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Bacterial isolates from lonar lake produce antimicrobial factors effective against MDR pathogen

Anushka Salvi* **and A. Harsulkar**

Department of Biotechnology, Poona College of Pharmacy, Maharashtra, India

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ABSTRACT

Lonar lake being one of its kind is a crater impact saline lake that harbors extremophile vegetation and organisms with unusual properties. Several microorganisms have been isolated from this lake and studied for their properties. The current project involved an antimicrobial lonar isolate: *Bacillus sp.* which was screened for its activity against six multi-drug resistant human pathogens and showed positive results against *Enterococcus faecalis*. The growth parameters of the antibacterial *Bacillus sp.* were optimized and the compound was extracted using chloroform as solvent. The crude extract and partially purified compound was further characterized using TLC and HRMS.

Keywords: Lonar lake; Extremophile; Alkaliphile; Antiniotic resistant human pathogen; Antibacterial

Address for correspondence:

Anushka Salvi,
Department of Biotechnology,
Poona College of Pharmacy,
Maharashtra, India;
E-mail: anushkasalvi@yahoo.com

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INTRODUCTION

Extremophiles are bacteria that thrive in extreme hot niches, ice, and salt solutions, as well as acid and alkaline conditions; some may grow in toxic waste, organic solvents, heavy metals or in several other habitats that were previously considered inhospitable for life. Alkaliphiles are organisms that grow optimally or very well at pH values above 9 but cannot grow or grow only slowly at the near-neutral pH value of 6.5. Haloalkaliphiles are mostly confined to very hyper-saline soda lakes and soda deserts. When needed to be cultured they require $NaHCO_3$-Na_2CO_3 buffer medium, to adjust the pH at around 10.5 which is done by the addition of 1% (w/v) Na_2CO_3. It is important to sterilize the Na_2CO_3 separately from the rest of the media components. Many of the diverse alkaliphiles displaying novel physiological features and potential applications were isolated from soda lakes found in various continents and settings. Soda lake alkaliphiles are found extensively in parts of Asia (e.g., China and Russia) and in Africa but there are other well-studied alkaliphiles that were found in places where soda lakes are less frequent, e.g., Lake Mono in California. As noted, the alkaliphiles isolated from soda lakes are often poly-extremophiles, e.g., *Natranaerobius thermophilus*, an anaerobe, which was isolated from the hypersaline lake Wadi El Natrun, Egypt and is resistant to high sodium-ion concentrations, pH and temperature.

In industries alkaliphiles contribute in production of enzymes which can work at alkaline pH with high or low temperature conditions which increase the range of activity of the bioreactors. Alkaliphiles also produce useful metabolites, including antibiotics. Antibiotics are any substance that inhibits the growth and replication of a bacterium or kills it outright. Antibiotics are antimicrobial designed to target bacterial infections within (or on) the body. Along with vaccines, antibacterial antibiotics are perhaps the most successful pharmacologic therapy of the modern era. The use of antibiotics has markedly diminished mortality from infectious diseases, reduced the burden of tuberculosis in the modern world, improved survival after trauma and allowed the development of therapies that intentionally or incidentally suppress the immune system for the treatment of autoimmune diseases and cancers. There exist different mechanisms by which antibiotics control infections by inhibiting the growth and proliferation of the bacteria. Some important mechanisms are inhibition of cell wall synthesis, breakdown of cell membrane structure or function, inhibition of the structure and function of nucleic acids, inhibition of protein synthesis and blockage of key metabolic pathways [1]. Lonar lake, also known as Lonar crater, is a notified national-geo-heritage monument located at Lonar in Buldhana district

of Maharashtra. Lonar lake was created by a meteor impact an event estimated to have happened during the Pleistocene Epoch. Microbial diversity assessment and isolation of amylase, protease, chitinase and antibiotic producers are reported by various researchers from Lonar lake. These microorganisms harbor several biotechnologically relevant enzymes and bio-molecules and there is the need to augment bio-prospecting efforts in soda lake environments with new integrated approaches.

Resistance to antimicrobials represents a worldwide health-care problem that affects therapy of infectious diseases caused by a large variety of organisms. Antimicrobial resistance happens when microorganisms (such as bacteria, fungi and viruses) change when they are exposed to antimicrobial drugs (such as antibiotics, antifungals and antivirals). Microorganisms that develop antimicrobial resistance are sometimes referred to as "superbugs". As a result, the medicines become ineffective and infections persist in the body, increasing the risk of spread to others. Bacteria have developed diverse means to circumvent the growth-inhibitory properties of antimicrobial agents. Major mechanisms of bacterial resistance to antimicrobial agents includes: Enzymatic drug inactivation, drug target modification, drug permeability reduction and active efflux of drugs. These resistance mechanisms allow bacteria to survive or even to actively grow, in the presence of a given antimicrobial agent and even certain bacterial variants have evolved mechanisms to resist multiple drugs, making such variants recalcitrant to chemotherapy against such bacterial strains [2].

Until 1984, *E. faecalis* was known as *Streptococcus faecalis*. Scientists previously categorized the bacteria as part of the genus Streptococcus. According to the Centers for Disease Control and Prevention (CDC), *E. faecalis* is responsible for approximately 80% of cases of human infection. The bacteria can cause infection in people when it enters through wounds, blood or urinary track. People with weakened immune systems are at risk. People in hospital settings are particularly vulnerable to *E. faecalis* infection because hospitalized patients tend to have reduced immunity. The common use of intravascular and urinary catheter devices can also contribute to the spread of infection, as these instruments frequently harbor the *E. faecalis* bacteria. It is believed to be one of the top three leading causes of hospital-acquired infection.

