

# Bacteria isolated from a limestone cave and its ability to produce industrially important enzymes

## Abstract

Caves have extreme environment with diverse microbial flora. In the present study a bacterial isolates, KC5- MRL, AF-3, AF-6 and AF-23 isolated from Kashmir Smast(cave), a limestone cave in PirsaiMardan,, KPK, Pakistan, was found to produce three industrially important enzymes; lipase, protease and amylase. The bacterial isolates were identified as *Lysinibacillus sphaericus* KC5-MRL, *Bacillus subtilis* AF-3, *Bacillus subtilis* AF-6 and *Bacillus subtilis* AF-23(Accession No. KF010827, MH027600, MH027601 and MH027602), respectively. Optimum pH for the growth of *Lysinibacillus sphaericus* KC5-MRL, was around 7 and grew best at 35°C. The optimum activities of lipase and amylase was observed at 30°C after 24 hr of incubation and pH 5 (42.23 U/ml) and (15U/ml) respectively. Maximum lypolytic activity (181.93 U/ml)was observed when 8% inoculum was used. Proteolytic activity of *L. sphaericus* KC5-MRL was found to be 59 U/ml, after 48 hr at 30°C.Highest stability of lipase, amylase and protease was observed at pH 10, 7 and 8 respectively. *L. sphaericus* KC5-MRL lipase, protease and amylase were stable at 35°C.Triton X-100 and sodium dodecyl sulfate (SDS) stimulated the lipase and protease activities, whereas, Triton X-100 and T-80 stimulated amylolytic activity. Lipase activity was stimulated by  $Mg^{++}$ ,  $NH_4^+$  and  $Ca^{++}$ . Amylase activity was stimulated by  $Na^+$  and  $Ca^{++}$ . Protease activity was stimulated by  $Na^+$  and  $Mg^+$ . Chloroform, formaldehyde, methanol and benzene stimulated amylase activity. Nitrobenzene, methanol, benzene, and acetone stimulated protease activity. Ethylenediaminetetraacetic acid (EDTA) showed stimulatory effect on lipase. EDTA, Trisodium citrate TSC, mercaptoethanol, Phenyl acetaldehyde (PAA) and PMSF reduced amylase activity. All the modulators reduced protease activity except TSC and PMSF which stimulated its activity. The study concludes that these enzymes can be used for different purposes in various industries such as food and detergent

**Keywords:** KC5- MRL, Kashmir smast, enzymes, limestone, Pakistan

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## Introduction

Caves are known to have very poor nutrient ecology and have constant low temperature and high humidity. Because of this environment, the cave organisms have some extraordinary properties to survive and live in such hard and crucial condition.<sup>1-3</sup> Cave ecology is rich in different types of microorganisms having outstanding characteristics. The most abundant organisms observed in caves are filamentous and belong to *Actinobacteria* group, followed by coccoid and bacilli forms.<sup>4</sup> A few pathogenic microorganisms have been reported from the Altamira cave.<sup>5</sup> Luonet al.<sup>6</sup> described for the first time, *A. altamirensis* recovered from human medical constituents. *E. coli* and *S. aureus* which are disease causing bacteria have also been isolated from caves,<sup>7</sup> species of *Pseudomonas*, *Sphingomonas* and *Alcaligenes sp*<sup>8</sup> and *Inquilinus*.<sup>9</sup> Nutrient recycling in the cave totally depends upon the bacterial and fungal activities.<sup>10</sup> Caves have fewer amounts of nutrients, less light or no light ecosystem, which have relatively low temperature and high humidity. From different caves, around the world, different types of microbial species have been isolated.<sup>11</sup> Most of the caves which have been studied are in Spain, Italy, France, Romania and USA. Altamira Cave in Spain and Lascaux Cave of France is the most microbiologically studied cave.<sup>12,13</sup> Significant growth in biospeleological research has been observed in the last two decades.<sup>14</sup> Caves are considered as extreme environments providing niches for highly specialized microorganisms.<sup>15</sup> Enzymes which are isolated from cave microbes are active at low temperature. Enzymes of psychrotrophs have great biotechnological importance.<sup>11</sup> These microbes survive in such environment because of production of

enzymes which function in such extreme conditions.<sup>16</sup> Our knowledge about cave microbial diversity is limited, so there is great potential to find novel microorganisms in caves.<sup>17</sup> Caves in Pakistan are less studied area especially with regards to industrially important enzymes from cave bacteria have not been reported or isolated so far. Therefore, in our study we focused, on the isolation of bacteria from Kashmir cave, and their potential to produce industrially important enzymes having some unique properties.

## Materials and methods

The present study was carried out in the Microbiology Research Laboratory (MRL), Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan. Production of lipase, amylase and protease by bacterial isolates, isolated from a limestone cave (Kashmir Smast Pirsai Mardan, Pakistan) (Figure 1) was investigated. The cave (GPS co-ordinates 34° 25' 48.24" N 72° 13' 43.18" E) is 188m long, 30m high and 25m wide, is located in Pirsai Mardan, Pakistan. Soil samples were collected in sterile bags under aseptic conditions.

## Qualitative tests for three enzymes

### Protease, Lipase and Amylase

Nutrient agar plates containing (1%) casein as substrate were used to check the proteolytic activity which was observed in the form of clear zones of hydrolysis around the colonies. For the screening of lipase, Tween-80 was used as lipid substrate. After incubation at 37°C for 24h, appearance of clear zones was observed. Starch was used as

substrate to detect the production of amylolytic enzyme. Inoculated plates were incubated at 37°C for 24h and clear zones of hydrolysis were observed.



**Figure 1** Limestone cave (Kashmir Smast Pirsai Mardan, Pakistan).

### PCR amplification and sequencing of 16S rRNA gene

To amplify the 16S rRNA gene the genomic DNA was extracted according to Roohiet al. (2012). Briefly, few isolated bacterial colonies were suspended in TE buffer in a micro-centrifuge tube. Followed by heating for 10 min at 95°C and centrifugation at 6,000 rpm for 5 min. to amplify the 16S rRNA gene the supernatant was used as template DNA. Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene, using primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTGTTACGA-3') using Premix Ex Taq (Takara, Japan) following protocol described previously. The amplification was carried out under the optimized PCR conditions: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1:30 min. The final extension was performed at 72°C for 5 min in ABI Veriti PCR Machine (Applied Biosystems, USA). The amplified PCR products of 16S rRNA gene of bacterial strain was purified and sequenced using the primers 27F (5' AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACCTGTTACGACTT-3') using commercial service of Macrogen Inc. Korea (<http://dna.macrogen.com/eng/>).

