ANTIMICROBIAL POTENTIALS OF PHOSPHOLIPID COMPOUND PRODUCED BY EXTREMOPHILIC BACTERIA ISOLATED FROM LONAR LAKE, INDIA

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Abstract: In present study antibiotic producing ability of the halophilic and alkaliphilic *Bacillus subtilis* were compared by screening the activity of the antimicrobial compound was determined against different microorganisms like, *Staphylococcus aureus, E. coli, P. aeruginosa, Candida tropicalis, Candida parapsilosis*. The halophilic and alkaliphilic *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 and Halophilic NG medium which supports the production of phospholipid antibiotic by respective organism and partial purification was done using thin layer chromatography. The partially purified extract of antimicrobial phospholipid compound from both the organisms were tested against different microorganisms and seen that halophilic *Bacillus subtilis* showed less antimicrobial activity than alkalophilic *Bacillus subtilis* isolated from Lonar Lake which are having moderate antibacterial activity against different microorganisms.

Keywords: **Halophiles, Alkalophiles, Phospholipid antibiotic, Thin layer chromatography, Antimicrobial Compound.**

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 [1]. The microbial diversity of saline lakes has been studied primarily by focusing on the isolation and characterization ofindividual organisms with potential industrial application [2, 3]. The spread of resistance to antibiotics undermines the therapeutic utility of Antiinfective drugs in current clinical use [4]. For example, *Staphylococcus aureus*, a major cause of community and hospital acquired infections, has developed resistance to most classes of antibiotics, and isolates exhibiting such resistance is drawing great concern. Methicillin - resistant *Staph. aureus* (MRSA) strains appeared in the hospital environment after introduction of the semi-synthetic Penicillin, Methicillin, leaving Vancomycin as the last line of defense for MRSA treatment [5] Search for new antibiotics effective against multi-drug resistant pathogenic bacteria is presently an important area of antibiotic research [6]. One strategy for enhancing the likelihood of obtaining novel antibiotic compounds and other secondary metabolites is to analyze uncommon ecosystems which exist under extreme conditions [7]. A series of 167 peptide antibiotics have been recently isolated from well-known *B. subtilis* strains [8]. The gram positive bacterium *Bacillus subtilis* produces a large number of antibiotics, which are classified as ribosomal or non-ribosomal. The non-ribosomal antibiotics may play a role in competition with other microorganisms during spore germination [9] The phospholipid antibiotic produced by *Bacillus subtilis* has the broad spectrum activity against gram positive and gram negative bacteria. This study is taken with the objective of isolation of halophilic and alkaliphilic *Bacillus subtilis* from the soil and comparative study of antimicrobial potentials of phospholipid compound produced by halophilic and alkaliphilic *Bacillus subtilis* 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 [10, 11]. 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. Nutrient agar having pH 10 with 15% NaCl was used for cultivation of halophilic bacteria and Nutrient agar having pH 10 was used for cultivation of alkaliphilic bacteria from Soil sample and was incubated at 30^{o}C for 48 hrs.

Isolation and identification of bacteria: Nutrient agar having pH 10 with 15% NaCl & Nutrient agar having pH 10 were used for isolation of halophilic and alkaliphilic *Bacillus* species respectively. Colonies showing characteristic feature were selected and confirmed by colony character and biochemical test. These strains were selected for further study. Bergeys manual of systematic bacteriology 9th edition was followed for confirmation [12].

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 A_{600} (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 $CaCl_2.2H_2O$; and 9 mg of $ZnSO_4.7H_2O$; (per liter)) supplemented with 50 µg for tryptophan per ml at 30°C for 24 hours [13]. 10% of this inoculum was reinoculated in fresh NG medium adjusted to pH 10 & fresh NG medium adjusted to pH 10 with 15% NaCl and incubated under shaking at 30°C for 72 Hours for alkaliphilic and halophilic *Bacillus subtilis* respectively. Then these production medium were 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 each aqueous layer were collected and evaporated to concentrate at room temperature [14]. Resulting crude extract were resuspended in 4 ml of Methanol; this crude sample were again extracted with ethyl acetate. The resulting crude extract were used for further purification. Purification was carried out using method for lipid extraction [15].

Bioassay of phospholipid antimicrobial compound: The crude extract of antimicrobial compound with ethyl acetate from halophilic and alkalophilic *Bacillus subtilis* were 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 extracts of phospholipid compound. The plates were kept in refrigerator for diffusion of compound. The plates were then incubated at 35±0.5 o C for 24 hours and then diameter of zone of inhibition was noted.

Purification of phospholipid antimicrobial compound: The crude extracts were 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 spots of Phospholipid antimicrobial compound from halophilic and alkalophilic *Bacillus subtilis*, the spots were removed and extracted with $CHCl_3$: Methanol [15].

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 ° C for 24 hours. After incubation diameter of zone of inhibition was recorded.

Result and discussion: The present research work was carried out to optimize the conditions for the production of bioactive microbial metabolites by *Bacillus subtilis*. Twenty five isolates from were isolated from halophilic and alkalophilic culture medium and identified according to Bergeys Manual of systematic bacteriology [12]. These isolates were screened for antimicrobial activity of crude phospholipid and three isolates from the halophilic and four isolates from the alkalophilic isolates showed activity against a variety of organisms like, *Staphylococcus aureus; E. coli, Pseudomonas aeruginosa, Candida tropicalis*, and *Candida parapsilosis* but Halophilic isolates exhibited better activity against gram positive *Staphylococcus aureus*; Gram negative *E. coli, Pseudomonas aeruginosa*, and *Candida parapsilosis* and weaker activity against *Candida tropicalis* and alkaliphilic isolates showed moderate activity against gram positive *Staphylococcus aureus*; Gram negative *E. coli*, and weaker activity against *Pseudomonas aeruginosa, Candida parapsilosis* and *Candida tropicalis*. Therefore, the isolates selected 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).

Table.1 Comparative Screening of Bacterial Isolates for phospholipid antimicrobial compound

Bacterial Isolates	Antimicrobial activity of phospholipid antimicrobial compound			
	SA	EC.	PA	CP
Halophilic Isolates				
BS 11	++	++	++	++
BS 18	++	++	++	++
BS 21	++	++	++	++
Alkaliphilic Isolates				
BS1	++	++	+	+
BS 2	++	++	+	+
BS 7	++	++	+	+
BS 8	++	++	+	+

SA-*S. Aureus*; EC-*E. coli*; PA-*P. aeruginosa* and CP-*Candida parapsilosis*

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 [16]. 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 [17]. 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* [18]. 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 [19].

In present study, the purified compound from halophilic and alkaliphilic isolates were 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 10^8 cells/ ml (Table-2).

Table.2 Comparative study of Antimicrobial activity of phospholipid antimicrobial compound produced by Halophilic and Alkaliphilic isolates.

Bacterial Isolates	Antimicrobial activity of phospholipid antimicrobial compound (in mm)			
	SA	EC.	PA	CP
Halophilic Isolates				
BS 11	25	22	23	10
BS 18	24	21	22	11
BS 21	26	21	22	10
Alkaliphilic Isolates				
BS1	21	18	15	11
BS 2	22	19	15	10
BS 7	21	18	11	10
BS 8	23	18	11	11

SA-*S. Aureus*; EC-*E. coli*; PA-*P. aeruginosa* and CP-*Candida parapsilosis*

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 [20].

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