



## ANTIBACTERIAL ACTIVITY OF A BACTERIOCCIN-LIKE INHIBITORY SUBSTANCE PRODUCED BY BACILLUS SP. FROM HALOPHILIC ENVIRONMENT

VISHAL R. DHUNDALE<sup>1</sup> AND VIJAYSHREE M. HEMKE\*<sup>2</sup>

<sup>1</sup>New Arts Commerce and Science College Ahmednagar, Maharashtra (India)

<sup>2</sup>Jijamata Mahavidyalay Buldhana, Maharashtra (India)

### ABSTRACT

The objective of this study was to investigate a bacteriocin like inhibitory substance (BLIS) produced by a *Bacillus* sp. isolated from the halophilic Lonar crater. In present study total fourteen *Bacillus* sp isolated, out of them twelve *Bacillus* species showed that the antimicrobial activity against the *E. coli* and *S. typhi* except the CW1 and CW3. The *Bacillus* sp. CW2 was found 24 mm zone of inhibition that considered as prominent BLIS producer against *E. coli* while the *Bacillus* DS3 was found 25 mm zone of inhibition that considered as prominent BLIS producer against *S. typhi*. The *Bacillus* Sp. GW3 and *Bacillus* Sp. HS4 were found moderate activity with 22 mm zone of inhibition against *P. aeruginosa* and 28 mm zone of inhibition against *P. vulgaris* respectively. Out of fourteen, nine *Bacillus* sp were found antibacterial activity against *B. subtilis*. The *Bacillus* Sp EW2 and *Bacillus* Sp. DS7 was found moderate activity with 21 mm zone of inhibition against *B. subtilis* and 22 mm against *B. megaterium* respectively. The isolated *Bacillus* sp. DS3 and DS2 were found antibacterial against all tested all gram positive bacteria. In conclusion, the studies suggested that halophilic environments could be sources of bacterial strains capable of synthesizing novel metabolites with antibacterial activity. These bacteria in general represent a new and rich source of BLIS that need to be explored

**KEY WORDS:** *Bacillus*, bacteriocin-like inhibitory substance, halophilic



**VIJAYSHREE M. HEMKE\***  
Jijamata Mahavidyalay Buldhana, Maharashtra (India)

Received on : 05-04-2017

Revised and Accepted on : 01-06-2017

DOI: <http://dx.doi.org/10.22376/ijpbs.2017.8.3.b356-362>

## INTRODUCTION

Hyper alkaline saline lakes, with salinity and alkalinity ranges at or near saturation are extreme environments; yet, they often harbours remarkably high microbial cell densities and are biologically very productive ecosystems<sup>1-3</sup>. To evolve to such conditions, bacteria - have developed various mechanisms to survive in such extreme environment. Halophilic bacteria from marine environment are also better sources of secondary metabolites that may have potential of pharmaceutical and biotechnological applications<sup>4</sup>. The development of resistance to multiple drugs is major issue in the medication of infectious disease caused by pathogenic microorganism. The multidrug resistance is presently targeted on exploration and therefore need to search new bioactive compound. To achieve this conditions and circumstances, there is concern to ameliorate novel class of antibiotics that have distinct mechanisms of activity worldwide. The investigations of such bacteria are of great importance due to produce medically important metabolites such as bacteriocin or bacteriocin like inhibitory substance (BLIS)<sup>5-6</sup>. The important 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<sup>7-8</sup>. Now a days it is seen the rapid emergence of antibiotic-resistant in pathogenic bacteria and there is an essential for discovering novel antimicrobial compounds<sup>9-10</sup>. The hypersaline environment including halophilic microorganisms may, as indicated, serve as source of antibacterial compounds<sup>11</sup>. Only a minor fraction of the halophilic and alkaliphilic microbiota is culturable and assessing the complete potential of bioactivity of the extremophilic microbial diversity would require inclusion of both cultured and uncultured organisms from marine environment<sup>4,12-13</sup>. The alkaline Lonar crater is a unique basaltic rock meteorite impact crater, ranking third in the world and is filled with saline water having an average pH of 9.5- 10. In Lonar, no significant studies have been conducted so far to isolate and produce useful antibiotics. Therefore, present study is intended to isolate, screen and characterize bacteriocin producing bacteria from Lonar Lake which could be further explored for a biotechnological potential.