### Phylogenetic analysis of bacterial isolates

BioEdit software (Hall, 1999) was used to assemble the fragment sequences of 16S rRNA gene. The 16S rRNA gene sequences were submitted to NCBI (<https://www.ncbi.nlm.nih.gov/>). Using 16S rRNA gene sequences, the strain was identified by BLAST search on EzBiocloud Server). Closely related type species sequences were retrieved to assess the molecular evolutionary relations and to construct phylogenetic tree using MEGA version.<sup>6</sup> A phylogenetic tree was constructed from unambiguously aligned nucleotides using the neighbour-joining (NJ) algorithm. The relationship stability was evaluated by boot strap analysis performed using 1000 re-samplings of the neighbour-joining data for the tree topology.

### Quantitative analysis of lipase

About 150ml of medium was added in four different Erlenmeyer flasks, autoclaved for 20 minutes at 121°C and 15lbs. About 1.5ml of olive oil was added to the above medium which was already sterilized in oven at 121°C for half an hour. Then medium was inoculated with two loops full of culture under sterilized conditions, and then flasks

were kept in shaking incubator at 37°C and 150rpm. Samples were drawn after 0, 24, 48 and 72h. Enzyme assay for lipase was performed by the method of.<sup>18</sup> Unit of enzyme is defined as the amount of enzyme that hydrolyzes 1µmol substrate in 1 minute.

### Quantitative test for protease

Medium (Gelatin: 15.0g/250ml, Casein hydrolysate: 0.5g/250ml, Glycerol: 3.0ml and pH 8) of 250ml was poured to four Erlenmeyer flasks, pH was adjusted by using 0.1N NaOH and 0.1N CH<sub>3</sub>COOH. The medium was autoclaved at 121°C 15lbs pressure for 20 minutes 3.0ml of 20% glycerol solution sterilized in dry oven at 100°C for half an hour was poured into the medium aseptically. Then two loops full of bacterial culture were inoculated in different flasks. Then flasks were kept in shaking incubator at 37°C with 150rpm. Samples were collected after 0, 24, 48 and 72hrs for quantitative analysis.

### Enzyme assay for protease

Enzyme assay for protease (Kunitz,1965), briefly 1ml of casein solution (1% casein solution in 0.02M Tris buffer, pH 8.5) was added into 1ml of crude enzyme. The mixture was incubated at 45°C for 1hour. After incubation 3ml of 5% TCA was added. Then tubes were placed in ice for 15minutes the precipitates were removed by centrifugation at 5000rpm for 15 minutes at 4°C. About 5% TCA was added in blanks before incubation. The optical density of the supernatant was measured at 280nm. Graph for standard curve was prepared using tyrosine as standard. One unit of enzyme caseinolytic activity defined as that amount of enzyme which releases 1µ mol tyrosine under standard conditions of assay method.

### Shake flask fermentation for optimization of different parameters

To determine the optimum pH for lipase production, fermentation was carried out at different pH (3, 4, 5, 6, 7, 8 and 9) and optimum temperature was determined by carrying out shake flask fermentation at various temperatures (15, 25, 30, 35 and 40°C). For the size of inoculum a 24h old bacterial culture grown in nutrient broth was added at a concentration of 1 to 10% to the production medium. About 150 ml of production medium was poured into four different flasks. Samples were taken after 0, 24, 48 and 72hr and assayed for lipase activity, and centrifuged at 10,000rpm for 30 minutes at 4°C. Precipitates were removed by filtration and supernatant was used as the crude enzyme for the estimation of enzyme activity.

### Effect of temperature, pH, metals ions, modulators and solvents on enzyme activity

Activity of crude enzyme was determined after incubating for 1hr at 30, 35, 40, 50, 60, 70 and 80 and 100°C. Effect of pH on the activity of crude enzyme extract was studied at different pH. The crude enzyme was incubated for 1h in buffers having different pH [0.02 M of acetate buffer (pH 4, 5, 6), phosphate buffer (pH 6, 7), Tris-HCl (pH 8), glycine-NaOH buffer (pH 9) and Na<sub>2</sub>HPO<sub>4</sub>/NaOH (pH 10)], and remaining enzyme activity was determined under standard conditions. Effect of different metals (100mM of HgCl<sub>2</sub>, NaCl, MgCl<sub>2</sub>, ZnCl<sub>2</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and CaCl<sub>2</sub> separately) on activity of enzymes. These metals were pre-incubated with crude extract (1:1) for 1hr at room temperature and then remaining activity was determined. Effect of different modulators on enzyme activity was determined for EDTA, Trisodium citrate, mercaptoethanol and PMSF. About 1% of these compounds were pre-incubated for 1h at room temperature (25°C) with crude enzyme (1:1). Effect of different solvents on activity

of lipase was determined after pre-incubating crude enzyme in the presence of (1% each, separately) xylene, benzene, chloroform, nitrobenzene, formaldehyde, methanol and acetone (1:1) for 1h at room temperature. For protein estimation, Lowry's method<sup>19</sup> was used.

### Statistical analysis

All statistical analyses were performed using SPSS version 20, and one-way analysis of variance (ANOVA) followed by LSD was used to check for the differences of enzymes activity under different treatments.  $P \leq 0.05$  and  $P \leq 0.01$  were statistically significant. The figures of differences of enzymes activity between treatments was prepared by Origin (Origin Pro 9.0 for Windows).

## Results

### Primary screening for the enzymes (lipase, amylase and protease)

All the strains were Gram positive rods. Phylogenetic analysis shows that AF-3, AF-6 and AF-23 closely related to *Bacillus subtilis* and KC5-MRL was closely related to *Lysinobacillus sparcicus*. On the basis of quantitative analysis *Bacillus subtilis* AF-6, *Bacillus subtilis* AF-3, *Lysinobacillus sparcicus* KC5-MRL, and *Bacillus subtilis* AF-23 strains were positive for the enzymes and selected for further study. Growth of all the strains were optimum at neutral pH 7, at 35°C and almost no growth at temperature 15 and 40°C, and size of inoculum 8% after 72h.

### Effect of pH, temperature and size of inoculum for the optimum lipase, amylase and protease activity

The optimum activity of *L.sphaericus* KC5-MRL lipase was observed at 30°C after 24 hr of incubation and pH 5 i.e. 42.23U/ml. Maximum lypolytic (181.93U/ml), proteolytic (59 U/ml) and amylolytic (15U/ml) activity was observed when 7% of inoculum was used at 30°C and 24 h of incubation (Fig.). *Bacillus subtilis* AF-3 produced maximum lipase (20U/ml), amylase (9.74U/ml) and protease (40.5U/ml) at 6% inoculum 24 and 72h of incubation at 35°C, 30°C respectively. *Bacillus subtilis* AF-6 produced maximum lypolytic (21U/ml), proteolytic (40.7U/ml) and amylolytic (15.59U/ml) at 6% of inoculum after 24, 48 and 72h of incubation at 30°C, and pH 5, respectively. *Bacillus subtilis* AF-23 produced maximum lypolytic (18U/ml), proteolytic (77.5U/ml) and amylolytic (9.78U/ml) at 5% inoculum after 48h of incubation at 25°C and 30°C and pH 6, respectively.

