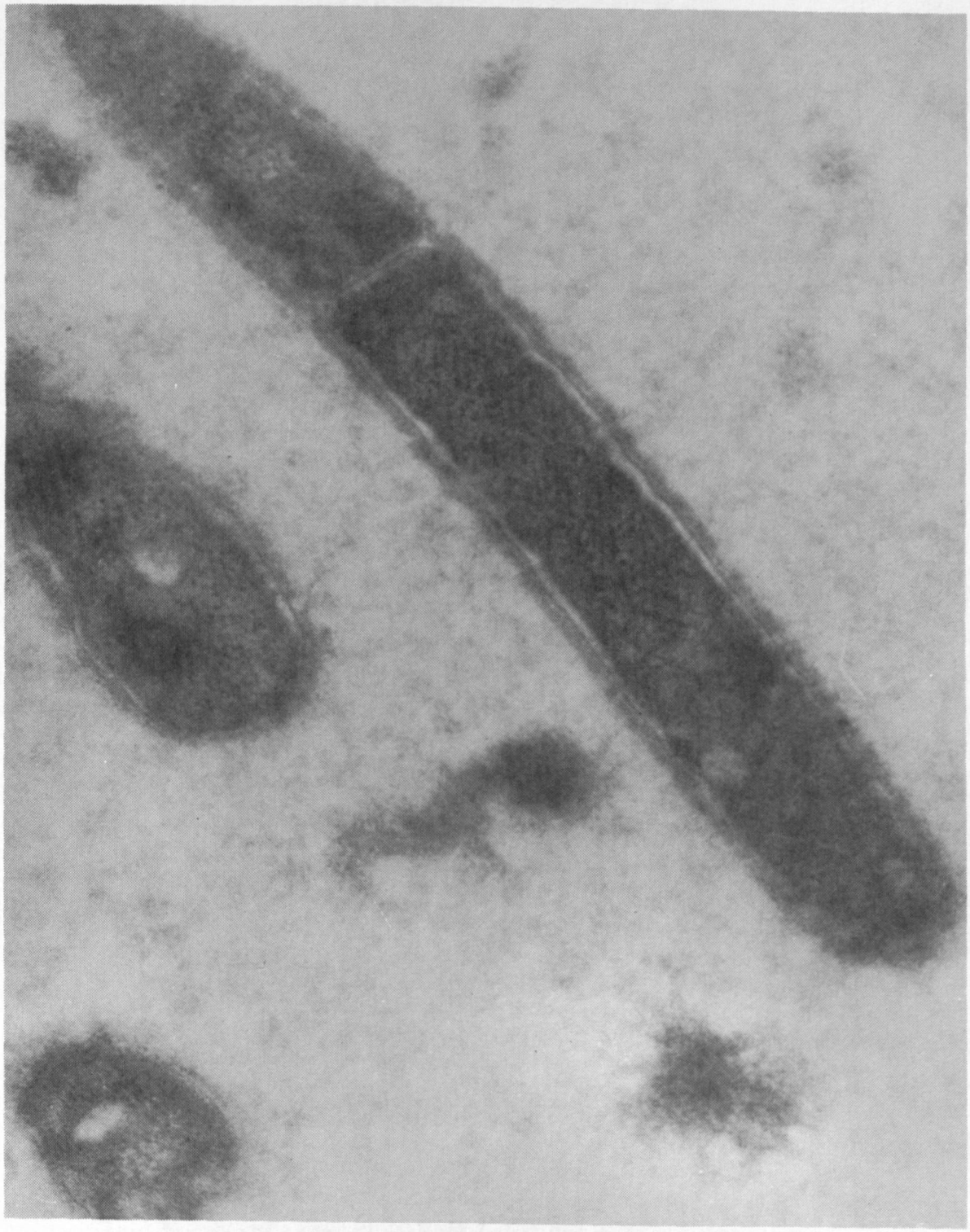
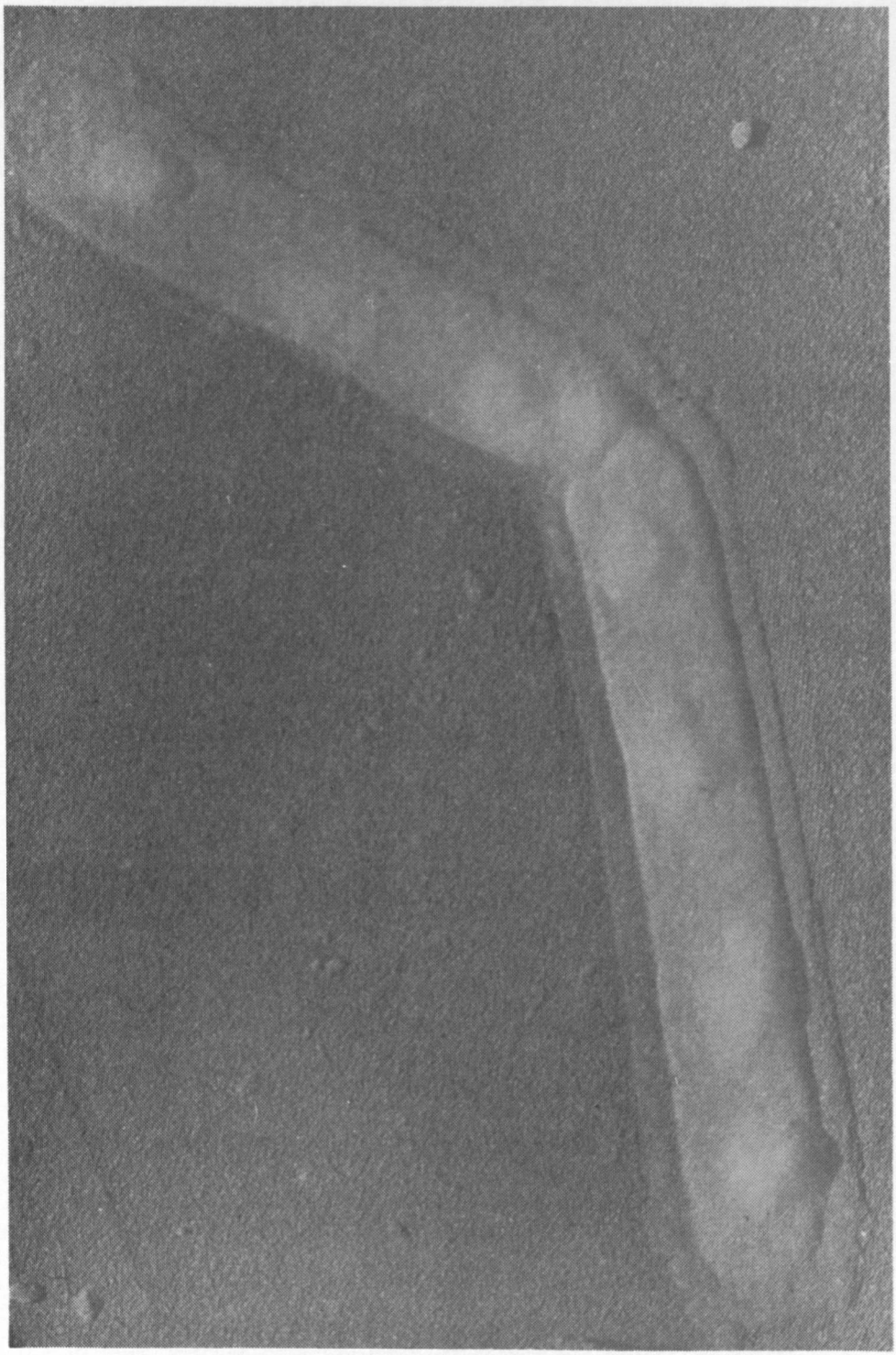


Figure 3 Electron micrograph of thin-sectioned organism.

Magnification: 48,000x.



envelope structure is of the general appearance of the complex envelope of gram-negative bacteria. The cell membrane appears to be the typical double track image observed for all biological membranes under the electron microscope. The nuclear material and cytoplasmic contents were similar to those of other bacteria. No spores or unusual structures were visible. Although an elaborate ultrastructural analysis of the organism was not carried out, the thermophile appeared to have no unique architectural features which vary from the mesophilic organisms.

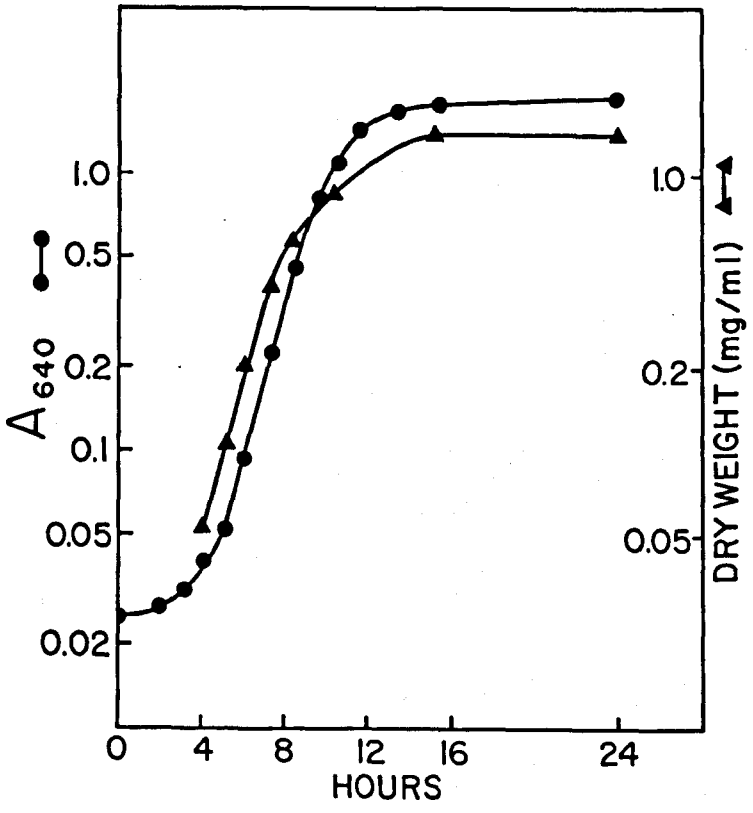
Physiological and nutritional characteristics: The thermophile grew optimally at 70 C, pH 7.7, in a dilute organic medium containing 0.2% yeast extract plus 0.2% tryptone in basal salts.

A typical growth curve shown in Fig. 4 indicates a generation time of approximately 60 minutes. The apparent discrepancy in the plots of the dry weight and turbidity curves at the end of the logarithmic phase of growth is perhaps explainable by a uniform shortening of the cells observed microscopically at that time. This shortening process would allow more cells to occupy a unit volume of medium, hence increasing the turbidity but allowing the dry weight per ml of culture to remain static. The addition of 1% concentrations of either glucose, lactose, or galactose to the standard growth medium did not change the growth curve kinetics. Generation times remained

Figure 4 Growth curve of thermophile. Turbidity readings of the culture and dry weight (mg/ml) cells plotted against time in hours.

● - A_{640} readings

▲ - dry weight (mg/ml) cells


1.0
0.5
0.2
0.1
0.05
0.02
A_{640}
1.0
0.2
0.05
DRY WEIGHT (mg/ml)
0
4
8
12
16
24
HOURS

around 60 min. and the logarithmic phase of growth lasted approximately 7 to 8 hours with each carbohydrate supplemented culture.

The pH optimum of the organism was between 7.5 - 7.8, which is in keeping with the alkaline conditions of their hot spring environment. No growth was observed below pH 6.0 or above pH 9.5.

The temperature growth range of the thermophile was from 45 to 78 C, with an optimum at 70 C.

There was no growth under anaerobic conditions and optimum growth was achieved only in shaken or aerated cultures.

The organism appeared to be heterotrophic, requiring organic compounds as a source of carbon. Growth in the presence of 0.2% glucose plus the standard growth medium produced acid and lowered the pH below 6.0, resulting in a sterile culture after 24 hours incubation. Good growth resulted in a defined medium containing 0.5% glutamate plus the growth factors, vitamin B_{12}, lipoic acid, PABA and biotin.

To determine what organic compounds the organism possibly utilizes in its natural environment, a sample of hot spring water was concentrated over 10-fold. The concentrate was passed through Dowex 50 and Dowex 1 ion exchange columns. Eluates from both columns and the neutral fraction were examined by paper chromatography using butanol, acetic acid, and water (4:1:5) as a solvent. The Dowex 50 eluate

revealed a single unidentified ninhydrin positive spot. The Dowex 1 eluate and the neutral fraction were negative when checked for organic acids and sugars, respectively.

