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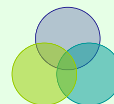

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Research article

Optimal conditions of phospholipid produced from *Bacillus subtilis*

Alaa Raheem Kazim[1]* and Sanaa Burhan alden[1]

ABSTRACT

Phospholipid contains antimicrobial activity against different kind of microorganisms. This article aimed to study the optimal conditions for the production and extraction of phospholipid produced from *Bacillus subtilis*. The result showed that the best production was obtained in NG medium containing 1% glucose, 50 µg/ ml tryptophan, pH 10, inoculated with 10% of bacterial culture and incubated at 30°C for 72 h in shaker incubator. The phospholipid was extracted with 50 % n-butanol three times, 10 % methanol, and then 10 % ethyl acetate. Fourier transform infrared spectroscopy (FTIR) analysis for *B. subtilis* Bf21 phospholipid showed peaks as 3460.06, 3359.77cm-1, 3228.62 cm-1, 2945.10 cm-1, 1728.68 cm-1, 1110.92 cm-1, and 1080,06 cm-1. These peaks indicating the presence of stretching (O-H, N-H, C-H, C=O, C-O, and P=O), which are functional groups present in phospholipid. Thin layer chromatography (TLC) analysis for *B. subtilis* Bf21 phospholipid was visualized after sprayed with spraying solution, as blue zone on a white background and Rf of phospholipid was 0.7.

Keywords: antimicrobial, *Bacillus subtilis*, FTIR, phospholipid.

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INTRODUCTION

Phospholipid is a class of lipids that are a major component of all cell membranes as they can form lipid bilayers [1]. Most phospholipid contains adiglyceride, a phosphate group, and a simple organic molecule such as choline; one exception to this role is sphingomyelin, which is derived from sphingosione instead of glycerol [2]. The first phospholipid identified as such in biological tissues was lecithin or phosphatidylcholine in egg yolk by Theodore Nicolas Gobley,a French chemist and pharmacist, in 1847. The structure of phospholipid molecule generally consists of hydrophobic tails and a hydrophilic head [3]. Biological membranes in eukaryotes also contain another class of lipid, sterol, interspersed among the phospholipid and together they provide membrane fluidity and mechanical strength. Phospholipid is com-

*Correspondence: alaa_alkenane@yahoo.com
Phone number: 009647711615099
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

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posed of 40 percent of bacterial membrane and play several biological activities [4]. Purified phospholipid is produced commercially and has found applications in nanotechnolog, Food technology, cosmetic industry, pharmaceutical industry, drug delivery system, and medical application [5]. *B. subtilis* known also as the hay Bacillus or grass Bacillus, is a rod shaped, and has the ability to form a tough protective endospore allowing the organism to tolerate extreme environmenttal conditions [6].

MATERIALS AND METHODS

Sample collection

Sixty-five samples were collected from different sources included: soil, water and food. Each sample was collected in sterile container and transported to the laboratory until usage (**table 1**).

Table 1. The sources of samples collection for bacterial isolates

Water Samples	Soil Samples	Food Samples			
Water tank	Grasses	Rice	Cream	Chicken	Apple
Pool	Elias	Meat	Cheese	Milk	Flour
Tap water	Eucalyptus	Red bean	Egg	Cucumber	Qrnabit
Sewage	different samples	Corn	Potato	Tomato	Un treated milk

Isolation of *Bacillus*

A. Soil samples and food samples

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.

B. Water samples

One-hundred ml was taken from each samples and centrifuged for 10 min, then most of supernatant was discarded and the pellet was shaked to get suspension then heated to 80 ^{0}C for 10 min. 0.1 ml of each sample was spread on nutrient agar and incubated at 45 ^{0}C for 24 h. Grown colony was streaked on nutrient agar plate and these steps were repeated until pure culture was obtained.

Detection and extraction of phospholipid produced from *Bacillus* isolates

To screen Bacillus isolates for their ability to produce phospholipid, following method was used as described by Tamehiro *et al.*, 2002 [7].

