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ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF MICROORGANISMS FROM SELECTED HOT SPRINGS IN SRI LANKA

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ABSTRACT

Thermophilic microorganisms have attracted most of the researchers in past decades as demands for the thermostable enzymes are steadily increasing to replace thermolabile enzymes. The purpose of this study was to isolate and identify the thermophilic microorganisms from local hot springs to achieve the above goal. *Mahaoya* and *Wahawa* hot springs selected for the study in *Ampara* district have water temperature ranging from 43 ^{0}C-57 ^{0}C, pH 7.6-7.8 and conductivity 1289-1350 µS cm^{-1}. Samples of water and soil with cyanobacteria were collected under sterile conditions and water samples were inoculated at the sampling site into three different broth culture media Luria Bertani (LB), Medium 13 and Medium 74 maintained at temperatures close to the concerned well water temperatures. Cultures were transported to the laboratory in such a way that prevents the significant heat loss and incubated at 43 ^{0}C. Seven bacterial strains isolated from water samples were stained gram positive, rod shaped, spore formers. Bacterial isolates capable of growing above 55 ^{0}C in LB medium were selected for further studies and they were subjected to a biochemical analysis. Almost all four bacterial isolates (MO3/ISO9, MO5/ISO10, WA2/ISO11, and WA2/ ISO13) showed amylase, protease and catalase activities at 43 ^{0}C. The staining, morphological and biochemical studies concluded that four bacteria belong to Genus Bacillus. Possibly nine Cyanobacterial genera were identified from Mahaoya and Wahawa hot springs; *Schytonema, Lyngbya, Stigonema, Nostoc, Gleocapsa, Chroococcus, Coelosphaerium, Phormidium* and *Calothrix*. Temperature effect on α-amylase enzyme production was studied for four bacterial strains grown in starch broth at different temperatures (40 ^{0}C, 43 ^{0}C, 45 ^{0}C and 50 ^{0}C) using starch iodine method. Highest enzyme activity (48.763 U mL^{-1}) was observed in WA2/ISO13 grown at 40 ^{0}C for 24 hrs. Isolate WA2/ISO11 showed a remarkable α-amylase enzyme activity (21.155 U mL^{-1} – 45.925 U mL^{-1}) when grown at temperature ranges of 45-40 ^{0}C for 24hrs. Considerably higher enzyme activity (54.3 U mL^{-1}) was observed for α-amylase produced by WA2/ISO11 grown at 40 ^{0}C for 24hrs, at reaction temperatures of 60 ^{0}C after 30 min of incubation. Reduced enzyme activity was observed when reaction temperature decreased to 40 ^{0}C - 50 ^{0}C. The optimum growth temperature of isolate WA2/ISO11 was observed at 43 ^{0}C after 24 hrs in LB broth medium using cell biomass concentrations *via* dry weight measurements (0.613 g L^{-1}). Majority of the bacteria (MO3/ISO9, MO5/ISO10, WA2/ISO11, and WA2/ISO13) in *Mahaoya* and *Wahawa* hot spring water samples belongs to genus *Bacillus* and all were found to be capable of producing thermophilic α-amylase enzyme, some of which showed activity even at 60 ^{0}C.

Key words: *Hot springs, thermophile, biochemical tests, thermostable, enzyme activity, α-amylase*

INTRODUCTION

Environmental conditions on earth vary dramatically up to extreme, mainly the physical conditions such as temperature, pressure or radiation *etc.* A long time ago it was believed that most extreme environments are too hostile to support any life form. During last few decades, it is proved that they actually provide a natural habitat for certain microorganisms (Islas *et al.*, 2007). Microorganisms that have evolved strategies to such extreme conditions are termed as extremophiles. Such microorganisms are adopted to survive in ecological niches such as hot springs, deep-sea hydrothermal vents, sea ice, sub surface regions and sulfataric fields. Hot springs are defined as springs where the temperature of water lies significantly above the mean of annual air temperature of that region. It is produced from emerging ground water from earth's crust which is heated geothermally (Sen *et al.*, 2010). There are about nine recognized thermal springs in Sri Lanka distributed across the country.

