# Characterization of Phospholipase C Productivity by *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus* isolates

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**Abstract:** Of 205 bacterial isolates, 28 have shown high phospholipase production. Three isolates, identified as *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Staphylococcus aureus*, have shown the highest PLC productivity compared to isolates from the same morphological class and were selected for characterization of enzyme production. PLC production by these isolates took place in phosphate starved media (PS-TMM). For *Pseudomonas aeruginosa* D183, PS- TMM supported maximum PLC production. Replacing glucose with other carbon sources at different concentrations, increasing glucose concentration, adding organic proteins (except for peptone at 0.5% and BSA at 1.5%), or replacing metal salt components of PS-TMM, inhibited PLC production. PS- TMM, to which BSA (1 or 1.5%) or sodium cholate (0.05%) was added, supported maximum PLC production by *Bacillus cereus* D101. PLC production by this isolate was increased with increasing glucose concentration in PS-TMM, while replacing glucose with other carbon sources either decreased or abolished PLC production. Adding BSA, triton X-100, sodium cholate (at 0.05 and 0.1%), tween 20, or tween 80 to PS-TMM or removing $CaCl_2$ from it increased PLC production by this isolate. Production of PLC by *Staphylococcus* isolate in PS-TMM occurred only when glucose concentration was increased to 110 mM, when BSA (0.5, 1 or 1.5%) or sodium cholate (1.25 or 2.5%) was added. PLC production by all three isolates was growth associated. Optimum pH for PLC production by the three isolates was pH 7-8 while optimum temperatures were 37˚C (*Pseudomonas aeruginosa* D183), 30˚C (*Bacillus cereus* D101) and 25-37˚C (*Staphylococcus aureus* D173).

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**Keywords:** Phospholipases C, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Production, Characterization.

**Abbreviations:** BSA, bovine serum albumin; CF, cystic fibrosis; CFU, colony forming unit; CSF, cerebrospinal fluid; DAG, diacyl Glycerol; NPPC, p-nitrophenyphosphorylcholine; PI-PLC, phosphatidylinositol-specific phospholipase; Pi, inorganic phosphate; PL, phospholipase; $PLA_1$, phospholipase $A_1$; $PLA_2$, phospholipase $A_2$; PLB, phospholipase B; PLC, phospholipase C; PLD, phospholipase D; PS-TMM, phosphate starved tris minimal medium; Pz, phospholipase activity; TMM, tris minimal medium

## 1. Introduction:

Phospholipases are a heterogeneous group of enzymes that share the ability to hydrolyze one or more ester linkage in glycerophospholipids. Qualifying letters are used to differentiate among phospholipases and to indicate the specific bond targeted in the phospholipid molecule. Phospholipase $A_1$ ($PLA_1$) hydrolyzes the fatty acyl ester bond at the sn-1 position of the glycerol moiety, while phospholipase $A_2$ ($PLA_2$) removes the fatty acid at the sn-2 position of this molecule. Phospholipase C (PLC) hydrolyzes the phosphodiester bond in the phospholipid backbone, whereas the second phosphodiester bond is cleaved by phospholipase D (PLD) (Ghannoum, 2000). The diversity of roles that phospholipases play in biology and medicine is exceptional. In the past decade, this class of enzymes has proven to be considerably more complex than initially perceived and their impact on an assortment of basic cellular processes in eukaryotes, including oncogenesis and inflammation has become widely appreciated. Likewise, there are sundry functions for phospholipases in prokaryotic biology, including their noteworthy contributions to microbial virulence (Vasil, 2006). The most important class of phospholipases that have been thus far shown to play a significant role in bacterial pathogenesis is the phospholipase C (PLC) (Johansen *et al.*, 1996). In 1941, Macfarlane and Knight demonstrated that the highly cytotoxic α-toxin of *Clostridium perfringens* has PLC activity. Since then, PLC activity has been demonstrated in a variety of other pathogenic bacteria, including *Pseudomonas aeruginosa* (Ostroff and Vasil, 1987; Stonehouse *et al.*, 2002; Barker *et al.*, 2004), *Bacillus cereus* (Verkleij *et al.*, 1973; Kreft *et al.*, 1983), *Staphylococcus aureus* (Marques *et al.*, 1989), *Legionella pneumophila* (Dowling *et al.*, 1992), *Helicobacter pylori* (Drazek *et al.*, 1995), *Mycoplasma* spp. (De Silva and Quinn, 1987), *Listeria*

*monocytogenes* (Camilli *et al.*, 1991; Marquis *et al.*, 1997), *Mycobacterium tuberculosis* (Johansen *et al.*, 1996), and others.

This study aimed at detecting the production of phospholipases among different bacterial isolates and characterizing the productivity of phospholipases C from some clinical bacterial isolates.

## 2. Material and Methods

### Chemicals

All chemicals were of high quality from available grades supplied (unless otherwise indicated) by El-Nasr Chemicals (Adwic), Egypt.

### Bacterial Isolates and their Maintenance

Isolates D101, D173, and D183 were obtained through screening of 205 clinical isolates for phospholipase production. The isolates were recovered from 210 pathological specimens of different sources. The isolates were maintained onto nutrient agar (Difco) slants at 4ºC and subcultured every month. Glycerol stocks were used for the long term preservation of the isolates (Miller, 1972).

### Growth Conditions.

1) **Primary Screening**

The solid medium used for screening the isolates was egg-yolk nutrient agar prepared by aseptically beating up one egg-yolk with 15 ml sterile saline using sterile magnet and a stirrer, then mixing with 85 ml sterile molten (60˚C) nutrient agar (Difco) and pouring in Petri dishes, 20 ml each (Collee *et al.*, 1996).

2) **Secondary Screening**

Trypticase soy broth (Difco) was used.

3) **Characterization of Enzyme Productivity**

Phosphate starved tris minimal medium was used in which the concentration of the phosphate component ($KH_2PO_4$) of TMM was reduced to 0.1 mM. TMM has the composition: 100 mM Tris-HCl [pH 7.2], 11 mM glucose, 5 mM $NH_4Cl$, 10 mM $KH_2PO_4$, 0.5 mM $K_2SO_4$, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$. For Mg(II)-starved cultures, the concentration of $MgCl_2$ was reduced to 0.01 mM and the $CaCl_2$ was omitted. For phosphate-starved cultures, $KH_2PO_4$ was reduced to 0.1 mM. For nitrogen-starved cultures $NH_4Cl$ was reduced to 0.5 mM (Norte *et al.*, 2003).

### Inoculum Preparation

Cells from 1-day-old cultures on nutrient agar (Difco) slants were harvested in sterile normal saline. The final count in the cell suspension was adjusted to about $2\times10^8$ CFU/ml turbidimetrically at 640 nm with reference to calibration curves constructed for each isolate between turbidity of the bacterial suspension and the bacterial count. For secondary screening and characterization of enzyme productivity aliquots (1 ml) from the inoculums prepared above was used to inoculate 9 ml culture medium within 50 ml Erlenmeyer flasks. Incubation was done at 37˚C in a platform orbital shaker (Orbital Shaker, SO1, Stuart Scientific, UK) at 225 rpm for 48 h.

. For studying the effects of carbon sources, protein sources, and surface active agents on productivity, the phosphate starved tris minimal medium (PS-TMM) was used as a basal medium. The effect of different metal ions was studied in two steps. The first step was the effect of the separate or combined removal of the metal salt components of phosphate-starved TMM leaving only $KH_2PO_4$ unchanged, as well as the replacement of $K_2SO_4$ by $Na_2SO_4$, at the same concentration. The second step was the effect of addition of metal ion salts to basal phosphate-starved TMM from which all metal ion salts have been removed except for $KH_2PO_4$. For investigation of the effects of inorganic phosphate, incubation time, initial pH and incubation temperature on productivity, PS-TMM was used for isolate D183, and was supplemented with 1.5% BSA for isolate D173 and with 0.05% sodium cholate.

### Enzyme Assays

Primary screening was done using the egg-yolk plate method where isolates were spot inoculated on the surface of egg-yolk agar plates and incubation was done 37ºC for 48-72 h. Phospholipase activity (Pz) was expressed in terms of the ratio between the diameter of the colony to the diameter of the zone of precipitation (Price *et al.*, 1982). The evaluation of phospholipase producing isolates was done as described by Dagdeviren *et al.* (2005) where a Pz of 1.0 was evaluated as negative (-), 0.99–0.9 as weak (+), 0.89–0.8 as mild (++), 0.79–0.7 as relatively strong (+++) and <0.69 as very strong (++++) activity. Isolates that showed very strong phospholipase productivity (Pz < 0.6) were further evaluated in shake-flask fermentations for their extracellular PLC production.

Phospholipase C assay was performed by the method of Kurioka and Matsuda (1976). The assay was adapted to a microtiter system by Berka and Vasil (1981). At the end of the incubation period, cells were removed from 1 ml of culture by centrifugation (Beckman Microfuge). After centrifuging, 10 µl of the clear supernatant fluid was added to 90 µl of NPPC reagent in a microtiter test plate (96 wells per plate). The NPPC reagent contained 250 mM tris(hydroxymethyl)- aminomethane-hydrochloride buffer (pH 7.2) (Oxoid), 60% glycerol (wt/wt), 1.0 µM $ZnCl_2$, and 10 mM NPPC (Sigma Chemical Co., St.

Louis, Mo.). The plates were then incubated at 37°C for 17 h before the absorbance at 405 nm was measured with MicroReader 4 plus microplate reader (Hyperion. Inc., USA). A yellow color was developed in positive cases. A blank containing 10 µl of the clear growth supernatant fluid and 90 µl of NPPC reagent that lacked the chromogenic substrate, and a control containing 10 µl of the uninoculated culture medium and 90 µl of NPPC reagent were treated similarly and included in runs of enzymatic assay.

**Biomass Determination**

The cell mass obtained by centrifugation of a known volume of culture broth at 12,000 rpm for 15 min was washed twice with sterile saline and resuspended in the same volume of sterile saline. Biomass was determined turbidimetrically at 640 nm in an Ultrospec 2000 spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with reference to calibration curves constructed for each isolate between turbidity of the bacterial suspension and the bacterial count determined using the viable count technique.

**Isolate Identification**

Identification was done according to Claus and Berkeley (1986), Schleifer (1986) and Palleroni (1989) in Bergey's Manual of Systemic Bacteriology.

## 3. Results

**Screening the Collected Isolates for Phospholipase Production**

Of the collected 205 bacterial isolates, 43 bacterial isolates having different morphological characters were found to produce phospholipases (Table 1). Phospholipase producing isolates were evaluated (Table 2) and isolates that showed very strong phospholipase productivity (Pz < 0.6) were further evaluated for their extracellular PLC production assay (Table 3). The isolate designated D183 gave the highest PLC productivity among the Gram-negative rods isolates, the isolate D173 gave the highest PLC productivity among the Gram–positive cocci in clusters isolates, and the isolate D101 was the highest PLC producer among the Gram-positive thick rods isolates. Therefore, these three isolates were selected for further studies and were identified as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus* respectively.