1. Bacillus isolates were activated in nutrient broth at 37 °C for 18 h, 0.2 ml of activated culture suspension was inoculated in 500 ml of nutrient glucose (NG) medium which contain of (Nutrient broth 10 gm, glucose 10 gm, NaCl 2 gm, CuSo4.5H2O 0.005 gm, FeSo4.7H2O 0.0075 gm, MnSo4.5H2O 3.6 gm, CaCl2 0.015 gm, ZnSo4.7H2O 0.009 gm, and 1000 ml distilled water, and tryptophan was added at concentration 50 µg/ml) and incubated at 30 °C for 24 h, absorbency at 600 nm for isolates were measured.
2. 10 % of 500 ml NG medium (optical density, 0.1) was re-inoculated in fresh NG medium (500 ml), and incubated under shaking at 30 °C for 72h. After incubation period bacterial culture was centrifuged at 10000 rpm for 10 min, cells were collected.
3. Cellular contents were extracted three times using 10 ml of 50 % n-butanol each time, then the aqueous layer was collected and evaporated to concentrate as crude extract at room temperature and the weight was measured.
4. Bacillus isolates, which gave the highest crud extract weight, were re-suspended in 4 ml of 10 % methanol, pH adjusted to 7 with NaOH and extracted three times with an equal volume of ethyl acetate.
5. The aqueous layer resulted from ethyl acetate was then adjusted to pH 2 with HCL and again extracted three times with ethyl acetate.
6. The aqueous layer was collected in sterilized Petri dishes and evaporated at room temperature.
7. The dry weight of extracted phospholipid was measured for each isolates.

Phospholipid analysis by FTIR

The highest dry weight of phospholipid obtained was analysis by FTIR spectroscopy as follows:
Sample of dried weight was mixed with potassium bromide (KBr) crystals at ratio 1:10 (w/w) using motor and pestle, placed in cap and compressed to form a thin pellet, the spectrum of the pellet was obtained by ashimadzu FTIR spectrophotometer [8].

Detection of phospholipid by thin layer chromatography (TLC)

Seperation and identification of phospholipid was performed by TLC using silica gel coated plate (TLC coated with silica gel) 20×20 cm, this method was applied by Dittmer and Lester [9] as following:

1. About 0.5 gm of phospholipid was dissolved in 1 ml of 10 % ethyl acetate.
2. About 10 µl was taken from the sample suspension by capillary tube and spotted many times at about 2 cm far from the lowest edge of TLC plate.

3. The plate was transferred to a closed jar containing separation system (containing Ammonium molybdate 0.5 gm was dissolved in 5 ml of distilled water, then 1.5 ml hydrochloric acid (25%) plus 2.5 ml perchloric acid (70 %) were added. The solution was cooled at room temperature; the volume was complete to 50 ml with acetone. The reagent solution can be used after standing for a day and it can be stored for about 3 weeks) and left to diffuse through silica gel plate to about 15 cm, after that the plate was dried at room temperature.
4. The dry plate was sprayed with reagent containing ammonium molybdate 0.5 gm was dissolved in 5 ml of D.W, then 1.5 ml hydrochloric acid (25%) and 2.5 ml perchloric acid (70%) were added. The volume was complete to 50 ml of acetone by a sprayer and placed in an oven at 110 °C for 5-10 min, to complete the reaction.
5. The position and distance of the spot was determined.
6. The relative flow (Rf) was estimated by dividing the distance of sample mobilized across the plate on the distance of the solvent [10].

Determination of optimal conditions for phospholipid production from Bacillus

Effect of different carbon sources

Five-hundred ml of sterilized NG medium containing 1% of different kinds of sugars instead of glucose (fructose, cellulose, and mannitol) were inoculated with 10% of activated bacterial culture broth (optical density, 0.1), and incubated at 30 °C for 72 h. Phospholipid was extracted and dry weight was measured.

Effect of nitrogen sources

Five-hundred ml of sterilized NG media with optimal carbon source were prepared with different nitrogen sources instead of tryptophan in concenteration of 50 µg/ml (glutamic acid, yeast extract, and peptone) inoculated with10% of activated bacterial culture (optical density, 0.1), and incubated at 30 °C for 72 h. Phospholipid was extracted and dry weight was measured.

Effect of pH

Five-hundred ml of sterilized NG media with optimal carbon and optimal nitrogen source were prepared at different pHs (8-12) adjusted with (1N) NaOH. The media were inoculated with 10% of activated bacterial culture and incubated at 30 °C for 72 h. Phospholipid was extracted and dry weight was measured.

Effect of temperature

Five-hundred ml of sterilized NG media with pH 10, were inoculated with 10% of activated bacterial culture (optical density, 0.1) incubated at different temperatures (30, 40, and 50 °C) for 72 h. Phospholipid was extracted and dry weight was measured.

