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Screening and Production of Antimicrobial Substances Produced by Haloalkaliphilic *Bacillus* Species

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ABSTRACT: Alkaline soda lake of Lonar at Buldhana district (Maharashtra, India) has a unique ecosystem with extremophilic environment. Due to this adverse condition, it can be a promising source of novel haloalkaliphilic bacterial species which is medically important to fight against by harboring different antimicrobial activity by the production of new class of biologically active secondary metabolites. Hence, in the present study different *Bacillus* species were isolated from the Lonar lake using Horikoshi medium (Horikoshi A, B, C and D) and three *Bacillus* species, confirmed as *Bacillus halodurans* (DHT 19), *Bacillus thuringiensis* (DHT 20) and *Lysinibacillus xylanilyticus* (DHT 21) by 16S rRNA gene sequencing which showed the effective antibacterial activity against the clinically important pathogens. Structural determination of the secondary metabolites which is obtained from this species was done by Gas Chromatography Mass Spectroscopy (GCMS) and this isolated Bacillus species were found to be a potent source of the secondary metabolites which can be useful in production of mechanically distinct activity of antibiotics globally.

KEYWORDS : Lonar Lake, *Bacillus sp.,* antimicrobial activity, GCMS)

I. INTRODUCTION

Alkaline soda lake of Lonar at Buldhana district (Maharashtra, India) has a unique ecosystem with extremophilic environment (pH 10.5) and harbors variety of halo-alkaliphilic microorganisms (Pathak & Rathod, 2013). These halophilic bacteria are good source of secondary metabolites that have potential pharmaceutical and biotechnological application (Tambekar *et al*., 2013). Due their adaptation in adverse condition they can be useful in the production of potent antimicrobial agents. Due to their wide ubiquity in nature and genetic and metabolic diversity leading the production of several antibiotics and enzymes, have become increasingly interesting for different biotechnological and pharmacological applications (Baruzzi *et al*., 2011)

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. Study showed that many chemically unique compounds of extreme condition like marine origin with different biologically activity have been isolated and a number of them are under investigation are being developed as new pharmaceuticals (Schwartsmann et al., 2000). 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 in bacteria, re-emergent and newly emerging infectious diseases and lead to serious global public health threat which affecting the economic development (Pavia, 1999). Thus, the successful identification and development of novel, potent and efficacious compounds will solve this problem (Sardari *et al*., 2011). Therefore, we tried to isolate new bioactive compound with distinct mechanism of activity against bacterial pathogens and can act antimicrobial therapy from Bacillus species from Lonar Lake which is ubiquitous in nature and showed the high potency of controling the pathogenic growth.

ISSN: 2350-0328

International Journal of Advanced Research in Science, Engineering and Technology

Vol. 3, Issue 11 , November 2016

II. MATERIALS AND METHODS

***A.* ENRICHMENT, ISOLATION AND IDENTIFICATION OF MICROORGANISMS: A** total of twelve samples, (four each of sediment, matt and water) were collected from different sites of Lonar Lake and were proceed further by heating at 80°C for removal of vegetative cell and the isolation of Bacillus sp. Enrichment of the culture were carried out in Horikoshi Medium A, B, C and D respectively, (Horikoshi, 1999). Inoculation of 10mL of each sample in 100 mL Horikoshi media A, B, C and D was done. All the flasks were incubated at room temperature on rotary shaker (100 rpm) for 3 days (72h). Continuous sub-culturing was done for 5 times. After enrichment, the organisms were isolated on respective media agar plates and incubated at 37^0C for 24h. Well isolated and differentiated colonies were transferred on the respective medium slants and cultures were maintained as stocks. Isolated *Bacillus* species were identified by cultural, morphological, biochemical test and finally by 16S rRNA gene sequencing from Agharkar Research Institute, Pune (India).

***B.* ANTIMICROBIAL ACTIVITY OF BACILLUS SPECIES:** The disc diffusion method was used to determine antimicrobial activity for antimicrobial properties of isolated three different bacillus species; 0.1mL of bacterial suspension of 105CFU mL-1 was uniformly spread on nutrient agar plate to form lawn cultures (Kirby et al., 1996). Sterile blotting paper discs were dipped into 48 h incubated culture broth and then placed on solidified Nutrient agar seeded with 3 h old culture of test organism, which includes *Escherichia coli* (MTCC 443), *Enterobacter aerogenes* (MTCC 111), *Klebsiella pneumonia* (MTCC 2653), *Proteus vulgaris* (MTCC 426), *Salmonella typhi* (MTCC 734), *Pseudomonas aeruginosa* (MTCC 424) and *Staphylococcus aureus* (MTCC 96) and plates were kept for incubation at 37°C for 24h. After complete incubation antimicrobial activity evaluate by measuring the diameter of zone of inhibition against given test pathogenic bacteria.

