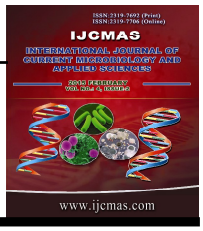
Int.J.Curr.Microbiol.App.Sci (2015) 4(2): 506-512

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 2 (2015) pp. 506-512**
http://www.ijcmas.com



Original Research Article

Antimicrobial Potentials of Phospholipid Compound Produced By Halophilic *Bacillus subtilis* isolated from Alkaline Meteorite Crater Lonar Lake, India

V.A.Shinde*, S.M.More and S.B.More

Department of Microbiology, Yeshwant Mahavidyalaya, Nanded- 431602, (M. S.), India
**Corresponding author*

Keywords

Phospholipid antibiotic, Thin layer chromatography, Antimicrobial compound

A B S T R A C T

In present study antibiotic producing ability of the halophilic *Bacillus subtilis* is screened and the activity of the antimicrobial compound was determined against different microorganisms like, *Staphylococcus aureus*, *E. coli*, *P. aeruginosa*, *Candida tropicalis*, *Candida parapsilosis*. The halophilic *Bacillus subtilis* isolates were isolated from Alkaline Meteorite Crater Lake Lonar situated in district Buldhana, India and were subjected to primary screening for antimicrobial compound production. The organisms were enriched in Alkaline NG medium which supports the production of phospholipid antibiotic and partial purification was done using thin layer chromatography. The partially purified extract of antimicrobial phospholipid compound was tested against different microorganisms and seen that halophilic *Bacillus subtilis* isolated from Lonar Lake are having antibacterial activity against different microorganisms.

Introduction

Hypersaline environments are widely distributed on the earth's continent where they exist either as natural water bodies such as permanent saline lakes, ephemeral saltpans and salt marshes,or as artificial solar salterns (Litchfield and Gillevet; 2002). The microbial diversity of saline lakes has been studied primarily by focusing on the isolation and characterization of individual organisms with potential industrial application (Horikoshi K., 1999; Jones et al, 1998). Microorganisms that thrive in these environments have been broadly classified into halophilic microorganism (that is, require salt for their viability) and halotolerant microorganisms which are able to grow in the absence as well as in the presence of NaCl. Halophiles can be further divided into slight halophiles that grow optimally in 3% (w/v) total salt, moderate halophiles optimal growth at 3-15% (w/v) salt and extreme halophiles that grow optimally at 25% (w/v) salt (Ventosa et al, 1998). The domain bacteria typically contains many types of halophilic and halotolerant microorganisms that spread over a large number of phylogenetic subgroups. Most of these are moderate rather than extreme halophiles (Oren, 2002). Scientific interest in extremophilic microorganisms, especially

hyperthermophiles, thermoacidophiles, archaebacterial anaerobes, and hyperhalophiles, has recently increased. One reason for this interest is the need to understand the biochemical mechanisms involved under extreme conditions because of possible biotechnological use of enzymes and antibiotics from such organisms (Ollivier et al, 1994). Search for new antibiotics effective against multi-drug resistant pathogenic bacteria is presently an important area of antibiotic research (Hamaki et al, 2005). One strategy for enhancing the likelihood of obtaining novel antibiotic compounds and other secondary metabolites is to analyze uncommon eco-systems which exist under extreme conditions (Okami and Hotta, 1988).

The word "antibiotic" is derived from Greek term antibiosis, which literally means "against life". It can be purified from microbial fermentation and modified chemically or enzymatically for either chemical use or for fundamental studies (Robbers et al., 1996; De Mondena et al., 1993). Antibiotics that are currently of greatest use have been derived from a relatively small group of microorganisms belonging to the genera *Penicillium, Streptomyces, Cephalosporium, Micromonospora* and *Bacillus* (Zinsser et al., 1988). More than 5000 different antibiotics have been isolated from cultures of bacteria, fungi and plant cells, 60% of them are contributed by the genus *Streptomyces* (Todar, 2002; Claus & Blackwill, 1989). Many species of *Bacillus* including *B. cereus, B. subtilis, B. mycoides* are known to suppress several fungal pathogens growth such as *Rhizoctonia, Sclerotinia, Fusarium, Gaeummanomyces, Nectria, Pythium* and *Phytophthora* (Cook and Baker, 1983; McKnight, 1993; Fiddman and Rossall, 1994). Bacilli exhibit an array of physiologic abilities that allow them to live in a wide range of habitats, including many extreme habitats such as desert sands, hot springs, and Arctic soils. Species in the genus *Bacillus* can be thermophilic, psychrophilic, acidophilic, alkaliphilic, halotolerant or halophilic and are capable at growing at pH values, temperatures, and salt concentrations where few other organisms can survive (Awais et al; 2007). Most of the peptide antibiotics produced bybacilli are active against gram-positive bacteria; however, compounds such as polymyxin, colistin and circulin exhibit activity almost exclusively upon gram-negative bacteria, whereas bacillomycin, mycobacillin and fungistatin are effective against molds and yeasts (Katz & Demain, 1977). A series of 167 peptide antibiotics have been recently isolated from well-known *B. subtilis* strains (Berdy, 1974). This study is taken with the objective of isolation of halophilic *Bacillus subtilis* from the soil and to assess the antimicrobial effect of antimicrobial compound produced and activity was tested against test organisms (*E.coli, Pseudomonas aeruginosa, Candida tropicalis, Staphylococcus aureus*).

