Research Article

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 35ºC. 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 35ºC.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

Volume 9 Issue 3 - 2021

Muhammad Afzal,[1,2,3] Ihsan-Ud-din,[4] Muhammad Rafiq,[2] Sahib Zada,[2] Naveed Hussain,[3] Muhammad Irfan,[2] Niaz Muhammad,[5] Aamer Ali Shah,[2] Fariha Hasan,[2] Sajid Muhammad[1]

[1]Zhejiang University, China
[2]Department of Microbiology, Quaid-i-Azam University, Pakistan
[3]Islamic girls public school and college, Pakistan
[4]Department of Biotechnology, Bacha Khan University, Pakistan
[5]Department of Microbiology, Kohat University of Science and Technology, Pakistan

Correspondence: Muhammad Afzal, Zhejiang Provincial Key Laboratory of Subtropic Soil and Plant Nutrition, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou, 310058, P.R. China, Tel +92-3348272264, Email mafzal.kust@gmail.com

Received: July 03, 2021 | **Published:** July 14, 2021

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.[1–3] 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.[4] A few pathogenic microorganisms have been reported from the Altamira cave.[5] Luonget al.[6] 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,7 species of *Pseudomonas, Sphingomonas* and *Alcaligenes sp*[8] and *Inquilinu*sp.[9] Nutrient recycling in the cave totally depends upon the bacterial and fungal activities.[10] 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.[11] 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.[12,13] Significant growth in biospeleological research has been observed in the last two decades.[14] Caves are considered as extreme environments providing niches for highly specialized microorganisms.[15] Enzymes which are isolated from cave microbes are active at low temperature. Enzymes of psychrotrophs have great biotechnological importance.[11] These microbes survive in such environment because of production of enzymes which function in such extreme conditions.[16] Our knowledge about cave microbial diversity is limited, so there is great potential to find novel microorganisms in caves.[17] Caves in Pakistan are less studied area especially with regards to industrially important enzymes from cave bacteria have not been reported or isolated so for. 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'-GGCTACCTTGTTACGA-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'-ACCTTGTTACGACTT-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.[6] 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.[18] 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_3COOH. 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°Cwith 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 inoculums a 24h old bacterial culture grown in nutrient broth was added at a concentration of 1to10% 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_2HPO_4/NaOH (pH 10)], and remaining enzyme activity was determined under standard conditions. Effect of different metals (100mM of $HgCl_2$, NaCl, $MgCl_2$, $ZnCl_2$, $(NH_4)_2SO_4$ and $CaCl_2$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[19] 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 sparicous.* On the basis of quantitative analysis *Bacillus subtilis* AF-6, *Bacillus subtilis* AF-3, *Lysinobacillus sparicous* 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 inoculum8% 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% inculum 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 inculum 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% inculum 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. sparicous* 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. sparicous* 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. sparicous* 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 satiability 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%), 70ºC(107%), 30°C (102%) and 40°C (122%). Lowest stability of amylases were noted at 100C (0%), 50ºC (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%), 80ºC (127%), 40°C (149%) and 100°C (111%), respectively. Lowest temperature stability was observed at 100C (31%), 60ºC (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 liypolytic activity of *L. sparicous* KC5-MRLup 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. sparicous* 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-6enzyme 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-23enzyme activity upto 4.3 and 1% and T-80 stimulate enzyme activity up to 9%.T-X-100 and T-80 showed stimulatory effect, stimulated *L. sparicous* 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 upto16% 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. sparicous* KC5-MRL as; 25.8%, 31.1% and 27.54%, respectively. Ammonium ion stimulated the lipase activity up to 31.7%. Hg^+Na^+ 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-6up 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. sparicous* 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. sparicous* 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. sparicous* 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 nitro-benzene (N.B) reduced lipase activity of *L. sparicous* 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. sparicous* KC5-MRL up to 33%, 5% and 50%, respectively. Chloroform, Frm, Met and B addition stimulated *L. sparicous* 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. sparicous* KC5-MRL protease activity upto 17%, 17%, 4% and 27%, respectively. Formaldehyde, Ch and X reduced the activity of protease from *L. sparicous* KC5-MRL up to 10%, 66% and 30%, respectively. Chloroform, Frm, Met, B, X and Ace reduced *B. subtilis* A F-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. Sparicous* 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. sparicous* 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. sparicous* KC5-MRL except TSC and PMSF which stimulated *L. sparicous* KC5-MRL protease a ctivity up to 24 and 5% respectively. Ethylenediamine Tetraacetic acid, Mer and PAA reduced *L. sparicous* 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.[20] So for different enzymes are isolated from different living organisms like plants, animals, fungi, yeast and bacteria.[21] Throughout the world only 2% enzymes are isolated from microorganisms.[22] 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.[23-25] Lipase from *Pseudomonas sp.*, which produced maximum lipase after 24h of incubation at 30-40°C.[26] *Pseudomonas* sp., produced maximum lipase at pH 5.5.[27] 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.,[28] reported maximum lipase production when 7% inoculum of *Geobacillus thermodenitrifican* was used in flask fermentation. Effect of different metal ions was checked on the basis of residual activity of crude extract. Mg^{++}, NH_4^+ and Ca^{++} stimulated lipase activity. Stimulation of crude extract activity have been reported by different investigators Li and Ziaobo (2005) reported that Ca^{++} and Mg^{++} stimulate the activity of *Geobacillus* sp.TW1 lipase. Our results are similar to that of Mobarak et al.[29] where *Pseudomonas aeruginosa* KM110 lipase was inhibited by Zn^{++} and Cu^{++}.[29] 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.[30] They reported benzene as stimulator for the lipase of thermophilic *Bacillus sp.* Schmidt et al.[31] reported that different organic solvents like methanol, acetone and ethanol enhanced lipase activity isolated from *B.thermocatenulatus.* Lee et al.[32] reported that different detergents change the activity of *Bacillus thermoleovorans* ID-1thermophilic 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. Mobarak et al.[29] reported that lipase of *Pseudomonas aeruginosa* KM110 showed optimum activity at pH8 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.[33] characterized a bacterial lipase of *Bacillus* strain A30-1 (ATCC 53841) which was stable at 75°C. Rathi et al.[34] 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[35] isolated amylase from *Bacillus subtilis* reported highest activity from the

