S. Zada, A.A. Naseem, S.-J. Lee, M. Rafiq, I. Khan, A.A. Shah, and F. Hasan – Geochemical and mineralogical analysis of Kashmir Cave (SMAST), Buner, Pakistan, and isolation and characterization of bacteria having antibacterial activity. *Journal of Cave and Karst Studies*, v. 78, no. 2, p. 94–109. DOI: 10.4311/2014MB0110

GEOCHEMICAL AND MINERALOGICAL ANALYSIS OF KASHMIR CAVE (SMAST), BUNER, PAKISTAN, AND ISOLATION AND CHARACTERIZATION OF BACTERIA HAVING ANTIBACTERIAL ACTIVITY

Sahib Zada[1], Abbas Ali Naseem[2], Seong-Joo Lee[2], Muhammad Rafiq[1], Imran Khan[1], Aamer Ali Shah[1], and Fariha Hasan[1]*

Abstract: Bacterial strains having the ability to inhibit the growth of other bacteria were isolated from soil samples collected from Kashmir Smast (smast is Pushto for cave), Khyber Pakhtunkhwa, Pakistan. The study includes mineralogical and geochemical analyses of soil sample collected from the cave, so as to describe the habitat from which the microorganisms have been isolated. Total bacterial count of the soil sample was 5.25×10^4 CFU mL^{-1}. Four bacterial isolates having activity against test organisms *Micrococcus luteus*, *Klebsiella* sp., *Pseudomonas* sp., and *Staphylococcus aureus* were screened out for further study. Two of the isolates were found to be Gram-positive and the other two Gram-negative. The four isolates showing antibacterial activity were identified as *Serratia* sp. KC1-MRL, *Bacillus licheniformis* KC2-MRL, *Bacillus* sp. KC3-MRL, and *Stenotrophomonas* sp. KC4-MRL on the basis of 16S rRNA sequence analysis. Although all isolates showed antibacterial activity, only *Bacillus licheniformis* KC2-MRL was selected for further study due to its large zone of inhibition. Antibacterial activity of *B. licheniformis* KC2-MRL was optimum when grown in nutrient broth adjusted to pH 5 and after 24 hours of incubation at 35 °C. The extracted antibacterial compound was stable at pH 5–7 and 40 °C when incubated for 1 hour. The strain was found resistant against cefotaxime (ctx). Atomic-absorption analysis of the soil sample collected from the cave showed high concentrations of calcium (332.938 mg kg^{-1}) and magnesium (1.2576 mg kg^{-1}) compared to the control soil collected outside the cave. FTIR spectrum of the concentrated protein showed similarity to bacitracin. The antibacterial compound showed activity against both Gram-negative and positive test strains. Mineralogy of Kashmir Smast is diverse and noteworthy. Different geochemical classes identified by X-ray diffraction were nitrates, oxides, phosphates, silicates, and sulfates. Weathered cave limestone contributes notably to the formation of these minerals or compounds. FTIR spectroscopic analysis helped to identify minerals such as quartz, clinochlore, vermiculite, illite, calcite, and biotite.

Introduction

Caves are characterized as having very low nutrient availability, constant low temperatures, and high humidity. Caves can be either terrestrial or aquatic and are usually oligotrophic in nature (i.e., nutrient limited). Some may be rich in specific natural minerals or be exposed to different nutrient-containing sources, therefore, different caves will have different types of microorganisms inhabiting various ecological niches. Fauna, environmental factors, temperature, and organic matter dictate the caves' biotic activities, such as nutrient cycling and geomicrobiological activities, including formation or alteration of cave structures (Adetutu and Ball, 2014).

Cave organisms have evolved some extraordinary abilities to survive and live in this inhospitable environment (Engel et al., 2005; Simmons et al., 2008; Northup and Lavoie, 2004). Cave microbial flora is rich in different types of microorganisms having some diverse and unique characteristics (Groth et al., 1999). The most abundant organisms observed in caves are filamentous and belong to the Actinobacteria group, followed by coccoid and bacilli forms (Cuezva et al., 2009). Some pathogenic microorganisms have been reported from Altamira Cave (Jurado et al., 2006). Luong et al. (2010) for the first time reported the recovery of *Aurantimonas altamirensis* from human medical samples, rather than from a cave. The disease-causing bacteria E. coli and S. aureus have also been isolated from caves (Lavoie and Northup, 2005), as well as species of *Pseudomonas*, *Sphingomonas*, and *Alcaligenes* sp. (Ikner et al., 2007), and *Inquilinus* sp. (Laiz et al., 1999).

* Corresponding author: farihahasan@yahoo.com
[1] Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan.
[2] Department of Geology, Kyungpook National University, Daegu 702 – 701, Korea

Caves can be a source of novel microorganisms and biomolecules, such as enzymes and antibiotics, that may be suitable for biotechnological purposes (Tomova et al., 2013). The influence of particular nutrients in antibiotic biosynthesis is caused by the chemical structures of antibiotic substances (Pereda et al., 1998). Rigali et al. (2008) provide evidence that certain substrates and oligotrophic conditions will lead to increased induction of secondary metabolites. Nitrogen from various sources may incorporate in antibiotic molecules as precursors, or their amino groups can transfer to specific intermediate products (Doull and Vining, 1990; Cheng et al., 1993). Nutrient deficiency is responsible for the onset of antibiotic biosynthesis (Demain et al., 1983; Doull and Vining, 1990; Sanchez and Demain, 2002). When carbon or nitrogen is a limiting factor, growth is rapidly reduced and antibiotic biosynthesis occurs in the stationary phase. In other cases, antibiotic production is associated with the growth phase. Due to the oligotrophic environment in cave ecosystems, microorganisms present in the cave compete for nutrients and produce antibiotics against other microbes. Wide-spectrum standard antibiotics, metabolic by-products (organic acids), lytic agents (lysozyme), and other biologically active compounds like exotoxins and bacteriocins are also produced by microbes (Riley and Wertz, 2002; Yeaman and Yount, 2003). The continuous job of scientists is to discover new antibiotics and new source microorganisms. Cave microorganisms can be used for the production of potential new antibiotics.

Antibiotic producing microbes mostly belong to the genera *Penicillium*, *Streptomyces*, *Cephalosporium*, *Micromonospora*, *Bacillus* (Park et al., 1998), and *Pseudomonas*, followed by the enterobacteria, lactobacilli, and streptococci (Bérdy, 2005). More than eight thousand antibiotics are known to exist and hundreds are discovered yearly (Brock and Madigan, 1991), but only a few prove to be commercially useful. About 17% of these antibiotics are produced by molds and 74% by actinomycetes (Zhang et al., 2008). *Bacillus* sp. mostly form peptides and phenazines, which are heterocyclic and derivatives of fatty acids, but the production of macrolactones is very rare (Bérdy, 2005). Gramicidins, polymixins, bacitracins, and some other antibiotics are formed non-ribosomally (Nissen-Meyer and Nes, 1997; Hancock and Chapple, 1999).

The number and species of microorganisms in soil vary in response to environmental conditions such as nutrient availability, soil texture, and type of vegetation cover (Atlas and Bartha, 1998). The soil composition and texture play important role in harboring microbes with unique characteristics. Thus it is important to know about the composition, type, structure, and texture of the soil from which the microorganisms are isolated for research or the production of metabolites such as antibiotics. A great number of antibiotics have been isolated from various microorganisms. Studies are still being conducted to isolate and identify novel antibiotics effective against pathogenic fungi and bacteria.

Microbial species adapt to caves by interacting with minerals there (Barton and Jurado, 2007). The geochemistry and metal content of the cave environment can influence the synthesis of antibiotics by cave bacteria, as metal ions are known to affect the synthesis of microbial metabolites *in vitro*. Tanaka et al. (2010) made a connection between the rare earth elements scandium and lanthanum and increased activation of the expression of nine genes belonging to nine secondary metabolite-biosynthetic gene clusters of *Streptomyces coelicolor* A3(2). Investigations on the effect of several metal ions indicated that Cu^{2+}, Mn^{2+}, and Fe^{2+} stimulated AK-111-81 biosynthesis by *Streptomyces hygroscopicus*, depending on their concentration (Gesheva et al., 2005). Divalent ions stimulated the production of polyenes (Georgieva-Borisova, 1974; Liu et al., 1975; Solivery et al., 1988; Park et al., 1998), and Fe^{2+} and Mn^{2+} have been found to favor niphimycin production. Soil texture and structure also strongly influence the activity of soil biota. For example, medium textured loam and clay soils enhance activity of microbes and earthworms, whereas fine textured sandy soils, with lower water retention potentials, are not very favorable. Alterations in pH of the soil can affect metabolism of species, enzyme activity, and availability of nutrients, and thus, are often lethal (Singh and Mishra, 2013).

The aim of the present study was to isolate microbes from the cave having antibacterial activity, identify them and their product, and investigate the geochemistry of the cave to understand the environmental conditions under which these microorganisms are living and producing compounds inhibitory for other microbes.