## MATERIALS AND METHODS

### *Enrichment and isolation of microorganisms*

The alkaline Lonar Lake in Buldhana district of Maharashtra is unique ecosystem and wonder in the India (latitude 19°58', Longitude 76°36'). 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

broth at pH 10, nutrient broth at pH 10.0 with 30 g l<sup>-1</sup> sodium chloride. 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<sup>14-15</sup>.

### *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.

### *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 organism, which included *Escherichia coli* (MTCC 443) *Proteus vulgaris* (MTCC 426), *Salmonella typhi* (MTCC 734), *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus*. The plates were kept for incubation at 37°C for 24h. Zones of inhibition were measured<sup>16</sup>.

### *Antibacterial assay of bacteriocin*

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<sup>17-18</sup>.

## RESULT AND DISCUSSIONS

The alkaline Lonar Lake is a unique basaltic rock meteorite impact crater. Lonar Lake is a one such soda Lake in which the indigenous microflora is present, and such microbial flora has ability thrives in alkaline condition. Total fourteen isolates obtained in the isolation exercise, cultural, morphological characteristics of all the strains were studied. The isolates were screened on the basis of biochemical characteristics as described earlier All the fourteen bacterial cultures were revealed to be *Bacillus* by their positive Gram reaction, spore bearing, motile, and rods in cell morphology. Then a various conventional phenotypic tests were used to identify them tentatively. In present investigation was to determine antibiotic substance producing diversity of Lonar Lake using different enrichment media with various substrates as peptone, yeast extract, glucose, and starch, Casein and egg yolk. The media composition seemed to have an effect on the recovery of different species of the *Bacilli*. There are evidence to show that the moderately halophilic bacteria have strong potable solutes, fermented foods, enzyme, polymers and degradation of toxic compounds<sup>19-22</sup>.