current study. Different investigators[36,37] have reported that protease is produced during stationary phase. They isolated these proteases from *Pseudomonas fluorescens* P26 and *Bacillus* spp. Amro et al.[38] 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 proteases activity. Overall, the isolated amylase in our study were stable from 30 to 80°C for 1h. Malhotra et al.[39] 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.[40] They isolated protease from *Bacillus sp.* BZI-2 and Femi-Ola & Oladokun[41] isolated protease from *Lactobacillus brevis.* Pathak & Deshmukh[42] reported a protease from *Bacillus licheniformis* which was active in the range of 20-90°C. Lee et al.[43] 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.[44] 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[45-50] 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 amylases activity. Ballschmiter et al.[51] 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.[52] 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[53] 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.[54] reported *Brevibacillus (Bacillus) brevis* to be inhibited by $CuSO_4$, $ZnCl_2$ and $HgCl_2$. Usharani and Muthuraj[55] 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.[56] 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 metalo proteases. Similar results also reported by Mabrouk et al.[57] 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).[58–63]
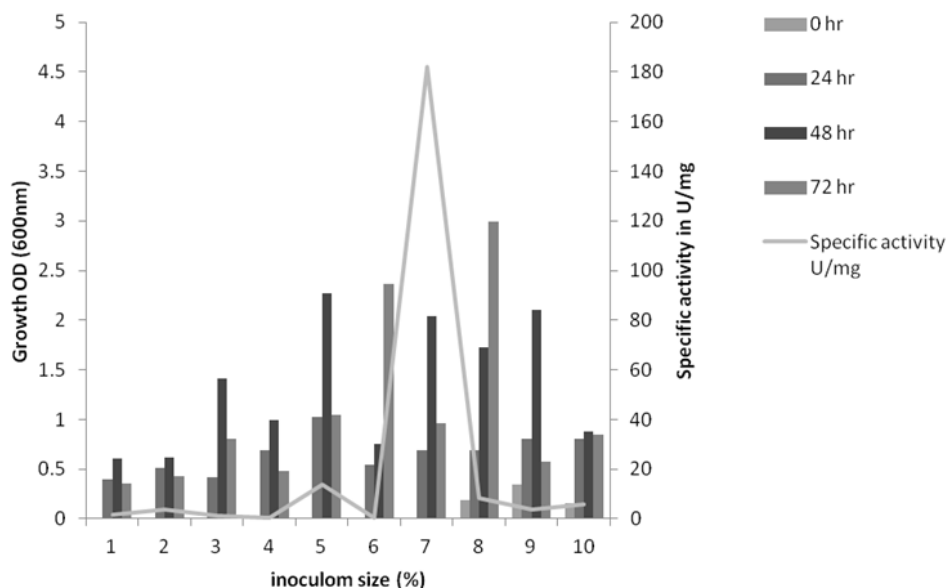
Figure 2 Growth of strain and lipase production of *Lysinibacillus sphaericus* KC5-MRL at different sizes of inoculum.
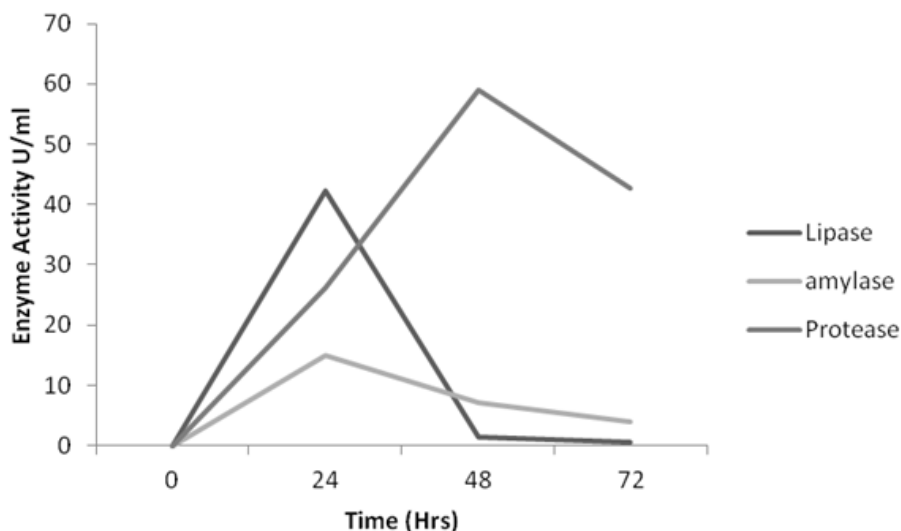

