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Production of phospholipase C from *Pseudomonas fluorescens* MICAYA 2023

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Abstract
Phospholipase C (PLC) producing bacteria were isolated from Egyptian soil and food sources. A potent *Pseudomonas* isolate was identified as *P. fluorescens* MICAYA by 16S rRNA sequencing and recorded in the GenBank with gene accession number (OQ231499). Optimization via Plackett Burman and central composite design found soybean meal, yeast extract, NaCl and egg yolk significantly enhanced phospholipase C production. Michaelis-Menten kinetics determined a K_m of 0.4 mg/ml egg yolk and V_{max} 287 U/ml. Box Behnken design identified optimal pH 6.5, 0.55 g/l $CaCO_3$, 1.05% egg yolk, 48.5°C for 395 U/ml phospholipase C production. The phospholipase showed low cytotoxicity against human fibroblast cells. Phospholipase C concentrations (0.2-1 ml) effectively degummed Sesame, Chamomile, Watercress, Jojoba, Olive, Nigella sativa and Castor oils. Phospholipase C concentrations of 0.4-0.8 ml/L gave the highest phospholipid reduction. *P. fluorescens* Phospholipase C offers a biodegradable alternative to chemical degumming. In summary, statistical optimization successfully increased phospholipase C yields. Characterization found the enzyme works best at alkaline pH, moderate temperature, and with egg yolk substrate. The bio-degumming capability was demonstrated for multiple plant seed oils. Further immobilization and protein engineering could improve phospholipase C industrial utility.
Keywords: Phospholipase C; *Pseudomonas fluorescens*; medium optimization; oil degumming; enzyme kinetics.

1. Introduction

Phospholipase (PLC) hydrolyzes the phosphodiester bond in the phospholipid backbone to yield 1, 2-diacylglycerol and a phosphomonoester depending on the specific phospholipid species involved. Microbial phospholipases are enzymes that catalyze the hydrolysis of phospholipids. They have diverse industrial applications due to their broad substrate specificity, high activity under mild conditions, and ease of mass production **[1].** Phospholipases have been used for modifying phospholipid structures to produce specific lipids, degumming vegetable oils, synthesizing cosmetic ingredients, improving the baking properties of dough, producing flavours and aromas, and more **[2].** Microbial sources of phospholipases like fungi, bacteria, and yeast offer advantages over plant and animal sources as they can be produced via fermentation in high yields and purity **[3].** The most efficient fungal producers are *Aspergillus niger*, *Penicillium cyclopium*, and *Rhizopus arrhizus*. *A. niger* produces high yields of phospholipase A1 and A2 **[4].** Immobilized *A. niger* phospholipase A2 showed good stability for repeated degumming of vegetable oils **[5].** The most common bacterial producers are *Pseudomonas* and *Bacillus* species. *Pseudomonas aeruginosa* and *Bacillus cereus* produce extracellular phospholipase C **[6,7].** *Bacillus subtilis* secretes phospholipase A2 and has been genetically engineered to improve production. During the the stationary phase, *Pseudomonas fluorescens* could produce various secondary metabolites with antimicrobial potentials, such as hydrogen cyanide (HCN), pyoluteorin (Pit), and 2, 4-diacetylphloroglucinol (Phi), as well as the iron-chelating metabolites **[8].** Pyoverdine, Salicylic acid and Pyochelin. Protease, Phospholipase C and Lipase are examples of three types of extracellular enzymes that *P. fluorescens* strains isolated from various environments have been found to produce **[9].** The highest levels of phospholipolytic activity were determined during the stationary growth phase, indicating that a growth-phase-dependent mechanism is responsible for inducing these enzymes. Moreover, yeast producers are *Cryptococcus humicolus* which was immobilized and used for soybean oil degumming. *Candida rugosa* is a lipase and phospholipase producer, with immobilized *C. rugosa* lipase used in structured lipids production **[10].**

Microbial phospholipases such as phospholipase A1, A2, C and D have shown promise in degumming, transesterification of oils, biosynthesis of lecithin, and wastewater treatment applications **[11].** Their enzymatic hydrolysis leads to partial hydrolysis of phospholipids, making separation of gum easier **[12].**

Response surface methodology (RSM) was effectively used for the optimization and modelling of a wide variety of microbial products **[13].** This method is a combination of statistical and mathematical techniques for model construction, assessing the effect of several independent variables and getting optimum values of variables. So, the aim of this work is to optimize the production and characterization of phospholipase C from *P. fluorescens* using the statistical approach of response surface methodology, as well as study its application in degumming some plant oils.

