The Journal of Animal & Plant Sciences, 27(6): 2017, Page: 2056-2062
ISSN: 1018-7081

ISOLATION AND IDENTIFICATION OF A CATALASE PRODUCING THERMODURIC ALKALOTOLERANT *BACILLUS* SP. STRAIN KP10 FROM HOT SPRINGS OF TATTA PANI, AZAD KASHMIR

N. Erum[1], Z. Mushtaq[1*] and A. Jamil[1]

[1]Molecular Biochemistry Lab, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.
Corresponding author's email: zahidmushtaquaf@uaf.edu.pk

ABSTRACT

A remarkable diversity of extremophilic bacteria has been reported which has significant applications in biotechnology, environment, industry and medicine. Hot springs are good example of naturally occurring extremophilic environment. In our research, a catalase producing thermoduric alkalotolerant bacterial strain was isolated from the hot springs of Tatta Pani, Poonch, Azad Kashmir where microbial flora is less explored. The thermoduric isolate was screened on basis of its pH tolerance. It was characterized biochemically and identified on basis of the 16S rRNA gene sequence. The cells of the isolate were aerobic, gram positive rods. The isolate was observed to grow at pH up to 11.0 and 65 °C whereas optimum conditions of pH and temperature were 10.0 and 50 °C, respectively. The isolate was oxidase and coagulase negative, non-motile and gave negative results for H_2S production and citrate utilization. The catalase activity of the isolate was determined using its crude enzyme extract which was maximum at pH 10.0 and 45 °C. The isolate showed maximum similarity with *Bacillus clausi* (~88% homology). The 16S rRNA gene sequence of the isolate, named as strain KP10, had been submitted in GenBank and accessioned as KX013388.

Keywords: Alkalophiles, *Bacillus,* hot springs, catalase, 16S rRNA gene.

INTRODUCTION

Extremophiles are organisms which can survive in extreme environmental conditions of temperature, pressure, pH, high metal and salt concentration, radiation, etc. (Morozkina *et al.,* 2010). A significant portion of this extremophile domain is covered by bacteria although the vast majority belongs to kingdom Archaea. Bacteria can be found in all possible locations on earth which include environments with conditions from normal to extreme (Rothschild *et al.,* 2001). The ability of extremophilic bacteria to thrive and live in extreme environments is strictly associated with their enzymes as well as biochemical pathways.

Extremozymes, enzymes produced by extremophiles, have proven to be of great importance in biotechnology and industry due to their ability to function under extreme conditions. The major reason of limited industrial usage of these important enzymes is their stability at limited ranges of temperature and pH as these are important parameters of industrial and biotechnological processes. Therefore, the exploration of microbial enzyme sources from extreme environments is significant (Ferrer *et al.,* 2007). Catalase finds extensive applications in diagnostic centers, synthesizing antioxidant drugs, biosensor technology and environmental monitoring. Thermoalkaliphilic catalases find promising applications as H_2O_2 detoxifying agents in food, semiconductor, paper and textile industries and in biotechnology. Little is known about thermo-alkali active catalases but this research area is being explored.

The accurate and definitive identification of microorganisms, including bacteria, is one of the cornerstones forming foundation of the field of microbiology (Janda and Abbott, 2002). Extremophilic bacteria can be identified by studying their morphology and biochemical characteristics following commonly used microbial identification manuals like Bergey's manual. Biochemical tests like catalase test, citrate utilization test, gram staining, indole test, oxidase test, starch hydrolysis test and urease test, etc. are performed to screen bacteria and check their enzymology (Reddy *et al.,* 2003). Molecular characterization of bacteria comprises of the sequence analysis of chromosomal and extra chromosomal DNA. At the present time, use of the 16S rRNA gene is thought to be a 'paragon' for identification and phylogenetic analysis of bacteria (Ludwig and Schleifer, 1999). In bacterial identification cases, the 16S rRNA gene is considered as a perfect molecular chronometer due to its convenient size (~1.5 kb), conserved nature, widely distributed database and high information content. (Clarridge, 2004).

