



Antimicrobial Activity of Phospholipid Compound Produced by Acidophilic *Bacillus subtilis* Isolated from Lonar Lake, Buldhana, India

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Abstract

Bacillus subtilis is an endospore forming rhizobacteria; produces several antibiotics with amazing structural variety viz. subtilisin, bacitracin, difficidin, fengycin, mersacidin, bacilysin and iturin. These antimicrobial compounds are effective against both gram positive and gram negative bacteria. *Bacillus subtilis* has ability to grow in extreme environments like alkaliphilic, acidophilic, acidophilic. These environmental conditions induce microorganisms to produce varied kinds of antimicrobials which have applications in chemotherapy. Therefore in the present study Acidophilic *Bacillus subtilis* (*B. subtilis*) strains were isolated from soil samples of Lonar lake and screened for production of phospholipid antibiotic. The purified phospholipid antibiotic showed broad spectrum activity against the test organisms i.e. *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Candida parapsilosis* (*C. parapsilosis*); of these test organisms *Staphylococcus aureus* showed higher sensitivity towards the phospholipids antimicrobial compound.

Keywords: *Bacillus subtilis*, acidophilic, phospholipid, bioactive compound.

Introduction

Soda lakes are the most stable and productive naturally occurring alkaline environments in the world, with pH values generally higher than 10 and occasionally reaching 12¹. These alkaline environments are caused by a combination of geological, geographical and climatic conditions. They are characterized by large amounts of sodium carbonate formed by evaporative concentration². The microbes present in such alkaline saline environments play an important role in the remineralization of organic matter within the ecosystem. They are the major contributors in the transformation of organic carbon, sulfur, nitrogenous compounds and metals with an important role in food webs and nutrient cycling. The ecology and diversity of an East African Soda Lake was studied and extensively reviewed for their biotechnological potential³. The microbial diversity of saline lakes has been studied primarily by focusing on the isolation and characterization of individual organisms with potential industrial application^{4,1}. However, there is meager data on the bacterial diversity of Lonar lake. Eutrophication and presence of blue green algae in Lonar lake have been described⁵. Some workers⁶ have studied the alkaline metalloprotease from alkaline *Streptomyces* isolated from Lonar lake silt sample. Bioremediation of phenol-using alkaliphilic bacteria isolated from Lonar lake sediments was an interesting finding⁷. A preliminary account of bacterial diversity of the Lonar Lake ecosystem has been reported⁸, which includes some of the biochemically identified isolates. Applied culture dependent phenotypic characterization and 16S rDNA-based phylogenetic analyses were applied to study aerobic, cultivable bacterial populations present in the alkaline Lonar Lake. The

isolates were further studied for their biotechnological potential⁷.

The spread of resistance to antibiotics undermines the therapeutic utility of anti-infective drugs in current clinical use⁹. 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 *S. 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 treatment¹⁰. With the appearance of vancomycin-resistant clinical isolates¹¹, no antibiotic class is effective against multiresistant *S. aureus* infections. Thus, new antibiotic and therapy options are urgently needed to improve the management of bacterial infections¹², and a major challenge is to find drugs that act against Methicillin-resistant *S. aureus* (MRSA).

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 germination¹³. The high proportion of antimicrobial compounds producing strains may be associated with ecological role, playing a defensive action to strains into an established microbial community¹⁴. It has been very recently shown that the biosynthesis of difficidin and bacillaene in *B. subtilis* A1/3 is dependent on a Sfp-homologous Ppan transferase¹⁵. A series of new antibiotics have been recently isolated from well-known *B. subtilis* strains. These include bacilysin, an anti-microbial

phospholipid that can be isolated from *B. subtilis* 168 cells by extraction with butanol¹⁶. Most probably bacilysocin is derived from the major *B. subtilis* phospholipid phosphatidylglycerol through YtpA-catalysed acyl ester hydrolysis¹⁶. Amicoumacins are produced by several *B. subtilis* strains excluding the 168 strain¹⁷. Their anti-bacterial and anti-inflammatory activities, as well as their action on *Helicobacter pylori* make the amicoumacins attractive for the treatment of chronic gastritis and peptic ulcer in humans¹⁸.

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 acidophilic *Bacillus subtilis* from the soil and to assess the antimicrobial effect of phospholipid compound produced and activity was tested against test organisms (*E. coli*, *Pseudomonas aeruginosa*, *Candida tropicalis*, and *Staphylococcus aureus*).

Methodology

Collection of water sample: Soil sample was collected from Lonar lake^{19,20}. 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 4 was used for cultivation of bacteria from Soil sample and was incubated at 30°C for 48 hrs.