E. faecalis has both natural and acquired immunity from antibiotic treatment. As a result, it is one of the most antibiotic-resistant types of bacteria. The bacteria can also tolerate a variety of conditions. It can survive in a range of temperatures and acidic or alkaline environments. Since naturally present in the gastrointestinal tract, *E. faecalis* is found in fecal matter. Improper cleaning of items containing fecal matter, or not washing hands after restroom use, can increase the risk of bacterial transmission. The bacteria can spread throughout hospitals if healthcare workers do not wash their hands between patients. If not thoroughly cleaned, catheters or dialysis ports can also transmit infection. In the current study 6 human pathogens were screened for their activity against an antibacterial compound extracted *Bacillus sp*. And an attempt was made to further characterize the crude metabolite.

MATERIALS AND METHODS

Procurement of bacterial strains and media preparation

Total 7 strains of bacteria were procured from National Centre for Microbial Resource-NCMR repository out of which 6 strains were pathogenic namely: (*Enterococcus fecalis* MCC-2409, *E. coli* MCC-2412, *E. coli* 2413, *K. pneumoniae* MCC-2451, *P. aeruginosa* MCC-2080, *S. aureus* MCC-2408) and one strain was a alkanine lonar isolate-*Bacillus sp*. MCC-2982. The lonar isolate was grown on nutrient agar as its basic media with pH adjusted to 9.5-10 with sodium carbonate (Na_2CO_3). The pathogenic strains required specific growth media as mentioned in Table 1. The optimum incubation temperature for pathogenic strains as well as lonar isolate MCC-2982 was 37°C+10°C (**Tab. 1.**) [3].

Tab. 1. Growth conditions employed for pathogenic strains.

MCC	Name	Media	Temperature	pH	Incubation period
2409	*Enterococcus faecalis*	Trypticase soy yeast extract or tryptone soy broth	37°C	7.2	24-48 hours
2412	*Escherichia coli*	Nutrient agar	37°C	7.2	24 hours
2413	*Escherichia coli*	Luria bertani Agar	37°C	7	24 hours
2451	*Klebsiella pneumoniae* sub sp. Pneumonia	Nutrient agar	37°C	7	24 hours
2080	*Pseudomonas aeruginosa*	Nutrient agar	37°C	7	24 hours
2408	*Staphylococcus aureus*	Trypticase soy yeast extract	37°C	7	24 hours

After preservation of the basic stock plates of the pathogenic strains Muller Hinton agar (MH) was found to support the growth of all the pathogens hence MH agar was used further to grow the pathogens.

Priliminary screening

The anti-microbial activity of MCC-2982 was estimated against the 6 procured pathogenic strains with well method initially using soft agar (1% agar) method. Briefly, a base layer of nutrient agar pH 9.5 and 2% agar was poured into clean autoclaved plates. Muller hinton with 1% agar infused with a thick suspension (500 ul) of pathogenic strains was poured upon the base layer. This was

allowed to set for 30 min. A well was bored in the base and top layers with a well borer. A thick suspension (50 ul) of MCC-2982 antimicrobial isolate was poured into the wells. The plates were kept at 4°C for 30 min to allow diffusion Plates were incubated at 37°C for 48 hours and results were observed.

Screening of resistance profile of MCC-2409

Growth *E. faecalis* MCC-2409 was inhibited by *Bacillus sp.* and hence its resistance profile was checked against other antibiotics. Dodeca discs were procured from HiMedia and resistance profile was assessed against universal 1- DE001, universal 2- DE007 and universal 3 minus-DE008. These discs are inert flat circular ring having 12 discs of 6 mm diameter on its projections and are coated with antibiotics that aid antibiotic susceptibility testing of a wide range of microorganisms. Briefly, 90 ml Mueller Hinton agar plates were poured with a depth of 4 mm agar and were incubated at room temperature for 24 hours to check their sterility. MCC-2409 was freshly grown in its Muller Hinton (MH) broth. After 24 hours under aseptic conditions using a sterilized swab MCC-2409 was spread on the MH agar plates. The Dodeca antibiotic discs were aseptically placed on the plates. The plates were kept at 4°C immediately for 30 min. To allow diffusion of the antibiotic in the agar. Post diffusion the plates were incubated at 37°C for 48 hours. Results were observed after 24 and 48 hours intervals [4].

Minimum inhibitory concentration

A stock solution (5 mg/ml) of filter sterilized 96 hours old culture of *Bacillus sp.* was prepared in 10% Dimethyl Sulfoxide (DMSO). Different concentrations of the extract (100 µg-1000 µg) were transferred to 96-well plate and 24 hour freshly grown test microbial strain MCC-2982 *Enterococcus fecalis* was added to each. 10% DMSO was used as negative control. The test for each concentration was carried out in triplicate. The plates were aseptically incubated for 24 hours at 37 degree Celsius and absorbance was measured at 620 nm using UV-Vis spectrophotometer. The plates were then incubated at room temperature for 2 hours after adding 0.015% resazurin. The concentration showing no change in color (Blue) was considered as the MIC value of the extract.

