RESEARCH PAPER | Microbiology | Volume : 4 | Issue : 8 | August 2014 | ISSN - 2249-555X

Studies on Production of Antimicrobial Substances from *BACILLUS* Species Isolated from Lonar Lake

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

Tambekar DH	Tiwari AA	Tambekar SD
PG Department of Microbiology SGB Amravati University, Amravati 444604 (India)	PG Department of Microbiology SGB Amravati University, Amravati 444604 (India)	Department of Microbiology, D.B. Science College, Gondia - 441614 (India)

ABSTRACT *The alkaline Lonar Lake is a unique ecosystem formed by meteor impact and situated in the Buldhana District, Maharashtra state (India). The development of resistance to multiple drugs is the major problem; therefore antimicrobial substances producing Bacillus sp. were isolated from the water, matt and sediment of alkaline Lonar Lake. Six Bacillus sp. were isolated using Horikoshi medium A, B, C and D. The isolated Bacillus sp. were identified by cultural, morphological, biochemical tests and by 16S rRNA gene sequencing, and found to be Bacillus fusiformis (CW4(1)), Bacillus circulance (CW2(2)), Bacillus fusiformis (CW1(3)), Bacillus cerecus (OCW3(1)), Bacillus krulwichiae (BW4(3)), Bacillus fusiformis (CW4(3)). All the selected Bacillus sp exhibited inhibitory effect against clinical isolates of pathogenic bacteria. Structural determination of the secondary metabolites was done by Gas Chromatography Mass Spectroscopy (GCMS) and results showed that OCW3(1) and CW4(1) showed strong, BW4(3) and CW4(3) showed moderate and CW1(3) and CW2(2) showed weak antimicrobial activity. Present study provides primary evidence that isolated Bacillus strains were promising sources for the antimicrobial bioactive substances and represent a new and rich source of secondary metabolites that need to be explored.*

INRODUCTION

The alkaline Lonar Lake a unique basaltic rocks form by meteorite impact around 52000 years ago, ranking third in the world. The uniqueness of the Lake water is its salinity and high alkalinity (pH of 9.5-10) (Fredriksson et al., 1973; Sengupta et al., 1997). Lonar Lake is a closed system without any outlet and unique due to its salinity, alkalinity and biodiversity. The Lonar Lake harbors diverse microbial flora of alkaliphilic microbes growing at pH 10 and/or at high salt concentrations and rich source of active biomolecules and secondary metabolites. Some studies have shown that alkaliphilic bacteria produce substances with antibacterial and antifungal activities and the alkaliphilic actenomycetes produced some potent antibiotics (Borgave et al., 2012; Joshi et al., 2007).

The Gram positive bacterium Bacillus subtilis produces a large number of antibiotics, which are classified as ribosomal or non-ribosomal antibiotics which may play a role in competition with other micro-organisms during spore germination (Shinde et al., 2012). Many antibacterial agents are available in the market now-a-days but they are less effective because of the indiscriminate use of these antimicrobial drugs in treatment of infectious diseases leading to increase in the resistance in bacteria. Hence, there is an urgent need to discover new antimicrobial compound with diverse chemical structure and novel mechanism of action for new and re-emerging infectious diseases, therefore researchers are increasingly turning their attention to new antibiotics for new treatments leads to develop better drug against microbial infections (Bandow et al., 2003).

The development of resistance to multiple drugs is the major issue in the medication of infectious diseases caused by pathogenic micro-organisms and therefore need to search new bioactive compound to achieve this conditions and circumstances there is concern to ameliorate or detect novel class antibiotics that have distinct mechanism of activity globally. Halophillic bacteria from Lonar Lake marine environment are also better source of secondary metabolites that may have potential of pharmaceutical and biotechnological application (Tambekar et al., 2013).Hence attempt has been made to isolated novel halophillic bacteria from Lonar Lake which can produce a potential antimicrobial agent.

MATERIALS AND METHODS

Enrichment and isolation of microorganisms:

Samples were collected from selected sites of Lonar Lake, during season 2013 by using sterilized spatula. Sediment and matt samples were collected in zip lock polythene bags and water samples in sterile bottles. All these water, matt and sediment samples were heated 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). All the flasks were incubated at room temperature on rotary shaker (100 rpm) for 72h. After enrichment, the organisms were isolated on respective media agar plates and incubated at 37^{0}C for 24h. Well isolated and differentiated colonies were transferred on the respective medium slants and cultures were maintained as stocks.