Isolation and identification of bacteria: Nutrient agar having pH 4 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 confirmation²¹.

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 triplicate and results are presented.

Production of Phospholipid antimicrobial compound: *Bacillus subtilis* was grown in NG medium adjusted to pH 4 (containing 10 gm (Gram) Nutrient broth; 10 gm Glucose; 2 gm Sodium chloride; 5 mg (miligram) of CuSO₄.5H₂O; 7.5 mg of FeSO₄.7H₂O; 3.6 gm of MnSO₄.5H₂O; 15 mg of CaCl₂.2H₂O; and 9 mg of ZnSO₄.7H₂O; (per liter)) supplemented with 50 µg for tryptophan per ml at 30°C for 24 hours²².

10% of this inoculum was reinoculated in fresh NG medium adjusted to pH 4 and incubated under shaking at 30°C 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 temperature¹⁶. 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 extraction²³.

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°C 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) CHCl₃: 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 CHCl₃: 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 CHCl₃: Methanol.

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.

Results 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%²⁴. The temperature and pH of Lonar lake was recorded. Twenty isolates were isolated and identified on the basis of biochemical characteristics (i.e. Catalase, Anaerobic growth Voges-Proskauer, Citrate utilization, growth at 55°C are Positive and egg yolk Lecithinase utilization negative; Fermentation of Glucose, Xylose Arabinose, Mannitol) and

named as BS 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 respectively²⁵. These isolates were screened for production of phospholipid antimicrobial compound and it was found that BS 14, 15, 16, 17 produced highest quantity of Antimicrobial compound, so these strains were subjected to production of antimicrobial compound.

The number of antibiotics produced by members of the genus *Bacillus* was 167. Of this total, 66 different peptide antibiotics are elaborated by strains of *Bacillus subtilis* and 23 are products of *Bacillus brevis*²⁹. Therefore in the present study, These three Acidophilic isolates were screened for antimicrobial activity of crude phospholipid and three of these acidophilic isolates showed activity against *Staphylococcus aureus*; *E. coli*, *Pseudomonas aeruginosa*, *Candida tropicalis*, and *Candida parapsilosis*. *Bacillus subtilis* 15, 16, 17 (acidophilic), showed good antimicrobial activity against *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, and *Candida parapsilosis* and weaker activity against *Candida tropicalis*. *Bacillus subtilis* 14 showed weak antimicrobial activity against all test organisms (i.e. *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Candida tropicalis*, and *Candida parapsilosis*) 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 and figure-1).

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 10^8 cells/ ml (table-2 and figure-2).

Table-1
Antimicrobial activity of Phospholipid antimicrobial compound

Bacterial Isolates	Antimicrobial activity of Phospholipid compound			
	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. parapsilosis</i>
BS15	26	14	16	15
BS16	30	21	28	18
BS17	28	18	15	20

Table-2
Antimicrobial activity of Phospholipid compound against *Staphylococcus aureus*

Sr. No.	Bacterial Isolate	Diameter of Zone of Inhibition (in mm)
1	BS15	24
2	BS16	34
3	BS17	28

The Phospholipid antimicrobial compound produced and purified should be further analyzed by using NMR and GC-MS to elucidate the detail structure that is helpful to conclude structure- antimicrobial activity relationship.