***C.* EXTRACTION OF ANTIMICROBIAL SUBSTANCES:** Extractions of antimicrobial substances from *Bacillus sp*. were done by centrifugation at 3000 rpm for 30 min. and chilled acetone treatment was also done at freezing temperature. Obtained precipitated was collected and treated with methanol, Chloroform to remove the impurity, dried it and collected in to the sterile glass bottles and store at 4^0C for further processing.

D.* STRUCTURAL CHARACTERIZATION OF ANTIMICROBIAL SUBSTANCES PRODUCED FROM *BACILLUS SP: The dried precipitates which was collected into sterile glass plates, structural determination was done by the highly sensitive technique Gas Chromatography Mass Spectroscopy (GCMS) at Institute of science, IIT, Bombay.

III. RESULT AND DISCUSSION

Cultural and morphological characteristics of the isolated *Bacillus* species were showed that all three isolates were Gram positive, long rod in case of DHT 19 and DHT 21 and short rod in case of DHT 20, as they grows in extreme alkaline environment by forming , endospore. They all are capsulated, motile and arranged as single. All these *Bacillus* species grow at the pH 7- 12, salt concentration at the 1% - 5% and temperature up to 60°C (Table 1).

The molecular detection of the isolated bacterial strains were done on the basis of 16S rRNA gene sequencing and this showed complete identification of the isolated species such as DHT19 as a *Bacillus halodurans* (Table 2), DHT 20 as *Bacillus thuringiensis* (Table 3) and DHT 21 as *Lysinibacillus xylanilyticus* (Table 4). All these isolated *Bacillus* species showed antimicrobial activity against the clinical test pathogens i.e. *S.typhi, P.aeruginosa, P.vulgaris, K. pneumonia, E.coli* and *S.aureus.*

The isolated *Bacillus* species from alkaline Lonar Lake showed the antimicrobial activity against all tested pathogenic organisms, and its antimicrobial sensitivity index against the pathogenic organism was maximum (Fig 1). The isolated DHT 19 and DHT 21 showed strong antimicrobial activity against *E. coli* and DHT 20 showed moderate antimicrobial activity against *E.coli* and showed relatively weak antimicrobial activity against *E.coli* by DHT 16 and DHT 22. DHT 16, DHT 22 showed less effective antimicrobial activity against S.*aureus* while DHT 21 high effectiveness of antimicrobial activity against S.*aureus* and DHT 19 and DHT 20 showed comparative antimicrobial activity against S.

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International Journal of Advanced Research in Science, Engineering and Technology

Vol. 3, Issue 11 , November 2016

aureus from other isolates. DHT 16, DHT 19 and DHT 22 showed weak antimicrobial activity against *P. vulgaris* except DHT 20 and DHT 21 which showed moderate antimicrobial activity against *P. vulgaris*, DHT 19 and DHT 21 showed moderate antimicrobial activity against *S. typhi* than the DHT 16, DHT 20 and DHT 22 which showed low antimicrobial activity against *S.typhi*. DHT 22 showed maximum antimicrobial activity against *K. pneumonia* and DHT 16, DHT 19, DHT 20 and DHT 22 showed less antimicrobial activity against *K. pneumonia*. Also DHT 19 and DHT 21 showed moderate antimicrobial activity against *Pseudomonas* and DHT 16, DHT 20 and DHT 22 showed weak antimicrobial activity against *Pseudomonas* (Fig 2).

After screening of secondary metabolites, by GCMS analysis, the report showed that DHT19 (*Bacillus halodurans)* produced secondary metabolites content Hexadecanoic acid, methyl ester, 9-Octadecenoic acid, methyl ester and Octadecenoic acid, methyl ester with concentration of the compound 73.2%, 11.4% and 61.4% respectively. All these compounds are the derivatives of fatty acid which show antimicrobial and antifungal activity.