Charecterization of *Bacillus sp.* MCC-2982 by scanning electron microscopy

The culture of *Bacillus sp.* MCC-2982 was observed under scanning electron microscope to observe its structure and predict any novel characteristics of the novel species. MCC-2982 was grown at 37°C for 48 hours on pH 10 Nutrient agar. A few bacterial colonies were suspended in sterile Phosphate Buffer Saline (PBS) and the pellet was washed and centrifuged 3 times at 8000 rpm for 5 minutes. Cells were then fixed immediately with 2.5% (v/v) glutaraldehyde in phosphate buffer saline for 2 hours at 4°C. The cells were further washed twice in buffer and dehydrated by sequentially increasing the concentration of ethanol (30%-90%) for 10 min each at 4°C. Subsequently the specimens were fixed on clean glass slides and air dried. The slides were then placed on microscope stubs and examined under Environmental scanning electron microscope. Environmental scanning electron microscopy of MCC2982 was carried out at Indian Institute of Science Education and Research, Pune [5].

Optimization of growth parameters of *Bacillus sp.* MCC-2982

MCC-2982 showed positive activity against a pathogenic fecalis strain and hence the following parameters essential for its growth were optimized:

Effect of incubation period: For optimization of time period, a 20 ml active culture of MCC-2982 was grown in Nutrient Broth (NB) pH 10 for 24 hours. This active culture was further inoculated in 180 ml NB pH 10 and was incubated in an incubator shaker (150 rpm) at 37°C for 96 hours. After every 24 hours 20 ml of the culture was transferred aseptically to sterilized tubes, grown culture was centrifuged at 38000 rpm for 40 min. Then the supernatant was filter sterilized and its activity was checked against the pathogens with disc bioassay.

Effect of pH of the growth medium: For optimizing pH of medium the bacteria was grown at different ph ranges: 7, 8, 9, 10, 11 and its activity against pathogen was checked upon. A 30% solution of Na2CO3 was used to adjust the pH of the medium. MCC-2982 was grown separately in 10 ml NB active broths of the different required ph ranges. The active cultures were then inoculated in 90 ml NB broth according to their corresponding pH ranges and were incubated at 37°C for 96 hours in an incubator shaker (150 rpm). The cultures were transferred aseptically to sterilized tubes, centrifuged at 38000 rpm for 40 min. Their respective supernatant was filter sterilized and its activity was checked was checked against the pathogens with disc bioassay.

Effect of salt concentration (NaCl) in the growth medium: For optimizing salt concentration of medium the bacteria were grown at varying salt (NaCl) concentrations *i.e*, 0%, 1% 3%, 5%, 7% and 10%. Similar to the above experiments10 ml active cultures of each salt concentration were grown for 24 hours at 37°C in an incubator shaker (150 rpm) and were further inoculated in their respective salt concentrations adjusted at pH 10. These broths were incubated at 37°C in an incubator shaker (150 rpm) for 96 hours. The cultures were transferred aseptically to sterilized tubes, centrifuged at 38000 rpm for 40 min. Their respective supernatant was filter-sterilized and its activity was checked against the pathogens with disc bioassay [6].

Innoculum density

McFarland standards were used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing. They provide a reference for standardization of bacterial suspensions and used for susceptibility testing and other procedures that require a standardized inoculum. They come in a set of 5 standards with increasing turbidity namely: 1, 2, 3, 4, 5.

MCC-2982 was grown initially in 10ml active culture in NB pH 10 at 37°C in an incubator shaker (150 rpm) for 48 hours. This active culture was then inoculated in 90 ml NB pH 10 and was grown for 48 hours. Five 90 ml NB pH 10 broths were separately made and the 100 ml active culture was used to adjust their turbidities to correspond to the 5 standards. These 5 standards were grown at 37°C for 96 hours, centrifuged at 3800, filter sterilized and each of their activity was assessed by Chloroform extraction and supernatant by well and disc assays.

Assessment of the antimicrobial metabolite

The activity of the antimicrobial metabolite was assessed after three steps of purification.

Lyophilization: MCC-2982 was grown in NB 100 ml of

NB; pH 10 in an Erlenmeyer flask at 37°C for 96 hours in an incubator shaker (150 rpm). After 96 hours, the culture broth was centrifuged at 38000 rpm for 40 min followed by filter sterilization using 0.22 micron syringe filters. This cell free supernatant was then transferred to sterile falcon tubes sealed with parafilm and kept overnight at -81°C. After freezing parafilm was punctured with toothpick and the tubes were subjected to lyophilization, wherein under vacuum, the frozen water is directly sublimated. The supernatant was concentrated until the volume was 4ml. The concentrate was then preserved at 5°C and was further used for determining the antimicrobial activity by disc diffusion assay and agar well diffusion assay.

Supernatent: MCC-2982 was grown initially in 10ml active culture in NB pH 10 at 37°C in an incubator shaker (150 rpm) for 24 hours. This active culture was then inoculated in 90 ml NB pH 10 and was grown for 96 hours in incubator shaker for 96 hours. The supernatant was then centrifuged at 3800 rpm and subjected to filter sterilization using 0.22 micron syringe filter. This cell free supernatant was then used to assess the antimicrobial activity [7].

Solvent extraction: MCC-2982 was grown initially in 10ml active culture in NB pH 10 at 37°C in an incubator shaker (150 rpm) for 24 hours. This active culture was then inoculated in 90 ml NB pH 10 and was grown for 96 hours in incubator shaker for 96 hours. The supernatant was then centrifuged at 3800 rpm and subjected to filter sterilization using 0.22 micron syringe filter. This cell free supernatant was then mixed with equal parts of chloroform and extracted twice with a separating funnel. The clear organic layer containing the crude antibiotic was then evaporated at 40°C to obtain a dry power of the antimicrobial metabolite. The activity was assayed with disc method re-dissolving the obtained metabolite with 10% DMSO (Dimethyl Sulfoxide). Extraction was done using 3 solvents *i.e.*, butanol, ethyl acetate and chloroform out of which only chloroform gave positive results.

