AEROBIC HETEROTROPHIC BACTERIAL FLORA OF ONE OF SHALLA HOT SPRINGS

A THESIS PRESENTED TO
THE SCHOOL OF GRADUATE STUDIES
ADDIS ABABA UNIVERSITY

IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE
OF MASTERS OF SCIENCE IN BIOLOGY

By

MULUGETA KIBRET

JUNE, 1991

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

AEROBIC HETEROTROPHIC BACTERIAL FLORA OF ONE OF SHALLA HOT SPRINGS

by

Mulugeta Kibret
Department of Biology
Science Faculty

Approved by:

Dr. Teferi Gemetchu
Chairman, Department Graduate Committee

Dr. Berhanu A. Gashe
Advisor

Dr. Haile Selassie Tesfaye
Examiner

Dr. Dawit Abate
Examiner

Dr. Zerihun Woldu
Examiner

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Berhanu Abegaz Gashe, my advisor, for his valuable comments, constant guidance and encouragement I received throughout the preparation of this thesis.

Due thanks go to Samuel Sahle, Adane Bitew, Berhanu Abrha and Erku Yimam who shared my problem in the laboratory. I thank Amare Gessese, Seyoum Ayehunie, Shibabaw Wondie, Menassie Ephrem and Afewoki Ghebrai for their moral and material support.

I express my sincere thanks to the Asmara University Computer Center for allowing me to use computer facilities. I am grateful to Temesgen Zewotir, Nigist Bezuayehu, Kelemwork Taddesse and others who contributed to the completion of this thesis.

The financial support made by the Swedish Agency for Research Cooperation with Developing Countries (SAREC) is greatly acknowledged. Last but not least I thank Arba Minch Water Technology Institute for the sponsorship.

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Four sites in one of the hot springs of Shalla were selected for the study. The water from the sites merge to form a stream which forms a temperature gradient. In this study four media were compared for the cultivation and isolation of aerobic heterotrophic bacteria. The pH of the hot spring was 8.7 with a temperature of 96-55°C. The formulated media were adjusted to pH values of 7.0-13.0. However,high counts of bacteria ($1x10^3$-$7x10^4$ cfu/ml) was only registered when the pH was adjusted between 8.0 and 9.0. A medium containing normal concentration of Trypton Soya Agar (TSA) was effective in supporting the growth of bacteria than a medium containing one-quarter strength of TSA or supplement of trace minerals. Replacing trace minerals with hot spring water improved the total number of bacteria growing and also the number of isolates.

Thermophiles were numerous ($1x10^3$-$7x10^4$ cfu/ml) than thermotolerants ($2x10^2$-$2x10^4$ cfu/ml) at all sites. The population of Bacillus brevis, Bacillus sphaericus, Bacillus stearothermophilus and Micrococcus sp. was found to be low at lower environmental temperatures than at higher environmental temperatures.

In the course of this study a total of 44 microorganisms were isolated and characterized. Twenty nine were identified to the species level and were found to be members of genus Bacillus.

Three isolates were Gram-positive cocci identified to the genus level - Micrococcus. The remaining 12 isolates were either Gram-positive, filamentous or non-filamentous rods which need a detailed biochemical analysis for identification.

To identify if any of the isolates may have industrial applications, they were checked for production of amylase and protease. Fifteen of the isolates produced protease, 18 amylase and eleven produced both amylase and protease.

1. INTRODUCTION

Natural and man-made thermal habitats exist world-wide. These habitats include : erupting volcanoes (1000°C), dry-steam fumaroles (up to 500°C), non-boiling hot springs (temperatures from near boiling to ambient), sun-heated substrates such as soils, litter, rock where temperatures reach 60 to 70°C or higher, self-heating organic rich materials such as compost piles, seaweed piles, and coal refuse piles (up to 70°C), hot water heaters (55 to 80°C),cooling waters from various industrial processes (from just above ambient to boiling), steam lines of steam-heated buildings (reaching up to 60°C) and many other habitats (Tansey and Brock, 1978).

Majority of these thermal habitats are difficult to study. However, research in hot springs is easier compared with other thermal habitats due to several reasons.

Hot springs form relatively well-defined outflow channels and as water cools along these channels, relatively stable thermal gradients are created. In the thermal gradients of a single hot spring under appropriate conditions, the only significant variable is temperature (Falk and Kell, 1966). Thus excellent naturally occurring experimental conditions can be made for ecological studies.

Measurements of temperature are easy to take and because of the high thermal capacity of water there exists a narrow fluctuation of temperature. In addition, the temperature

of the micro-environment is the same as that of the macro-environment. Conversely, in thermal soils, compost and other terrestrial thermal habitats temperature is variable and also the temperature of the sub-merged organisms may be different from the measured temperature (Brock, 1967).

Hot springs have relatively constant chemical and physical properties. Water samples for chemical and microbiological analyses are easy to obtain and repeated measurements can be made (Brock, 1986).

Temperature is not the sole factor that limit microbial growth in hot springs. Other environmental parameters such as pH, available energy sources, ionic strength also influence microbial growth in hot springs (Zeikus,1980).

Hot springs are low in organic matter and high in dissolved solids (Castenholz, 1969). Microbiologically important minerals dissolved in water include: hydrogen sulphide (H_2S), carbon dioxide (CO_2), low molecular weight organic compounds, methane (CH_4), hydrogen (H_2), ammonia (NH_3), and trace elements. Chlorides (Cl^-) and bicarbonates (HCO_3^-) are usually the dominant anions (Brock, 1986). Some springs precipitate silica, others deposit travertine ($CaCO_3$), and still others form elemental sulphur (Brock, 1967).

The pH of hot springs varies and values as low as 1.0 and greater than 9.0 have been recorded. The distribution of pH values of hot springs is bimodal, with many values between 2.0-4.0 and 7.0-9.0 but few between the two regions (Brock, 1971). This has led to the division of hot springs into acid and alkaline hot springs.

When one considers the chemical, hydrologic, thermal, and geographic variation, every hot spring can be considered as an individual, different in minor or major ways from other springs. However, many springs are more similar than different. Most springs have been remarkably constant in thermal, chemical, and hydrologic properties for many years, although minor variations do occur (Munzel, 1947; cited in Brock, 1967).

The presence of organisms in hot springs was noted in antiquity. Plinius (1944 ; cited in Brock, 1967) noted that plants grew in the hot sprigs of Padua, frogs in those of Pisa, at Vetulonia in Etruria, near the sea. Earlier studies (Brock, 1967; Bott and Brock, 1969) have shown that in neutral or alkaline hot springs, bacteria live and grow in boiling water. Studies in New Zealand and Iceland, where the boiling springs are located at low altitude and having temperatures of 95 to 101^{0}C indicated that every spring in neutral and alkaline pH range has high populations of bacteria. On the other hand, bacterial species diversity in acid hot springs is more limited than in neutral and alkaline habitats. Only at temperatures around 60 to 65^{0}C do macroscopically visible accumulations of bacteria appear in acid hot springs (Kaplan, 1956; cited in Brock and Darland, 1970). Thermophilic extreme alkalophiles have also been described (Sonnleitner and Fiecher, 1983).

Under favorable environmental conditions certain non-photosynthetic bacteria can grow in most hot springs where the

temperature is below 90°C, and a few even up to the boiling point of water (Brock, 1967). Among bacteria living in boiling water are: sulphur bacteria, hydrogen oxidizing bacteria, elemental sulphur respiring bacteria,obligate anaerobic and aerobic heterotrophs. The photosynthetic prokaryotes (Blue green algae and photosynthetic bacteria) are unable to grow at temperatures as high as that of non-photosynthetic prokaryotes, even if other environmental conditions are favorable. Generally, there exist an upper temperature limit for photosynthetic life and it is at about 70 to 73°C (Castenholz, 1969).

Eukaryotic microorganisms are much more restricted in their distribution than prokaryotic microorganisms. The upper temperature limit is approximately 60 to 62°C (Tansey and Brock, 1972) at which only few species of fungi can grow. the upper temperature limit for eukaryotic algae and protozoa are slightly lower, 55 to 60°C and 56°C respectively. Those for metazoans and higher plants are below 50°C (Brock, 1985).

All species capable of reproducing at temperatures greater than 90°C are members of the Archaebacteria. The spore-forming bacteria that are thermophilic have generally lower temperature maxima than the species that do not form spores.The Eubacteria living at the highest temperatures almost without exception do not form spores;they are Gram-negative and of uncertain taxonomic affiliation (Brock, 1978; cited in Brock,1985; Saiki et al., 1972).

Three broad classes of organisms have been recognized

on the basis of their cardinal temperatures;i.e their minimum, optimum,and maximum growth temperatures: psychrophiles,with growth ranges from -5 to 22°C, mesophiles from 10°C to 47°C and thermophiles between 40°c to 80°C (Stanier et al., 1970). Considering the cardinal growth temperature of microorganisms, their division into psychrophiles, mesophiles, and thermophiles is not satisfactory. Many organisms would fall on a border line and may be classified, for instance, as either mesophiles or thermophiles depending on the investigators point of view. The cardinal temperature may also vary a few degrees depending on the culture medium and growth conditions. Further more, thermophiles cover a growth range between 40 and 100°C, and thermophiles in the lower temperature range have been considered different from those in the upper temperature range. Consequently, several attempts have been made to sub-divide the thermophilic bacteria.

Three groups have been proposed by Farrel and Campbell (1969):

1.Strict or obligate thermophiles which demonstrate optimum growth temperature at 65°C to 70°C but which do not grow below 40 to 42°C.

2.Facultative thermophiles have maximam growth temperatures between 50°C and 65°C but also are capable of reproducing at room temperature though at a very low rate.

3.Thermotolerant bacteria have growth maxima at 45°C to 50°C and can also grow at room temperature fairly well.

Since many extreme thermophiles have growth optima above 80°C, Heinen and Heinen (1972) have introduced the term "caldoactive"(caldus from latin meaning hot) to describe extremely thermophilic bacteria. The following sub-division has been proposed by Williams (1975).

a) caldoactive bacteria-maximum growth temperature above 80°C optimum above 65°C, and minimum above 40°C. This sub-division has validity as an addition to the original classification of Farrel and Campbell(1969).

b). thermophilic bacteria-maximum growth temperature above 60°C, optimum above 50°C and minimum above 30°C. This sub-division ignores certain facultative thermophiles such as Bacillus coagulans having a minimum growth temperature of about 58°C, and does not provide maximum, optimum, and minimum growth temperatures for the thermotolerant bacteria.

All these differences in the classification of thermophiles imply that strict adherence to any of them is difficult.

Prior to 1969, the bacterium with the highest known optimum growth temperature in the laboratory culture was Bacillus stearothermophilus and majority of the work on "thermostable" enzymes and other sub-cellular components was carried out with this bacterium. However, the description of an extremely thermophilic bacterium, Thermus aquaticus, from a hot spring in Yellowstone National Park by Brock and Freeze (1969) has given a new impetus to the study of thermophiles and

provided new material for the isolation of thermostable enzymes. Since then thermophilic bacteria have been isolated world-wide from neutral and alkaline hot springs (Ramaley and Hixon, 1970; Saiki et al., 1972; Jackson et al., 1973; Kristjan and Alfredson, 1983; Oshima and Imahori, 1971; Egorova and Loginova, 1974; Taya et al., 1988; Heinen and Heinen, 1972; Cometta, 1982, Hudson et al., 1990; Pask-Hughes and Williams, 1977). The distribution of organisms in acid hot springs has been studied (Brock and Darland, 1970; Belly and Brock, 1974). Tansey and Brock (1978) have listed all known species of thermophilic bacteria.

It is apparent that thermophilic bacteria are able to grow and reproduce at temperatures ranging from slightly below freezing to the boiling point of water. However, cultures have been obtained only of organisms able to grow at somewhat lower temperatures. The highest temperature at which it has been possible to grow a bacterial culture continuously and reproducibly is about 85°C (Heinen, 1971).

The existence of microorganisms which grow and actually require temperatures above the upper limit of most life has stimulated research in the fundamental aspect of these organisms in order to a) learn more about their role in the environment b) understand more about their mechanism of existence and c) study the thermal stability of macromolecule of these organisms. From the applied point of view, interest in thermophiles has increased from time to time because of their potential application in biotechnology and economic advantages

in the use of the organisms or their enzymes in commercial and other processes.