### Characterization of crude lipase, amylase and protease produced by the isolates

**Stability of crude enzymes at different pH:** Amylolytic activity of *L. sparcicus* KC5-MRL, *B. subtilis* AF-3, *B. subtilis* AF-6 and *B. subtilis* AF-23 showed highest stability at pH 7, 10, 8 and 10 i.e 99.4%, 101%, 99.89% and 107%, respectively. Proteolytic activity of *L. sparcicus* KC5-MRL, *B. subtilis* AF-3, *B. subtilis* AF-6 and *B. subtilis* AF-23 were more stable at pH 8, 7, 8 and 10 i.e 115%, 127%, 106% and 102 respectively. Proteases of all the four strains were stable at all pH values. Lipases of all the strains were not stable at pH 4. Highest stability of *L. sparcicus* KC5-MRL, *B. subtilis* AF-3, *B. subtilis* AF-6 and *B. subtilis* AF-23 lipases were observed at pH 10, 7,8 and 6 i.e. 42%, 40.5%, 34.5% and 41% respectively.

**Stability of crude enzymes at different temperature:** Lowest stability of lipase of all the isolates was observed at 100°C. *L.*

*sphaericus* KC5-MRL, *B. subtilis* AF-3, *B. subtilis* AF-6 and *B. subtilis* AF-23 lipases highest stability were observed at 35°C (118%), 40°C (80%), 30°C(105%) and 40°C (40%), respectively. The highest temperature stability of *L. sphaericus* KC5-MRL, *B. subtilis* AF-3, *B. subtilis* AF-6 and *B. subtilis* AF-23, amylases were observed at 35°C (107%), 70°C( 107%), 30°C (102%) and 40°C (122%). Lowest stability of amylases were noted at 100C (0%), 50°C (94%), 100C (14%) and 50C (33%) for *L. sphaericus* KC5-MRL, *B. subtilis* AF-3, *B. subtilis* AF-6 and *B. subtilis* AF-23, respectively. The highest temperature stability of proteases of *L. sphaericus* KC5-MRL, *B. subtilis* AF-3, *B. subtilis* AF-6 and *B. subtilis* AF-23, were observed at 35°C(104%), 80°C (127%), 40°C (149%) and 100°C (111%), respectively. Lowest temperature stability was observed at 100C (31%), 60°C (25%), 70C (55%) and 50C (83%) for *L. sphaericus* KC5-MRL, *B. subtilis* AF-3, *B. subtilis* AF-6 and *B. subtilis* AF-23, respectively.

### Effect of detergents on enzymes activity

Addition of Tween-80 reduced lypolytic activity of *L. sparcicus* KC5-MRL up to 4%. Triton X-100 (T X-100) and sodium dodecyl sulfate (SDS) stimulated the lipase activity up to 4% and 3%, respectively. Triton X-100, Tween-80 (T-80) and SDS reduced the *B. subtilis* AF-3 lipase activity up to 4%, 6% and 9% respectively. While reduced the activity up to 4%. While Triton X-100 stimulated the lipase activity up to 6%. Tween-80 and SDS stimulated the *B. subtilis* AF-6 lipase activity up to 5% and 7% respectively. While Triton X-100 reduced the activity up to 2%. Triton X-100 and SDS stimulated the *B. subtilis* AF-23 lipase activity up to 5% and 4% respectively. While Tween-80, reduced lipase activity up to 2%. SDS reduced amylase activity of *L. sparcicus* KC5-MRL upto 70%. TX-100 and T-80 stimulate enzyme activity 19 and 23% respectively. SDS completely inhibited amylase of *B. subtilis* AF-3 enzyme activity. TX-100 and T-80 showed inhibitory effect inhibited activity upto 98 and 19%. SDS reduced *B. subtilis* AF-6 enzyme activity upto 45.5%. TX-100 and T-80 stimulate enzyme activity 5 and 8.5% respectively. SDS and TX-100 reduced *B. subtilis* AF-23 enzyme activity upto 4.3 and 1% and T-80 stimulate enzyme activity up to 9%. TX-100 and T-80 showed stimulatory effect, stimulated *L. sparcicus* KC5-MRL protease activity upto 9 and 23% respectively. SDS reduced lipase activity up to 70%. Protease of *B. subtilis* AF-3 stimulated by TX-100 upto 82%. SDS and T-80 reduced protease activity of *B. subtilis* AF-6 up to 31 and 4%, respectively. Triton X-100 and SDS showed stimulatory effect, stimulated *B. subtilis* AF-6 protease activity upto 48.7 and 28%, respectively while T-80 reduced enzyme activity 62.3%. TX-100 and T-80 showed stimulatory effect, stimulated *B. subtilis* AF-23 protease activity upto 16% and 31%, respectively, while SDS reduces enzyme activity up to 66.5%.

**Effect of metal ions on enzymes activity:** All the metals were inhibitory for all proteases and amylases of *B. subtilis* AF-3 and *B. subtilis* AF-6. For the rest of enzymes the some metals showed stimulatory and some inhibitory effect. The metal ions e.g.  $Mg^{++}$ ,  $NH_4^+$  and  $Ca^{++}$  stimulated lipase activity of *L. sparcicus* KC5-MRL as; 25.8%, 31.1% and 27.54%, respectively. Ammonium ion stimulated the lipase activity up to 31.7%.  $Hg^+$  and  $Zn^{++}$  inhibited lipase activity as 2%, 7% and 11.31%, respectively.  $Zn^{++}$  showed highest inhibitory effect on lipase activity. The metal ions e.g.  $Na^+$ ,  $Mg^{++}$  and  $Ca^{++}$  stimulated lipase activity of *B. subtilis* AF-3 up to 20%, 27% and 31%, respectively. Ammonium ion,  $Hg^+$  and  $Zn^{++}$  inhibited lipase activity as 6%, 10% and 18%, respectively.  $Zn^{++}$  showed highest inhibitory effect on lipase activity. The metal ions e.g.  $Mg^{++}$  and  $Ca^{++}$  stimulated lipase activity of *B. subtilis* AF-6 up to 26% and 30%,