Table 1: Characteristic of *Bacilli* Isolated from Lonar Lake

Bacterial Character	Isolation code			**Biochemical Character**	Isolation code		
	DHT 19	**DHT 20**	**DHT 21**		**DHT19**	**DHT 20**	**DHT 21**
Gram character	+	+	+	Maltose	-	+	+
Shape of Bacteria	LR	SR	LR	Fructose	-	+	-
Size of Bacteria	5.2 µm	3.1 µm	5.6 µm	Dextrose	+	+	+
Arrangement of Cell	S	S	S	Galactose	-	-	-
Spore bearing	+	+	+	Adonitol	-	-	-
Motility	+	+	+	Arabitol	-	-	-
Growth at Temp.				Erythritol	-	-	-
37°C	+	+	+	Rhamnose	-	-	-
45°C	+	++	+	α-Methyl-D glucoside	-	-	-
50°C	++	++	+	α-Methyl-D-mannoside	-	+	-
60^0C	+	+	++	Raffinose	-	-	-
Growth at pH				Trehalose	-	+	+
pH 7	+	+	+	Melibiose	-	-	-
pH 8	+	+	+	Sucrose	-	-	-
pH 9	++	+	+	L- Arabinose	-	-	-
pH 10	+	++	++	D-Arabinose	-	-	-
pH 12	+	+	+	Mannose	-	-	-
Growth at NaCl				Inulin	-	-	-
1% NaCl	++	+	++	Sodium gluconate	-	-	+
2% NaCl	++	++	+	ONPG	-	-	-
3% NaCl	+	+	+	Salicin	-	+	-
4% NaCl	+	+	+	Dulcitol	-	-	-
5% NaCl	+	+	+	Inositol	-	-	-
Biochemical Character				Esculin hydrolysis	-	+	+
Indole	-	-	-	Sucrose	-	-	-
Methyl Red	-	-	-	Xylose	-	-	-

ISSN: 2350-0328

International Journal of Advanced Research in Science, Engineering and Technology

Vol. 3, Issue 11 , November 2016

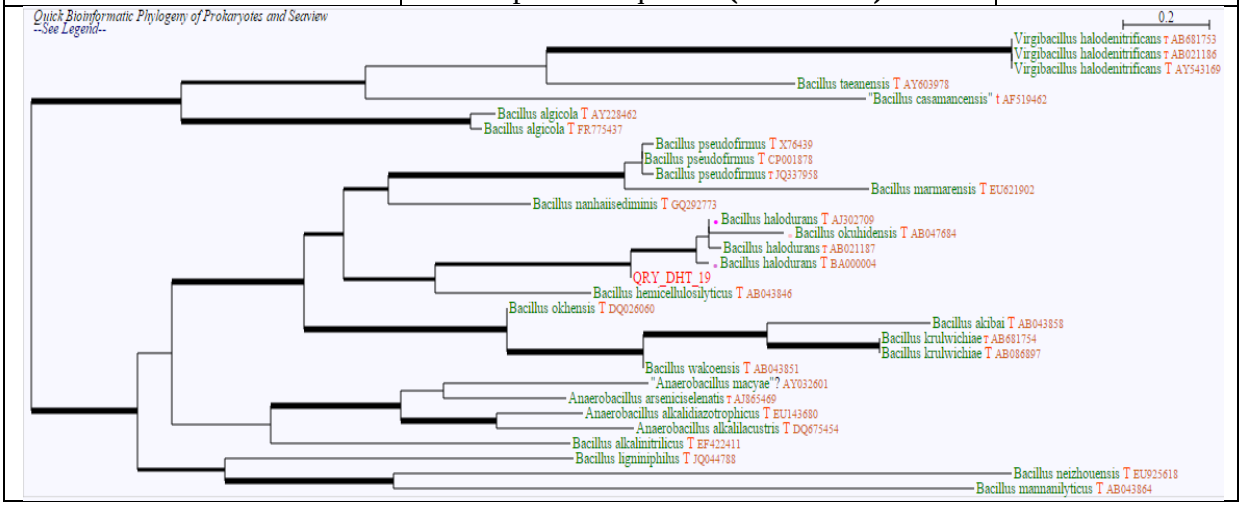
Voges Proskauer	-	-	-	Glycerol	-	+	-
Citrate Utilization	-	+	-	Sorbitol	-	-	-
Catalase	-	+	-	Malonate Utilization	-	-	-
Oxidase	-	-	-	Cellobiose	-	-	-
Arginine	+	-	-	Xylitol	-	-	-
Lactose	-	-	-	Nitrate reduction	-	-	-
Bacterial isolates on the basis of 16S rRNA sequencing				**DHT 19 - *Bacillus halodurans* DHT20 - *Bacillus thuringiensis* DHT 21 - *Lysinibacillus xylanilyticus***			
Note: LR- Long Rod, SR- Short Rod, S-Single, (+)=Positive, (-)=Negative							

Screening of antimicrobial substances which is extracted from the DHT 20 (*Bacillus thuringiensis)* was done by GCMS analysis and showed that, there are number of secondary metabolites consisting of Methoxy-phenyl oxime (84.4%); Pyrrolo(1,2-a)pyrazine-1,4-dione, hexahydro-3(2- methoxy propyl) (82.4%) and Pyrrolo(1,2-a)pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) (78.8%). Presence of similar antioxidative agent, Pyrrolo (1, 2-a) pyrazine-1, 4-dione, hexahydro- in newly isolated *Streptomyces mangrovisoli* species was also reported by Ser *et al*., (2015).