Hot springs present a good example of extreme (geothermal) environments and are habitats of variety of thermoduric, thermophilic and hyperthermophilic microorganisms (Chaban *et al.,* 2006). Molecular characterization of bacteria based on sequence analysis of the 16S rRNA gene (16S rDNA) has been used to examine the microbial diversity of various hot springs

such as Yellowstone National Park in USA, Bakreshwar hot spring in India, Grensdalur hot spring in Iceland, Gargahot spring in Russia, El Biban hot spring in Northeast of Algeria, Rotoura hot spring in New Zealand (Selim *et al.,* 2014). Pakistan is located over the intersections of the tectonic plates of Indo-Pak subcontinent and this location makes the country rich in geothermal resources (Bakht, 2000). In the present research, an attempt has been made to isolate and identify catalase producing extremophilic, in terms of pH and temperature, microbial flora of hot springs of Tatta Pani, Azad Kashmir, Pakistan. The aim of the study is to isolate and identify a new thermoduric bacterial strain capable to grow under alkaline conditions as alkaline catalase producer.

MATERIALS AND METHODS

The present research work was carried out in Molecular Biochemistry Lab, Department of Biochemistry, University of Agriculture, Faisalabad.

Sample collection and isolation of catalase producing alkalophilic bacteria: Water samples from five hot springs were collected in sterile bottles and brought to laboratory. The pH and temperature of the water bodies were measured at the spot using pH paper strips and mercury thermometer (Roohi *et al.,* 2012). Basal medium containing lab-lamco powder (1 g/L), yeast extract (2 g/L), peptone (5 g/L), NaCl (5 g/L) and 30% H_2O_2 (5 mL/L) was used to screen catalase producing bacterial isolates. The samples were incubated at 45 °C and 150 rpm for 18 hours (Nakayama *et al.,* 2008). Bacterial isolates obtained were further proceeded for isolation of extremophilic bacteria (in terms of pH) by growing in nutrient broth (Oxoid) of different alkaline pH values ranging from 8.0 to 12.0. Isolates that grew at maximum values of pH were selected; distinct bacterial colonies on nutrient agar plates were screened, purified by reculturing and stored at 4 °C on nutrient agar plates and in 80% glycerol stock solution at -40 °C.

Morphological Identification and Biochemical Characterization of Isolated Bacteria: Morphological studies of isolated bacterial colonies were done on basis of color, shape, margin and elevation of colonies. Morphological characterization was also carried out on basis of cell morphology and Gram's staining observed under 100X light microscope (Olympus). General biochemical characterization was also done.

Growth conditions of isolated bacterial strain: The isolate was grown at different ranges of pH (4-12) and temperature (35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C and 70 °C) to measure the effect of these physical parameters on its growth and optimum values were determined. Optimal growth conditions were determined by measuring optical density (OD) of cell culture in LB broth at 600 nm (Olofsson *et al.,* 2003). Spectrophotometric measurements were performed with a Gene Quant spectrophotometer using a 1-cm-light-path cuvette.

Catalase Activity Assay: The catalase assay was based on decrease in the absorbance of hydrogen peroxide at λ_{max} = 240 nm. The crude enzyme extract was prepared from overnight grown bacterial cultures at their optimum pH and temperature. Bacterial culture in nutrient broth (Oxoid) was centrifuged at 8000 rpm, cell mass obtained was washed and re-suspended in 0.05 M phosphate buffer of pH 7.0. The cell lysis was carried out by sonication at 4 °C with pulse-on time 15 sec for every 60 seconds. The cell lysate was collected after centrifugation at 12,000 rpm for 25 minutes at 4 °C and used as crude enzyme extract for catalase assay (Li and Schellhorn, 2007).

Thirty five percent hydrogen peroxide was diluted in 0.05 M phosphate buffer (pH 7.0) to a final concentration of 5mM. A volume of 200 µL of substrate solution was added to 100 µL of crude enzyme extract in a 1-cm-light-path cuvette and after 1 minute scanned via spectrophotometer at 240 nm. Catalase activity was calculated on basis of rate of decomposition of hydrogen peroxide, which is proportional to the reduction in absorbance at λ_{max} = 240 nm (Bonnineau *et al.,* 2011). The enzyme assay was carried out in triplicates.