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From the beginning of nineteenth century, existence of microorganisms in hot springs was reported and it includes the members of algae, bacteria and fungi (Farrel and Rose 1967). The ability of thermophiles to thrive in extremely hot environments clarifies that the bimolecular constituents in these microorganisms are designed to function at high temperatures. They are usually known as extremozymes, enzymes which can function even at extremely high temperatures where other enzymes denatured. Heat stable enzymes discovered from thermophiles received special attention from scientists as they are very important in biotechnology field (Malkawi and Al-Omari 2010).

Objective of this study is to isolate and identify thermophilic or thermotolerant microorganisms from Sri Lankan hot springs and to study the growth temperature and enzyme activity of such thermophilic bacteria.

METHODOLOGY

Sampling and Isolation

Mahaoya and *Wahawa* thermal springs were selected as sampling spots for this study. Thermal spring water samples were collected from, three wells of *Mahaoya* and three wells of *Wahawa* and they were inoculated into three different broth media LuriaBertani (LB-1.0% Bacto tryptone, 0.5% Bacto yeast, 1.0% NaCl), Medium13(0.1% yeast extract, 0.02% $(NH_4)_2SO_4$, 0.05% $MgSO_4.7H_2O$, 0.025% $CaCl_2.2H_2O$, 0.06% KH_2PO_4, 0.1% glucose. pH 7.0) and Medium74 (0.4% yeast extract, 0.8% polypeptone, 0.2% NaCl. pH 7.0) at the site itself under aseptic condition. Cyanobacteria grown along the wall of the hot springs were collected from *Mahaoya* and *Wahawa* hot springs into sterilized flasks. The thermal water temperature of each spring was measured. All the broth cultures were incubated at 43°C, and the cyanobacterial samples were placed near the window pane to get enough sunlight. Electrical conductivity and pH of collected thermal spring water samples were measured using EC and pH meter. Isolation of thermophilic bacteria was done in three different solid media namely LB agar, Medium13 agar and Medium74 agar and the plates were incubated at 43°C for 24 hrs.

Identification of Bacterial Isolates and Cyanobacteria

Seven different bacterial isolates were identified and they were labeled properly as follows: MO1/ISO8, MO3/ISO9, MO5/ISO10, WA2/ISO11, WA2/ISO12, WA2/ISO13, and WA3/ISO14. Out of the seven bacterial isolates, four were selected based on their ability to grow above 55°C namely, MO3/ISO9, MO5/ISO10, WA2/ISO11, and WA2/ISO13, for further studies. These four bacterial isolates were identified to their generic level according to their colony characteristics, gram properties, endospore production and standard biochemical characteristics. Cyanobacteria were identified based on their size, shape and morphological features using available keys (Abeywickrama *et al.*, 1986 and Deshikachary 1959).

Determination of Optimum Temperature for α Amylase Production

Selected four bacterial isolates (MO3/ISO9, MO5/ISO10, WA2/ISO11, and WA2/ISO13) were inoculated into flasks containing about 50 mL of starch broth medium. An un-inoculated starch broth was served as the control. Inoculated starch broths were incubated at 40°C in an incubated shaker at 100 rpm for 24 hrs. α-Amylase enzyme activity was determined by a modified method of Fuwa (Sohail *et al.*, 2005).Cell free culture supernatants were obtained by centrifuging 1.0 mL of broth cultures for 5 min at 3000 rpm. To determine the amylase enzyme activity 60 µL of crude enzyme was mixed with 200 µL of reaction mixture containing of 0.5M Sodium acetate buffer (pH 5.9) and 0.5% starch solution. Then the mixture was incubated at 40°C for 30 min. The reaction was stopped after incubation period by adding 200 µL of 1M acetic acid and the volume of the solution was made up to 10 mL using sterile distilled water and the spectrophotometer readings were recorded at 660nm after adding 200 µL of iodine solution. Separate blanks were used for each and every bacterial culture. The same volume of distilled water was used for blanks instead of 0.5% starch solution.