**Table 1. Prevalence of phospholipase production among screened bacterial isolates.**

| Isolate Category | Microscopical Characters | Total number of screened isolates | Number of PL producing isolates | Number of PL producing isolates in each category (% of PL producing isolates in each category) |
|---|---|---|---|---|
| **Gram-negative** | Rods | 103 | 26 | 115 (22.6) |
| | Coccobacilli | 12 | 0 | |
| **Gram-positive** | Cocci in clusters | 60 | 12 | 90 (18.9) |
| | Thick rods | 5 | 5 | |
| | Diplococci | 22 | 0 | |
| | Cocci in chains | 2 | 0 | |
| | Cocci in tetrads | 1 | 0 | |
| | | **∑ 205** | **∑ 43** | |

**Table 2. The level of phospholipase production by the producing isolates.**

| Microscopical Characters | Number of isolates showing phospholipase activity of: | | | |
|---|---|---|---|---|
| | ++++ | +++ | ++ | + |
| **Gram-negative rods** | 14 | 1 | 9 | 2 |
| **Gram-positive cocci in clusters** | 9 | 2 | 1 | 0 |
| **Gram-positive thick rods** | 5 | 0 | 0 | 0 |
| | ∑ 28 | ∑3 | ∑ 10 | ∑ 2 |

++++, very strong activity (Pz <0.69); +++, relatively strong activity (Pz = 0.79–0.7); ++, mild activity (Pz = 0.89–0.8); +, weak activity (Pz = 0.99–0.9).

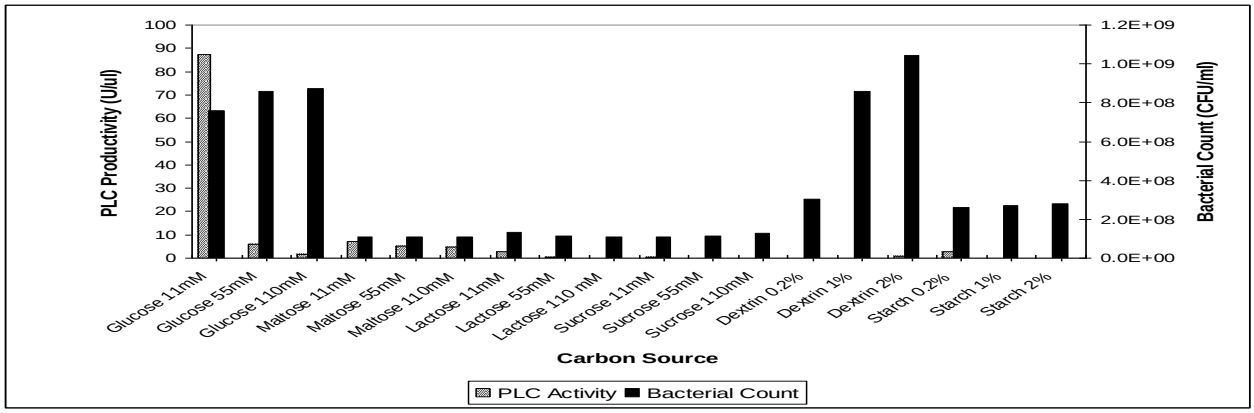
**Table 3. PLC production by selected isolates in secondary screening tests.**

| Microscopical Character | Isolate Code | Viable Count | Production (Unit/ml) | Specific Productivity (Unit/$10^6$ CFU) |
|---|---|---|---|---|
| **Gram- negative rods** | D23 | 1.25E+10 | 9.72E+04 | 7.775 |
| | D52 | 1.47E+10 | 8.73E+04 | 5.936 |
| | D53 | 1.59E+10 | 6.64E+04 | 4.175 |
| | D54 | 1.00E+10 | 9.02E+04 | 9.020 |
| | D71 | 1.25E+10 | 9.90E+04 | 7.919 |
| | D94 | 1.08E+10 | 5.88E+03 | 0.545 |
| | D117 | 1.16E+10 | 1.22E+04 | 1.055 |
| | D155 | 1.63E+10 | 4.93E+04 | 3.027 |
| | D169 | 1.47E+10 | 5.23E+04 | 3.557 |
| | D180 | 1.28E+10 | 1.31E+05 | 10.263 |
| | D182 | 1.09E+10 | 9.23E+04 | 8.466 |
| | D183 | 1.35E+10 | 1.69E+05 | 12.569 |
| | D203 | 1.34E+10 | 1.70E+04 | 1.272 |
| | N10 | 1.50E+10 | 4.70E+04 | 3.133 |
| **Gram-positive cocci in clusters** | D95 | 3.56E+08 | 2.97E+03 | 8.344 |
| | D119 | 3.43E+08 | 4.15E+03 | 12.099 |
| | D163 | 3.70E+08 | 3.17E+03 | 8.567 |
| | D166 | 3.75E+08 | 3.87E+03 | 10.328 |
| | D167 | 3.88E+08 | 1.93E+03 | 4.966 |
| | D172 | 3.29E+08 | 2.68E+03 | 8.138 |
| | D173 | 3.38E+08 | 5.67E+03 | 16.776 |
| | D193 | 3.07E+08 | 1.31E+03 | 4.279 |
| | D195 | 3.31E+08 | 3.04E+03 | 9.174 |
| **Gram-positive thick rods** | D92 | 3.00E+09 | 3.57E+04 | 11.892 |
| | D93 | 2.17E+09 | 7.91E+03 | 3.639 |
| | D99 | 2.35E+09 | 6.37E+03 | 2.712 |
| | D101 | 2.48E+09 | 3.87E+04 | 15.615 |
| | N9 | 2.63E+09 | 1.32E+04 | 5.002 |

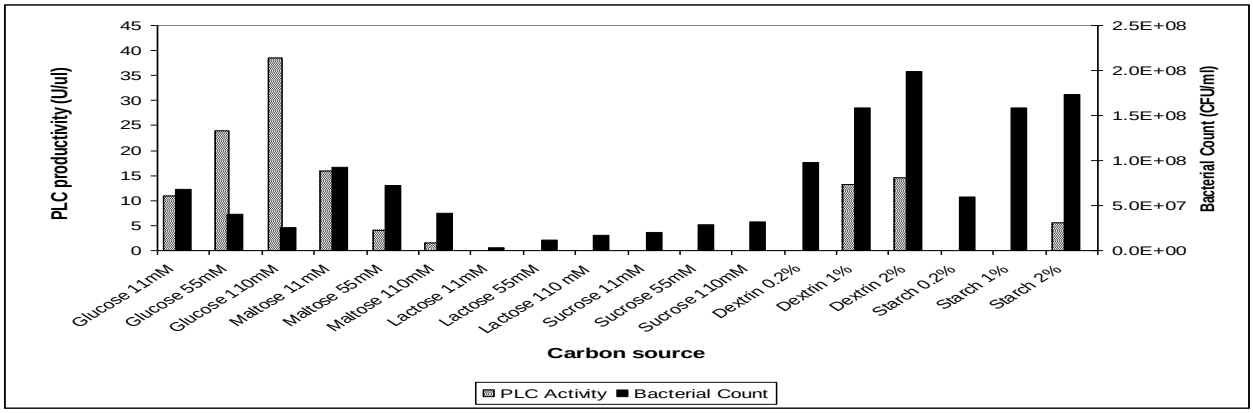
**Effect of Different Carbon Sources and their Concentrations on the Production of PLC**

For ***Pseudomonas aeruginosa*** **D183**. Increasing the concentration of glucose or replacing it with any of the tested carbon sources, at any of the tested concentrations, has led to a sharp decrease in PLC production. Higher growth levels were obtained with glucose (at all three tested concentrations) and with dextrin (at 1% and 2% concentrations) than any other tested carbon source (Figure 1a). However for ***Bacillus cereus*** **D101**, the increase in glucose concentration of phosphate-starved TMM was accompanied by an increase in PLC production. Yet, this wasn't the case with maltose which induced a higher level of PLC production than glucose at 11 mM concentration, but lower levels at 55 and 110 mM (Figure 1b). The replacement of glucose with lactose or sucrose promoted low levels of growth and no PLC production. Alternatively, dextrin and starch promoted high levels of growth but PLC production was detected only at the higher concentrations (1 and 2% for dextrin and 2% only for starch). For ***Staphylococcus aureus*** **D173,** poor microbial growth and PLC production were observed with all the tested carbohydrate sources, except for glucose at the higher concentrations (Figure 1c). When glucose at 55 mM was used, good growth was observed but without PLC production, while at 110 mM, good growth and PLC production were detected.

**a)** ***Pseudomonas aeruginosa*** **D183.**




**b)** ***Bacillus cereus*** **D101.**




**c)** ***Staphylococcus aureus*** **D173.**

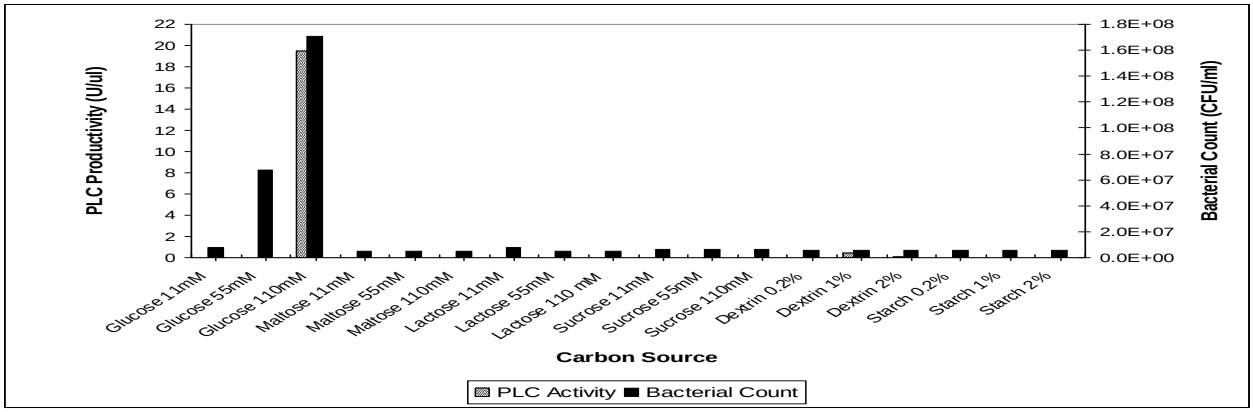


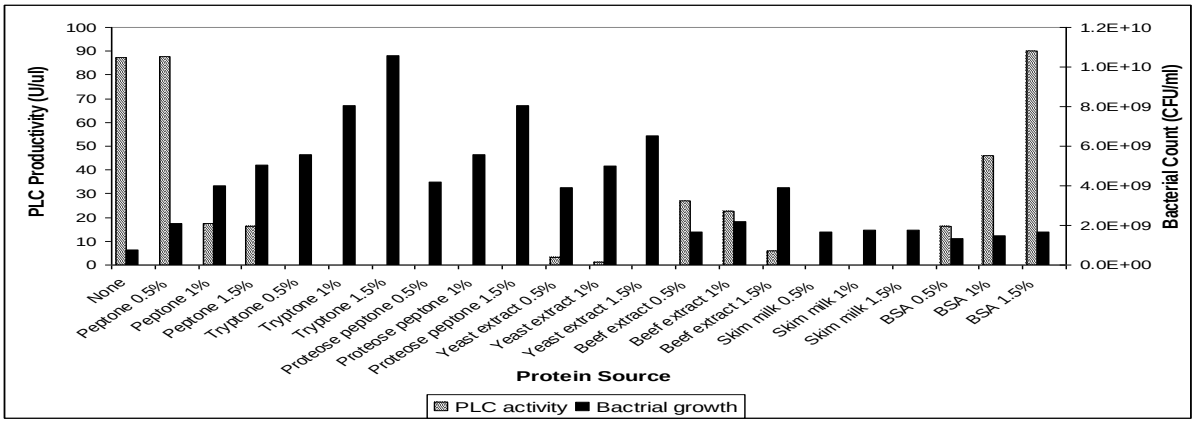

**Figure 1.** Effect of different carbon sources on growth and PLC production by *Pseudomonas aeruginosa* D183, *Bacillus cereus* D101 and *Staphylococcus aureus* D173.