Materials and Methods

Sampling Site and Collection of Soil Samples

Two soil samples were collected from Kashmir Smast (*smast* in local language means cave), Nanser, Buner, Khyber Pakhtunkhwa (GPS coordinates 34°25′42.12″N 72°13′10.82″E) (Fig. 1). The cave is 188 m long, with average height and width about 28 m and 25 m, respectively. The Kashmir Smast is one of a series of natural caves in limestone, probably of marine origin, located in the Babozai Mountains between Mardan and Buner in northern Pakistan. According to study of a rare series of bronze coins and artifacts found in the region, the caves and their adjacent valley probably composed a sovereign kingdom in Gandhara, which maintained at least partial independence for almost 500 years, from the fourth to the ninth centuries AD (Ziad, 2006). It is a limestone cave with internal temperature around 10 °C. The interior of the cave was muddy due to dripping of water from the surface, the only source of water. Soil samples were collected from the cave wall (sample smast-7) and floor (sample smast-5) in sterile Falcon

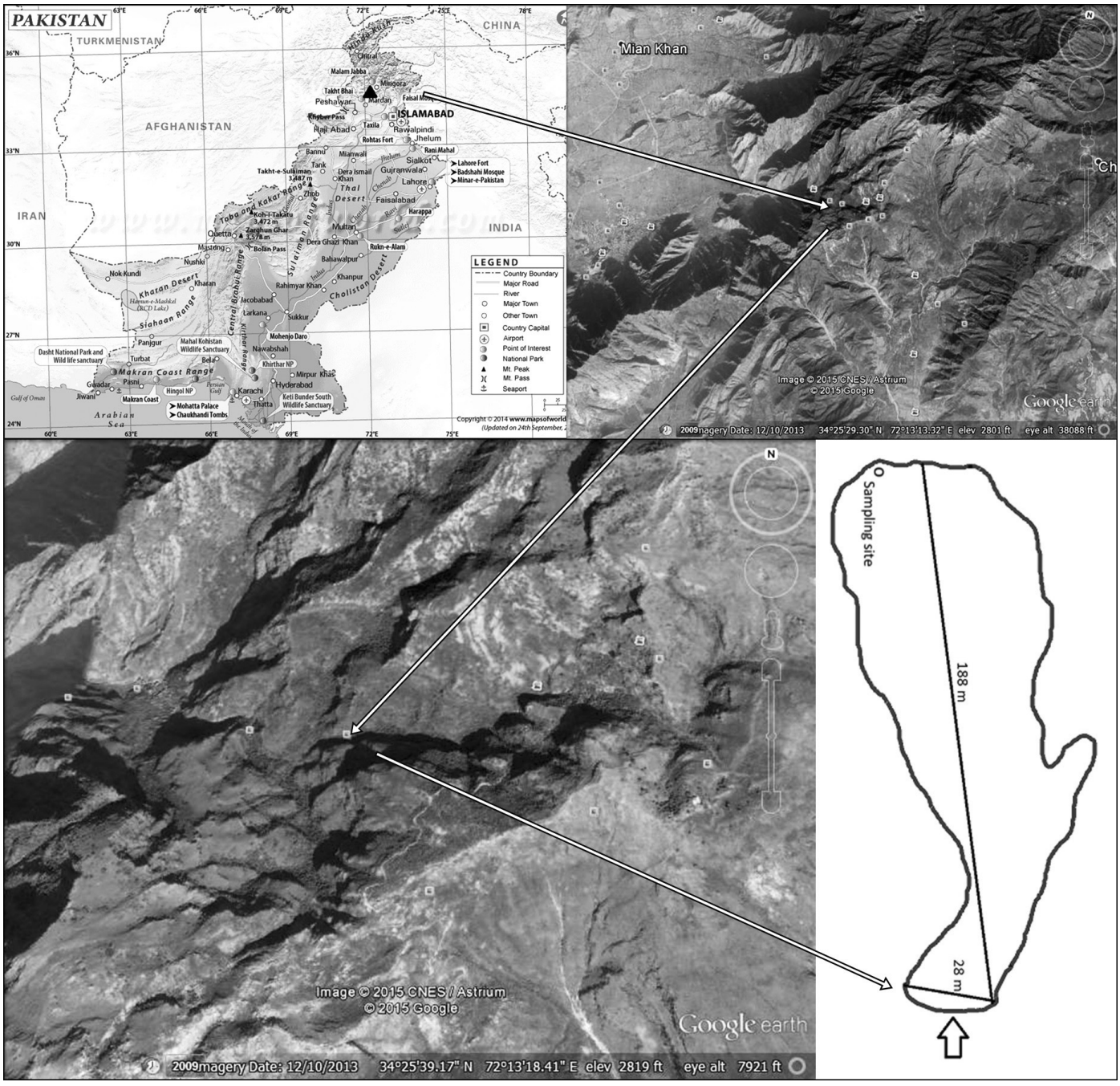

Figure 1. Location and plan map of Kashmir Smast (Cave), Nanseer Buner, Khyber Pakhtunkhwa, Pakistan. White arrows show location of the cave; large arrow shows entrance to the cave. (Pakistan full map from http://www.mapsofworld.com/pakistan/; aerial images Google Earth.)

tubes under aseptic conditions. The samples were collected from the dark end of the cave about 188 m from the entrance. This cave is located far away from human travel routes, so human intervention is negligible. The samples were then brought to the laboratory in an icebox and stored at 4 °C for further processing. These soil samples were screened for the antibiotic-producing isolates within 24 hours.

MINERALOGICAL ANALYSIS

For the quantitative analysis of elements (Ni, Cr, Co, Cu, Zn and Pb) in the soil sample, Atomic Absorption (AA240FS Fast Sequential Atomic Absorption Spectrophotometer) spectrophotometry was performed. To prepare the sample for this analysis, soil digestion was performed.

One gram each of soil from the cave floor and control soil from outside the cave were ground separately and

were then mixed in 15 mL aqua regia, heated at 150 °C, and left overnight. Then 5 mL of $HClO_4$ was added and again heated at 150 °C. The solution almost became dry until brown fumes were produced. Whatman filter paper (No. 42) was used for filtration, and the volume was made up to 50 mL using double-distilled water (Jensen et al., 1983).

X-ray powder diffraction is a rapid analytical technique used for phase identification and characterization of unknown crystalline materials such as minerals and inorganic compounds and identification of fine-grained minerals such as clays and mixed-layer clays that are difficult to determine optically (Dutrow and Clark, n.d.). XRD patterns were obtained from the samples using X'Pert-APD (Philips, The Netherlands) with an X-ray generator (1.2 kW) and anode (LFF Cu). The Cu Kα radiation had a wavelength of 1.54 Å. The X-ray generator voltage and current were 40 kV and 30 mA, respectively. The step-scan data were continuously collected over the range of 5 to 80°2θ.

Mineral proportions were calculated using SIROQUANT, a commercially available MS-Windows program for standardless mineral quantification. Weight-percent mineral phase contents were estimated. Using calculated hkl mineral library files, refinement stages were optimized for the smallest possible χ^2 goodness-of-fit parameter for the associated Rietveld peak pattern match (Taylor, 1991; Taylor and Clapp, 1992).

Thermogravimetric analysis records change in mass from dehydration, decomposition, or oxidation of a sample as a function of heating time and temperature (Voitovich et al, 1994). TGA was performed on a high-resolution thermogravimetric analyzer (Staram TGA Instruments, series Q500) in a flowing nitrogen atmosphere (60 cm^3 min^{-1}). Approximately 35 mg of sample underwent thermal analysis, with a heating rate of 5 °C min^{-1} within the range of 25 to 1000 °C. With the isothermal, isobaric heating program of the instrument the furnace temperature was regulated precisely to provide a uniform rate of decomposition in the main decomposition stage.

The field-emission cathode in the electron gun of a scanning electron microscope provides narrower probing beams at low, as well as high, electron energy that results in improved spatial resolution and minimizes sample charging and damage (Stranks et al., 1970). FE-SEM with EDS analysis of the samples was performed for the determination of thickness, structure uniformity, and elemental composition, using S-4800 and EDX-350 (Horiba) FE-SEM (Hitachi, Tokyo, Japan). Samples were spread on a glass plate that was fixed onto a brass holder and coated with osmium tetraoxide (OsO_4) using a VD HPC-ISW osmium coater (Tokyo, Japan) prior to FE-SEM analysis

About 2 mg of the soil sample was mixed with 40 mg of KBr in ratio 1:20 using mortar and pestle. KBr powder had been dried at 120 °C in an oven to avoid the broad spectral peak. A 1 by13 mm pellet was prepared. The pellet was placed in a holder and introduced in the infrared beam for analysis through Fourier Transform Infrared Spectrometer (Jasco FT/ IR – 620).

Microbiological Studies

For isolation of bacteria from the cave soil, 1 g of each soil sample was serially diluted in normal saline and then was spread on nutrient-agar plates aseptically, and plates were incubated aerobically for 24 hrs at 35 °C. Viable cell count was calculated as CFU mL^{-1}.

