ABSTRACT

The present study is concerned with the isolation of thermoduric microflora from the hot springs of Azad Kashmir. A total of seventy two water samples mixed with some mud were collected from nine hot springs in Tatta Pani (Azad Kashmir) at four different time intervals depending upon seasonal variation, and were analyzed *in-situ* and *ex-situ* in terms of pH, temperature, soluble anions and cations, biological oxygen demand, chemical oxygen demand and electric conductivity. The temperature and pH of Tatta Pani hot springs (Azad Kashmir) ranged from 38-110°C and 6.82-7.18, respectively. Thirty seven pure cultures were isolated from the said hot springs. Based on morphological, physiological and biochemical characterization, the isolates were divided into seven groups. One representative isolate from each group was further subjected to molecular characterization. All isolates showed thermoduricity, TP-1, TP-2, TP-3, TP-4 and TP-5 isolates tolerated 100°C for 30 min, 105°C for 20 min and 110°C for 10 min while isolates TP-33 and TP-37 tolerated 110°C for 30 min and 115°C for 10 min.

Isolate TP-1 was facultative anaerobic bacterium that formed pale yellow, round, smooth, flat and slimy colonies while the cells were Gram positive rods, about 3.5-5.0 μm in length to 0.6-0.7 μm in width and were motile. It showed growth within the temperature range of 35-80°C with optimum growth observed at 65°C. It grew within the pH range of 5.5-8.5 with optimal growth observed at pH 7.0. It tolerated NaCl within the range of 0-4.5% (w/v) with optimum growth observed at 1%. It showed growth on maltose, fructose, lactose, starch, xylan and CMC used as sole carbon source. It was oxidase and catalase positive and gave positive tests for *o*-nitro phenyl β-D-galactopyranoside, gelatin hydrolysis and produced acid from maltose. Almost complete 16S rRNA gene sequence analysis showed that it had 97% similarity with *Geobacillus pallidus*.

TP-2 isolate was aerobic, Gram positive, motile, rod shaped bacterium that ranged in size from about 2.1-3.6 μm to 0.2-0.3 μm in width. It formed cream colored, round, smooth, flat and slimy colonies. The temperature and pH range for growth was found to be 45-75°C and 5.5-8.5, respectively with optimum growth observed at 65°C and pH 7.0. It showed growth within the NaCl concentration of 0-3.5% (w/v) with optimal growth

observed at 0.5%. It was capable of growing on CMC, lactose, sucrose, starch, glucose, maltose, xylan, fructose and filter paper used as sole carbon source. It was catalase and oxidase positive and gave positive test for *o*-nitro phenyl β-D-galactopyranoside, gelatin hydrolysis and nitrate reduction and produced acid from glucose, maltose and sucrose. Based on 16S rRNA gene sequence analysis, isolate TP-2 gave low level of similarity (89%) with *Geobacillus debilis*.

Isolates TP-3 and TP-4 were facultative anaerobic, Gram positive, catalase and oxidase negative, motile, rod shaped bacteria that showed growth within the temperature range and pH range of 45-75°C and 5.5-9.0, respectively with optimal growth observed at 70°C and pH 7.0. Isolates TP-3 and TP-4 showed optimal growth at 1.5% and 1.0% NaCl concentration, respectively and produced acid from maltose, sucrose and mannose while isolate TP-3 produced acid from glucose also. Isolate TP-3 utilized glucose, maltose, fructose, lactose, sucrose, starch, CMC, wheat bran extract and filter paper for growth while isolate TP-4 showed growth on maltose, fructose, lactose, sucrose, starch, wheat bran extract, xylan and CMC. 16S rRNA gene sequences showed that isolates TP-3 and TP-4 displayed 94% and 96% similarity, respectively with *Geobacillus vulcani*.