Figure 3 Lipolytic, Amylolytic and proteolytic activity of strain *Lysinibacillus sphaericus KC5*-MRL at 30°C.
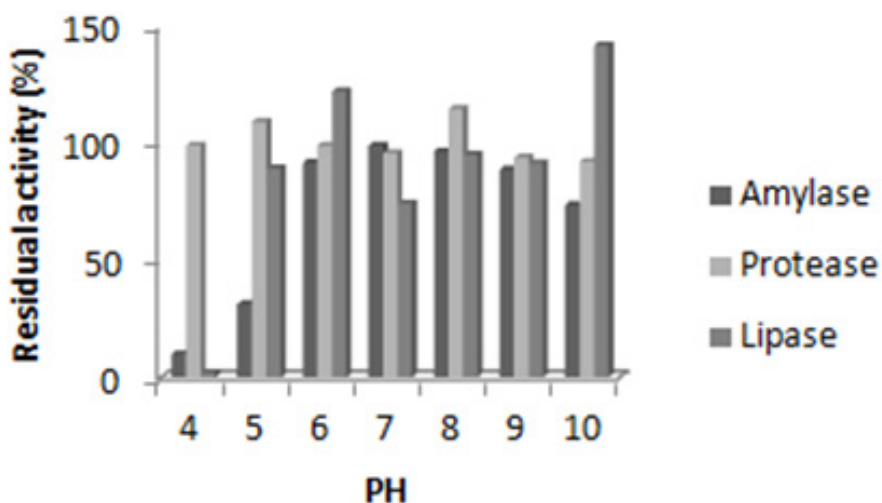