respectively.  $Hg^{+}$ ,  $Na^{+}$  and  $Zn^{++}$  inhibited lipase activity as 4%, 8% and 12%, respectively. Ammonium ion and  $Zn^{++}$  showed highest inhibitory effect on lipase activity. The metal ions e.g.  $Mg^{++}$  and  $Ca^{++}$  stimulated lipase activity of *B. subtilis* AF-23 as; 27% and 31%, respectively. Ammonium ion,  $Hg^{+}$ ,  $Na^{+}$  and  $Zn^{++}$  inhibited lipase activity as 3%, 6%, 10% and 23%, respectively.  $Zn^{++}$  showed highest inhibitory effect on lipase activity. Some metals were inhibitory and some stimulatory for amylase enzyme. *L. sparcicus* KC5-MRL amylase showed reduced activity under  $Hg^{++}$ ,  $Mg^{++}$ ,  $Zn^{++}$  and  $NH_4^{+}$  treatment as; 69%, 11%, 26% and 55% respectively.  $Na^{+}$  and  $Ca^{++}$  showed stimulatory effect up to 11 and 6% respectively. Metals ions showed inhibitory effect on amylase activity of *B. subtilis* AF-3 crude extracts.  $Ca^{++}$  completely inhibits the enzyme activity.  $Hg^{++}$ ,  $Na^{+}$ ,  $Mg^{++}$  reduced enzyme activity up to 33, 54 and 25% respectively. *B. subtilis* AF-6 amylase activity was reduced by  $Hg^{++}$ ,  $Na^{+}$ ,  $Mg^{++}$ ,  $Zn^{++}$   $NH_4^{+}$  and  $Ca^{++}$  up to 72%, 12%, 2%, 28%, 18% and 21% respectively. *B. subtilis* AF-23 amylase activity was reduced by  $Hg^{++}$ ,  $Na^{+}$ ,  $Mg^{++}$ ,  $Zn^{++}$  and  $NH_4^{+}$  up to 79%, 44%, 100%, 81.5% and 75.5% respectively while  $Ca^{++}$  showed stimulatory effect up to 84%. All metals were inhibitory for *B. subtilis* AF-3 protease enzyme.  $Hg^{++}$ ,  $Na^{+}$ ,  $Mg^{++}$ ,  $Zn^{++}$ ,  $Ca^{++}$  and  $NH_4^{+}$  reduced enzyme activity up to 17%, 49%, 74%, 64%, and 64% respectively. All metals were inhibitory for *B. subtilis* AF-6 protease.  $Hg^{+}$ ,  $Na^{+}$ ,  $Mg^{++}$ ,  $Zn^{++}$ ,  $Ca^{++}$  and  $NH_4^{+}$  reduced enzyme activity up to 72%, 12%, 1.4%, 72%, 21% and 18%, respectively. Some metals were inhibitory for *L. sparcicus* KC5-MRL protease.  $Hg^{++}$ ,  $Zn^{++}$ ,  $Ca^{++}$  and  $NH_4^{+}$  reduced enzyme activity up to 57%, 46%, 47% and 15%, respectively.  $Na^{+}$  and  $Mg^{++}$  stimulate *L. sparcicus* KC5-MRL protease activity up to 8% and 16% respectively. All metals were inhibitory for *B. subtilis* AF-23 protease except  $Na^{+}$ , which stimulated enzyme up to 65%.  $Hg^{++}$ ,  $Mg^{++}$ ,  $Zn^{++}$ ,  $Ca^{++}$  and  $NH_4^{+}$  reduced *B. subtilis* AF-23 protease activity 43%, 50%, 23.4%, 60.3% and 38.2%, respectively.

**Effect of organic solvents on enzymes activity:** Presence of nitrobenzene (N.B) reduced lipase activity of *L. sparcicus* KC5-MRL up to 3.4%. Other organic solvents stimulated lipase activity. Highest stimulation was observed in case of benzene (B) 14.32%. Addition of xylene (X) showed no effect on the activity of lipase while chloroform (Ch), formaldehyde (Frm), methanol (Met) and acetone (Ace), stimulated lipase activity as 2.5%, 10.5%, 4.2% and 3%, respectively. Presence of N.B reduced lipase activity of *B. subtilis* AF-3 up to 5%. Other organic solvents stimulated lipase activity. Highest stimulation was observed in case of Ace 16%. Addition of X and methanol showed no effect on the activity of lipase while Ch, Frm and B stimulated lipase activity as 3%, 9%, 5% and 4%, respectively. Presence of N.B reduced lipase activity of *B. subtilis* AF-6 up to 8%. Other organic solvents stimulated lipase activity. Highest stimulation was observed in case of Ace 11%. Addition of X and methanol showed no effect on the activity of lipase while Ch, Frm and benzene stimulated lipase activity as 2%, 4%, 6% and 3%, respectively. Presence of N. B and Ch reduced lipase activity of *B. subtilis* AF-23 up to 6% and 8%. Other organic solvents stimulated lipase activity. Highest stimulation was observed in case of Ace 13%. Addition of X and methanol showed no effect on the activity of lipase while Frm and B stimulated lipase activity as 7% and 4%, respectively. Nitrobenzene X and Ace reduced amylase activity of *L. sparcicus* KC5-MRL up to 33%, 5% and 50%, respectively. Chloroform, Frm, Met and B addition stimulated *L. sparcicus* KC5-MRL amylase activity upto 9%, 4%, 3.5%, 2% and 7%, respectively. Benzene and Ch reduced *B. subtilis* AF-3 amylase enzyme activity up to 18 and 90% respectively. Nitro Benzene stimulation was highest 86%, X, Frm, Ace and Met stimulate enzyme activity up to 70%, 60%, 56% and 48%, respectively. All organic solvents reduced *B. subtilis* AF-6 enzyme activity. Chloroform completely inhibited enzyme activity. Nitro Benzene, X, Frm, Ace,

Met and B reduced *B. subtilis* AF-6 enzyme activity up to 16%, 19%, 13%, 15% and 17%, respectively. Chloroform, Frm, Met, B, X and Ace reduced *B. subtilis* AF-23 amylase activity up to 87.5%, 34%, 66%, 32% and 63%, respectively. Nitrobenzene, Met, B, and Ace stimulated *L. sparcicus* KC5-MRL protease activity upto 17%, 17%, 4% and 27%, respectively. Formaldehyde, Ch and X reduced the activity of protease from *L. sparcicus* KC5-MRL up to 10%, 66% and 30%, respectively. Chloroform, Frm, Met, B, X and Ace reduced *B. subtilis* AF-3 protease activity up to 17%, 100%, 77%, 33%, 45% and 74.5%, respectively. Nitrobenzene, Frm, B, and Ace stimulate *B. subtilis* AF-6 enzyme activity up to 22%, 1.5%, 37% and 20.5%, respectively. Methanol, Ch and X reduced the activity of protease from *B. subtilis* AF-6 up to 5%, 63.4% and 34.5%, respectively. Chloroform showed no effect on the activity on *B. subtilis* AF-6 protease. Nitrobenzene, X and Frm reduced *B. subtilis* AF-23 protease activity up to 42%, 66.5% and 30.4%, respectively. Acetone and Met stimulated *B. subtilis* AF-23 protease activity up to 15.5% and 30.4% respectively.