Effect of different chemical compounds

Five-hundred ml of NG media with pH 10 containing 1% of different chemical compounds (methanol, chloroform, and tween 80) were inoculated with 10 % activated bacterial culture and incubated at 30 °C for 72 h. Phospholipid was extracted and dry weight was measured.

RESULTS AND DISCUSSIONS

Isolation and identification of *B. subtilis*

Sixty-five samples were collected from different sources: food, soil, and water (35 samples of food, 20 samples of soil, and 10 samples of water). Eighty bacterial isolates were belonged to Bacillus spp. depending on morphological and microscopic examination [6]. 45 isolates were isolated from food (56.25 %), 15 isolates were isolated from soil (18.75 %), and 20 isolates (25 %) were isolated from water.

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), an irregular in shapes. Microscopic examination showed gram positive rod bacilli, may occur singly, pairs, chain and as filaments. Spore former bacteria. Gram stain was sufficient to determine the presence of spore because the spore remains non stainable, while the vegetative cell will stain [11].

Biochemical tests

The result showed that 37 isolates from 80 isolates were identified as *B. subtilis*, they were Gram positive, rod shaped, spore former bacteria, catalase positive, motile, indole test positive, urease negative, fermented carbohydrate, triple sugar iron (TSI) acid/acid (slant/butt), simmon citrate positive, lecithinase negative, resist to penicillin, gelatin liquefication, vogas-proskuer positive, starch hydrolysis, and nitrate reduction to nitrite was positive [12].

Detection and extraction of phospholipid production from *B. subtilis* isolates

The results showed that all isolates were phospholipid production with different ranged between (0.002-0.185) gm/500 ml according to the crude extract resulting from evaporated of n-butanol (**tabel 2**).
From the result above ten isolates (Bw1, Bw2, Bf1, Bf3, Bf8, Bf17, Bf20, Bf21, Bs5, and Bs7), which gave highest crude extract resulting from evaporated of n-

butanol were extracted with 10% ethyl acetate to obtain phospholipid dry weight. Phospholipid dry

Table 2. Crude extract produced from *B. subtilis* after evaporated of n-butanol**.**

No. of isolates	Crude extract gm/500ml	No. of isolates	Crude extract gm/500ml	No. Of isolates	Crude extract Gm/500ml	No. of isolates	Crude extract gm/500ml
B_w1	0.162	B_f5	0.002	B_f16	0.117	B_s7	0.164
B_w2	0.161	B_f6	0.980	B_f17	0.178	B_s8	0.120
B_w3	0.110	B_f7	0.145	B_f18	0.083	B_s9	0.073
B_w4	0.128	B_f8	0.174	B_f19	0.032	B_s10	0.086
B_w5	0.124	B_f9	0.161	B_f20	0.172	B_s11	0.136
B_w6	0.102	B_f10	0.103	B_f21	0.185	B_s12	0.157
B_f1	0.165	B_f11	0.087	B_s1	0.098	B_s13	0.124
B_f2	0.127	B_f12	0.132	B_s2	0.074		
B_f3	0.163	B_f13	0.095	B_s3	0.106		
B_f4	0.137	B_f14	0.139	B_s5	0.176		

weights were varied from (0.021-0.11) gm/500 ml and *B. subtilis* (B f 21) isolated from food (potato) produc-ed maximum phospholipid dry weight (0.110 gm /500 ml) (**table 3**).

Table 3. *B. subtilis* phospholipid dry weights resulting from evaporated of 10% ethyl acetate**.**

B. subtilis isolates	Phospholipid dry weight gm /500ml	*B. subtilis* isolates	Phospholipid dry weight gm /500ml
B_w1	0.062	B_f17	0.098
B_w2	0.021	B_f20	0.087
B_f1	0.084	B_f21	0.110
B_f3	0.072	B_s5	0.093
B_f8	0.091	B_s7	0.078

Membrane associated lipids such as phospholipid are more polarized and require polar solvents such as ethanol, butanol, and methanol to disrupt hydrogen bondings or electrostatic forces. The crude extracted resulting from evaporated of n-butanol is an indication of phospholipid producing organisms [4]. Ethyl acetate is an organic compound with antioxidant effect used as a solvent and diluent. It is preferable because of its low cost, low toxicity, and agreeable odor and the organic desolvents evaporate quickly [13]. All lipids must be protected against degradation through oxidation by solvent, oxygen, enzymes in combination with temperature. Belguith *et al.*, (14) used ethyl acetate as antioxidant agent when extracted cholesterol from rats fed fenugreek seeds.

