ANTIMICROBIAL POTENTIAL OF HALOALKALIPHILIC *BACILLUS THERMOTOLERANS* FROM ALKALINE ENVIRONMENT

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Abstract: Day-by-day, because of the irregular and indiscriminate use of the antimicrobial drugs in treatment of infectious diseases leading to increase in the rapid spread of multidrug-resistant bacteria, re-emergent infections, and newly emerging infectious diseases and have become a serious global public health threat, specifically affecting the economic development Therefore, the study was undertaken to find new antibacterial producer from extreme environment like Lonar Lake. In present study, Bacillus *thermotolerans*, isolated from Lonar Lake, showed the most prominent antimicrobial activity and maximum antimicrobial sensitivity index against most of the enteric pathogens. GCMS analysis recorded the presence of antimicrobial components such as Ethyl cyclopropanecarboxylate, Squalene, Decanedioic acid, bis (2-ethylhexyl) ester, 5-Cyclopropylcarbonylontaxypentadecane, Octadecanoic acid, Z-10-Tetradecen-1-ol acetate, n-Hexadecanoic acid, Pyrrolo (1,2-a) pyrazine-1,4 dione, hexahydro-3(2-methylpropyl) and 4-Heptanol, 2,6-dimethyl. Our study provides primary evidence that *Bacillus thermotolerans* from Lonar Lake has the capacity to provide active and high antimicrobial secondary metabolites which have potential in medical and pharmaceutical industry.
Keywords: Bioactive compound, Pharmaceutical, *Bacillus*,Antimicrobial activityand Lonar Lake

Introduction:

Lonar Crater, an impact crater situated in Buldhana District (Maharashtra, India), is unique in its salinity, alkalinity and microbial extremophilic biodiversity. Due to its uniqueness, the crater has evoked much scientific attention as of wet land, ecology, biology and limnology or hydrology (Tambekar and Dhundale, 2013). Being an alkaline ecosystem, Lonar Lake harbors variety of extremophilic microorganisms including alkaliphilic and halophilic bacteria that have potential in producing antibacterial substances (Kanekar *et al.*, 2008; Deshmukh *et al., 2*011). Most of these haloalkaliphilic bacteria produced broad spectrum classical antibiotics, organic acids and lytic agents such as lysozyme. The Gram positive bacterium produces a large number of antibiotics, which are classified as ribosomal or non-ribosomal antibiotics which may play a role in competition with other microorganisms during spore germination (Shinde *et al.*, 2012). Antibiotics are usually assumed as secondary metabolites produced during the microbial growth and play important role in preventing the host against the various infectious diseases (Tambekar *et al.,* 2016). Among these extremophilic microorganisms, the *Bacillus*species which are ubiquitous in nature, having ability to produce secondary metabolite like bacteriocin and various type of antibacterial substances (Tambekar *et al.,* 2014). Day-by-day, because of the irregular and indiscriminate use of the antimicrobial drugs in treatment of infectious diseases leading to increase in the rapid spread of multidrug-resistant bacteria, re-emergent infections, and newly emerging infectious diseases and have become a serious global public health threat, specifically affecting the economic development (Bandow *et al.,* 2003). Therefore, the study was intended to find the novel secondary bioactive compounds for treating the diseases caused by enteric pathogens from *Bacillus* species from Lonar lake environment.

Materials and Methods:

Enrichment and Isolation of microorganism: Total 12 samples; (Sediment, matt and water) were collected from Lonar Lake in zip lock polythene bags and sterile plastic bottles. All the samples were further heated at 80°C to kill the vegetative cells and then enriched repeatedly five times in Horikoshi medium (A, B, C and D) on rotary shaker (100 rpm) for 72h (Horikoshi. 1999). After enrichment, it was inoculated on nutrient agar plates (pH 10) and isolated colonies were then further studied for production of antibacterial substance. The antibacterial substance producing isolates were identified culturally, morphologically, biochemically and by 16S rRNA gene sequencing.