Material and Methods

Collection of water sample: Soil sample was collected from Lonar lake (Narayan et al, 2008; Abou-Shanab, 2007). Temperature and pH of Lonar lake was recorded. Same sample was used for further study.

Media: Nutrient agar and broth was used for isolation of *B. subtilis* and production of antimicrobial compound. Same media having pH 10 with 15% NaCl was used for cultivation of bacteria from Soil sample and was incubated at 30^0C for 48 hrs.

Isolation and identification of bacteria: Nutrient agar having pH 10 with 15% NaCl was used for isolation of *Bacillus* species. Colonies showing characteristic feature were selected and confirmed by colony character

and biochemical test. These strains were selected for further study. Bergey's manual of systematic bacteriology 9th edition was followed for confirmation (Bergey et al, 1994).

Inoculum: Bacterial suspension was prepared by adding 10 ml sterile water to a 4- day- old slant culture and 5 ml of this was used as inoculum in all experiments unless and otherwise stated. In each case the bacterial suspension was standardized to have 0.5 O. D. at A600 (McFarland Standards). All experiments were conducted in the triplicates.

Production of phospholipid antimicrobial compound: *Bacillus subtilis* was grown in NG medium adjusted to pH 10 (containing 10 gm (Gram) Nutrient broth; 10 gm Glucose; 15% Sodium chloride; 5 mg (miligram) of $CuSO_4.5H_2O$; 7.5 mg of $FeSO_4.7H_2O$; 3.6 gm of $MnSO_4.5H_2O$; 15 mg of CaCl2.2H2O; and 9 mg of $ZnSO_4.7H_2O$; (per liter)) supplemented with 50 µg for tryptophan per ml at 30^oC for 24 hours (Hosoya et al, 1998). 10% of this inoculum was reinoculated in fresh NG medium adjusted to pH 10 with 15% NaCl and incubated under shaking at 30^0C for 72 hours. Then this production medium was centrifugation at 10000 rpm for 10 minutes, (Using Cooling centrifuge, REMI) cells were collected.

This cellular contents were extracted three times using 10ml of 50% n- butanol each time, then aqueous layer was collected and evaporated to concentrate at room temperature (Tamehiro et al, 2002). Resulting crude extract was resuspended in 4 ml of Methanol; this crude sample was again extracted with ethyl acetate. The resulting crude extract was used for further purification. Purification was carried out using method for lipid extraction (Bligh and Dyer, 1959).

Bioassay of phospholipid antimicrobial compound: The crude extract of antimicrobial compound with ethyl acetate was used for bioassay. The 24 hours old cultures of test organisms, *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa, Candida tropicalis*, and *Candida parapsilosis* were streaked on sterile Muller-Hinton (MH) agar with sterile swabs. Then wells were made on MH agar. The wells were filled with 100 µl of crude extract of phospholipid compound. The plates were kept in refrigerator for diffusion of compound. The plates were then incubated at 35 ± 0.5^0C for 24 hours and then diameter of zone of inhibition was noted.

Purification of phospholipid antimicrobial compound: The crude extract was taken 1 ml and to it 3.75 ml 1:2 (v/v) $CHCl_3$: Methanol was added and vortex well. Finally 1.25 ml distilled water was added and mix well and then centrifuged at 1000 rpm for 5 minutes at room temperature, to give two phase system. The bottom phase was removed and then TLC (Thin Layer Chromatography) was performed using silica gel. The plates were spotted with the bottom phase and plates were developed with $CHCl_3$: Methanol: water (65:25:04 v/v). The phospholipid spots were located on chromatogram by placing the plates in iodine chamber to treat with iodine vapour. After locating spot of Phospholipid antimicrobial compound it is removed and extracted with $CHCl_3$: Methanol (Bligh and Dyer, 1959).