To establish the overall metabolic pattern of the organism, preliminary studies included enzyme determinations on cell-free extracts. Cell-free preparations precipitated with 50-80% ammonium sulfate saturation were active when assayed for succinic dehydrogenase, malic dehydrogenase, and isocitric dehydrogenase. Activity could be demonstrated for all three enzymes after 30 min incubation at 80 C. No activity, either before or after incubation at 80 C, could be shown for glucose 6-phosphate dehydrogenase.

Table I summarizes the morphological, cultural, and physiological features of the organism.

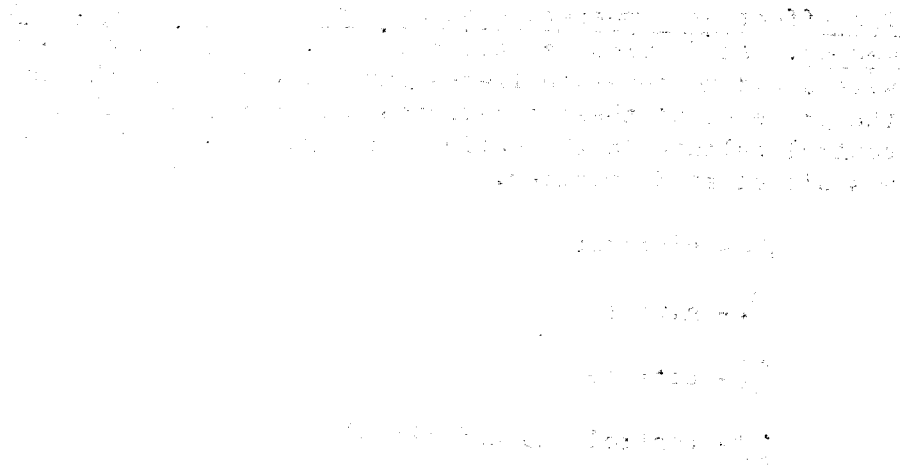
Respiration and cytochrome studies: Figure 5 shows that the rate of oxygen uptake of the organism was 3-fold greater at 70 C than at 50 C. This finding was of interest because the organism is an obligate aerobe and the concentration of dissolved oxygen decreases markedly in this temperature range. The addition of glutamate and glucose effected an increase in the rate of oxygen uptake at 50, 60, and 70 C when compared with the control. Citrate addition decreased the respiration rate at 60 C and 70 C, while increasing the rate at 50 C.

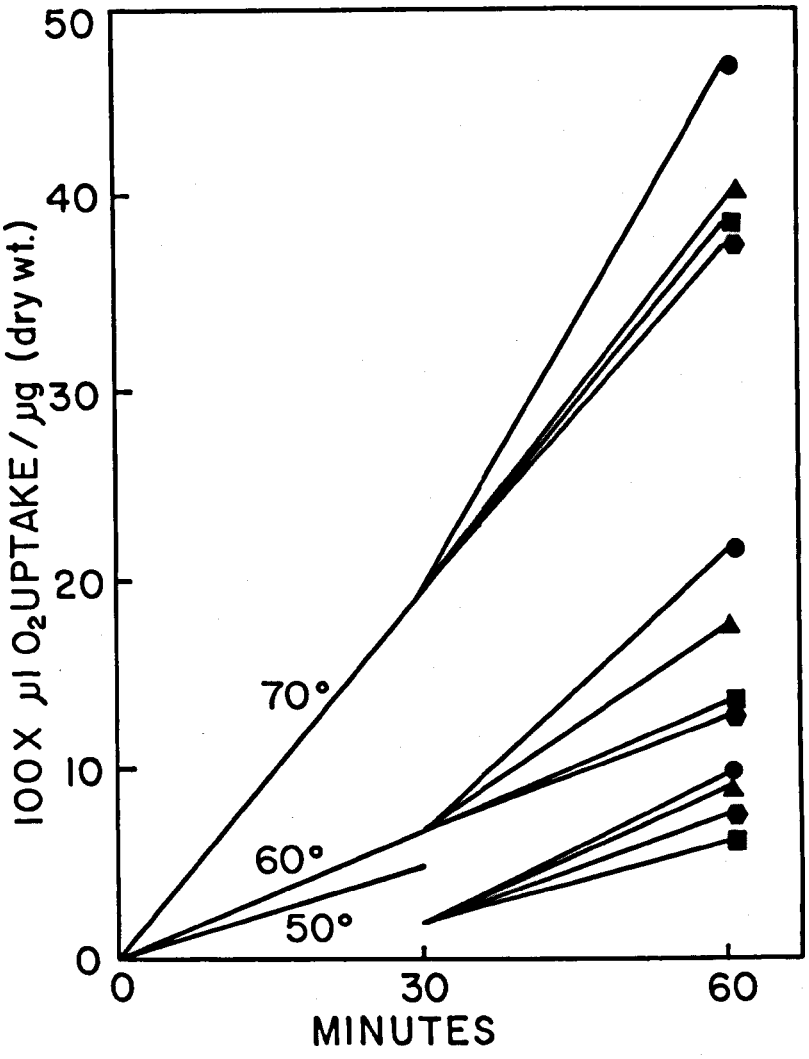
TABLE I

Summary of the morphological, cultural, and physiological features of the T2 isolate of the genus Thermus.

MORPHOLOGY:	Gram-negative rods and filaments, 0.4 - 0.6 µm in diameter, and 5 to 8 µm in length, with early cultures showing filaments up to 150µm. Organisms occur singly or in aggregates, with occasional rosette-like forms. Flagella and endospores absent.
TEMPERATURE RANGE:	Minimum 45 C to a maximum of 78 C, with an optimum at 70 C.
pH RANGE:	Optimum 7.5 - 7.8. No growth below pH 6.0 or above 9.5.
COLONY CHARACTERISTICS:	Dense, spreading, yellow pigmented colonies on 3% agar containing 0.2% tryptone and 0.2% yeast extract in basal salts.
LIQUID CULTURES:	Yellow surface pellicle formed in unshaken liquid cultures.
RELATION TO OXYGEN:	Obligately aerobic.
NUTRITION:	Grows best in complex media containing 0.2% tryptone or casamino acids plus 0.2% yeast extract. Good growth on defined medium containing 0.5% glutamate plus growth factors: vitamin B_{12}, lipoic acid, PABA, and biotin. Acid produced in medium containing 0.2% glucose.

Figure 5 The effect of temperature on oxygen uptake in a defined medium. At 30 minutes, glutamate, glucose, and citrate were added to separate flasks and the oxygen uptake in the presence of these substrates was compared with a control culture in the defined medium. The 50 C curve was offset at 30 minutes.




50
40
30
20
10
0
100X µl O2 UPTAKE / µg (dry wt.)
70°
60°
50°
0
30
60
MINUTES

The increased respiratory activity observed at 70 C obviates the unique ability of this organism to function under conditions of low oxygen tension. Smith (79) has reviewed examples of alterations in the cytochrome content of cells grown under low oxygen tension. To test whether this phenomenon was occurring in this thermophile, cells were grown to late log phase at 50, 60, and 70 C in the complete medium and their cytochrome content was spectrophotometrically analyzed.

The ambient spectrum seen in Figure 6 shows the bands that are characteristic for cytochromes *a* and a_3, *b*, and *c* in cells grown at 60 C. The alpha peaks at 603 nm and 555 nm represent cytochromes *a* + a_3 and *b* + *c*, respectively. Spectra done at liquid nitrogen temperatures, which sharpen and intensify the peaks, clearly revealed both alpha and Soret peaks for *b* and *c*. The Soret peak at 430 nm with a shoulder at 445 nm represents the cytochromes *b* and a_3. Analysis of cells that were grown at 50 C and 70 C also confirmed the presence of these cytochromes. These data reflect similarities between the cytochromes present in this thermophile and those of *B. stearothermophilus* (24) in that the predominant respiratory pigments of both are a_3, *b*, and *c*.

Figure 7 shows the effect of growth temperature on the various cytochrome concentrations. The concentrations of cytochromes a_3, *b*,

Figure 6 The oxidized vs dithionite reduced difference spectrum at room temperature. Cells were grown at 60 C and suspended at 1.25 mg dry weight cells/ml in 0.01M phosphate buffer, pH 7.6.

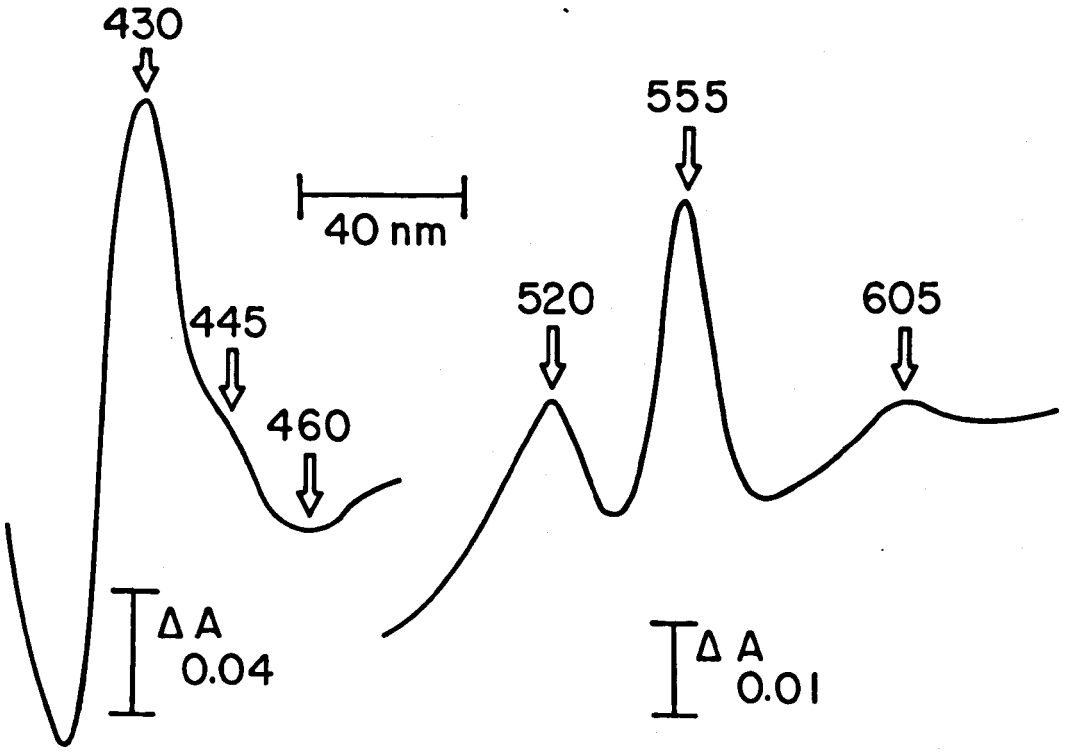
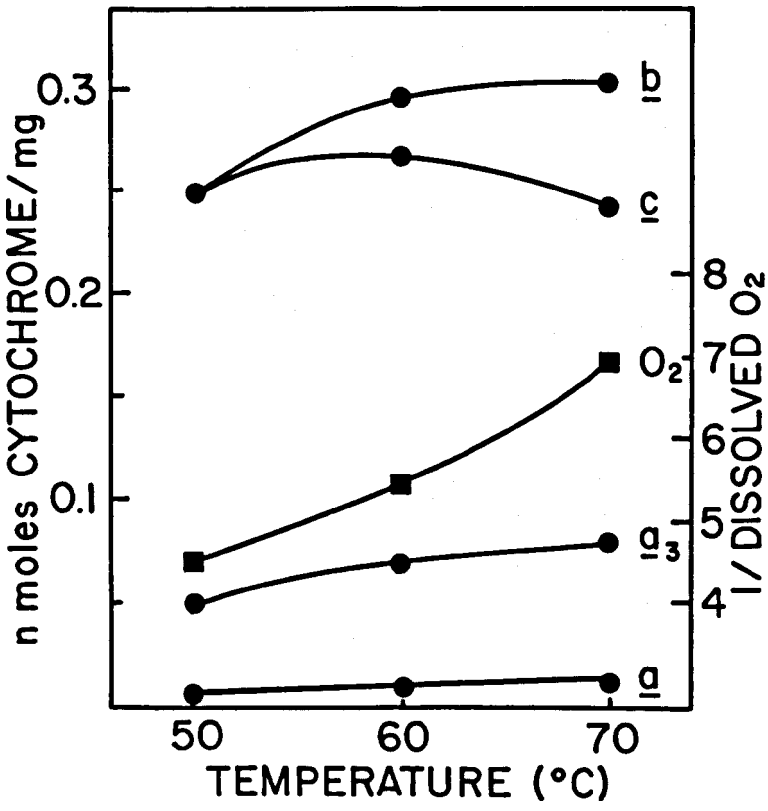

430
445
460
40 nm
555
520
605
Δ A
0.04
Δ A
0.01

Figure 7 The effect of temperature on cytochrome concentration and oxygen solubility. Cytochrome concentrations were determined by appropriate extinction coefficients. The dissolved oxygen was calculated by correcting the oxygen solubility in water at each temperature to the appropriate atmospheric pressure. Dissolved oxygen is expressed as $(mg/l)^{-1}$.


n moles CYTOCHROME/mg
0.3
0.2
0.1
b
c
O_2
a_3
a
8
7
6
5
4
1/ DISSOLVED O_2
50
60
70
TEMPERATURE (°C)

and c are within the ranges reported for many mesophilic microbial systems (34, 79). The concentration of cytochrome a, however, is reduced. There is no significant variation of the concentration of any individual cytochrome species with a decrease in oxygen tension.