The enzyme activity was calculated as relative activity based on the following equation (Bautista *et al.*, 1978):

$$\text{Unit Enzyme Activity} = \frac{(OD_{control} - OD_{test})}{OD_{control}} \times 100 \times \frac{1}{t}$$

t- Incubation time (min).

One unit of enzyme activity was defined as the quantity of enzyme able to hydrolyze 0.1 mg of starch per minute under standard assay conditions (Sohail *et al.*, 2005). Then, the average unit of enzyme activity per mL (U/mL) of crude enzyme was determined. Above procedure was repeated at different growth temperatures such as 43°C, 45°C and 50°C and their enzyme activities were calculated as described above.

Effect of Reaction Temperature in α Amylase Enzyme Activity

Effect of reaction temperature in α Amylase enzyme activity was studied for the crude enzyme obtained from WA2/ISO11 bacterial isolate as it showed an indicative α-amylase enzyme activity at ranges of growth temperature compared to other isolates. Cell free culture supernatant was obtained from starch broth cultures of WA2/ISO11 grown at 40 °C for 24 hrs. The unit enzyme activity was determined after incubating the reaction mixture at different temperatures (40°C, 50°C & 60°C), as described above.

Determination of Optimum Temperature for the Growth of WA2/ISO11

Fresh culture of WA2/ISO11 was inoculated into three flasks containing 100 mL of Luria Bertani medium. The flasks were incubated at different temperatures 40°C, 43°C and 45°C for 24 hrs. Optimum temperature for the growth of WA2/ISO11 was determined by the measurements of cell biomass concentration as grams of dry weight per liter using averages of values obtained at different temperatures (Wang 1996).

RESULTS AND DISCUSSION

The temperature of these two hot springs ranges from 43°C-57°C. Their pH ranges from 7.6-7.8, are somewhat closed to neutral and their conductivities ranges from 1289-1350 µS/cm, are higher than that of pure water (EC of pure water is 0.055 µS/cm). The conductivity of *Wahawa* well No.03 (212 µS/cm) out lays this range. Electrical conductivity is estimated by the total amount of dissolved inorganic solids in water. It is generally affected by water temperature; warmer the water higher the conductivity. Very low conductivity of *Wahwa* well No. 03 may be due to the low levels of dissolved solids as it is a still emerging thermal spring.

Table 1: Temperature, pH and conductivity of water in *Mahaoya* hot springs

Well No.	Temperature/°C	Special note	pH (30.3°C)	Conductivity/ µS/cm
1	55	Erupt bubbles	7.81	1343
3	57	Erupt bubbles	7.72	1299
5	55	Erupt bubbles	7.72	1350

Bacterial growth above temperatures of 55°C, suggested that the selected four bacterial isolates (MO3/ISO9, MO5/ISO10, WA2/ISO11 and WA2/ISO13) are thermophiles and capable of producing enzymes such as amylase, protease and catalase. As these enzymes are produced by organisms capable of growing at higher temperatures, they were assumed as thermostable enzymes which are active even at high temperatures.

Table 2: Temperature, pH and conductivity of water in *Wahawa* hot springs

Well No.	Temperature /^{o}C	Special note	pH (30.3^{o}C)	Conductivity/µS/cm
1	45	The flowing artesial well	7.61	1253
2	47	Built in the form of tanks	7.58	1289
3	47	Originating spring in a paddy field	7.63	212

These four bacterial strains were identified as Gram positive, rod shaped spore formers *via* Gram stain and spore stain procedures (Table 3). Most of the characters of these selected bacterial strains such as morphological, staining and biochemical characters were similar to genus *Bacillus*. Therefore, these strains were confirmed as species of *Bacillus*.