Thin layer chromatography

To analyze the metabolite, two solvent systems were used. The first solvent system contained petroleum ether (30%) and ethyl acetate (70%)-(30:70). This solvent system was prepared and kept aside in a beaker for an hour for saturation. Silica coated aluminum sheet was used as a stationary phase. Dried chloroform extract of the metabolite was re-dissolved in chloroform and was spot on silica plates with the help of capillary tube. These plates were then placed in the saturated solvent system and the mobile phase was allowed to run upto 80%. The silica plates were then removed from the chamber, observed under UV and dipped in a mixture of 90% ethanol and 10% H_2SO_4. The plates were then dried with a hot air gun at 110°C, spots were observed and Rf value was calculated. The second solvent system contained n-butanol (2%), Acetic acid (1%) and water (1%)-(2:1:1), the rest of the conditions being the same as those used for the first solvent system.

High resolution mass spectrometry (hr-ms)

Mass Spectrometry (MS) measures the mass (m) to charge (z) ratio-m/z of the ions in a sample. Those ratios provide an atomic signature for what makes up a sample. (HR-MS) allows detection of analytes to the nearest 0.001 atomic mass units. The crude antibacterial metabolite was analyzed by HR-MS at Savitribai Phule Pune university to determine its atomic mass. Two batches were given for the analysis.

RESULTS

Priliminary screening of pathogens

Six human pathogens, *Enterococcus fecalis* MCC-2409, *E. coli* MCC-2412, *E. coli* 2413, *K. pneumoniae* MCC-2451, *P. aeruginosa* MCC-2080, *S. aureus* MCC-2408 were screened for their activity against *B. sp*. MCC-2982. Their activity was assessed by agar well diffusion assay and out of the six pathogens, clear zone of inhibition of 20 mm was found around *Enterococcus fecalis* MCC-2409. Rest of the five pathogens remained resistant and did not show zone of clearance (**Fig. 1.**).

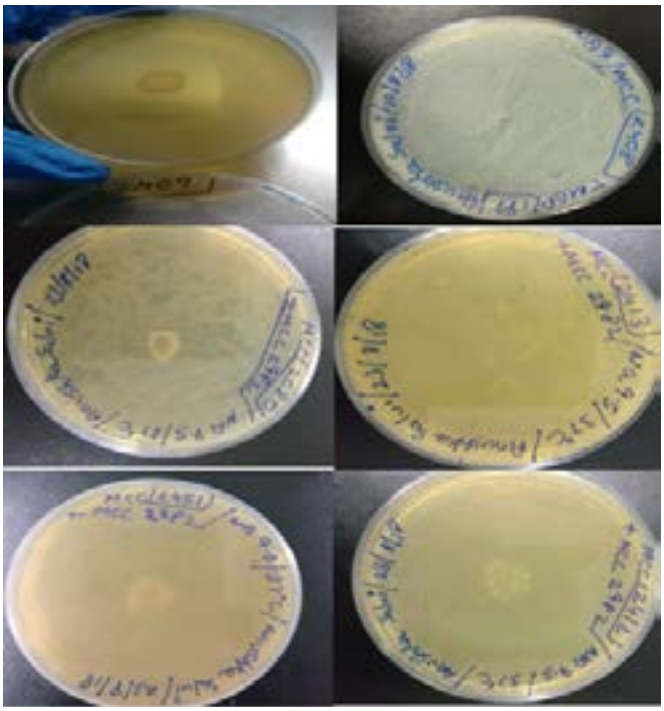


Fig. 1. Six human pathogens, *Enterococcus fecalis* MCC-2409, *E. coli* MCC-2412, *E. coli* 2413, *K. pneumoniae* MCC-2451, *P. aeruginosa* MCC-2080, *S. aureus* MCC-2408 were screened for their activity against *B. sp.* MCC-2982. Only *Enterococcus fecalis* MCC-2409 showed zone of clearance.

Screening of resistance profile of MCC-2409

As the growth of pathogen *Enterococcus fecalis* MCC-2409 was inhibited by antibacterial MCC-2982 it was necessary to check the resistance profile of the pathogen against the available standard antibitiotics. This was done using the Himedia Dodeca Universal 1, 2 and 3 discs. The discs contained 12 antibiotics each and the results ensured that the human pathogen was indeed resistant against 60% of the market available antibiotics and our antibacterial was able to restrict its growth (**Tab. 2-4. and Fig. 2.**).

Tab. 2. Universal-I DE001.

Sr. no	Antibitic	Symbol	Concentration	Standard zones	Zone in mm		Results
					plate 1	plate 2	
1	Cefpodoxime (CPD)	CPD	10 ug	23 mm-28 mm	0 mm	0 mm	Resistant
2	Chloramphenico (C)	CPD	30 ug	21 mm-27 mm	25 mm	27 mm	Sensitive
3	Vancomycin (VA)	VA	30 ug	17 mm-21 mm	22 mm	21 mm	Sensitive
4	Streptomycin (S)	SYMBOL	10 ug	12 mm-20 mm	17 mm	17 mm	Sensitive
5	Rifampicin (RIF)	RIF	5 ug	8 mm-10 mm	21 mm	23 mm	Sensitive
6	Levofloxacin (LE)	LE	5 ug	29 mm-37 mm	32 mm	29 mm	Sensitive
7	Ceftriaxone (CTRE)	CTR	30 ug	29 mm-35 mm	0 mm	0 mm	Resistant
8	Clindamycin (CD)	CD	2 ug	21 mm-30 mm	18mm	17 mm	Sensitive
9	Augmentin (AMC)	AMC	30 ug	18 mm-24 mm	0 mm	0 mm	Resistant
10	Amikacin (AK)	AK	30 ug	19 mm-26 mm	12mm	11 mm	Slightly sensitive
11	Cefixime (CFM)	CFM	5 ug	23 mm-27 mm	0 mm	0 mm	Resistant
12	Tetracyclin (TE)	TE	30 ug	23 mm-25 mm	18 mm	22 mm	Sensitive

Tab. 3. Universal-I DE007.