Screening and Identification of antimicrobial producing *Bacillus* species: The isolated *Bacillus* species were screened for the antimicrobial activity by observing the zone of growth inhibition against the clinical pathogens by disc diffusion method. The 1mL of bacterial suspension (10^5CFU mL^{-1}) was uniformly spread on nutrient agar plate to form lawn (Kirby *et al.,* 1996) and sterile blotting paper discs dipped in 48 h incubated culture broth was placed on solidified nutrient agar seeded with 3h old culture of test organism. The organisms used for test includes *Escherichia coli, Staphylococcus aureus, Salmonella typhi*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia.* After incubation antimicrobial activity was measured as zone of inhibition against test pathogenic bacteria and Antimicrobial sensitivity index (ASI) was calculated as under:

Formula for calculation of Antimicrobial sensitivity index (ASI):

$$\text{Antimicrobial sensitivity Index} = \frac{\text{Total zone of growth inhibition}}{\text{No. Antimicrobial agent tested}} \times \text{No. of pathogenic organisms tested}$$

Extraction and characterizations of antimicrobial substances: Extraction of antimicrobial substances from *Bacillus sp*. was done by centrifugation of 48 hr nutrient broth at 3000 rpm for 30 min. The obtained pellet then treated with chilled acetone. The settle precipitate in chilled acetone was collected and treated with methanol and chloroform to remove the impurity, dried and collected in sterile glass bottles and store at 4^0C for further processing. The antibacterial precipitate obtained by *Bacillus* sp was structurally determined by Gas Chromatography Mass Spectroscopy (GCMS).

Results and discussion:

In the present study, total twelve samples (4 each, sediments, matt and water) were collected from different sites of alkaline Lonar Lake. The samples were enriched on Horikoshi medium (A, B, C and D). Media A and B favored the growth of several moderate Halophilic alkali tolerant bacteria and Medium C favors the growth of moderate alkaliphilic bacteria and Medium D support growth of Halophilic bacteria (Horikoshi, 1999). From 12 selected samples, four bacilli were isolated. Out of these bacilli, DHT 26 was found to be prominent antimicrobial producer. This bacilli was identified by cultural, morphological and biochemical characteristic and finally by 16s rRNA gene sequencing as *Bacillus thermotolerans* (Table 1).

The antibacterial activity for antimicrobial substance produced by isolate was done by disc diffusion method, using blotting paper discs and zones were measured (Fig.1). It showed that isolate *Bacillus thermotolerans* (DHT 26) was prominent antibacterial substance producing with constant zones and showed good antibacterial activity against *E.coli*. As isolated *Bacillus thermotolerans* from alkaline Lonar Lake showed strong antimicrobial activity towards all the pathogenic organisms, its activity was calculated by antimicrobial sensitivity index (Fig.2). Gupta *et al.*, (2013) studied the assessment of antimicrobial potential of *Bacillus spp* isolated from extreme environmental of silicate factory (high temperature and high alkali) and was screened for antimicrobial activity against bacterial pathogens (*Staphylococcus aureus, E. coli, Vibrio cholerae)* and fungal pathogens (*Aspergillus niger, Candida albicans, Penicillium chrysogenum*).

Gas Chromatography Mass Spectroscopy (GCMS) analysis of antimicrobial substances of extracted from *Bacillus thermotolerans* (DHT 26) showed number of secondary metabolites consisting of Ethyl cyclopropanecarboxylate, Squalene, Decanedioic acid, bis (2-ethylhexyl) ester, 5-Cyclopropylcarbonylontaxypentadecane, Octadecanoic acid, Z-10-Tetradecen-1-ol acetate, n-Hexadecanoic acid, Pyrrolo (1,2-a) pyrazine-1,4 dione, hexahydro-3(2-methylpropyl) and 4-Heptanol, 2,6-dimethyl (Table.2). All these compounds are the derivatives of fatty acid which show antimicrobial and antifungal activity. Similarly, Ser *et al.*, (2015) reported some

antioxidative agents like Pyrrolo (1, 2-a) pyrazine-1, 4- dione, hexahydro- in newly isolated *Streptomyces mangrovisoli* species.