The isolate *Bacillus licheniformis* KC2-MRL was incubated at 25, 35 and 45 °C. A growth curve was constructed by taking values of cell concentration on y-axis versus time along x-axis. Using a standard formula, growth rate and generation time was calculated from the graph.

Nutrient agar medium was used for isolation of antibiotic-producing bacteria. Lawns of susceptible test organisms *Micrococcus luteus* (ATCC 10240), *Klebsiella* sp., *Pseudomonas* sp., and *Staphylococcus aureus* (ATCC 6538) were made on nutrient agar plates (Gauthier, 1976) that were then sprinkled with 20 to 25 particles of soil. All the plates were gently shaken so that the soil particles spread uniformly. Plates were then incubated at 35 °C for 24 hours, lid side up so that the soil particles would not fall off the agar. After 24 hours of incubation, plates were checked for antibacterial activity shown by the formation of clear zone of inhibition around the KC2-MRL bacteria colony. Zone-producing isolates were purified and stored at 4 °C. Colony morphology, Gram-staining, and biochemical tests (citrate utilization, oxidase and catalase production, nitrate and sulfate reduction, H_2S production, and carbohydrate fermentation) were performed according to *Bergey's Manual of Determinative Bacteriology* (Holt, 2012).

The DNA extraction from bacteria was done by spinning 1 mL of culture at 10,000 rpm for about 3 min, after which the cells were pelleted out and rinsed twice in 400 µL TE buffer after removing the supernatant. Then the cells were centrifuged at 10,000 rpm for 3 min, and the pellets were resuspended in 200 µL TE buffer. Then 100 µL Tris-saturated phenols of pH 8.0 were added to these tubes, followed by a vortex-mixing step of 60 sec, to lyse the cells. To separate the aqueous and organic phases, the samples were centrifuged at 13,000 rpm at 4 °C for 5 minutes. Then 160 µL of upper aqueous phase was taken in a 1.5 mL Eppendorf. About 40 µL of TE buffer was added to make 200 µL, which was then mixed with 100 µL of 24:1 chloroform:isoamyl alcohol and centrifuged for 5 min at 13,000 rpm at 4 °C. Chloroform:isoamyl alcohol (24:1) extraction was used for the purification of lysate, until there was no longer a white interface, and the same method was repeated twice or thrice (Aitken, 2012). Purified DNA was present in the aqueous phase and was stored at −20 °C for further use. The purified DNA was analyzed through agarose gel 1.5 g in 1X TBE and staining with ethidium bromide.

Phylogenetic analysis was performed with a ClustalW program implemented in MEGA4.0 (Thompson et al., 1994). The similar sequences were downloaded from NCBI.

All sequences were aligned, and the phylogenetic tree was constructed using the neighbor-joining method. Bootstrap analysis with 1000 replicates was performed for the significance of the generated tree.

An inoculum of *B. licheniformis* KC2-MRL, selected after screening on the basis of its larger zone of inhibition against test strains, was prepared in nutrient broth. First about 50 mL of nutrient broth was prepared in 250 mL flask, autoclaved, and incubated at 35 °C overnight to check the sterility. The nutrient broth was taken in 100 mL flasks and its pH was adjusted to 5 (pH of sampling site was 5). Approximately 10% inoculum was added to each flask and incubated at 35 °C in orbital shaker at 150 rpm. After every 24 hrs, samples were collected and centrifuged at 10,000 rpm for 16 minutes, for a total of 96 hrs to obtain cell free supernatant that was checked for antibacterial activity by agar-well diffusion assay (Haque et al., 1995). About 80 µL of cell-free supernatant was added in the wells and the plates were incubated at 35 °C for 24 hours. After 24 hrs, the zones of inhibition were observed and the diameter of the zone of inhibition was measured.

Different media were used for the production of antibacterial compounds by *B. licheniformis* KC2-MRL, including Trypticase soya broth, nutrient broth and Luria Bertani broth. Inoculum (10%) was added and incubated at 37 °C and 150 rpm. The cell growth was measured by optical density at 600 nm, and antimicrobial activity was checked by agar-well diffusion assay.

To check the effect of time of incubation on the antimicrobial activity, the strain was incubated at 37 °C in orbital shaker at 150 rpm and samples were drawn after every 24 hours from 0 to 96 hours. The antimicrobial activity of all the collected cell-free supernatants was checked against *S. aureus*, *M. luteus*, *Klebsiella* sp., and *E. coli*.

The effect of temperature (15, 25, 35, and 45 °C) on optimum antibacterial activity was studied by inoculating *B. licheniformis* KC2-MRL in nutrient broth and incubating at 15, 25, 35 and 45 °C at 150 rpm. Samples were drawn every 24 hours from 0 to 96 hrs. Centrifuged cell-free supernatants were used for further analysis using *S. aureus*, *M. luteus*, *Klebsiella* sp., and *Pseudomonas* sp. as test strains.

The effect of pH (5, 6, 7, and 8) on the production of antibiotics was studied by inoculating *B. licheniformis* KC2-MRL in the growth medium adjusted to those values. Samples were drawn every 24 hours from 0 to 96 hours, and centrifuged and cell free supernatants were used for further analysis.

The standard Kirby-Bauer disk-diffusion assay (Koneman, 2006) was performed to check the sensitivity of the selected strains against various broad-spectrum antibiotics to check for the intrinsic ability of the microorganisms to resist antibiotics.

Cell-free supernatant of *B. licheniformis* KC2-MRL culture grown under optimized conditions was used for the precipitation of antibacterial compounds using increasing concentrations of 10 to 80% of ammonium sulfate. The pellet was kept at −20 °C in 10 mL of 0.1M phosphate buffer, pH 7. FTIR was performed to identify unknown compounds. Spectrum of the antibacterial compound produced by *Bacillus licheniformis* KC2-MRL was compared with that of bacitracin as a control. Samples were scanned from 4000-400 cm^{-1} at resolution of 6.0 cm^{-1}.

RESULTS

MINERALOGICAL ANALYSIS

Observed X-ray diffraction patterns of samples smast-5 and smast-7 along with the Inorganic Crystal Structure Database reference data of different minerals are shown in Figures 2a and 2b. In Figure 2a, two prominent peaks at 2θ 26.624 and 29.420 were observed. The observed peaks match with the ICSD Reference codes 03-065-0466 Quartz and 01-086-1385 Muscovite-2M1. Along with these peaks, some other weak peaks matched with reference peaks of 01-075-8291 Chlorite-ll-4, 01-080-1108 Biotite, 01-075-1656 Dolomite, 01-077-0022 Vermiculite-2M, and 01-075-8291 Clinochlore-llb-4. Figure 2b indicates three prominent peaks at 2θ 26.661, 29.442, and 30.984. These matched with ICSD Reference codes 01-087-2096 Quartz, 01-072-4582 Calcite, and 01-076-6603 Vermiculite. Silicate minerals found in the cave were illite, muscovite, vermiculite, chlorite, clinochlore, and quartz. The chemical composition of the minerals is given in Table 1.

Weight-percent mineral phases were used to estimate the SIROQUANT (Fig. 3), considering 100% crystalline compound to calculate the quantitative analysis. Figure 3a shows that vermiculite, illite, and chlorite were the most abundant minerals in smast-5. Similarly, Figure 3b shows that the vermiculite-2M1, muscovite, and clinochlore-llb are the most abundant minerals in smast-7.

The Fourier-transform infrared absorption peaks from the cave were observed to determine the major and minor constituent minerals present in the sample smast-7 (Fig. 4). The samples analyzed were mixtures of minerals such as silicon oxide, calcite, quartz, muscovite, clinochlore, nimite, biotite, and vermiculite. Various peaks appeared indicating the presence of a variety of minerals.

Mass loss steps were observed from Figure 5 at 77, 200 and 280, 400 and 790 °C, with mass losses of 10.23, 21.55, 5.20 and 7.58% recorded due to carbonates.

Scanning electron microscope observations (Fig. 6) suggest that cave's clay particles are poorly crystallized clasts with angular, irregular outlines, and swirly texture with face-to-face arrangement of clay grains. Si, Al, and Fe were found enriched within the samples.