Analysis of *B. subtilis* (Bf21) isolate phospholipid by using FTIR

The result showed that the presence of O-H stretching group in 3460.06-3359.77 cm-1, bending groups N-H and C-H in 3228.62 cm-1 and 2945.10 cm-1, The C=O of the carboxylic groups or ester groups stretching vibration appears at 1728.68 cm-1, C-O stretching in 1110.92 cm-1, P=O aliphatic strong phosphate group stretching vibration appears at 1080.06 cm-1 (**fig. 1**).

Detection of phospholipid producing from *B. subtilis* Bf21 by TLC

Phospholipid produced from *B. subtilis* Bf21 was visualized after sprayed with spraying solution, as blue zone on a white background. Rf of phospholipid was 0.7 (**fig. 2**).

Effect of different culture conditions on phospholipid produced from *B. subtilis* Bf21

Effect of carbon sources

The result showed that NG medium containing glucose was the best medium for phospholipid production, and there were no production of phosph-olipid in NG medium contaning cellulose or mannitol (**fig. 3**).

B. subtilis appeared depend on the growth conditions, addition of glucose to the medium reduced pH during growth this was accompanied with increasing in the amount of phospholipid [15].

Effect of nitrogen sources

The result showed that the highest phospholipid extract obtained from NG medium containing tryptophan followed by glutamic acid and yeast extract, while the lowest production in NG medium with pepton, the dry weights were 0.170, 0.112, 0.1, and 0.074 gm/500 ml, respectively (**fig 4**).

