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ISOLATION AND CHARACTERIZATION OF ANTIBACTERIAL SUBSTANCE PRODUCED BY *BACILLI* ISOLATED FROM LONAR LAKE

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ABSTRACT

By screening for antibacterial substance producing twenty nine bacilli isolated from Lonar lake. In present study all the bacillus species showed that the antimicrobial activity against the E. coli (100%), out of twenty nine, twenty seven (93%) bacillus species showed antimicrobial activity against to K. pneumoniae, seven (24%) were antimicrobial against to P. vulgaris and nine (31%) were found antimicrobial against to E. aerogenes and twelve (41%) Bacillus species showed antimicrobial activity against to S. aureus The antibacterial substance produced by B. asahii, B. gibsonii, B. pseudofirmus, B. pumilus, B. flexus were found heat stable upto 121° C and showed that the antibacterial activity against to E. coli. Antibacterial substance produced by B. flexus, B. lehensis, B. asahii were found heat stable after incubation at 121°C for 10 min and showed antibacterial ac:tivity against to K. pneumoniae. Twenty six bacilli species were active at pH 10-12 and inhibit the growth of E. coli, twenty one (72%) Bacillus species showed antimicrobial activity against to K. pneumoniae, seven (24%) were found antibacterial against S. typhi and eleven (37%) Bacillus species showed antimicrobial activity against to S. aureus. But all antibacterial substance was not showed the activity against to P. vulgaris and E. aerogenes. The antibacterial substance not showed the antibacterial activity against to S. aureus, S. typhi, P. vulgaris, and E. aerogenes at pH 7-9. This study provides primary evidence that haloalkaliphilic bacilli strains are promising sources for antibacterial substance. These bacteria in general represent a new and rich source of secondary metabolites that need to be explored

INTRODUCTION:

Bacteriocin are antimicrobial peptides widespread produced among bacteria (Cotter *et al*. 2005). The attractive features of bacteriocin, such as their natural sources, wide range of activities, and their proteinaceous nature, which implies a putative degradation in the gastrointestinal tract of man and animals, have interested researchers seeking to develop new antimicrobial agents (Cleveland *et al*. 2001; Asaduzzaman and Sonomoto 2009). Those produced by lactic acid bacteria (LAB) are largely studied with the perspective to search for safe and food-grade preservatives of biological origin (O'Sullivan *et al*. 2002; Calo-Mata *et al*. 2008). Despite the intensive work on bacteriocin produced by LAB, the genus *Bacillus* comprises a variety of industrially important species and has a history of safe use in both food and pharmaceutical industry (Paik *et al*. 1997; Pedersen *et al*. 2002; Bizani *et al*. 2005; Motta *et al*. 2007; Hong *et al*. 2008; Vaucher *et al*. 2010). The genus *Bacillus* encompasses a number of bacteriocinogenic species, such as *B. subtilis* which produces subtilin, (Jansen and Hirschmann 1944) and subtilosin (Zheng and Slavik 1999), *B. coagulans* which produces coagulin (Hyronimus *et al*. 1998), and *B. megaterium* which produces megacin (von Tersch and Carlton 1983). During the past two decades, marine bacteria have highlighted the tremendous potential of the microorganisms as a source of new bioactive secondary metabolites (Ahmed *et al.* 2000). There is growing awareness of the need for development of new antimicrobial agents for the treatment of human, animal and plant diseases. No reports documenting the presence of haloalkaliphilic *Bacillus* sp. from the Indian soda lake. We have applied this strategy to characterized antibacterial activity of *Bacilli* isolated from Lonar Lake.

The alkaline Lonar Lake (Latitude 19 ° 58′, Longitude 76 ° 36′) is a unique basaltic rock meteorite impact crater, ranking third in the world. Lonar crater is filled with saline water. The uniqueness of the lake water is its salinity and high alkalinity. The lake water is alkaline having an average pH of 9.5- 10. Lonar Lake is a closed one without any outlet and unique due to its salinity, alkalinity and biodiversity. Due to the uniqueness, the lake has evoked much scientific value among researchers and continues to site of attraction for many (Tambekar *et al*. 2010). Chaphalkar and Dey (1994), have studied the metalloprotease from alkaline Streptomyces isolated from Lonar lake silt sample. Methanol degrading microorganism also isolated and identified from Lonar Lake water and sediment samples was good finding (Tambekar *et al*. 2011; Surakashi *et al*. 2010). Kharat *et al.* (2009); Deshmukh and Puranik (2010), have studied on the antibacterial activity of alkaliphilic *Streptomyces and* cyanobacteria isolated from Lonar Lake respectively. The purpose of this work was to evaluate the potential antimicrobial activity of a bioactive compound produced by a *Bacillus sp*. isolated from Lonar Lake.