β-Galactosidase activity in lactose-induced cells: To establish a suitable assay for β-galactosidase in induced cells, it was necessary to determine the effect of toluene-treatment and temperature on the permeability of the cell to the test substrate, ONPG. The specific activity of a whole cell suspension, a toluene-treated whole cell suspension, and a completely disrupted sonicated cell suspension was determined at various temperatures and are shown in Table II. Both toluene treatment and increased assay temperatures enhanced the permeability of ONPG. The sonicated preparation (essentially cell-free enzyme) assayed at 90 C appears to have undergone some loss of activity.

Optimal enzyme assay conditions: Lactose-induced cells of a 12 hour culture and a 52-fold purified preparation of the cell-free extract of these induced cells were used to determine the optimal conditions for the assay of the enzyme. Table III shows the effect on enzyme activity of various buffers used to prepare the assay solution. Assays performed at pH 7.0 in sodium phosphate buffer resulted in the highest

TABLE II

The effect of assay temperature on the permeation of ONPG into untreated and toluene-treated induced cells.

Assay Temperature	Treatment	Specific Activity eu/mg
70 C	None[a]	3.1
	Toluene[b]	8.8
	Sonicated[c]	10.8
80 C	None[a]	12.0
	Toluene[b]	16.0
	Sonicated[c]	19.3
90 C	None[a]	19.3
	Toluene[b]	25.0
	Sonicated[c]	18.5

[a] No treatment of whole cells prior to the assay.

[b] One ml suspensions of whole cells were treated with 0.1 ml of toluene-acetone (2:1) and the mixture was allowed to incubate for 5 min at 25 C prior to the assay.

[c] One ml aliquots of a cell suspension which was sonicated for 4 min prior to the assay. This sample served as a control to measure 100% accessibility of the ONPG to the enzyme.

TABLE III

Effect of various buffers on enzyme activity.[a]

Assay Buffer Solution	β-Galactosidase Specific Activity eu/mg
0.05M Sodium phosphate	9.0
0.05M Sodium plus potassium phosphate	8.5
0.05M Potassium phosphate	7.7
0.05M TRIS (hydroxymethyl) aminomethane	6.0

[a] All assays were performed at 70 C and pH 7.0 with toluene treated cells of a 12 hour lactose induced culture.

enzyme activity while TRIS resulted in the lowest of those buffers tested. The addition of magnesium ion (as 1×10^{-3}M $MgSO_4 \cdot 7H_2O$) had no effect.

The optimum temperature for activity of the enzyme in both toluene-treated whole cells and a cell-free enzyme preparation is shown in Figure 8. Although the temperature optimum for the whole-cell system is over 90 C, this result may possibly be due to an increased permeability of the substrate at elevated temperatures or to the intracellular environment providing necessary factors for optimum activity. The optimum temperature for the cell-free enzyme is 80 C. The addition of 5×10^{-3}M cysteine to the preparation enhanced the activity as well as stabilizing the enzyme. At temperatures below 50 C no activity was observed in either the whole-cell or cell-free assay. From this data a temperature of 70 C was chosen as the standard assay temperature. Although this was not optimal, it had the virtue of providing a stable environment for the enzyme and test substrate (ONPG) when assayed at pH 7.0.

Figure 9 demonstrates the pH optimum observed in both the whole-cell assay and the cell-free assay in both sodium phosphate and sodium citrate buffer. Although the pH optimum is approximately 5.0, in both the cell-free and whole-cell assay, a pH of 7.0 was chosen for the standard assay. This pH (7.0) was selected because at assay

Figure 8 Effect of temperature on the activity of β-galactosidase.

A. Optimum temperature for activity assayed in whole cells.

B. Optimum temperature for activity of 52-fold purified enzyme. Protein concentration was 10 μg/ml.

● - Toluene-treated cells or cell-free enzyme in 0.05M sodium phosphate buffer, pH 7.0.

■ - Cell-free enzyme in 0.05 sodium phosphate buffer, pH 7.0, plus 5×10^{-3}M cysteine.

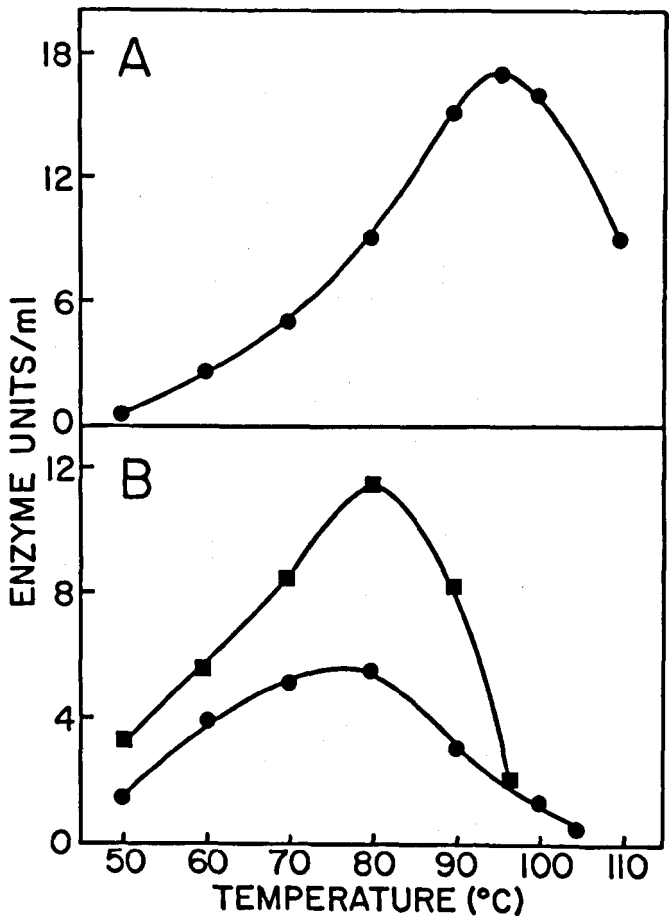

A
B
18
12
6
0
12
8
4
0
ENZYME UNITS/ml
50
60
70
80
90
100
110
TEMPERATURE (°C)

Figure 9 Effect of pH on the specific activity of β-galactosidase.

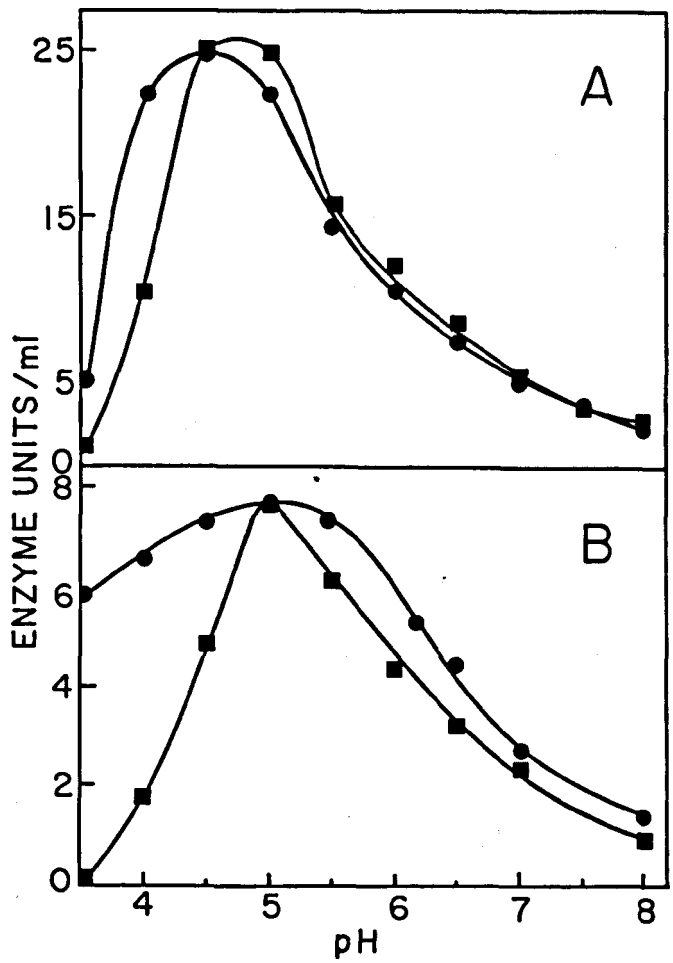
A. Optimum pH for enzyme activity assayed in whole cells.

B. Optimum pH for activity of 52-fold purified enzyme. Protein concentration was 10 µg/ml.

Assays were conducted at 70 C.

● - Toluene-treated cells or cell-free enzyme in 0.05M sodium phosphate buffer adjusted to indicated pH.

■ - Toluene-treated cells or cell-free enzyme in 0.05M sodium citrate buffer adjusted to indicated pH.


25
15
5
0
A
8
6
4
2
0
B
ENZYME UNITS/ml
4
5
6
7
8
pH

temperatures in excess of 60 C (Figure 10), enzymatic activity was rapidly lost under low pH conditions.

Figure 10 shows the effect of temperature on the loss of enzymatic activity. The cell-free enzyme preparation was preincubated at various temperatures for timed intervals. At pH 7.0, temperatures in excess of 70 C rapidly destroyed enzyme activity. The enzyme preincubated at 70 C in pH 5.0 buffer showed a 20% loss of activity after 30 minutes when compared to the enzyme preincubated at pH 7.0 and 70 C. The thermostability of the enzyme was quite apparent when compared to the β-galactosidase of E. coli. This mesophilic enzyme species showed approximately 70% loss of activity after 15 min incubation at 50 C; and after 5 min at 60 C, less than 1% activity remained. The addition of 5×10^{-3}M cysteine to the cell-free thermophilic enzyme provided 100% thermostability for up to 60 min preincubation at 80 C.

Specificity of enzyme induced with various substrates: Table IV shows the substrate specificity of the enzyme induced with various carbohydrates and tested for activity with alpha and beta linked ONPG, and alpha and beta linked p-nitrophenyl-D-galactopyranoside (PNPG). The enzyme induced by either lactose or the α-galactoside, melibiose, shows only beta hydrolytic specificity. Galactose induced cells, however, exhibit some alpha hydrolytic activity when tested with both

Figure 10 Effect of time and temperature on the residual activity of β-galactosidase. One ml aliquots of 52-fold purified enzyme preparation (protein concentration 15 µg/ml) in 0.05M sodium phosphate buffer (pH 7.0) were incubated for various time intervals at the temperatures indicated. The assays for residual activity were conducted at 70 C.

▲ - Purified E. coli β-galactosidase (protein concentration 25 µg/ml) assayed for residual activity at 37 C.

■ - 52-fold purified thermophilic β-galactosidase preincubated in 0.05M sodium phosphate (pH 5.0) at 70 C. Residual activity assayed at 70 C.