Antimicrobial activity of purified phospholipid antimicrobial compound: The extracted purified phospholipid antimicrobial compound was filled in well prepared in MH agar plates inoculated with *Staphylococcus aureus* and incubated at 35 ± 0.5^oC for 24 hours. After incubation diameter of zone of inhibition was recorded.

Result and Discussion

The alkaline Lonar lake a unique basaltic rock meteorite impact crater, ranking third in the world (Latitude 19°58′ and Longitude 76°36′). Lonar crater is filled with saline water. The uniqueness of the lake water is its salinity and high alkalinity. A review of literature revealed that its salinity was 40.78, 31.52 and 30.87 in 1910, 1958 and 1960, respectively. The salinity of lake is now lowered down to 7.9% (Malu et al, 2000). The temperature and pH of Lonar lake was recorded.

The rhizobacterium *Bacillus subtilis* is an endospore forming bacteria. Several hundred wild-type B. subtilis strains have been collected, with the potential to produce more than two dozen antibiotics with an amazing variety of structures. *Bacillus subtilis* also produces several other antibiotics: subtilin, a 32-residue peptide; bacilysin, a dipeptide; subsporins A–C, lipooligopeptides; and rhizocticins A–D, phosphooligopeptides (Priest, 1992; DeFuria & Claridge, 1976).

The present research work was carried out to optimize the conditions for the production of bioactive microbial metabolites by *Bacillus subtilis*. Twenty five isolates were isolated and identified according to Bergey's Manual of systematic bacteriology (Bergey et al, 1994). These isolates were screened for antimicrobial activity of crude phospholipid and three of these halophilic isolates showed activity against a variety of organisms like, *Staphylococcus aureus; E. coli, Pseudomonas aeruginosa, Candida tropicalis,* and *Candida parapsilosis* but they exhibited better activity against gram-positive *Staphylococcus aureus;* Gram negative *E. coli, Pseudomonas aeruginosa,* and *Candida parapsilosis* and weaker activity against *Candida tropicalis*. Therefore, the three isolates were used for phospholipid antimicrobial compound production and the purified phospholipid was tested against *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa*, and *Candida parapsilosis* (Table-1). In the search for antibiotics produced by *Bacillus species*, especially *Bacillus cereus, Bacillus subtilis* and *Bacillus licheniformis*, several antifungal compounds, mainly peptides, have also been described (Katz & Demain, 1977; Kugler et al., 1990; Lebbadi et al., 1994; Silo-Suh et al., 1994). Some researchers have reported a compound produced by *Bacillus* pumilus (MSH) that inhibits Mucoraceae and Aspergillus species. Also different scientists have reported inhibition of various organisms (Bottone & Peluso, 2003). Some researchers isolated a strain of *Bacillus subtilis* C126 from sugar cane fermentation, which produced a polypeptide antibiotic, bacitracin, which inhibited the growth of Micrococcus flavus (Marahiel et al., 1997). A *Bacillus* licheniformis strain, 189, isolated from a hot spring environment in the Azores, Portugal, was found to strongly inhibit growth of Gram-positive bacteria by producing peptide antibiotic (Mendo et al., 2004).

In present study, the purified compound was further purified by using thin layer chromatography. The spot was detected and the compound was fractionated. The antimicrobial activity of the collected fraction was determined against most sensitive organism *Staphylococcus aureus* at 10^8cells/ ml (Table-2 and Graph-1). Multiplicity of antibiotic production obviously complicates attempts to identify antibiotics in unfractionated material. However, even in whole cultures similarities or differences between known and unidentified antibiotics may be noted by means of chromatography (Snell et al., 1955).

