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Research article

Antimicrobial effect of Phospholipid produced from *Bacillus subtilis*

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ABSTRACT

Bacillus subtilis produces several antimicrobial compounds with different structurs. These antimicrobial compounds showed antimicrobial effect against Gram positive and Gram negative bacteria. The present study aims to evaluate the antimicrobial effect of phospholipid produces from Alkaliphilic *B. subtilis* isolated from different samples of food against different species of bacteria (Gram positive, Gram negative and actinomycetes) and fungi, and study the effect of pH and temperature on the antimicrobial activity of phospholipid. The results showed that the phospholipid produced from *B. subtilis* inhibited the growth of Gram negative bacteria (*Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*), Gram positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*), Actinomyces sp. and fungi (*Aspergillus niger*, *Candida albicans*) with different inhibition zones. It was observed that the antimicrobial activity of phospholipid was decreased at high temperature (50 ^{0}C) and pH (10). It can be concluded that the phospholipids produced from *B. subtilis* has anti-microbial activity and that affected by incubation condition (temperature and pH).

Keywords: Actinomyces, Anti-microbial activity, *Bacillus subtilis*, Bacteria, Fungus, Phospholipids.

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INTRODUCTION

Bacillus subtilis produces a large number of antibiotics, which are classified as ribosomal or non ribosomal antibiotics: The non ribosomal antibiotics may play a role in competition with other microorganism during spore germination [1]. The high proportion of antimicrobial compounds producing strains may be associated with ecological role, playing a defensive action to strains into an established microbial community [2].

A series of new antibiotics have been recently isolated from well known *B. subtilis* strain, these include Bacilysocin, an antimicrobial phospholipid that can be isolated from *B. subtilis* [3]. The phospholipid antibiotic produced by *B. subtilis* has broad spectrum activity against Gram positive and Gram negative bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida tropicalis*, and *Staphylococcus aureus* [4]. Moreover, another study highlighted the role of ph-

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ospholipid as an anti-fungal antibiotic produced from *B. subtilis* belongs to the family of anti-fungal agents and acts with a strict sterol phospholipid dependence on biomembranes [5]. The present study aimed to evaluate the anti-microbial activity of phospholipids produced from *B. subtilis* on different microorganisms and role of incubation condition on this activity.

MATERIALS AND METHODS

Sample collection

Sixty-five of different food samples (rice, meat, red bean, corn, cream, cheese, egg, potato, chicken, milk, cucumber, tomato, apple, flour and ornabit) were collected in sterile container and transported to the laboratory until using.

Isolation and identification of *Bacillus*

One gram of each sample was added to 9 ml of sterilized distilled water in sterile test tube, mixed thoroughly then heated to 80 ^{0}C for 15 min in water bath, serial dilution were prepared and 0.1 ml of each dilution spread on nutrient agar plates and incubated at 45 ^{0}C for 24 h [6]. Grown colony was streaked on nutrient agar plate and these steps were repeated until pure culture was obtained. Biochemical texts were used to identify *B. subtilis* [6].

Detection and extraction of phospholipid produced from *Bacillus* isolates

To screen Bacillus isolates for their ability to produce phospholipid, the standard method of Kazim and Alden, (2014) was used [7].

Detection of antimicrobial activity of phospholipid

The antimicrobial activity of phospholipid that produced from highest phospholipid production isolate was tested against different microorganisms. The standard method of agar well diffusion method [7]:

- Five ml of nutrient broth medium in test (three test tubes) tubes was inoculated with a single colony of each of Gram positive bacteria, Gram negative bacteria, and actinomycetes as indicator cultures and incubated at 37 °C for 18 hrs.
- Five ml of potato-dextrose broth (P.D.B) media was inoculated with a single colony of each of mold and yeast as indicator cultures. The test tubes were incubated at 28°C for 3 days.
- Decimal dilutions of growth were prepared to obtain dilution corresponding to turbidity of Macfrland tube (tube number 0.5).
- 100 µl of bacterial dilution was inoculated on the Muller -Hinton agar media, and 100 µl of mold and yeast dilution were inoculated on potato-dextrose agar and laid on agar by glass spreader.
- Two - wells were done on Muller-Hinton agar and potato-dextrose agar media with sterilized cork borer (6 mm).
- One of these well was filled with 100 µl of crude phospholipid, and the second well filled with100 µl of 10 % ethyl acetate as control.
- The plates were kept in refrigerator for 10 min for diffusion of compounds.
- The plates were incubated at right side at 37 °C for 18 h for bacterial and actinomycetes growth and at 28 °C for 3 days for mold and yeast growth.
- The inhibition zone around the extract wells were measured by millimeter (mm), using calipers.

Effect of temperature on the antimicrobial activity of phospholipid

About 0.2 gm of crude phospholipid was dissolved in 1 ml of 10 % ethyl acetate at pH 4.6 and incubated for 30 min at different temperatures (4°C and 50°C), after that the phospholipid activity was measured by agar well diffusion method, briefly. Muller-Hinton agar and potato-dextrose agar plates were seeded with 10^8 cell/ml of indicator isolates by spreading method. Wells were made and filled with 100 µl of treated phospholipid; the plates were incubated at 37 ^{0}C for 24 h for bacterial growth, and at 25 ^{0}C for 3 days for mold and yeast growth. Inhibition was detected by the diameter of inhibition zone around the wells. The results were compared with control, which contained 0.2 gm phospholipid dissolved in 1 ml of 10% ethyl acetate pH, 4.6.

Effect of pH on the antimicrobial activity of phospholipid

About 0.2 gm of phospholipid was dissolved in 1 ml of different buffer at different pH. (pH 5, 0.2 M acetate buffer; pH 7, 0.1 M phosphate buffer and pH 10, 0.2 M glycin buffer). Control composed of 0.2 gm phospholipid dissolved in 1 ml of 10% ethyl acetate with pH 4.6. Tubes were incubated for 30 min in a water bath at 37°C and then the activity of phospholipid was measured by agar well diffusion method.

RESULTS AND DISCUSSION

Isolation and identification of *B. subtilis*

Thirty-five samples were collected from different food samples. Forty-five bacterial isolates were belonged to Bacillus spp. depending on morphological and microscopic examination [8]. *Bacillus* isolates on nutrient agar showed variability in size and morphology. They were varied from moist to wrinkled, with different color of colonies (off white to creamy) and an irregular shape. Microscopic examination showed Gram positive rod bacilli arranged singly, pairs, chain (as filaments). Spore former bacteria.

Gram stain is sufficient to determine the presence of spore because the spore remains none stained, while the vegetative cell appeared stained [9]. According to biochemical stain the bacteria was identified as *B. subtilis* [10].

Production and extraction of phospholipid

The results showed that all isolates 100% were produced phospholipid with different ranged between 0.002- 0.185 gm/500 ml according to the crude extract resulting from evaporated of n-butanol. From these result ten isolates which gave highest crude extract resulting from evaporated of n-butanol were extracted with 10 % ethyl acetate to obtain phospholipid dry weight. Phospholipid dry weights were varied from 0.021 to 0.11 gm/500 ml. *B. subtilis* isolated from potato produced the maximum phospholipid dry weight (0.110 gm /500 ml).

Membrane associated lipids such as phospholipid are more polar and require polar solvents such as ethanol, butanol, and methanol to disrupt hydrogen bonds or electrostatic forces. The crude extract resulting from evaporated of n-butanol is an indication of phospholipid producing organisms [4]. Ethyl acetate is organic compound with antioxidant effect used as a solvent and diluent. It is preferable in present study because of its low cost, low toxicity, and agreeable odor, evaporates quickly [11]. All lipids must be protected against degradation through oxidation by solvent, oxygen, enzymes in combination with temperature. Belguith *et al*., [12] used ethyl acetate as antioxidant agent when extracted cholesterol from rats fed fenugreek seeds.