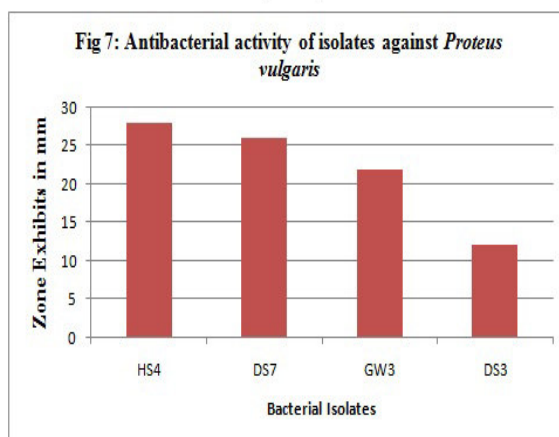
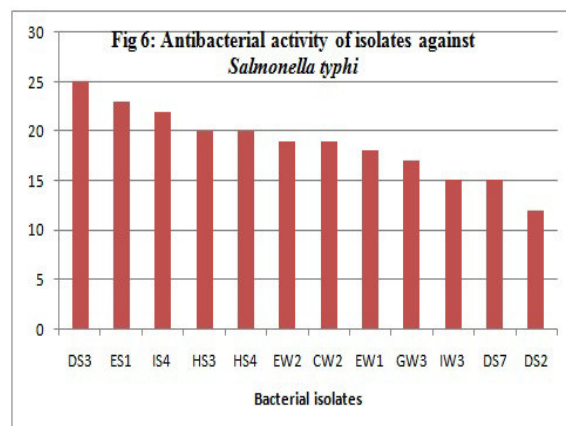
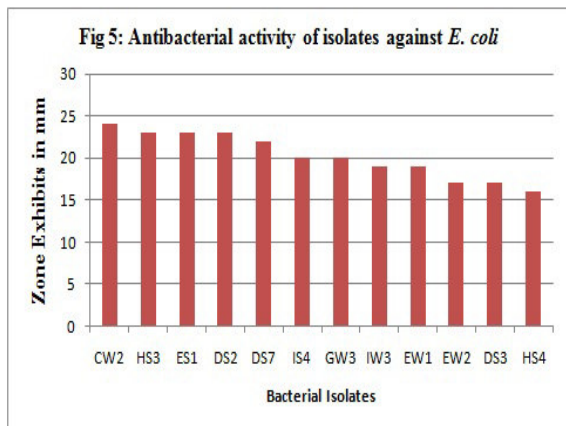
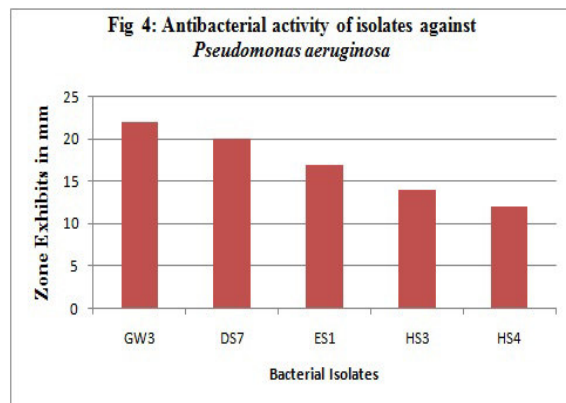
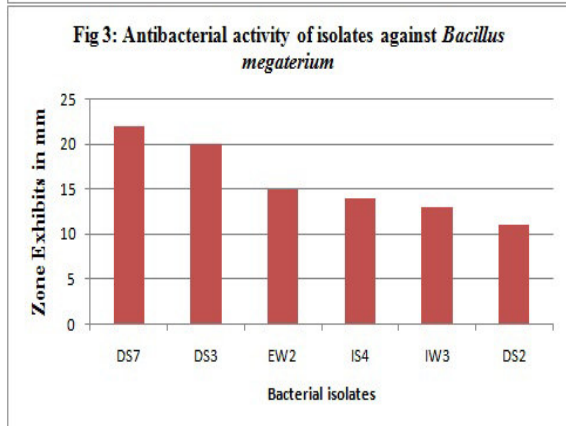
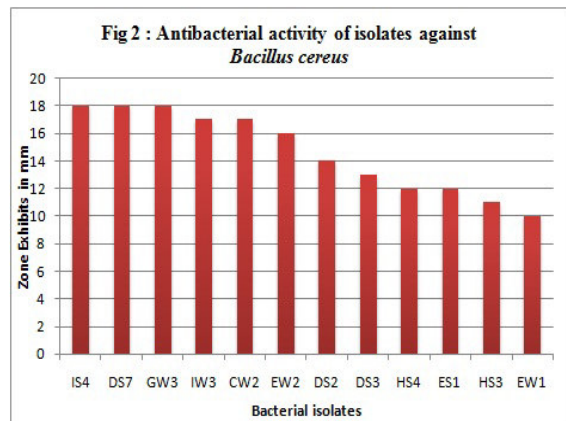
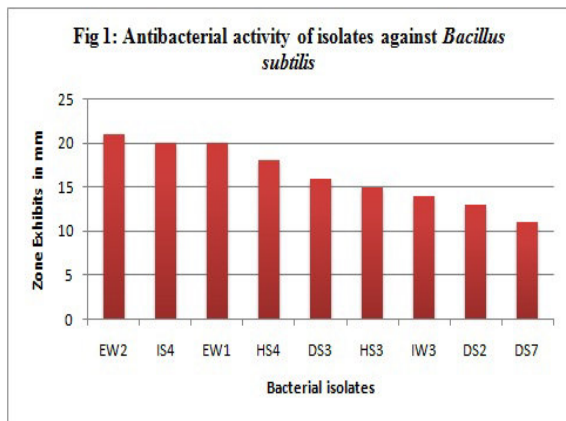
Table 1  
**Biochemical characteristic of Bacilli  
 isolated from Lonar Lake**