**Effect of modulators on enzymes activity:** Phenyl acetaldehyde (PAA) inhibited the activity of crude extract of lipase of *L. sparcicus* KC5-MRL up to 17.3%. Ethylenediamine Tetraacetic acid (EDTA) stimulated lipolytic activity up to 24.4%. Mercaptoethanol (Mre) showed no effect on lipolytic activity, whereas, Tri-sodium citrate (TSC) and phenylmethylsulfonyl fluoride (PMSF) both inhibited lipase activity up to 14%. Phenyl acetaldehyde inhibited the activity of crude extract of lipase of *B. subtilis* AF-3 up to 18%. Ethylenediamine Tetraacetic acid stimulated lipolytic activity up to 24%. Mercaptoethanol showed no effect on lipolytic activity, whereas, TSC and PMSF both inhibited lipase activity up to 11% and 13%, respectively. Phenyl acetaldehyde inhibited the activity of crude extract of lipase of *B. subtilis* AF-6 up to 18%. Ethylenediamine Tetraacetic acid stimulated lipolytic activity up to 23%. Mercaptoethanol showed no effect on lipolytic activity, whereas, TSC and PMSF both inhibited lipase activity up to 15%. Phenyl acetaldehyde inhibited the activity of crude extract of lipase of *B. subtilis* AF-23 up to 20%. Ethylenediamine Tetraacetic acid stimulated lipolytic activity up to 21%. Mercaptoethanol showed no effect on lipolytic activity, whereas, TSC and PMSF both inhibited lipase activity up to 18% and 23%, respectively. All the inhibitors reduced the amylases activity of all the strains except for amylase of *B. subtilis* AF-3 which was stimulated by PAA up to 43%. *L. sparcicus* KC5-MRL amylase activity inhibited by EDTA, TSC, Mer, PAA and PMSF up to 18%, 84%, 01%, and 16%, respectively. The amylase *B. subtilis* AF-3 activity inhibited by EDTA, TSC, Mer and PMSF up to 93%, 43%, 59%, and 51%, respectively. *B. subtilis* AF-6 amylase activity was reduced by EDTA, TSC, Mer, PAA and PMSF up to 55.5%, 54%, 32%, 16% and 37%, respectively. EDTA, The amylase activity of *B. subtilis* AF-23 reduced by TSC, Mer, PAA and PMSF up to 01%, 01%, 79%, and 100% and 100%, respectively. All the inhibitors reduced the protease activity of *L. sparcicus* KC5-MRL except TSC and PMSF which stimulated *L. sparcicus* KC5-MRL protease activity up to 24 and 5% respectively. Ethylenediamine Tetraacetic acid, Mer and PAA reduced *L. sparcicus* KC5-MRL protease activity up to 34, 37, and 26%, respectively. All the inhibitors reduced *B. subtilis* AF-3 protease enzyme activity EDTA, TSC, Mer, PAA and PMSF reduced enzyme activity up to 64%, 66%, 26%, and 81% and 56%, respectively. All the inhibitors reduced *B. subtilis* AF-6 enzyme activity except PAA which stimulated enzyme activity up to 69%. Ethylenediamine Tetraacetic acid, TSC, Mer and PMSF reduced enzyme activity up to 61%, 36%, 25%, and 68%, respectively. All the inhibitors reduced *B. subtilis* AF-23 enzyme activity. Tri-sodium citrate, PMSF, EDTA and Mer reduced enzyme activity up to 46%, 50%, and 33% respectively.

## Discussion

Enzymes are known as nature's catalysts.<sup>20</sup> So for different enzymes are isolated from different living organisms like plants, animals, fungi, yeast and bacteria.<sup>21</sup> Throughout the world only 2% enzymes are isolated from microorganisms.<sup>22</sup> These microorganisms are isolated from different ecosystems. The hidden potential of microorganisms in sense of valuable industrial enzymes exploration is still on wait. These enzymes have number of applications in different industries like food, medicine, cosmetics, textile, leather, detergents, oleo chemical and for different diagnostic tools (Hasan et al., 2005). Caves are extreme environment with poor nutrient content. Caves still have to be explored for new horizons in Microspeleology sectors. In the world different antibiotics are isolated from different caves. From ice caves different types of industrially important enzymes have also been reported. Our present study is focused on caves bacteria to find out industrially important enzymes. *Lysinibacillus sphaericus* is a gram positive, rod shaped mesophilic bacterium that naturally occur in soil. *Lysinibacillus sphaericus* KC5-MRL showed maximum lipase production 42.23U/ml at 30°C and pH 5, after 24h of incubation. Similar specific enzyme activity was reported by different investigators for lipase.<sup>23-25</sup> Lipase from *Pseudomonas sp.*, which produced maximum lipase after 24h of incubation at 30-40°C.<sup>26</sup> *Pseudomonas sp.*, produced maximum lipase at pH 5.5.<sup>27</sup> Variation in pH, incubation time and incubation temperature may be due to isolate specificity for different conditions.

Maximum lipase activity was observed at a size of 7% inoculum. Balan et al.,<sup>28</sup> reported maximum lipase production when 7% inoculum of *Geobacillus thermodenitrificans* was used in flask fermentation. Effect of different metal ions was checked on the basis of residual activity of crude extract. Mg<sup>++</sup>, NH<sub>4</sub><sup>+</sup> and Ca<sup>++</sup> stimulated lipase activity. Stimulation of crude extract activity have been reported by different investigators Li and Ziaobo (2005) reported that Ca<sup>++</sup> and Mg<sup>++</sup> stimulate the activity of *Geobacillus* sp. TW1 lipase. Our results are similar to that of Mobaraket al.<sup>29</sup> where *Pseudomonas aeruginosa* KM110 lipase was inhibited by Zn<sup>++</sup> and Cu<sup>++</sup>.<sup>29</sup> Organic solvents mostly showed some kind of stimulatory effects. Highest stimulation noted in case of benzene while nitrobenzene was inhibitory for enzyme. Our results are similar with that of Nawani et al.<sup>30</sup> They reported benzene as stimulator for the lipase of thermophilic *Bacillus sp.* Schmidt et al.<sup>31</sup> reported that different organic solvents like methanol, acetone and ethanol enhanced lipase activity isolated from *B. thermocatenuatus*. Lee et al.<sup>32</sup> reported that different detergents change the activity of *Bacillus thermoleovorans* ID-1 thermophilic lipase. They reported no activity of Triton X-100 but in contrast to our study, it stimulated the lipase activity and SDS also stimulated enzyme activity. *Bacillus thermoleovorans* ID-1 lipase activity were reduced by Tween 80. Mobaraket al.<sup>29</sup> reported that lipase of *Pseudomonas aeruginosa* KM110 showed optimum activity at pH 8 i.e. 27%. Our isolated lipase also showed 142% activity at alkaline pH 10. This was maximum activity for the pH value tested. Kojima et al., (1994) isolated bacterial lipase from *Pseudomonas fluorescens* AK102 which were stable at pH 4-10 and maximum stability was noted at pH 8-10. Lipase retained its activity at all temperatures tested. The highest activity was observed at 35°C. The enzymes were stable at 30-80°C. Wang et al.<sup>33</sup> characterized a bacterial lipase of *Bacillus* strain A30-1 (ATCC 53841) which was stable at 75°C. Rathiet al.<sup>34</sup> isolated a lipase from *Pseudomonas sp.*, which was stable at 90°C for 3h. In our research, lipase was stable for 1 h at 80°C. Demirkan<sup>35</sup> isolated amylase from *Bacillus subtilis* reported highest activity from the