Isolate TP-5 was facultative anaerobic, motile, Gram positive, catalase positive, oxidase negative, rod shaped bacterium, 2.7-3.8 µm in length to about 0.6-0.7 µm in width. It formed whitish, round, smooth, convex and slimy colonies. It grew optimally at 70°C and pH 7.0 and tolerated NaCl concentration of 0-4% (w/v) with optimum growth observed at 0.5%. Isolate TP-5 utilized all the carbon sources (glucose, maltose, fructose, lactose, sucrose, starch, xylan, wheat bran extract, CMC and filter paper) for growth. It gave positive results for gelatin hydrolysis and nitrate reduction and produced acid from glucose, maltose, sucrose and mannose. 16S rRNA gene sequence analysis displayed 94% similarity with *Geobacillus stearothermophilus*.

Based on phenotypic (morphological, physiological and biochemical) and genotypic (16S rRNA gene sequence analysis) characterization, and taking phylogenetic analysis into consideration, it was concluded that isolates TP-1, TP-3, TP-4 and TP-5 belonged to the genus *Geobacillus* while isolate TP-2 was quite distinct in its characters from the known *Geobacillus* species as well as to other established genra, so it may

represent a novel strain. Further, intracellular protein profiling of bacterial isolates using SDS-PAGE analysis with the type strain of *Geobacillus pallidus* displayed that intracellular protein pattern of isolate TP-1 was most closely related to the intracellular protein pattern of *Geobacillus pallidus* ATCC 51176 (type strain), isolates TP-3, TP-4 and TP-5 displayed intermediate level of differences in protein pattern in comparison to that of type strain while the intracellular protein pattern of isolate TP-2 was most distant as compared to type strain.

Isolates TP-33 and TP-37 were anaerobic archaea that formed off-white, round colonies. The cells were Gram negative cocci having diameter of 0.7-1.5 and 0.7-1.7 µm for isolates TP-33 and TP-37, respectively. Isolates TP-33 and TP-37 grew optimally at 80°C and 75°C, respectively and at pH 7.0. The optimal NaCl concentration for growth was determined to be 0.5% and 0.3% for isolates TP-33 and TP-37, respectively. Isolates TP-33 and TP-37 grew on complex proteinaceous substrates i.e, peptone, tryptone and yeast extract while were unable to grow in the absence of cystine. Isolate TP-33 utilized maltose and starch for growth while isolate TP-37 was unable to grow on any of the carbon sources tested in the absence of proteinaceous substrates but grew on xylan and glucose in the presence of 0.1% peptone. 16S rRNA gene sequence analysis showed that isolates TP-33 and TP-37 displayed 97% and 95% similarity with *Thermococcus waiotapuensis* and *Thermococcus zilligii,* respectively. Based on morphological, physiological and molecular characterization as well as phylogenetic analysis it was found that isolates TP-33 and TP-37 belonged to the genus *Thermococcus*.

Isolate TP-1 produced extracellular α-amylase, CMCase, xylanase, lipase and protease, isolate TP-2 produced extracellular α-amylase, CMCase, FPase, xylanase, lipase and protease, isolate TP-3 gave extracellular activities for α-amylase, CMCase, FPase, lipase, protease and phytase enzymes, isolate TP-4 gave extracellular activities for α-amylase, CMCase, lipase, protease and phytase and isolate TP-5 gave extracellular activities for α-amylase, CMCase, FPase, xylanase, lipase and protease. Intracellular CMCase activity was recorded for isolates TP-1, TP-2, TP-3, TP-4 and TP-5 while intracellular FPase activity was observed for isolates TP-2, TP-3 and TP-5. Isolate TP-33 gave positive result for extracellular α-amylase while isolate TP-37 gave positive result

for extracellular xylanase and protease. Maximum α-amylase activity (0.993 U/ml/min) was given by isolate TP-5, isolate TP-1 gave maximum production of extracellular CMCase (0.091 U/ml/min), intracellular CMCase (0.025 U/g/min), extracellular xylanase (0.587 U/ml/min), extracellular lipase (0.23 U/ml/min) and extracellular protease (0.314 U/ml/min). Maximum extracellular FPase activity (0.021 U/ml/min) was given by isolate TP-5 while isolate TP-3 gave maximum production of intracellular FPase (0.009 U/g/min) and extracellular phytase (0.023 U/ml/min).