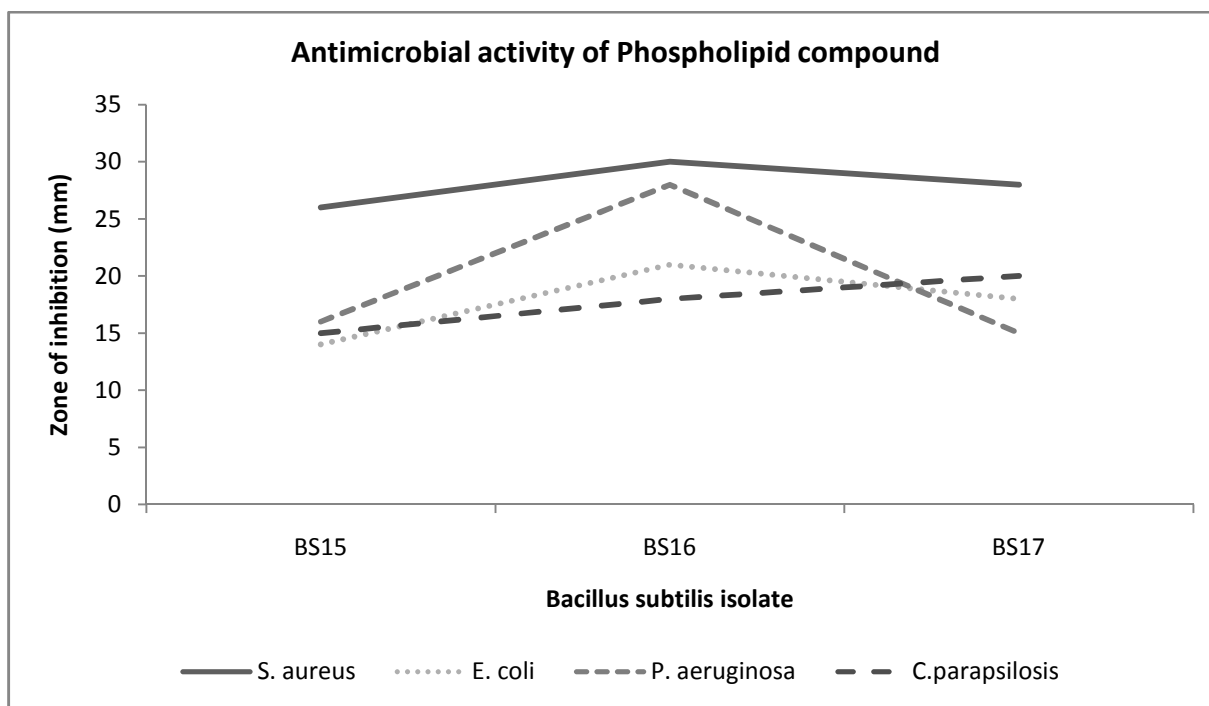


Figure-1
Antimicrobial activity of phospholipid antimicrobial compound

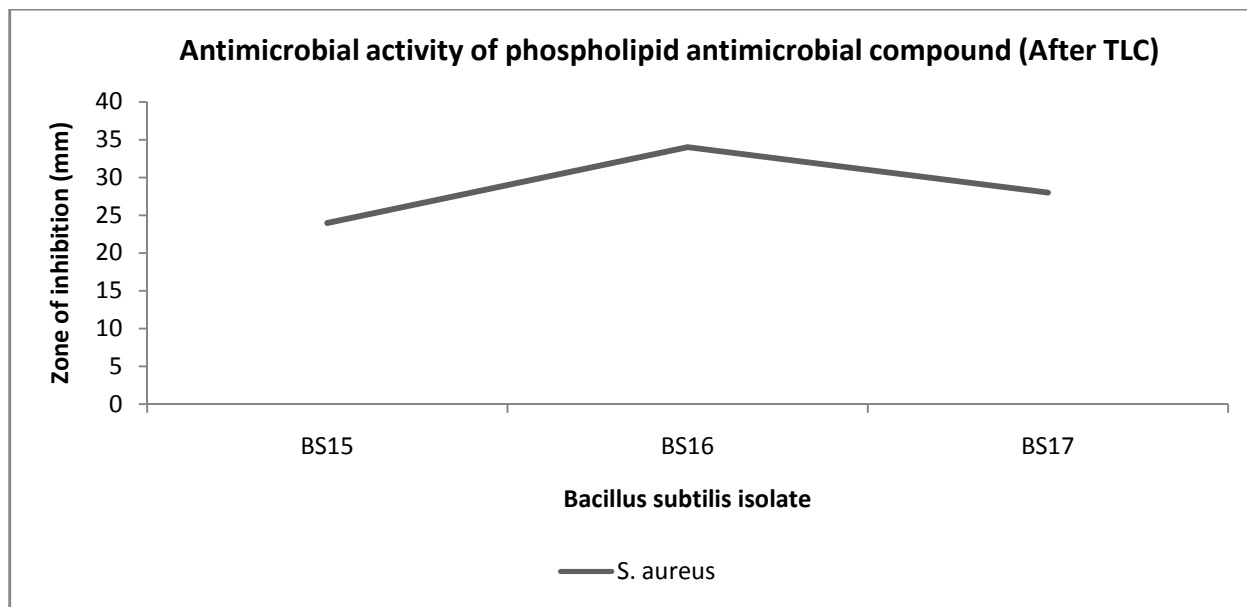


Figure-2
Antimicrobial activity of phospholipid antimicrobial compound (After TLC)

Conclusion

The result of this study indicate that the phospholipid antimicrobial compound produced by acidophilic *Bacillus subtilis* was found to be strongly effective against commonly occurring Gram positive (*Staphylococcus aureus*) and Gram negative (*E. coli*, *Pseudomonas aeruginosa*) and *Candida parapsilosis* but weakly effective against *Candida tropicalis* and thus, this compound can be used against infection caused by *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*. the phospholipids compound shows higher activity towards *Staphylococcus aureus* this compound can be used against infection caused by *Staphylococcus aureus*.

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