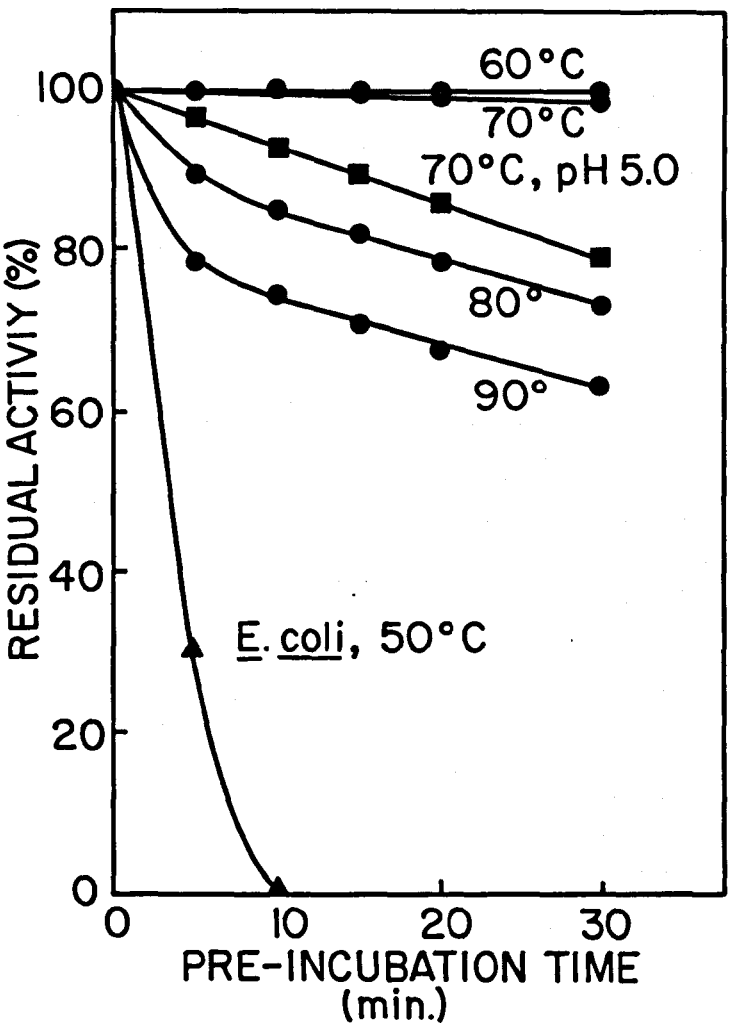

60°C
70°C
70°C, pH 5.0
80°
90°
E. coli, 50°C
RESIDUAL ACTIVIY (%)
100
80
60
40
20
0
0
10
20
30
PRE-INCUBATION TIME
(min.)

TABLE IV

Comparison of alpha and beta galactosidase activity in lactose, galactose, and melibiose induced cells.[a]

Inducer	Test Substrate	A_{420} Reading
Galactose	α-ONPG	0.08
	β-ONPG	0.45
	α-PNPG	0.11
	β-PNPG	1.00
Melibiose	α-ONPG	0.00
	β-ONPG	0.15
	α-PNPG	0.00
	β-PNPG	0.67
Lactose	α-ONPG	0.00
	β-ONPG	0.15
	α-PNPG	0.00
	β-PNPG	0.60
None[b]	α-ONPG	0.00
	β-ONPG	0.02
	α-PNPG	0.00
	β-PNPG	0.16

[a] Twelve hour cultures of cells induced by each of the above carbohydrates were washed in sodium phosphate buffer and resuspended to the same turbidity. The cells were toluene-treated and assayed at 70 C, pH 7.0. The reactions were read colorimetrically at A_{420}.

[b] Non-induced (basal level) culture used as a control.

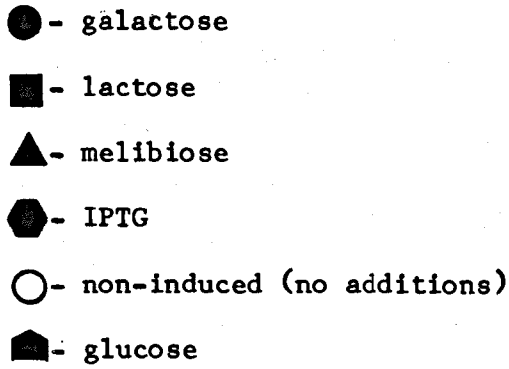
α-ONPG and α-PNPG. Non-induced cells exhibit only slight beta hydrolytic activity, which indicates the presence of basal levels of the enzyme.

Differential induction rates: Figure 11 shows the induction rates of β-galactosidase in cells after the addition of equimolar concentrations of various compounds. The induction rates appear linear throughout the logarithmic growth phase of the cells, with galactose serving as the most effective inducer. The α-galactoside, melibiose, served almost as effectively as an inducer of β-galactosidase synthesis as did the natural substrate, lactose. Induction was also achieved by the use of isopropyl-thio-β-D-galactopyranoside (IPTG), a gratuitous compound. It was not determined if the concentration of IPTG was optimal. Glucose repressed the basal (non-induced) level of enzyme.

To determine the optimal temperature for the synthesis of enzyme, induction rates were followed over the temperature range of growth of the organism. Figure 12 demonstrates that the induction response with both galactose and lactose is dependent on temperature. The optimum temperature for induction with galactose appears to be 70 C and that for lactose at 75 C.

Repression of enzyme synthesis: Figure 13 relates the effect of equimolar concentrations of exogenously supplied glucose, pyruvate,

Figure 11 Relative rates of β-galactosidase induction using various compounds. Cells were grown to the early logarithmic phase of growth (OD .10), at which time 3×10^{-2}M concentrations of lactose, galactose, melibiose, glucose, and a 4×10^{-3}M concentration of IPTG were added to separate flasks. Samples were removed from each flask and the OD determined. The samples were then cooled in an ice bath, washed, and resuspended in 0.05M sodium phosphate buffer (pH 7.0). After the collection of all samples, the cells were toluene-treated and assayed for enzyme activity at 70 C.



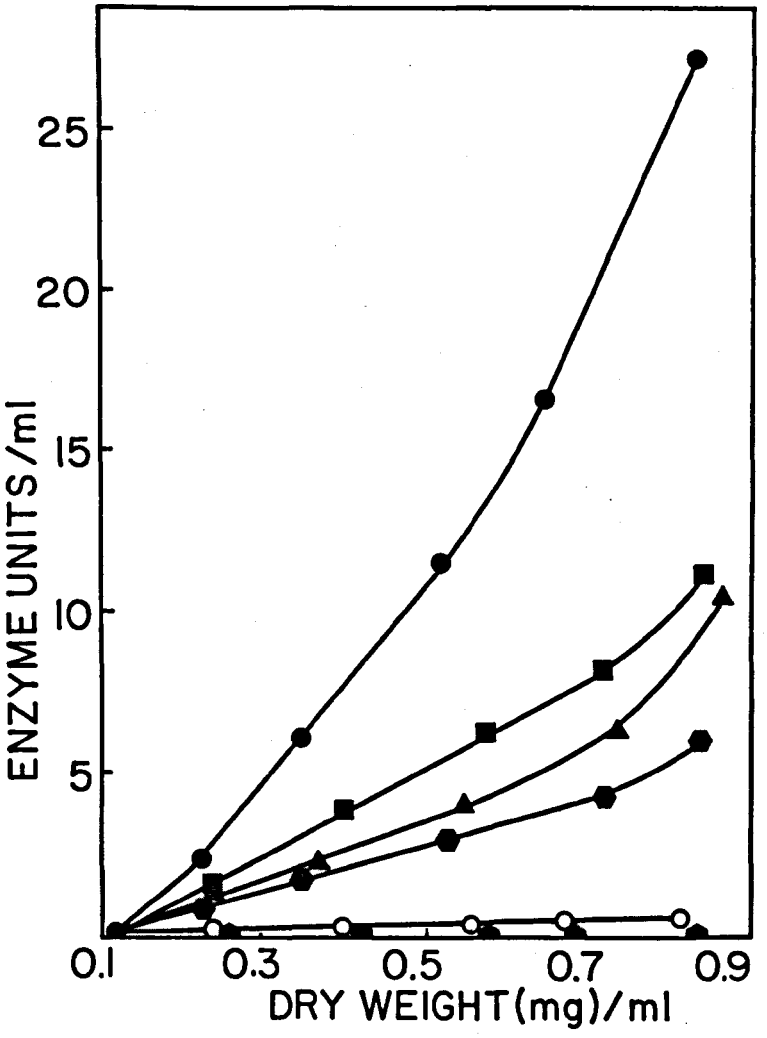

25
20
15
10
5
ENZYME UNITS/ml
0.1
0.3
0.5
0.7
0.9
DRY WEIGHT(mg)/ml

Figure 12 Effect of temperature on the differential rate (P) of β-galactosidase induction. Equimolar concentrations (3×10^{-2}M) of galactose and lactose were used to induce cells at each indicated temperature. The differential rate (P) of β-galactosidase induction was obtained from the slope of a plot of specific activity vs time using each inducer substrate at each temperature.

● - galactose

■ - lactose

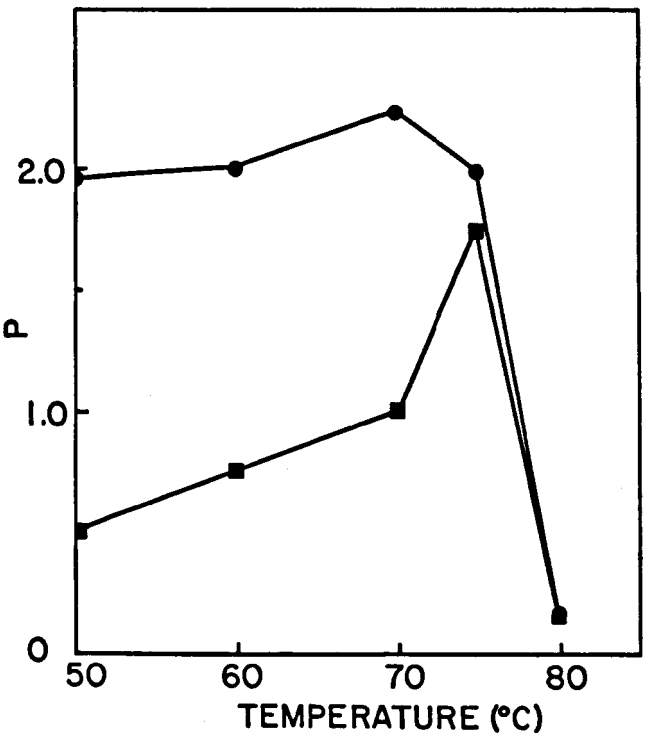
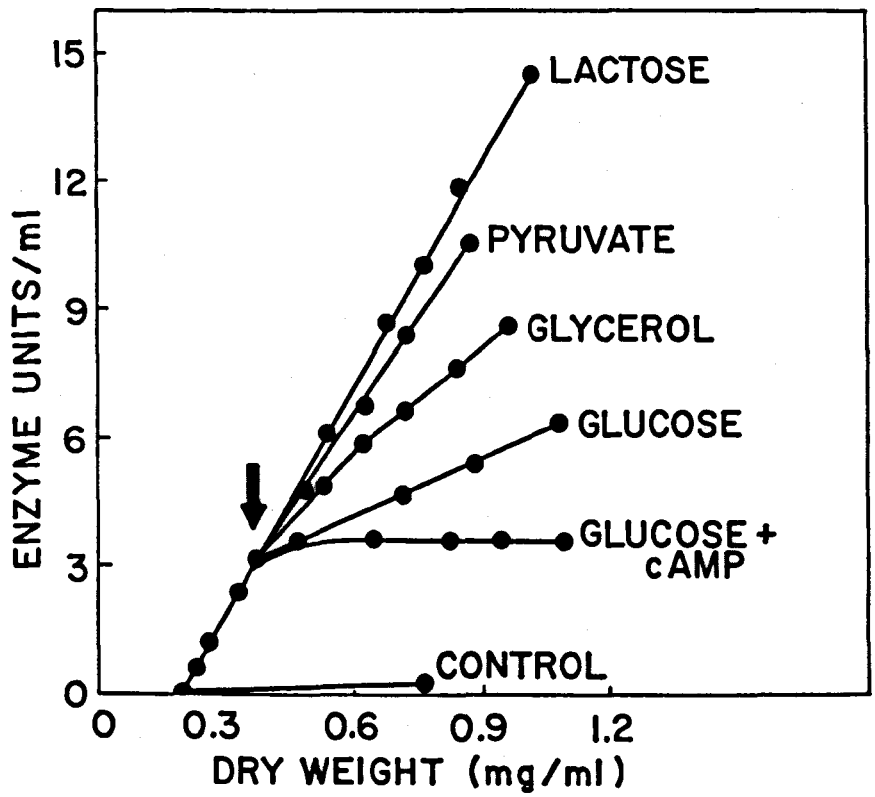

2.0
1.0
0
P
50
60
70
80
TEMPERATURE (°C)

Figure 13 Effect of the addition of equimolar concentrations of various compounds on the synthesis of β-galactosidase. An early-log (OD .18) culture was split into 6 equal portions and 3×10^{-2}M lactose was added to 5 of the cultures. After approximately 1 generation time at 70 C, 3×10^{-2}M glucose, 3×10^{-2}M pyruvate, 3×10^{-2}M glycerol, and 3×10^{-2}M glucose plus 7.5×10^{-3}M cAMP were added separately (arrow) to 4 of the 5 induced cultures. The cultures were sampled at various time intervals, washed, resuspended in 0.05M sodium phosphate buffer (pH 7.0), toluene-treated, and assayed at 70 C. The non-induced culture served as a control to measure the basal level of enzyme.