SOIL ANALYSIS

Atomic-absorption spectroscopy was performed to determine the concentration of elements in the cave soil sample smast-5. Ca was 332.938 mg kg^{-1} as compared to 121.65 mg kg^{-1} in control soil from the surface, Mg was

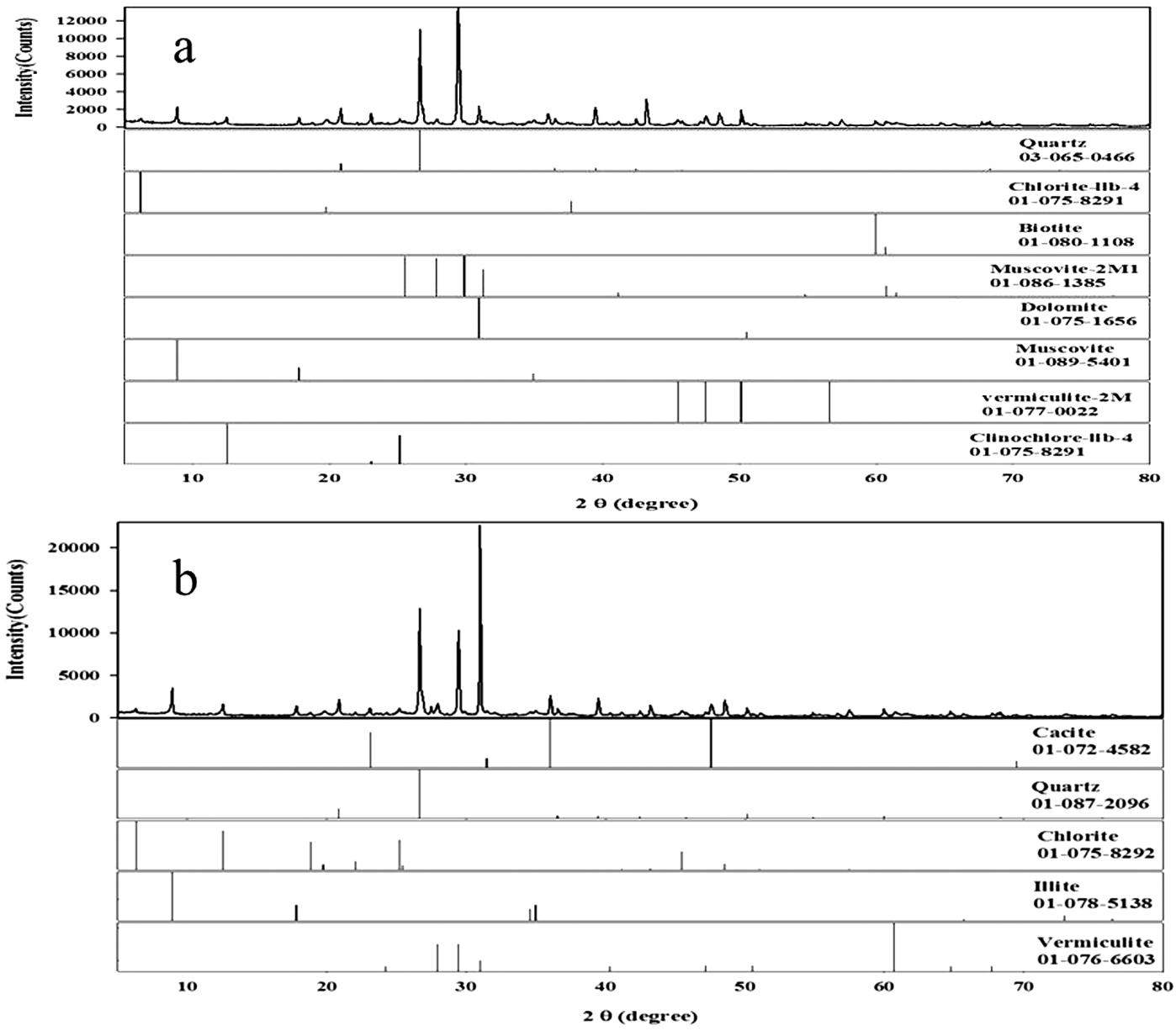

Figure 2. X-ray diffraction patterns of Kashmir Smast soil samples smast-5 from the floor (a) and smast-7 from the wall (b) with spectra from the Inorganic Crystal Structure Database for comparison and identification.

1.2576 mg kg^{-1} in cave soil and 1.023 mg kg^{-1} in control soil, and that of Ni, Cr, Co, Cu, Zn, and Pb were much lower than those found in the control soil (Table 2).

Microbiology Results

Numbers of viable cells per mL were calculated for the smast-7 floor-soil sample collected from Kashmir Cave. The bacterial count (CFU) was 5.25×10^4 mL^{-1}.

Initial screening resulted in isolation of four phenotypically distinct bacterial strains showing antimicrobial activity against four test organisms. Figure 7 shows a typical nutrient-agar plate with zones of inhibition. Of the four, the strain *B. licheniformis* KC2-MRL showed the largest zones of inhibition, 28 mm against *Micrococcus*, 20 mm against *E. coli*, 14 mm against *Staphylococcus aureus*, and 15 mm against *Klebsiella*). Therefore it was selected for further analysis.

The 16S rRNA gene sequences of the antibiotic-producing cave bacteria have been submitted to NCBI GenBank. The isolates KC1-MRL, KC2-MRL, KC3-MRL and KC4-MRL were identified as *Serratia* sp. KC1-MRL (Accession No. KC128829.1), *Bacillus licheniformis* KC2-MRL (Accession No. KC128830.1), *Bacillus* sp. KC3-MRL (Accession No. KC128831.1), and *Stenotrophomonas* sp. KC4-MRL (Accession No. KC128832.1) (Fig. 8).

Maximum antimicrobial activity was found when *B. licheniformis* KC2-MRL was cultured in nutrient broth

Table 1. List of minerals identified in samples from the wall and floor of Kashmir Smasta.

Mineral	Chemical Formula
Calcite	$CaCO_3$
Quartz	SiO_2
Dolomite	$CaMg(CO_3)_2$
Muscovite-2M1	$K_{0.86}Al_{1.94}(Al_{0.965}Si_{2.895}O_{10})((OH)_{1.744}F_{0.256})$
Muscovite	$KAl_{2.20}(Si_3Al)_{0.975}O_{10}((OH)_{1.72}O_{0.28})$
Clinochlore-llb	$(Mg_{4.715}Al_{0.394}Fe_{0.109}Cr_{0.128}Nl_{0.011})(Si_{3.056}A_{1.944})O_{10}(OH)_8$
Biotite	$KFeMg_2(AlSi_3O_{10})(OH)_2$
Vermicullite-2M	$(Mg_{2.36}Fe_{0.48}Al_{0.16})Mg_{0.32}(Al_{1.28}Si_{2.72})O_{10}(OH)_2(H_2O)_{4.32}Mg_{0.32}$
Vermicullite	$Mg_3((AlSi_3O_{10})(OH))(H_2O)$
Chlorite-llb-4	$(Mg_{11.06}Fe_{0.94})((Si_{5.22}Al_{2.78})O_{20}(OH)_{16})$
Illite	$(K_{0.71}Ca_{0.01}Na_{0.01})(Al_{1.86}Mg_{0.15}Fe_{0.04})((Si_{3.27}Al_{0.73})O_{10}(OH)_2)$

after 24 hours of incubation, with zone of inhibition of 28 mm against *M. luteus*, 20 mm against *S. aureus*, 11 mm against *Klebsiella* and 8 mm against *E. coli*. The antibacterial activity decreased with passage of time in all media except the nutrient broth.

Best antimicrobial activity (21 mm) of *B. licheniformis* KC2-MRL was observed against *M. luteus*, 14 mm against *S. aureus*, 12 mm against *Klebsiella*, and 8 mm against *E. coli* after 48 hours of incubation, while there was a decrease in the sizes of zones after 48 hours showing decrease in antimicrobial activity of *B. licheniformis* KC2-MRL (Fig. 9).

Maximum antibacterial activity of 28 mm and 22 mm was observed against *S. aureus* and *M. luteus*, respectively, with 17 mm against *E. coli* and 9 mm activity against *Klebsiella*, at 35 °C after 48 hrs of incubation. The activity in terms of zones of inhibition decreased with further increase in temperature (45 °C) (Fig. 9).

Effect of pH (5, 6, 7, and 8) on the production of antibiotics was studied. Activity in terms of zones of inhibition was measured against the same test organisms. Best activities were observed at pH 5, 23 mm against *S. aureus*, followed by *M. luteus*, *E. coli* and *Klebsiella* after 24 hrs of incubation. The second best activity was observed at pH 6, and a gradual decrease in activity was observed with increase in pH (Fig. 9).

To check the stability of antimicrobial compounds at different temperatures, the cell free supernatant was treated at 15, 25, 35, and 45 °C for 1 hour. Antibacterial activity (26 mm) was observed until 40 °C, but the activity decreased at a temperature above 40 °C and was totally lost with further rise in temperature. The antimicrobial compound produced by *B. licheniformis* KC2-MRL was stable at pH 5–8, although highest activity was observed at pH 5 and 6, whereas activity decreased at pH 7 and 8.

Vancomycin, nalidixic acid, cefotoxime, ampicillin, amoxicillin, imipenem, methicillin, cefotetan, and levofloxacin were tested to check the susceptibility of *Bacillus licheniformis* KC2-MRL. The organism was more susceptible to levofloxacin, which produced a 40 mm zone of inhibition (Fig. 10).