In the present study, nine cyanobacterial genera were identified from *Mahaoya* and *Wahawa* hot springs namely, *Schytonema, Lyngbya, Stigonema, Nostoc, Gleocapsa, Chroococcus, Coelosphaerium, Phormidium* and *Calothrix*. A study conducted by Wanigatunge *et al.* (2012), on microbial diversity of thermal springs using 16S rRNA analysis, revealed the presence of *Burkholderia* a proteobacteria from *Mahaoya* and *Wahawa*, archaea from *Nelum-wewa* and *Wahawa* and eubacteria and cyanobacteria such as *Chroococcidiopsis, Oscillatoria, Calothrix, Leptolyngbya* and *Xenococcus*in from *Kanniya, Nelum-wewa, Mahaoya, Wahawa, Kapurella* and *Rangiriulpotha*.

Table 3: Summary of characteristics of selected bacterial isolates from *Mahaoya* and *Wahawa* hot springs

Organisms	Fermentation			H_2S Production	Indole Production	MR Reaction	VP Reaction	Citrate Use	Urease Activity	Catalase Activity	Gelatin Liquefaction	Starch Hydrolysis	Casein Hydrolysis	Gram's stain	Sporulation
	Lactose	Dextrose	Maltose												
MO3/ISO 9	-	A	A	-	-	-	+	-	-	+	-	+	+	Rod +	+
MO5/ISO 10	-	A	-	-	-	-	+	-	-	+	-	+	+	Rod +	+
WA2/ISO 11	-	A	-	-	-	-	+	-	-	+	+	+	+	Rod +	+
WA2/ISO 13	-	A	-	+	-	-	+	-	-	+	-	+	+	Rod +	+
Bacillus sp.	-	**A**	-	-	-	-	+	-	-	+	+	+	+	**Rod** +	+

A-Acid formation, (-) – Negative, (+) – Positive

Determination of Optimum Temperature for α-Amylase Production

Bacterial isolate WA2/ISO13 showed the highest enzyme production at growth temperatures of 40^{0}C and when the growth temperature was increased, the enzyme production gradually decreased. According to unit enzyme activities obtained for four bacterial isolates at different growth temperatures, isolate WA2/ISO11 showed high rate of enzyme production at different temperature ranges compared to other three isolates (Figure 1).