DNA extraction and PCR amplification: The DNA of the isolated bacterial strain was extracted by CTAB method as described by Griffiths *et al.* (2000). The 16S rRNA gene of the isolated DNA was amplified by (PCR) using universal primers, forward LRF1 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse LRR1 5'-AAG GAG GTG ATC CAG CC-3'. Amplification was carried out for 4 minutes of initial denaturation followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 1 minute. The PCR products were examined by agarose gel electrophoresis and detected using ethidium bromide fluorescence. The amplified PCR product was purified using Favorgen PCR clean up kit (Ramutton *et al.,* 2012) according to manufacturer's instructions.

Identification and Phylogenetic Analysis: Identification of the isolated bacterial strain was carried out by bioinformatics analysis of 16S rRNA gene. The sequencing was carried out availing commercial services of Center of Applied Molecular Biology (CAMB), Ministry of Science and Technology, Lahore, Pakistan. The sequence was matched with the Genbank database that was available at http://www.ncbi.nlm.nih.gov /BLAST/ to check the maximum similarity. The sequence was then undergone multiple sequence alignment with 16S rRNA gene sequences of bacteria showing maximum homology and phylogenetic tree was constructed. The

sequence of strain KP10 was aligned with sequences obtained from the GenBank database. The inference of evolutionary distance of KP10 isolate was made using the Neighbor-Joining method and the distances were computed using the Tamura-Nei model. The analysis involved 20 nucleotide sequences. Phylogenetic analysis was made using MEGA 7 (Kumar *et al.,* 2016).

RESULTS

Isolation of catalase producing alkalophilic bacteria: The water samples collected were named as KP1, KP2, KP3, KP4 and KP5, where KP refers to "Kashmir Poonch". Out of these five sample, bacterial growth was obtained from sample KP1 only in H_2O_2 containing screening basal medium. Bacterial cultures of KP1 sample were proceeded further for isolation of extremophilic bacteria (in terms of pH). The maximum pH value at which KP1 bacterial isolates were found able to grow was 11.0. These bacteria were grown on nutrient agar plate and one distinct colony was selected and purified by reculturing. The isolated bacterial strain was named as KP10 since it grew best at pH 10.0.

Morphological Identification and Biochemical Characterization of Isolated Bacteria: The morphology of the isolated bacterial strain on blood agar has been described in Table 1 and shown in Figure 1.

Cell morphology (Figure 2) and general biochemical characterization of KP10 have been summarized in Table 2.

Growth characteristics of isolated bacterial strain: KP10 strain was found to grow best at pH 10 and temperature 50 °C (Figure 3 a & b). The maximum values of pH and temperature that the organism survived were 11.0 and 65 °C, respectively. Whereas, no growth was observed beyond these values.

Catalase Activity Assay: The pH and temperature of the culture medium plays a critical role for the optimal physiological performance of the cells (Kumar *et al.,* 2004). An optimal catalase activity (3555 U/mL) was observed when the isolate was incubated at 45 °C and pH 10.0 for 24 hours.

Phylogenetic analysis: The identification of the isolated strain was executed on basis of phylogenetic analysis of its 16S rRNA gene sequence which consisted of 1488bp. BLAST results showed that KP10 strain was most closely related to *Bacillus clausii* (~88 %). According to phylogenetic tree based on 16S rRNA gene sequence KP10 strain showed close relation to *Bacillus clausii* strain EHYL2, accession no DQ866812 (Figure 4). The evolutionary history was inferred using neighbor-joining method and the evolutionary distances were computed using Tamura-3-parameter model.

Table 1. Colony morphology of the isolated bacterial strain, KP10

Color	Edge	Elevation	Form	Size	Surface
Translucent Grey white	Lobate	Flat	Irregular	2-3mm	Slimy smooth

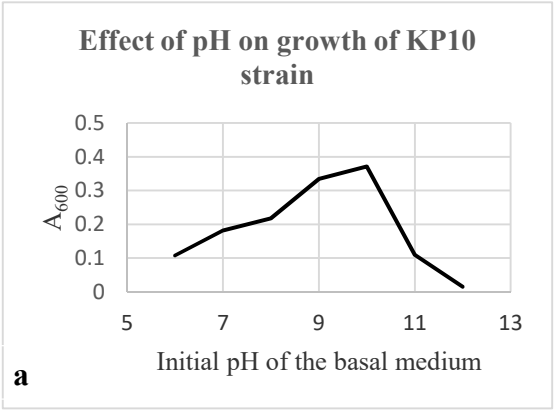
Table 2. Cell morphology and general biochemical characterization of KP10.