Sr.NO	Isolation Code	Morphological characteristicBiochemical characteristicBacteriocin-like inhibitory substance against																	
		Gram	Shape of Bacteria	Colony Size	Catalase	Oxidase	Glucose	Arabinose	Mannitol	Xylose	Sucrose	Maltose	Fructose	<i>B. subtilis</i>	<i>B. cereus</i>	<i>P. vulgaris</i>	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>S. typhi</i>
1	IS4	+	SR	1.5	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
2	IW3	+	SR	1	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
3	EW1	+	SR	1	+	+	+	+	-	+	-	-	+	+	-	-	+	+	-
4	EW2	+	SR	1	+	+	+	+	-	+	-	-	+	+	-	-	+	+	+
5	DS3	+	SR	1.5	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
6	DS7	+	SR	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	CW1	+	SR	1	+	+	+	+	-	+	+	-	+	-	-	-	-	-	-
8	CW2	+	SR	1	+	+	+	+	-	+	+	-	+	-	-	-	+	+	-
9	CW3	+	SR	1	+	+	+	+	-	+	+	-	+	-	-	-	-	-	-
10	HS3	+	LR	2.5	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
11	HS4	+	LR	3	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
12	ES1	+	LR	1	+	+	+	+	-	+	-	-	+	+	-	+	+	+	-
13	DS2	+	LR	1	+	+	+	+	-	+	-	-	+	+	-	-	+	+	+
14	GW3	+	LR	1.5	+	+	+	-	+	+	+	+	+	-	+	+	+	+	-

SR- Short Rod, LR- Long Rod, + Positive, - Negative

Lei chen *et al.*,<sup>23</sup> were studied on the antimicrobial and cytotoxic activities of moderately halophilic bacteria isolated from the Weihai Solar saltern. In present study twelve Bacillus species showed that the antimicrobial activity against the *E. coli* and *S. typhi* except the CW1 and CW3 were not showed activity. The Bacillus CW2 was found 24 mm zone of inhibition that considered as prominent antibiotic substance producer against *E. coli* followed by the HS3, ES1 and DS2 were shows 23mm zone of inhibition. While the dissimilar result was observed by Motta *et al.*,<sup>24</sup>, when the antibacterial substance produced by Bacillus sp BLS P34 was co-added with EDTA showed antibacterial activity but BLS or EDTA alone showed no inhibitory effect on the *E. coli* and *S. typhi*. The Bacillus DS3 was found 25 mm zone of inhibition that considered as prominent antibiotic substance producer against *S. typhi* followed by the ES1 was shows 23mm zone of inhibition. Few attention have been addressed to their application as antimicrobials in clinical studies. Because attention towards the bacteriocin is important, effective and not harmful to human perspectives, it has been already investigated as an another choice for disease control<sup>25-26</sup>. The antibacterial substance produced by Bacillus sp. may represent an antimicrobial substance with potential application in the prevention and treatment of *E. coli* and *S. typhi*. The prominent antibacterial activity of *Oceanobacillus iheyensis* was found against *E. coli* and moderate activity against *K.*

*pneumoniae*, *S. typhi*, *S. aureus* and poor antibacterial activity against *P. vulgaris* and *E. aerogenes* while no antibacterial activity found against *P. aeruginosa*<sup>11</sup>. In the present studies, five Bacillus sp. were found antibacterial activity against *P. aeruginosa*. The Bacillus Sp GW3 was found moderate activity with 22 mm zone of inhibition towards the *P. aeruginosa*. Followed that the Bacillus Sp. DS7 was found 20mm zone of inhibition while HS4 was found lowest activity. These results against *P. aeruginosa* indicate that the these antibacterial substance may be effective against *P. aeruginosa* which is responsible for the biofilm formation. Similar results were observed by Nakamura *et al.*,<sup>27</sup> the antibacterial activity of violet pigment produced from psychrotropic bacterium RT102 Strain. These antibacterial substance produced by Bacillus sp. may represent an antimicrobial substance with potential application in the prevention of biofilm formation and treatment of *P. aeruginosa*. Shanks *et al.*,<sup>28</sup> was performed the isolation and identification of a bacteriocin with antibacterial and antibiofilm activity from *Citrobacter freundii*. The Bacillus Sp. HS4 was found moderate activity 28 mm zone of inhibition towards the *P. vulgaris*. Followed that the Bacillus Sp. DS7 was found 26mm zone of inhibition while DS3 was found lowest activity. Similar results were observed by Tambekar and Dhundale<sup>5</sup> against *E. coli*, *K. pneumoniae*, *P. vulgaris* and *E. aerogenes*.