Table.1 Screening of Bacterial Isolates for phospholipid antimicrobial compound

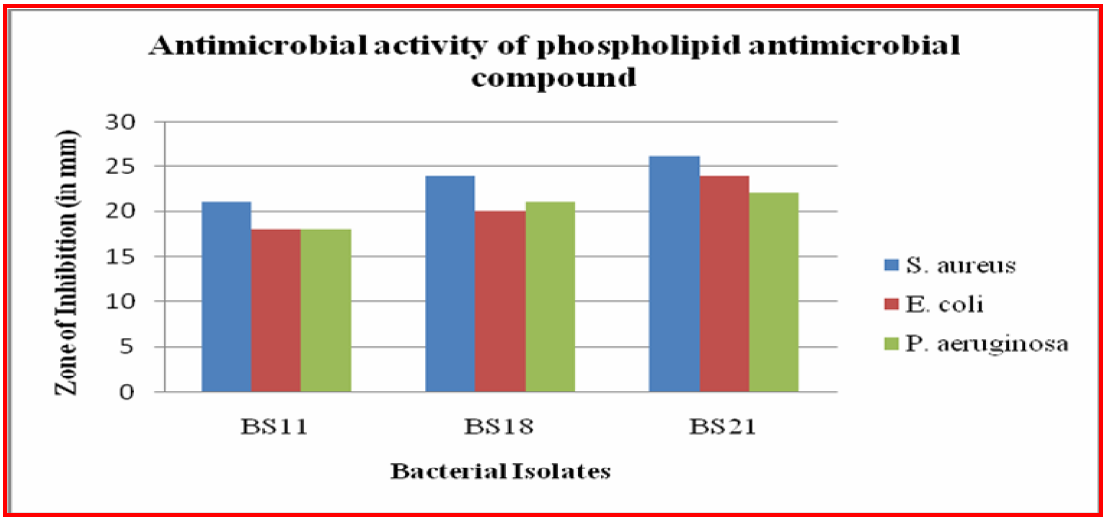
Bacterial Isolates	Antimicrobial activity of phospholipid antimicrobial compound				
	Staphylococcus aureus	*E. coli*	*Pseudomonas aeruginosa*	*Candida parapsilosis*	*Candida tropicalis*
BS11	++	++	++	++	-
BS18	++	++	++	++	-
BS21	++	++	++	++	-

Strong activity = ++; Weak activity = –

Table.2 Antimicrobial activity of phospholipid antimicrobial compound

Bacterial Isolates	Antimicrobial activity of phospholipid antimicrobial compound (in mm)		
	Staphylococcus aureus	*E. coli*	*Pseudomonas aeruginosa*
BS11	21	18	18
BS18	24	20	21
BS21	26	24	22

Graph.1 Antimicrobial activity of phospholipid antimicrobial compound



References

Abou-Shanab R.A.I. 2007. Characterization and 16S rDNA identification of thermo-tolerant bacteria isolatedfrom hot spring, J. appl. Sci. Res., 3,994-1000.

Berdy, J., 1974. Recent developments of antibiotic research and classification of antibiotics according to chemical structure. Adv. Appl. Microbiol., 18: 309-406.

Bergey D.H., Holt J.G., Krieg N.R. and Sneath P.H.A. 1994. Bergey's Mannual of Determinative Bacteriology, 9th ed., (Breed RS, Murray EGD and Smith NR, eds.) Williams and Wilkins, Baltimore.

Bligh E.G. and Dyer W.J. 1959. A rapid method for total lipid extraction and purification, Can.J.Biochem.Physiol., 37: 911-917.

Bottone, E.J. and R.W. Peluso. 2003. Production by *Bacillus* pumilus(MSH) of an antifungal compound that is active against Mucoraceae and Aspergillusspecies: preliminary report. J. Med. Microbiol., 52: 69-74.

Claus, G.W. and D. Balckwill. 1989. Antibiotic Evaluation by Kirby-Bauer Method. In: Understanding Microbes: A Laboratory Textbook for Microbiology, pp. 405. U.S.A.

Cook R.J., Baker K.F. 1983. The Nature and Practice of Biological Control of PlantPathogens. APS Press, St. Paul, Minnesota.

De Mondena, J.A., S.A.J. Guttierrez, R.A Falchini, J.L. Gallazo, D.E. Hughes,J.E. Bailey and J.F. Martin. 1993. Intracellular expression of vitreoscilla haemoglobin improves cephalosporin C production by Acremonium chrysogenum. Biotech., 11: 926-929.

Defuria, M.D. and C.A. Claridge. 1976. Aminoglycoside antibiotics produced by the genus *Bacillus*. In:Microbiology. (Ed.): M. Schlessinger, pp. 421-436. American Society of Microbiology, Washington, D.C.

Fiddman P.J., Rossall S. 1994. Effects of substrate on the production of antifungal volatiles by *Bacillus subtilis*. J. Appl. Bacteriol., 76: 395-405.

Hamaki T, Suzuki M, Fudou R, Jojima Y, Kajiura T, Tabuchi A, Sen K, Shibai H. 2005. Isolation of novel bacteria and actinomycetes using soil extract agar medium. J. Biosci. Bioengineer. 99: 485-92.

Horikoshi K. 1999. Alkaliphiles: Some applications of their products for biotechnology, Microbiol. Mol. Biol. Rev., 63: 735–750.

Hosoya Y., Okamoto S., Muramatsu H. and Ochi K. 1998. Acquisition of certain streptomycin-resistant (str) mutations enhances antibiotic production in bacteria, Antimicrob. Agents. Chemother., 42: 2041–2047.