**Effect of Addition of Different Protein Sources on the Production of PLC**

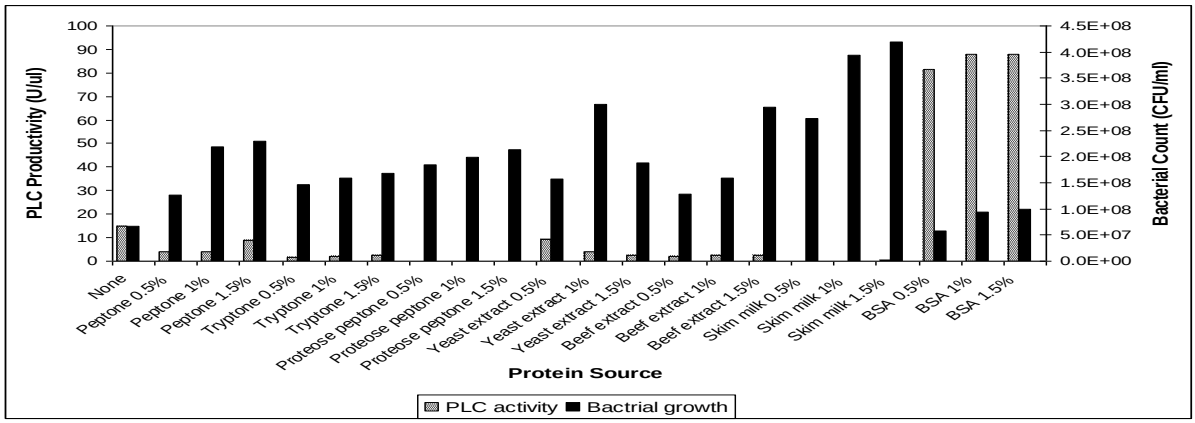
The growth of ***Pseudomonas aeruginosa*** **D183** increased upon the addition of the tested protein sources increased; however, with the exception of peptone, beef extract and bovine serum albumin, nearly no PLC production was observed by the addition of the tested protein sources at all the tested concentrations (Figure 2a). The production has fallen to zero with the addition of tryptone, proteose peptone and skim milk. The addition of beef extract resulted in low levels of PLC production and the levels decreased as the concentration of beef extract increased. Peptone at 0.5% did not affect enzyme production, but at higher concentrations, growth increased and enzyme production decreased. The increase in concentration of bovine serum albumin did not lead to a significant increase in growth; however, the inhibitory effect on PLC production has decreased until production restored its

levels as in phosphate-starved TMM at bovine serum albumin concentration 1.5%. As per ***Bacillus cereus* D101,** all the tested protein sources had a positive effect on the growth of the test isolate; however, their effects on PLC production were variable (Figure 2b). The addition of peptone, tryptone, yeast extract and beef extract has led to a decrease in PLC production. Both proteose peptone and skim milk when separately added to the phosphate-starved TMM abolished PLC production. On the other hand, the addition of bovine serum album resulted in a marked increase in PLC production (88 U/µl at 1 and 1.5% concentrations). For ***Staphylococcus aureus* D173**, although good microbial growth was obtained with all the protein sources tested, high yields of PLC were obtained only when bovine serum albumin was added to the phosphate-starved TMM (Figure 2c). Both growth and PLC production increased gradually by increasing the concentration of added bovine serum albumin.

a) ***Pseudomonas aeruginosa* D183.**




b) ***Bacillus cereus* D101.**




c) ***Staphylococcus aureus* D173**

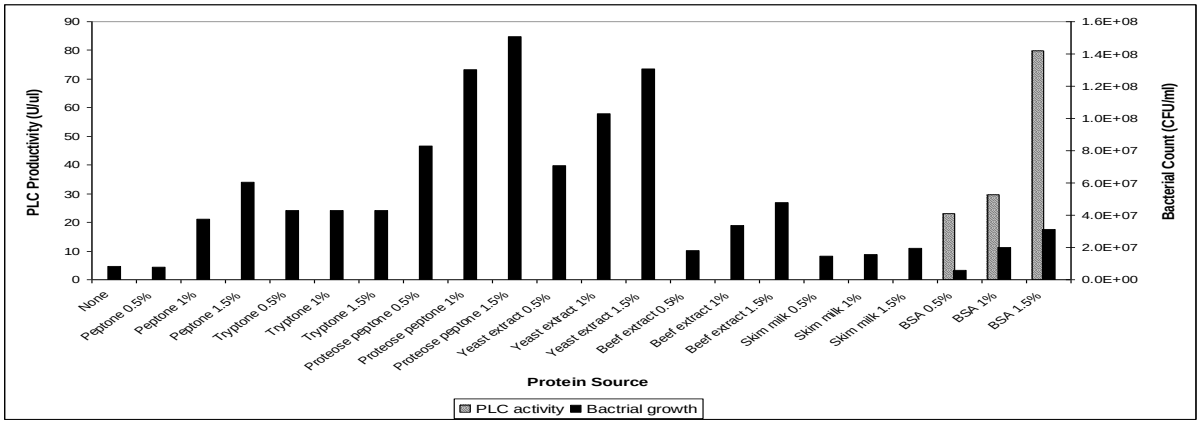
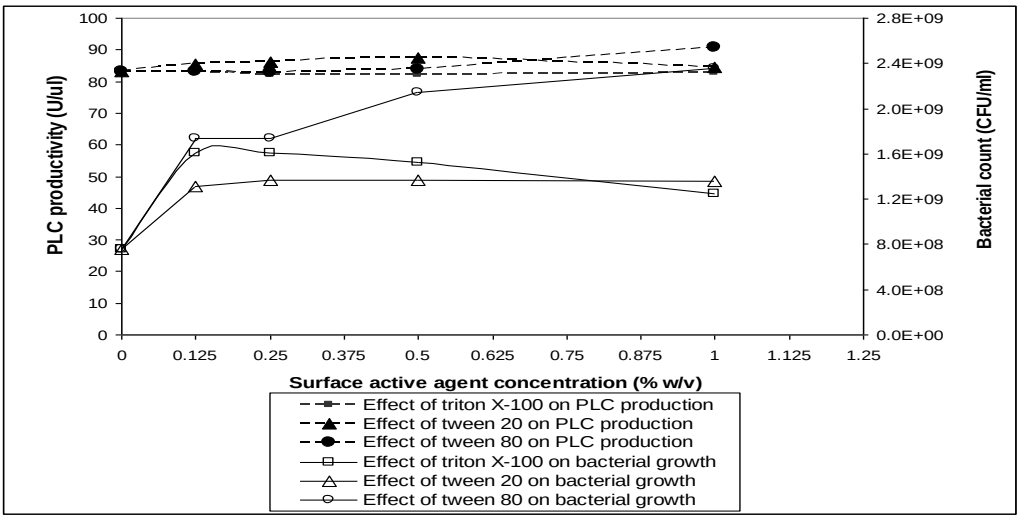



**Figure 2.** Effect of different protein sources on growth and PLC production by *Pseudomonas aeruginosa* D183, *Bacillus cereus* D101 and *Staphylococcus aureus* D173.

**Effect of Addition of Different Surface Active Agents on the Production of PLC**

The addition of triton X-100 or tween 20 to phosphate-starved TMM did not affect PLC production. However, the addition of tween 80 and sodium cholate slightly increased PLC production (less than 15% increases in both cases) by ***Pseudomonas aeruginosa*** **D183** (Figure 3). However for ***Bacillus cereus*** **D101**, the addition of triton X-100 to phosphate-starved TMM has led to a sharp decrease in growth of the test isolate, accompanied by an increase in PLC production. Tween 20 and Tween 80 have also increased PLC production by the test isolate, but the increase was accompanied by a gradual decrease in growth (Figure 4a). Sodium cholate had a positive effect on both growth and enzyme production at 0.05% (w/v) concentration, but at 0.1% (w/v) a sharp decline in growth was detected. At higher concentrations, 0.2 and 0.4% (w/v), neither growth nor enzyme production was detected (Figure 4b). As per ***Staphylococcus aureus*** **D173,** all the tested surface active agents caused a slight increase in growth of the test isolate, except for tween 20, which caused a considerable increase in growth at 0.125 and 0.25% (w/v) as compared to the other tested concentrations. While triton X-100**,** tween 80 and sodium cholate were unable to support PLC production at the tested concentrations, tween 20 supported very low levels of production at 0.125% and 0.25% (w/v) concentrations (Figure 5).

**a) Effect of triton X-100, tween 20 and tween 80.**




**b) Effect of sodium cholate**

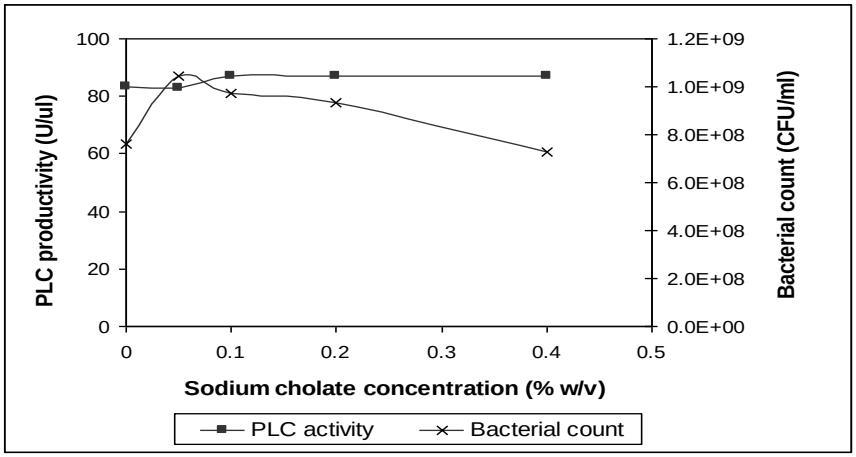
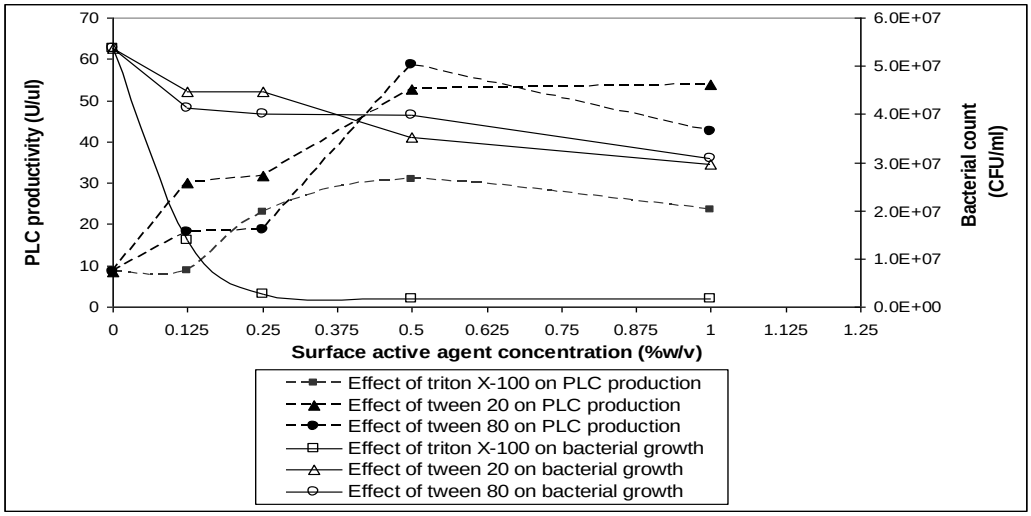



**Figure 3.** Effect of triton X-100, tween 20, tween 80 and sodium cholate on growth and PLC production by *Pseudomonas aeruginosa* D183.