The antibacterial components of DHT 21 (*Lysinibacillus xylanilyticus*) analysed by GCMS showed highest production is of 2-piperidinon, 1,3- benzylhexapyrrolo(1,2-a), pyrazine-1,4-dione, 3-methyl butanoic acid and Methoxy-phenyl oxime (81 to 82.5%). Pramitha and Kumari, (2016) also found the similar compound like 1, 3-benzylhexapyrrolo (1, 2-a) pyrazine-1, 4-dione after GCMS analysis of marine Brown Macroalga, *Sargassum wighti*.

Table: 2. The 16S rRNA gene sequencing closest phylogenetic affiliation, pair similarity and ribosomal database project report of isolated organism DHT 19 from Lonar Lake

Strain Designation	Closest Phylogenetic affiliation	Max ident
DHT 19	***Bacillus halodurans*** (T) 16S rRNA gene partial sequence **(AJ302709)**	**99.0%**



ISSN: 2350-0328

International Journal of Advanced Research in Science, Engineering and Technology

Vol. 3, Issue 11 , November 2016

Table: 3. The 16S rRNA gene sequencing closest phylogenetic affiliation, pair similarity and ribosomal database project report of isolated organism DHT 20 from Lonar Lake		
Strain Designation	**Closest Phylogenetic affiliation**	Max ident
DHT 20	***Bacillus thuringiensis (T) 16S rRNA gene partial sequence (CP003889)***	**100%**

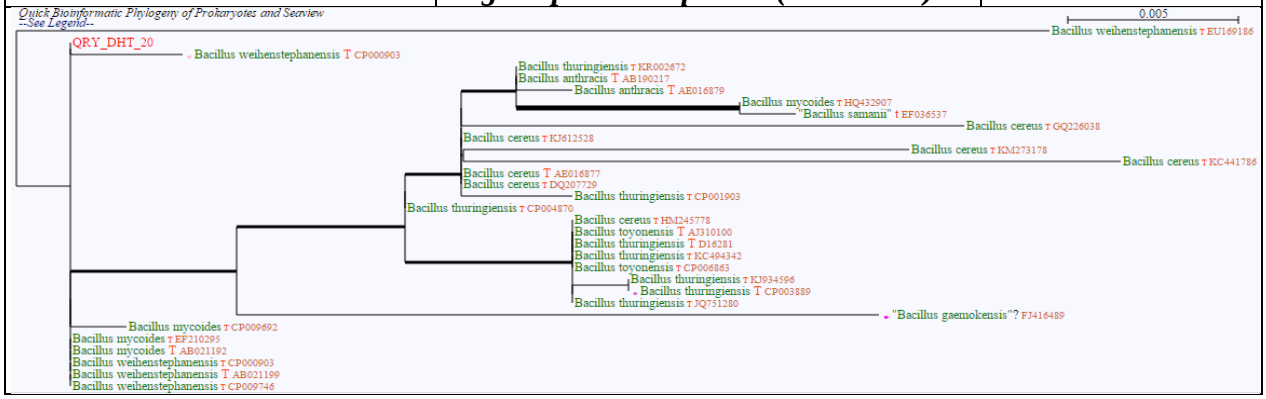
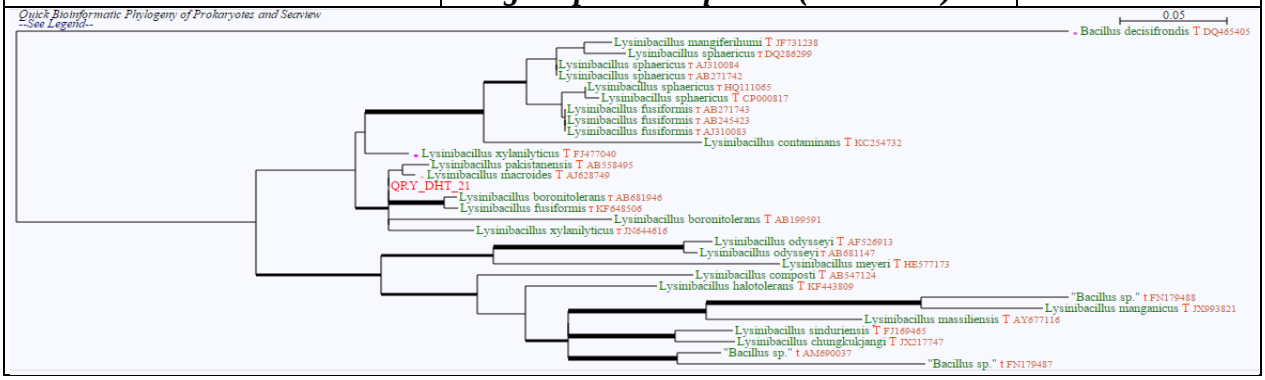