Jones B.E., Grant W.D., Duckworth A.W. and Owenson G.G. 1998. Microbial diversity of soda lakes, Extremophiles 2: 191–200.

Katz, E. and A.L. Demain 1977. The peptide antibiotics of *Bacillus*; chemistry, biogenesis, and possible functions. Bacteriol. Rev., 41: 449-474.

Kugler, M., W. Loeffler, C. Rapp, A. Kern and A.G. Jung. 1990. Rhizocticin A, an antifungal phosphono-oligopeptide of *Bacillus subtilis*ATCC 6633: biological properties. Arch. Microbiol., 153: 276-281.

Lebbadi, M., A. Galvez, M. Maqueda, M. Martinez-Bueno and E. Valihuia. 1994. Fungicin M4: a narrow spectrum peptide antibiotic from *Bacillus* licheniformisM-4. J. Appl. Bacteriol., 77: 49-53.

Litchfield CD, Gillevet M P. 2002. Microbial Diversity And Complexity In Hypersaline Environments: A Preliminary Assessment. Ind. Microbiol.Biotechnol 28: 48-55.

Malu R.A., Dhabhade D.S. and Kodarkar M.S. 2000. Diversity in Lonar lake, J. Aquat. Bio., 15: 16–18.

Maraheil, M.A., T. Stachelhaus and H.D. Mootz. 1997. Modular peptide synthetases involved in non-ribosomal pepetide syhtesis. Chem. Rev., 97: 2651-2673.

McKnight S.E. 1993. Effects of *Bacillus subtilis*on Cotton Seedling Development: University of Nottingham, PhD Thesis, Nottingham.

Mendo, S., N.A. Faustino, A.C. Sarmento, F. Amado and A.J. Moir. 2004. Purification and characterization of a new peptide antibiotic produced by a thermotolerant *Bacillus* licheniformisstrain. Biotechnol. Lett., 26: 115.

Muhammad Awais, Aamer Ali Shah, Abdul Hameed and Fariha Hasan. 2007. Isolation, Identification and Optimization of Bacitracin Produced By *Bacillus* species. Pak. J. Bot., 39(4): 1303-1312.

Narayan V.V., Hatha M.A., Morgan H.W. and Rao D. 2008 Isolation and characterization of aerobic thermophilic bacteria from the Savusavu Hot spring in Fiji, Microbes Environ., 23: 350-352.

Okami Y, Hotta K. 1988 Search and discovery of new antibiotics. In: Goodfellow M, Williams ST, Mordarski M. (eds.) Actinomycetes in Biotechnology. Academic Press, London, pp. 41.

Ollivier B, Caumette P, Garcia J., Mah R. 1994. Anaerobic Bacteria From Hypersaline Environments. American Society For Microbiology 58: 1.

Oren A. 2002. Diversity Of Halophilic Microorganisms: Environments, Phylogeny, Physiology And Applications. Ind. Microbiol Biotechnol 28: 56-63.

Priest, F.G. 1992. Biology of Bacilli. In: Bacilli: applications to industry. (Eds.): R.H. Doi and M. Mcgloughlin. pp. 293- 320. Butterworth-Heinemann, Boston, Mass.

Robbers, E. J., S. K. Marilyn and T. E. Varro. 1996. Antibiotics. In: Pharmacognosy and Pharmacobiotechnology, University School of Pharmacy and pharmaceutical Sciences, pp. 219- 220. West Lafayette, Indiana.

Silo-Suh, L.A., B.J. Lethbridge, S.J. Raffel, J. Clardy and J. Handelsman. 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus* cereus UW 85. Appl. Environ. Microbiol., 60: 2023-2030.

Snell, N., K. Ijichi and J.C. Lewis. 1955. Paper Chromatographic Identification of Polypeptide Gram Positive Inhibiting Antibiotics. Western Utilization Research Branch, U.S Department of Agriculture, Albany, California.

Tamehiro N., Okamoto-Hosoya Y., Okamoto S., Ubukata M., Hamada M., Naganawa H. and Ochi K. 2002. Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168, Antimicrob. Agents Chemother., 46: 315– 320.

Todar, K. 2002. Antimicrobial Agents used in the treatment of infectious disease. Department of Bacteriology University of Wisconsin-Madison.

Ventosa A, Joaquin JN, Oren A. 1998. Biology of Moderately Halophilic Aerobic Bacteria. Microbiol Mol Rev 62: 504-544.

Zinsser, H. 1988. Antimicrobial Agents. In: Zinsser Microbiology. (Eds.): H. Zinsser, W.K. Joklik, H.P. Willett, D.B. Amos and C. Wilfert. pp. 128-160. Prentice Hall International, UK.