The present knowledge about the inhibitory range of bacteriocins from Gram-positive bacteria is that they are narrow to inhibit the other Gram-positive bacteria. The range of sensitive strains can vary significantly, from relatively narrow as in the case of lactococcins which have been found to kill only *Lactococcus*, to extraordinarily broad<sup>29</sup>. In the present study, the isolated *Bacillus* sp were produced antibacterial substance against *B. subtilis*, *B. cereus* and *B. megaterium*. Out of fourteen, nine bacillus sp were found antibacterial activity against *B. subtilis*. The *Bacillus* Sp EW2 was found moderate activity with 21 mm zone of inhibition towards the *B. subtilis*. The *Bacillus* Sp IS4 and EW1 were found 20mm zone of inhibition while DS7 was found lowest activity. Similar results were observed by Nakamura et al.,<sup>27</sup> the antibacterial activity of violet pigment showed antibacterial activity against gram-positive bacteria such as *S. aureus*, *B. licheniformis*, *B. subtilis*, *B. megaterium*. The *Bacillus* sp. DS7 was exhibit 22 mm and *Bacillus* sp. DS3 (20mm) the prominent zone of inhibition against *B. megaterium*. The isolated *Bacillus* sp. DS3 and DS2 were found antibacterial against all tested gram positive bacteria such as *Bacillus subtilis*, *B. megaterium* and *B. cereus*, but the degree of activity were found variable towards the different bacillus sp. The twelve isolated bacillus sp were found antibacterial activity including IS4, DS7 and GW3 were exhibit the optimum antibacterial activity against the *B. cereus* while the *Bacillus* sp EW1 display lowest activity. Similar result was found to Motta et al.,<sup>24</sup> bacteriocin like substance (BLS) produced by a novel *Bacillus* sp. strain P34 isolated from the Amazon basin.

The effect of the BLS was tested against *Listeria monocytogenes*, showing a bactericidal effect, while dissimilar results were found against *Bacillus cereus*. The isolation, identification and biochemical characterization of bacteriocins produced by *Bacillus* sp., and exploration of their important use in the control of pathogenic and spoilage microorganisms. The genus *Bacillus* comprises a variety of industrially important species and has a history of safe use in both food and pharmaceutical industry<sup>30-35</sup>. The genus *Bacillus* encompasses a number of bacteriocinogenic species, such as *B. subtilis* which produces subtilin<sup>36</sup> and subtilosin<sup>37</sup>, *B. coagulans* which produces coagulin<sup>38</sup>, and *B. megaterium* which produces megacin<sup>39</sup>.

## CONCLUSION

In conclusion, antibacterial activity of Bacteriocin like inhibitory substance isolated from Lonar Lake bacterial culture. The comparative results, between various bacterial cultures, suggested that EW2, IS4, DS7, GW3, CW2, DS3 and HS4 were revealed important to the antibacterial performance with different tested bacterial cultures. In future the genomic data will be determined for the presence of genes encoding possible antibiotic activity.

## CONFLICT OF INTEREST

Conflict of interest declared none.