Identification of antimicrobial compound producing Bacillus:

Identification of Isolates by cultural, morphological, biochemical and 16S rRNA gene sequencing from NCCS, Pune, (India).

Antimicrobial activity of Bacillus species:

The disc diffusion method was used to determine antimicrobial activity for antimicrobial properties, 0.1mL bacterial suspension of 105CFU mL-1 was uniformly spread on nutrient agar plate to form lawn cultures (Kirby et al., 1996). The discs were applied to the surface of the nutrient agar plates seeded with 3h culture of test bacterium which includes Escherichia coli (MTCC443), Staphylococcus aureus (MTCC96), Salmonella typhi (MTCC734), Proteus vulgaris (MTCC426), Pseudomonas aeruginosa (MTCC424), Klebsiella pneumonia (MTCC109). The plates were kept for in-

Volume : 4 | Issue : 8 | August 2014 | ISSN - 2249-555X

cubation at 37^{0}C for 24h. After complete incubation zone of inhibition was measured.

Extraction of antimicrobial substances:
Extraction of antimicrobial substances from Bacillus sp. were done by chilled acetone treatment and 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^{0}C for further processing.

Structural characterization of antimicrobial substances produced from Bacillus sp:
The precipitates were collected in to a sterile glass bottles and then dried it, after this the Structural determination was done by the very sensitive technique Gas Chromatography Mass Spectroscopy (GCMS) for all the six Bacillus sp.

RESULTS AND DISCUSSION
To survive in the environment and compete with other microorganisms for resources, many bacteria produce antimicrobial compounds to inhibit the human and animal pathogens. Search for new antimicrobial compound is very essential, as day by day resistance of the bacterial pathogens get increased therefore isolated Bacillus sp. from the from extremophilic environment of alkaline Lonar Lake possesses the varied microbial flora which produces antimicrobial compounds.

Table 1 : Characteristic of Bacilli Isolated from Lonar Lake

Isolation code→ / Bacterial Character	CW4(1)	CW2(2)	CW1(3)	OCW3(1)	CW4(3)	BW4(3)
Gram character	+	+	+	+	+	+
Shape of Bacteria	LR	LR	LR	LR	LR	LR
Arrangement of Cell	Single	Single	Single	Chain	Single	Single
Spore bearing	+	+	+	+	+	+
Capsule	+	+	+	+	+	+
Motility	+	+	+	+	+	+
Growth at Temperature						
37°C	+	+	+	+	+	+
45°C	+	+	+	+	+	+
50°C	+	-	+	+	+	+
55°C	-	-	-	-	-	+
Growth at pH						
pH 7	+	+	+	+	+	+
pH 8	+	+	+	+	+	+
pH 9	+	+	+	+	+	+
pH 10	+	+	+	+	+	+
pH 12	+	+	+	+	+	+
Growth at NaCl						
1% Nacl	+	+	+	+	+	+
2% Nacl	+	+	+	+	+	+
3% Nacl	+	+	+	+	+	+
4% Nacl	+	+	+	+	+	+
5% Nacl	+	+	+	+	+	+
6% Nacl	+	+	+	+	+	+
7% Nacl	+	+	+	+	+	+
Biochemical character						
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	-	+	+
Indol	-	-	-	-	-	-
MR	-	+	-	-	-	-
VP	-	+	-	+	-	-
Citrate Utilization	-	-	+	+	-	-
Urea Hydrolysis	-	-	-	-	-	-
Nitrate reduction	-	-	-	+	-	+
Glucose	+	+	+	+	+	+
Arabinose	-	+	-	+	-	-
Mannitol	+	+	+	+	+	+
Xylose	-	-	-	-	-	-
Lactose	-	+	-	-	-	-
Trehalose	-	-	-	+	-	-
Sucrose	-	+	-	+	-	+
Cellobiose	-	+	-	-	-	-
Galactose	-	+	-	-	-	+
Maltose	-	-	-	-	-	-
Fructose	-	-	-	+	-	-
Salicin	-	+	-	-	-	-
Sorbitol	-	+	-	-	-	-
Raffinose	-	+		-	-	-
Hydrolysis						
Starch	-	+	-	+	-	+
Lipid	-	-	-	+	-	-
Casein	-	-	-	+	-	-
Bacterial identified based 16S rRNA sequencing	B. fusiformis	B.circulance	B. fusiformis	B.cerecus	B. fusiformis	B.krulwichiae