current study. Different investigators<sup>36,37</sup> have reported that protease is produced during stationary phase. They isolated these proteases from *Pseudomonas fluorescens* P26 and *Bacillus* spp. Amroet al.<sup>38</sup> isolated bacterial proteases from different bacteria which have maximum protease activity in the range of 34-44 U/ml. In our study we reported 59 U/ml protease activity. Overall, the isolated amylase in our study were stable from 30 to 80°C for 1h. Malhotra et al.<sup>39</sup> reported α-amylase of *Bacillus thermooleovorans* NP54 which was active at 100°C for 10 minutes. The enzymes they reported were stable at 40-100°C.

Our results are similar to that of Adinarayana et al.<sup>40</sup> They isolated protease from *Bacillus sp.* BZI-2 and Femi-Ola & Oladokun<sup>41</sup> isolated protease from *Lactobacillus brevis*. Pathak & Deshmukh<sup>42</sup> reported a protease from *Bacillus licheniformis* which was active in the range of 20-90°C. Lee et al.<sup>43</sup> isolated a protease from *Pseudoalteromonas sp.* A28, which is in accordance with our study. Some metals were found inhibitory and some stimulatory for the *Lysinibacillus sphaericus* KC5-MRL amylase. Hailemariam et al.<sup>44</sup> also reported amylase from a *Bacillus sp.* which was inhibited by different metals ions. Activity of amylase from different bacterial isolates was reduced by different divalent cations<sup>45-50</sup> reported two different types of bacterial amylases whose activities were inhibited by chloroform and other organic solvents while alcohol and acetone stimulated enzymes activity. Similar observation was reported in our study. All the inhibitors reduced the amylase activity. Ballschmiter et al.<sup>51</sup> reported an amylase from hyper thermophile bacteria with reduced activity in the presence of EDTA. They reported that amylase activity was slightly stimulated by the presence of mercaptoethanol which contradicts with our results. None of the surfactants tested had a pronounced inhibitory effect on amylase activities. Oliveira et al.<sup>52</sup> reported amylase from *Rhizobia* strains. Amylase activity was not inhibited by detergents/ surfactants which they used in their experiments. They reported that Triton X-100 moderately inhibited enzyme activity and SDS and Tween-80 showed stimulatory effect on the enzyme activity. Similar results were noted in the present study. Addition of surfactants in case of proteases showed some stimulatory and as well as inhibitory effect. Nascimento & Martins<sup>53</sup> isolated protease from *Bacillus sp.*, which was inhibited by the addition of surfactants like SDS and Triton X-100. This result is similar to the present study.

All metals were inhibitory for the protease. Banerjee et al.<sup>54</sup> reported *Brevibacillus (Bacillus) brevis* to be inhibited by CuSO<sub>4</sub>, ZnCl<sub>2</sub> and HgCl<sub>2</sub>. Usharani and Muthuraj<sup>55</sup> reported *Bacillus laterosporus* protease whose activity was inhibited by the presence of different metals ions. The inhibitory effect of heavy metal ions is well documented in the literature. In the present study most of the metal ions inhibited the enzyme activity. Organic solvents showed stimulatory and as well as inhibitory effects in case of proteases. Rahman et al.<sup>56</sup> isolated protease from *Pseudomonas aeruginosa* strain K whose activity was inhibited by 1-pentanol, benzene, toluene, p-xylene and n-hexane up to 33%, 35%, 30%, 48% and 36% respectively. Protease activity was stimulated by metals ions. *Lysinibacillus sphaericus* KC5-MR showed that this protease belong to metallo proteases. Similar results also reported by Mabrouket al.<sup>57</sup> they isolated protease from *Bacillus licheniformis* ATCC 21415, which was inhibited by EDTA. The hydrolytic activity of different enzymes (Lipase, protease, amylase) was characterized. *Lysinibacillus sphaericus* KC5-MRL has shown varying activities of enzymes. *Lysinibacillus sphaericus* KC5-MRL enzymes properties indicated possibilities to be used for enzyme biotechnology in order to enhance catalytic efficiency of enzymes in industry (Figure 2 – Figure 10).<sup>58-63</sup>

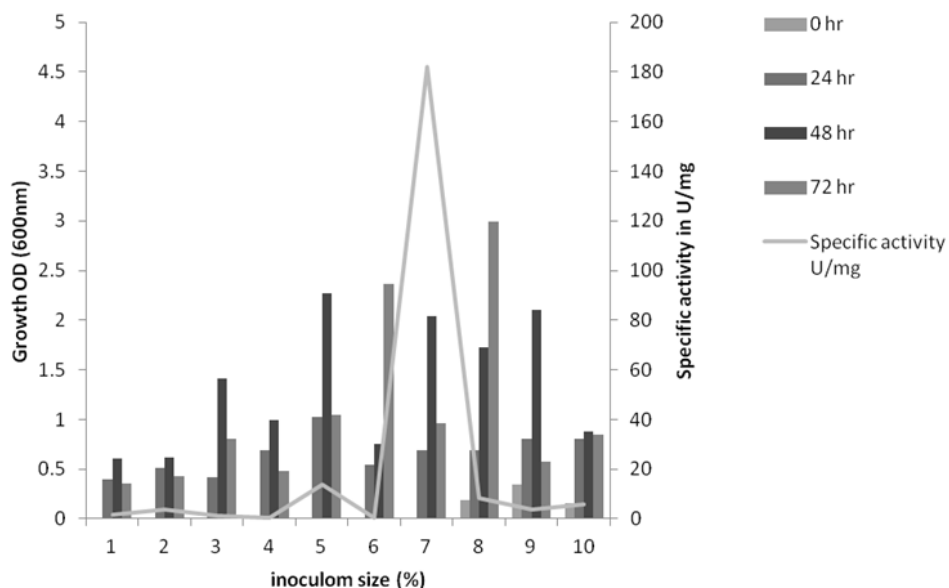


Figure 2 Growth of strain and lipase production of *Lysinibacillus sphaericus* KC5-MRL at different sizes of inoculum.