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2. Materials and methods

2.1. Collection of samples

Sixty samples were collected from food and soil, food specimens including spoiled wheat, rice, tomato, ketchup, mayonnaise, and soybean gathered from various local markets across Cairo, Egypt. The samples were collected in sterile plastic bags and transported cooled to the Agricultural Microbiology laboratory at the Faculty of Agriculture, Ain Shams University in Cairo for further analysis. Additionally, soil samples were collected from agricultural fields at the Faculty of Agriculture at a depth of 25 cm below the surface using sterile bags. The soil samples were also transported cooled to the same laboratory and stored refrigerated at 7°C along with the food samples until they were studied further.

2.2. Media used

1- Nutrient agar: **[14].** It was used for the isolation and maintenance of bacterial isolates. Its composition was as follows: g/l beef extract 3.0, Yeast extract 1.0, Peptone 5.0, Sodium chloride (NaCl) 5.0, and agar 20.0.
2- Potato dextrose agar (PDA): **[14].** It was used for the isolation and maintenance of fungal isolates. Its composition was as follows: g/l Glucose 20.0, peeled potato 200.0 and, agar 20.0.
Media to produce phospholipase C:
3- Luria-Bertani medium (LB): **[14].** Its composition was as follows: g/l
Tryptone 10.0, Sodium chloride (NaCl) 10.0, and yeast extract 5.0.
4- Penassay medium: **[14].** Its composition was as follows: g/l
Beef Extract 1.5, Yeast Extract 1.5, Peptone 5.0, Dextrose 1.0, Sodium Chloride (NaCl) 3.5, Dipotassium Phosphate 3.68, and Monopotassium Phosphate 1.32.
5- tryptone soya broth and agar: It was purchased from Himedia Laboratories.

2.3. Screening for the phospholipase C production by *P. fluorescens*

Tryptone soya broth was prepared, and each collected food and soil sample was individually inoculated into the medium, and then incubated at 28°C for 24 hours. Colonies that displayed clear zone formation were isolated and purified by subculture on tryptone soya agar. The purified bacterial isolates were maintained on nutrient agar slants at 7°C, with monthly subculture for further studies. To test phospholipase C production efficiency, all bacterial isolates were individually inoculated into Tryptone soya broth supplemented with 2% egg yolk and incubated at 28°C for 24 hours. The isolate demonstrating the highest phospholipase production was selected for identification via 16S rRNA gene sequence analysis. Maintenance on slants and monthly subculture ensured pure cultures were preserved for ongoing studies **[15].**

2.4. Molecular identification for phospholipase C PLC 32 isolate using 16S rRNA gene analysis

Genomic DNA was extracted from the selected isolate to perform a polymerase chain reaction for amplifying the 16S rRNA gene sequence, using the universal primers F1 and R1. The PCR product containing the 16S rRNA gene sequence was used for BLAST searches against the NCBI database as per the method of Al-Dhabi and Esmail to identify similarities to existing sequences. A phylogenetic tree was constructed with the MEGA 11 software using the neighbour-joining tree-building algorithm. The 16S rRNA sequence of the isolate was compared to the ten most closely matching *Pseudomonas fluorescens* sequences in the NCBI Gene Bank to determine phylogenetic relationships. This allowed molecular identification of the bacterial isolate through 16S rRNA sequencing, PCR amplification, NCBI sequence comparison, and phylogenetic analysis with related reference strains.

2.5. Preparation of Standard Inoculum

Fifty millilitres of Luria-Bertani medium (LB) were prepared in 250 ml Erlenmeyer flasks. The flasks were inoculated with a full loop of bacteria taken from subcultured slants. The inoculated flasks were incubated at 28°C at 120 rpm agitation in a rotary shaking incubator (Lab-Line Ltd.) for 24 hours. This yielded a standard inoculum containing 7.0 x 105 CFU/ml, which was used for subsequent shake flask experiments. All experiments were performed in triplicate flasks inoculated with this standardized culture.