MATERIALS AND METHODS:

Enrichment and isolation of microorganisms: Lonar lake water and sediment sample were collected in sterile bottles and polythene bags respectively, from defined sampling site. Enrichment of water samples and sediment samples were carried out in Horikoshi I, Horikoshi II, and nutrient agar at pH 10, nutrient agar at pH 10.0 with 30 g l−1 sodium chloride (Table 1). All the flasks were incubated at room temperature (RT) on a rotary shaker (100 rpm) for 48h. After enrichment, the organisms were isolated on respective media agar plates and incubated at 37°C for 24h. Well isolated and differentiated colonies from these enrichment media were transferred on the respective medium slants and cultures were maintained as stocks.

Media Composition

	Horikoshi I g/L (A)	Horikoshi II g/L (B)	Nutrient agar g/L (C)	Nutrient agar g/L(D)
Glucose	10	-	-	-
Soluble starch	-	10	-	-
Peptone	5	5	5	5
Yeast extract	5	5	1.5	1.5
Beef extract	-	-	1.5	1.5

KH_2PO_4	1	1	-	-
$MgSO_4.7H_2O$	0.2	0.2	-	-
Na_2CO_3	10	10	-	-
Sodium Chloride	-	-	5	35
Agar	20	20	20	20

Identification of the bacterial culture

The *Bacillus* culture isolated from Lonar lake water and sediment samples were identified by conventional biochemical tests in according with Bergey's Manual of systematic bacteriology (Sneath *et al.*1986).

Detection of antagonistic activities: The antagonistic properties of *Bacillus* preparations were determined by modifying the disc diffusion method. Sterile blotting paper discs (6mm) were dipped into 48h incubated cell free culture broth and then placed on solidified Nutrient agar seeded with 3h old culture of test oraganism, which included *Escherichia coli* (MTCC 443), *Enterobacter aerogenes* (MTCC 111), *Klebsiella pneumoniae* (MTCC 2653), *Proteus vulgaris* (MTCC 426), *Salmonella typhi* (MTCC 734). The plates were kept for incubated at 37°C for 24h. Zones of inhibition were measured (Kirby *et al.* 1996).

Preparation of bacteriocin assay: As potential bacteriocin producers grown in respective broth at 37°C for 48h. Cell suspensions were centrifuged at 5000 rpm for 15 min. The antagonistic activity of antibacterial substance was determined by disc diffusion method (Tagg *et al.* 1971; Lade *et al*. 2007).

Thermal and pH stability: Analyze thermal stability, aliquots of cell-free supernatant were exposed to temperatures ranging from 60°C, 70°C, 80°C, 90°C, 100°C and 121°C for 10 min. Similarly sensitivity of antibacterial substance to different pH was tested by adjusting the pH of culture supernatant in the range of pH 7.0 and 12 then antibacterial activity was detected by disc diffusion method against test organism.

RESULT AND DISCUSSIONS

The alkaline Lonar Lake is a unique basaltic rock meteorite impact crater, ranking third in the world. Lonar crater is filled with saline water. Lonar Lake is a one such alkaline saline Lake in which the indigenous microflora is present, and such microbial flora has ability thrives in alkaline condition. Soda lakes are a specific type of salt lake with high to extremely high carbonate alkalinity, a pH from 9 to 11, and a moderate to extremely high salinity. From a total fifty five isolates obtained in the isolation exercise, cultural, morphological characteristics of all the strains were studied and twenty nine bacilli isolates were selected on the basis of gram positive *Bacillus* and spore bearing for further identification and antibacterial activity. The phenotypic characterization indicated that bacterial strains were related to genera *Bacillus and species* were *Bacillus flexus, Bacillus cellulosilyticus, Bacillus pseudofirmus, Bacillus clausii, Bacillus krulwichiae, Bacillus pumilus, Bacillus lehensis, Bacillus halodurans, Bacillus circulans, Bacillus cereus, Bacillus agaradhaerens, Bacillus sphaericus, Bacillus fusiformis,* and *Bacillus asahii*, *Bacillus pseudalcalophilus, Bacillus okuhidensis and Bacillus gibsonii.*