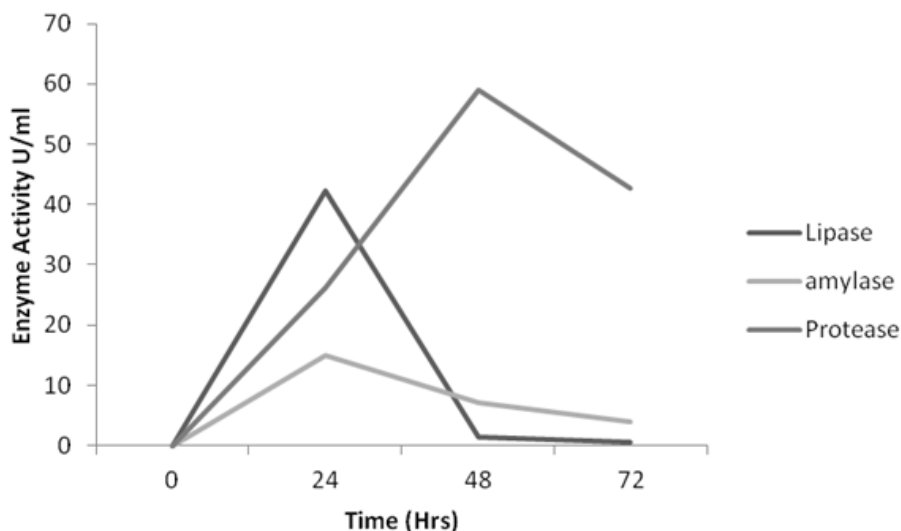


Figure 3 Lipolytic, Amylolytic and proteolytic activity of strain *Lysinibacillus sphaericus* KC5-MRL at 30°C.

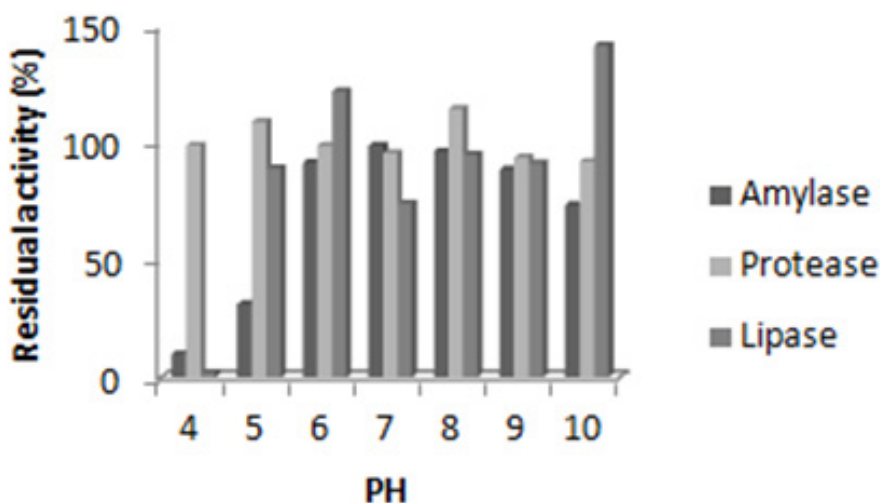


Figure 4 Stability of crude extract of lipase, amylase and protease of *Lysinibacillus sphaericus* KC5-MRL at different pH.

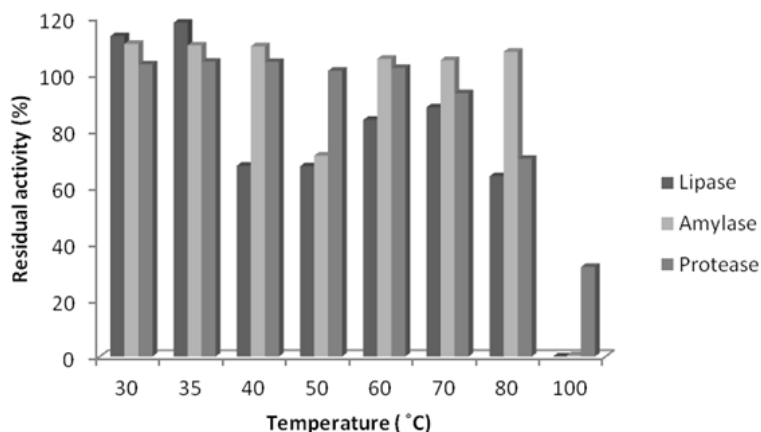


Figure 5 Stability of crude extracts of lipase, amylase and protease of *Lysinibacillus sphaericus* KC5-MRL at different temperature.

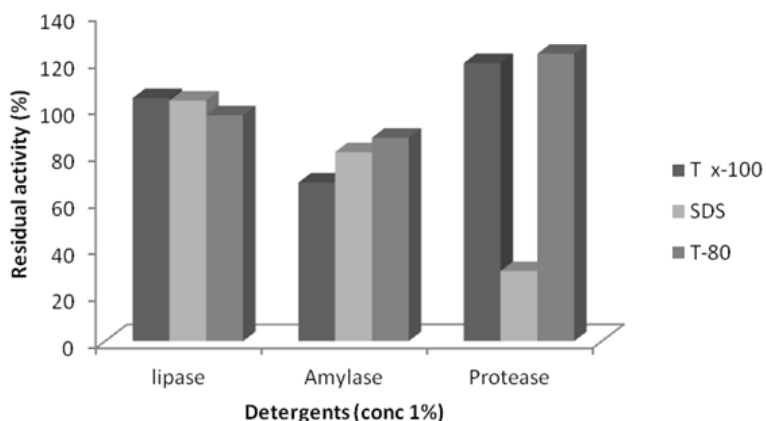


Figure 6 Effects of Detergents on the lipolytic, amylolytic and proteolytic activity of isolate *Lysinibacillus sphaericus* KC5-MRL.

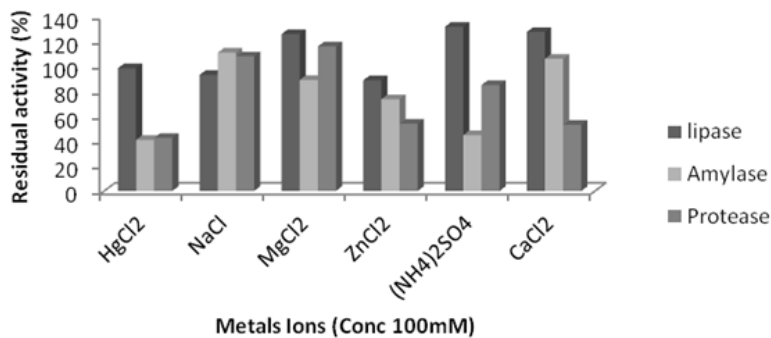


Figure 7 Effects of metal ions on lipase, Amylase and protease activity of isolate *Lysinibacillus sphaericus* KC5-MRL.