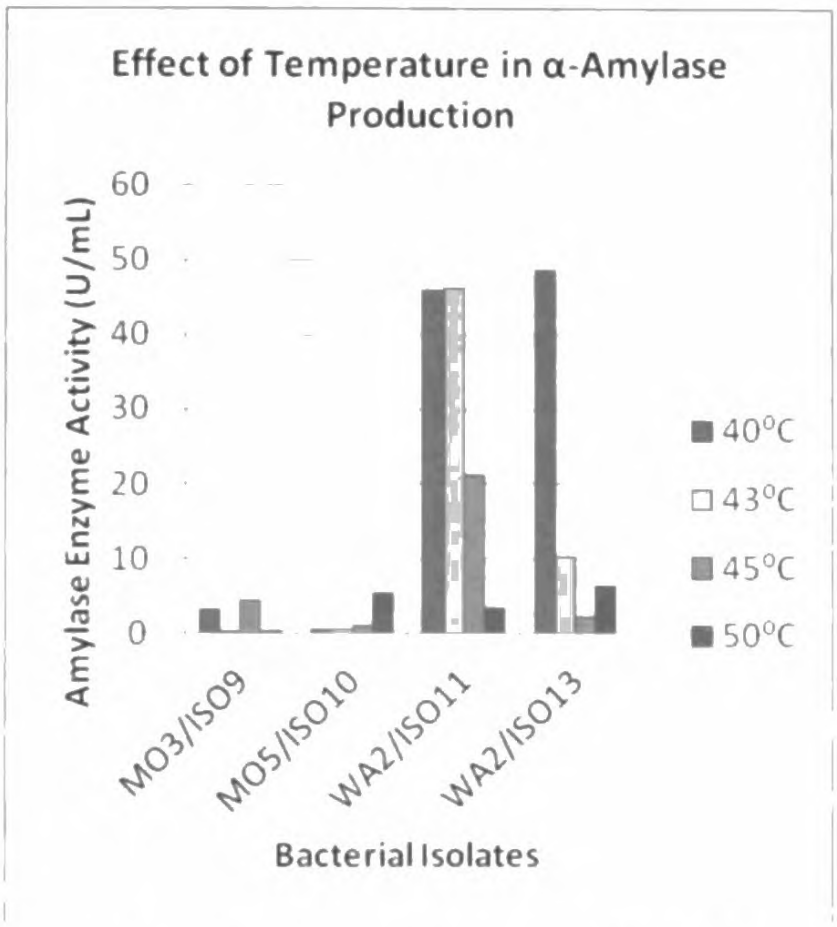
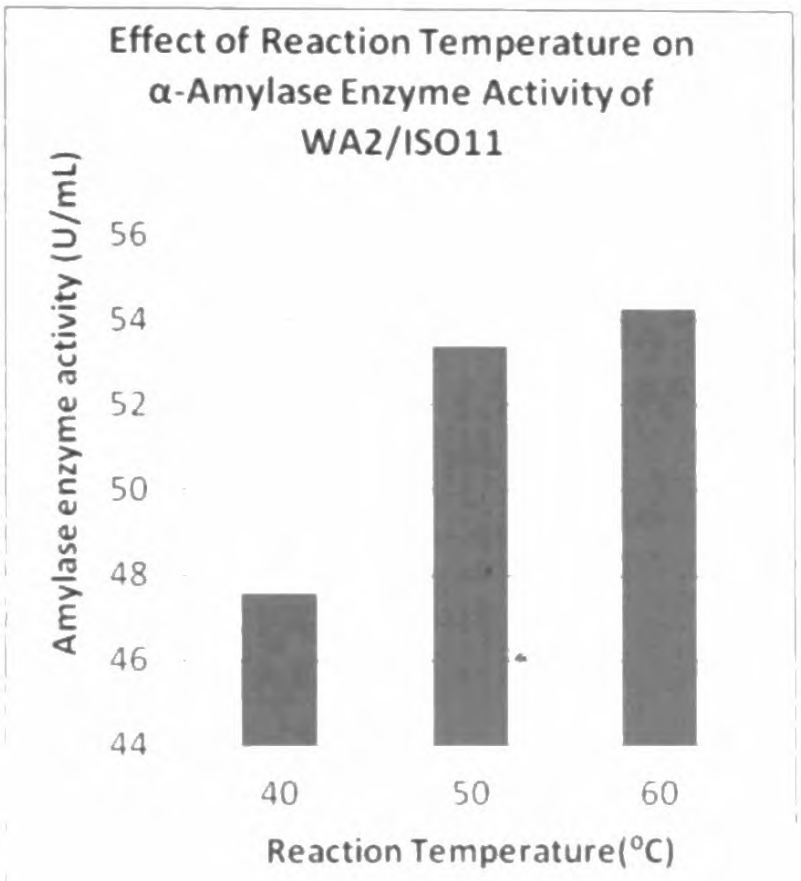


Figure 1: α-Amylase enzyme activities of four bacterial isolates grown at different temperatures.