Thermophilic and thermotolerant organisms are expected to produce qualitatively new enzymes. Since they can grow at high temperatures their cellular constituents must function at similarly high temperatures. Enzymes from thermophiles are promising not only because of their thermostability and activity at high temperatures but also because of their resistance to high solute (reactant) concentration. Thermolysin, the protease from Bacillus thermoproteolyticus, for instance, retains 86% of its activity after 30 hours at 70°C. Caldolysin, a Thermus protease has a half life of 30 hours at 80°C, 30 times longer than thermolysin under the same conditions (Sonnleitner and Fiechter, 1983).

Amylase of microbial and plant origin are nowadays used in the starch processing industries. Most widely used are amylases produced by Bacillus amyloliquefaciens and Bacillus licheniformis, the glucoamylases of Aspergillus niger and Rhizopus sp. Starch processing industries require highly thermostable and thermoactive amylases. Plant amylases are expensive and unstable. Therefore, thermophiles are often the first choice sources when looking for new enzymes (Buonocore et al., 1976).

Enzymes from thermophiles are stable at conventional temperatures than enzymes from mesophiles thus prolonging the shelf life of commercial products (Ng and Kenealy, 1986).

An increase in temperature results in increase in the

diffusion rate and the solubility of most non-gaseous compounds, allowing operations at high concentrations of reactants. An increase in temperature also reduces the viscosity and surface tension of water. It also increases the quality of mixing and allows easier liquid-solid separation (Ng and Kenealy, 1986).

Fermentation reactions result heat production. Extensive effort must therefore be made to cooling the fermentation process when heat sensitive bacteria are used. Heat of fermentation is normally removed by circulating water through coils or the fermentor jacket. For this method to be effective, the water must be substantially cooler than the operating temperature of the fermentor and this gradient must be larger when the rate of heat evolution is greater. Evaporative cooling towers are ineffective because of high atmospheric humidity. Therefore, mechanical refrigeration with consequent high operating cost have to be considered. If thermophilic organisms could be used, the problem of fermentor cooling would be simplified,since a large gradient between the operating temperature of the fermentor and the temperature of the cooling water would be available (Mateles et al., 1967).

There has been a considerable interest in the use of thermophiles for the production of fuel and bulk chemicals. A potentially major microbial process is the production of ethanol. At present ethanol production is made by the fermentation of sugars with yeast. Because a number of thermophilic bacteria produce ethanol as a metabolic product,

the use of thermophilic bacteria for ethanol production has been proposed. There are several advantages for its use. The elevated incubation temperature makes distillation of the ethanol product more efficient. The cooling requirement which is necessary when yeast are used is obviated. In addition, some thermophilic bacteria can carry out direct fermentation of polysaccharides to ethanol. Yeasts however are incapable of hydrolysing polysaccharide polymers; hence they can not produce ethanol. The most popular thermophilic bacterium of this kind is Clostridium thermocellum, a bacterium capable of fermenting cellulose to ethanol (Weimer, 1986).

Several other products might be produced by using thermophilic bacteria. Among these are lactic acid produced by bacteria of genera Clostridium ,Thermoanaerobacterium Thermoanaerobacter, Bacillus coagulans; carotenoids produced by Thermus aquaticus; amino acids produced by Bacillus coagulans (Weimer, 1986).

Thermostable enzymes generally are more resistant to the denaturing effects of detergents and organic solvents. The consequence is the possibility of applying higher concentrations of reactants, the choice of not necessarily highly aquatic reaction systems, or the possibility of efficient cleaning of the system with organic solvents (Daniel et al., 1981).

Thermophilic anaerobic digestion process has advantages over the mesophilic process. These are increase in reaction rate and destruction of pathogenic microorganisms that

might be present in the sewage and lower viscosity which requires less energy for mixing (Zinder, 1986).

Mass cultivation of thermophilic bacteria would be cheaper than that of mesophilic bacteria due to reduced contamination problem. Production would be increased as reaction rates of organisms and enzymes increases (Sonnleitner, 1984).

Because of the high temperature of operation thermophilic enzyme reactors would not be prone to contamination problems. Lastly, thermophilic bacteria would not be pathogenic to man (Sonnleitner, 1984).

In Ethiopia hot springs are abundant. Industrially important microorganisms could be present in these hot springs. However, there has not been any study on the microflora of these hot springs. Hence this research was geared to study the microflora of one of the hot springs in Ethiopia. The objectives of this study include the following:

1. to select and/or formulate media which favor greatly the growth of aerobic heterotrophic microorganisms present in one of Shalla hot springs .
2. to study the influence of media pH and incubation temperature on the growth of the microorganisms.
3. to isolate and characterize the microorganisms which grew on various media.
4. to identify potentially useful isolates which could be good sources of for biotechnological application.

might be present in the sewage and lower viscosity which requires less energy for mixing (Zinder, 1986).

Mass cultivation of thermophilic bacteria would be cheaper than that of mesophilic bacteria due to reduced contamination problem. Production would be increased as reaction rates of organisms and enzymes increases (Sonnleitner, 1984).

Because of the high temperature of operation thermophilic enzyme reactors would not be prone to contamination problems. Lastly, thermophilic bacteria would not be pathogenic to man (Sonnleitner, 1984).

In Ethiopia hot springs are abundant. Industrially important microorganisms could be present in these hot springs. However, there has not been any study on the microflora of these hot springs. Hence this research was geared to study the microflora of one of the hot springs in Ethiopia. The objectives of this study include the following:

1. to select and/or formulate media which favor greatly the growth of aerobic heterotrophic microorganisms present in one of Shalla hot springs .
2. to study the influence of media pH and incubation temperature on the growth of the microorganisms.
3. to isolate and characterize the microorganisms which grew on various media.
4. to identify potentially useful isolates which could be good sources of for biotechnological application.

2. MATERIALS AND METHODS

2.1 STUDY AREA.

The hot spring where samples were collected is located in the eastern shore of lake Shalla. Water samples were collected at four sites established in the spring and three sites identified as (3a, 3b and 3c) where the water forms a thermal gradient (Fig. 1).

Temperature at the various sites was measured during sample collection using a thermometer. pH was measured with Griffin pH meter Model 140.(Griffin, England).

2.2 SAMPLE COLLECTION.

Water samples (about 40 ml) for microbiological analysis were collected during three different trips from each site with sterile plastic centrifuge tubes. Samples were transported to the laboratory on the same day of sampling and kept at 55^{0}C in an incubator.

2.3 CULTURAL METHODS.

The media selected and /or formulated for isolation of bacteria had the following composition (in grams/l of distilled water):

Medium 1: Tryptone Soya Agar (TSA) (Oxoid, England),10; Yeast Extract (Oxoid,England),1; Glucose,1; Agar (Oxoid,England),25; and trace minerals solution,10 ml. The trace minerals solution

had the following composition (µg/100 ml medium): $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 1; KNO_3, 10; K_2HPO_4, 2; NaCl, 0.08; H_3BO_3, 0.5; $CaCO_3$, 10; $CuSO_4.5\ H_2O$ 1; $FeSO_4(NH_4)SO_4$, 50; KI, 1; $MnSO_4$, 2; MoO_3, 1; and $ZnSO_4.7H_2O$, 5.

Medium 2: TSA, 40; and Yeast Extract, 1 Agar, 10.

Medium 3 Yeast Extract, 1; Glucose, 1; Agar, 25; and trace minerals solution, 10 ml.

Medium 4: Hot spring water, 650 ml; Glucose, 1; Yeast Extract, 1; and Agar, 25.

In the case of Medium 4, hot spring water was filter sterilized with Carlson filters (Gallenkamp, England). The other constituents were autoclaved separately and added to the filter sterilized hot spring water.

All media were adjusted to pH values of 6.0 to 13.0 with 6N HCl or 6N NaOH. The required amounts of sterile NaOH and HCl were added to the media aseptically after sterilization. Water samples were serially diluted in sterile water. After thorough mixing, 1 ml portion of water was seeded into duplicate sterile plates and the media were poured into each of the plate. Plates were incubated in inverted position at 7-10°C for about one week for psychrophilic count, and at 32°C for 24-48 hours for thermotolerant count, and at 55°C for 24-48 hours for thermophilic count. Enrichment for extreme thermophiles was

carried out on Tryptone Soya Broth (TSB) at 70°C until the appearance of heavy turbidity.

Colonies were counted with a Quebec colony counter (Quebec,USA) and described based on their morphology, color, size,texture etc. Colonies were recorded as colony forming units (cfu) per ml of sample. Representative pure colonies were maintained on agar slants and used for biochemical tests.

The population of the bacteria isolated using the four media adjusted to the different pH values were determined for each site. The population distribution of microorganisms in the thermal gradient was also determined.

2.4. CHARACTERIZATION AND NAMING OF THE ISOLATES

Biochemical, morphological and growth characteristics in liquid medium were tested on the pure isolates according to the procedures of Smibert and Krieg (1981), Collins and Lyne (1976) and Mitruka and Bonner (1976).The tests include; utilization of sugars activity on macromolecules,enzyme production, citrate utilization, and nitrate reduction . Naming of the characterized bacteria was done based on the schemes of Buchanan and Gibbons (1974).

2.5. TEST FOR AMYLOLYTIC AND PROTEOLYTIC ACTIVITY

Amylolytic and proteolytic activities were tested according to the methods of Collins and Lyne (1976). Amylase

production was tested on TSA supplemented with 10% soluble starch. Plates were streaked with the bacteria and incubated for 48 hours. The cultures were flooded with dilute iodine. Appearance of a clear brown or dark brown zone around the bacterial colony after the addition of iodine was recorded as a positive test for amylase production.

Protease production was tested on TSA containing 5% gelatin. After 48 hours of incubation, the plates were flooded with saturated solution of ammonium sulphate. The appearance of a clear zone of various sizes around the colony after the addition of ammonium sulphate was taken as a positive test for protease production.

3. RESULTS

3.1. STUDY AREA

The hot spring located in the eastern shore of lake Shalla has a slightly alkaline pH (8.7) with maximum temperature of 94-96°C at site 2 and a minimum temperature of 55-58°C at site 4. The water flowing from sites 1,2, and 3 to site 4 forms a thermal gradient from site 3a to site 3c (Fig.1). The temperature of the hot spring at the four sites selected for this study is shown on Table 1.

3.2. THE INFLUENCE OF MEDIUM COMPOSITION ON THE GROWTH OF BACTERIA.

Four media were employed for cultivation and isolation of the bacteria from the hot spring. The media were selected and/or formulated on the basis of their chemical composition and their relative capacity to support growth of various microorganisms.

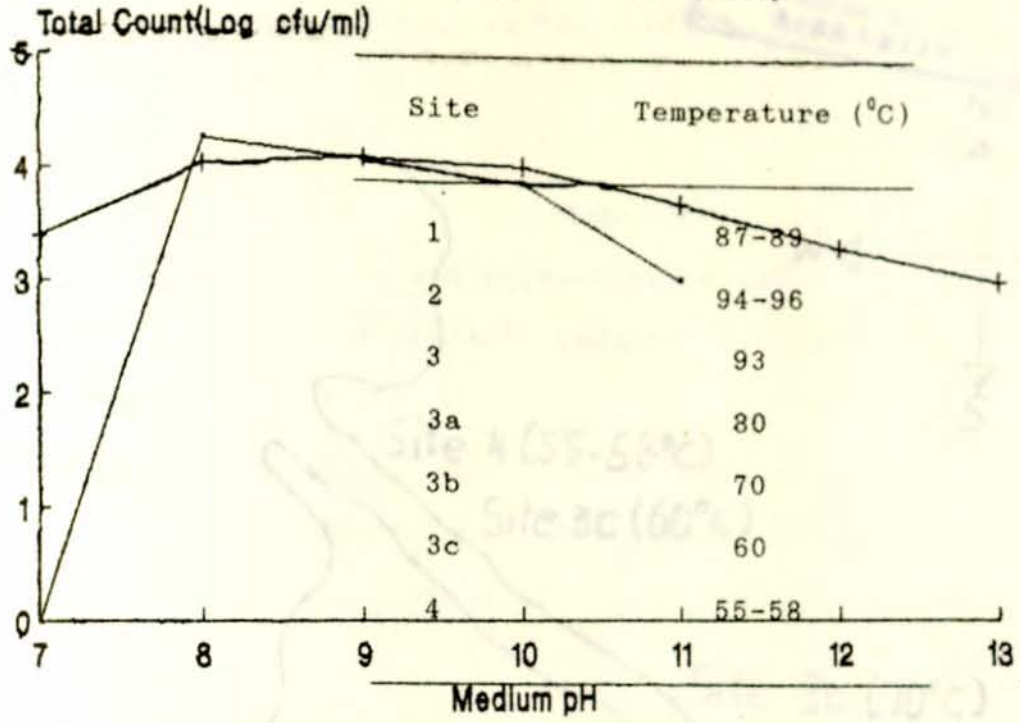
When the media were adjusted to pH of 6.0 there was some growth. There was no growth at the temperature of 7-10°C after one week of incubation. Therefore, media adjusted to pH values of 7.0-13.0, and incubation temperature of 32°C and 55°C were used for isolation of bacteria in the course of the study.