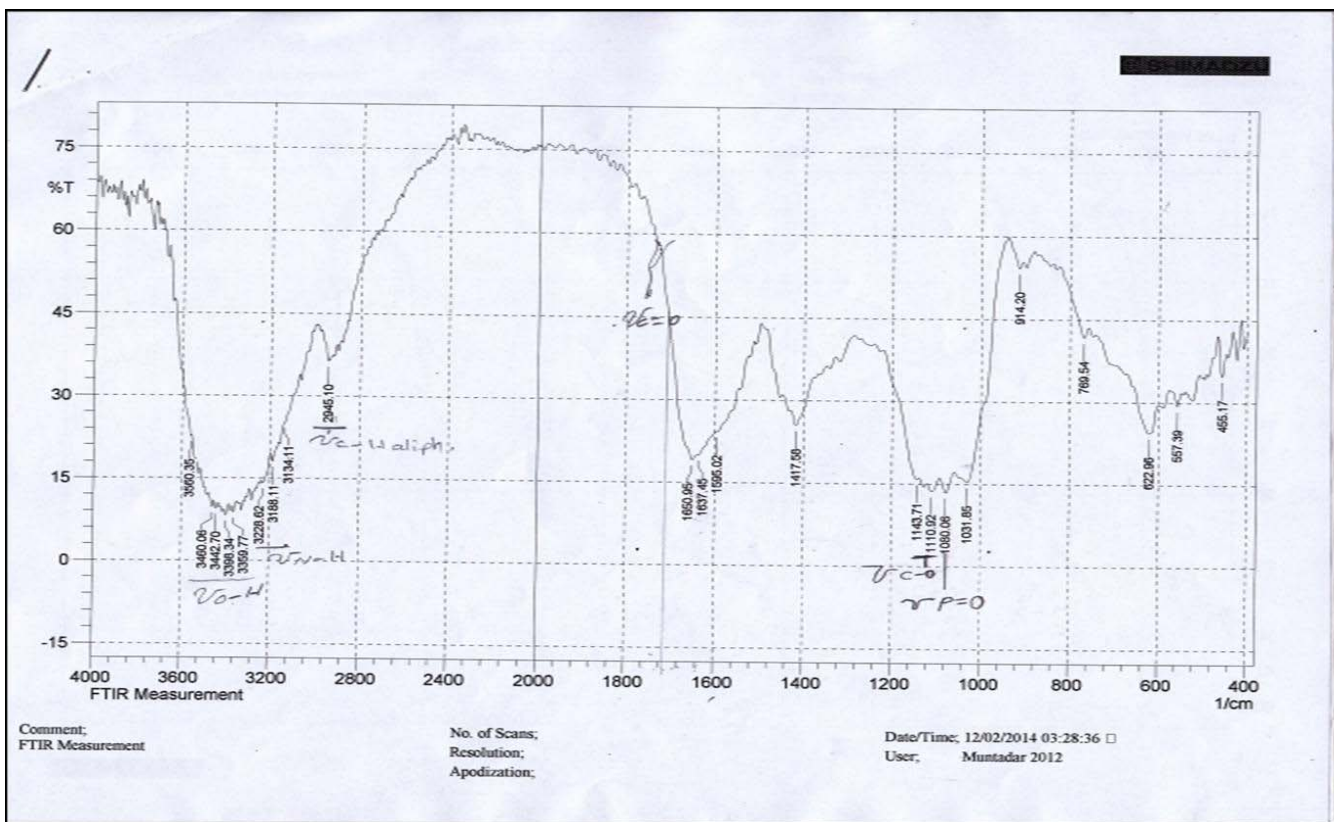

Fig 1. FTIR analysis of phospholipid produced from *B. subtilis* (B_f2).

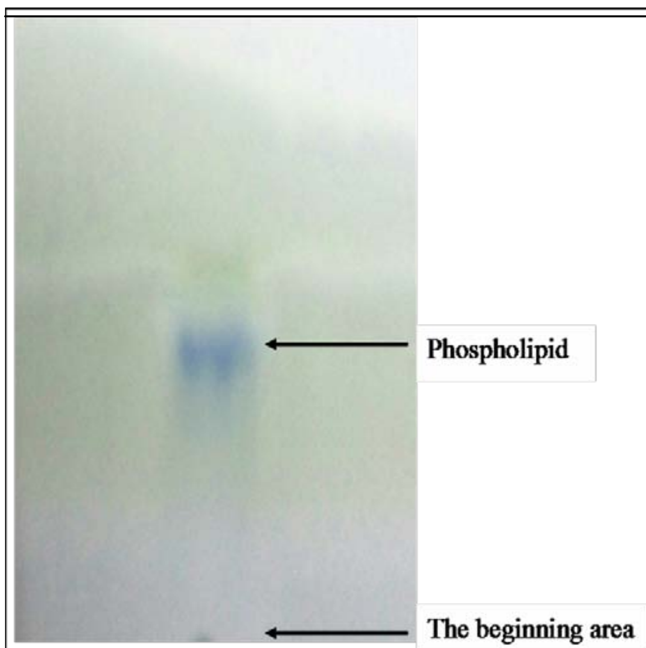

Fig 2. TLC analysis for detection phospholipids produced from *B. subtilis* B_f21 using silica gel plate (20*20) cm with solvent system (0.5 gm Ammonium molybdate, 5 ml distilled water, 1.5 ml hydrochloric acid (25%), 2.5 ml perchloric acid (70%), and 50 ml aceton), at dark room temperature.

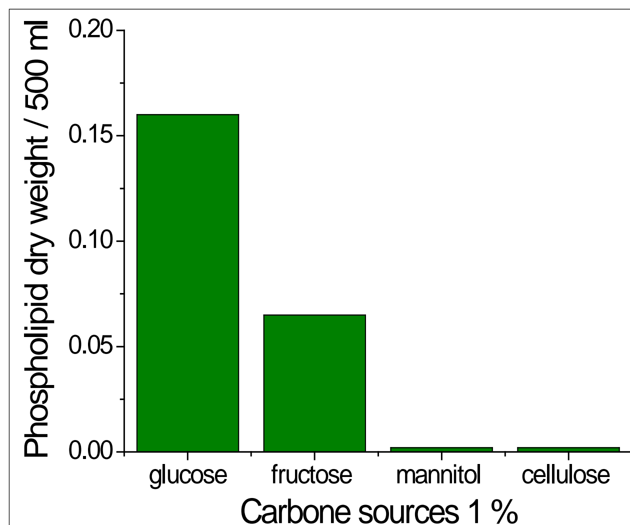

Fig 3. Production of phospholipid from *B. subtilis* B_f21 in NG medium containing different carbon sources, pH 10, inoculum size 10%, incubated at 30 ^{0}C for 72 h.

Nitrogen is one of the important factors that effected bacterial growth, bacteria required nitrogen for metabolic pathways, also nitrogen enhanced and increased bacterial growth, and required for biosynthesis of an important molecules such as (nucleic acid, protein and other important components) [16].