Sr. no	Antibitic	Symbol	Concentration	Standard zones	Zone in mm		Results
					plate 1	plate 2	
1	Amikacin (AK)	AK	30 ug	19 mm-26 mm	13 mm	14 mm	Sensitive
2	Co-Trimoxazole (COT)	COT	25 ug	23 mm-29 mm	35 mm	35 mm	Sensitive
3	Colistin (CL)	CL	10 ug	11 mm-17 mm	0 mm	0 mm	Resistant
4	Augmentin (AMC)	AMC	30 ug	18 mm-24 mm	0 mm	0 mm	Resistant
5	Netillin (NET)	NET	10 ug	22 mm-30 mm	0 mm	0 mm	Resistant
6	Norfloxacin (NX)	NX	10 ug	26 mm-30 mm	26 mm	25 mm	Sensitive
7	Ceftriaxone (CTR)	CTR	10 ug	29 mm-35 mm	0 mm	0 mm	Resistant
8	Ciprpfloxacin (CIP)	CIP	5 ug	30 mm-40 mm	28 mm	28 mm	Sensitive
9	Cefotaxime (CTX)	CTX	30 ug	29 mm-35 mm	15 mm	16 mm	Slightly sensitive
10	Gentamicin (GEN)	GEN	10 ug	19 mm-26 mm	21 mm	20 mm	Sensitive
11	Furazolidone (FR)	FR	50 ug	20 mm-25 mm	13 mm	12 mm	Slightly sensitive
12	Amoxycillin (AMX)	AMX	10 ug	19 mm-25 mm	0 mm	0 mm	Resistant

Tab. 4. Universal-I DE008.

Sr. no	Antibitic	Symbol	Concentration	Standard zones	Zone in mm		Results
					plate 1	plate 2	
1	Ampicillin (AMP)	AMP	10 ug	16 mm-22 mm	0 mm	0 mm	Resistant
2	Cefuroxime (CXM)	CXM	30 ug	20 mm-26 mm	0 mm	0 mm	Resistant
3	Cefedroxil (CFR)	CFR	30 ug	12 mm-18 mm	0 mm	0 mm	Resistant
4	Augmentin (AMC)	AMC	30 ug	18 mm-24 mm	0 mm	0 mm	Resistant
5	Penicillin (P)	p	10 units	26 mm-37 mm	0 mm	0 mm	Resistant
6	Cefotaxime (CTX)	CTX	30 ug	29 mm-35 mm	11 mm	11 mm	Resistant
7	Cefeclor (CF)	CFR	30 ug	23 mm-27 mm	0 mm	0 mm	Resistant
8	Azithromycin (AZM)	AZM	15 ug	21 mm-26 mm	12 mm	11 mm	Resistant
9	Erythromycin (E)	E	15 ug	22 mm-30 mm	19 mm	21 mm	Sensitive
10	Cefoperazone (CPZ)	CPZ	75 ug	28 mm-34 mm	0 mm	0 mm	Resistant
11	Clarithromycin (CLR)	CLR	15 ug	26 mm-32 mm	18 mm	18 mm	Slightly sensitive
12	Ciprofloxacin (CIP)	CIP	5 ug	30 mm-40 mm	28 mm	28 mm	Slightly sensitive

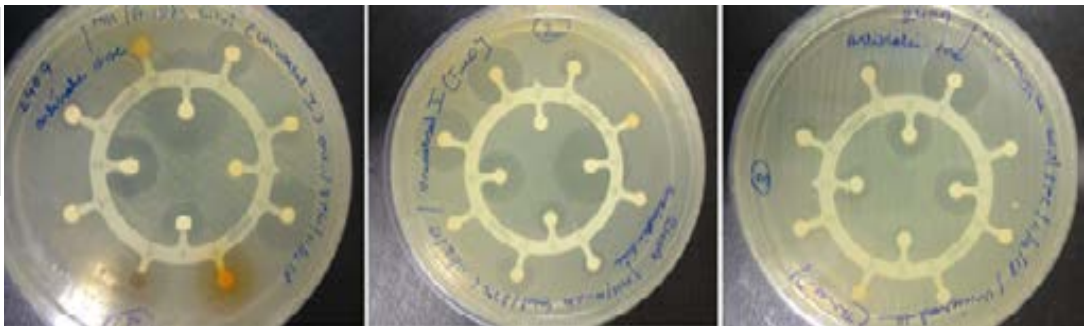


Fig. 2. Resistance profile of the pathogen *Enterococcus fecalis* MCC-2409 against Himedia Dodeca universal 1, 2, and 3 discs showed that it is resistant to 60% of the antibiotics.

Minimum inhibitory concentration

Crude extract of the antibacterial compound was used to determine the minimum inhibitory concentration against the pathogen. The results of MIC were found to be in the range of 100 ug-200 ug which indicates repetition of the MIC to be done using the purified version of the antibacterial compound [8].