We used a solution of bacitracin as a standard. FTIR spectrum of *B. licheniformis* KC2-MRL's precipitated protein was compared with the standard. The FTIR spectrum

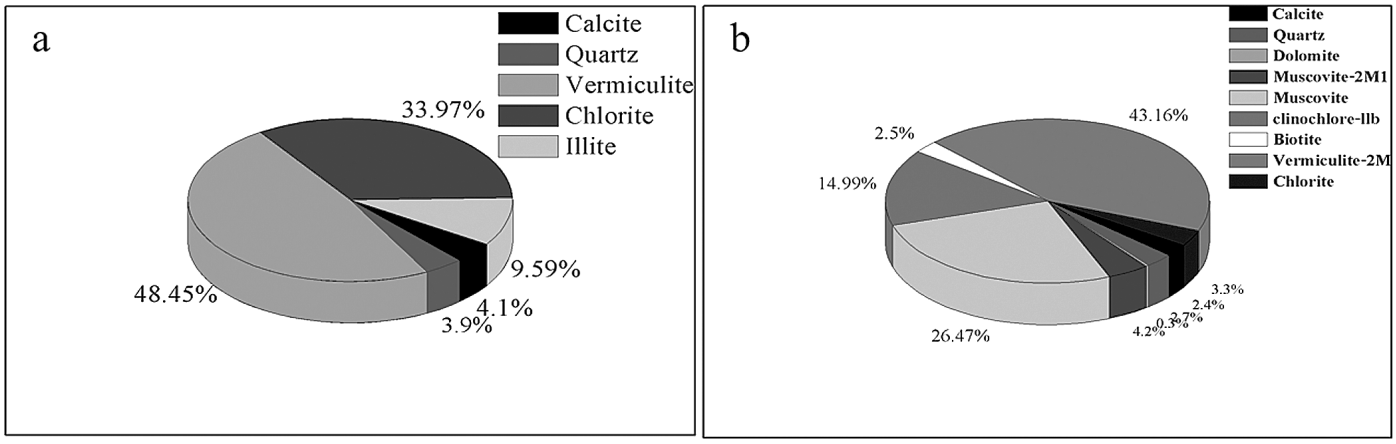

Figure 3. Distribution of minerals identified in soil sample smast-7 from wall (a) and smast-5 from floor (b).

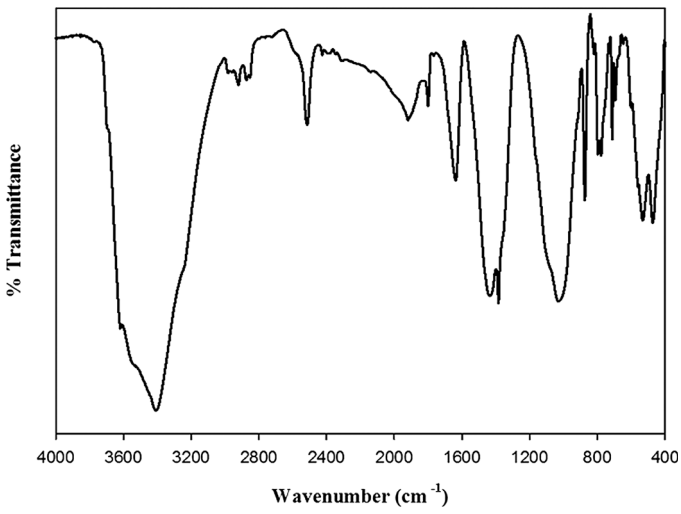

Figure 4. Fourier-transform infrared absorption spectrum of soil sample smast-7 from the cave wall.

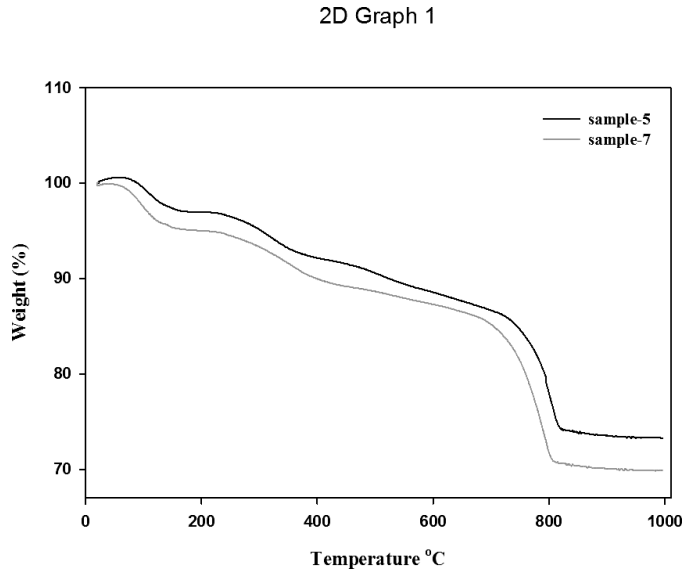

Figure 5. Thermogravimetric analysis plots of Kashmir Smast soil samples 5, cave soil, and 7, cave wall.

of bacitracin showed the absorption bands at 3295.63, 3016.9, 2133.64, and 1635 cm^{-1} that correspond to NH, CH, C–C, and C=C groups. Similarly, in the case of *B. licheniformis* KC2-MRL protein the absorption bands appeared at 3271.98, 3016.90, 2120.12, 1635.20 and 1076.22 cm^{-1} which were attributing to NH, CH, C=C and C–N (Fig. 11).

Discussion

Solution caves are formed in carbonate and sulfate rocks such as limestone, dolomite, marble, and gypsum by the action of slowly moving groundwater that dissolves the rock to form tunnels, irregular passages, and even large caverns along joints and bedding planes (Davies and Morgan, 2000). Caves usually have very low nutrient availability, but they still contain diverse, and often unique, microbial communities (Barton, 2006). Caves on other worlds such as Mars may provide protected sites for extraterrestrial life forms (Nelson, 1996). The subsurface of Earth is considered as the best possible site to look for microbial life and the characteristic lithologies that indicates the remnants of life (Boston et al., 2001). Microbial analysis of caves showed *Bacillus* as the most commonly detected microbial genus (Adetutu et al., 2012). It is important to understand how the ecosystems are operating and accommodating microbial diversity. The rock composition and mineralogy can be helpful to understand the geomicrobiology and potential metabolic capabilities of the microorganisms to use ions within the rock as nutrients and for chemolithotrophic energy production. Cave sediments can therefore act as reservoirs of microorganisms (Adetutu et al., 2012). The use of these ions may be supported by the formation of a corrosion residue, through microbial scavenging activities (Barton, 2006). Cave microorganisms also have potential to produce unique antibiotics and cancer treatment drugs (Onaga, 2001). Minerals have profound effect on the production of antibiotics by microorganisms. Basak and Majumdar (1975) reported that kanamycin production by *Streptomyces kanamyceticus* ATCC 12853 required magnesium sulfate and potassium phosphate (0.4 and 1.0 g L^{-1}, respectively) and Fe and Zn (0.25 and 0.575 μg mL^{-1}, respectively), amounts of Mn and Ca did not have any effect, and Cu, Co, Ni, and V have inhibitory effect. Divalent ions as Mn^{2+}, Cu^{2+}, Fe^{2+} stimulated AK-111-81 antibiotic biosynthesis by *Streptomyces hygroscopicus* 111-81 (Gesheva et al., 2005). The divalent metal ions (Mg, Fe and Mn) sodium dihydrogen phosphate were found essential for bacitracin production by *Bacillus licheniformis*,

Table 2. Concentrations of some metals from soil sample collected from the floor of Kashmir Smast and control sample from outside the cave, determined by atomic-absorption spectroscopy.

	Metals, mg kg^{-1}							
Soil Samples	Ni	Cr	Co	Cu	Zn	Ca	Mg	Pb
Cave Soil	0.965	0.571	0.266	1.824	12.7311	332.938	1.2576	1.31
Control Soil	10.4	8.74	0.810	4.7	36.41	121.65	1.023	8.14