Cell morphology	Gram's staining reaction (+/-)	O_2 utilization	Citrate utilization (Y/N)	Indole formation (Y/N)	Glucose, Lactose and Sucrose utilization (Y/N)	H_2S production (Y/N)	Motile (Y/N)	Coagulase Test (+/-)	Oxidase Test (+/-)
Bacillus	+	Aerobic	N	N	N	N	N	-	-

Y: Yes, **N:** No, **+:** positive, **-:** negative

Figure 1. Colony morphology of KP10: The isolate, KP10, grew in form of grey white translucent colonies on blood agar

Figure. 2. Cell morphology of KP10 strain: Gram positive rod cell of isolate KP10 under 100X lens of light microscope



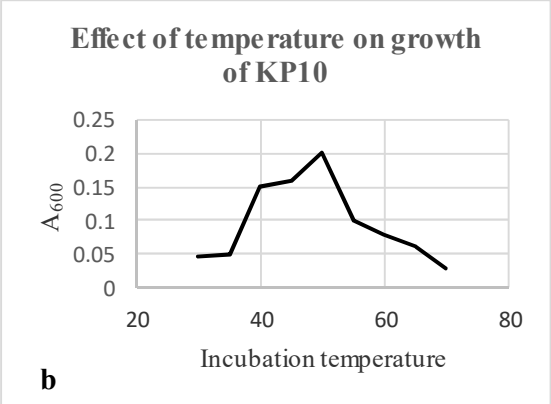


Figure. 3 (a) Effect of pH on growth of KP10 strain: The peak shows that the strain grew best at pH 10 whereas no growth at pH 12 **(b) Effect of temperature on growth of KP10 strain:** The peak shows that maximum growth of the strain was obtained at temperature 50 °C.

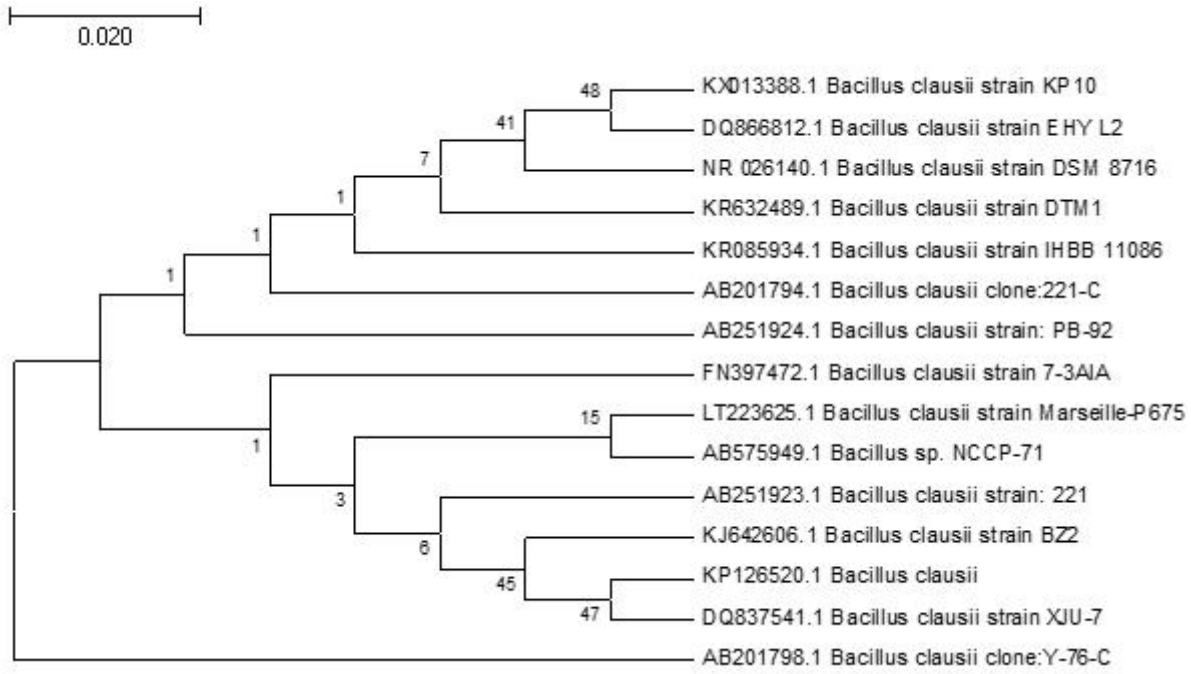


Figure 4. Phylogenetic relationship of the isolate based on analysis of 16S rRNA gene sequence: The scale bar = 0.02 substitutions per nucleotide position (evolutionary distance). The 16S rRNA gene sequences used for the alignment are shown with their GenBank accession numbers.