Figure 4 Stability of crude extract of lipase, amylase and protease of *Lysinibacillus sphaericus* KC5-MRL at different pH.
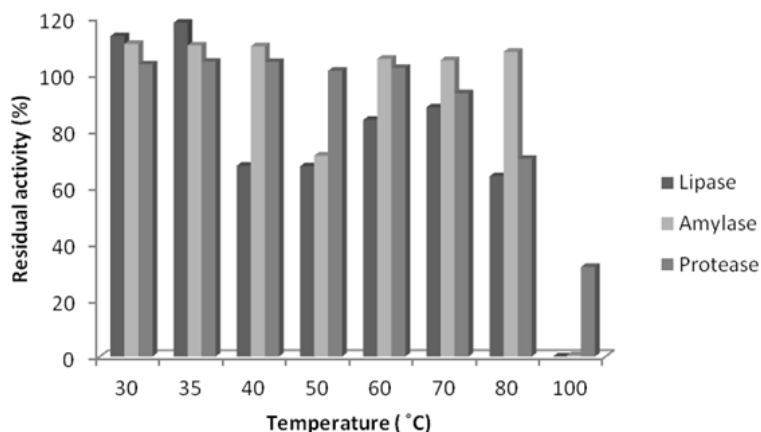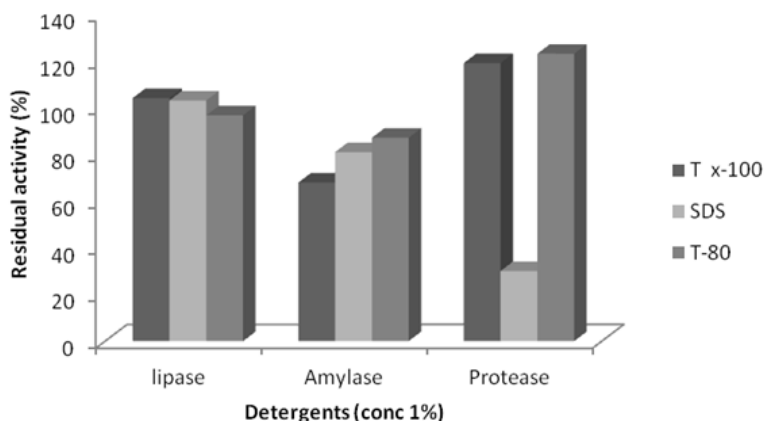
Figure 5 Stability of crude extracts of lipase, amylase and protease of *Lysinibacillus sphaericus KC5-MRL* at different temperature.

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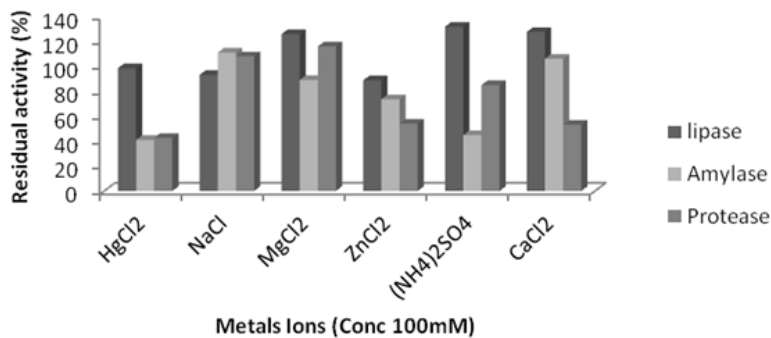

Figure 7 Effects of metals ions on lipase, Amylase and protease activity of isolate *Lysinibacillus sphaericus* KC5-MRL.
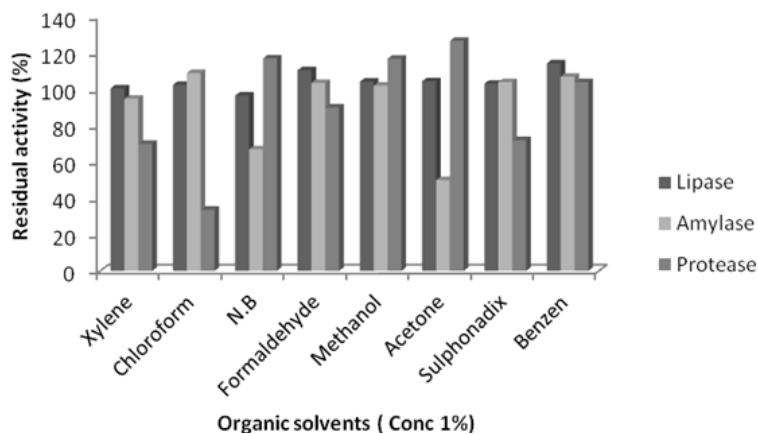

