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THERMOPHILIC BACTERIA FROM THE HOT SPRINGS OF GILGIT (PAKISTAN)

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ABSTRACT

The importance of thermostable biomolecules in the field of biotechnology has spurred research into organisms capable of growth at high temperatures. Three thermophilic bacterial strains GCTP-1, GCM_{B}-1 and GCDP-1 were isolated from the hot springs of Tatta Pani, Murtazabad & Darkut Pass respectively in the surroundings of Gilgit. All isolates have entire and slimy colonies while the cells were small rods, gram-negative non-motile and semi aerobic. Strains GCTP-1 showed positive results of ortho nitrophenyl-β-D-galactopyranosidase (ONPG) and gelatin hydrolysis (GEL) tests other isolates gave negative results in all tests such as ortho nitrophenyl-βD-galactopyranosidase, arginine dihydrolases, lysine decarboxlase, ornithine decarboxylases, citrate utilization, H_2S production, urease, tryptophan deaminases, indole production, acetoin production, gelatin hydrolysis, Fermentation/oxidation (glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose) and cytochrome oxidase. All isolates were grown well at pH range 7–9, with optimum pH 7.2. However, isolates were highly thermophilic and showed optimum growth temperature 65-70°C

Key words: Thermophiles, Pakistan, Bacterial strains, hot springs.

INTRODUCTION

Organisms often live in places where seem to be unlivable. Thermophiles are heat-loving, with an optimum growth temperature of 70°C or more, but these are only approximate. Some hyperthermophiles require a very high temperature 80°C to 105°C for growth (Brock and Freeze, 1969; Hamilton-Brehm *et al.,* 2005). Hyperthermophiles were first discovered in the 1960s from hot springs in Yellowstone National Park. Since then, more than fifty species have been discovered. The most extreme/hyper-thermophiles thus known live on the superheated walls of deep-sea hydrothermal vents, requiring temperatures of at least 90°C for survival (Zierenberg *et al.,* 2000). The most heat tolerant hyperthermophile that was recently discovered has been able to double its population during 24 hours in an autoclave at 121°C. However, it is thought unlikely that microbes could survive at temperatures above 150°C, as the cohesion of DNA and other vital molecules begins to break down at this point (*Madigan and Martinko, 2005).*

For instance, thermophilic bacteria live in and around hot springs and deep-sea hydrothermal vents where temperatures can exceed 100ºC (Brock, 1970). It's surprising that how they can live in an environment with such high temperatures and what factors determine the temperature limit for life? Thermophilic bacteria are a key to the birth of living organisms in the primordial earth (Kimura *et al.,* 2006). As thermophiles grow at high temperature, so they must contain metabolites that can function at high temperature. The enzymes isolated from some extremophiles have proven to be of great use in the modern fields of biological sciences (for example heat stable DNA polymerases for polymerase chain reaction), medicine and in surfactants as they are able to do work under such conditions that would denature enzymes taken from most "normal" organisms (Mattila *et al*., 1991).

The importance of thermostable biomolecules in the growing field of biotechnology has spurred research into organisms capable of growth at high temperatures. During the last two decades, many reports have described the isolation of novel thermophilic organisms from both the domains archaea and bacteria. Thermophiles belonging to the bacterial domain have received attention for their potential in the bioconversion of substrates of plant origin to end products such as lactate and ethanol, compounds with potential for the production of bulk chemicals and fuels (Wiegel and Ljungdahl, 1981; Jones *et al*., 1983; Schink and Zeikus, 1983; Fiala and Stetter, 1986; Freier *et al*., 1988; Cayol *et al*., 1995; Cook *et al*., 1996; Engle *et al*., 1996; Liu *et al*., 1996).

Pakistan is situated between latitude 24º to 37º North and longitude 62º to 75º East. It lies over the junctions of the tectonic plates of the sub-continent and is rich in geothermal resources. Major tectonic elements during the Cenozoic and Mesozoic era have shaped the Geological structures that observed in Pakistan today. Tectonic movements along these plates give rise to localization of hot springs. A large number of hot springs are scattered along the Main Mantle Thrust and Main Karakoram Thrust in Chilas and Hunza areas respectively and their surface water temperature ranges up to 96°C. Keep in mind the biotechnological importance of present study was initiated to isolate thermophilic bacteria for their exploitation as a source of potential agents.

MATERIALS AND METHODS

Water samples were collected in aseptic culture tubes containing 1.0% nutrient broth medium at different distances from the source (where the water oozes out) of the Hot springs selected on the base of geological survey of Gilgit. Serial dilution method was used to isolate thermophilic bacteria in 1.0% LB medium supplemented with sterile water of the hot springs (which contain all the naturally occurring salts) for aerobic isolates and YE medium used for anaerobic bacteria according to Clark *et al*. (1958) and Takahata *et al.* (2000).