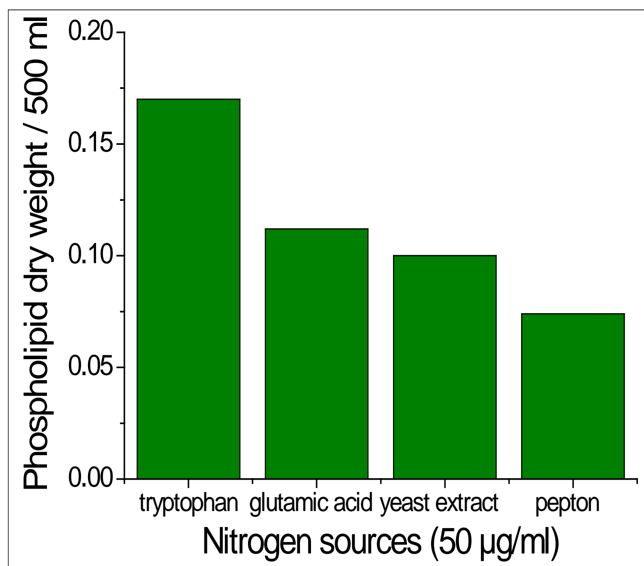

Fig 4. Production of phospholipid from *B. subtilis* B_f21 in NG media containing 1 %glucose and different nitrogen sources, pH 10, inoculum size were 10 %, incubated at 30^0C for 72 h.

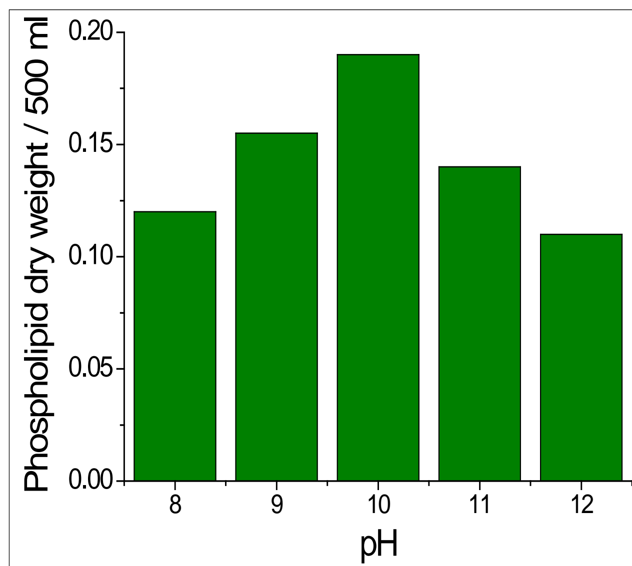

Fig 5. The effect of pH on phospholipid production from *B. subtilis* B_f21 in NG media containing 1% glucose, 50 µg tryptophan /ml, inoculum size 10% and incubated at 30^0C for 72 h.

Tryptophan is an amino acid, acts as building block in protein biosynthesis. The distinguishing structural characteristic of tryptophan is an indole functional group. Indole group acts as a signal molecule regulates various aspects of bacterial physiology including spore formation, plasmid stability, resistance to drugs, biofilm formation, and virulence [17]. Yeast extract served to be a better nitrogen sources for the production of crude extract than peptone. Yeast extract contain glutamic acid and the average content of this amino acid about 5% [18]. Hsieh and Labbe [19] investigated the effectiveness of peptone on the sporulation of Clostridium perfringens organism and they used peptone as a convenient sporulation, they investigated that peptone stimulated the sporulation, and there were increase in sporulation rates. Phospholipid as antimicrobial compound is synthesized after growth ceased and before the sporulation processes [7]. The nitrogen source (glutamic acid) concentration effected the production of phospholipid and it was shown that increased concentration improve the production of antimicrobial compound [20].

Effect of pH on phospholipid production

The results showed that good productions were obtained at all pH values, and maximum productions were observed at alkaline pH (9 and 10), the dry weights ranged from 0.11 to 0.190 gm/500 ml (**fig. 5**).
Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites [21,22]. Growth of the bacteria at neutral pH resulted in a high ratio of Zwitter-ionic phospholipid induced by the fermentation of glucose [15].

Effect of temperature on phospholipid production

The result showed that the best temperature for phospholipid production was at 30 ^{0}C (dry weight was 0.193 gm /500ml), and then in 40 ^{0}C (0.152 gm/500ml), sharp decrease in phospholipid production was noticed at 50 ºC (0.11 gm/500ml) (**fig. 6**).