Figure 8 Effects of Organic solvents on Lipase, amylase and protease activity of isolate *Lysinibacillus sphaericus* KC5-MRL.
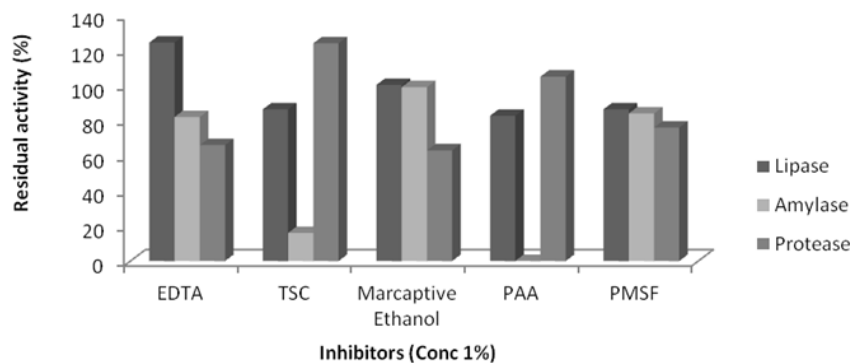
Figure 9 Effects of inhibitors on lipase, amylase and protease activity of isolate *Lysinibacillus sphaericus* KC5-MRL.
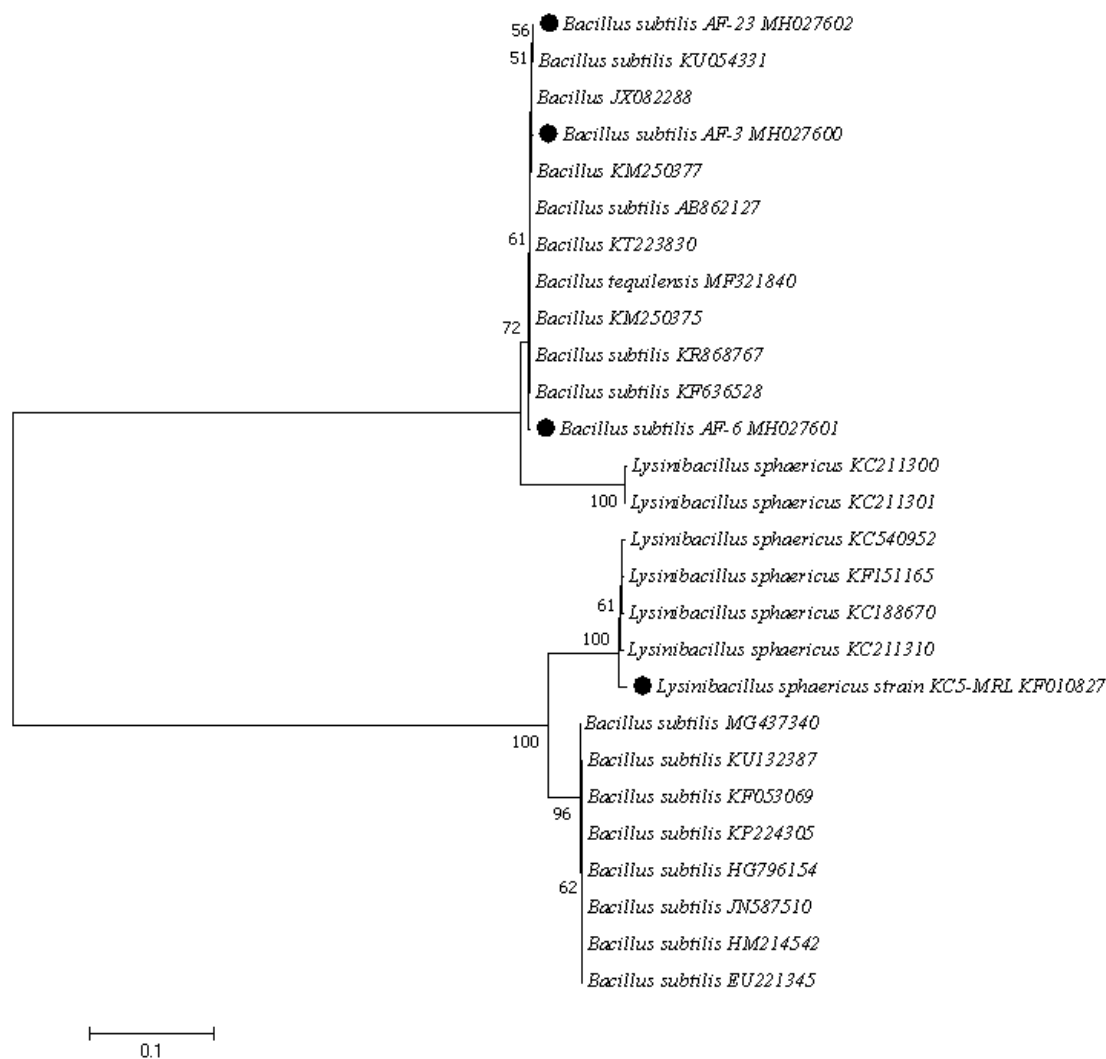

Figure 10 Evolutionary relationships of taxa isolated from limestone cave (Kashmir Smast) Pirsai Mardan, Pakistan.