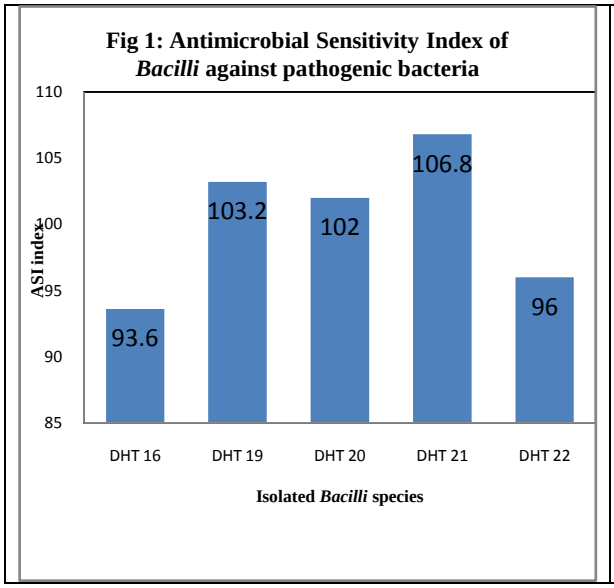
Table: 4 The 16S rRNA gene sequencing closest phylogenetic affiliation, pair similarity and ribosomal database project report of isolated organism DHT 21 from Lonar Lake		
Strain Designation	**Closest Phylogenetic affiliation**	Max ident
DHT 21	***Lysinibacillus xylanilyticus (T) 16S rRNA gene partial sequence (FJ477040)***	**100%**

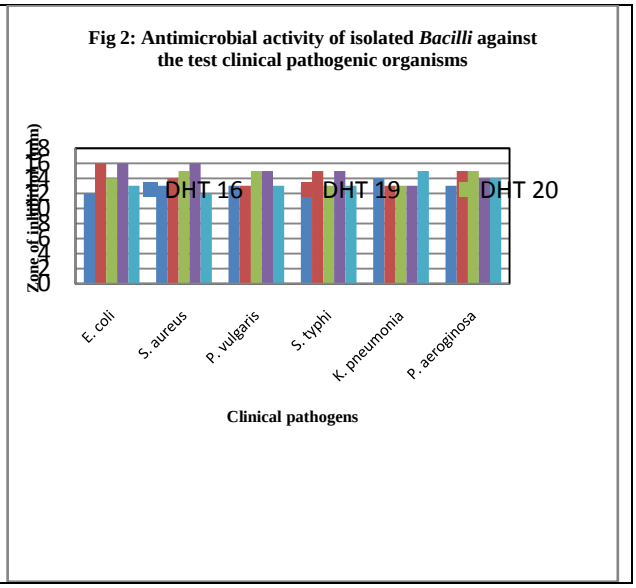


ISSN: 2350-0328

International Journal of Advanced Research in Science, Engineering and Technology

Vol. 3, Issue 11 , November 2016





IV. CONCLUSION

The present study revealed that, out of the isolated five *Bacillus* species, three isolates i.e. *Bacillus halodurans* (DHT19), *Bacillus thuringiensis* (DHT 20) and *Lysinibacillus xylanilyticus* (DHT 21) gave the highest prominent zone of inhibition as antimicrobial activity as compare to other two isolates DHT 16 and DHT 22 and produced secondary metabolite Pyrrolo (1,2-a) pyrazine-1,4-dione, hexahydro-3(2- methoxy propyl) by botjh DHT 20 (*Bacillus thuringiensis)* and DHT 21 (*Lysinibacillus xylanilyticus*) which is potent antioxidant agent as well as antibacterial agent. Our study provides primary evidence that *Bacillus* strains of Lonar Lake has the capacity of forming new and active secondary metabolites which has highly medically and pharmaceutically importance due their genetic makeup in adverse condition and it is need to be further studied for its potency and broad spectrum activity for the improvement in the antimicrobial activity and production of new drugs.

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Vol. 3, Issue 11 , November 2016

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