Detection of antimicrobial activity of phospholipid

The result showed antimicrobial activity of phospholipid produced from *B. subtilis* against all indicator isolates with range of inhibition zone diameters from 18 to 35 mm. The highest inhibition zone was found in case of Actinomyces sp. with diameter 35 mm followed by *S. aureus* with 31 mm. The Lowest phospholipid activity was observed against *C. albicans* with diameter of inhibition zone 18 mm **table 1, fig 1.**

Bacteria vary widely in the lipid composition of their membranes and would therefore be expected to exhibit different sensitivities to antimicrobial compounds that acts at the cell surface [13]. Stein et al. [14] stated that phospholipid compounds affect on the permeability of membrane and act on the lipid part of cell membranes or outer proteins and causing structural fluctuations in the membranes. Actinomycetes are susceptible to a wide range of antibiotics that are used to treat bacterial diseases. The fungi possess cell wall composed of carbohydrate layers, long chain of polysaccharides, as well as glycoprotein and lipid. Phospholipid affects on the lipids and alters the membrane fluidity, perhaps produces pores in the membrane in which ions and molecules maybe lost [15].

Table 1. Antimicrobial activity of phospholipid produced from *B. subtilis* against indicator isolates by agar well diffusion method

Indicator isolates	Diameter of inhibition zone (mm)
E. coli	23
P. mirabilis	21
P. aeruginosa	25
S. aureus	31
E. faecalis	21
C. albicans	18
A. niger	20
Actinomyces sp.	35