## REFERENCES

- Rohban R, Amoozegar MA, Ventosa A. Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. *Journal of industrial microbiology & biotechnology*. 2009 Mar 1;36(3):333-40.
- Ramos-Cormenzana A. Ecology of moderately halophilic bacteria. *The Biology of Halophilic Bacteria*. 1993:55-86.
- Ventosa A, Nieto JJ, Oren A. Biology of moderately halophilic aerobic bacteria. *Microbiology and molecular biology reviews*. 1998 Jun 1;62(2):504-44.
- Gram L, Melchiorson J, Bruhn JB. Antibacterial activity of marine culturable bacteria collected from a global sampling of ocean surface waters and surface swabs of marine organisms. *Marine biotechnology*. 2010 Aug 1;12(4):439-51.
- Tambekar DH., Dhundale VR. Isolation and characterization of Antibacterial substance produced by *Bacilli* isolated from Lonar Lake. *Int J Res Reviews Pharmacy Appl Sci*. 2012, 2(1): 41-54
- Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology*. 2005 Oct 1;3(10):777-88.
- Cleveland J, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: Safe natural antimicrobials for food preservation. *International J Food Microbiol*; 2001,71: 1–20
- Asaduzzaman SK, Sonomoto K. Lantibiotics: Diverse activities and unique modes of action. *J Biosci Bioengineering*; 2009, 107: 475–487
- Vicente M, Hodgson J, Massidda O, Tonjum T, Henriques-Normark B, Ron EZ. The fallacies of hope: will we discover new antibiotics to combat pathogenic bacteria in time? *FEMS Microbiol Rev* 2006, 30:841 – 852.
- Wagner-Döbler I, Biebl H. Environmental biology of the marine *Roseobacter* lineage. *Ann Rev Microbiol* 2006,60:255-280.
- Tambekar DH and Dhundale VR. Screening of antimicrobial potentials of haloalkaliphilic bacteria isolated from Lonar lake. *IJPCBS*. 2013,3(3), 820-825.
- Kogure K, Simidu U, Taga N. Distribution of viable marine bacteria in neritic seawater around Japan. *Can J Microbiol* 1980, 26:318 – 323.
- Bernard L, Schäfer H, Joux F, Courties C, Muyzer C, Lebaron P. Genetic diversity of total, active and culturable marine bacteria in coastal seawater. *Aquat Microb Ecol* 2000,23:1 – 11.
- Joshi AA, Kanekar PP, Kelkar AS, Shouche YS, Wani AA, Borgave SB, Sarnaik. Cultivable Bacterial Diversity of Alkaline Lonar Lake, India. *Microb Ecol*, 2007,55:163–172.
- Tambekar DH, Dhundale VR. Studies on the