Charecterization of *Bacillus sp*. MCC-2982 by scanning electron microscopy

Environmental scanning electron microscopy of a 48 Hours old culture of Bascillus sp. was carried out under magnification of 5.70KX, 7.90KX and 10.53KX. A typical morphology of bacilli was observed with rod shaped cells (**Fig. 3.**).

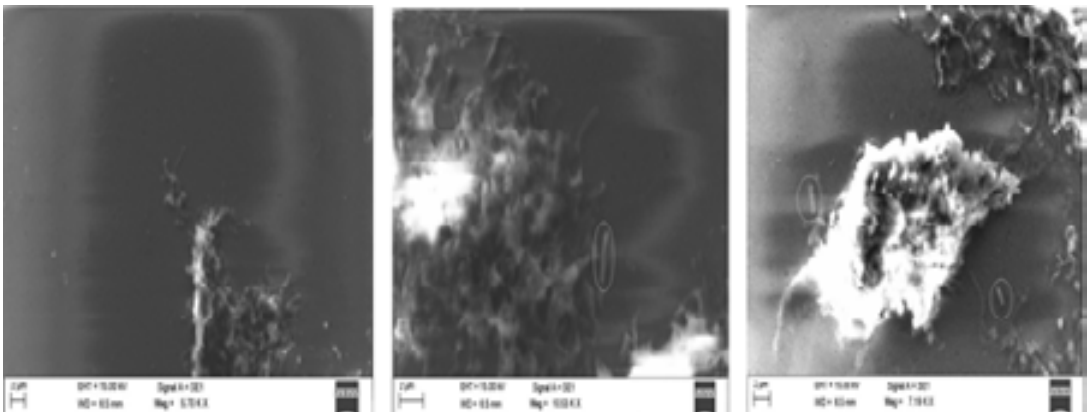


Fig. 3. SEM photographs of 48 hours old culture of *Bascillus sp*.

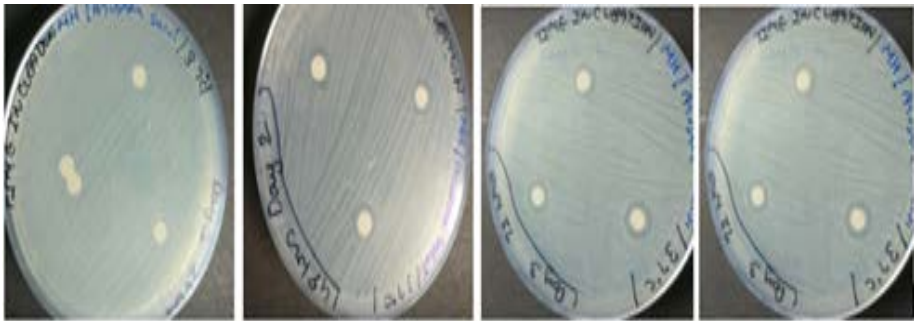
Optimization of growth parameters of *Bacillus sp*. MCC-2982

Effect of incubation period: The experiment was carried out in triplicate to determine the number of hours required for production of the metabolite. Zones start appearing from the 2nd day and increase till the 4th day (**Tab. 5. and Fig. 4.**).

Tab. 5. Incubation time period.

Day	Zone (d1)	Zone (d2)	Zone (d3)
1 (24 Hours)	10 mm	10 mm	10 mm
2 (48 Hours)	11 mm	11 mm	11 mm
3 (72 Hours)	12 mm	12 mm	12 mm
4 (96 Hours)	13 mm	13 mm	13 mm

Fig. 4. *Bacillus sp.* MCC-2982 required 96 hours for optimum growth.



Effect of pH of the growth medium: *Bacillus sp*. was grown in Nutrient Broth with varying pH values from 7 to 11(using Na_2CO_3). Our organism being an alkaliphilic extremophile grew and produced the secondary metabolite showing zones of inhibition only at pH 10. This experiment was also set in triplicate.

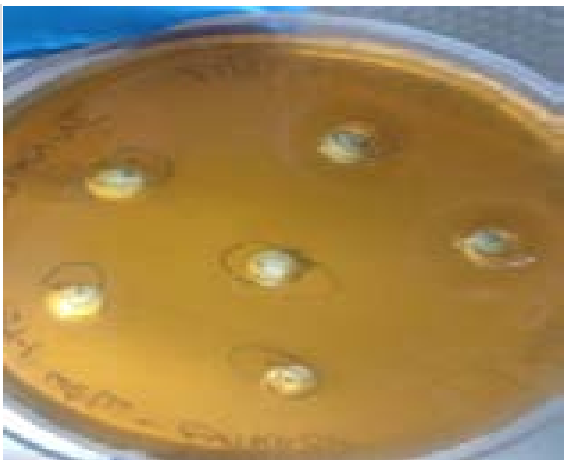
Effect of salt concentration (NaCl) in the growth medium: The salt concentration was varied from 0% to 10% (0%, 1%, 3%, 5%, 7% and 10%). Zones appeared at 7% and 10% indicating the growth and production of the secondary metabolite [9].

Innoculum density

Using Mcferland standards 1, 2, 3, 4 and 5 ab pattern of metabolite production was observed. In the case of the chloroform extract the inhibition zones were not directly proportional to the increasing order of the cell density whereas in the case of supernatant the zones were found to be linear throughout (**Fig. 5. and 6.**). Chloroform extract showed clear zones of inhibitions as compared to dusky and unclear zones portrayed by supernatant (**Tab. 6. and 7.**).

Fig. 5. Determination of Inoculum density of chloroform using disc assay.

Fig. 6. Determination of Inoculum density of chloroform using well assay.