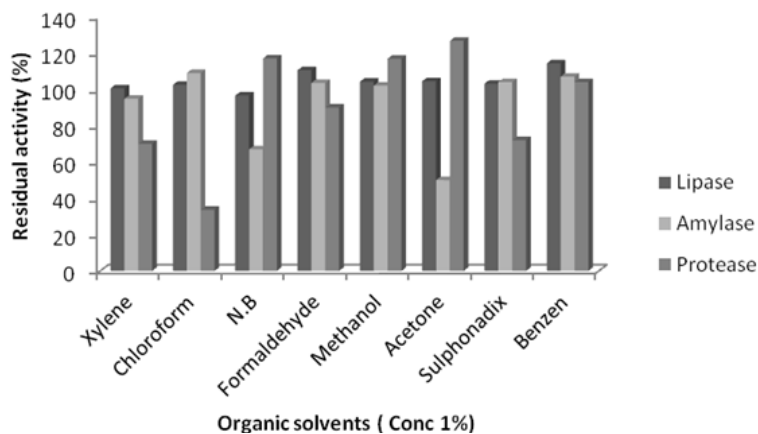


Figure 8 Effects of Organic solvents on Lipase, amylase and protease activity of isolate *Lysinibacillus sphaericus* KC5-MRL.

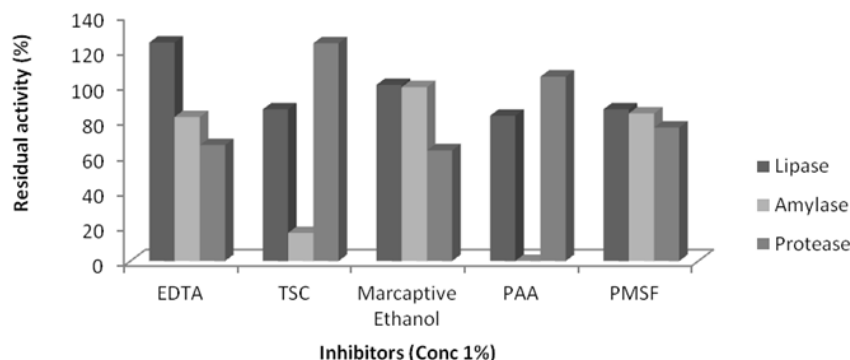


Figure 9 Effects of inhibitors on lipase, amylase and protease activity of isolate *Lysinibacillus sphaericus* KC5-MRL.

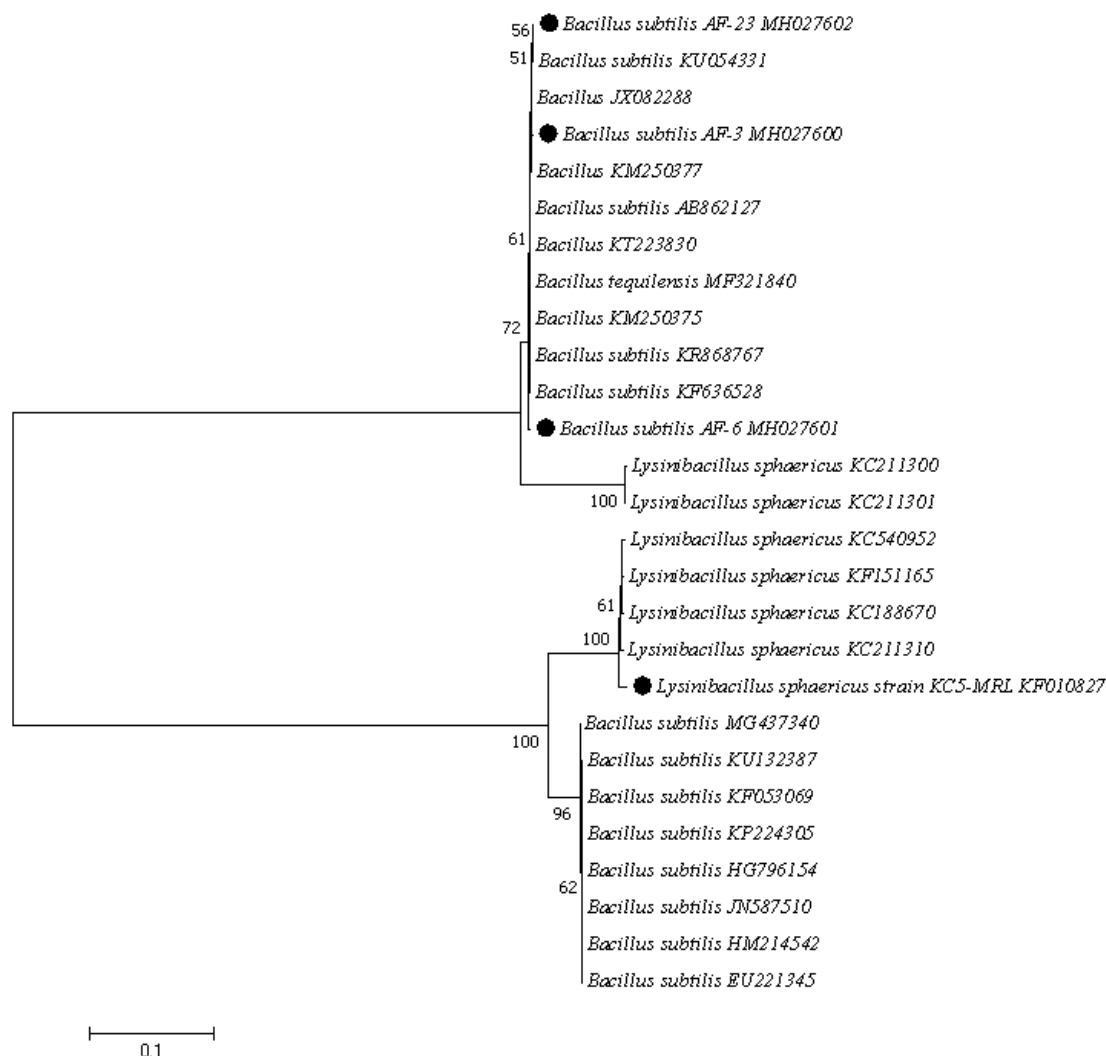


Figure 10 Evolutionary relationships of taxa isolated from limestone cave (Kashmir Smast) Pirsai Mardan, Pakistan.

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### Conflicts of interest

The authors declare that there is no conflict of interest.

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