**a) Effect of triton X-100, tween 20, tween 80.**




**b) Effect of sodium cholate**

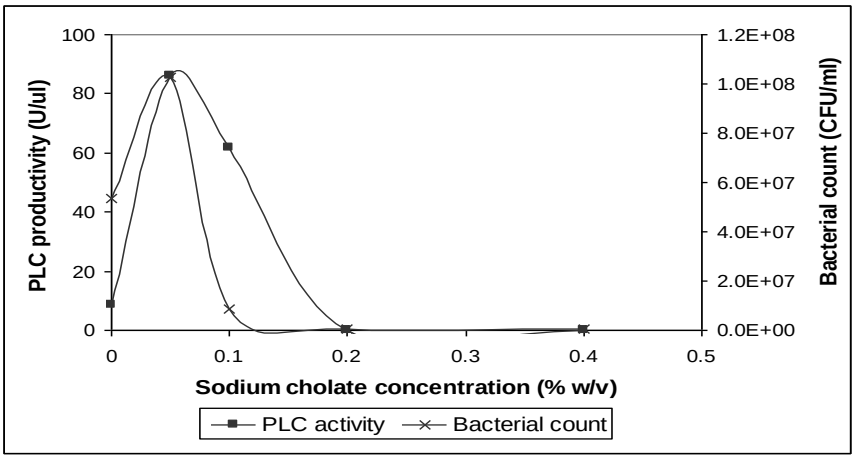
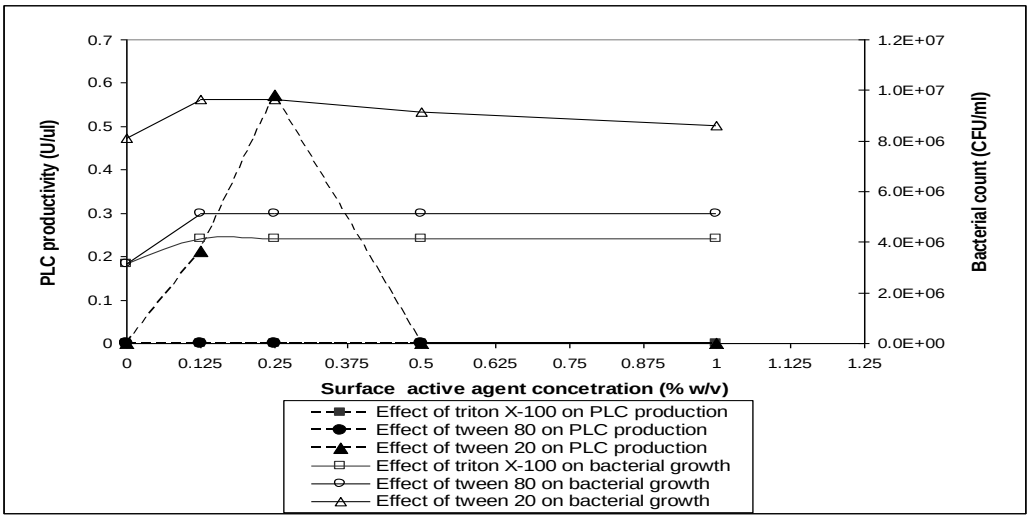



**Figure 4.** Effect of triton X-100, tween 20, tween 80 and sodium cholate on growth and PLC production by *Bacillus cereus* D101.

**a) Effect of triton X-100, tween 20, tween 80**




**b) Effect of sodium cholate.**

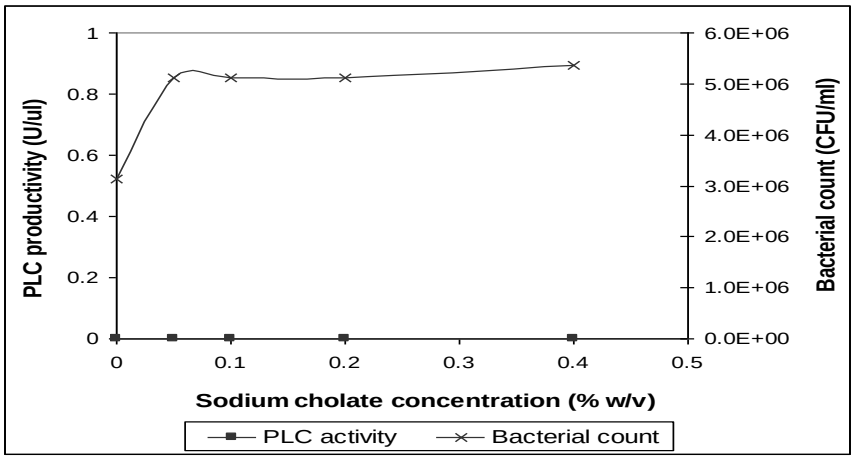



**Figure 5.** Effect of triton X-100, tween 20, tween 80 and sodium cholate on growth and PLC production by *Staphylococcus aureus* D173.

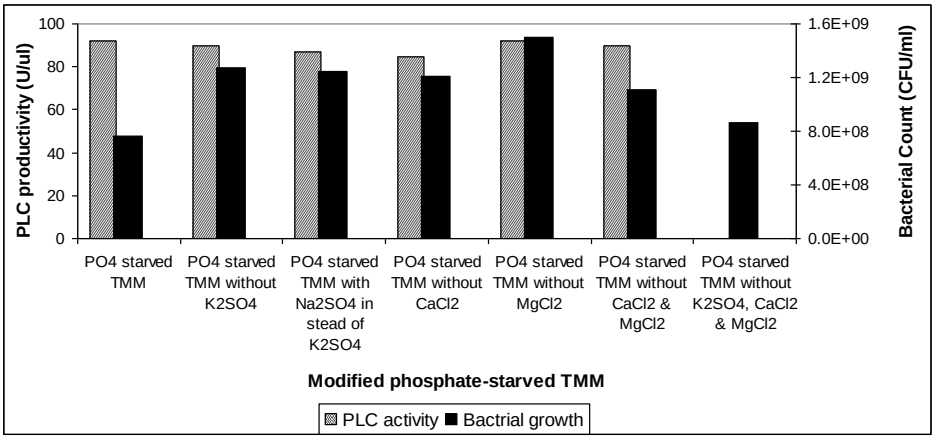
**Effect of Different Metal Ions on the Production of PLC**

**1. Effect of Removal or Replacement of Metal Salt Components of Phosphate-Starved TMM**

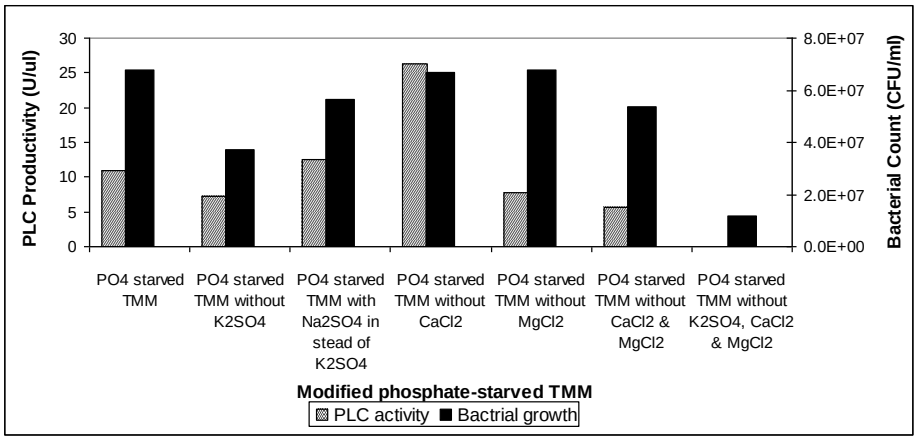
The effect of the metal salt components of phosphate-starved TMM on growth and PLC production from the test isolates was studied by removing them, one at a time, leaving only $KH_2PO_4$ unchanged, and measuring growth and PLC production in each case. So, in the first case, $K_2SO_4$ was removed, in the second, $CaCl_2$ was removed, the third, $MgCl_2$ was removed, the forth, both $CaCl_2$ and $MgCl_2$ were removed, and in the fifth case, $K_2SO_4$, $CaCl_2$ and $MgCl_2$ were removed (basal phosphate-starved TMM). In another experiment, $K_2SO_4$ was replaced with $Na_2SO_4$. The removal of $K_2SO_4$, $CaCl_2$, $MgCl_2$, both $CaCl_2$ and $MgCl_2$, or the replacement of $K_2SO_4$ by $Na_2SO_4$ showed a little effect on PLC production by ***Pseudomonas aeruginosa* D183**, although such removal increased its growth (Figure 6a). However, the removal of all three metal salts (basal phosphate-starved TMM) caused PLC production to drop to zero, yet no pronounced effect on growth was detected. With ***Bacillus cereus* D101,** unlike the case with *Pseudomonas aeruginosa* D183, removing $K_2SO_4$, replacing it with $Na_2SO_4$, or removing $MgCl_2$, $CaCl_2$ or both $CaCl_2$ and $MgCl_2$ caused a variable effect on both growth and PLC production by *Bacillus cereus* D101 (Figure 6b). Removal of $K_2SO_4$ caused a considerable decrease in both growth and PLC production while its replacement with $Na_2SO_4$ had a little effect on both parameters. PLC production by the test isolate, and not its growth, was obviously affected by the separate or combined removal of calcium and magnesium salts; where calcium removal caused more than two fold increase in PLC production, the separate removal of magnesium or the combined removal of calcium and magnesium salts decreased the enzyme production. The triple removal of potassium, calcium and magnesium ions drastically decreased bacterial growth and abolished PLC production. For

***Staphylococcus aureus* D183.** As mentioned earlier phosphate-starved TMM did not support PLC production by this test isolate although it potentiated bacterial growth. Interestingly, the removal of $K_2SO_4$ from such medium, its replacement with $Na_2SO_4$, or the separate removal of $CaCl_2$ or $MgCl_2$ relieved this inhibition and supported low levels of PLC production (Figure 6c). The highest enzyme production was observed upon the replacement of $K_2SO_4$ with $Na_2SO_4$. The combined removal of $CaCl_2$ and $MgCl_2$ did not enhance PLC production by the isolate, and neither did the combined removal of $K_2SO_4$, $CaCl_2$ and $MgCl_2$. In all cases, the resultant modified phosphate-starved TMM caused little reduction in bacterial growth.