ENZYME UNITS/ml
15
12
9
6
3
0
0
0.3
0.6
0.9
1.2
DRY WEIGHT (mg/ml)
LACTOSE
PYRUVATE
GLYCEROL
GLUCOSE
GLUCOSE + cAMP
CONTROL

and glycerol on β-galactosidase synthesis in lactose-induced cells.

As is shown the addition of 7.5×10^{-3}M adenosine 3'-5' cyclic monophosphate (cAMP) together with glucose completely prevented further synthesis of β-galactosidase. This result was surprising as the current concepts of catabolite repression of inducible enzyme systems indicate that cAMP reverses the glucose effect (68).

In order to more fully evaluate the effect of glucose on β-galactosidase synthesis in this thermophilic system, the glucose concentration was reduced 6-fold (5×10^{-3}M) and added stimultaneously with 3×10^{-2}M lactose to an early logarithmic culture. In addition, 5×10^{-3}M glucose was added to cells preinduced with 3×10^{-2}M lactose. The results shown in Figure 14 indicate that decreasing the concentration of glucose in the presence of lactose allows enzyme induction, whereas, equimolar concentrations of glucose and lactose added simultaneously (Figure 11) allowed no induction of enzyme synthesis.

Purification of the thermophilic β-galactosidase: The purification of the thermophilic β-galactosidase (E.C. 3.2.1.23 β-D-galactoside galactohydrolase) followed a scheme as outlined in Table V. Early attempts at purification utilized DEAE-cellulose, in place of DEAE-Sephadex A50, with great losses of both enzyme activity and protein. Table VI shows that the enzyme was purified approximately 78-fold

Figure 14 Effect of decreasing the concentration of glucose added to lactose-induced cells. An early-log (OD .18) culture was split into 4 equal portions and 3 x 10^{-2}M lactose was added to 2 of the 4 cultures. After approximately one-half of a generation time at 70 C, 5 x 10^{-3}M glucose was added (arrow) to 1 of the 2 induced cultures. To a third culture 3 x 10^{-2}M lactose and 5 x 10^{-3}M glucose were added simultaneously at time zero. A fourth culture received no additions. The cultures were sampled at various time intervals and treated as described in Figure 13.

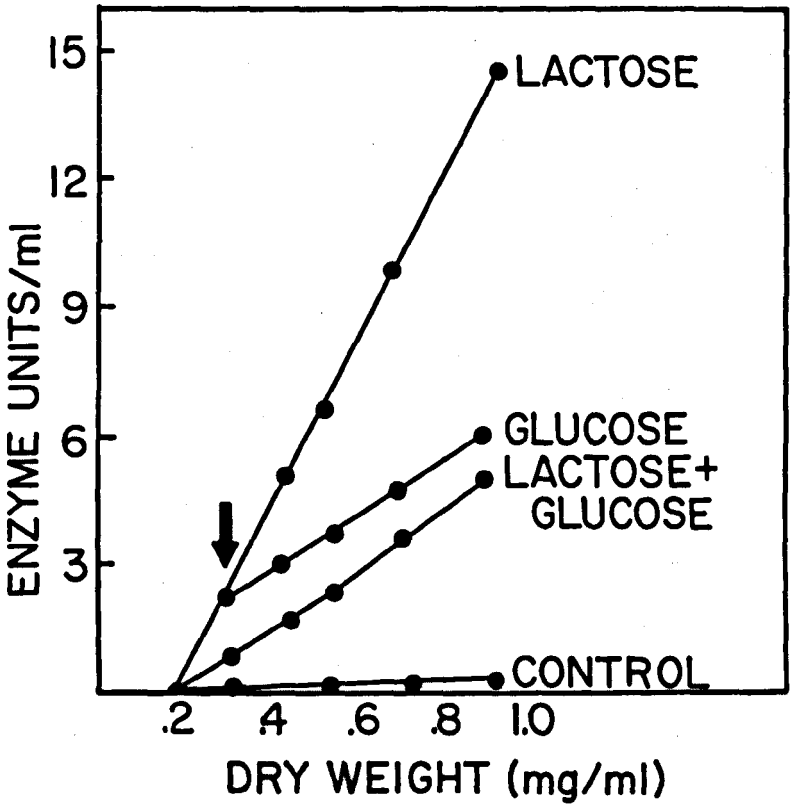

15
12
9
6
3
ENZYME UNITS/ml
LACTOSE
GLUCOSE
LACTOSE+
GLUCOSE
CONTROL
.2
.4
.6
.8
1.0
DRY WEIGHT (mg/ml)

TABLE V

Purification scheme for the thermostable β-galactosidase.

(1)	CELL EXTRACT FROM FRACTIONATION OF INDUCED CELLS Centrifugation at 40,000 x g for 60 minutes - debris discarded - supernatant dialysed against sodium phosphate buffer for 24 hours.
(2)	ACETONE PRECIPITATION Precipitate centrifuged out - resuspended in sodium phosphate buffer - dialysed for 24 hours - insoluble debris centrifuged out and discarded.
(3)	AMMONIUM SULFATE FRACTIONATION Activity in 22.5 - 35% saturation step. Precipitate resuspended in sodium phosphate buffer - dialysed for 24 hours.
(4)	SEPHADEX G 200 GEL FILTRATION Eluent - sodium phosphate buffer.
(5)	DEAE - SEPHADEX A 50 ION EXCHANGE Enzyme eluted with a linear gradient of NaCl in sodium phosphate buffer. Dialysed against sodium phosphate buffer for 24 hours.

TABLE VI

Effectiveness of each step in the purification of the thermostable β-galactosidase.

Step	Volume (ml)	Total Enzyme Units	Total Protein (mg)	Specific Activity (eu/mg)	X Purified
(1) Crude extract	140	126,000	5,600	22.5	-
(2) Acetone Precipitate	115	123,000	5,250	23.0	1.1
(3) $(NH_4)_2SO_4$ Precipitate	15	118,100	288	410.0	18.3
(4) Sephadex G 200	21	100,500	86	1160.0	52.0
(5) DEAE - Sephadex A 50	41	61,000	35	1750.0	78.0

with a 48% recovery of the original enzyme activity.

Molecular exclusion column chromatography using Sephadex G200 was employed as a purification tool as well as to provide knowledge of the molecular weight-range of the molecule. Figure 15 demonstrates the elution profile of the enzyme from the Sephadex G200 column. The void volume of the column was measured at 45 ml (tube 15) by passing a solution of Blue Dextran 2000 (2×10^6 daltons) through the column and collecting the eluent in 3 ml volumes. Blue Dextran was determined colorimetrically at 625 nm. The enzyme activity was eluted at void volume indicating a molecular weight of greater than 10^5 daltons.

DEAE-Sephadex A50 was employed as an ion exchanger and had the added advantage of providing molecular sieving. Figure 16 shows the elution profile of enzyme activity from the DEAE-Sephadex A50 column. A linear salt gradient was used to accomplish elution. Due to a change in ionic strength, the column bed had a tendency to shrink which might account for the loss of some enzyme activity at this purification step. Subsequent attempts to further purify the enzyme using preparative gel electrophoresis were unsatisfactory.

The effect of divalent cations and sulfhydryl containing compounds on enzyme activity: The effects of certain divalent cations on enzyme activity added to the assay mixture are shown in Table VII. Cobalt and magnesium had no effect, and a combination of ferrous iron plus

Figure 15 Enzyme elution profile from Sephadex G 200. The enzyme was eluted in 0.05M sodium phosphate buffer (pH 7.0) and each tube contained 3 ml fractions. The column size was 2.5 x 35 cm and the void volume (V_o) as determined by Blue Dextran was 45 ml (tube 15).

● - enzyme units/ml

▲ - A_{280} readings

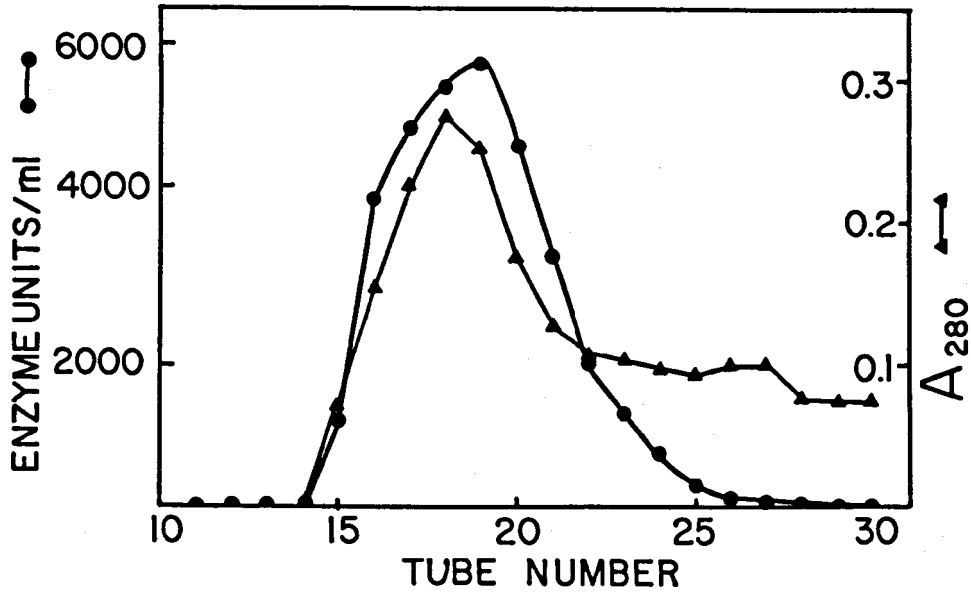

6000
4000
2000
ENZYME UNITS/ml
0.3
0.2
0.1
A280
10
15
20
25
30
TUBE NUMBER

Figure 16 Enzyme elution profile from DEAE - Sephadex A50. The enzyme was eluted by a linear salt gradient (0.0 - 0.5M NaCl) in 0.05M sodium phosphate buffer (pH 7.0) and each tube contained 3 ml fractions. The column size was 2.5 x 35 cm.