There are evidence to show that the moderately halophilic bacteria have strong potable solutes, fermented foods, enzyme, polymers and degradation of toxic compounds (Canovas *et al*. 1998 Ventosa *et al*. 1998; Mata *et al*. 2002; Sanchez – Porro *et al*, 2003). Lei chen *et al*. (2010), were studied on the antimicrobial and cytotoxic activities of moderately halophilic bacteria isolated from the Weihai Solar saltern. In present study all the *Bacillus* species showed that the antimicrobial activity against the *E. coli* (100%), out of twenty nine, twenty seven (93%) *Bacillus* species showed antimicrobial activity against to *K. pneumoniae*, seven (24%) were antimicrobial against to *P. vulgaris* and nine (31%) were found antimicrobial against to *E. aerogenes* and twelve (41%) *Bacillus* species showed antimicrobial activity against to S. aureus (Table 2). Although inhibition to Gram-negative strains is less common, this has been previously reported for a BLIS produced by a *B. cereus* isolate that was also able to inhibit a Gram-negative, also *E. coli* (Torkar and Matijasic 2003). In present study the antibacterial activity by *Bacillus* were mostly inhibitory to Gram-negative strains and were less effective against Gram-positive bacteria. Lei Chen *et al*. (2010), isolate revealed that, *Bacillus hwajinpoensis, HaloBacillus trueperi, Planococcus rifietoensis, Salinicoccus halodurans* and *Halomonas spp*. They were found 23 halophilic bacterial strain inhibited *B. subtilis* and thirteen halophilic bacterial strain displayed antifungal activity against human pathogenic fungus, *Candida albicans.*

The heat sensitivity of the antimicrobial substance was determined by measuring its activity after incubation for 10 min at different temperatures. It was stable at 60° C against the *E.coli* and *K. pneumoniae* and not showed the antibacterial activity against to the *S. aureus, S. typhi, E. aerogenes* and *P. vulgaris*. At 60° C almost all the *Bacillus* showed the antibacterial activity against the *E. coli* except *B. cellulosilyticus. B. halodurans, B. agaradhaerens. B. gibsonii, B. sphaericus, B. pseudofirmus, B. cereus* and *B. pseudalcalophilus* not showed that the antibacterial activity against the *K. pneumoniae*. The antibacterial substance produced by *B. krulwichiae, B. fusiformis, B. sphaericus, B. circulans, B. cereus, B. agaradhaerens* were found heat sensitive above the 70° C and not showed the antibacterial activity. The antibacterial substance produced by *B. asahii, B. gibsonii, B. pseudofirmus, B. pumilus, B. flexus* were found heat stable upto 121° C and showed that the antibacterial activity against to *E. coli*. Antibacterial substance produced by *B. flexus, B. lehensis, B. asahii* were found heat stable after incubation at 121°C for 10 min and showed antibacterial activity against to K. pneumoniae and it was remarkable property (Table 2).The antibacterial substance isolated in the present study had shown thermo stability thus it was placed under class II of bacteriocin which was found to be heat stable in nature (Klaenhammer 1993). Similar result was observed by Sharma *et al*. (2005) in the retention of activity of bacteriocin of *B. lentus* till 100° C for 10 min was a remarkable property. Naclerio *et al*. (1993) isolated Cerein from *B. cereus* which was partially stable to heat treatment. They revealed that, the activity was maintained during treatment up to 75° C and disappeared only after 15 min incubation at 90° C. Thermo stability of inhibitory compound was also detected by Hyronimus *et al*. (1998) of coagulin produced by *B. coagulans*.