Effect of Reaction Temperature on A - Amylase Enzyme Activity of WA2/ISO11

Thermostable enzymes have received the attention of most of the researchers in recent years as plenty of industrial enzymatic processes operate at high temperatures. Amylases are starch degrading enzymes and they have numerous applications in various fields including industries, analytical, pharmaceutical and clinical applications (Sohail *et al.*, 2005). As isolate WA2/ISO11 is capable of producing α-amylase enzyme at a range of temperatures 40^{0}C-45^{0}C (Figure 1), during the industrial production, if there is any fluctuation in the system temperature within the range, it may cause only a minor effect in enzyme production. Although the observed enzyme production of isolates MO3/ISO9 and MO5/ISO10 were comparatively lower than other two isolates, the isolate MO5/ISO10 showed a gradual increase in enzyme activity at very low level (0.507 U/mL – 5.517 U/mL) when the growth temperature is increased (Figure 1). Further increase in temperature may increase the enzyme production rate. However, the requirement of high temperature for industrial enzyme production is not an advantage as it costs energy. Most constrains in enzyme production can be overcome using molecular microbiology. The gene encodes for thermozymes can be cloned and expressed in mesophiles such as *Escherichia coli* (Gomes and Steiner 2004). As unique thermal properties are encoded in genes, it is possible to express them in mesophilic host organisms (Sellek and Chaudhuri 1999).

Effect of reaction temperature on α -Amylase enzyme activity

The crude α amylase enzyme obtained from WA2/ISO11 showed higher enzyme activity at 60^{0}C. The enzyme activity decreased gradually when the temperature was decreased (Figure 2). Bacterial isolate WA2/ ISO11 showed relatively high activity (54.3 U/mL) even at high temperature 60^{0}C, indicating that α-amylase

produced by particular isolate is thermostable. As the temperatures higher than 60^{0}C was not used during the present study the maximum temperature for α-amylase enzyme activity was not determined. However, thermostable amylase enzyme produced by the bacterial isolate WA2/ISO11 may be able to replace thermolabile α-amylases used in various industrial processes. A study conducted by Sohail *et al.* (2005) found that the maximum enzyme activity of bacterial origin α-amylase at 60^{0}C, supports the results obtained during the present study, and some of the α-amylases found to be remain active even at 80^{0}C which was not determined during the present study. A separate study conducted by Cordeiro *et al.* (2002) revealed that a thermophilic *Bacillus* sp. Strain SMIA-2 has optimum α-amylase activity at 70^{0}C. A recent study carried out by Mathew *et al.* (2012) found that *Bacillus licheniformis* isolated from *Nelum-wewa* hot water spring produced an extracellular amylase enzyme that has the maximum activity (4300 U/L) at 50^{0}C and their activity was reduced when the temperature was increased up to 60^{0}C-70^{0}C. But, amylase enzyme of WA2/ ISO11 showed higher activity at 60^{0}C.

Determination of the optimum temperature for the growth of WA2/ISO11

The optimum growth (0.613 g/L) of bacterial isolate WA2/ISO11 was observed at 43^{0}C (Figure 3). The optimum temperature for α-amylase enzyme production can be correlated with the optimum temperature of its growth, as both were observed at 43^{0}C. High amount of enzyme production (46.262 U/mL) at 43^{0}C may be due to the optimum growth of bacteria. A study conducted by Fooladi and Sajjadian (2010) reported that the effect of temperature on α-amylase enzyme production can be related to the growth of particular organism. But, it is not a norm that optimum growth temperature of all α-amylase producing strains induces the optimum enzyme production always. In a study conducted by Windish and Mhatre (1965) it is reported that there is an inverse ratio between the growth and α-amylase production of *Bacillus stearothermophilus*.