Temperature of the water was measured on the spot where it oozes out, while pH & EC was measured when temperature was cooled down up to 25^{o}C. All other characterization/analysis was made in the laboratories (Allen, 1974). Different morphological, physiological and biochemical tests like Gram's staining, colony morphology, motility test, oxidation fermentation test and QTS-20 Test were studied according to Wiegel and Ljungdahl (1981) and MacFaddin (2000).

The temperature ranges for growth were determined at varied incubation in 40, 50, 60, 70, 80, 90 and 100^{o}C. The pH range for growth was studied by growing them overnight (16 hours) at 60±1^{o}C in LB medium adjusted to different pH (4, 5, 6, 7, 8, 9 and 10) separately (Anton *et al*., 2002). Optical density was monitored at 600nm on a double beam UV/VIS scanning spectrophotometer (Model: CECIL-7200 series, UK).

RESULTS AND DISCUSSION

A large number of hot springs occur in the Gilgit, Hunza and Yasin valleys in the Northern areas of Pakistan. The Tatta Pani (TP) hot springs are located on the Karakoram Highway at the right bank of the Indus River. The springs & seepages are numerous, and stretch along wide zone located one or two Km near Rai Kot bridge, approximately 80 Km SW from the Gilgit town. These springs are located at the altitude of 1200m. There are two hot springs in Murtazabad (M), located in the Hunza valley, downward near the bank of the Khunjerab River, Murtazabad Zairen (MZ) and Murtazabad Balai (MB) hot springs. Murtazabad Balai hot spring is located somewhat upper as compared to the Murtazabad Zairen hot spring. Other hot springs are located 3.0 km earlier from Darkut Pass in Yasin valley upper to the Rawat base camp. It is situated at the height of about 4650m from the sea level. Two hot springs are oozing out here, which seem to have the same origin

Shuja (1986) and Bakht (2000) have also found numerous hot springs along the Main Mantle Thrust and Main Karakoram Thrust in Chilas and Hunza areas respectively. The geothermal system here is the result of the collision of the Indian and Eurasian plates. Hot springs are scattered and their temperature ranges up to 91°C. Three parts of Pakistan i.e. Kashmir, North West Frontier Province and Balochistan are the potential zones where geothermal resources are located. Major tectonic elements during the Cenozoic and Mesozoic era have shaped the Geological structures that are observed in Pakistan today. These structural elements are indicators for delineating and developing the potential geothermal resources of the country. The zone of deformation extends from the Makran region in the southwest, to the Hazara-Kashmir syntaxial bend in the north. These features are produced by interaction of the Indian, Arabian and Eurasian Plates. Tectonic movements along these plates give rise to localization of hot springs.

Three thermophilic bacterial strains GCTP-1, GCM_{B}-1 and GCDP-1 were isolated from the hot springs (Tatta Pani, Murtazabad & Darkut Pass respectively) of the Gilgit. Water analysis was done to check the chemical characteristics of the hot springs water. All the hot springs studied, had alkaline pH and temperature higher than 80^{o}C (Tatta Pani, Mutazabad Balai and Murtazabad Zairen) while Darkut Pass hot spring had 62^{o}C at the source (Table I **).** All isolates have entire and slimy colonies while the cells were small rods, gram-negative non-motile and semi aerobic. Strains GCTP-1 showed positive results of ortho Nitrophenyl-β-D-Galactopyranosidase (ONPG) and Gelatin hydrolysis (GEL) tests while other isolates gave negative results in all tests such as Ortho Nitrophenyl-β-D-Galactopyranosidase, Arginine Dihydrolases, Lysine Decarboxlase, Ornithine Decarboxylases, Citrate utilization, H_2S Production, Urease, Tryptophan Deaminases, Indole production, Acetoin production, Gelatin Hydrolysis, Fermentation/oxidation (Glucose, Mannitol, Inositol, Sorbitol, Rhamnose, Sucrose, Melibiose, Amygdalin, Arabinose) and Cytochrome Oxidase. All isolates grew well at pH range 7–9, with optimum pH 7.2 (Figure-1 a-c) However, isolates were highly thermophilic and showed optimum growth temperature 65- 70^{o}C (Figure-2a-c). These characteristics showed that the isolates have a good resemblance with the habitat environment (Table I). Brock and Freeze, (1969) also isolated and described thermophilic bacterium *Thermus aquaticus*, which had growth temperature range 70°C to 75°C. The organism was an obligate aerobe and had a pH optimum of 7.5 to 7.8. Isolates of *T. aquaticus* were gram-negative, non-sporulating, non-motile rods, which frequently form long filaments at supra-optimal temperatures or in the stationary phase. All isolates formed a yellow cellular pigment, probably a carotenoid. Antoine *et al.,* (1997) isolated *Bathymodiolus brevior* resembled with *Thermosipho africanus* which was gram-negative and rod shaped and generally occurred singly or in pairs, rarely occurring as chains with a maximum of five rods, at the optimum temperature for growth 70°C and optimum pH 6.5.