Tab. 6. Determination of Inoculum density of chloroform using disc assay.

Mc Farland standard no	Zones of inhibition
1	15 mm
2	17 mm
3	17 mm
4	13 mm
5	15 mm
Chloroform (Blank)	0 mm

Tab. 7. Determination of Inoculum density of chloroform using well assay.

Mc Farland standard no	Zones of inhibition
1	17 mm
2	16 mm
3	11 mm
4	10 mm
5	10 mm
Chloroform (Blank)	0 mm

Assessment of the antimicrobial metabolite

The lyophilized extract showed maximum *i.e.* 16 mm but a dusky zone of inhibition. Supernatant showed a dusky 11 mm zone of inhibition. The Chloroform extract showed 13 mm and a clear zone of inhibition (**Fig. 7.**).

Fig. 7. Assessment of metabolite using supernatant, solvent extract and lyophilized extract.

Thin layer chromatography

TLC was carried out of the crude dried chloroform extract. Two solvent systems were run and the results obtained are as follows:

System 1: Petroleum ether (30%) and ethyl acetate (70%)-(30:70). Rf value: 0.79

System 2: n-butanol (2%), Acetic acid (1%) and water (1%)-(2:1:1). Rf value: 0.82

Spots with tailing were observed in both the TLC chromatograms. This can be attributed to the crude nature of the metabolite (**Fig. 8.**).

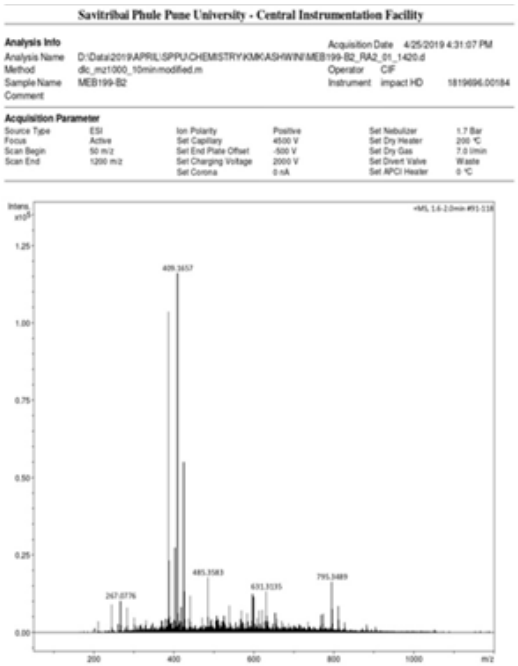
Fig. 8. TLC photographs of system 1 and 2.

Fig. 7. Assessment of metabolite using supernatant, solvent extract and lyophilized extract.

High resolution Mass spectrometry (Hr-Ms)

Two batches of the crude antibacterial metabolite were analyzed for Hr-MS characterization and the images above depict their results. Two peaks of similar molecular weights were observed in both the figures. In the 1st figure molecular weight of major peak was 409.1653 and its base peak was 795.3489. In the 2nd figure molecular weight major peak was 409.1651 and base peak was 795.3451. These recurring peaks assure the presence of a compound which can be assumed as the compound of our interest (**Fig. 9. and 10.**).

Fig. 9. HRMS graphs of 1st batch.



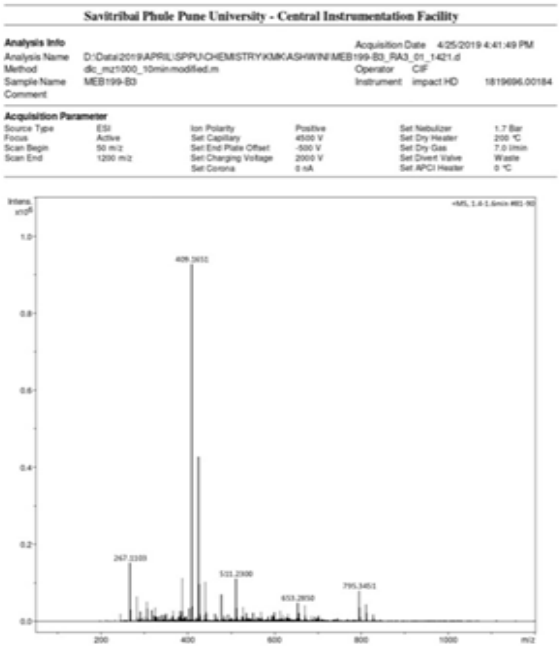


Fig. 10. HRMS graphs of 2nd batch.

DISCUSSION

Lonar crater is believed to be originated due to meteoritic impact and is the third biggest in the world and the only such in the great basaltic province of India. The remarkable shape, size and uniqueness of crater lake at crater basin being saline has attracted the attention of geologist, ecologists, archaeologists, naturalists, astronomists, biologists and has been the subject of several studies on various aspects of crater ecosystem. This inland lake with no effluent is fed by a seasonal drainage mainly confined to its periphery and also by number of fresh water springs. The crater contains many sub-ecosystems, each constituting a subtle combination of floral and faunal species, due to localized variations in the conditions of soil, water and humidity. The Lonar ecosystem has evolved in a unique way due to the unusual geohydrological and climatic conditions.