Attempts to isolate extreme thermophiles after enrichment at 70°C was made several times. However, upon transfer to solid media the microorganisms failed to grow.

Figure 1. Map of Shalla hot spring showing the sampling sites.

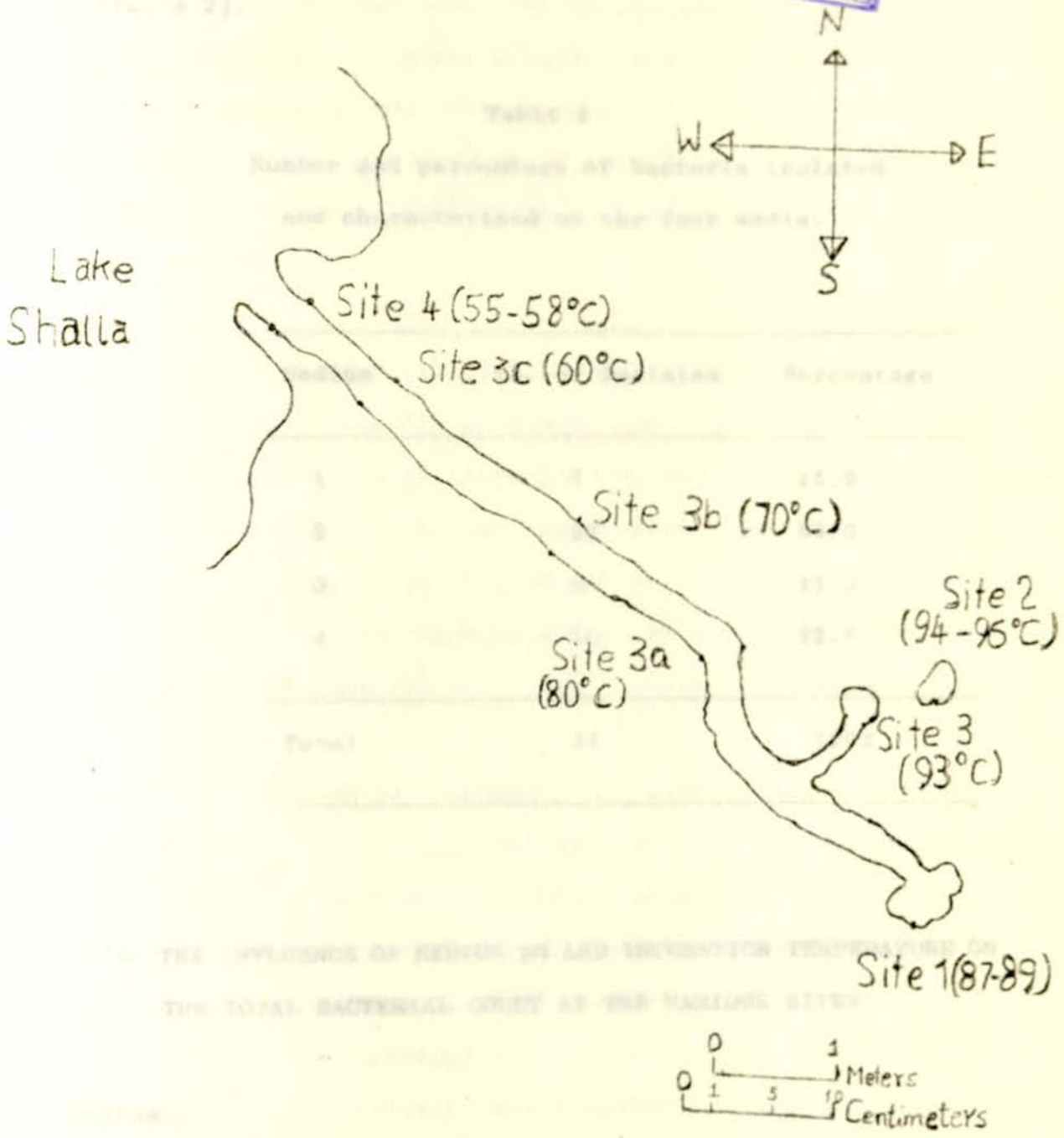
Table 1

Temperature of the hot spring at the various sites selected for study



Medium 2 which contained the recommended concentration of TSA supplemented with yeast extract supported the best growth of the different types of bacteria. As can be seen in Table 2, the number of species isolated using medium 2 was the highest. All of the isolates from the other media at the different sites also grow in this medium. Since most media used for isolation of aquatic microorganisms are generally low in chemical composition, medium 1 containing one-quarter the strength of medium 2 was used in this study. However, medium 1 performed poorly (Table 2).

The only difference between medium 3 and medium 4 was the use of trace minerals and hot spring water as sources of chemicals for growth in medium 3 and medium 4 respectively.



chemicals for growth in medium 3 and medium 4 respectively. Medium 4 which contained the hot spring water favored the growth of more species of the isolated bacteria than medium 3 (Table 2).

Table 2

Number and percentage of bacteria isolated and characterized on the four media.

Medium	No. of Isolates	Percentage
1	7	15.9
2	22	50.0
3	5	11.3
4	10	22.6
Total	44	100%

3.3. THE INFLUENCE OF MEDIUM pH AND INCUBATION TEMPERATURE ON THE TOTAL BACTERIAL COUNT AT THE VARIOUS SITES

Medium-1

The total bacterial count on medium 1 at site 1 is shown on Fig.2. At pH values greater than 9.0 the facultative thermophilic count was greater than the thermotolerant count.

Facultative thermophiles grew from pH 7.0-13.0 with the highest (1×10^4 cfu/ml) being at pH 9.0. The population decreased at pH values greater than 9.0. The thermotolerant bacteria grew between pHs of 8.0-11.0 the highest (2×10^4 cfu/ml) being at pH 8.0. Thermotolerants were apparently absent at medium pH greater than 11.0. and pH values less than 8.0. The population of the thermotolerant bacteria decreased at pH values greater than 8.0.

Facultative thermophiles at site 2 grew from pH 7.0-13.0 in medium 1 (Fig.3). They were the most abundant (1×10^5 cfu/ml) at pH 9.0. The pH range for thermotolerant bacteria was however narrow (7.0-10.0) and the highest count registered (1×10^4 cfu/ml) was at pH 9.0. There was a decrease in total count with further increase in medium pH above 9.0. Even then facultative thermophiles were more numerous than thermotolerants.

The facultative thermophilic count at site 3 was the highest (2×10^4 cfu/ml) in medium 1 when the pH was adjusted to 8.0 (Fig.4). Increases in medium pH above 8.0 resulted in decreased total count of thermophiles. Thermotolerant bacteria were found in abundance (8×10^3 cfu/ml) at pH 9.0.

Facultative thermophilic members did not grow in medium 1 at site 4. Only thermotolerant bacteria were cultivated at site 4 and the highest count recorded (5×10^3 cfu/ml) was at pH 9.0 (Fig.4).

Figure 2. Total number of bacteria cultivated on medium 1 at site 1; facultative thermophiles (—•—) and thermotolerant (—▲—).

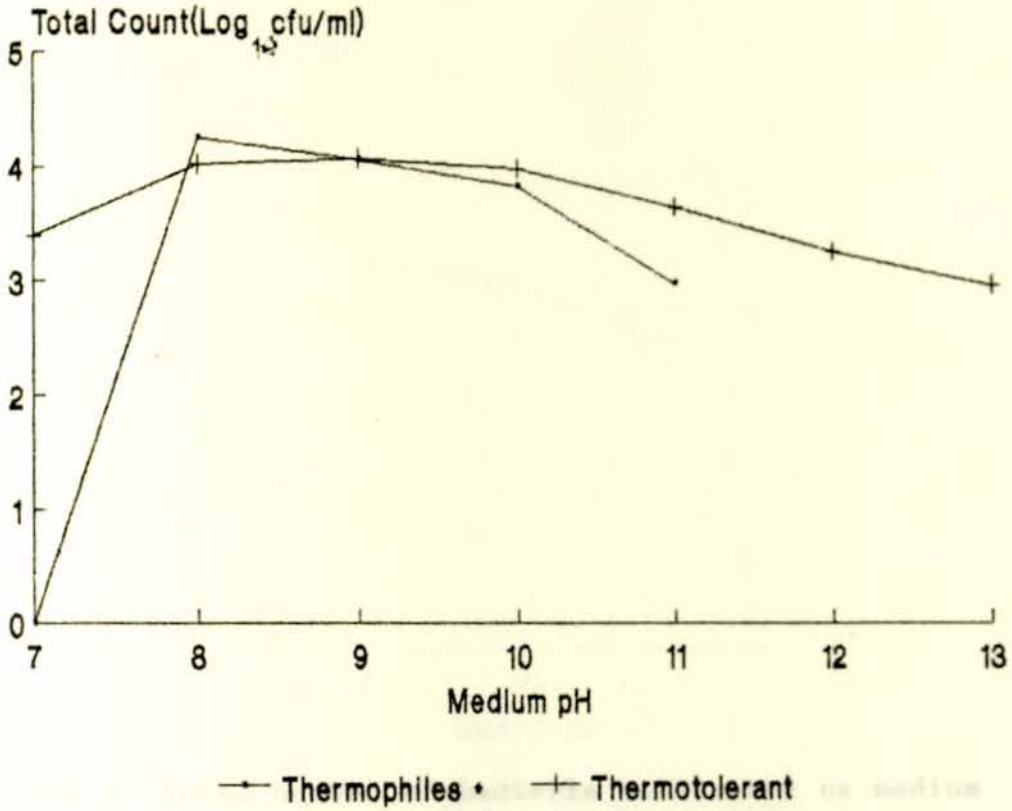

Total Count(Log cfu/ml)
5
4
3
2
1
0
7
8
9
10
11
12
13
Medium pH
Thermophiles
Thermotolerant

Figure 3. Total number of bacteria cultivated on medium 1 at site 2; facultative thermophiles (—•—) and thermotolerant (—•—).