Acknowledgments

Conflicts of interest

The authors declare that there is no conflict of interest.

References

1. Northup E, Diana, Kathleen H, et al. Microorganisms in Caves. Encyclopedia of Caves and Karst. 2009.

2. Engel A, Zondervan I, Aerts K, et al. Testing the direct effect of CO_2 concentration on a bloom of the coccolithophoridEmilianiahuxleyi in mesocosm experiments. *A Socity Limnol Oceanogr*. 2004;50(2):493–507.

3. Simmons JA, Currie WS, Eshleman KN, et al. Forest To Reclaimed Mine Land Use Change Leads To Altered Ecosystem Structure and Function. *Ecological Applications*. 2008;18(1):104–118.

4. Cuezva S, Sanchez-Moral S, Saiz-Jimenez C, et al. Microbial communities and associated mineral fabrics in Altamira cave, Spain. *International Journal of Speleology*. 2009;38:83–92.

5. Jurado, Gonzalez, Laiz, et al. Aurantimonas altamirensis sp. nov., a member of the order Rhizobiales isolated from Altamira Cave. *International Journal of Systematic and Evolutionary Microbiology*. 2006;56:2583–2585.

6. Luong P, Kinch LN, Brautigam CA, et al. Kinetic and structural insights into the mechanism of AM Pylation by VopSFic domain. *Journal of Biolological Chemistry*. 2010;285(26):20155–20163.

7. Lavoie, Kathleen H, Diana, et al. Bacteria as Indicators of Human Impact in Caves. *Nat Cave and Karst Management Symposium*. 2005.

8. Ikner LA, Toomey RS, Nolan G Neilson, et al. Culturable microbial diversity and the impact of tourism in Kartchner Caverns, Arizona. *Microbial Ecology*. 2007;53:30–42.

9. Laiza L, Grothb I, Gonzaleza I, et al. Microbiological study of the dripping waters in Altamira cave (Santillana del Mar, Spain). *Journal of Microbiology*. 1999;36:129–138.

10. Prabhavathi P, Rajendran R, Kuberan T, et al. Degradation of organic wastes and recycling of nutrients enhanced by microbes in subterranean habitat. *African Journal of Microbiology Research*. 2012;6(21):4449–4456.

11. Tomova I, Irina L, Anna T, et al. Diversity and biosynthetic potential of culturable aerobic heterotrophic bacteria isolated from Magura Cave, Bulgaria. *International Journal of Sepeleology*. 2013;42:65–76.

12. Bastian F, Alabouvette C, Saiz-Jimenez C. Bacteria and free-living amoeba in the Lascaux Cave. *Journal of Research in Microbiolology*. 2009;160:38–40.

13. Schabereiter GC, Saiz JC, Piñar G, et al. Altamira cave Paleolithic paintings harbor partly unknown bacterial communities. *FEMS Microbiology Letter*. 2000a;211:7–11.

14. Urzì C, Leo FD, Bruno L, et al. Microbial diversity in Paleolithic caves: A study case on the phototrophic biofilms of the Cave of Bats Zuheros, Spain. *Journal of Microbial Ecology*. 2010;60:116–129.

15. Schabereiter GC, Saiz JC, Piñar G, et al. Culture-independent analyses of bacterial communities on paleolithic paintings and surrounding rock walls in karstic caves (Altamira, Tito Bustillo, La Garma and Llonin). *Coalition Newsletter*. 2000b;5:7–10.

16. Burg BVD. Extremophiles as a source for novel enzymes. *Current Opinion in Microbiology*. 2003;6:213–218.

17. Engel AS. Microbial diversity of cave ecosystems. Geomicrobiology: Molecular and Environmental Perspective. New York. Springer; 2010:219-238.

18. Lesuisse E, Schanck K, Coloson C. Purification and preliminary characterization of the exracellular lipase of Bacillus subtilis 168, an exteremly basic tolerant enzyme. *European Journal of Biochemistry*. 1993;216:155–160.

19. Lowry OH, Nira JR, Farr AL, et al. Protein Measurement with The Folin Phenol Reagent. *Journal Biological Chemistry*. 1951:265–275.

20. Louwrier A. Industrial products: the return to carbohydrate-based industries. *Biotechnology Applied Biochemestry*. 1998;27:1–8.

21. Wiseman A. Introduction to principles. *Handbook of enzyme biotechnology*. 1995:3–8.

22. Foret GM, Moss DA. Production of enzymes by fermentation. In: Rehm HJ, Reed G, editors. Biotechnology, Weinheim Ver-lag Chemie. 1987:65–211.