Conclusion:

The present study reveals that, *Bacillus thermotolerans*, showed the most prominent antimicrobial activity and optimum antimicrobial sensitivity index. GCMS analysis showed the presence antimicrobial components such as Ethyl cyclopropanecarboxylate, Squalene, Decanedioic acid, bis (2-ethylhexyl) ester, 5-Cyclopropylcarbonylontaxypentadecane, Octadecanoic acid, Z-10-Tetradecen-1-ol acetate, n-Hexadecanoic acid, Pyrrolo (1,2-a) pyrazine-1,4 dione, hexahydro-3(2-methylpropyl) and 4-Heptanol, 2,6-dimethyl. Our study provides primary evidence that *Bacillus thermotolerans* from Lonar Lake has the capacity to provide active and high antimicrobial secondary metabolites which have potential in medical and pharmaceutical industry.

Table-1 (a). Cultural, morphological and biochemical characteristics of Antimicrobial substance producing isolate

Gram character	+	Glucose	+	α-Methyl-D-glucoside	-
Shape of Bacteria	LR	Dextrose	+	Rhamnose	-
Arrangement of Cell	Single	Galactose	-	Cellobiose	-
Spore	+	Raffinose	-	Melezitose	-
Motility	+	Trehalose	-	α-Methyl-D-mannoside	-
Catalase	+	Melibiose	-	Xylitol	-
Oxidase	+	Sucrose	+	ONPG	-
Voges Proskauer	-	L- Arabinose	-	Esculin hydrolysis	-
Citrate utilization	+	Mannose	-	D-Arabinose	-
Nitrate reduction	-	Inositol	-	Malonate Utilization	+
Arginine	+	Sorbitol	-	Sorbose	-
Lactose	-	Mannitol	+	Inulin	-
Xylose	-	Adonitol	-	Sodium gluconate	-
Maltose	+	Arabitol	-	Glycerol	-
Fructose	-	Erythritol	-	Salicin	-
Note: LR- Long Rod, (+) = Positive, (-) = Negative					

Table-1. (a) 16S rRNA sequencing of *Bacillus thermotolerans* (DHT 26)

TGCGGCCGTACTCCCCAGGCGGAGTGCTTATTGCGTTAGCTGCGGCACTGAGGATTGGAGTCCCCAACACCTAGCACTCAAC
GTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGGCCAGAGA
GCCGCCTTCGCCACGGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCGCTCTCCTCTCCTGCACTCA
AGCTTCCCAGTTTCAAGTGGCCCTCCACGGTTGAGCCGTGGGCTTTCACACCTGACTTAAGAAGCCGCCTGCGCGCGCTTTA
CGCCCAATAATTCCGGACAACGCTTGCCCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTTCTCGTTAG
GTACCGTCAGACCGGGAGGTCATCCCGGCGGTTCTTCCCTAACAACAGAACTTTACGATCCGAAAACCTTCATCGTTCACGCG
GCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCC
CAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTAGGCCGTTACCCCACCAACTAGCTAATGCGCCG
CAGGCCCATCTGTACGTGACCAAARGTCTTTCCCTTTCAGACCATGCGGTCTGAAAGAAGTATCCCGGTATTAGCTCCCGGTT
TCCCGGGAGTTATCCCAGTCGTACAGGCAGTTGC

Results for Query Sequence: seqmatch_seq, 738 unique oligos
rootrank Root (20) (match sequences)
domain Bacteria (20)
phylum Firmicutes (20)
class Bacilli (20)
order Bacillales (20)
family Bacillaceae 1 (6)
genus Bacillus (6)
S000016369 - not_calculated 0.682 1351 Bacillus badius (T); ATCC 14574; X77790
S000383283 - not_calculated 0.686 1461 Bacillus mannanilyticus (T); AM-001; AB043864
S000437736 - not_calculated 0.672 1417 Bacillus sporothermodurans (T); M215; U49078
S003303796 - not_calculated 0.680 1381 Bacillus thermotolerans (T); SgZ-8; JX261934
S003613950 - not_calculated 0.678 1389 Bacillus composti (T); SgZ-9; JX274437
S004052356 - not_calculated 0.683 1421 Bacillus encimensis (T); SGD-V-25; KF413433