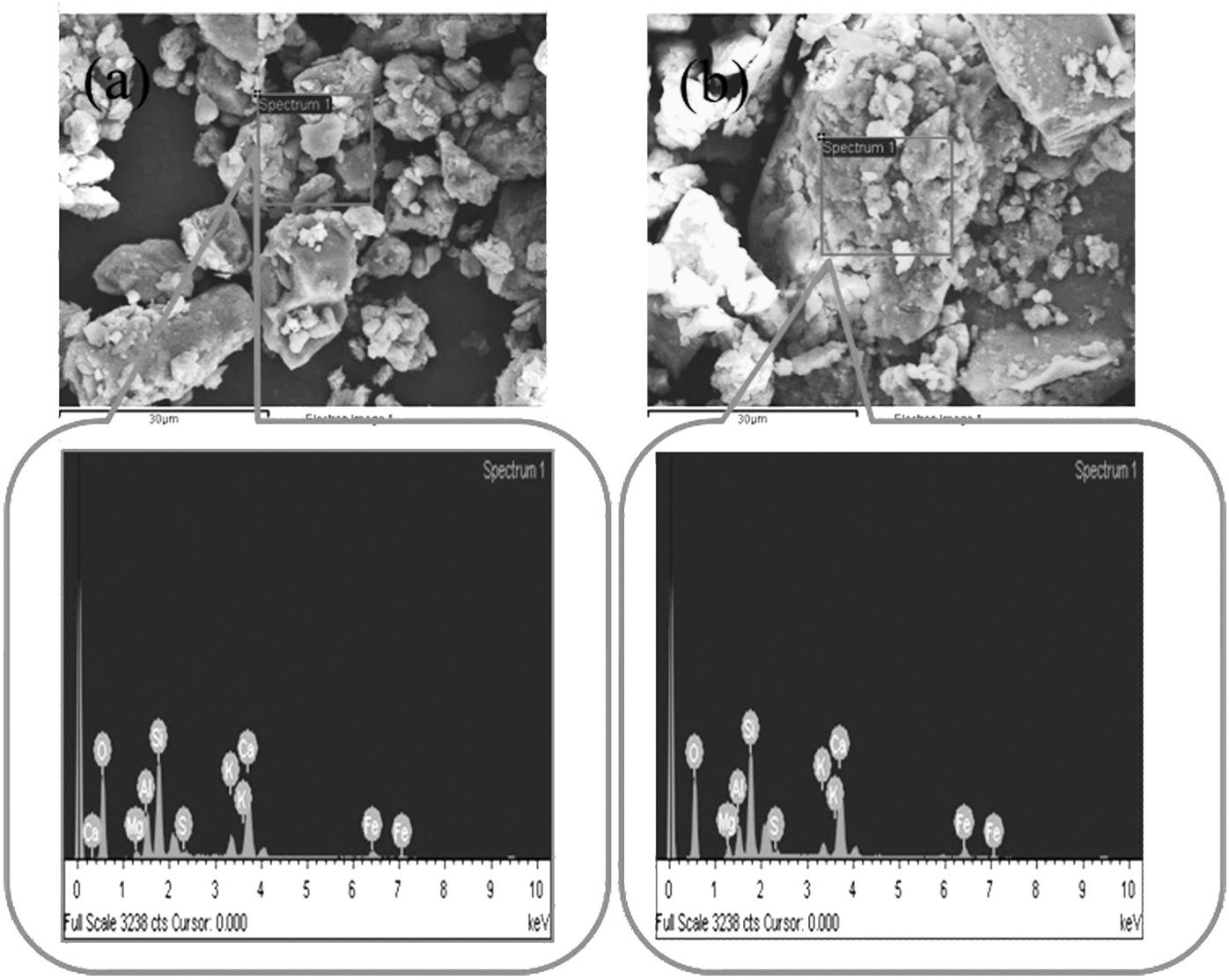

Figure 6. Scanning electron micrograph and energy-dispersive X-ray spectroscopy results for samples smast-7 from cave wall (a) and smast-5 from cave floor (b).

whereas Na_2SO_4 and $CaCl_2$ decreased the bacitracin yield (Yousaf, 1997).

The soil sample from which *B. licheniformis* KC2-MRL was isolated was reddish-brown in color. Brown soils are usually low in organic matter. Terra rossa is a soil that is heavy and clay-rich soil, strongly reddish, developed on limestone or dolomite, usually derived from the insoluble residue of the underlying rock. Following dissolution of calcium carbonate by rain, clay contained in limestone sediments, along with other insoluble substances or rock fragments, forms discontinuous residual layers variable in depth. Under oxidizing conditions iron oxides appear that produce the characteristic red color. According to this theory, terra rossa is usually considered a polygenetic relict soil, formed during the Tertiary and subjected to hot and humid periods during the Quaternary (Jordán, 2014).

X-ray diffraction analysis of the cave sample confirmed the presence of clay minerals, carbonates, and silicates (Hill, 1999). Minerals are produced as a result of intense chemical weathering on land under possibly tropical conditions, where abundant rainfall favored ionic transfer and pedogenic development (Millot, 1970).

Carbonates found in Kashmir Smast are predominantly calcite and traces of dolomite (Vogel et al., 1990; Schwabe et al., 1993). In caves, illite is found mostly in fault zones and also occurs as clay floor deposits (Hill, 1999). Illite is commonly present as little-altered, disintegrated particles (Weaver, 1989). Pedogenic clay minerals are derived from moderate chemical weathering and generally develop in poorly drained tropical to subtropical areas of low relief, marked by flooding during humid seasons and subsequent concentration of solutions in the soil during dry seasons.

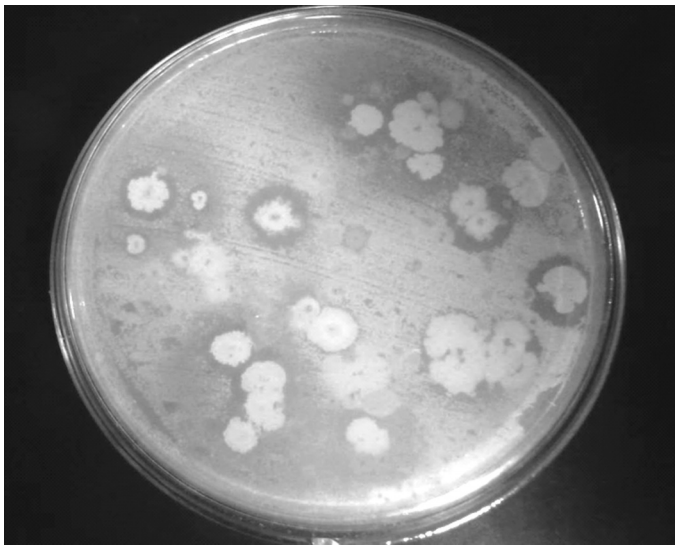

Figure 7. Nutrient-agar plate showing typical zones of inhibition due to antimicrobial activity against a test strain.

Al, Fe, and Si are transported by means of water saturation during wet seasons; concentration for mineral growth takes place during in dry seasons (ChamLey, 1989). During pedogenesis, chlorite transforms into kaolinite, and in intensely weathered laterite soils chlorite would be completely eliminated (Vicente et al., 1997). The accumulations of illite, kaolinite, chlorite, dolomite, and muscovite in Kashmir Smast are probably indicative of changes in degree of weathering, and thus reflect the changes in climatic conditions. The degree of weathering related to the presence of SiO_2 and Al_2O_3 shows a similar pattern to clay minerals (Tardy and Nahon, 1985; Zhao and Yang, 1995). The mineral assemblages investigated in the cave are diverse.

The quantitative mineral analysis technique SIROQUANT determined mineral compositions of rocks, including clay mineral content. Thermal analysis offers an important technique for the determination of thermal stability of minerals and roughly estimating organic content of samples. Importantly, the decomposition curves can be obtained and mechanism of decomposition of the mineral determined. Generally, the theoretical mass loss of water is 10.46%, and structural disorganization upon thermal treatment may occur in response to the loss of hydration water, which could provoke collapse of the crystalline structure (Doak et al., 1965). The two overlapping mass loss steps at 263 and 280 °C are attributed to the hydroxyl group (Palmer and Frost, 2010). The higher mass loss at 280 °C is believed to be due to the loss of both OH^- and CO_3^{2+}. The broad mass loss at 485 °C is ascribed to the loss of carbonate as carbon dioxide (CO_2) (Frost et al., 2009). The higher temperature mass loss at 828 °C is attributed to the Mg.