***a) Pseudomonas aeruginosa* D183.**




***b) Bacillus cereus* D101.**




**c) *Staphylococcus aureus* D173.**

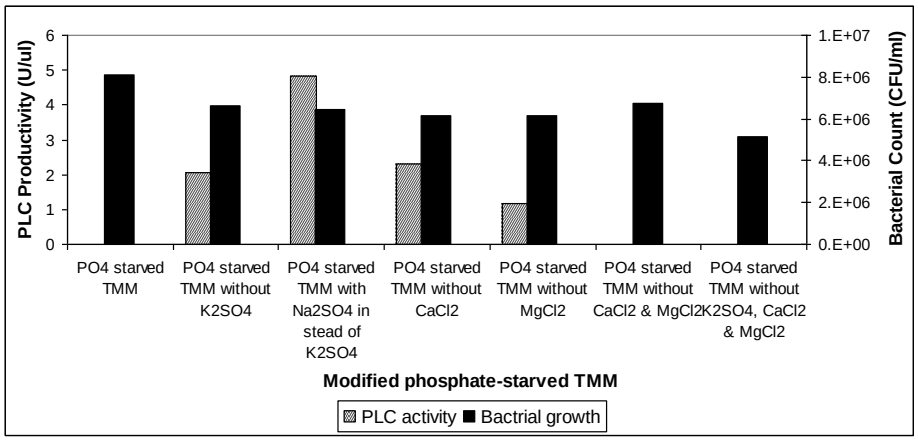
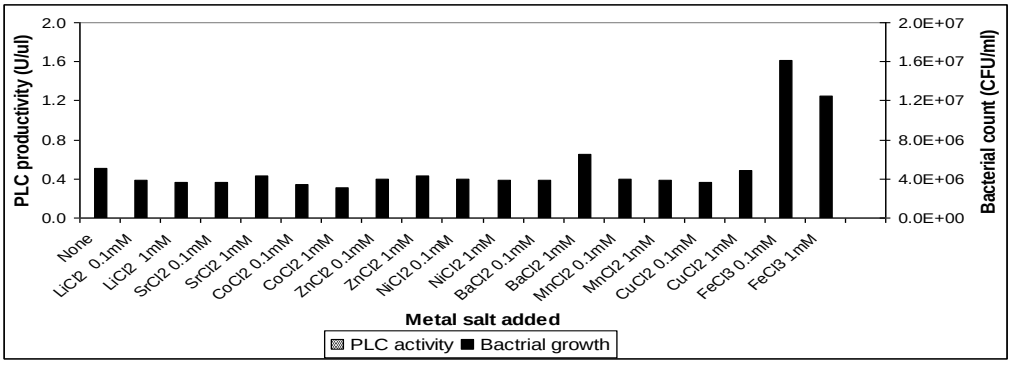



**Figure 6.** Effect of removal or replacement of metal salt components of phosphate-starved TMM on growth and PLC production by *Pseudomonas aeruginosa* D183, *Bacillus cereus* D101, and *Staphylococcus aureus* D173.

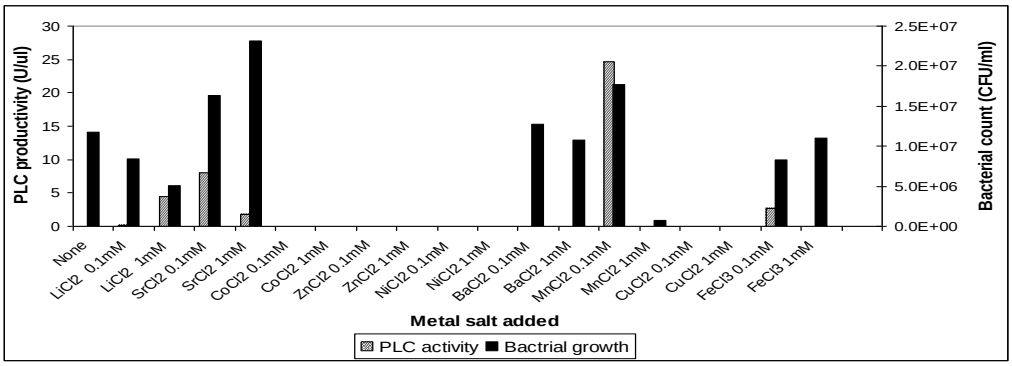
**2. Studying the Effect of Addition of Other Metal Salts to Basal Phosphate-Starved TMM.**

This was carried out by adding different metal salts to basal phosphate-starved TMM and measuring their effects on growth and PLC production. For ***Pseudomonas aeruginosa* D183,** when the chloride salts of different metals were added to basal phosphate-starved TMM, only $LiCl_2$ and $FeCl_3$ were able to restore some PLC production at the two concentration levels tested (Figure ***7***a). Nonetheless, the effect of these chloride metal salts on growth was variable; only ferric chloride at low concentration (0.1 mM) increased growth. The chloride salts of barium, lithium, and strontium had a little effect on growth while a sharp decline (≥70%) in growth was observed with cobalt, zinc and nickel chlorides. Both copper and manganese chlorides caused about 50% reduction in growth. However for ***Bacillus cereus* D101**, the addition of metal salts to basal phosphate-starved TMM was accompanied by PLC production only in case of $LiCl_2$ (1mM), $SrCl_2$ (0.1 and 1 mM), $MnCl_2$ (0.1 mM) and $FeCl_3$ (0.1 mM) (Figure 7b). On the other hand, the isolate was unable to grow when $CoCl_2$, $NiCl_2$, $ZnCl_2$ and $CuCl_2$ were added. For ***Staphylococcus aureus* D183**, none of the added metal salts was able to support PLC production from the isolate under the tested concentrations (Figure 7c). As per bacterial growth, ferric chloride showed high growth levels and still reasonable growth was obtained with other metal salts.

***a)*** ***Pseudomas aeruginosa* D183.**




***b)*** ***Bacillus cereus* D101.**




**c)** ***Staphylococcus aureus* D173.**

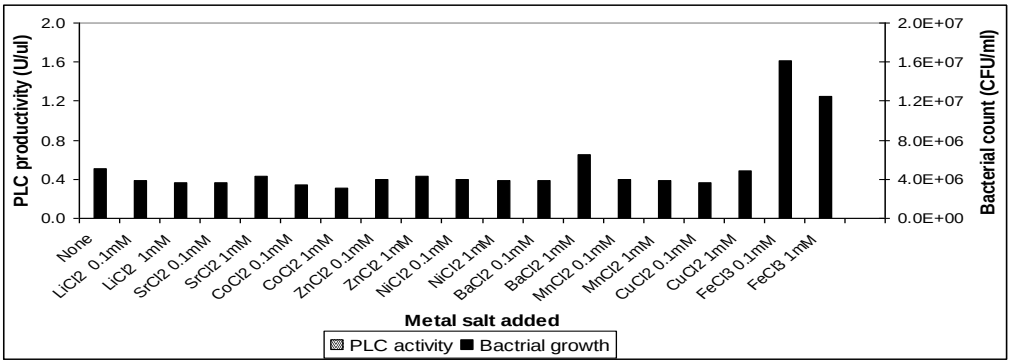


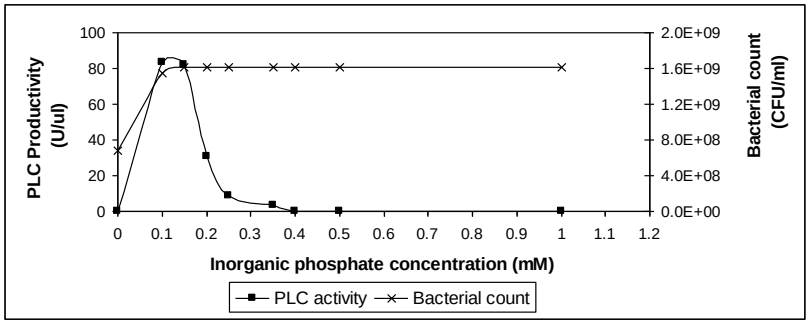

**Figure 7.** Effect of addition of metal salts to basal phosphate-starved TMM on growth and PLC production by *Pseudomonas aeruginosa* D183, *Bacillus cereus* D101, and *Staphylococcus aureus* D173.

In the forecoming experiments, for each isolate, the medium that gave maximum or comparable PLC production, compared to the other tested media, was used for studying the effect of other factors on production. Accordingly, phosphate-starved TMM was used for *Pseudomonas aeruginosa* D183, phosphate-starved TMM supplemented with 0.05% (w/v) sodium cholate for *Bacillus cereus* D101, and phosphate-starved TMM supplemented with 1.5% (w/v) BSA for *Staphylococcus aureus* D173.

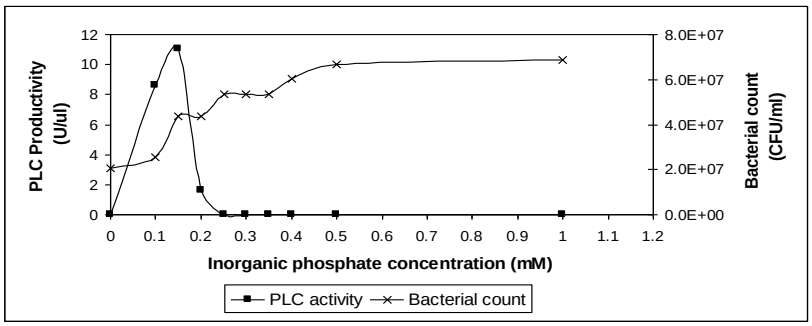
**Effect of Inorganic Phosphate Concentration on the Production of PLC**

As per ***Pseudomonas aeruginosa* D173 and *Bacillus cereus* D101**, as shown in Figure 8 (a) and (b), the optimum concentrations of inorganic phosphate for PLC production by the two isolates were 0.1 and 0.15 mM after which PLC production dropped sharply although considerable growth was detected. While for ***Staphylococcus aureus* D173**, high PLC production by the isolate was detected at inorganic phosphate concentrations in the range from 0.1 - 0.25 mM (Figure 8 c), and production decreased gradually by increasing the concentration until it reached zero at 1 mM concentration. On the other hand, growth increased with increasing inorganic phosphate concentration to reach its maximum level at 1 mM concentration.

a) ***Pseudomonas aeruginosa* D173 in phosphate-starved TMM.**




b) ***Bacillus cereus* D101 in phosphate-starved TMM supplemented with 0.05% sodium cholate.**




c) ***Staphylococcus aureus* D173 in phosphate-starved TMM supplemented with 1.5% BSA.**

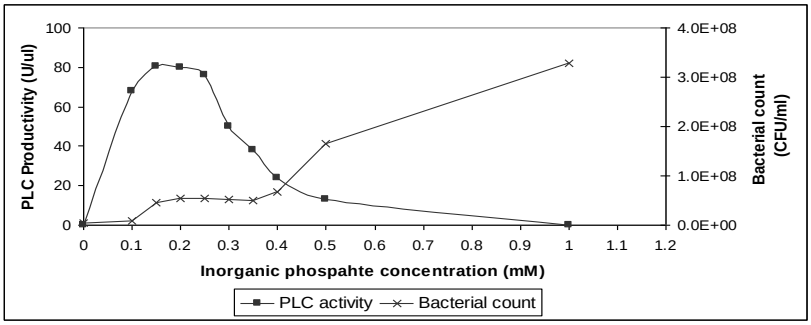
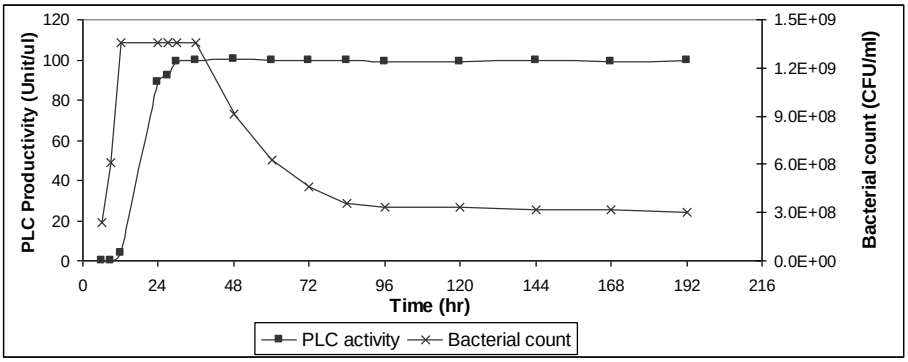