DISCUSSION

Extremophiles are thought to be the ancestors of all life forms on earth including the extinct and extant. Since life on earth is believed to be emerged in extremely high temperatures, thermophiles appear to be the first organisms on earth (Qazi, 2013). Extremophilic microorganisms find their use in industrial and biotechnology sector in terms of enzymes production and utilization because the environment in these sectors favours their growth and metabolism.

In Pakistan there are many extreme natural environments are present especially salt mines and hot springs. Khewra, Kalabagh and Warcha are three important salt mine ranges. Various *Bacillus* species have been isolated from Khewra salt mine range (Akhtar *et al.,* 2008). A number of geothermal resources is present in Pakistan. Baluchistan, Gilgit, Azad Kashmir and Khyber Pakhtunkhwa (KPK) are four potential zones of Pakistan where hot springs are located. The presence of *Thermus aquaticus* and *Thermosipho africanus* has been reported by Javed *et al.* (2012) on basis of biochemical

characterization. Zahoor *et al.* (2012) has reported *Geobacillus pallidus* found in Tatta Pani hot springs of Azad Kashmir. There is still gap and need to explore the microflora of the geothermal environments of Pakistan and this microflora can be exploited in terms of enzyme applications.

According to earlier studies and research made on extremophilic bacteria, *Bacillus* strains have been found to survive under adverse conditions (Roohi *et al.,* 2012). Alkaline *Bacillus* strains produce industrially and domestically useful enzymes such as xylanases, cellulases, amylases and proteases (Senesi *et al.,* 2001). GMBAE strain of *Bacillus clausii* has been reported by Denizci *et al.* (2004) to produce extracellular alkaline protease with maximum activity at pH 11 and 60 °C. Catalases are important enzymes but less reported in extremophiles. In the present research work, the isolate KP10 strain grew best at pH 10.0 and 50 °C whereas it could survive at pH values ranging from 6.0 to 11.0 and temperature 35-65 °C. The growth was inhibited beyond these limits of temperature and pH. Catalase activity was maximum at pH 10.0 and 45 °C. Phenotypic and phylogenetic analysis show that KP10 strain is closely related to *Bacillus clausii* but it can be identified completely using other techniques such as cellular fatty acids composition and G+C content studies.

In conclusion, enzymes from alkaliphilic *Bacillus* species have found great industrial application potential. Since the maximum enzyme activity of catalase from isolated strain KP10 strain was observed at pH 10 and 45 °C, this may find applications in paper pulp and textile industry where H_2O_2 is used as a bleaching agent and the process is carried out at alkaline pH and high temperature. The strain can further be explored in aspects of production of other useful enzymes such as cellulases, proteases and amylases.

REFERENCES

Akhtar, N., M. A. Ghauri, A. Iqbal, M. A. Anwar and K. Akhtar. (2008). Biodiversity and phylogenetic analysis of culturable bacteria indigenous to Khewra salt mine of Pakistan and their industrial importance. Braz. J. Microbiol. 39: 143–150.

Bakht, M. S. (2000). An overview of geothermal resources of Pakistan. *In* Proceedings of the World Geothermal Congress.

Bonnineau, C., B. Bonet, N. Corcoll and H. Guasch. (2011). Catalase in fluvial biofilms: a comparison between different extraction methods and example of application in a metal-polluted river. Ecotoxicology 20: 293–303.

Chaban, B., S.Y. Ng and K.F. Jarrell. (2006). Archaeal habitats-from the extreme to the ordinary. Can. J. Microbiol. 52: 73-116.

Clarridge, J.E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17: 840-862.

Denizci, A.A., D. Kazan, E.C.A. Abeln and A. Erarslan. (2004). Newly isolated Bacillus clausii GMBAE 42: an alkaline protease producer capable to grow under highly alkaline conditions. J. Appl. Microbiol. 96: 320–327.