● - enzyme units/ml

▲ - A_{280} readings

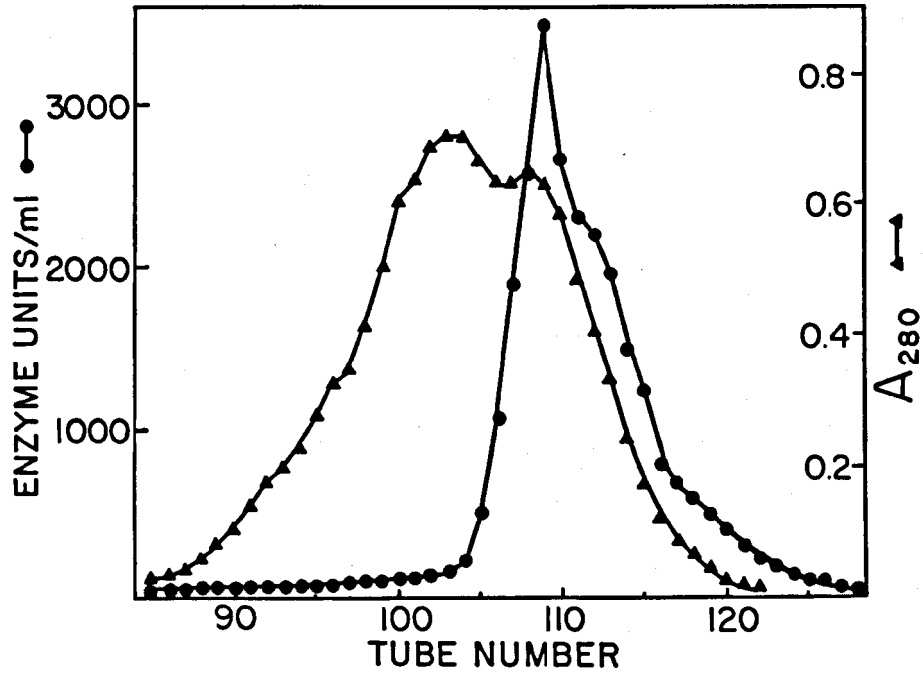

ENZYME UNITS/ml
3000
2000
1000
0.8
0.6
0.4
0.2
A_{280}
90
100
110
120
TUBE NUMBER

TABLE VII

Effect of divalent cations on β-galactosidase activity.[a]

Cation	Concentration	Relative Activity
None	-	1.0
Mn^{2+}	1×10^{-3}M	1.2
Mg^{2+}	1×10^{-3}M	1.0
Fe^{2+}	1×10^{-3}M	1.3
Co^{2+}	1×10^{-3}M	1.0
$Mn^{2+} + Fe^{2+}$	ea. 5×10^{-4}M	1.6
$Mn^{2+} + Mg^{2+}$	ea. 5×10^{-4}M	1.3
$Fe^{2+} + Mg^{2+}$	ea. 5×10^{-4}M	0.9

[a] The enzyme used was 52-fold purified material at a concentration of 20 µg/ml of protein in 0.05M sodium phosphate, pH 7.0. Assay temperature was 70 C. Mn^{2+} was added as $MnCl_2 \cdot 4H_2O$; Mg^{2+} as $MgCl_2 \cdot 6H_2O$; Fe^{2+} as $FeCl_2 \cdot 6H_2O$; and Co^{2+} as $CoCl_2 \cdot 6H_2O$.

magnesium had an apparent inhibitory effect. The most effective activators of the enzyme were manganese and ferrous iron when used separately or together. Rickenberg (72) has shown the β-galactosidase of E. coli to be activated by manganese ion, inhibited by nickel and zinc, with magnesium having no effect.

The activation of the enzyme by both cysteine and β-mercaptoethanol is shown in Table VIII. Cysteine serves the enzyme in a dual capacity, both in activation and in stabilization. Sulfhydryl compounds have been used routinely to stabilize mesophilic β-galactosidases during purification (19).

Kinetic evaluation of the thermostable β-galactosidase: The effect of substrate concentration on the velocity of the enzyme reaction at 80 C is shown in Figure 17. In the presence of cysteine, the enzyme kinetic data can be plotted to conform to the relationships described by the Michaelis-Menten equation. However, the enzyme in the absence of cysteine shows a curve which never reaches zero order kinetics (maximum velocity) in the substrate (ONPG) concentration followed. The shape of this curve is best explained in terms of enzyme denaturation occurring at 80 C. The data from low substrate concentrations was plotted by the method of Lineweaver and Burk as seen in Figure 18. A K_m value in the presence of cysteine was 2.0×10^{-3}M,

TABLE VIII

Effect of SH-containing compounds on β-galactosidase activity.[a]

Compound	Concentration	Relative Activity
None	-	1.0
Cysteine	5×10^{-3}M	3.3
Cysteine	1×10^{-3}M	2.5
Cysteine	5×10^{-4}M	1.1
β-mercaptoethanol	5×10^{-3}M	3.0
β-mercaptoethanol	1×10^{-3}M	2.4
β-mercaptoethanol	5×10^{-4}M	1.5

[a] The enzyme used was 52-fold purified material at a concentration of 20 µg /ml of protein in sodium phosphate, pH 7.0. Assay temperature was 70 C.

Figure 17 Effect of substrate concentration on the velocity of the β-galactosidase reaction. A 78-fold purified enzyme preparation in 0.05M sodium phosphate buffer (pH 7.0) was used at a protein concentration of 5 µg/ml in the presence of 5×10^{-3}M cysteine or 10 µg/ml in the absence of cysteine. The assays were performed at 80 C with the indicated concentrations of ONPG.

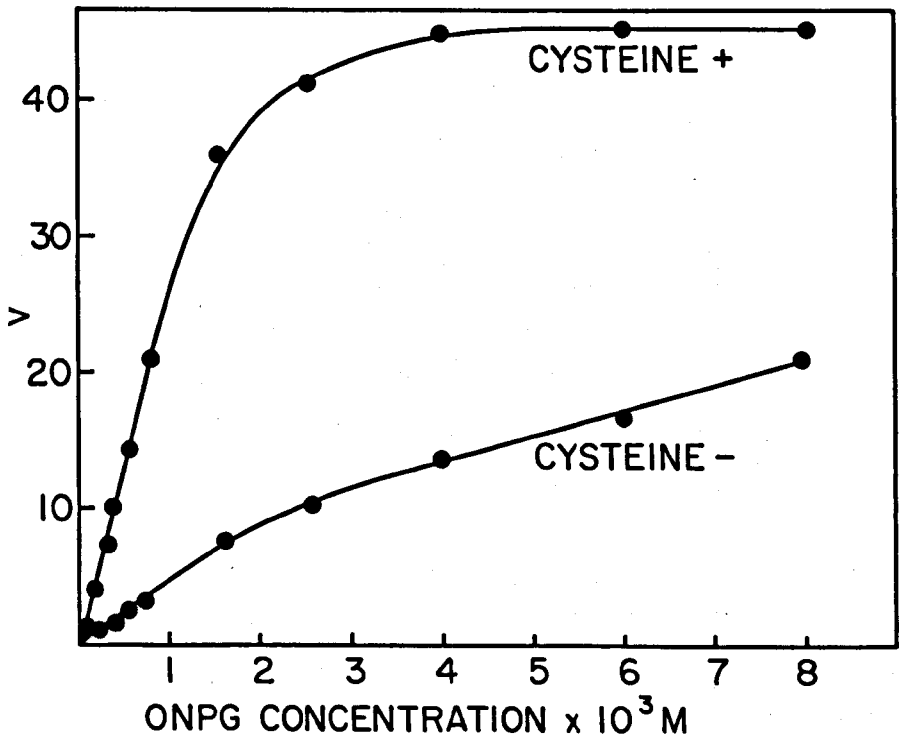
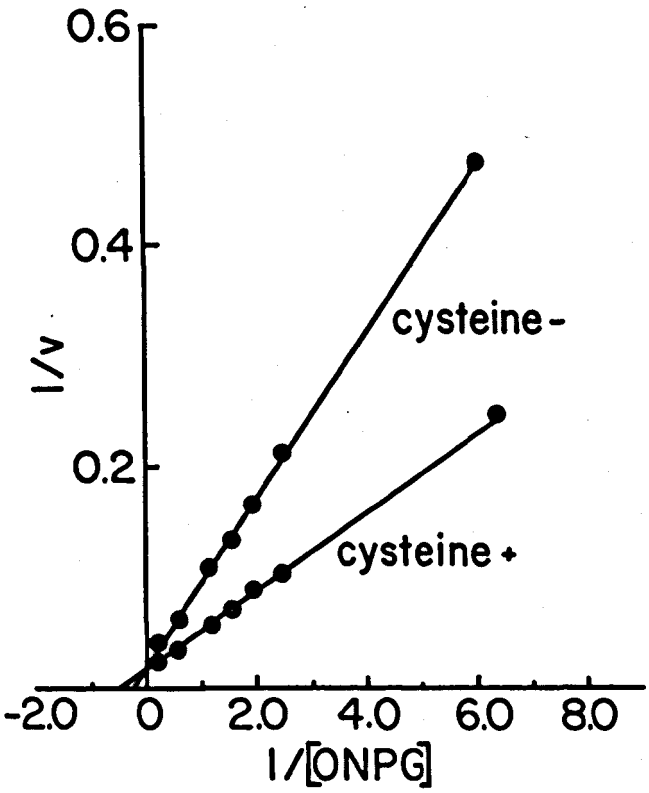

CYSTEINE +
CYSTEINE -
40
30
20
10
V
1
2
3
4
5
6
7
8
ONPG CONCENTRATION x 10³ M

Figure 18 Lineweaver-Burke plot of data from Figure 17.


0.6
0.4
0.2
1/v
cysteine -
cysteine +
-2.0
0
2.0
4.0
6.0
8.0
1/[ONPG]

and that in the absence of cysteine was 4.0×10^{-3}M.

Although the colorimetric assay using ONPG does not permit a test of the efficiency of splitting other galactosides, their affinity for the enzyme has been evaluated by treating them as competitive inhibitors of ONPG (48). The binding function of lactose was evaluated from the inhibition kinetics of ONPG activity. The K_i for lactose of 7.5×10^{-3}M, as determined by the method of Dixon, is seen in Figure 19. This determination was made at 80 C, using cysteine treated enzyme.

Arrhenius energy of activation: To determine if the high temperatures necessary for optimum activity of this thermophilic enzyme implied a high energy of activation, the velocity constants of the enzyme were determined at various temperatures. Figure 20 shows the Arrhenius curve plotted as the logarithm of the velocity constants versus the reciprocal of the absolute temperature. The Arrhenius energy of activation as calculated from this data was 13,700 cal/mole. This value is in keeping with the 1,000-25,000 cal/mole range considered for the activation energies of mesophilic enzymes. In addition, the Arrhenius constant of the thermostable β-galactosidase is not significantly higher than the value of 12,400 cal/mole reported for E. coli β-galactosidase (47).

Figure 19 Dixon - plot of enzyme activity in the presence of lactose used as a competitive inhibitor. The velocity of the enzyme reaction was measured by varying the concentration of lactose in the presence of 2 concentrations of ONPG. Assays were at 80 C in the presence of 5 x 10^{-3}M cysteine.

● - 5 x 10^{-4}M ONPG

■ - 1 x 10^{-3}M ONPG

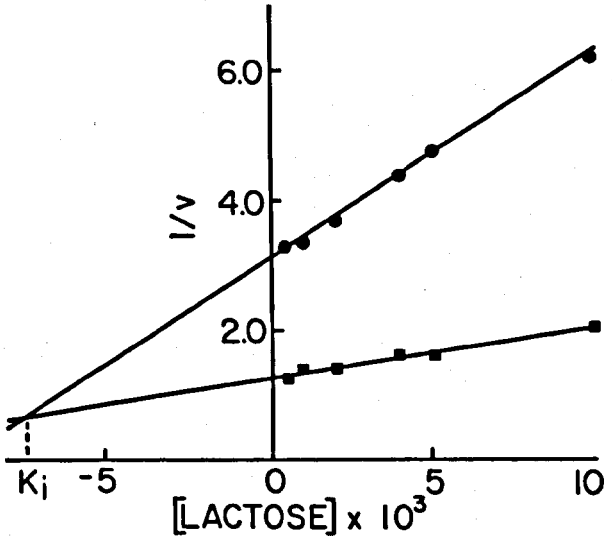
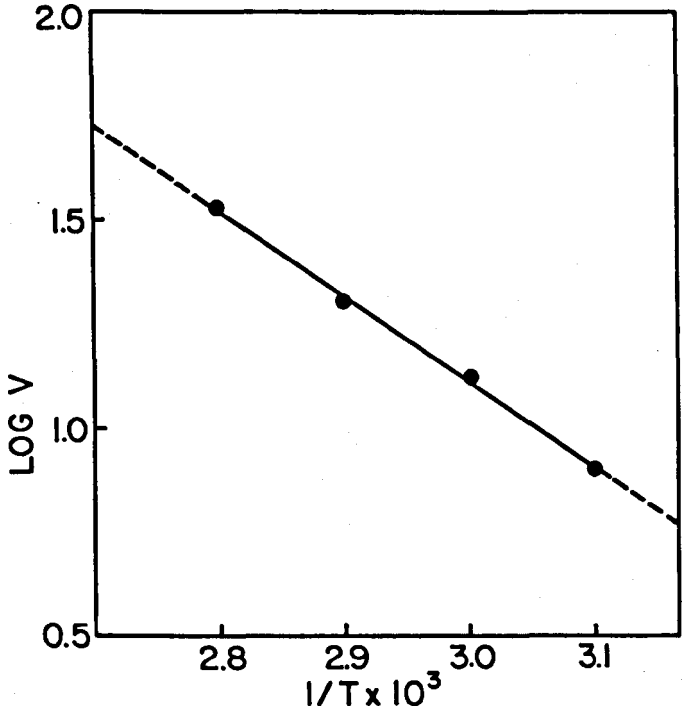

6.0
4.0
2.0
1/v
K_i
-5
0
5
10
[LACTOSE] x 10^3

Figure 20 Plot of the logarithm of the initial velocity vs the reciprocal of the absolute temperature.