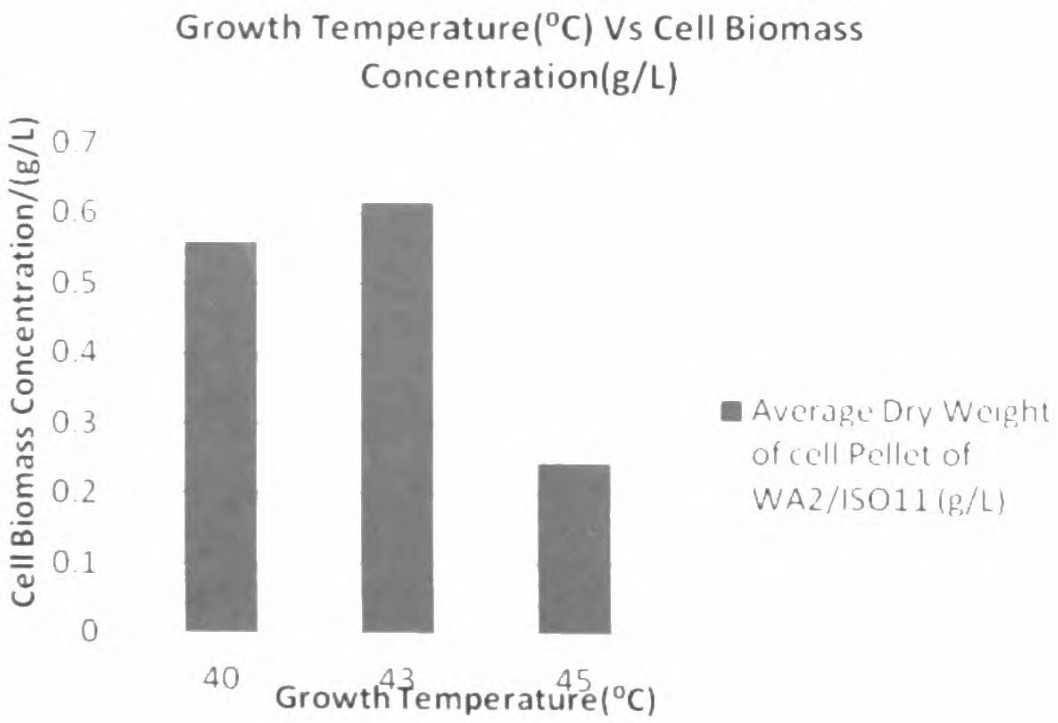


Figure 3: Average cell biomass concentration of WA2/ISO11 at different growth temperatures

Optical density (OD) measurement is the most often used method in determination of bacterial cell growth (Wang 1996). However, selected four thermophiles were not capable of producing a homogeneous culture broth. Flocculation of bacterial cells was observed within 24 hrs of inoculation resulting in a non homogeneous culture. Therefore, it was impossible to measure OD values using a spectrophotometer. It was observed that the growth of bacteria changed the pH of the medium in to alkaline range. Therefore, several drops of diluted acid were added into the sample culture broth in order to dissolve the flocks of bacterial cells. But, it was not successful even at increased acidic concentrations to remove flocculation of bacterial cells.

CONCLUSIONS AND RECOMMENDATIONS

Majority of bacteria in *Mahaoya* and *Wahawa* hot spring water samples belong to genus *Bacillus. Schytonema, Lyngbya, Stigonema, Nostoc, Gleocapsa, Chroococcus, Coelosphaerium, Phormidium* and *Calothrix* are the nine cyanobacterial genera identified from hot spring samples of *Mahaoya* and *Wahawa.* Bacterial isolates MO3/ISO9, MO5/ISO10, WA2/ISO11 and WA2/ISO13 were found to be thermophilic and some were capable of producing thermostable enzymes. The α-amylase enzyme production by isolate WA2/ ISO11 was found to be optimum at its optimum growth temperature 43 ^{0}C. Crude α-amylase enzyme of WA2/ISO11 was found to be active even at 60 ^{0}C and the activity decreased with decrease in temperature.

There are disputed conceptions about the promising applications of thermophiles and their enzymes in various fields. Due to the complications in the manufacturing processes only a small extent of thermostable enzymes enters into the current market. However, it opens a wide avenue to investigate the potentials of thermophilic bacteria and cyanobacteria for their ability to produce other products with vital biotechnological importance.

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