Fig.-1. Antimicrobial activity of isolated *Bacilli* against the test clinical pathogenic organisms

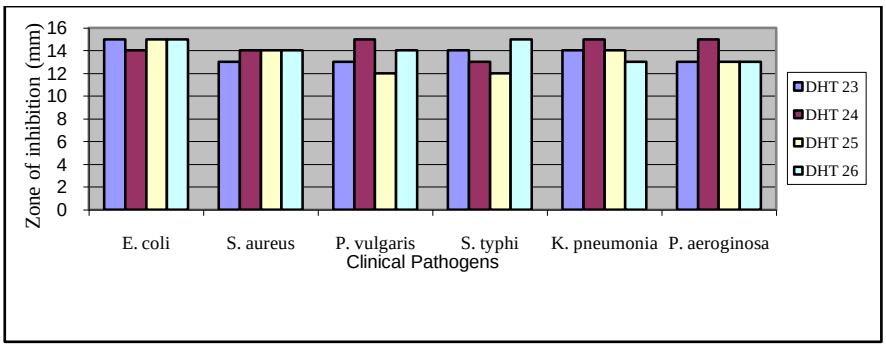

Fig.-2. Antimicrobial sensitivity index of Bacillus species against pathogenic bacteria

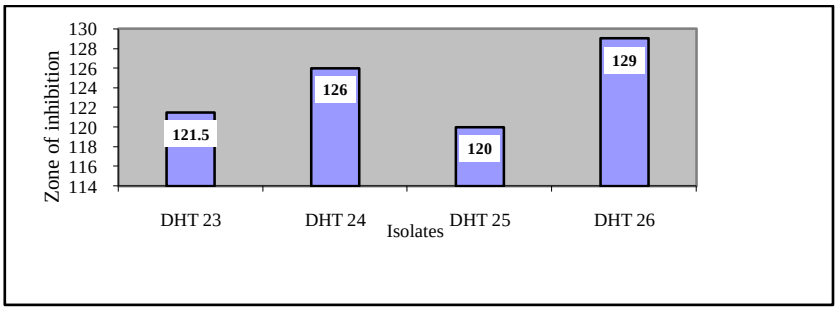

Table-2.Results of GCMS of *Bacillus thermotolerans* (DHT 26)

Peak No.	Compound Name	Formula	Molecular weight	Percentage of Compound	Properties of all compounds
1	Ethyl cyclopropanecarboxylate	$C_6H_{10}O_2$	114	25.9	Saturated fatty acid ester, used in the treatment of schizophrenia, act as a long-acting release carrier medium. Also used in treatment of breast cancer and also act as an insect pheromone, pesticidal agent. Cyclic dipeptide, used as potential antimicrobial agent, therapeutic agent and antioxidant agent.
2	Squalene	$C_{30}H_{50}$	410	32.6	
3	Decanedioic acid, bis (2-ethylhexyl) ester	$C_{26}H_{50}O_4$	426	89.8	
4	5-Cyclopropylcarbonylontaxypentadecane	$C_{19}H_{36}O_2$	296	7	
5	Octadecanoic acid	$C_{18}H_{36}O_2$	284	21.1	
6	Z-10-Tetradecen-1-ol acetate	$C_{16}H_{30}O_2$	254	9.81	
7	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	27.6	
8	4-Heptanol,2,6-dimethyl	$C_9H_{20}O$	144	15.4	
9	Pyrrolo (1,2-a) pyrazine-1,4 dione, hexahydro-3(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	210	22.2	

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