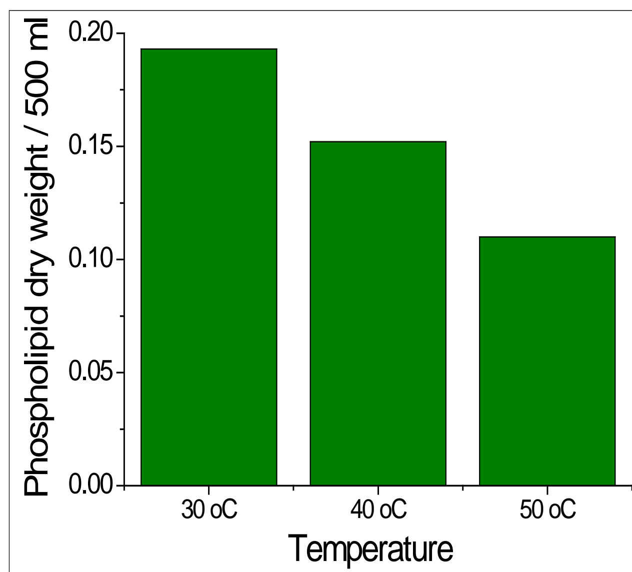

Fig 6. The effect of temperature on phospholipids production from *B. subtilis* B_f21 in NG media containing 1% glucose, 50 µg tryptophan/ml, inoculum size 10%, incubated at 30 ^{0}C for 72 h.

These results indicating that the optimum temperature for phospholipid production is 30 ^{0}C and this may be due to that this temperature may be the optimum

temperature for lysophospholipase (LPL), enzyme that responsible for biosynthesis of phospholipid and from these result we showed that when temperature increased the production of phospholipid decreased, these may be resulted from the effected of temperature on the activity of the enzyme (23). Ray *et al.*, [24] reported that the temperature regulate the synthesis and secretion of extracellular enzymes that responsible for biosynthesis.

Effect of chemical compounds on phospholipid production

The result showed that the dry weights were 0.05, 0.04, and 0.03 gm/500 ml in NG medium contaning methanol, chloroform, and tween 80, respectivily, comparing with control NG medium with out any addition of chemical compounds and the dry weight was 0.2 gm/500ml (**fig 7**). These result of the effect of organic solvents on bacterial membranes, both chloroform and methanol are hydrophilic and cell membranes are made up of phospholipid bilayer which can be disrupted when surrounded by hydrophilic molecules such as chloroform and methanol. Methanol and chloroform are polar solvent effect on the permeability of membranes [25]. Brown and Winsley [26] stated that polysorbate 80 alters cell permeability and viability of bacteria, the loss by leakage of constituents, including purine and pyrimidine compounds.

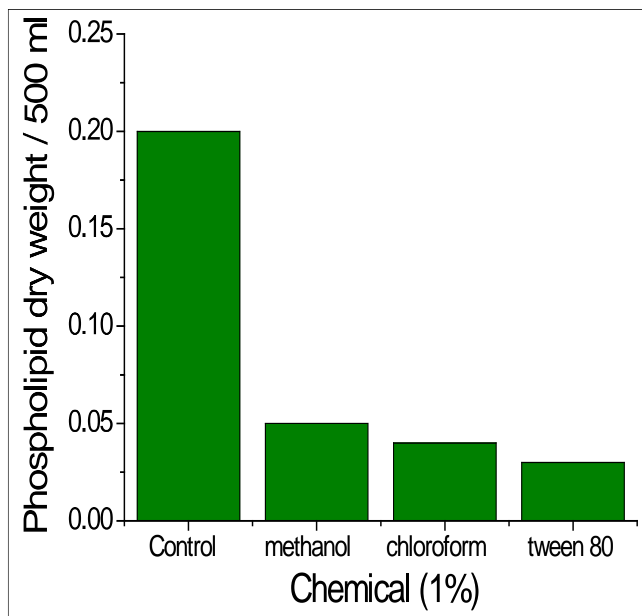

Fig 7. Production of phospholipid from *B. subtilis* B_f21 in NG media containing 1% glucose, 50 µg/ml tryptophan with addition of 1% of different chemical compounds, pH 10, inoculum size 10%, incubated at 30 ^{0}C for 72 h.

Conflict of interest:

The authors declare that they have no conflict of interests.

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Author affiliation:

1. *Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.*