- physiological and cultural diversity of *Bacilli* characterized from Lonar Lake. Biosci Discovery. 2012,3(1): 34-39.
16. Kirby MM, Baur AW, Sherris JC, Tuurck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clinical Pathol. 1966, 45:493-496.
  17. Lade HS, Chitanand MP, Kadam TA, Gyanath G. Antibiotic resistance of bacteriocin producing *Lactobacillus* species isolated from curd and vegetables waste. Bioscan Int Quarterly J Life Sci; 2007, 2(3):185-188.
  18. Tagg JR. McGiven. Assay system for bacteriocins. Appl Microbiol; 1971,21:943
  19. Canovas D, Vargas C, Calderon MI, Ventosa A, Nieto JJ. Characterization of the genes for the biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halomonas elongata* DSM 3043. System Appl Microbiol; 1998,21:487-497 .
  20. Ventosa A, Quesada E, Rodriguez-Valera F, Ruiz-Berraquero F, Ramos-Cormenzana A. Numerical taxonomy of moderately halophilic Gram-negative rods. J Gen Microbiol; 1982, 128:1959- 1968 doi:10.1099/00221287-128-9-1959.
  21. Mata JA, Martı́nez-Ca´novas J, Quesada E, Be´jar V. A detailed phenotypic characterisation of the type strains of *Halomonas* species. Syst Appl Microbiol; 2002, 25:360-375doi:10.1078/0723-2020-00122
  22. Sanchez-Porro C, Martin S, Mellado E, Ventosa A. Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. J Appl Microbiol; 2003, 94:295-300 doi:10.1046/j.1365-2672.2003.01834.x
  23. Lei Chen, Guangyu W, Tong B, Yunbin Z, Yixin W, Ming L, Xiukun Lin. Phylogenetic analysis and screening of antimicrobial and cytotoxic activities of moderately halophilic bacteria isolated from the Weihai Solar Saltern (China). World J Microbiol Biotechnol; 2010, 26:879-888 DOI 10.1007/s11274-009-0247-4.
  24. Motta AS, Cannavan FS, Tsai SM, Brandelli A. Characterization of a broad range antibacterial substance from a new *Bacillus* species isolated from Amazon basin. Archives Microbiol; 2007, 188: 367-375.
  25. Oliveira SS, Abrantes J, Cardoso M, Sordelli D, Bastos MCF. Staphylococcal strains involved in mastitis are inhibited by Staphylococcus aureus antimicrobial peptides. Lett Appl Microbiol 1998, 27:287-291
  26. Twomey DP, Wheelock AI, Flynn J, Meaney WJ, Hill C, Ross RP. Protection against Staphylococcus aureus mastitis in dairy cows using a bismuth-based teat seal containing the bacteriocin lacticin 3147. J Dairy Sci 2000, 83:1981-1988.
  27. Nakamura Y, Asada C, Sawada T. Production of Antibacterial Violet Pigment by Psychrotropic Bacterium RT102 Strain. Biotechnology and Bioprocess Engineering 2003, 8: 37-40
  28. Shanks RMQ, Dashiff A, Alster JS, Kadouri DE. Isolation and identification of a bacteriocin with antibacterial and antibiofilm activity from *Citrobacter freundii*. Arch Microbiol 2012, 194:575-587DOI 10.1007/s00203-012-0793-2
  29. Ross RP, Galvin M, McAuli V e O, Morgan SM, Ryan MP. Developing applications for lactococcal bacteriocins. Antonie Van Leeuwenhoek 1999,76:337-346
  30. Paik HD, Bae SS, Park SH, Pan JG. Identification and partial characterization of tochicin, a bacteriocin produced by *Bacillus thuringiensis* subsp *tochigiensis*. J Industrial Microbiol Biotechnol; 1997,19: 294-298.
  31. Pedersen PB, Bjørnvad ME, Rasmussen MD, Petersen JN. Cytotoxic potential of industrial strains of *Bacillus* spp. Regulatory toxicology pharmacol; 2002,36: 155-161
  32. Bizani D, Dominguez APM, Brandelli A. Purification and partial chemical characterization of the antimicrobial peptide cerein 8A. Letters Appl Microbiol ; 2005,41: 269-273.
  33. Motta AS, Flores FS, Souto AA, Brandelli A. Antibacterial activity of a bacteriocin-like substance produced by *Bacillus* sp. P34 that targets the bacterial cell envelope. Antonie van Leeuwenhoek 2008 93:275-284. DOI 10.1007/s10482-007-9202-2
  34. Hong HA, Huang JM, Khaneka R, Hiep LU, Urdaci MC, Cutting SM. The safety of *Bacillus subtilis* and *Bacillus* in food safety indicus as food probiotics. J Appl Microbiol; 2008, 105: 510-520.
  35. Vaucher RA, Teixeira ML, Brandelli A. Investigation of the cytotoxicity of antimicrobial peptide P40 on eukaryotic cells. Curr Microbiol; 2010, 60(1):1-5.
  36. Jansen EF, Hirschmann DJ. Subtilin, an antibacterial substance of *Bacillus subtilis*. Culturing condition and properties. Archives Biochemistry; 1944, 4: 297-309
  37. Zheng G, Slavik MF. Isolation, partial purification and characterization of a bacteriocin produced by a newly isolated *Bacillus subtilis* strain. Letters Applied Microbiol 1999,28(5): 363-367
  38. Hyronimus B., Marrec C., Urdaci MC. Coagulin, a bacteriocin-like inhibitory substance produced by *Bacillus coagulans* I4 J Appl Microbiol; 1998, 85: 42-50
  39. Von Tersch MA. Carlton BC. Bacteriocin from *Bacillus megaterium* ATCC 19213: comparative studies with megacin A-216. J Bacteriol, 1983,155(2):866-871

## Reviewers of this article

### **Dr.Kapil Kample**

Associate professor  
Department of microbiology  
Amravathi university  
Amravati, Maharashtra 444602



**Prof.Dr.K.Suriaprabha**

Asst. Editor , International Journal  
of Pharma and Bio sciences.

### **Asso.Prof.Dr. R. Usha, MSc, M.Phil, Ph.D.**

Associate Professor, Department of  
Microbiology,  
Karpagam University, Eachanari (PO),  
Coimbatore - 641 021, Tamil Nadu, India.



**Prof.P.Muthuprasanna**

Managing Editor , International  
Journal of Pharma and Bio sciences.

We sincerely thank the above reviewers for peer reviewing the manuscript