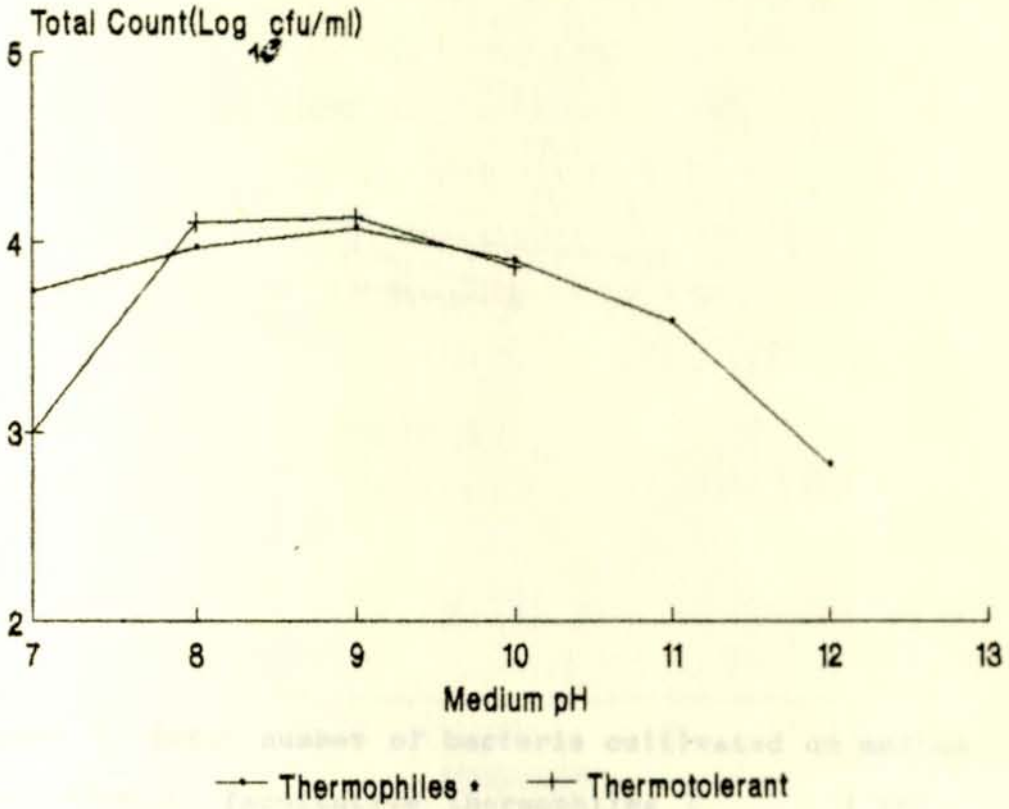
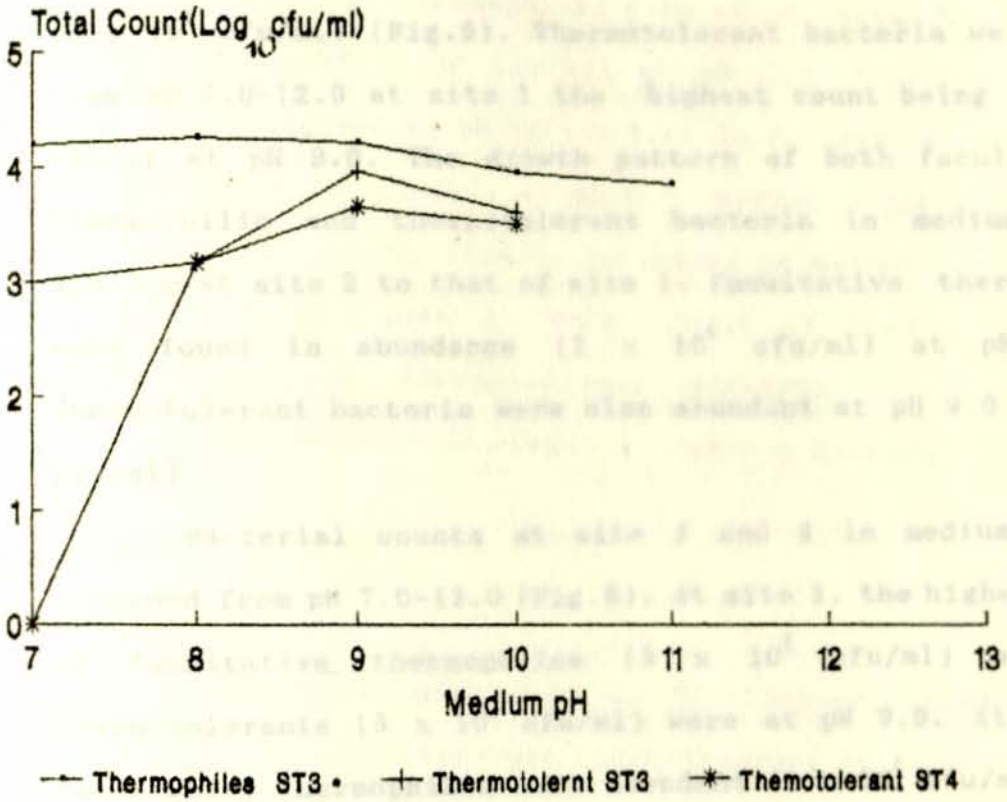

Total Count(Log cfu/ml)
5
4
3
2
7
8
9
10
11
12
13
Medium pH
Thermophiles
Thermotolerant

Figure 4. Total number of bacteria cultivated on medium 1 at site 3; facultative thermophiles (—•—),thermotolerant (—+—) at site 4;thermotolerant(—*—)


Total Count(Log10 cfu/ml)
5
4
3
2
1
0
7
8
9
10
11
12
13
Medium pH
Thermophiles ST3
Thermotolernt ST3
Themotolerant ST4

Medium-2

Facultative thermophilic bacteria were more numerous than thermotolerant bacteria in medium 2 at all pH values and at all sites. At site 1, facultative thermophiles were cultivated from pH 7.0-13.0. They were abundant at pH 9.0 (2×10^4 cfu/ml) (Fig.5). Thermotolerant bacteria were grown from pH 7.0-12.0 at site 1 the highest count being 1×10^4 cfu/ml at pH 9.0. The growth pattern of both facultatively thermophilic and thermotolerant bacteria in medium 2 was similar at site 2 to that of site 1. Facultative thermophiles were found in abundance (2×10^4 cfu/ml) at pH 9.0. Thermotolerant bacteria were also abundant at pH 9.0 (9×10^3 cfu/ml).

Bacterial counts at site 3 and 4 in medium 2 were obtained from pH 7.0-13.0 (Fig.6). At site 3, the highest count of facultative thermophiles (3×10^4 cfu/ml) and thermotolerants (5×10^3 cfu/ml) were at pH 9.0. At site 4, facultative thermophiles were abundant (1×10^4 cfu/ml) at pH 9.0. At the pH of 9.0 thermotolernt bacteria were abundant (5×10^3 cfu/ml). Increases in medium pH above 9.0 resulted in decreases in total counts of bacteria.

Medium-3

Medium-3 supported the growth of facultative thermophilic bacteria at pH values 8.0-12.0 and thermotolerant bacteria at pH 8.0-10.0 at site 1 (Fig. 7). The highest count of facultative thermophiles (2×10^4 cfu/ml) and that of

thermotolerants (5 x 10^3 cfu/ml) were recorded at pH 9.0. Facultative thermophiles at site 2 grew at the pH values 8.0-12.0 with the highest count (6 x 10^3 cfu/ml) at pH 9.0(Fig. 8). Thermotolerant bacteria on the other hand grew from pH 7.0-11.0. They were found in abundance at pH 9.0 (6 x 10^3 cfu/ml).

Facultative thermophiles grew at pH values 8.0-12.0 the highest being (6 x 10^3 cfu/ml) at pH 9.0. Thermotolerant bacteria were grown at pH values 8.0- 10.0. (Fig. 9). They were abundant (2 x 10^3 cfu/ml) at pH 9.0. Medium 3 did not support growth of bacteria at site 4. Increases in medium pH above 9.0 resulted in decreases in total count of both facultative thermophiles and thermotolerant bacteria. In medium 3, facultative thermophiles were more numerous than thermotolerant bacteria.

Medium-4

In medium 4, more numerous facultatively thermophilic bacteria were cultivated than thermotolerant bacteria. At site 1, facultative thermophiles grew from pH 7.0-11.0 while thermotolerants grew from pH values 8.0-11.0. Facultative thermophiles were abundant (7 x 10^4 cfu/ml) at pH 9.0. Thermotolerant bacteria were also abundant at pH 9.0 (3 x 10^4 cfu/ml) Fig. 10. Facultayive thermophiles at site 2 grew from pH 7.0-10.0 with the highest count (4 x 10^4 cfu/ml) at pH 9.0. Thermotolerants on the other hand grew at the pH of 8.0 and 9.0.

The growth pattern of bacteria in medium 4 at site 3 was restricted to pH values of 8,0-10.0 (Fig.11). The highest facultative thermophilic count (3×10^4 cfu/ml) and thermotolerant count (3×10^3 cfu/ml) being at pH 8.0. At site 4, growth of bacteria were obtained at pH values 7.0-9.0. Facultative thermophiles were abundant (5×10^3 cfu/ml) at pH 8.0. The population of thermotolerant bacteria at pH 8.0 and 9.0 was the same (2×10^3 cfu/ml).

3.4. THE SPECTRUM OF MICROORGANISMS ISOLATED AND CHARACTERIZED FROM THE HOT SPRING.

In the course of this study 44 microorganisms were isolated and characterized from the hot spring. Of these isolates, 29 were identified to the species level and 3 to the genus level (Table 3). The remaining 12 isolates were not identified (Table 4). These isolates require detailed analyses to determine their taxonomic position. Among the unidentified isolates, six were Gram-positive, non-spore forming, catalase and oxidase positive, indole negative motile rods. The remaining six were filamentous rods having features similar to the rods mentioned above. Besides the biochemical and morphological characteristics shown in Table 4, tests like utilization of sugars (e.g xylose, trehalose, sucrose, raffinose,maltose) were performed on the isolates.

Following the classification of Farrel and Campbell (1969) and Stanier et al. (1970) the microorganisms were placed into two categories: 1) facultative thermophiles and

thermotolerant. Based on this growth temperature dependent scheme, Bacillus megaterium, Bacillus brevis, Bacillus lentus, Bacillus stearothermophilus and Bacillus marcoccanus were placed in the category of facultative thermophiles. On the other hand Bacillus circulans, Bacillus cereus, Bacillus pacificus, Bacillus panthothenicus, Bacillus firmus, Bacillus subtilis, Bacillus pumilis and Micrococcus species were identified as thermotolerant.

The most frequently isolated bacteria from the hot spring was Bacillus stearothermophilus. Bacillus brevis, and Bacillus circulans and Mirococcus species were the second most abundant isolates. These were followed by Bacillus megaterium, Bacillus sphaericus, and Bacillus subtilis. Other species belonging to genus Bacillus were also isolated from the hot spring during the study period.

Figure 5. Total number of bacteria cultivated on medium 2 at site 1; facultative thermophiles (—•—), thermotolerant (—/—) at 2; facultative thermophiles (—▲—) thermotolerant (—✱—).