**Figure 8.** Effect of inorganic phosphate on growth and PLC production by the test isolates.

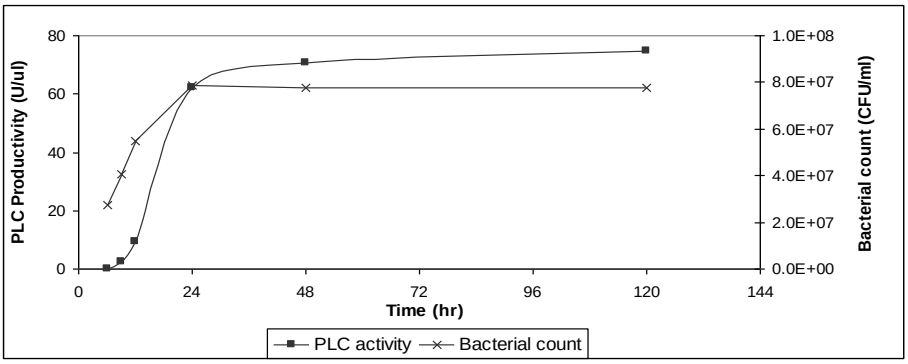
**Effect of Incubation Period on the Production of PLC**

For ***Pseudomonas aeruginosa*** **D183**, the results (Figure 9a) showed that after 12 h of incubation, cell growth reached its maximum and PLC production started to appear. Maximum enzyme production was achieved after around 30 h followed by a plateau, while cell growth showed a gradual decline after a 24 h stationary phase to reach a constant level after 84 h of incubation. While for ***Bacillus cereus*** **D101**, as shown in Figure 9b, bacterial cell growth reached its maximum after 24 h of incubation which was concomitant with the start of PLC production at high levels. Maximum PLC production was reached after 36 h. For both growth and PLC production plateau levels were observed after reaching the maxima. For ***Staphylococcus aureus*** **D173**, growth of the isolate maximized after 24 h and started to drop after 48 h of incubation. PLC production was recorded after 24 h and maximized after 36 h with no change in its level thereafter (Figure 9c).

**a)** ***Pseudomonas aeruginosa*** **D173 grown in phosphate-starved TMM**




**b)** ***Bacillus cereus*** **D101 in phosphate-starved TMM supplemented with 0.05% sodium cholate**




**c)** ***Staphylococcus aureus*** **D173 in phosphate-starved TMM supplemented with 1.5% BSA**.

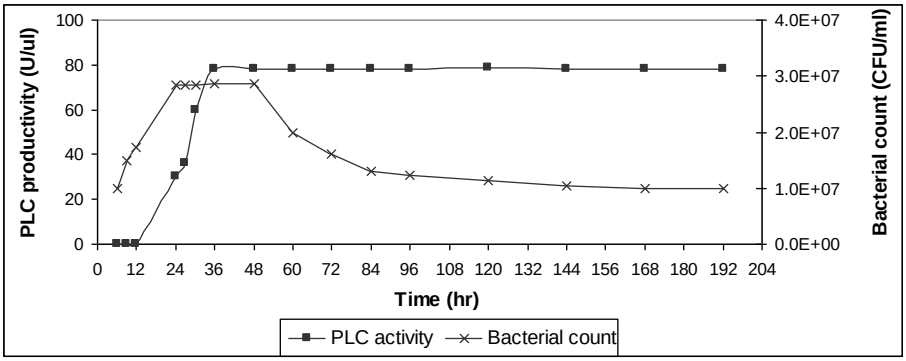
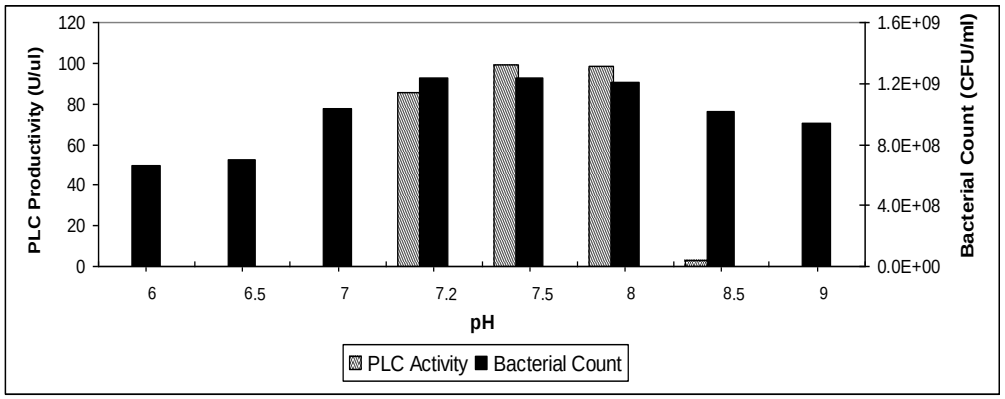



**Figure 9.** Effect of incubation period on growth and PLC production by the test isolates.

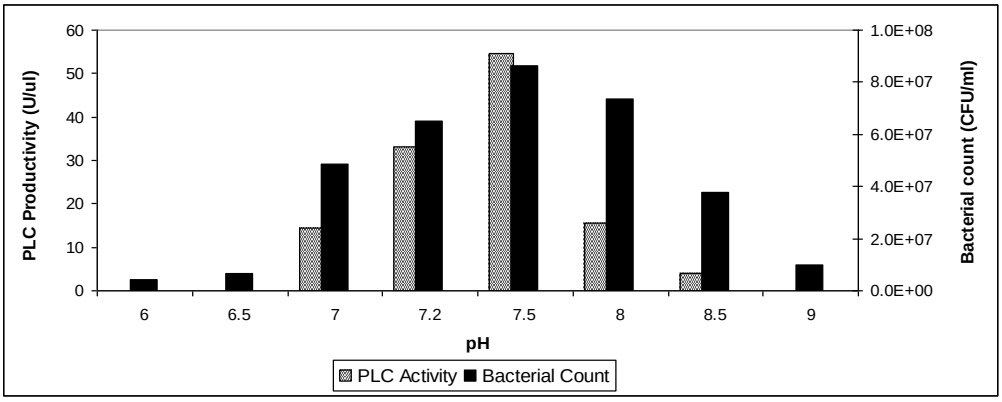
**Effect of Initial pH on the Production of PLC**

***Pseudomonas aeruginosa* D183** could grow at all the pH values tested, with maximum growth at pH 7.2, 7.5 and 8; whereas PLC production was detected only at pH values 7.2, 7.5, 8 and at a very low level at 8.5 (Figure 10a). ***Bacillus cereus* D101** showed reasonable cell growth, as well as PLC production at the tested pH values between 7 and 8.5 and maximum values for both parameters were at pH 7.5 (Figure 10b). Low cell growth levels with no PLC production were recorded at the other tested pH values. ***Staphylococcus aureus* D173** could grow at all the pH values tested; with maximum growth at pH values 7, 7.2 and 7.5 (Figure 10c). PLC production was detected only at pH values 7, 7.2, and 7.5.

**a) *Pseudomonas aeruginosa* D173 grown in phosphate-starved. TMM.**




**b) *Bacillus cereus* D101 grown in phosphate-starved TMM supplemented with 0.05% sodium cholate.**




**c) *Staphylococcus aureus* D173 in phosphate-starved TMM supplemented with 1.5% BSA.**

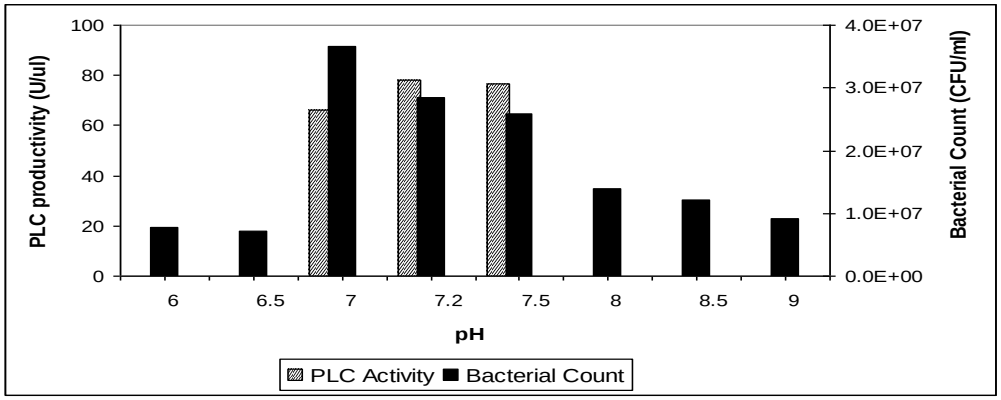
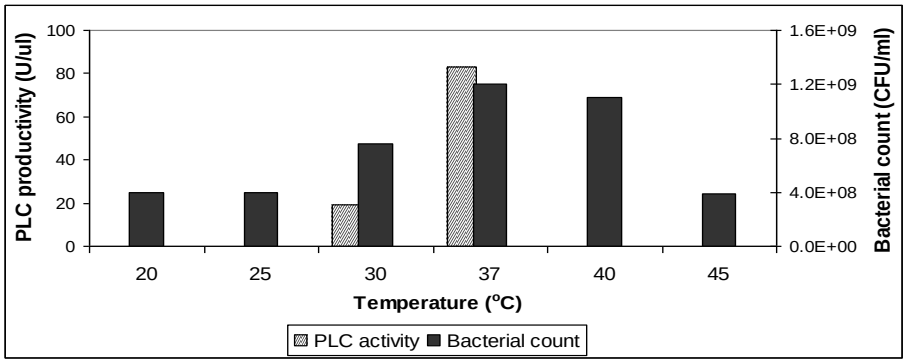



**Figure 10.** Effect of initial pH on growth and PLC production by the test isolates.

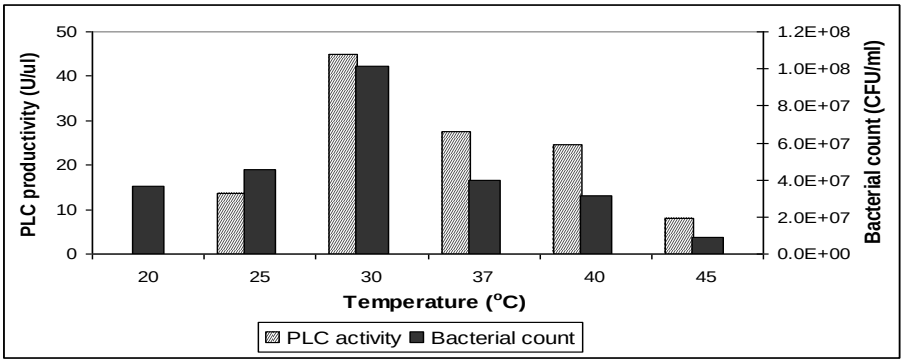
**Effect of Incubation Temperature on the Production of PLC**

***Pseudomonas aeruginosa* D183** could grow at all the tested temperatures while PLC production occurred only at 30 and 37°C. Maximum cell growth and PLC production were obtained at 37°C (Figure 11a). As per ***Bacillus cereus* D101**, although with different degrees, all the tested temperatures could promote growth of the isolate and maximum growth and PLC production were obtained at 30°C (Figure 11b). With the exception of 20°C, PLC production was detected at all tested temperatures. ***Staphylococcus aureus* D173** showed considerable growth with PLC production only at incubation temperatures 25, 30 and 37°C. However, at 20, 40 and 45°C low levels of growth with no PLC production were detected (Figure 11c).