23. Hasan F, Hameed A. Optimization of lipase production from Bacillus sp. *Pakistan Journal of Botany*. 2001;33:789–796.

24. Ankit M, Sunil KY, Mishra P, et al. Screening and Temperature Optimization for Lipase-Producing Bacteria from Waste Contaminated Water. *Asian Journal of Biochemical and Pharmaceutical Research*. 2011;1:62–69.

25. Ozgur K, Nilufer C. Comparison of three Yarrowia lipolyticastrains for lipase production: NBRC 1658, IFO 1195, and a local strain. *Turkish Journal of Biology*. 2012;36:15–24.

26. Kathiravan T, Marykala J, Sundaramanickam A, et al. Studies on nutritional requirements of Pseudomonas aeruginosa for lipase production. *Advances in Applied Science Research*. 2012;3:591–598.

27. Kavitha M, Shanthi C. Isolation and Characterization of Cold active lipase producing Pseudomonas sp. 4 from Marine samples of Tamilnadu Coast. *Research Journal of Biotechnology*. 2013;8:57–62.

28. Balan A, Magalinggam N, Ibrahim D, et al. Thermostable Lipase Production by Geobacillus thermodenitrificans In A 5-L Stirred-Tank Bioreactor. *International Journal of Microbiolog*. 2010;8:1937–8289.

29. Mobarak QE, Kasra KR, Moosavi NZ. Isolation and identification of a novel, lipase-producing bacterium, Pseudomnas aeruginosa KM110. *Iranian Journal of Microbiology*. 2011;3:92–98.

30. Nawani N, Singh R, Kaur J. Immobilization and stability studies of a lipase from thermophilic Bacillussp: The effect of process parameters on immobilization of enzyme. *Journal of Microbial Biotechnology*. 2006;9:0717–3458.

31. Schmidt DC, Sztajer H, Stocklin W, et al. Screening purification and properties of a thermophilic lipase from Bacillus themocatenulants. *Biochimica et Biophysica Acta*. 1994;1214:43–53.

32. Lee DW, Koh YS, Kim KJ, et al. Isolation and characterization of a thermophilic lipase from Bacillus thermoleovorans ID-1. *FEMS Microbiology Letters*. 1999;179:393–400.

33. Wang Y, Srivastava KC, Shen GJ, et al. Thermostable alkaline lipase from a newly isolated thermophilic Bacillus strain A30-1 (ATCC 53841). *Journal of Fermentation and Bioengineering*. 1995;79:433–438.

34. Rathi P, Bradoo S, Saxena RK, et al. A hyperthermostable, alkaline lipase from Pseudomonas sp. with the property of thermal activation. *Journal of Biotechnology Letter*. 2000;22:495–498.

35. Demirkan E. Production, purification, and characterization of α-amylase by Bacillus subtilis and its mutant derivates. *Turkish Journal of Biology*. 2011;35:705–712.

36. Mayerhofer HJ, Marshall RT, White H, et al. Characterization of a Heat-Stable Protease of Pseudomonas fluorescens P261. *Journal of Applied Microbiology*. 1973;25(1):44–48.

37. Patil CS, Gangawane AK, Shankerappa SH. Production and characterization of alkaline thermostable protease from newly isolated Bacillus sp. *Journal of Plant Genetics*. 2010;1:09–17.

38. Amro AA, Soheir RS, Mohamed SSA. The Possibility to Use Bacterial Protease and Lipase as Biodetergent. *Global Journal of Biotechnology & Biochemistry*. 2009;4:104–114.

39. Malhotra R, Noorwez SM, Satyanarayana T. Production and partial characterization of thermostable and calcium-independenta-amylase of an extreme thermophile Bacillus thermooleovorans NP54. *Letters in Applied Microbiology*. 2000;31:378–384.

40. Adinarayana K, Ellaiah P, Prasad DS. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated Bacillus subtilis PE-11. *American association of pharmaceutical scientists Pharm Sci Tech*. 2003;4:1–9.

41. Femi-Ola TO, Desmond OO. Partial purification and characterization of a thermo stable alkaline protease from Lactobacillus brevis. *Malaysian Journal of Microbiology*. 2012;8:1–5.

42. Pathak PA, Deshmukh KB. Alkaline protease production, extraction and characterization from alkalophilic Bacillus lichenuiformis KBDL4. A Lonar soda lake isolate. *Indian Journal of Experimental Biology*. 2012;50:569–576.