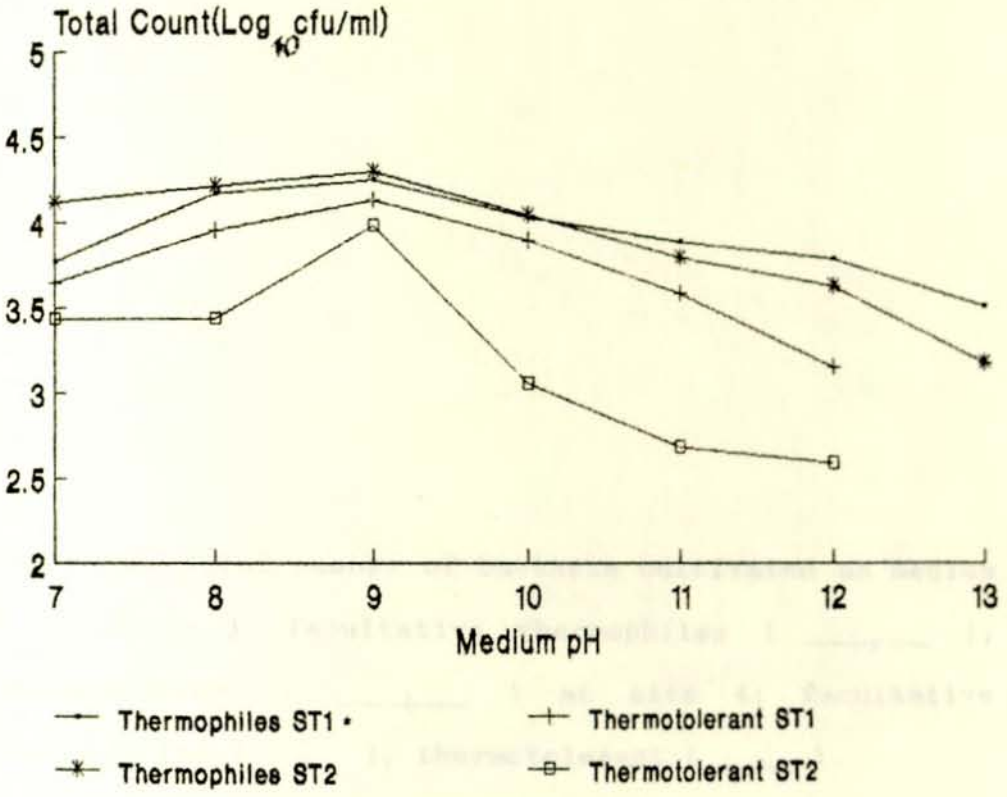
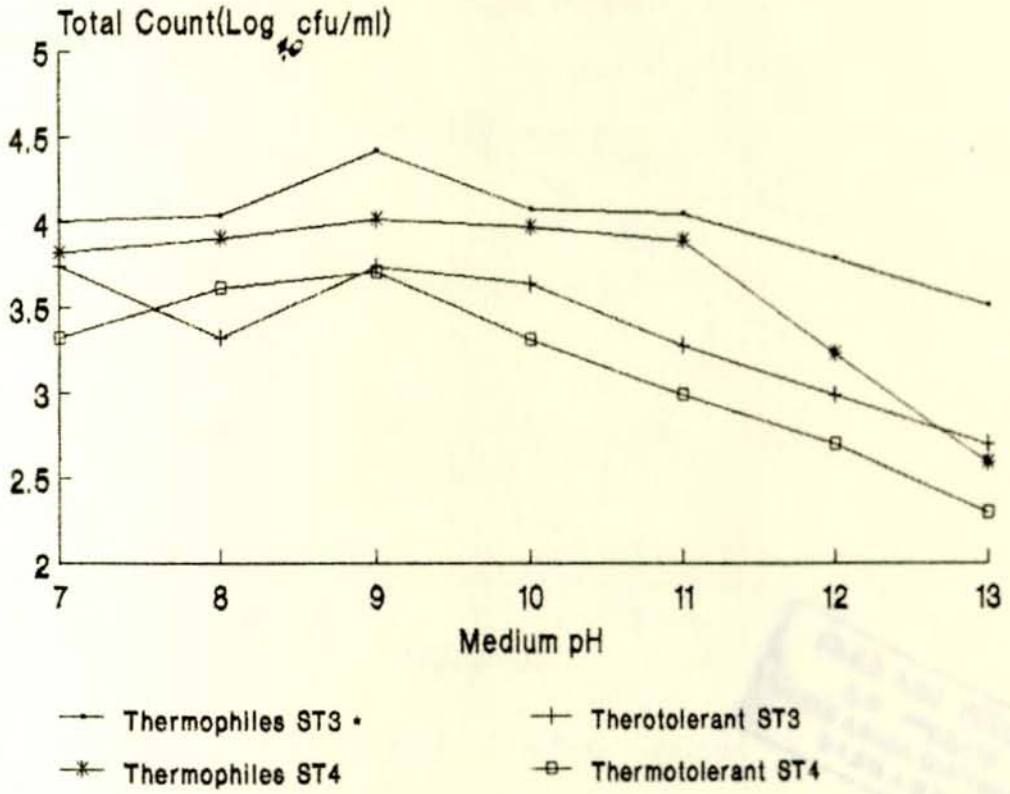

Total Count(Log10 cfu/ml)
5
4.5
4
3.5
3
2.5
2
7
8
9
10
11
12
13
Medium pH
Thermophiles ST1
Thermophiles ST2
Thermotolerant ST1
Thermotolerant ST2

Figure 6. Total number of bacteria cultivated on medium 2 at site 3; facultative thermophiles (—•—), thermotolerant (—+—) at site 4; facultative thermophiles (—*—), thermotolerant (—›—).


Total Count(Log cfu/ml)
5
4.5
4
3.5
3
2.5
2
7
8
9
10
11
12
13
Medium pH
Thermophiles ST3
Therotolerant ST3
Thermophiles ST4
Thermotolerant ST4

• • Facultative

This thesis has been submitted for examination with my approval as a University advisor.

Name : DR. BERHANU A. GASHE.

Signature : _____.

Date : _____.

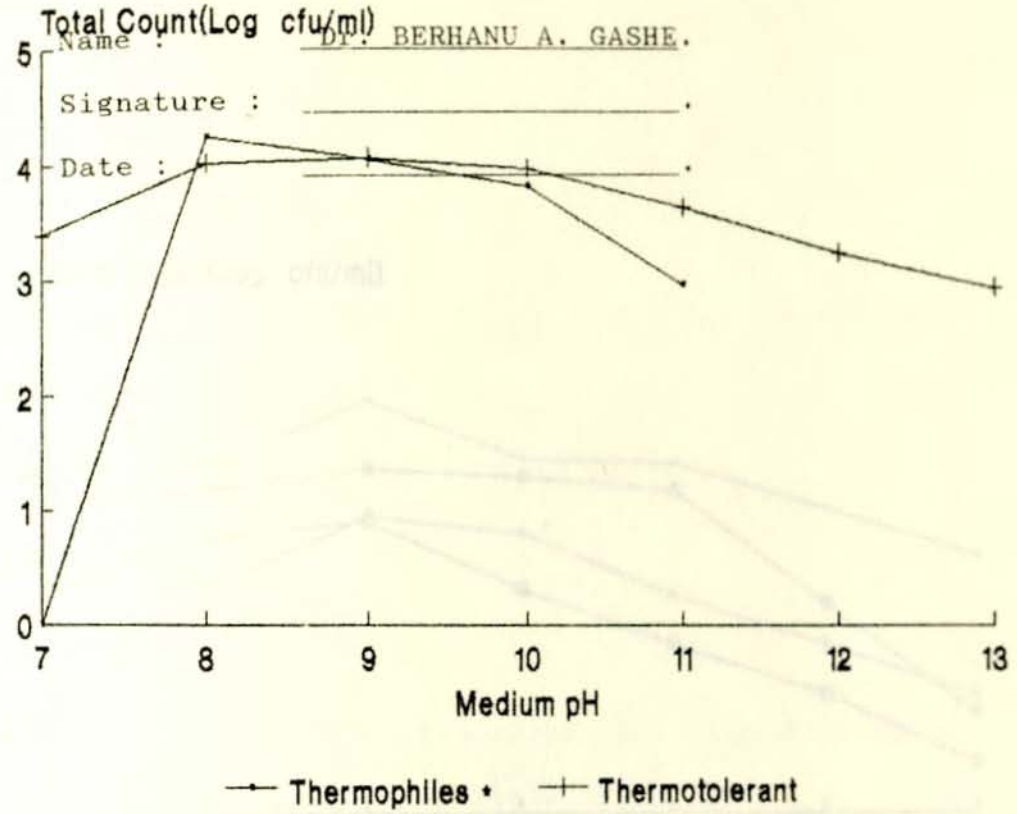
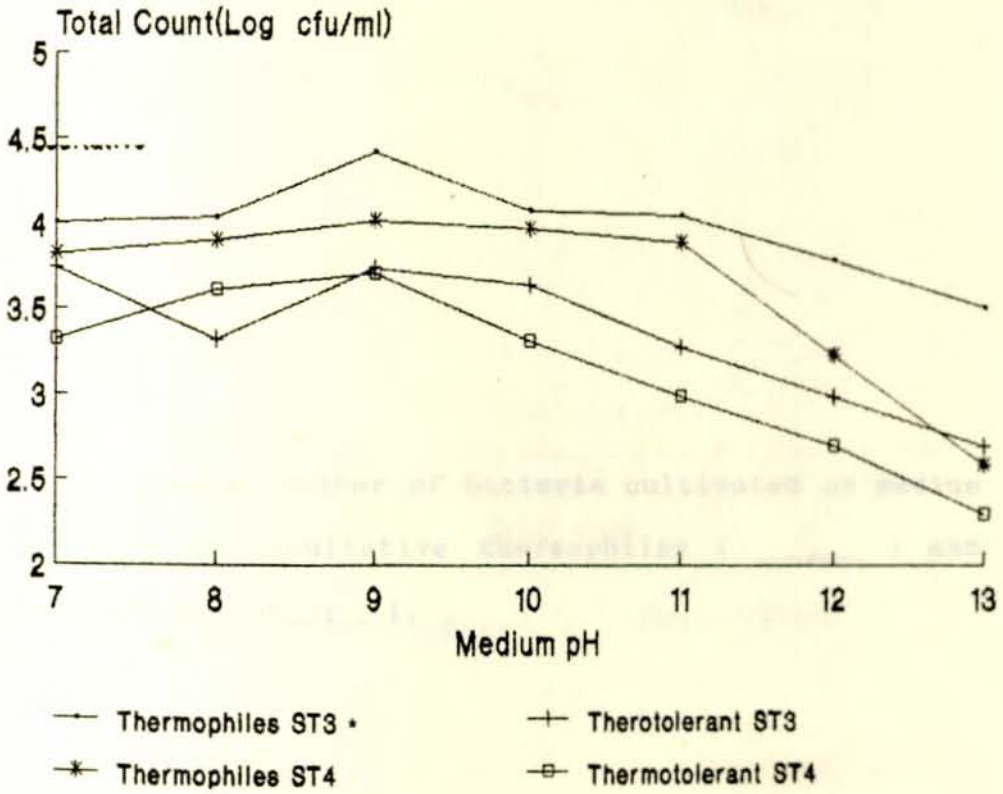


Figure 11. Total number of bacteria cultivated on medium 4 at site 3; facultative thermophiles (_____), thermotolerant (____); at site 4; facultative thermophiles (____), thermotolerant (____).



• • Facultative

Figure 7. Total number of bacteria cultivated on medium 3 at site 1; facultative thermophiles (—•—) and thermotolerant (—+—).

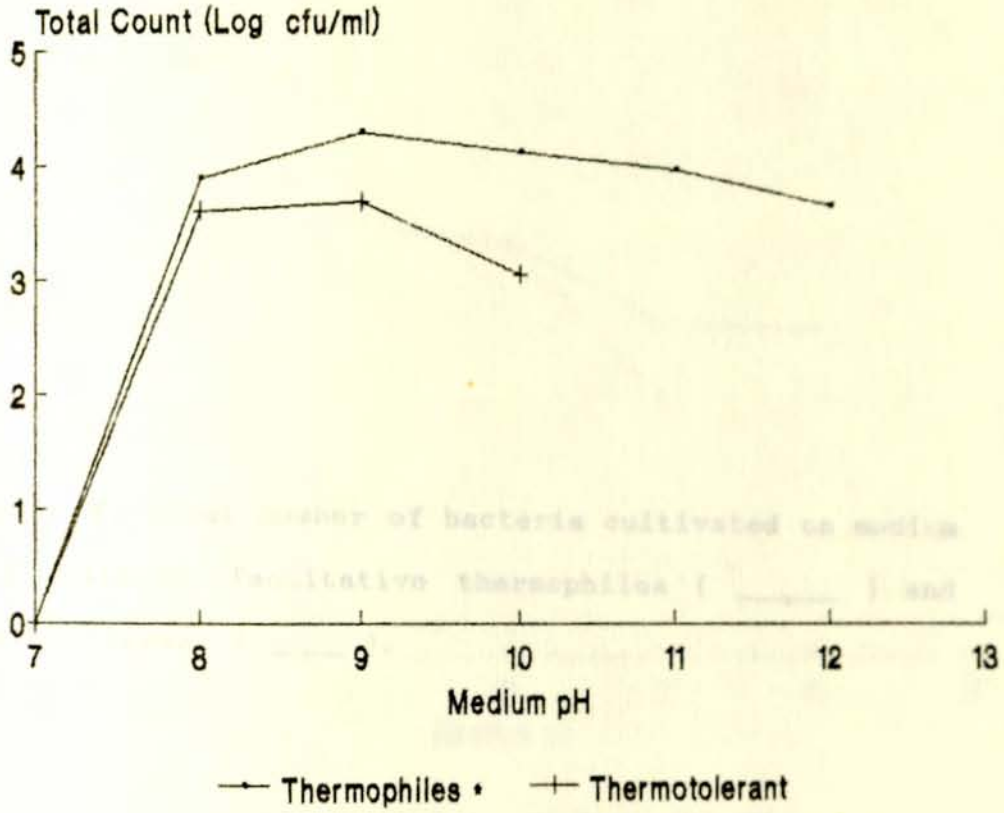

Total Count (Log cfu/ml)
5
4
3
2
1
0
7
8
9
10
11
12
13
Medium pH
Thermophiles
Thermotolerant

Figure 8. Total number of bacteria cultivated on medium 3 at site 2; facultative thermophiles (—•—) and thermotolerant (—+—).