Table I: Physical and chemical properties of the water samples

Type of Study		Samples			
		TP	M_B	M_Z	DP
Ca^{+2} meq/l		1.4	0.7	0.5	0.67
Mg^{+2} meq/l		1.2	3.56	2.13	0.96
Na^{+} ppm		223	355	178	142
K^{+} ppm		4	108	6	3
CO_3^{-2} meq/l		4.2	9.3	3.1	3.6
HCO_3^{-} meq/l		16.5	99	14.7	11.4
Cl meq/l		0.81	0.97	1.02	0.86
TDS (g)		0.026	0.083	0.017	0.011
S.A.R.		195.61	243.15	154.78	157.25
Temperature (°C)	Upper	85	91	89	62
	Lower	63	77	64	44
EC ms		1.15	1.84	2.56	1.016
pH		7.9	8	8.14	7.18

M_Z = Murtazabad Zareen; **M_B** = Murtazabad Balai; **DP** = Darkut Pass
Temperature upper = Temperature of hot spring water at the source
Temperature Lower = Temperature of hot spring water at 10 feet away from the source

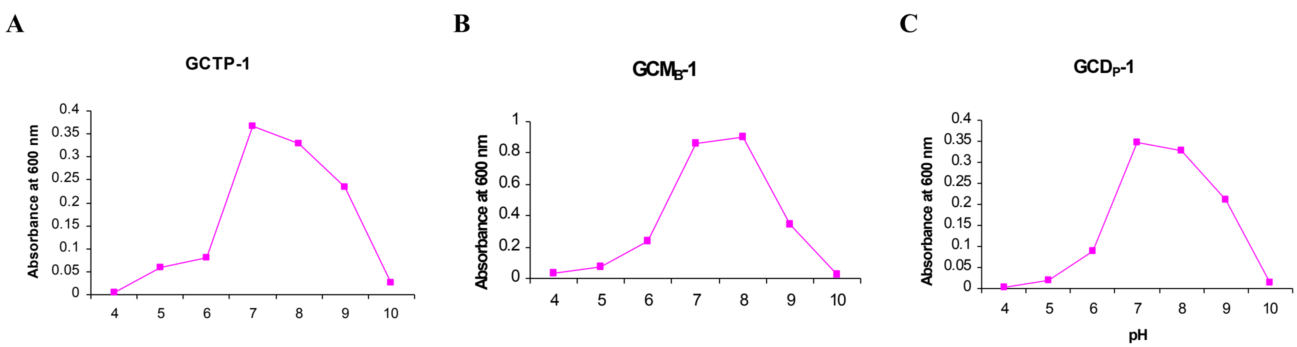

Figure 1 (a-c): Effect of pH on the growth of thermophilic bacterial isolates

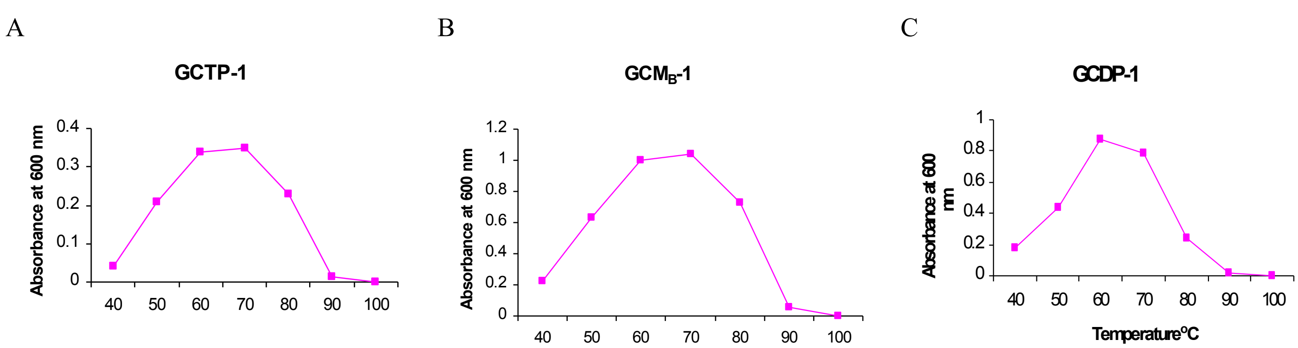

Figure 2 (a–c): Effect of temperature on the growth of thermophilic bacterial isolates.

As thermophiles grow at high temperature, so they must contain metabolites that can function at high temperature. The enzymes isolated from some extremophiles have proven to be of great use in the modern fields of biological sciences (for example heat stable DNA polymerases for polymerase chain reaction), medicine and in surfactants as they are able to do work under such conditions that would denature enzymes taken from most "normal" organisms (Mattila *et al*., 1991). These isolates can be exploited in various useful processes after studying and cloning their gene. Further study can also help to draw evolutionary links with modern organisms.

Based on the morphological, biochemical and physiological chahracteristics and comparison with the characteristics given in Bergy's manual, all the strains showed similarities with *Thermus aquaticus*, though the full taxonomic description is pending which will be finalized after scanning electron microscopy and comparison of the base sequences of the ribosomal RNA.

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