43. Lee SO, Kato J, Takiguchi N, et al. Involvement of an Extracellular Protease in Algicidal Activity of the Marine Bacterium Pseudoalteromonas sp. Strain A28. *Journal of Applied & Environmental Microbiology*. 2000;66:4334–4339.

44. Hailemariam AT, Tilahun BG, Gulelat DH. Isolation, Purification and Characterization Thermostable Alpha-Amylase from Geothermal Sites of Ethiopia (Afar Region). *Greener Journal of Biological Sciences*. 2013;3:061–073.

45. Najaf MF, Deobagkar D, Deobagkar D. Purification and characterization of an extracellular α-amylase from Bacillus subtilis AX20. *Protein Expression and Purification*. 2005;41:349–354.

46. Goyal N, Gupta JK, Soni SK. A novel raw starch digesting thermostable α-amylase from Bacillus sp. I-3 and its use in the direct hydrolysis of raw potato starch. *Journal Enzyme and Microbial Technology*. 2005;37:723–734.

47. Ramesh MV, Lonsane BK. Effect of metal salts and protein modifying agents on activity of thermostable α-amylase produced by Bacillus licheniformis M27 under solid state fermentation. *Chemie, Mikrobiologie, Technologie der Lebensmittel*. 1990;12:129–136.

48. Koch R, Spreinat A, Lemke K, et al. Purifcation and properties of a hyperthermoactive α-amylase from the archaeo bacterium Pyrococcuswoesei. *Journal of Archives of Microbiology*. 1991;155:572–578.

49. Božić N, Ruiz J, López SJ, et al. Production and properties of the highly efficient raw starch digesting α-amylase from a Bacillus licheniformis ATCC 9945a. *Biochemical Engineering Journal*. 2011;53:203–209.

50. Prakash B, Prakash P, Vidyasagar M, et al. Effect of organic solvents and osmolytes on extremely halotolerant and thermostable α-amylases from Chromohalobacter sp. TVSP 101. *International Journal of Biotechnology and Biochemistry*. 2009b;44:210–215.

51. Ballschmiter M, Futterer O, Liebl W. Identification and Characterization of a Novel Intracellular Alkaline-Amylase from the Hyperthermophilic Bacterium Thermotogamaritima MSB8. *Journal of Applied & Environmental Microbiolology*. 2006;72:2206–2211.

52. Oliveira AND, Oliveira LAD, Andrade JS. Partial Characterization of Amylases of two Indigenous Central Amazonian Rhizobia Strains. *Brazilian Archives of Biology and Technology*. 2010;53(1):35–45.

53. Nascimento WCA, Martins MLL. Studies on the stability of protease from bacillussp. And its compatibility with commercial detergent. *Brazilian Journal Microbiology*. 2006;37:307–311.

54. Banerjee UC, Sani RK, Azmi W, et al. Thermo stable alkaline protease from Bacillus brevis and its characterization as a laundry detergent additive. *Journal of Process Biochemistry*. 1999;35:213–219.

55. Usharani B, Muthuraj M. Production and characterization of protease enzyme from Bacillus laterosporus. *Africation Journal of Microbiology Research*. 2010;4:1057–1063.

56. Rahman RN, Ra Z, Geok ALP, et al. An organic solvent-stable alkaline protease from Pseudomonas aeruginosa strain K: Enzyme purification and characterization. *Enzymes and Microbial Technology*. 2006;39:1484–1491.

57. Mabrouk SS, Hashem AM, El-Shayed NMA, et al. Optimization of alkaline protease productivity by Bacillus licheniformis ATCC 21415. *Bioresources Technology*. 1999;69:155–159.

58. Allemand L, Bahn PG. Best way to protect rock art is to leave it alone. *Nature*. 2005;433:800.

59. Cañaveras JC, Sanchez MS, Sloer V, et al. Microorganisms and microbially induced fabrics in cave walls. *Geomicrobiology Journal*. 2001;8:223–240.

60. Fox JL. Some say Lascaux Cave paintings are in microbial "crisis" mode. *Microbe*. 2008;3:110–112.

61. Hasan F, Aamer AS, Hameed A. Industrial applications of microbial lipases. *Enzy Microb Technol*. 2006;39:235–251.

62. Li H, Ziaobo Z. Characterization of thermostable lipase from thermophilic Geobacillus sp. TW1. *Protein Expression and Purification*. 42:153-159.

63. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*. 2016;33:1870–1874.