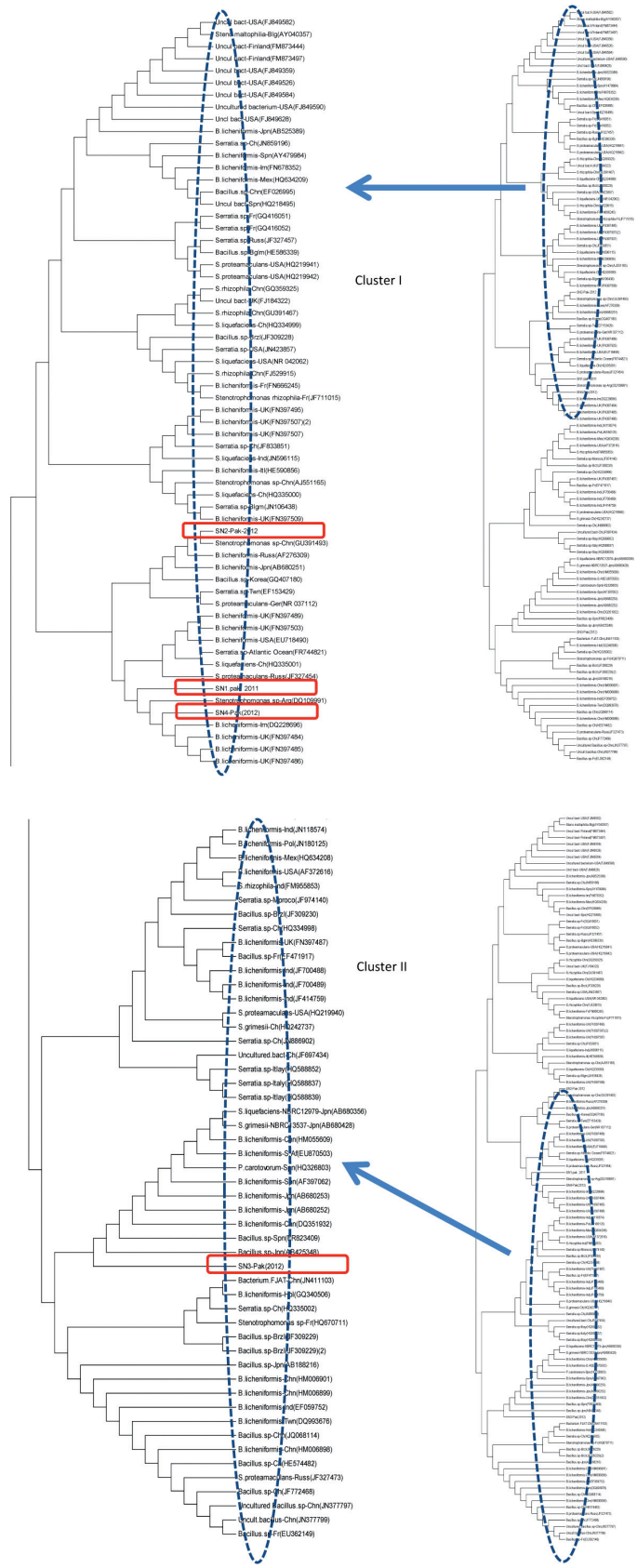

Figure 8. Phylogenetic tree showing all four isolates with related sequences in NCBI.

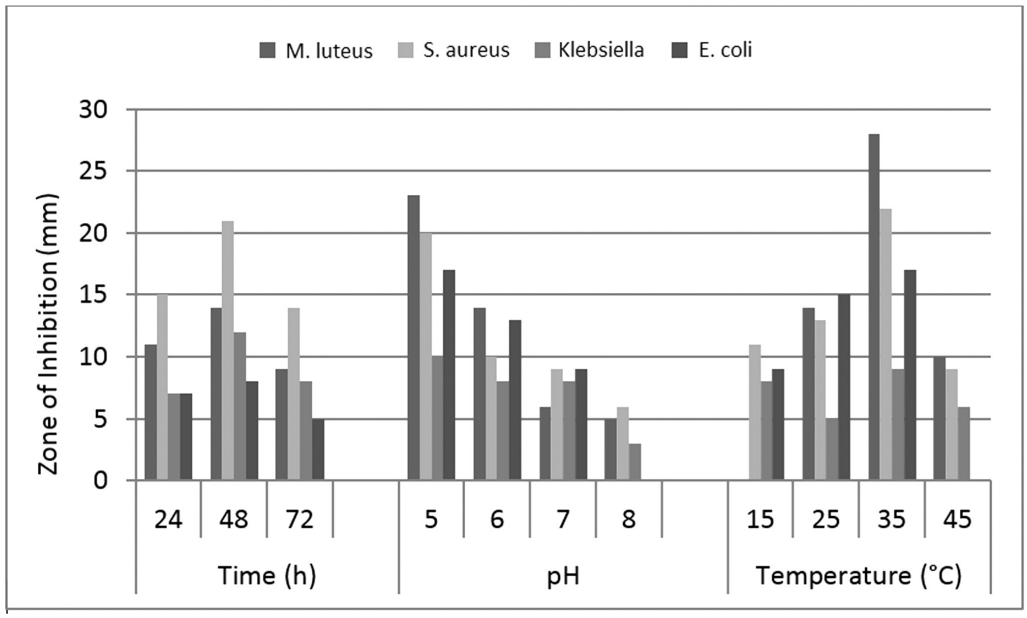

Figure 9. Effect of time of incubation, pH, and temperature on the growth and antimicrobial activity produced by growth of *Bacillus licheniformis* KC2-MRL against *Micrococcus luteus*, *Staphylococcus aureus*, *Klebsiella* sp., and *E. coli*.

Clay particles were observed to have poorly crystallized clasts with angular, irregular outlines, and swirly texture with face-to-face arrangement of clay grains, as also reported by Manju et al. (2001) in the Madayi kaolin deposit, North Kerala, India. Generally, intensely weathered clay flakes show ragged edges, exhibit a rounded outline or bay-shaped edges, and poor lateral dimension, with a particularly small platy thickness. Analysis shows that Si, Al, and Fe were enriched within the samples, which probably reflects minerals such as quartz, feldspar, clay minerals, and iron oxide (Jeong et al., 2003).

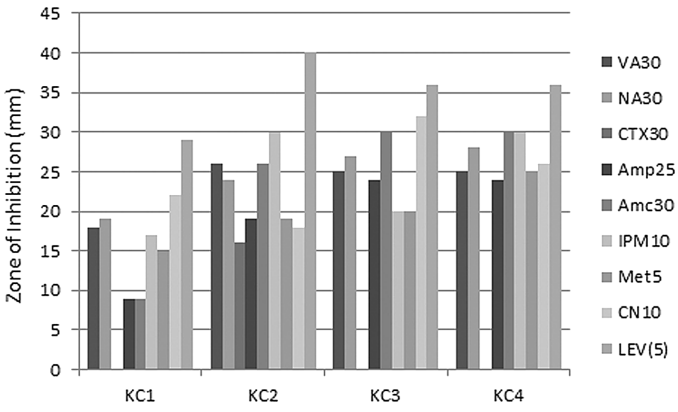

Figure 10. The results of disk-diffusion assay of the susceptability of our four antibiotic producing strains (*Serratia* sp. KC1-MRL, *Bacillus licheniformis* KC2-MRL, *Bacillus* sp. KC3-MRL and *Stenotrophomonas* sp. KC4-MRL) to selected antibiotics. Names of antibiotics can be found in the Results section of the text.

Fourier-transform infrared spectroscopy analysis showed peaks at 885 cm^{-1}, 746 cm^{-1}, and 715 cm^{-1} because of presence of dolomite (White, 1964; Van Der Marel and Beutelspacher, 1976). A wide band around 1020 cm^{-1} is assigned to quartz, SiO_2 (Russell, 1987; Ravisankar et al., 2012), and the peak at 1646 cm^{-1} is attributed to the bending vibration modes of water (Manoharan et al., 2007). Peaks in the region of 2800–3000 cm^{-1} are ascribed to the C–C stretching that is present in the form of organic matter in the mineral contribution (Maritan et al., 2005) or may be due to P–OH bond stretching around 2845 cm^{-1} and 2935 cm^{-1}. The sharp peak at 2513 cm^{-1} is due to the presence of silicate minerals like quartz, nimite, musciovite, and vermiculite (Vedder, 1964). The appearance of broad band in the region of 3000 cm^{-1} to 3700 cm^{-1} is attributed to the structural water present in the mineral vermiculite and to the moisture present in the sample (Zadrapa and Zykova, 2010). The hydroxyl and water-stretching region near 3200 cm^{-1} for most hydrated carbonates usually consists of one or two broad bands shifted somewhat to lower frequencies due to hydrogen bonding (Nakamoto, 2008; Schrader, 1995), but the appearance of the broad band is due to the interpretation OH^- and H_2O in a mineral in which some minerals were participating in hydrogen bonding and some were not involved, e.g., non-hydrogen-bonded Al-OH units (White, 1964; Van Der Marel and Beutelspacher, 1976). Atomic absorption spectroscopy was performed to determine the concentrations of the elements calcium, magnesium, chromium, cobalt, nickel, zinc, copper, and lead in the cave floor soil sample, and it was found that the soil contained very high amount of calcium compared to outside soil.

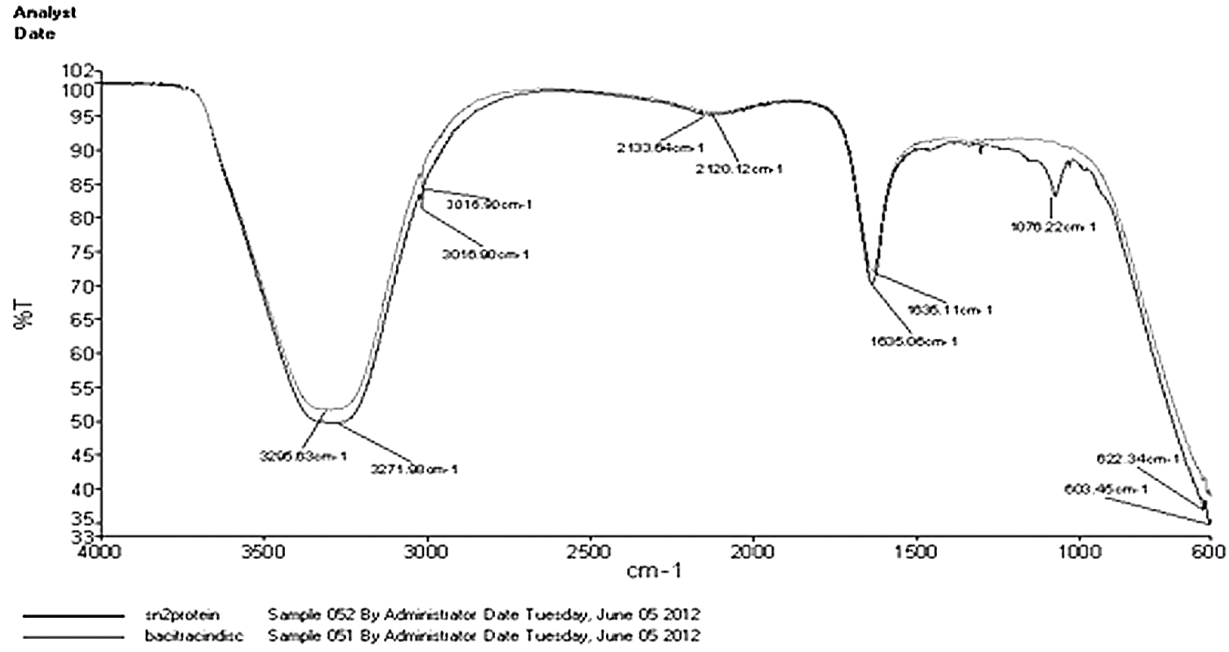

Figure 11. Comparison of Fourier-transform infrared spectra of bacitracin (lighter line) and the antibacterial compound produced by *Bacillus licheniformis* KC2-MRL (darker line).