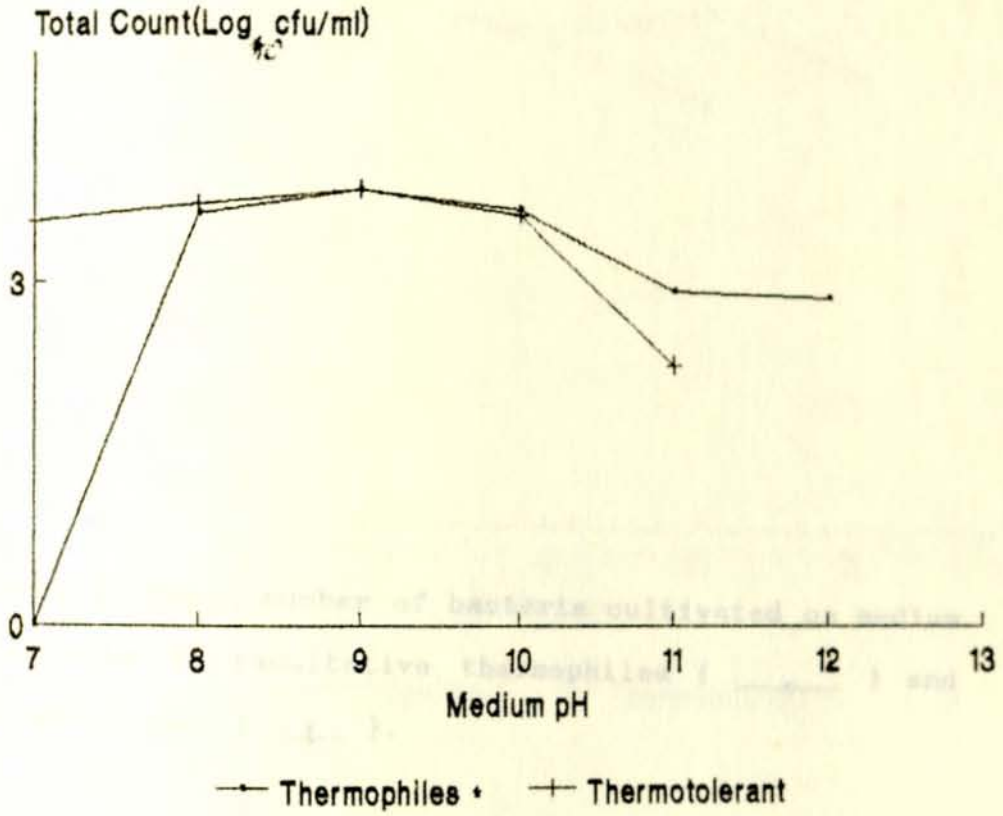

Total Count(Log cfu/ml)
3
0
7
8
9
10
11
12
13
Medium pH
Thermophiles
Thermotolerant

Figure 9. Total number of bacteria cultivated on medium 3 at site 3; facultative thermophiles (—•—) and thermotolerant (—+—).

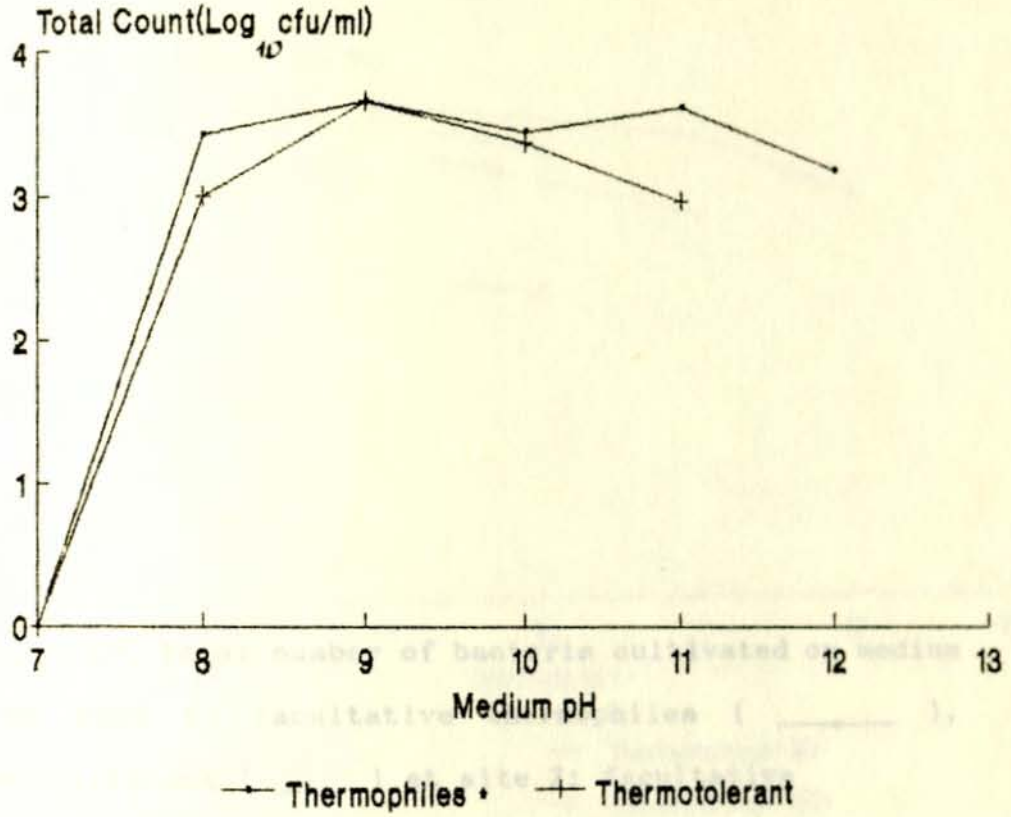

Total Count(Log cfu/ml)
4
3
2
1
0
7
8
9
10
11
12
13
Medium pH
Thermophiles
Thermotolerant

Figure 10. Total number of bacteria cultivated on medium 4 at site 1; facultative thermophiles (——•——), thermotolerant (—+—) at site 2; facultative thermophiles (—*—), thermotolerant (—○—).

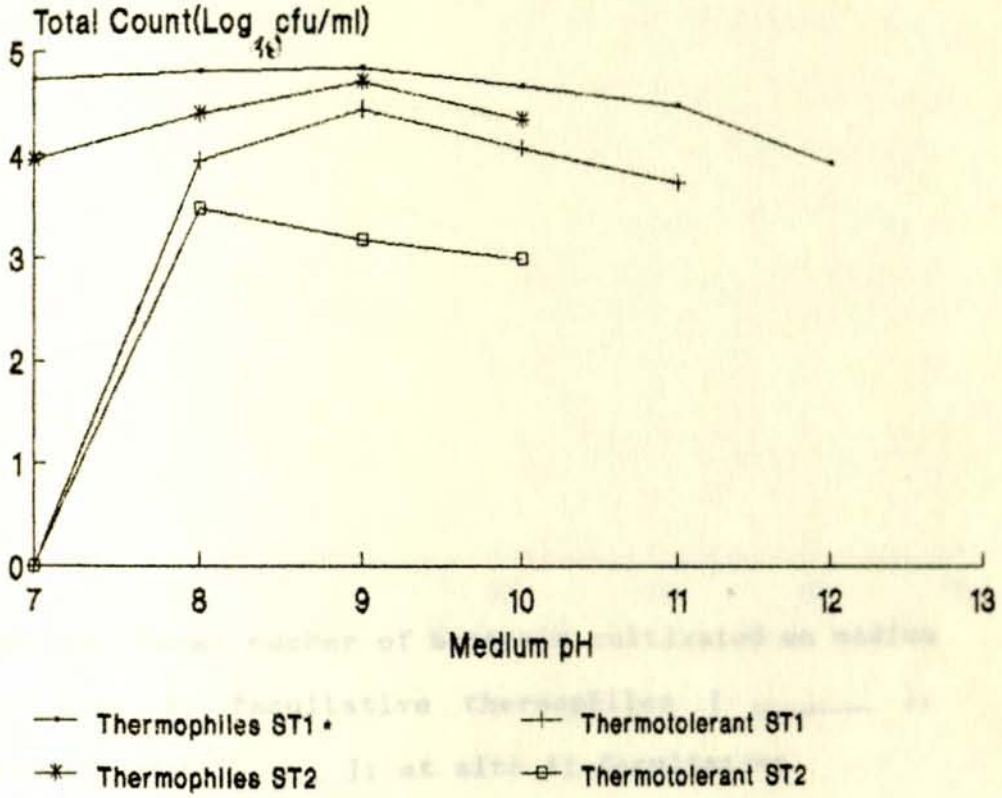

Total Count(Log cfu/ml)
5
4
3
2
1
0
7
8
9
10
11
12
13
Medium pH
Thermophiles ST1
Thermophiles ST2
Thermotolerant ST1
Thermotolerant ST2

Figure 11. Total number of bacteria cultivated on medium 4 at site 3; facultative thermophiles (——•——), thermotolerant (—▲—); at site 4; facultative thermophiles (——×——) and thermotolerant (—○—).

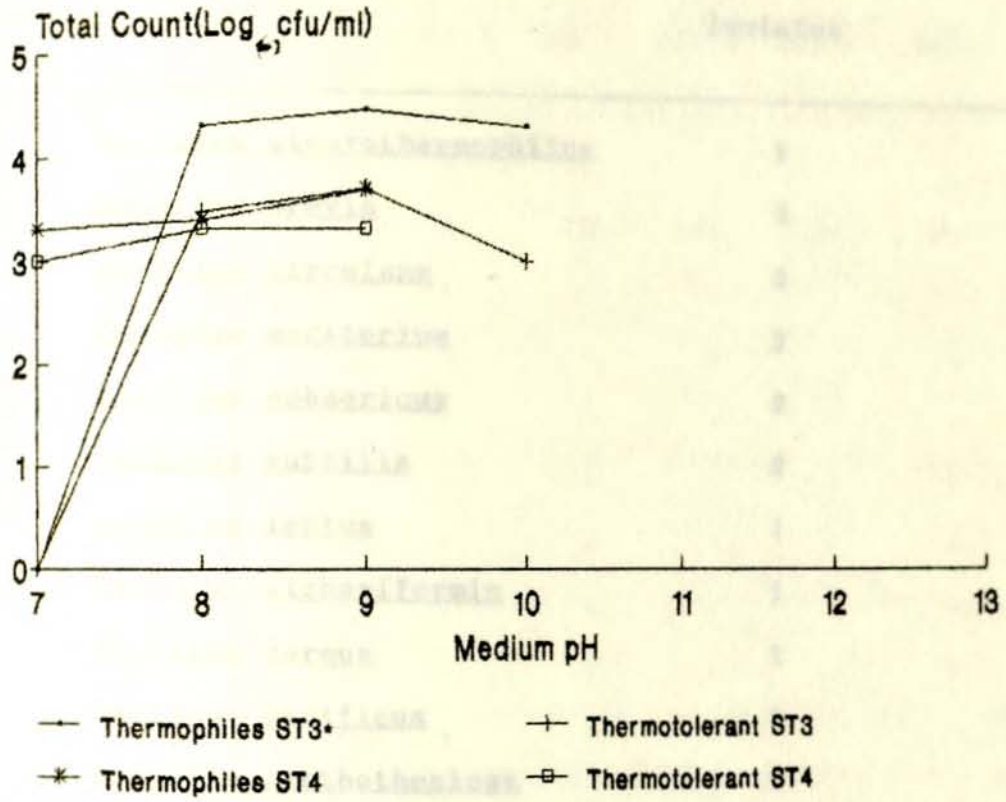

Total Count(Log cfu/ml)
5
4
3
2
1
0
7
8
9
10
11
12
13
Medium pH
Thermophiles ST3
Thermotolerant ST3
Thermophiles ST4
Thermotolerant ST4

Table 3

List of bacteria identified to the species and the genus level

Name of organism	No. of Isolates	%
Bacillus stearothermophilus	9	28.1
Bacillus brevis	3	9.3
Bacillus circulans	3	9.3
Bacillus megaterium	2	6.2
Bacillus sphaericus	2	6.2
Bacillus subtilis	2	6.2
Bacillus lentus	1	3.1
Bacillus licheniformis	1	3.1
Bacillus cereus	1	3.1
Bacillus pacificus	1	3.1
Bacillus panthothenicus	1	3.1
Bacillus marcoccanus	1	3.1
Bacillus pumilis	1	3.1
Bacillus firmus	1	3.1
Micrococcus sp	3	9.3
Total	32	100%

Table 4

Unidentified isolates and their major biochemical and morphological characteristics

	Code No.							
	UR-1	UR-2	UR-3	UFR-1	UFR-2	UFR-3	UFR-4	UFR-5
01	R	R	R	FR	FR	FR	FR	FR
02	+	-	+	-	-	-	+	-
03	-	-	-	+	+	-	-	+
04	-	-	+	-	-	-	-	-
05	+	-	+	-	-	+	+	+
06	+	+	+	+	-	-	+	+
07	-	-	+	+	-	-	+	+
08	+	+	-	+	+	+	+	+
09	+	+	+	-	+	-	-	-
10	+	-	+	+	+	+	+	+

01 = morphology, 02 = methyl-red test, 03 = Voges-Prskaur's test, 04 = citrate utilization, 05 = nitrate reduction, 06 = urease test, 07 = anaerobic growth, 08 = starch hydrolysis, 09 = = esculin hydrolysis, 10 = gelatin hydrolysis, R = rod, FR = filamentous rod, UR = unidentified rod, UFR = unidentified filamentous rod, + = positive reaction, - = negative reaction.