2.0
1.5
1.0
0.5
LOG V
2.8
2.9
3.0
3.1
1/T x 10³

Molecular weight determination: Figure 21 shows the elution profile of the thermophilic β-galactosidase and E. coli from a Sepharose 4B column. The void volume of the column was measured at 48 ml (tube 16) using Blue Dextran as a marker. By eluting a series of internal standards (globular proteins) of known molecular weights, a plot of elution volumes (V_e) versus the logarithm of the molecular weights was constructed. The results shown in Figure 22 provide an estimated molecular weight of 5.7×10^5 daltons for the thermophilic enzyme.

Disc gel electrophoresis: Figure 23 shows densitometer scans of the 78-fold purified enzyme. The enzyme migrated in an anodal direction at a pH 7.0. The densitometer scan of biological activity shows only one band of enzymatic activity when tested with the beta-linked substrate ONPG. There was no activity when the migrated protein was tested for activity with the alpha-linked substrate PNPG. The densitometer scan of the Coomassie Blue stained tube revealed that the greatest amount of protein in the preparation was β-galactosidase, however, a certain amount of unidentified protein was also present. This contamination precluded a meaningful amino acid analysis of the enzyme.

Thermostability studies on the purified enzyme: The 78-fold purified enzyme preparation was treated in the various manners as described in

Figure 21 Sepharose 4B elution profile of thermophilic β-galactosidase and E. coli β-galactosidase. A 1.0 ml sample containing 3 mg/ml (protein) of thermophilic β-galactosidase and 5 mg/ml (protein) of E. coli β-galactosidase was layered on a 2.5 x 35 cm Sepharose 4B column and eluted in 3 ml fractions/tube with 0.05M sodium phosphate buffer (pH 7.0). The void volume (V_o) of the column was 48 ml (tube 16) as determined by Blue Dextran.

▲- E. coli β-galactosidase

●- thermophilic β-galactosidase

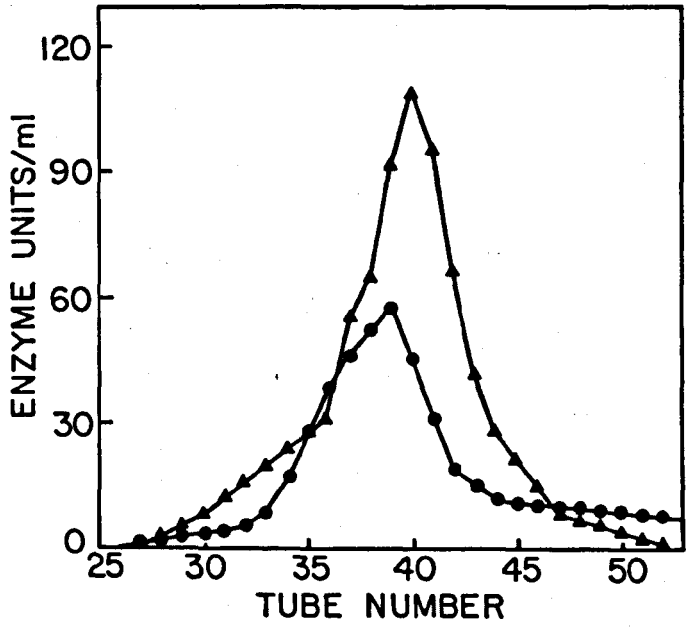

120
90
60
30
0
ENZYME UNITS/ml
25
30
35
40
45
50
TUBE NUMBER

Figure 22 Molecular weight standard curve. Elution volumes (V_e) of known molecular weight globular proteins from a Sepharose 4B 2.5 x 35 cm column were plotted vs the logarithm of the molecular weights. All samples were layered on the column in 5 mg/ml concentrations and collected in 3 ml fractions.

1 - catalase

2 - urease (monomer)

3 - *E. coli* β-galactosidase

4 - thermophilic β-galactosidase

5 - urease (dimer)

6 - Blue Dextran 2000

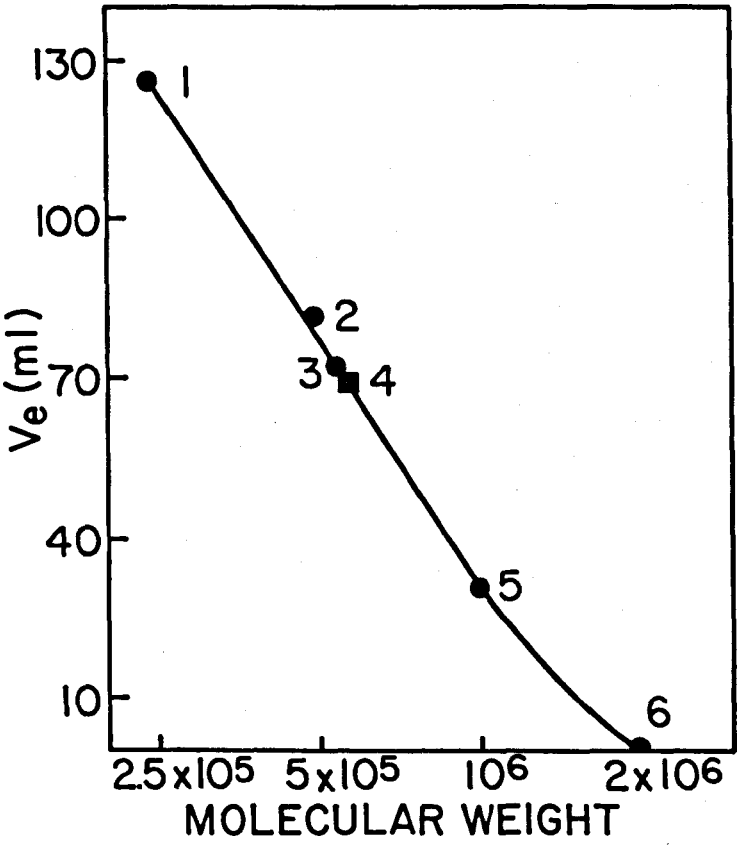

130
100
70
40
10
Ve (ml)
1
2
3
4
5
6
2.5x10⁵
5x10⁵
10⁶
2x10⁶
MOLECULAR WEIGHT

Figure 23 Densitometer tracings of disc gel electrophoresis of the enzyme. A 50 µg/ml sample of 78-fold purified enzyme was layered on each of 3 polyacrylamide gels and subjected to electrophoresis for 3.5 hours.

A. Drawing of Coomassie brilliant blue stained 5% polyacrylamide gel.

B. A densitometer tracing of the yellow chromophore produced by incubating the gel in a 5×10^{-3}M solution of β-ONPG at 70 C. A separate gel incubated with α-PNPG produced no chromophore.

C. A densitometer tracing of the protein stain (Coomassie brilliant blue) of a gel.

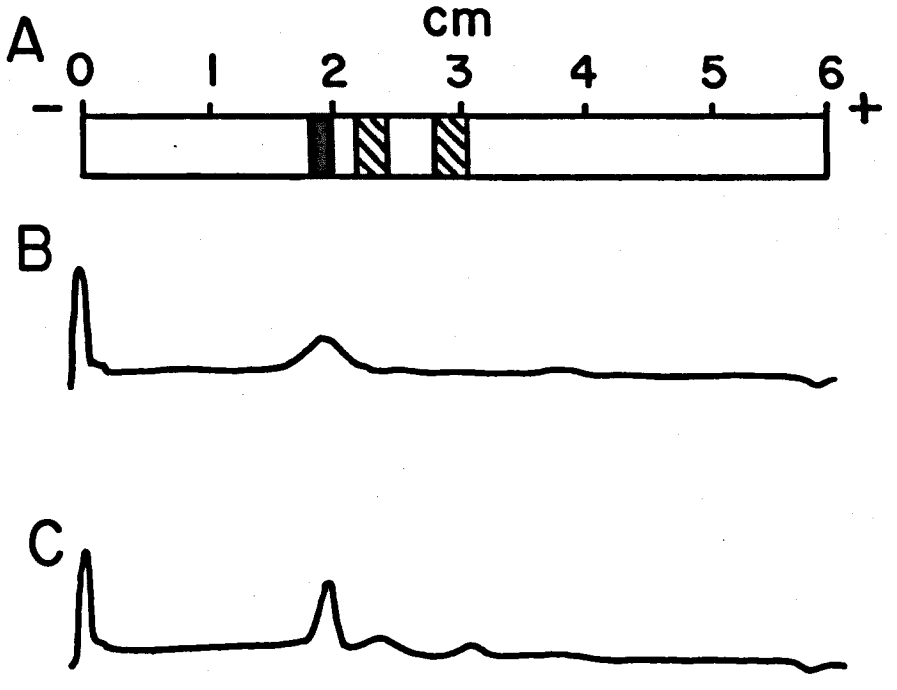

A
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Table IX. There appears to be no demonstrable loss of enzyme thermostability associated with any of these procedures although loss of total enzyme activity is apparent in both pronase digestion and the alkaline pH treatment.

TABLE IX

The effect of pronase digestion[a], alkaline pH[b], and charcoal adsorption[c] on the thermostability of purified β-galactosidase.

Treatment	% Residual Activity[d] after 15 min	% Residual Activity[d] after 30 min	% of original enzyme activity remaining after treatment[e]
Pronase Digestion	100	97	42
Alkaline pH (11.0)	100	100	60
Charcoal Adsorption	100	100	100

[a] A 78-fold purified enzyme preparation containing 3 mg/ml protein was incubated at 37 C with 500 µg Pronase for 16 hours.

[b] The pH of a 78-fold purified enzyme preparation containing 0.15 mg/ml protein was raised to 11 by the addition of 2N NaOH. The mixture was incubated at 4 C for 48 hours and the pH adjusted to 7.0 by the addition of 1 N HCl.

[c] One mg of Norit A activated charcoal was added to a 78-fold purified enzyme preparation containing 0.15 mg/ml protein and allowed to incubate at 4 C for 10 hours. Prior to assay the charcoal was removed by centrifugation.

[d] Each treatment sample was preincubated at 70 C for 15 and 30 minutes. The sample were cooled, ONPG added and the residual activity assayed at 70 C, pH 7.0. Aliquots of the treated samples were left at 25 C during the preincubation periods and assayed as controls containing 100% residual activity.

[e] A sample of the original untreated enzyme preparation was assayed at the time of each treatment assay to determine the loss of total enzyme activity due to each treatment.

DISCUSSION

In the past, most of the laboratory studies done on thermophilic bacteria have been limited to the spore-forming species, especially, B. stearothermophilus (14). The need, therefore, has been obvious for studies with other species of bacteria in order to expand knowledge of life at high temperatures.

The results of this study have described the presence of a non-sporeforming gram-negative bacterium living at extremely elevated temperatures. The organism appears to have no unique morphological features which reveal any obvious information as to its ability to survive at high temperatures. Enrichment for the organism requires fairly low concentrations of organic constituents which is in keeping with the low organic content of its natural aquatic habitat. The organism is heterotrophic for its carbon needs and requires oxygen for respiration. Although a defined medium has been described, optimum growth occurs in the presence of yeast extract and tryptone, which indicates a dependency on one or more unknown factors or simply the need for certain concentrations of nutrients not achieved in this study. The metabolic map of the organism appears complete in terms of the tricarboxylic acid cycle, as activity was found for three key enzymes, isocitrate dehydrogenase, succinic dehydrogenase, and malic dehydrogenase. Glucose 6-phosphate dehydrogenase, the first enzyme of the hexose monophosphate shunt, could not be demonstrated. The

organism has a wide temperature range for growth (45-78 C) and the addition of various exogenous substrates at different temperatures expressed differences in the rates of oxygen uptake. Citrate, for instance decreased the respiration rate at 60 and 70 C while increasing it at 50 C. This observation could reflect an altered metabolic scheme in the lower growth temperatures of this organism. Altered metabolic pathways are explainable by the direct effect of the temperature on the relative activities of the enzyme systems composing a particular pathway.