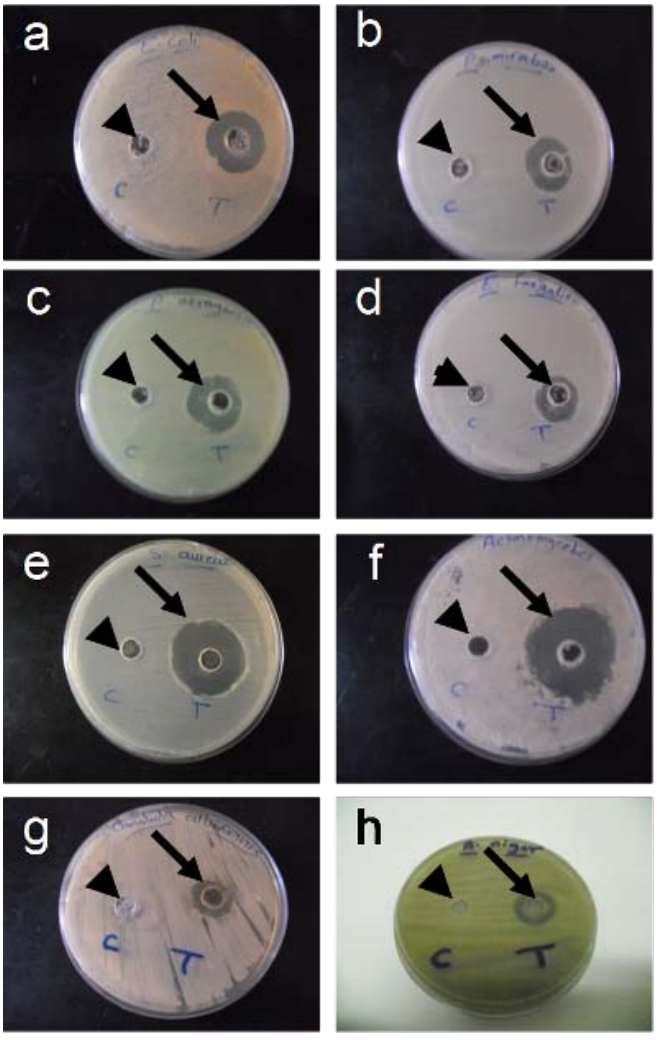

Fig. 1 Antimicrobial activities of phospholipid produced from *B. subtilis* B_i21 against different Gram negative (a, *E. coli*; b, *P. mirabilis;* c, *P. aeruginosa*), Gram positive bacteria (d, *E. faecalis*; e, *S. aureus*) Actinomycetes (f) and fungi (g, *C. albicans*; *A. niger*), well diffusion method was used to specify antimicrobial activity of phospholipids. C: Control (1ml of 10% ethyl acetate); T: Test (0.2 gm of *B. subtilis* phospholipid dissolved in 1ml of 10 % ethyl acetate). Arrow, test; head of arrow control.

Effect of temperature on the antimicrobial activity of phospholipid

The results showed that at high temperature the activity of tested phospholipid as antimicrobial agent was reduced against indicator isolates when compared with the activity of tested phospholipid at low temperature (**table 2, fig 2**).

Table 2. Influence of temperature on *B. subtilis* phospholipid activity against indicator isolates.

Indicator microorganism incubated at 37 ^{0}C for 24 h	Diameter of inhibition zone in (mm)		
	Control phospholipid incubated at 37^{0}C for 30 min	Tested phospholipid incubated at 4^{0}C for 30 min	Tested phospholipid incubated at 50^{0}C for 30 min
E. coli	17	17	16
P. mirabilis	18	21	20
P. aeruginosa	18	24	15
S. aureus	21	27	24
E. faecalis	16	18	16
C. albicans	18	22	19
A. niger	20	25	19
Actinomyces	20	18	16

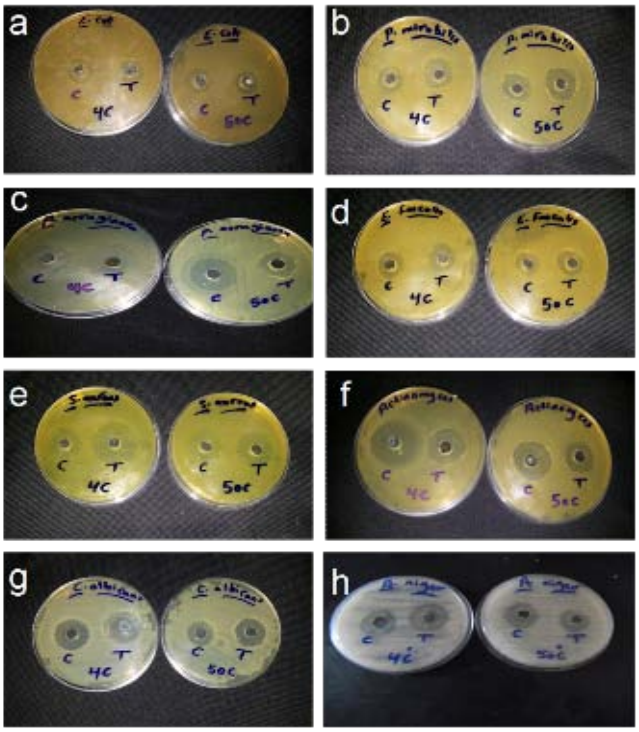

Fig. 2 Effect of exposed temperatures (4 ^{o}C and 50 ^{o}C) for 30 min on antimicrobial activity of phospholipid against different Gram negative (a, *E. coli*; b, *P. mirabilis*; c, *P. aeruginosa*), Gram positive bacteria (d, *E. faecalis*; e, *S. aureus*) Actinomycetes (f) and fungi (g, *C. albicans*; *A. niger*). Control (C), 0.2 gm of phospholipid dissolved in 1 ml of 10% ethyl acetate incubated at 370C for 30 min; test (T), 0.2 gm of phospholipid dissolved in 1 ml of 10% ethyl acetate and incubated at 4 ^{o}C and 50 ^{o}C for 30 min) before checking the antimicrobial activity by well diffusion method.