Table 4 (continued)

	Code No.							
	UR-1	UR-2	UR-3	UFR-1	UFR-2	UFR-3	UFR-4	UFR-5
11	+	+	-	+	+	-	+	+
12	-	+	+	-	+	+	+	+
13a	20	30	20	20	20	20	35	30
13b	45-50	50-55	45-50	50-55	45-55	45-55	55-60	45-50
13c	55	65	65	65	65	65	65	60
14	5-10	6-10	5-10	5-10	5-10	5-10	6-10	5-9
15	2-7	2-7	2-5	2-5	2-7	2-7	2-7	2-5

11 = hippurate hydrolysis, 12 = poly-B-hydroxy butyrate accumulation test, 13 = growth temperature(^{0}C);a = minimum, b = optimum, c = maximum,14 = pH range for growth, 15 = growth in NaCl(%), UR = rod, UFR = filamentous rod, + = positive reaction, - = negative reaction.

3.5. POPULATION DISTRIBUTION OF THE MICROORGANISMS AT THE VARIOUS SITES

The population distribution of facultatively thermophilic and thermotolerant bacteria from the hot spring was found to be variable (Table 5). The occurrence of some

isolates was not site specific. Bacillus stearothermophilus, Bacillus brevis and Micrococcus species were isolated from all sites where the temperatures were between 96-55°C. On the other hand the distribution of some of the bacteria was restricted only to one, two or three sites. Bacillus megaterium, Bacillus lentus, Bacillus licheniformis, Bacillus pacificus, Bacillus sphaericus were isolated at sites 1, 2, and 3; Bacillus subtilis at sites 1, 3 and 4; Bacillus pumilis at sites 1, 2 and 4. Bacillus circulans and Bacillus cereus were isolated at two sites. Bacillus firmus and Bacillus panthothenicus were isolated at one site only.

Among the unidentified microorganisms, UR-1 and UFR-1 were isolated from all sites. UR-3 was isolated at sites 1, 2 and 3 and UR-2 at sites 1, 2 and 4. UFR-2 was isolated at sites 1 and 2. In addition, UFR-4 and UFR-5 were restricted to one site only.

Generally, there had been decreases in number of bacteria per ml of water samples of most bacteria from higher temperature (96°C) to lower temperature (55°C). The population of some microorganisms was stable at all environmental temperature

Table 5

Population distribution of the microorganisms at the various sites (cfu/ml of water sample)

Name of Organism	Site 1			Site 2			Site 3			Site 4		
	Medium pH			Medium pH			Medium pH			Medium pH		
	>6<8	8-10	>10	>6<8	8-10	>10	>6<8	8-10	>10	>6<8	8-10	>10
Bacillus megaterium	-	$7x10^3$	$1x10^3$	-	$2x10^4$	-	-	$2x10^4$	-	-	-	-
B.brevis	$1x10^3$	$8x10^3$	$5x10^3$	-	$1x10^4$	$5x10^3$	$4x10^3$	$1x10^4$	2x10	-	7x103	-
B.lentus	-	$2x10^3$	-	-	$2x10^3$	-	-	$3x10^3$	-	-	-	-
B.lichenifomis	-	$4x10^3$	-	-	$6x10^3$	$8x10^3$	-	$5x10^3$	-	-	-	-
B.circulans	-	$1x10^4$	-	-	$1x10^4$	-	-	-	-	-	-	-
B.cereus	-	-	-	-	-	-	-	-	$6x10^3$	-	$2x10^3$	-

Table 5 (cotinued)

Name of organism	Site 1			Site 2			Site 3			Site 4		
	Medium pH			Medium pH			Medium pH			Medium pH		
	>6<8	8-10	>10	>6<8	8-10	>10	>6<8	8-10	>10	>6<8	8-10	>10
Bacillus pacificus	-	-	$1x10^3$	$2x10^3$	$5x10^3$	-	$4x10^3$	$2x10^3$	-	-	-	-
B.sphaericus	-	$7x10^6$	-	$1x10^3$	$1x10^4$	-	-	$5x10^3$	$1x10^3$	-	-	-
B.pathoth enicus	-	-	-	1x103	-	-	-	-	-	-	-	-
B.stearoth-ermophilus	$1x10^4$	$6x10^4$	-	$7x10^3$	$8x10^4$	$7x10^3$	$8x10^3$	$6x10^4$	$1x10^4$	$2x10^3$	$3x10^4$	-
B.marcocanus	-	$5x10^3$	-	-	-	-	-	-	-	$1x10^3$	$2x10^3$	-
B.pumilis	-	$9x10^3$	-	-	$1x10^4$	-	-	-	-	-	$4x10^3$	-
B.subtilis	-	$9x10^3$	-	-	-	-	$1x10^3$	$3x10^3$	-	-	$3x10^3$	-
B.firmus	-	$1x10^3$	-	-	-	-	-	-	-	-	-	-
Micrococcus sp.	$5x10^3$	$1x10^4$	-	-	$2x10^4$	$4x10^3$	-	$1x10^4$	$1x10^3$	$1x10^3$	$4x10^3$	-

Table 5 (continued)

	Site 1			Site 2			Site 3			Site 4		
	Medium pH			Medium pH			Medium pH			Medium pH		
Code No.	>6<8	8-10	>10	>6<8	8-10	>10	>6<8	8-10	>10	>6<8	8-10	>10
UR-1	-	-	-	$1x10^4$	$2x10^4$	-	-	-	$1x10^3$	-	$3x10^3$	-
UR-2	-	$2x10^3$	$1x10^3$	-	-	-	-	-	$1x10^3$	-	$2x10^3$	-
UR-3	-	$1x10^3$	-	-	-	-	-	-	-	-	$2x10^3$	-
UFR-1	-	$1x10^3$	$1x10^4$	$2x10^4$	$4x10^3$	-	-	$2x10^3$	-	-	$1x10^3$	-
UFR-2	-	$3x10^3$	-	-	$3x10^3$	-	-	-	-	-	-	-
UFR-3	-	$2x10^3$	-	-	$3x10^3$	-	-	$4x10^3$	-	-	-	-
UFR-4	-	$1x10^3$	-	-	-	-	-	-	-	-	-	-
UFR-5	-	$3x10^3$	-	-	-	-	-	-	-	-	-	-

3.6 POPULATION DISTRIBUTION OF BACTERIA IN THE THERMAL GRADIENT.

The distribution pattern of six isolates in the thermal gradient is shown in Table 6. Decreases in the population of bacteria were observed as the temperature of the hot spring water in the gradient decreased. Bacillus brevis, Bacillus stearothermophilus, and Micrococcus sp. were found throughout the thermal gradient. On the other hand, Bacillus lentus, and Bacillus licheniformis were isolated at water temperatures above 80°C. Bacillus sphaericus was isolated at temperatures above 70°C.

3.7 TEST OF ISOLATES FOR AMYLOLYTIC AND PROTEOLYTIC ACTIVITY

Proteolytic activity was demonstrated by 15 isolates (Table 7). Bacillus stearothermophilus, Bacillus brevis, Bacillus sphaericus, Micrococcus sp. and UR-1 were shown to have high proteolytic activities. Eighteen isolates were positive for starch hydrolysis indicating that they produced the enzyme amylase. Isolates with high amylolytic activity were Bacillus megaterium, Bacillus licheniformis, Bacillus panthothenicus, Bacillus pacificus, Bacillus firmus, UR-2 and UFR-1. Bacillus cereus, Bacillus pacificus, Bacillus panthothenicus, Bacillus pumilis, Bacillus subtilis, Bacillus firmus, Bacillus circulans, UR-1, UFR-1, UFR-2, UFR-3 and UFR-4 were positive for both amylase and protease. No isolate showed negative activity to both enzymes.

Table 6

Disribution of bacterial isolates in the thermal gradient

Name of Organism	Site 3a			Site 3b			Site 3c		
	Medium pH			Medium pH			Medium pH		
	>6<8	8-10	>10	>6<8	8-10	>10	>6<8	8-10	>10
Bacillus brevis	$1x10^3$	$7x10^3$	-	-	$3x10^3$	-	-	$3x10^3$	-
B.stearothermophilus	$6x10^3$	$2x10^4$	-	$7x10^2$	$8x10^3$	-	$8x10^3$	$7x10^3$	-
B.lentus	-	$5x10^3$	-	-	-	-	-	-	-
B.licheniformis	$1x10^3$	$2x10^3$	-	-	-	-	-	-	-
B.sphaericus	-	$9x10^3$	-	-	-	-	-	-	-
Micrococcus sp.	$2x10^3$	$3x10^3$	-	$2x10^3$	$3x10^3$	-	$1x10^3$	$2x10^3$	-

Table 7

List of isolates showing enzyme activity.

Isolate	amylase	protease
Bacillus megaterium	+	-
Bacillus brevis	-	+
Bacillus lentus	+	-
Bacillus licheniformis	+	-
Bacillus cereus	+	+
Bacillus pacificus	+	+
Bacillus panthothenicus	+	+
Bacillus sphaericus	-	+
B.stearothermophilus	+	-
Bacillus marcocanus	+	-
Bacillus pumilis	+	+
Bacillus subtilis	+	+
Bacillus firmus	+	+
Bacillus circulans	+	+
Micrococcus sp.	-	+
UR-1	+	+
UR-2	+	-
UR-3	-	+
UFR-1	+	+
UFR-2	+	+
UFR-3	+	+
UFR-4	+	+
UFR-5	+	-

4. DISCUSSION

The maximum temperature of the hot spring (96°C) and the pH (7.0) measured during the study period were comparable to the values (97°C and pH 8.5) registered by Pitwell in 1971 of the hot spring at Shalla. There existed only a negligble difference in temperature of 1° and pH of 0.2 units. This is not unusual because many hot springs remained constant in physical, chemical and other features for several years (Brock, 1967). Hence the temperature and the pH of the hot spring under study changed little in the last 20 years since Pitwell (1971) took the measurements.