Ferrer, M., O. Golyshina, A. Beloqui and P.N. Golyshin. (2007). Mining enzymes from extreme environments. Curr. Opin. Microbiol. 10: 207-214.

Griffiths, R.I., A.S. Whiteley, A.G. O'Donnell and M.J. Bailey. (2000). Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Appl. Environ. Microbiol. 66: 5488-5491.

Janda, J.M. and S.L. Abbott. (2002). Bacterial Identification for Publication: When Is Enough Enough? J. Clin. Microbiol. 1887–1891.

Javed, M. M., S. Zahoor, H. Sabar, I. U. Haq and M. E. Babar. (2012). Thermophilic bacteria from the hot springs of Gilgit (Pakistan). The J. Anim. Plant. Sci. 22(1): 83-87.

Kumar, C.G., H.S. Joo, Y.M. Koo, S.R. Paik and C.S. Chang. (2004). Thermostable alkaline protease from a novel marine haloalkalophilic *Bacillus clausii* isolate. World. J. Microbiol. Biotechnol. 20: 351–357.

Kumar, S., G. Stecher and K. Tamura. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 33: 1870-1874.

Li, Y. and H.E. Schellhorn, H.E. (2007). Rapid kinetic microassay for catalase activity. J. Biomol. Tech. 18: 185–187.

Ludwig, W. and K.H. Schleifer. (1999). Phylogeny of bacteria beyond the 16S rRNA standard. ASM. News. 65: 752-757.

Morozkina, E.V., E.S. Slutskaya, T.V. Fedorova, T.I. Tugay, L.I. Golubeva and O.V. Koroleva. (2010). Extremophilic microorganisms: Biochemical adaptation and biotechnological application (review). Appl. Biochem. Microbiol. 46: 1-14.

Nakayama, M., T. Nakajima-Kambe, H. Katayama, K. Higuchi, Y. Kawasaki and R. Fuji. (2008). High catalase production by rhizobium radiobacter strain 2-1. J. Biosci. Bioeng. 106: 554–558.

Olofsson A. C., M. Hermansson and H. Elwing. (2003). N-acetyl-L-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces. Appl. Environ. Microbiol. 69: 4814-4822.

Qazi, J., I. (2013). Biotechnological potential and conservatory of extremophiles from climatically wide ranged developing countries: Lesson from Pakistan. Crit. Rev. Microbiol. 39: 1-8.

Ramutton, T., I.C.E. Hendriksen, J. Mwanga-Amumpaire, G. Mtove, R. Olaosebikan, A.K. Tshefu, M.A. Onyamboko, C. Karema, K. Maitland, E. Gomes and S. Gesase. (2012). Sequence variation does not confound the measurement of plasma PfHRP2 concentration in African children presenting with severe malaria. Malar. J. 11: 1.

Reddy, G.S.N., P.U.M. Raghavan, N.B. Sarita, J.S.S. Prakash, N. Nagesh, D. Delille and S. Shivaji. (2003). Halomonas glaciei sp. nov. isolated from fast ice of Adelie Land, Antarctica. Extremophiles. 7: 55-61.

Roohi, A., I. Ahmed, N. Khalid, M. Iqbal and M. Jamil. (2012). Isolation and phylogenetic identification of halotolerant/halophilic bacteria from the salt mines of Karak, Pakistan. Int. J. Agric. Biol. 16: 564-570.

Rothschild, L.J., and L.M. Rocco. (2001). Life in extreme environments. Nature. 409: 1092-1101.

Selim, S., M.E. Sherif, S. El-Alfy and N. Hagagy. (2014). Genetic diversity among thermophilic bacteria isolated from geothermal sites by using two PCR typing methods. Geomicrobiol. J. 31: 161-170.

Senesi, S., F. Celandroni, A. Tavanti and E. Ghelardi. (2001). Molecular characterization and identification of Bacillus clausii strains marketed for use in oral bacteriotherapy. Appl. Environ. Microbiol. 67: 834–839

Zahoor, S., M. M. Javed, M. N. Aftab and I. Haq. (2012). Isolation and molecular identification of a facultatively anaerobic bacterium from the hot spring of Azad Kashmir. Pakistan J. Bot. 44: 329-333.