Cell wall of Gram negative bacteria contain two membranes, cytoplasmic membrane in addition an outer membrane, therefore, it is expected to be more resistances than Gram positive bacteria [13]. At lowest temperatures, membrane fluidity must increase in order to avoid transition from a liquid crystalline into a gel-like phase state of the lipid bilayer [16]. The activity of purified phospholipid was reduced immediately when exposure to temperature above 420C for short time because of the disorders within the arrangement of its composition which were reversible according to the short exposure time [17].

Effect of pH on the antimicrobial activity of phospholipid

The results showed no antimicrobial effect of phospholipid at pH, 5 and pH, 7 on the, but the activity was reduced when exposure to pH above 7 (**table 3, fig 3**).

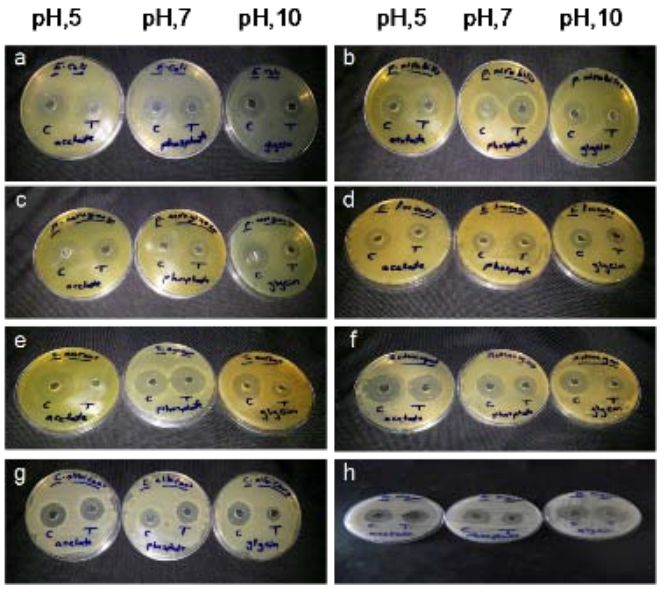

Fig. 3 Effect of different incubation pH (5, 7 and 10) for 30 min on the antimicrobial activity of phospholipid against different Gram negative (a, *E. coli*; b, *P. mirabilis*; c, *P. aeruginosa*), Gram positive bacteria (d, *E. faecalis*; e, *S. aureus*) Actinomycetes (f) and fungi (g, *C. albicans*; *A. niger*), Control (C), 0.2 gm of phospholipid dissolved in 1 ml of 10% ethyl acetate incubated at pH, 4.6 for 30 min; test (T), 0.2 gm of phospholipid dissolved in 1 ml of 10% ethyl acetate and incubated in 1 ml of different buffers (5, 7 and 10) before checking the antimicrobial activity by well diffusion method.

Miller et al. [18] stated the effect of different pH values on antimicrobial activity of phospholipid, and he mentioned that phospholipid molecules were still active in low and high pH values because different pH led to reversible changes in the phospholipid structure. In the present study the concept can be proved, the phospholipids that extracted from *B. subtilis* have antimicrobial activity and this activity sensitive to

temperature and pH. Thus this kind of material can be used in future as an antimicrobial agents post checking the toxicity.

Table 3. Influence of pH on *B. subtilis* phospholipid antimicrobial activity against different bacterial species

pH value	Inhibition zone in mm resulted from *B. subtilis* phospholipid against indicator microorganism							
	E. coli	*P. mira-bilis*	*P. aerug-inosa*	*S. aureu*	*E. face-alis*	*C. albic-ans*	*A. niger*	*Actin-omyces*
pH 4.6*	23	22	25	32	21	20	23	25
pH 5	24	21	23	31	20	19	22	24
pH 7	24	23	23	33	20	21	22	22
pH 10	20	19	18	16	15	14	20	16

*control phospholipid (0.2gm of phospholipid dissolved in 1 ml of 10% ethyl acetate pH, 4.6).

Conflict of interest:

The authors declare that they have no conflict of interests.

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