**a) *Pseudomonas aeruginosa* D183 grown in phosphate-starved TMM.**




**b) *Bacillus cereus* D101 in phosphate-starved TMM supplemented with 0.05% sodium cholate.**




**c) *Staphylococcus aureus* D173 grown in phosphate-starved TMM supplemented with 1.5% BSA.**

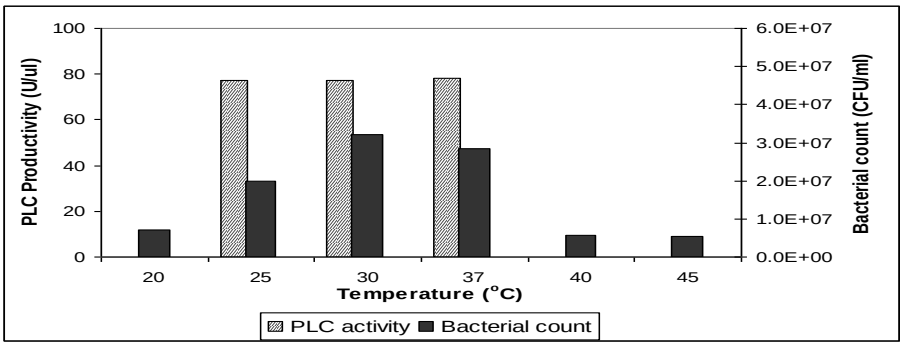



**Figure 11.** Effect of incubation temperature on growth and PLC production by the test isolates.

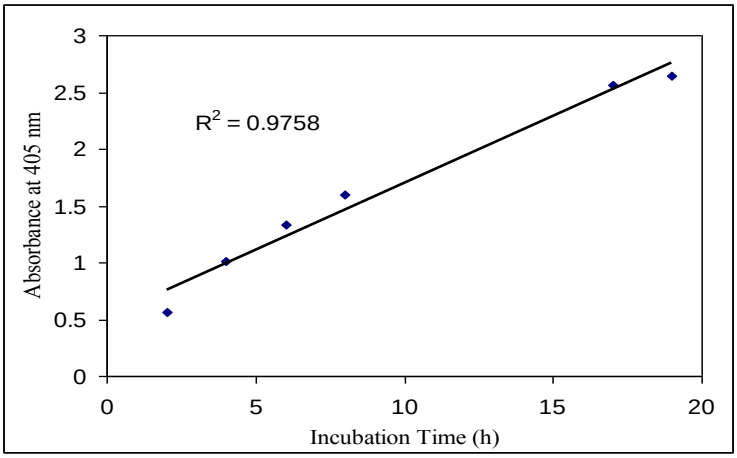



**Figure 12.** Relationship between incubation time and absorbance of yellow color liberated by a constant amount of the clear supernatant of a selected isolate using chromogenic assay of PLC activity.

**4. Discussion**

A variety of pathological specimens was collected to recover clinical isolates and evaluate their ability to produce phospholipases. The goal was to find strong PLC producing isolates from different bacterial species, then to evaluate the effect of different factors on the productivity of PLC by a few of them. The recovered bacterial clinical isolates, 205 isolates, were primarily classified by Gram staining and microscopical examination. Of these isolates, 103 were Gram-negative rods, 60 were Gram-positive cocci with the characteristic morphology of genus *Staphylococcus*, 22 isolates were Gram-positive diplococci, 12 isolates were Gram-negative coccobacilli, 5 were Gram-positive thick bacilli with the characteristic morphology of genus *Bacillus*, 2 isolates were Gram-positive cocci arranged in chains, and one isolate was Gram-positive cocci arranged in tetrads. Primary screening for phospholipase production was performed by the egg-yolk agar plate method described by Price *et al.* (1982) and the level of phospholipase production was determined as described by Dagdeviren *et al.* (2005). About 25% of the screened isolates were able to produce phospholipase; of these positive isolates 64% were very strong producers, 12% were strong producers, 19% were mild producers, and 5% were weak producers. Phospholipase production prevails among Gram-negative rods, but not among Gram-negative coccobacilli, and among Gram-positive thick rods and Gram-positive cocci in clusters but not among Gram-positive diplococci, cocci in chains, or in tetrads. Phospholipase production is also common among yeasts.

Of the 43 phospholipase producing isolates obtained in the primary screening, 28 were considered very strong phospholipase producers. Therefore, these isolates were subjected to a more quantitative secondary screening test for characterizing their PLC productivities using the chromogenic assay in which NPPC was used as a substrate for the enzyme. Not all the very strong phospholipase producing isolates obtained from primary screening exhibited the same high PLC productivities when assayed by the chromogenic assay method. This may be attributed to the fact that the formation of opacity on egg-yolk plates may be caused by numerous substances (Flieger *et al.*, 2000). The use of NPPC as an artificial substrate to detect PLC activity is very common (Flieger *et al.*, 2000). NPPC is cleaved by PLC to phosphocholine and p-nitrophenol. The optical density of the yellow compound p-nitrophenol (p-NP) is determined colorimetrically at 405 nm. Since the rate of hydrolysis of NPPC by PLC is very low in aqueous media, 60% sorbitol was added to the reagent to improve the hydrolytic rate. Such an effect of sorbitol may be related to an improvement in the characteristics of the reaction medium; a hydrophobic environment surrounding NPPC and PLC is suspected to exist as a result of hydration of sorbitol added to the reaction medium (Kurioka and Matsuda, 1976). In this study, NPPC was used at a low concentration (10 mM), since even at this concentration the time course curve was always linear during the period of measurement. The reaction mixture also included $ZnCl_2$, since the maximal catalytic activity of PLC is obtained in the presence of $10^{-4}$ M $ZnCl_2$ as this divalent metal cation, at the specified concentration, increases the $V_{max}$ of the catalytic reaction (Kurioka and Matsuda, 1976).

From each morphological class tested (Gram-negative rods, Gram-positive thick rods arranged in chains, and Gram-positive cocci arranged in clusters), the isolate that gave the maximum PLC production, as well as maximum specific productivity, was selected for further studies. These isolates were identified, according to Burgey's Manual of Systemic

Bacteriology (1989), with the help of Baird (1996) for the selected Gram-positive cocci in clusters isolate, and Govan (1996) for the selected Gram-negative rods isolate. The isolates were identified as *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus*, respectively.

After the biological entities for PLC production from different species have been selected and identified, the next task was to study the effect of different factors on PLC production. A basal synthetic medium that contains the basic requirements for bacterial growth was used. Tris Minimal Medium (TMM) contained ammonium chloride as an inorganic source of nitrogen; glucose as a source of carbon; tris-HCl as a buffer; $MgCl_2$, $CaCl_2$ and $K_2SO_4$ as mineral sources; and $KH_2PO_4$ at 10 mM concentration as a phosphate source (Norte *et al.*, 2003). When tested, TMM was able to support growth of the selected isolates but was unable to support PLC production by them. Testing modifications of TMM, including phosphate-starved TMM which contained only 0.1 mM $KH_2PO_4$, nitrogen-starved TMM which contained one tenth the amount of ammonium chloride in TMM, and magnesium-starved TMM at which the concentration of $MgCl_2$ is 1/1000 that in TMM and $CaCl_2$ was omitted (Norte *et al.*, 2003), the problem appeared to be in the phosphate concentration. Only phosphate-starved TMM was able to support PLC production from *Pseudomonas aeruginosa* D183 and *Bacillus cereus* D101; however production of PLC from *Staphylococcus aureus* D173 needed further adjustment of conditions. Phosphate-limiting conditions induce the expression of PLC as well as several other phosphate-regulated products such as alkaline phosphatase, and phosphate transport proteins (Titball, 1993). This has led many authors to suggest that the major role of PLC, along with phosphatases, is to scavenge phosphate by degrading host phospholipids (Pritchard and Vasil, 1986; Guddal *et al.*, 1989; Ostroff *et al.*, 1989; Hansen *et al.*, 1992; Vasil, 2006). The absence of one of these phosphate-regulated components may impede the ability of the organism to acquire phosphate during phosphate limitation (Titball, 1993).

Based on the above mentioned discussion, phosphate-starved TMM was selected as the basal medium for studying the effect of different factors on PLC production by the selected isolates. In an attempt to study the effect of the carbon source and its concentration on PLC production, the concentration of glucose in phosphate-starved TMM was increased 5 folds and 10 folds. It was also replaced by other carbon sources at the same three concentrations. These tests revealed that glucose was the most suitable carbon source for PLC production; though its optimal concentration varied among the tested isolates; for *Pseudomonas aeruginosa* D183, maximal PLC production occurred at 11 mM glucose (the concentration used in phosphate-starved TMM) rather than the higher glucose concentrations (55 and 110 mM) (Figure 1a). These findings come in agreement with the results obtained by Stinson and Hayden (1979) who studied the conditions necessary for the secretion of phospholipase C by *Pseudomonas aeruginosa* and recorded that maximum PLC production, in tryptose minimal medium, occurred when the concentration of glucose was 10 mM. On the other hand, *Bacillus cereus* D101 and *Staphylococcus aureus* D173 needed higher glucose concentrations (110 mM) for maximal PLC production (Figure 1b and c).

Although the addition of organic nitrogen sources to phosphate-starved TMM supported excellent growth of the test isolates, an inhibition of PLC production could be detected in all such preparations, except those containing BSA at 1.5% and peptone at 0.5% for *Pseudomonas aeruginosa* D183, BSA at 0.5, 1 and 1.5% for *Bacillus cereus* D101 and *Staphylococcus aureus* D173. For *Staphylococcus aureus* D173, BSA was the only additive that could support high levels of PLC production. This inhibition of PLC production by the addition of most protein sources may be attributed to the high content of organic phosphates present in these protein sources (Hasegawa *et al.*, 1982). These organic phosphates are hydrolyzed by the periplasmic enzyme alkaline phosphatase (Cheng *et al.*, 1970) into inorganic phosphates which in turn inhibit PLC production. This inhibitory effect did not accompany the addition of BSA since it is free from organic phosphates (Enomoto *et al.*, 2008); besides, BSA is known to cause inactivation of alkaline phosphatase activity (Henneman *et al.*, 1955;Foster and Bannister, 1976). These results may suggest that BSA has a positive effect on the production of PLC from the test isolates. The results also indicate that PLC production by the test isolates is favored at nutrient limiting media and that a low growth rate favors PLC production (Bjorn *et al.*, 1979; Hirata *et al.*, 1995; Ouhib *et al.*, 2006).