In present study the antibacterial substance was also active over a wide range of pH from 7 -12. twenty six bacilli species were active at pH 10-12 and inhibit the growth of *E. coli* , twenty one (72%) *Bacillus* species showed antimicrobial activity against to *K. pneumoniae*, seven (24%) were found antibacterial against *S. typhi* and eleven (37%) *Bacillus* species showed antimicrobial activity against to S. aureus (Table 2). But all antibacterial substance was not showed the activity against to *P. vulgaris* and *E. aerogenes* (Table 3). The antibacterial substance not showed the antibacterial activity against to *S. aureus, S. typhi, P. vulgaris,* and *E. aerogenes* at pH 7-9. Almost all the bacilli showed the antibacterial activity against to *E. coli* and *K. pneumoniae* at pH 8-9 except some species. In present study we revealed that the antibacterial activity of *B. circulans, B. pumilus, B. pseudofirmus and B. agaradhaerens* were loss when the pH of the inhibitory substance were neutral against to E.coli. *B. krulwichiae, B. halodurans, B. pumilus, B. pseudofirmus* were loss their antibacterial activity of substance at pH neutral against to *K. pneumoniae* (Table 3). Hyronimus *et al*. (1998), were studied on the coagulin and they revealed that the inhibitory substance was also active over a wide range of pH, as full activity was retained at pH values between 3 and 8. Activity was reduced to 75% at pH 1 while it was severely altered for pH over 9 against to *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Listeria* and *Pediococcus*. In conclusion, our study reveals the diversity of haloalkaliphilic bacilli in a Lonar lake, and these kinds of bacteria could produce broad spectrum antimicrobial agents. However, further studies are needed to identify the components of the halophilic *Bacillus* responsible for the biological activity. Our study provides primary evidence that *Bacillus* strains were promising sources for the bioactive substances. These bacteria in general represent a new and rich source of secondary metabolites that need to be explored.

Table2: Effect of temperatures on the antibacterial substance produced by bacilli

							60		70		80		90		100		121	
	E.coli	*S. aureus*	*S. typhi*	*pneumoniae*	*P. vulgaris*	*E. aerogenes*	*E.coli*	*pneumoniae*	*E.coli*	*pneumoniae*	*E.coli*	*pneumoniae*	*E.coli*	*pneumoniae*	*E.coli*	*pneumoniae*	*E.coli*	*pneumoniae*
Bacillus cellulosilyticus	8	-	-	9	-	-	-	8	-	8	-	8	-	8	-	8	-	8
Bacillus asahii	8	8	-	8	9	-	8	8	8	8	8	8	8	-	8	-	8	-
Bacillus clausii	8	-	-	8	-	-	8	8	8	8	8	-	8	-	8	-	-	-
Bacillus asahii	8	8	-	8	9	-	8	8	8	8	8	8	8	-	8	-	8	-
Bacillus asahii	10	8	8	8	-	-	8	8	9	8	9	8	-	9	-	8	-	8
Bacillus krulwichiae	9	8	8	10	-	-	9	9	-	-	-	-	-	-	-	-	-	-

Bacillus gibsonii	10	8	-	-	-	-	10	-	10	-	10	-	10	-	10	-	9	-
Bacillus krulwichiae	9	8	8	10	-	-	9	9	-	-	-	-	-	-	-	-	-	-
Bacillus fusiformis	8	11	-	9	9	10	8	10	-	-	-	-	-	-	-	-	-	-
Bacillus fusiformis	8	11	-	9	9	10	8	10	-	-	-	-	-	-	-	-	-	-
Bacillus sphaericus	10	-	8	9	8	8	10	-	8	-	-	-	-	-	-	-	-	-
Bacillus fusiformis	8	11	-	9	9	10	8	10	-	-	-	-	-	-	-	-	-	-
Bacillus pumilus	9	-	-	8	-	-	9	8	9	8	9	-	9	-	9	-	9	-
Bacillus pseudofirmus	9	-	-	9	-	-	8	8	8	8	9	-	8	8	8	-	-	-
Bacillus flexus	9	9	-	8	-	10	9	8	9	8	9	8	9	8	9	8	9	8
Bacillus okuhidensis	9	-	-	8	-	-	9	9	-	8	-	8	-	-	-	-	-	-
Bacillus pseudofirmus	9	-	-	9	-	-	8	8	8	9	8	8	8	8	8	8	-	8
Bacillus lehensis	8	8	-	8	-	-	8	8	8	8	8	8	8	-	8	-	-	-
Bacillus halodurans	9	9	-	8	11	8	-	8	-	8	-	8	-	-	-	-	-	-
Bacillus circulans	9	-	10	9	-	-	9	11	-	-	-	-	-	-	-	-	-	-
Bacillus pumilus	9	-	-	8	-	-	9	8	8	8	9	8	9	8	9	8	9	-
Bacillus pseudofirmus	9	-	-	8	-	-	8	-	8	-	9	-	8	-	8	-	-	-