It was not the purpose of this research to establish the taxonomical relationships of this extreme thermophile. However, the morphological and cultural features of this organism are strikingly similar to those described for a new genus, Thermus, by Brock and Freeze (12). The authors have discussed the possible relationship with other aquatic organisms, such as the genus, Flavobacterium, and the genus, Flexibacter; however, the flavobacteria do not have the property of forming long filaments, and the gliding motility characteristic of the flexibacteria has not been observed in this new thermophilic species. Thus the taxonomical relationships of this gram-negative non-sporeforming thermophile remain obscure.

Consideration of the respiration of thermophiles necessarily becomes a question of how these bacteria have resolved the problem

of lowered oxygen tensions at elevated temperatures. Studies with B. stearothermophilus (23, 24, 25) have revealed that nitrate reduction assumes an increasingly important role in the energy metabolism of that organism as oxygen availability diminishes. This finding was not unexpected since B. stearothermophilus has the ability to use nitrate, as well as oxygen, as a terminal oxidant. Nitrate reductase is induced under conditions of low oxygen tension.

The present studies with a strictly aerobic thermophilic organism indicate that the lowered concentrations of solubilized oxygen present at elevated temperatures appeared not to be a rate limiting factor in respiration. The presence of cytochromes of the a, a_3, b, and c types is certainly not surprising in view of the widespread occurrance of these heme pigments in microorganisms. In as much as the cytochrome concentrations remained quite uniform over the 20 degree temperature range studied, it may be speculated that the optimal respiration rates seen at 70 C are a reflection of the average optimal temperatures for activity of all enzyme systems, including the cytochromes, in this unique thermophile. The rate limiting step in respiration could be postulated as the availability of the reduced diphosphopyrindine nucleotides to the electron transport system. If the optimum temperatures for activity of the enzyme systems providing reducing power is achieved, an efficient state of electron transfer

would allow optimum respiratory activity.

The induction of enzyme synthesis is a well established biochemical control mechanism in many mesophilic systems. In particular, the induction of β-galactosidase in E. coli served as a model system for the development of the genetic control hypothesis of Jacob and Monod (42). It is not implausible, therefore, that an organism living under optimal conditions, in this case very high temperatures, should have an operable control mechanism involving induction of a thermostable enzyme.

The results of these studies show that a thermostable β-galactosidase was induced. The induction response to the α-galactoside, melibiose, was not without precedent, as melibiose has been reported to be as effective as lactose in inducing β-galactosidase in E. coli (46) and Shigella sonnei (71); and induced nearly 40 times more enzyme than lactose in S. paradysenteriae (71). The current concepts of β-galactosidase induction in mesophiles indicate that lactose itself is not the inducer of the lac operon, but galactose, a product of lactose hydrolysis, is transferred to some receptor to provide inducer function (35). Galactose was the most effective inducer of β-galactosidase tested in this unique thermophilic system. This response is similar to the effect of galactose in the mesophilic system of Staphylococcus aureus (60) and is contrary to the poor

response observed in E. coli (46) and S. sonnei (17). The inducer effect of galactose may be the result of an inability of the organism to utilize galactose, hence the inducer would reach high concentrations inside the cell and provide all the inducer function necessary. Llanes and McFall (51) have shown galactose to be a potent inducer of the lac operon in galactokinaseless mutants of E. coli. Conversely, the lower differential rate of enzyme synthesis in the presence of lactose, may be due to the accumulation of glucose, a product of lactose hydrolysis and an inhibitor of β-galactosidase synthesis.

The specificity of the enzyme induced in the presence of both lactose and melibiose was for the beta-linked substrates. Although galactose induced 3-fold more beta specific enzyme than lactose, a measurable amount of alpha specific enzyme activity was also detected. This result is paradoxical considering that melibiose, an α-galactoside, induced no detectable α-galactosidase activity.

Several investigators have reported the temperature dependence of the regulation of certain repressible and inducible enzyme systems in mesophilic organisms. Marr, Ingraham, and Squires (59) have shown that the differential rates of synthesis of E. coli β-galactosidase decreased progressively with a decrease in growth temperature. They suggested that temperature strongly affects the concentration of specific repressor. Horiuchi and Novick (38) isolated a mutant of

E. coli which was inducible for β-galactosidase at 14 C, but is constitutive at 43.5 C. They assumed that these findings reflected a loss of the thermal stability of the repressor. The increased inducibility of this thermophilic organism with an increase in temperature may reflect a decrease in the concentration of the repressor, but does not preclude the possibility of impaired inducer permeability at lower temperatures.

The effect of glucose on β-galactosidase synthesis has been shown to operate by 3 mechanisms in mesophilic systems. Nakada and Magasanik (63) have shown that a product of glucose metabolism was actually responsible for the repression of β-galactosidase synthesis in E. coli. This regulatory effect of glucose metabolism has been termed catabolite repression (53). The concept of catabolite repression has been enlarged recently by studies with cAMP (68). These studies have shown that glucose metabolism reduces the intracellular pool of cAMP, and that high levels of cAMP are necessary for the initiation of the synthesis of the lactose operon mRNA. A second method by which glucose effects the synthesis of β-galactosidase was recently demonstrated by Riggs, et al (73). It was shown that glucose interacts directly with the lac repressor protein in vitro and behaves as an anti-inducer. An inducer-exclusion effect of glucose has also been reported for E. coli β-galactosidase synthesis (18). In

this system low levels of lactose permease are found and glucose acts competitively with lactose for permeation into the cells.

The results of the present study indicate that glucose severely inhibited β-galactosidase synthesis in lactose-induced cells. Although an anti-inducer effect or catabolite repression effect of glucose cannot be ruled out, the following evidence is suggestive of inducer-exclusion as means of control of induced enzyme synthesis in this thermophilic organism: (i) the addition of cAMP to glucose repressed cells failed to reverse the effect of glucose, (ii) cells preinduced with lactose were not completely repressed by the addition of equimolar concentrations of glucose, and (iii) when equimolar concentrations of glucose and lactose were added simultaneously to early logarithmic cells, no induction of enzyme was observed throughout the remainder of the growth curve, however, when the concentration of glucose was reduced 6-fold under these same conditions, a low level of enzyme was induced. These results taken collectively indicate that glucose competes with lactose for permeation into the cells and that this process was responsible for the repressive effects of glucose in this thermophilic system.

The optimal temperature for activity (80 C) and the high temperatures necessary to achieve denaturation reflect the unique thermostability of this thermophilic species of β-galactosidase. A

comparison of the optimum activity of the enzyme assayed in whole cells and as the cell-free enzyme, indicates (i) that the internal environment of the cell offers some heat stabilizing quality to the enzyme or (ii) that optimum conditions were not achieved in the *in vitro* assay system. The pH optimum was lower than that observed for *E. coli* (46) and *Paracolobactrum aerogenoides* (6), however, the requirement of sodium ion for maximum enzymatic hydrolysis of ONPG reflects the results obtained for *E. coli* (46), *P. aerogenoides* (6) and *Streptococcus lactis* 7962 (16).

Purification of the thermostable β-galactosidase resulted in a 78-fold purified preparation. Several physicochemical properties of the purified enzyme were examined to allow comparisons between this unique thermophilic species and the many mesophilic species of the enzyme. The activation of the enzyme by manganese ion, with magnesium ion having no effect, is in agreement with the effects of these cations on *E. coli* β-galactosidase (72). Activation by manganese ion suggests that this cation plays a role in maintaining the intactness of the catalytic site of the enzyme molecule. The effect of cysteine suggests that the thermophilic β-galactosidase is a sulfhydryl enzyme requiring a reducing agent for stability and maximal activity.

It is of interest that the aldolase enzyme of *T. aquaticus* is activated by cysteine, ferrous ion, and manganese ion; substances

which have the greatest effect on the activation of the β-galactosidase from this bacterium which is, presumably, the same thermophilic organism.

Kinetic analysis of this thermostable β-galactosidase indicated a K_m of 2.0×10^{-3}M, a value which is 20-fold higher than that for E. coli (48) and nearly the same as that reported for S. lactis 7962 (62). The K_i for lactose of 7.5×10^{-3}M was 5-fold greater than that reported for E. coli (48). The Arrhenius activation energy was commensurate with the value reported for E. coli β-galactosidase (47).

The molecular weight of the thermophilic β-galactosidase was estimated to be 5.7×10^5 daltons which is quite similar to that of 5.4×10^5 daltons for E. coli. It must be pointed out that several methods of molecular weight determination must be used to accurately describe the molecular weight of this thermostable β-galactosidase.

The comparisons made above indicate no major differences, except in thermostability, between the thermophilic and mesophilic β-galactosidases. Although differences do exist, they seem to be no greater than the difference between any two mesophilic proteins isolated from separate species. The unique thermostable property of the enzyme would appear to be intrinsic to the molecule itself, as alkaline hydrolysis, pronase digestion, and charcoal adsorption had no measurable effect on its ability to withstand temperatures as high

as 70 C for up to 30 minutes duration. This conclusion is supported by the results of other studies with thermostable proteins (4, 39, 78). It should be pointed out that the non-covalent interaction between the S-peptide and S-protein in ribonuclease-S, provide both strength and thermostability to this enzyme (77, 84), indicating that a few strategically located hydrophobic (or other) interactions may be all that is required for the increased thermostability of thermophilic molecules.

The results of this thesis research have described a gram-negative non-sporulating thermophile which is apparently adapted to the low concentrations of dissolved oxygen in its natural high temperature environment. In addition, evidence has been given for an inducible and repressable enzyme system operating in a thermophile. The induction of β-galactosidase provides indication that thermophiles are not restricted to only the essential biosynthetic systems, but have very complex biological control mechanisms. From a taxonomical viewpoint, existence of the lactose operon in this organism and the probable non-existence of lactose in its natural habitat seemingly point to an adaptation from a mesophilic ancestry, rather than to the organism being a relic of times when the earth was hotter than it is at present.

SUMMARY

A gram-negative non-sporeforming extreme thermophilic bacterium was isolated from an alkaline hot spring in Yellowstone National Park. The morphology of the organism examined by both light and electron microscopy showed no unusual features which would provide an ability to survive at high temperatures. The organism had a temperature range of growth from 45 C to 78 C with an optimum at 70 C. The pH optimum was 7.5 - 7.8, with no growth occurring below pH 6.0 or above 9.5. Growth was optimal in 0.2% tryptone and 0.2% yeast extract in shaken or aerated cultures. No growth was demonstrated under anaerobic conditions. The metabolic pattern of the organism appeared complete in terms of the tricarboxylic acid cycle (TCA), but glucose 6-phosphate, the first enzyme of the hexose monophosphate shunt could not be demonstrated.

Respiration studies with the organism revealed optimal oxygen uptake at 70 C. Spectrophotometric analysis of the cells grown at 50, 60, and 70 C showed the presence of cytochromes of the *a*, a_3, *b*, and *c* types. Although the oxygen tension decreased inversely with the increase in temperature, the concentrations of each cytochrome remained relatively uniform over the 20 degree temperature range studied.

The organism was found to be inducible for a thermostable β-galactosidase using the carbohydrates lactose, melibiose, and galactose as inducer substrates. Induction was found to be dependent on the

growth temperature of the cells, with optimum induction occurring at 75 C in lactose induced cells and at 70 C in galactose-induced cells. Glucose repressed induced enzyme synthesis suggesting a model of biosynthetic control mechanisms operating at high temperatures in a very similar fashion to those seen in mesophilic systems.

Purification of the thermostable β-galactosidase resulted in a 78-fold purified preparation. The optimum pH of the enzyme was 5.0, with optimum temperature for activity at 80 C. There was a metal ion dependence for manganese and ferrous iron. The effect of cysteine and β-mercaptoethanol suggested that the thermophilic β-galactosidase is a sulfhydryl enzyme requiring a reducing agent for stability and maximal activity.

Kinetic observations at 80 C provided a Michaelis constant of 2.0×10^{-3}M and a K_i for lactose of 7.5×10^{-3}M. The Arrhenius activation energy was calculated as 13,700 cal/mole.

The molecular weight of the enzyme was estimated at 5.7×10^5 daltons using Sepharose 4B molecular exclusion chromatography in the presence of internal globular protein standards.

Treatment of the purified enzyme with pronase digestion, charcoal adsportion, and alkaline hydrolysis resulted in no loss of thermostability of the enzyme.

These results provided evidence that the enzyme molecule is inherently thermostable and not dependent on an extrinsic stabilizing factor.

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