Most investigators have used diluted media or media with low concentrations of nutrients to cultivate microorganisms from bodies of water (Egorova and Loginova, 1974; Ramaley and Hixon, 1970). This line of approach was considered to determine which medium supported excellent growth and also favored the isolation of numerous but different kinds of bacteria. In addition, other possibilities in media formulations were also included. As a result, the media employed and put into comparison contained: a) normal strength of Tryptone Soya Agar (TSA) b) one-quarter strength of TSA supplemented with trace minerals c) trace minerals with out TSA d) sterile hot spring water instead of trace minerals with out TSA. Equal concentrations of yeast extract was common to all media. Glucose was included in all media except in the nomal strength of TSA. Unlike the findings of Egorova and Loginova

(1974) and Ramaley and Hixon (1974), the medium which supported the growth of different kinds of bacteria was medium 2 which contained the normal strength of TSA. Medium 2 supported growth of 22 isolates which represent 50 % of the total isolates(Table 2). This was followed by medium 4 which contained hot spring water. Ten bacteria were isolated on medium 4. Employing one-quarter strength TSA supplemented with trace minerals solution (medium 1) was better in supporting growth than using trace minerals solution in the absence of TSA (medium 3). Medium 1 favored growth of 7 bacteria while medium 3 performed poorly; only 5 bacteria were isolated on medium 3 (Table 2). Though incorporating hot spring water (medium 4) improved on the number of isolates, it did not in any way compare with the use of normal strength of medium (medium 2).

In this study, higher total facultative thermophilic count was recorded than thermotolerant count (Fig 2-11). Among the identified isolates, the number of facultative thermophiles was also more numerous than the number of thermotolerants. No pschrophile or bacteria capable of growing at 10°c or less were isolated. The population of bacteria isolated from the region of higher temperature in the thermal gradient was much higher than from the region with lower temperature in the gradient. That is, the population of the isolates at site 3a was higher than those at sites 3b or 3c (Fig.1). As a result, the bacteria isolated were mostly thermophilic. Bacteria such as Bacillus lentus, Bacillus licheniformis and to some extent Bacillus sphaericus, which were present at site 3a were absent at site

3c which had a temperature of about 60°C (Table 6). On the other hand Bacillus stearothermophilus and a Gram-positive coccus identified as Micrococcus sp. were present at all sites in the thermal gradient (Table 6). Studies on the activities of organisms in natural geothermal habitats suggest that organisms living at high temperatures are optimally adapted to those temperatures and are mostly thermophilic (Brock, 1986). Therefore, it can be concluded that the microorganisms in the hot spring under study are adapted to high temperatures. Moreover, the temperature of the habitat can be considered also as the best incubation temperature for cultural studies (Brock, 1986).

The media pH were adjusted to 7.0-13.0. However, the pH with the highest total count regardless of the kind of media employed was between 8.0 and 9.0 (Fig. 2-11). The highest population of the microorganisms isolated and characterized were also obtained at media pH between 8.0 and 9.0 (Table 5). Decreases in media pH below and above the optimum (8-9) resulted in decreases in the population of bacteria (Table 5). This suggested that the pH of the water was the best medium pH for culturing the microorganisms from the hot spring. Thermophiles had wider pH range (7-13) for growth than the thermotolerants (7-11). Also, thermotolerant bacteria tended to grow at media pH lower than that of thermophiles.

All isolates from Shalla hot spring which grew in media 1-4 were Gram-positive and mostly rod-shaped (Table 3 and 4). Therefore comparison of the spectrum of the microorganisms

isolated in this study with microorganisms isolated earlier from other hot springs was difficult to make because no study has been done in hot springs of Ethiopia in general and Shalla hot spring in particular. Saiki et al. (1972) and Brock (1985) found out that Gram-negative bacteria grew at temperatures higher than those of Gram-positive bacteria. The absence of Gram-negative bacteria may be due to either the nature of media employed, the incubation temperature or both. Gram-negative extreme thermophiles were isolated after enrichment in liquid culture at incubation temperature of 70°C. However, attempts to sub-culture these organisms on solid media led to culture failure. Bacillus stearothermophilus was the most abundant bacterium ($7x10^3$-$8x10^4$cfu/ml) isolated during this study (Table5). Bacillus stearothermophilus is the most studied thermophile, and many strains have been isolated from hot springs (Ramaley and Bitziger, 1975; Pozmogova,1975; cited in Ljungdahl, 1979).

The bacteria isolated in this study demonstrated activity to either amylase or protease. Some of the isolates showed activity to both enzymes (Table 7). Amylase and protease are widely used enzymes in various industries. The microorganisms isolated or their enzymes could find application in various industries such as textiles, leather and detergent.

5. CONCLUDING REMARKS

In this study attempts have been made to isolate and characterize some members of the aerobic heterotrophic microorganisms in one hot spring. The media and incubation conditions employed can not provide all the necessary conditions for the growth of all heterotrophic microorganisms. Heterotrophic microorganisms are potentially good sources of industrial enzymes. Extension of this work is therefore required to isolate and characterize more and more microorganisms from the hot spring for this will in turn increase the chance of getting new thermostable enzymes.

For culturing microorganisms from the hot spring, incubation temperatures similar to the temperature of the natural environment, media adjusted to the pH of the water, would give a better picture of the resident microorganisms than under other incubation conditions.

6. REFERENCES

Belly, R.T., and T.D.Brock. (1974). Widespread occurrence of acidophilic strains of Bacillus coagulans in hot springs. J.Appl.Bacteriol. 37: 175-177.

Bott, T.L., and T.D. Brock. (1969). Bacterial rates above 90°C in Yellow-stone hot springs. Science. 164: 1411-1412.

Brock, T. D. (1967). Life at high temperatures: evolutionary, ecological and biochemical significance of organisms in hot sprigs. Science. 158: 1012-1019.

_____. (1971). Bimodal distribution of pH values of thermal springs of the world. Bull.Geol.Soc. Am. 82: 1393-1394

_____. (1985). Life at high temperatures. Science. 230: 132-138.

_____. (1986). In " Thermophiles:general, molecular and applied microbiology." T.D. Brock ed., pp 15-17.John Willy and Sons. New York.

Brock, T.D., and G.K. Darland. (1970). Limits of microbial existence:temperature and pH. Science.(NY) 169: 1316-1318.

Brock, T.D. and H. Freeze. (1969). Thermus aquaticus gen. n. and sp. n., a nonsporulating extreme thermophile J.Bacteriol. 98: 289-297.

Buchanan, R.E., and N.E. Gibbons. (1974). Bergey's Manual of Determinative Bacteriology.8th ed., Williams and Wilkins company. Baltimore.

Buonocore, V.,C. Caporale, M. Denosa, and A. Gambacorta. (1976). Stable, inducible thermophilic amylase from Bacillus acidocaldarius. J. Bacteriol. 128: 515-521.

Castenholz, R.W. (1969). Thermophilic blue-green algae and the thermal environment. Bacteriol. Rev. 33: 476-504.

Collins, C.H., and P.M. Lyne. (1976). Microbiological Methods, 4th ed. Butter Worths, London.

Cometta, S.,B. Sonnleitner, W. Sidler, and A. Fiechter. (1982). Population distribution of aerobic extremely thermophilic microorganisms in an Icelandic natural hot springs. Eur. J. Appl. Microbiol. Biotechnol. 16:151-156.

Daniel, R.M., D. A. Cowan, and H. W. Morgan. (1981). The industrial potential of enzymes from extremely thermophilic bacteria. Chemist. New Zealand. 45: 94-97

Egorova, L.A., and L.G. Loginova. (1974). Nonsporulating bacteria from Kamchatka Hot sources with 70-75°C optimum growth temperature. Microbiol. 43: 758-762.

Falk, M., and G.S. Kell. (1966). Thermal properties of water:discontinuities questioned. Science. 154: 1013-1014.

Farrel, J., and L.L. Campbell. (1969). Thermophilic bacteria

and bacteriophages. Ad. Microbiol.Physiol.3:83-109

Heinen, W. (1971). Growth conditions and temperature dependent substrate specificity of two extremely thermophilic bacteria. Arch. Mikrobiol. 76: 2-17.

Heinen, U.J., and W. Heinen. (1972). Characteristics and properties of a caldo-active bacterium producing extracellular enzymes and two related strains. Arch.Mikrobiol. 82: 1-23.

Hudson, J.A., H.W. Morgan, and R.M. Daniel. (1990). A survey of cellulolytic anaerobic thermophiles from hot springs. System. Appl. Microbiol. 13: 72-76.

Jackson, T.J., R.F. Ramaley, and W.G. Meinschein. (1973). Thermomicrobium rosem a new genus of extremely thermophilic bacterium. Internat. J.Syst. Bacteriol. 23: 28-36.

Kristjan, J.K., and G.A. Alfredsson. (1983). Distribution of Thermus spp. in Icelandic hot springs and a thermal gradient. Appl. Environ. Microbiol. 45: 1785-1789.

Ljungdahl, L.G. (1970). Physiology of thermophilic bacteria. Ad. Microbiol. Physiol. 19: 149-243.

Mateles,R.I., J.N. Baruah, and S.R. Tannebaum. (1967). Growth of a thermophilic bacterium on hydrocarbons:a new source of single-cell protein. Science. 157: 1322-1323.

Mitruka, B.M., and M.J. Bonner. (1976). Methods of Detection and Identification of Bacteria. CRC Press, Inc. Ohio. USA.

Ng, T.K. and W.F. Kenealy. (1986). In "Thermophiles: general, molecular and applied microbiology." T.D.Brock. ed., pp. 217-256. John Wiley and Sons. New York.

Oshima, T., and K. Imahori. (1971). Isolation of an extreme thermophile and thermostability of its transfer ribonucleic acid and ribosomes. J.Gen.Appl. Microbiol. 17: 513-517.

Pask-Huighes, R.A., and R.A.D. Williams. (1977). Yellow-pigmented strains of Thermus spp. from Icelandic Hot Springs.J.Gen. Microbiol. 102: 375-383.

Pitwell, L.R. (1971). Analysis of Ethiopian and other natural waters. Haile Sellassie I University. Addis Ababa, Ethiopia.

Ramaley, R.F. and K.Bitzinger. (1975). Types and distribution of obligate thermophilic bacteria in man-made and thermal gradient. Appl. Microbiol. 30: 152-155.

Ramaley, R.F. and J.Hixon. (1970). Isolation of a non-pigmented thermophilic bacterium similar to Thermus aquaticus. J. Bacteriol. 103: 627-528.

Saiki, T. R. Kimura, and K. Arima. (1972). Isolation and characterization of bacteria from hot springs. Agricul.Biol. Chem. 36: 2357-2366.

Smibert, R.M., and N.R.Krieg. (1981). Manual of Methods in General Bacteriology, American Society for Microbiology, Washington DC.

Sonnleitner, B. (1984). Biotechnology of thermophilic bacteria:

growth, products and application. Adv. Biochem. Eng.30: 70-138.

Sonnleitner, B., and A.Fiecher. (1983). Advantages of using thermophiles in biotechnological processes: Expectation and reality. Trend. Biotechnol. 1: 741-780.

Stanier, R.Y., M. Doudoroff, and E.A. Adelberg. (1970). The Microbial World, 3rd ed. Prentice-Hall Inc., Eaglewood Cliffs, NJ, USA.

Tansey, M.R., and T.D.Brock. (1972). The upper temperature limit for eukaryotic organisms. Proc. Natl. Acad.Sci.USA, 69: 2426-2428.

Tansey, M.R., and T.D. Brock. (1978). In " Microbial Life in Extreme Environments. " D.J.Kushner, ed., pp. 159-216. Academic Press, New York.

Taya, M., H. Hinoki, T. Yagi, and T. Kobayashi. (1988). Isolation and characterization of extremely thermophilic, cellulolytic, anaerobic bacterium. Appl.Microbiol.Biotechnol. 29: 474-479.

Weimer, P.J. (1986). In " Thermophiles: general, molecular and applied microbiology." T.D.Brock ed., pp 217-256. John Wiley and Sons. New York.

Williams, R.A.D. (1975). Caldoactive and thermophilic bacteria and their thermostable proteins. Sci. Prog. Oxford. 62: 373-393.

Zeikus, J. G. (1980). Thermophilic bacteria: ecology, physiology and technology. Enzyme. Microbiol. Technol. 1: 243-253.

Zinder, S. H. (1986). In "Thermophiles: general, molecular and applied microbiology." T.D.Brock, ed., 257-278. John Wiley and Sons. New York.

DECLARATION

I, the undersigned, declare that this thesis is my work and that all sources of materials used for this thesis have been duly acknowledged.

Name : MULUGETA KIBRET.

Signature : Mulugeta Kibret

date : 9 July 1991 .

This thesis has been submitted for examination with my approval as a University advisor.

Name : Dr. BERHANU A. GASHE.

Signature : Berhanu A. Gashe

Date : 9 July 1991 .