Bacillus cereus	9	-	-	8	-	8	9	-	-	-	-	-	-	-	-	-	-	-
Bacillus pseudofirmus	9	-	-	9	-	-	9	8	9	8	9	-	8	-	8	-	-	-
Bacillus pumilus	9	-	-	8	-	-	9	8	8	8	9	-	9	-	9	-	9	-
Bacillus agaradhaerens	8	-	8	8	-	8	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus agaradhaerens	8	-	8	8	8	8	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus pumilus	9	-	-	8	-	-	9	8	8	8	9	-	9	-	9	-	9	-
Bacillus pseudalcalophilus	8	-	-	-	-	-	8	-	-	-	-	-	-	-	-	-	-	-

Table3 : Effect of pH on the antibacterial substance produced by bacilli

	pH12				pH10				pH 9		pH 8		pH7	
	E.coli	*S.aureus*	*S.typhi*	*K. pneumoniae*	*E.coli*	*S.aureus*	*S. typhi*	*K. pneumoniae*	*E.coli*	*K. pneumoniae*	*E.coli*	*K. pneumoniae*	*E.coli*	*K. pneumoniae*
Bacillus cellulosilyticus	-	-	-	8	-	-	-	8	-	8	-	8	-	8

Bacillus asahii	8	8	-	8	8	8	-	9	10	10	8	12	8	10
Bacillus clausii	8	-	-	8	8	-	-	8	10	10	8	11	8	10
Bacillus asahii	8	8	-	8	8	8	-	9	10	10	8	12	8	10
Bacillus asahii	10	8	8	8	10	8	8	9	10	8	10	8	10	8
Bacillus krulwichiae	9	-	8	10	9	-	-	9	10	9	8	9	8	-
Bacillus gibsonii	10	8	-	-	10	-	-	-	-	-	-	-	-	-
Bacillus krulwichiae	9	9	8	10	9	8	-	10	8	9	8	-	8	-
Bacillus fusiformis	8	10	-	9	8	-	-	9	10	9	9	9	9	8
Bacillus fusiformis	8	10	-	9	8	-	-	9	10	9	9	9	9	8
Bacillus sphaericus	10	-	8	9	10	-	8	9	-	-	-	-	-	-
Bacillus fusiformis	8	10	-	9	8	-	-	9	10	9	9	9	9	8
Bacillus pumilus	9	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus pseudofirmus	9	-	-	-	9	-	-	-	8	-	8	8	-	-
Bacillus flexus	8	8	-	8	8	8	-	8	8	8	8	8	8	8
Bacillus okuhidensis	9	-	-	8	8	-	-	8	8	9	8	9	8	8

Bacillus pseudofirmus	9	-	-	9	9	-	-	9	8	9	8	10	9	10
Bacillus lehensis	8	8	-	8	8	8	-	8	9	10	9	9	8	9
Bacillus halodurans	9	9	-	8	9	8	-	9	11	8	8	8	-	-
Bacillus circulans	9	-	9	9	9	-	8	9	-	10	-	-	-	-
Bacillus pumilus	9	-	10	8	9	-	9	9	8	8	8	9	-	-
Bacillus pseudofirmus	8	-	-	8	8	-	-	8	9	9	8	11	-	-
Bacillus cereus	9	-	-	8	9	-	-	8	-	9	-	9	-	9
Bacillus pseudofirmus	9	-	8	8	9	-	-	8	8	8	8	10	9	-
Bacillus pumilus	9	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus agaradhaerens	-	-	-	-	-	-	-	-	-	9	-	9	-	8
Bacillus agaradhaerens	-	-	-	-	-	-	-	-	-	9	-	9	-	8
Bacillus pumilus	9	-	-	-	-	-	-	-	-	-	-	8	-	-
Bacillus pseudalcalophilus	8	-	-	-	-	-	-	-	-	8	-	11	-	10

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