Note: LR- Long Rod, S-Single, C-Chain, (+)=Positive, (-)=Negative

Volume : 4 | Issue : 8 | August 2014 | ISSN - 2249-555X

In the present study, total twelve samples; four each, sediments, matt and water were collected from different sites of alkaline Lonar Lake. The isolation of Bacillus sp. was done on Horikoshi media (A, B, C and D). Media A and B favoured the growth of several moderate Halophilic alkali tolerant bacteria and Medium C favours the growth of moderate alkaliphilic bacteria and Medium D support growth of Halophilic bacteria (Horikoshi, 1999). Bacillus sp. was identified on the basis of cultural, morphological, biochemical characteristics and 16S rRNA gene sequencing from NCCS, Pune. Cultural and morphological characteristics of the isolated Bacillus sp. were showed that all were Gram positive, long rod and as they grows in extreme alkaline environment endospore formation occurred, capsulated, motile and arrangement of Bacillus sp. was single only OCW3(1) arrange in chain, and all Bacillus sp. grows at the pH7 to 12 and temperature up to 50°C . Biochemical analysis showed that, all the six isolated Bacillus are aerobic showed catalase, oxidase positive and Indole, MR, VP are negative except CW2(2) which showed MR and VP positive. The 16S rRNA results were found to be Bacillus fusiformis (CW4(1)), Bacillus circulance (CW2(2)), Bacillus fusiformis (CW1(3)), Bacillus cerecus (OCW3(1)), Bacillus krulwichiae (BW4(3)), Bacillus fusiformis (CW4(3)) (Table 1). All these isolated Bacillus sp. were screened for the production of antimicrobial substances.

Antimicrobial activity of the isolated Bacillus sp.:-

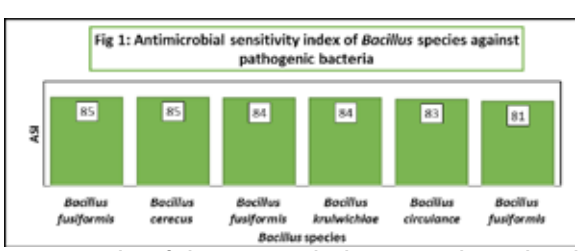
In the present study all the six isolated Bacillus sp. from Lonar Lake India showed antimicrobial activity against the Escherichia coli, Staphylococcus aureus, Salmonella typhi, Proteus vulgaris, Pseudomonas aeruginosa and Klebsiella pneumonia.

Previously, Borgave et al., (2012) studied Alkaliphilic bacteria isolated from Lonar Lake exhibited inhibitory effect against clinical isolates of pathogenic bacteria and phytopathogenic fungi. Yilmaz et al., (2006) also studied the Bacillus sp. isolated from the soil sample by agar diffusion method and found that all isolates are effective against Gram positive and Gram negative bacteria and also found Bacillus cerecus M15 strain has an inhibitory effect against both Gram positive and Gram negative bacteria. And Shinde et al., (2012) studied on antimicrobial activity of alkaliphilic Bacillus subtilis isolated from Lonar Lake and it is phospholipid in nature. Tambekar and Dhundale, (2013) isolated O. iheyensis produce broad spectrum of antimicrobial agents from Lonar Lake, which can be exploited for biotechnological potential and improve as promising sources for new antibacterial compound.

Table 2 : Zone of inhibition against pathogenic organism (mm)

Isolation code	E. coli	S. aureus	P. vulgaris	S.typhi	K.pneumonia	P. aerogenosa
B. fusiformis CW4(1)	16mm	14mm	14mm	14mm	14mm	13mm
B.circulance CW2(2)	15mm	14mm	13mm	14mm	13mm	14mm
B. fusiformis CW1(3)	14mm	15mm	13mm	13mm	13mm	13mm
B.cerecus OCW3(1)	13mm	16mm	14mm	15mm	13mm	14mm
B. fusiformis CW4(3)	13mm	15mm	14mm	13mm	15mm	14mm
B.krulwichiae BW4(3)	13mm	14mm	14mm	13mm	16mm	14mm

In the present study six Bacillus sp. were isolated, out of six Bacillus sp. four Bacillus were gives very prominent antimicrobial activity in which OCW3(1) and CW4(1) showed strong antimicrobial activity, BW4(3) and CW4(3) showed moderate antimicrobial activity and CW2(2) and CW2(2) showed weak antimicrobial activity (Fig 1).