Microbiology

The capacity of bacteria inhabiting karstic caves to produce valuable biologically active compounds has still not been investigated much (Tomova et al., 2013). Soil is a natural reservoir for microorganisms and their antimicrobial products (Dancer 2004). The four selected strains isolated from cave soil were screened for the production of antibiotics by using agar-well diffusion assay against *Staphylococcus aureus*, *Klebsiella*, *E. coli* and *Micrococcus luteus*. *B. licheniformis* KC2-MRL was selected for further analysis on the basis of the greatest zone of inhibition. In the present study, *B. licheniformis* KC2-MRL showed the best antimicrobial activity against *M. luteus*, followed by *S. aureus*, *Klebsiella* and *E. coli* after 48 hrs of incubation.

Studies show that caves are inhabited by different types of microorganisms having unique characteristics. A cave ecosystem has a deficiency of nutrients, which is why microorganisms present in the cave compete for the nutrients and fight for survival. Due to this struggle among microbes, they have the potential to produce antibiotics against other microbes. There are nine different groups of bacteria that have been reported to be present in caves, Proteobacteria, Acidobacteria, Planctomycetes, Chloroflexi, Bacteroidetes, Gemmatimonadetes, Nitrospirae, Actinobacteria and Firmicutes (Zhou et al., 2007; Porttillo et al., 2008). Proteobacteria are the dominant bacteria in caves (Zhou et al., 2007). The 16S rRNA gene sequences of our antibiotic producing cave bacteria have been submitted to NCBI GenBank. The isolates KC1-MRL, KC2-MRL, KC3-MRL and KC4-MRL were identified as *Serratia* sp. KC1-MRL (Accession No. KC128829.1), *Bacillus licheniformis* KC2-MRL (Accession No. KC128830.1), *Bacillus* sp. KC3-MRL (Accession No. KC128831.1), and *Stenotrophomonas* sp. KC4-MRL (Accession No. KC128832.1). In Magura Cave, Bulgaria, Tomonova et al. (2013) reported that Gram-positive bacteria were represented by the genera *Bacillus*, *Arthrobacter*, and *Micrococcus*.

Soil bacterial genera such as *Bacillus, Streptomyces*, and *Pseudomonas* synthesize a high proportion of agriculturally and medically important antibiotics (Hosoya et al., 1998; Sharga et al., 2004). Peptide antibiotics are the major group of antibiotics (Pinchuk et al., 2002). Antibiotic-producing microorganisms can be found in different habitats, but the majority are common inhabitants of soil. Caves contain abundant *Actinobacteria*, which are valuable sources of novel antibiotics that can replace currently ineffective antibiotics (Montano and Henderson, 2012). Molecular analysis of a sample from Kashmir cave showed the presence of different bacterial strains.

Isolated strains were screened for the production of antimicrobial compounds by using agar-well diffusion assay. Ducluzeau et al. (1978) isolated *Bacillus licheniformis* that was active against *Clostridium perfringens* or *Lactobacillus* sp. Muhammad et al. (2009) also observed that *Bacillus* metabolites showed activity against *M. luteus* and *S. aureus*. Bacitracin is a major polypeptide antibiotic produced by *Bacillus licheniformis* and *Bacillus subtilis*, based on using *M. luteus* as a test organism (Vieira et al., 2011). *B. licheniformis* isolated from marine sediments showed best antimicrobial activity against pathogenic test strains *S. aureus*, *E. coli* and *P. aeruginosa* (Hosny et al., 2011). Antibiotic

production depends upon the composition of the medium, which is required for cell biomass and for its maintenance (Stanbury et al., 1995, chap. 4). Maximum activity was found when *Bacillus licheniformis* was grown in nutrient broth. Similarly, Vieira et al. (2011) used nutrient broth for the growth of *B. licheniformis* when incubated at 46 °C in a shaking incubator at 150 rpm. Al-Janabi (2006), Yilmaz et al. (2006), and Al-Ajlani and Hasnain. (2010) also reported maximum production of antimicrobial compound by *Bacillus* sp. in nutrient broth medium at varying temperatures.

External factors can also affect the growth of microorganisms and the production of antibiotics (Marwick et al., 1999). It has been reported that environmental factors such as temperature, pH, and incubation duration influence antibiotic production (Iwai et al., 1973). In our study, the optimum temperature for antimicrobial compound production was observed to be 30 to 35 °C. Béahdy (1974) and Haddar et al. (2007) observed production of bacitracin and other antibiotics by *B. licheniformis* (Zarei, 2012) at 37 °C, and it was also seen at 30 °C by Hosny et al. (2011).

We found that our selected organism showed optimum activity at pH 5–6. Flickinger and Perlman (1979) reported pH 6.5 for the optimum production of antibiotics by *B. licheniformis*. Haddar et al. (2007) found maximum bacitracin production rate (192 units/mL) at pH 7.5. A similar study was conducted by Gulahmadov et al. (2006) that found antimicrobial activity was best at the wide pH range of 6–8 by *Bacillus* sp. Newly emergent infectious diseases, re-emerging diseases, and multidrug-resistant bacteria mean that there is a persistent need to produce novel antimicrobial compounds (Uzair et al., 2009).

We performed an antibiotic susceptibility test in which *B. licheniformis* KC2-MRL was found resistant to cefotaxime, but was more susceptible to levofloxacin, which produces a 40 mm zone of inhibition. *B. thuringiensis* RSKK 380 was reported to be unaffected by cephazolin, cefoxitin, and cefamandole (Yilmaz et al., 2006).

Our results show that the antibacterial activity was stable up to 45 °C. A similar study by He et al. (2006) reported *B. licheniformis* to be stable at 25 °C for 6 hrs and inactivated above 40 °C. However, in some cases the antimicrobial compounds retained their activity even after autoclaving the sample at 121 °C (Fontoura et al., 2009; Tabbene et al., 2009; Uzair et al., 2009; Ebrahimipour et al., 2010). At the same time, sensitivity to different pH values was also evaluated in the present study, and the antimicrobial compound was found to be stable at pH 5–7. A similar study, in which antimicrobial activity was found to be stable at pH 7, was reported by He et al. (2006). The stability of antibacterial activity at pH 7 and after heat-treatment might be useful in several industrial applications (Tabbene et al., 2009). Our study showed best activity against *M. luteus*, *S. aureus*, and *E. coli* after 48 hours of incubation. A similar study by Aslim et al. (2002) showed the maximum zone of inhibition after 24 to 48 hrs.

Bacillus licheniformis KC2-MRL was further tested for antibiotic sensitivity by using the antibiotics vancomycin, nalidixic acid, cefotoxime, ampicillin, amoxicillin, imipenem, methicillin, Cefoten, and levofloxacin. It was found that the selected strain was more susceptible to levofloxacin, which produced a 40-mm zone of inhibition (Fig. 10).

Sirtori et al. (2006) reported clear absorption peaks at 3,500, 2,925, 1,639, and 1,546 cm^{-1} corresponding to the O–H, C–H, C–N and angular deformation of the N–H bond. Kong and Yu (2007) also detected bands at peaks of 3100, 1600–1690, 1480–1575 and 1229–1301 cm^{-1} that are assigned to N–H, C=O, C–N and N–H. Kumar et al. (2010) reported absorption bands at 1670, 1539, 1418, and 1488 cm^{-1} attributing to N–H, C=O, O–H and CO.

CONCLUSION

Our study explored the ability of cave microorganisms to produce antibiotics and characterized of the producing strain. Due to the internal acidic environment and high calcium concentration in the cave, *Bacillus licheniformis* KC2-MRL grew better under acidic conditions at temperatures higher than that in the cave. Caves of Pakistan had never been explored for the presence of bacteria with regards to diversity or having ability to produce novel antimicrobial metabolites. These metabolites, as well as those produced in other caves, can be further investigated to find bioactive compounds with unique characteristics.

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