The addition of the tested surface active agents to phosphate-starved TMM did not have a noteworthy effect on growth or PLC production from *Pseudomonas aeruginosa* D183 or *Staphylococcus aureus* D173, at the tested concentrations (Figures 3,5). Conversely, the addition of triton X-100, tween 20 and tween 80 decreased growth and increased PLC production by *Bacillus cereus* D101. Sodium cholate addition supported high levels of growth and PLC production by the *Bacillus cereus* isolate only at low concentrations (0.05 and 0.1% w/v for PLC production and 0.05% for growth), while at higher concentrations (0.2 and 0.4%), both growth and production leveled off (Figure 4). According to Chin and Watts (1988) the

increase in PLC production caused by the addition of surface active agents is due to the perturbation of the membrane system of the isolate resulting in increased production and release of PLC. Morotomi *et al.* (1990) and Vulevic *et al.* (2004) reported that these results could be of biological significance since bile salts are present in the lumen of the colon at the concentration range found to enhance PLC production and thus DAG formation by fecal bacteria. DAG is a normal physiological activator of protein kinase C, an enzyme that plays a key role in growth control and tumor promotion, therefore, DAG produced by the intestinal microflora might stimulate growth of colonic epithelial cells. Thus an interaction between dietary lipids, bile salts, and PLC production by specific bacteria in the intestinal lumen could contribute to the risk of colon cancer development in humans.

The effect of metal ions on the growth and production of PLC by the selected isolates was tested on two steps; first, the effect of the stepwise removal of the metal salt components, with the exception of $KH_2PO_4$, from phosphate-starved TMM. The effect of replacement of $K_2SO_4$ with $Na_2SO_4$ at the same concentration was also tested. Second, was the effect of addition of different metal ion salts to phosphate-starved TMM from which all its metal ion components have been removed leaving only $KH_2PO_4$, named basal phosphate-starved TMM. For *Pseudomonas aeruginosa* D183, the removal of metal salts from phosphate-starved TMM and the replacement of $K^+$ by $Na^+$ did not affect PLC production, nonetheless, production was lost, although considerable growth was detected, when all three metal salts ($KSO_4$, $MgCl_2$, $CaCl_2$) were removed leaving only $KHPO_4$ (Figure 6a). This proves that cations are required for PLC secretion by cultures of *Pseudomonas aeruginosa* (Stinson and Hayden, 1979). Only $Li^+$ and $Fe^{3+}$ ions could restore some PLC productivity when added to basal phosphate-starved TMM (Fig. 7a).

For *Bacillus cereus* D101, PLC production was lost only when all three metal ion salts ($KSO_4$, $MgCl_2$, and $CaCl_2$) were removed; however, PLC production increased, without an increase in bacterial growth, when $CaCl_2$ was omitted (Figure 6b). The addition of Co, Ni, Zn and Cu chlorides, at the tested concentrations, to basal phosphate-starved TMM completely inhibited the growth of the microorganism. Similar results about the toxicity of these metal ions on growth of *Bacillus* sp. were reported by several researchers including (Babich and Stotzky, 1978; Freitas et al., 2008; Kamala-Kannan et al., 2008). Although, the mechanisms responsible for the toxicity of these metal ions are essentially undefined, it has been suggested that they may interfere with the metabolism of $Mg^{2+}$ (Babich and Stotzky, 1978). On the other hand, the addition of Li, St, Mn and Fe chlorides could restore some PLC production by the *Bacillus cereus* isolate (Fig. 7b). No PLC production was detected when phosphate-starved TMM, phosphate-starved TMM without both $CaCl_2$ and $MgCl_2$, phosphate-starved TMM without the three metal salts $KSO_4$, $MgCl_2$, and $CaCl_2$ were used with *Staphylococcus aureus* D173. When $K_2SO_4$ was omitted from phosphate-starved TMM, replaced with $Na_2SO_4$, or when $CaCl_2$ or $MgCl_2$ were omitted from such medium, low levels of PLC production were detected (Figure 6c). None of the metal salts tested by addition to basal phosphate-starved TMM was able to support PLC production by this isolate (Fig. 7c).

The production of PLC by the three tested isolates was found to be dependent on the level of inorganic phosphate in the production medium. The optimum levels were found to be 0.1 and 0.15 mM $KH_2PO_4$ for *Pseudomonas aeruginosa* D183 and *Bacillus cereus* D101, and 0.1-0.25 mM $KH_2PO_4$ for *Staphylococcus aureus* D173. At higher concentrations a sharp decline in PLC production occurred. The phosphate regulation of PLC production has been previously reported by many workers including (Pritchard and Vasil, 1986; Ostroff *et al.*, 1989; Hansen *et al.*, 1992; Kaenjak *et al.*, 1993;Gabdrakhmanova *et al.*, 2002). This suggests that PLC may be a component of a phosphate-scavenging mechanism (Stinson and Hayden, 1979; Vulevic *et al.*, 2004). Under cultural conditions where inorganic phosphate has been depleted, the PLC genes become derepressed and synthesis and secretion are initiated. The nutrient requirement for phosphate is subsequently satisfied by the release of phosphomonoesters from phospholipids by the action of PLC, followed by the liberation of free phosphate by the periplasmic enzyme alkaline phosphatase (Cheng *et al.*, 1970). This has led more than a few authors to surmise that the major purpose of PLC, along with phosphatases, is to scavenge Pi by degrading host phospholipids. While this may indeed be of benefit to the PLC producing organism in a phosphate-limiting environment, it is a very limited perspective *vis-à-vis* the real potential of this virulence determinant. Vasil (2006) has put forward a scenario whereby this virulence determinant, through its ability to generate another moiety from phospholipids (choline phosphate), would provide much more than just a single ion (i.e. Pi) for the survival of the bacteria. First, the organism can utilize choline phosphate as a sole source of carbon, nitrogen and Pi. Consequently, it would be possible for this organism to survive solely on phospholipids for these needs if it is expressing PLC. A further benefit of choline phosphate is that it will enable the organism to survive and grow in high osmolarity environments. Choline, once it is generated from choline phosphate by a phosphatase, can be readily taken up and converted to the osmolyte, glycine

betaine. Under conditions of high osmolarity (e.g. lungs of CF patients for *Pseudomonas aeruginosa*) the ability of the organism to generate choline, and convert it to glycine betaine, through the hydrolysis of phospholipids could allow it survive in this hostile environment. Other organisms that do not produce PLCs would be unable to survive in such a harsh milieu where phospholipids might be the only source of choline.

The test isolates secreted PLC during late logarithmic and early stationary growth phases. There is precedence for this production pattern recorded by Stinson and Hayden (1979) for *Pseudomonas aeruginosa* in tryptose minimal medium, Durban *et al.* (2007) for *Bacillus cereus* in Luria Bertani broth, Marques *et al.* (1992) for *Staphylococcus aureus* PI-PLC in dialysate of proteose peptone broth at both pH 5.0 and 7.4, and Coffey *et al.* (1996) for *Listeria monocytogenes* in a chemically defined minimal medium. The fact that most enzyme secretion systems in bacteria have been shown to be regulated by either catabolite or end product repression (Glenn, 1976; Stinson and Hayden, 1979) suggested that the delay in the appearance of PLC was because its synthesis was repressed by some components in the culture medium. It may well be that limiting growth substrates at the late logarithmic and early stationary phases stimulate the expression of PLC (Coffey *et al.*, 1996). The increase in the activity of PLC in the medium followed the rate of bacterial growth through the logarithmic phase and was leveled off in the late stationary phase. This type of fermentation was classified as growth associated (Shiloach *et al.*, 1973). The kinetics of PLC production might indicate that the enzyme is not associated with autolysis of the cells and that bacterial phospholipids probably are not hydrolyzed by the extracellular enzyme in intact bacterial structures (Shiloach *et al.*, 1973). Moreover, the finding that PLC activity appeared to be strongly related to cell density and as cultures entered the exponential phase activity increased in a linear fashion over time supports the possibility that PLC expression is likely under the control of quorum sensing systems (Glessner *et al.*, 1999; Dong *et al.*, 2002 ).

The optimum pH for PLC production by the test isolates was found to be 7.2 and/or 7.5. This data comes in agreement with the data reported by Stinson and Hayden (1979) who had studied of the effect of pH on PLC production by *Pseudomonas aeruginosa* and indicated an optimum range of pH 7.0 to 8. Good growth of *Pseudomonas aeruginosa* D183 was detected over the pH range tested (6-9). Similar results were obtained by Wolski *et al.* (2006) who recorded good growth of *Pseudomonas* sp. cells at pH values within a range of 6 to 8 in a glucose containing minimal salt medium. However, PLC production occurred only at pH values 7.2, 7.5, 8 and at a very low level at 8.5. This indicates that extremes of pH may be an inhibitory factor for PLC production by this isolate (Vasil, 2006). For *Bacillus cereus* D101, very low levels of growth and no PLC production were recorded at pH values 6, 6.5 and 9. The results about the growth of the isolate are not in line with the earlier findings that growth of *Bacillus cereus* was possible in the pH range 4.5-9.5 (Sutherland and Limond, 1993). However, the results about PLC production by the same isolate are in line with the enterotoxin production results recorded by Beattie and Williams (2002), who reported that the amount of enterotoxin produced by the steady state cultures of *Bacillus cereus* was pH-dependent. The greatest amounts of toxin detected were at pH 7 and 8, while at the pH extremes examined (pH 5 and pH 9), very low levels of toxin were detected. As per *Staphylococcus aureus* D173, PLC production occurred only at pH values 7, 7.2 and 7.5 which were the same pH values at which considerable growth occurred. These results come opposing to those obtained by Marques *et al.* (1992) on the production of enzymatically active PI-PLC by *Staphylococcus aureus*. They reported that lowering the pH of *S. aureus* cultures from 7.0 to 5.4 progressively increased the yield of PI-PLC. And that the final yield of PI-PLC was at least five-fold greater when the initial culture pH was 5.4 compared with 7.0. They added that low pH enhanced PI-PLC activity recovered from two *S. aureus* strains capable of high PI-PLC production, but not from a strain producing little PI-PLC.

Regarding production temperature, the optimum temperatures for PLC production by the test isolates were 37˚C for *Pseudomonas aeruginosa* D183, 30˚C for *Bacillus cereus* D101 and 25-37˚C for *Staphylococcus aureus* D173. Similar results were reported by Coffey *et al.* (1996) for PLC from *Listeria monocytogenes*, where maximum PLC production was at 30-37˚C. The maximum production of protease and lipase by *Pseudomonas aeruginosa* occurred at the same temperature (37˚C) as recorded by Kanwar *et al.* (2002) and Abd Rahman *et al.* (2005), respectively. Similarly, Agata *et al.* (2002) and Nilegaonkar *et al.* (2007) reported that the optimum production temperature for emetic toxin and protease, respectively, from *Bacillus cereus* was 30˚C. Herten *et al.* (2008) has also reported that the production of enterotoxin by *Staphylococcus aureus* occurred at temperatures between 20 and 37˚C.

In conclusion, the results presented herein provide evidence that media composition, nutrient availability, inorganic phosphate concentration, pH, and temperature all have a significant effect on microbial PLC production and such effect is bacterial species dependant.

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