GCMS results of the antimicrobial compounds produced by Bacillus sp.

Lonar Lake ecosystem having tremendous amount of extremophilic organisms which grows on extreme conditions and produce various useful secondary metabolites like enzymes, peptides, antimicrobial compounds etc. Bacteriocin are the antimicrobial peptides widespread produced among bacteria (Cotter et al., 2005). In the present study structural determination of the antimicrobial compounds were performed by GCMS and results showed production of secondary metabolites from Bacillus sp. some of them are very good antimicrobial compound (Table 3) this similar secondary metabolites were also reported by Selim et al., (2013) from the oil extract of medicinal plants. Mabrouk et al., (2008) studied marine-derived fungus Varicosporina ramulosa and found similar secondary metabolites from marine-derived.

Table 3: Gas Chromatography Mass spectroscopy (GCMS) Analysis Bacillus circulance (CW2(2))

Peak No.	Compound Name	Formula	Molecular weight	Percentage in compound	Properties
1	Cholesta-8,24-dien-3-ol, 4-methyl-, (3β,4α)	$C_{28}H_{46}O$	398	65.48%	Tetra unsaturated Sterols
2	2-Nonadecanone 2,4-dinitriphenyl-hydrazine	$C_{25}H_{42}N_4O_4$	446	7.15%	Pesticidal agent
3	Cis-13-Eicosenoic acid	$C_{20}H_{38}O_2$	310	6.74%	Free fatty acid
4	Tetrapentacontane, 1,54-dibromo	$C_{54}H_{10}8Br_2$	834	15.48%	Antimicrobial and antifungal agent
Bacillus fusiformis (CW4(1))					

Volume : 4 | Issue : 8 | August 2014 | ISSN - 2249-555X

Peak No.	Compound Name	Formula	Molecular weight	Percentage in compound	Properties
1	Benzenepropanoic acid	$C_9H_{10}O_2$	150	5.20%	Antioxidant and thermal stabilizer
2	Sucrose	$C_{12}H_{22}O_{11}$	324	41.76%	dextrorotatory disaccharide sugar
3	Benzenpropionic acid,3,5-bis(1,1-dimethylethyl)-4hydroxy-,methyl ester	$C_{18}H_{28}O_3$	292	2.54%	Volatile organic compound
Bacillus krulwichiae (BW4(3))					
1	Benzenpropionic acid,3,5-bis(1,1-dimethylethyl)-4hydroxy-,methyl ester	$C_{18}H_{28}O_3$	292	2.54%	Volatile organic compound
2	9-Octadecenoic acid (Z)-,methyl ester	$C_{19}H_{36}O_2$	296	6.61%	Fatty acid ester
3	Glycidol stearate	$C_{21}H_{40}O_3$	340	3.58%	Fatty acid
4	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278	12.16%	Anti-tumour, Chemo-preventive, Pesticidal and Sun-screen properties
5	Squalene	$C_{30}H_{50}$	410	12.71%	Unsaturated oil
6	i-Propyl 11 , 12-methylene-octadecanoate	$C_{22}H_{42}O_2$	338	26.32%	Ethyl esters of fatty acids
Bacillus fusiformis (CW1(3))					
1	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278	12.16%	Anti-tumour, Chemo-preventive, Pesticidal and Sun-screen properties
2	i-Propyl 11 , 12-methylene-octadecanoate	$C_{22}H_{42}O_2$	338	26.32%	Ethyl esters of fatty acids
Bacillus fusiformis (CW4(3))					
1	Cholestane, 3,5-dichloro-6-nitro-,(3β,5α,6β)	$C_27H_45CI2NO2$	485	0.013%	Cytotoxic steroid
2	2,2-Bis[4-[(4,6-dichloro-1,3,5-triazin-2-yl)oxy]phenyl]-1,1,1,3,3,3-hexafluoropane	$C_{21}H_8Cl_4F_6N_6O_2$	624	1.463%	-
3	Tert-Hexadecanethiol	$C_{16}H_{34}S$	258	9.47%	Used to prepare nanoparticle
4	i-Propyl 11 , 12-methylene-octadecanoate	$C_{22}H_{42}O_2$	338	26.32%	Ethyl esters of fatty acids
Bacillus cerecus (OCW3(1))					
1	1 ,3-Propanediol, 2-ethyl-2-(hydroxymethyl)-	$C_6H_{14}O_3$	134	27.05%	Polyalcohol
2	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	3.52%	Plasticizer and Ectoparasiticide
3	9-Octadecenoic acid (Z)-,methyl ester	$C_{19}H_{36}O_2$	296	6.24%	Monounsaturated fatty acid ester
4	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	294	3.10%	Estrogen-dependent urinary sex pheromone of female mice.
5	Decanedioic acid,bis(2-ethylhexyl) ester	$C_{26}H_{50}O_4$	426	7.06%	Xenobiotic organic compound
6	17-Pentatriacontene	$C_{35}H_{70}$	490	17.30%	Semi volatile and volatile organic compound