Multi drug resistance is an emerging crisis of the modern world. The resistance among various microbial species (infectious agents) to different antimicrobial drugs has emerged as a cause of public health threat all over the world at a terrifying rate. Due to the pacing advent of new resistance mechanisms and decrease in efficiency of treating common infectious diseases, it results in failure of microbial response to standard treatment, leading to prolonged illness, higher expenditures for health care and an immense risk of death. Almost all the capable infecting agents (e.g., bacteria, fungi, virus and parasite) have employed high levels of Multidrug Resistance (MDR) with enhanced morbidity and mortality; thus, they are referred to as "super bugs." Although the development of MDR is a natural phenomenon, the inappropriate use of antimicrobial drugs, inadequate sanitary conditions, inappropriate food-handling, and poor infection prevention and control practices contribute to emergence of and encourage the further spread of MDR. Our microbe of interest, *B. alkalinitrilicus* being an extremophile found in the Lonar habitat is a novel species with proven antibacterial activity against a human pathogen proves that the crater does harbor the growth of peculiar flora and fauna [10].

Six human pathogens were screened for their resistance out of which *Enterococcus fecalis* showed sensitivity towards this novel abtibacterial extracted from Lonar lake. *Enterococcus fecalis* is one of the most resistant type of bacteria which usually resides in a healthy gut causing no harm but is proven to be life threatening when it enters other body parts causing infections of the urinary tract, meningitis or blood poisoning. It immediately affects people with weakened immunity and hence is the most common hospital acquired infection. Putting in more efforts to examine the habitat and finding the different properties of the species residing in the crater formed saline Lonar lake will surely prove to be beneficial for mankind.

A variety of parameters influence the production of antibacterial metabolites by bacteria. Both nutritional and environmental parameters like carbon sources, nitrogen sources, inorganic salt concentration, fermentation time, temperature, pH etc have significant effect on production of active metabolites. Together with these parameters, the combination of media components also influences growth and metabolite production. *Bacillus sp*. produces the antibacterial as a secondary metabolite in 96 hours in an incubator shaker (150 rpm) and requires nutrient broth with its pH adjusted to 10 with Na_2CO_3 which was optimized during the course of this project.

The crude antibacterial metabolite was extracted from the broth by solvent extraction using chloroform in a separating funnel in 1:1 ratio. This metabolite was then obtained as a dry powder after evaporating the chloroform at 40 [11].

Different ways to characterize the antibacterial secondary metabolites can be TLC, MIC, mass spectrometry, HPLC, LCMS out of which TLC and HRMS were used to characterize the obtained crude metabolite. For carrying out TLC the solvent system was developed from scratch identifying the polarity of the compound. The compound being soluble and extracted by chloroform ensured it being moderately polar, hence solvents like toluene, ethyl acetate, petroleum ether, acetic acid were used in different concentrations multiple times in the solvent systems until a single spot was observed in the two shortlisted solvent systems

i.e ethyl acetate:petroleum ether in 70:30 and n-butanol:acetic acid:water in 2:1:1. The spots observed in TLC were clear but with tailing due to the presence of many other metabolites being present in the crude unpurified product. Further analysis of 2 batches of this crude metabolite by high resolution mass spectroscopy showed 2 recurring base peaks and major peaks. HRMS detects all the components present in the sample with accurate molecular masses and picks up even the slightest of impurities or variation present in the sample. In our case both the batches showed 2 recurring peaks each with consistant molecular mass till the third decimal in the case of major peaks and second decimal in the case of base peaks (major peak-409.1653 and 409.1651 and the base peaks-795.3489 and 795.3451). Hence we can assume our metabolite of interest being portrayed by one of the two recurring peaks. Further purification of the compound with HPLC can be done which will improve the potency of the antibacterial and further characterization using NMR, IR will help to identify the exact composition and nature of the antibacterial compound [12].

In one of the earlier researches seventy four bacteria were isolated from Lonar which belonged to phylum *Firmicutes* and proteobacteria. Majorities (eight) were *Firmicutes* and three were proteobacteria. For the first time *Alcanivorax spp*. was reported, a genus well known for its oil degradation capacity which indicate the probable existence of oil reservoir in vicinity of Lonar lake. In addition, all the eleven bacteria are potential producers of industrially important enzymes, pigments, antibiotics as well. Studies carried out by also revealed haloalkaliphilic *Oceanobacillus* and other similar bacteria could produce broad spectrum of antimicrobial agents.

Another study revealed that Lonar lake harbors a wealth of diverse microorganisms with useful commercial properties and alkaliphilic isolates belonging to genera *Bacillus*, *Paenibacillus*, *Alkalibacillus*, *Halomonas*, *Alkalimonas, Planococcus, Alcaligenes, Arthrobacter, Exiguobacterium, Cellulosimicrobium, Marinobacter, Roseinatronobacter* and *Rhodobaca* exhibited antibiosis against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi and Klebsiella pneumonia* procured from KEM hospital Pune.

The Lonar lake habitat exhibits the growth of unique flora fauna with desirable properties, one of them being the antibacterial properties exhibited by the bacteria growing there as proved by many researchers. Our study was concentrated on the antibacterial property of *Bacillus sp*. which inhibited the growth of *Enterococcus fecalis*-MCC 2409, a human MDR pathogen. No similar evidence of a lonar alkaliphile inhibiting the growth of MDR was found *via* literature survey.

CONCLUSION

The Lonar lake should be taken into consideration for exploring newer antimicrobial entities. Studies on microbial diversity of alkaline/saline environments are important for two reasons. First, some of the earliest microbial life on earth might have been haloalkaliphiles, thus research on microbial community in soda lakes may give clues into the evolution of life on earth. Secondly, because of the presence of hyper-saline conditions on Mars, terrestrial saline environments may act as good models for studies on life on Mars. Studying extremophiles from different environment can help explore their valuable properties which are unknown to mankind as of now.

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