CONCLUSION

In the present study six Bacillus sp. were isolated, out of six Bacillus sp. four Bacillus were gives very prominent antimicrobial activity in which OCW3(1) and CW4(1) showed strong antimicrobial activity, BW4(3) and CW4(3) showed moderate antimicrobial activity and CW2(2) and CW2(2) showed weak antimicrobial activity. Our study provides primary evidence that Bacillus strains were promising sources for the bioactive substances. These Bacillus sp. in general represent a new and rich sources of secondary metabolites that need to be explored.

Volume : 4 | Issue : 8 | August 2014 | ISSN - 2249-555X

REFERENCE

1. Borgave SB, Joshi AA, Kelkar AS and Kanekar PP, (2012). Screening of Alkaliphilic, Haloalkaliphilic bacteria and Alkalithermophilic Actinomycetes isolated from alkaline Soda Lake of Lonar, India for antimicrobial activity. Int. J Pharma Bio Sci 3(4):258-274. | 2. Cotter PD, Hill C and Ross P, (2005). Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3:777–788. | 3. Fredriksson A, Dube A, Milton DJ and Balasundaram MS, (1973). Lonar Lake, India: an impact crater in basalt, Sci, 180: 862-864. | 4. Horikoshi K, (1991). General view of alkaliphiles and thermophiles in superbugs: Microorganisms in extreme environments, (Eds. Horikoshi K and Grant WD), Springer Verlag Berlin, 3-13. | 5. Horikoshi K, (1999). Extracellular enzymes In Horikoshi K, (ed). Alkalophiles Harwood Acad Pub Japan, 147-285. | 6. Joshi, AA, Kanekar PP, Kelkar AS, Shouche YS, Wani AA, Borgave SB and Sarnaik SS, (2007). Cultivable bacterial diversity of alkaline Lonar Lake, India Microbe Ecol, 55(2): 163-172. | 7. Mabrouk AM, Kheiralla ZH, Hamed ER, Youssry AA and Abeer, (2008). Production of some biologically active secondary metabolites from marine-derived fungus Varicosporina ramulosa. Malaysian J Microbiol; 4(1): 14-24. | 8. Shinde, Vinod A, More SM and Kadam TA, (2012). Antimicrobial activity of Phospholipid compound produce by alkaliphilic Bacillus subtilis isolated from Lonar Lake. Int J Pharma Bio Sci. 2(4):172-175. | 9. Selim SA, Abdel Aziz MH, Mashait MS and Warrad MF, (2013). Antibacterial activities, chemical constitutes and acute toxicity of Egyptian Origanum majorana L., Peganum harmala L. and Salvia officinalis L. essential oils. Afr J of Pharma and Pharmacology. 7(13): 725-735. | 10. Sengupta D, Bhandari N and Watanabe S, (1997). Formation age of Lonar meteor crater, India. Revista de Fisica Aplicada e Instrumentacao, 12: 1–7. | 11. Tambekar DH and Dhundale VR, (2013). Screening of antimicrobial potentials of haloalkalophilic bacteria isolated from Lonar Lake Int. J Pharma chem Bio Sci 3(3) 820-825. | 12. Yilmaz M, Soran H and Beyatli Y, (2006). Antimicrobial activity of some Bacillus species Strains isolated from the soil Microbiol research. 161:127-131. |