2.6. Effect of incubation period on phospholipase production using *P. fluorescens* MICAYA2023

To assess phospholipase C production, Tryptone soya medium supplemented with 2% sterile egg yolk was utilized. Fifty millilitres of the prepared medium in flasks was inoculated with 2.5 ml of the standardized inoculum. The inoculated flasks were incubated at 28°C at 120 rpm shaking for 24-hour. To determine enzyme activity over time, 10 ml samples were collected from the flasks at 4 h. intervals over the 24 h period. The samples were centrifuged at 10,000 rpm for 20 min. The supernatant was collected for analyzing phospholipase C enzyme activity. The cells were collected to determine cell dry weight (g/l). To calculate the growth kinetics of *P. fluorescens* during the logarithmic phase, the specific growth rate (μG h−1), doubling time (td), and multiplication rate (MR) were determined using to the formula as according to**[16].**

$$\mu G = \frac{\ln x - \ln x_0}{t - t_0}$$

$$t_d = \frac{\ln_2}{\mu G}$$

$$MR = \frac{1}{t_d}$$

Where:X: cell dry weight (g) after t time (t) of incubation.X0: cell dry weight (g) at the beginning time (t0) of incubation.

Table (1): Levels of different factors tested in Plackett–Burman design (PBD).

Factor	Unit	Symbol	Coded levels	
			-1 (Low)	+1(High)
Soybean meal	g/l	(A)	20	40
Egg yolk	ml/l	(B)	20	40
Tryptone	g/l	(C)	15	35
Yeast extract	g/l	(D)	15	35
NaCl	g/l	(E)	0.2	2
MnSO4	g/l	(F)	0.2	2
NH4Cl	g/l	(G)	0.2	2
Agitation	rpm	(H)	100	200
Inoculum size	%	(J)	3	7
pH		(K)	6	8

2.7. Plackett- Burman Design (PBD) for screening the most significant parameters affecting phospholipase C production using *P. fluorescens* MICAYA2023

Plackett-Burman experimental design (PBD) was utilized to screen and evaluate the effect of different nutritional and physical parameters on phospholipase production by the selected bacterial strain**.** The parameters included nitrogen sources, mineral salts, agitation speed, incubation time and pH. As described by **[17]**, a PBD was implemented using Design Expert® 12 software. A total of 10 factors were studied at two levels - high (+1), and low (-1) - in 18 experimental runs, as shown in **Table (1)**. The main variables selected were soybean meal, egg yolk, tryptone, yeast extract, NaCl, MnSO4, NH4Cl, agitation speed, incubation time, inoculum size and pH. All media were inoculated with 5% standard inoculum containing 7.0x105 CFU/g cell dry weight. Post-inoculation, the flasks were incubated under shaking conditions using a rotary shaker at 30°C and the designated agitation speed per run (rpm). Phospholipase activity was assayed after 48 hours of incubation. The experiments were performed in duplicate. Phospholipase activity was determined.

2.8. Central composite design (CCD) for optimizing the most significant parameters affecting phospholipase C production using *P. fluorescens* MICAYA2023

Central composite design was utilized to optimize phospholipase C production by the *P. fluorescens* MICAYA2023 using the two most significant variables identified from the previous Plackett-Burman design. These two variables were studied at three levels, low (-1), medium (0), and high (+1), in a 13-run experiment. The variables were: (A) soybean meal, tested at 10.8579, 15, and 30.1421 g/l; and (B) egg yolk, tested at 2.5, 3, and 5 ml/l, as shown in **Table (2).** All trials were performed in duplicate and phospholipase C activity was measured. After completing the experiments, the maximum phospholipase C activity was considered as the response variable (Y). A second-order polynomial equation was fitted to the data using multiple regression analysis. For the two-factor system, the model equation was: Y= β0 + β1A +β2B + β11A2+β22B2+ β12AB, where Y is the predicted response; β0 is the intercept; β1, β2 are linear coefficients; β11, β22 are squared coefficients; and β12 is the interaction coefficient. The Fisher test evaluated the statistical significance of the model equation and terms. The coefficient of determination (R2) determined how well the second-order polynomial model fit the data. Three-dimensional response surface plots were generated from the fitted model equation to illustrate the relationship between phospholipase C production and the tested levels of the parameters.

Table 2 : Levels of nutritional factors tested in CCD design.

Factor	Symbol	Levels of variables tested in CCD		
		-1 (Low)	0 (Mid)	+1 (High)
Soybean meal	(A)	10.8579	15	30.1421
Egg yolk	(B)	2	2.5	5
NaCl	(C)	0.01	0.02	0.05

2.9. Effect of egg yolk substrate concentration on enzyme velocity

To determine the kinetic parameters Vmax and Km, the effect of substrate concentration on phospholipase activity was investigated. The partially purified enzyme was incubated with varying volumes of egg yolk substrate (0, 0.5, 1, 1.5, 2, 2.5, 3%) in the reaction mixture and assayed under standard conditions at each concentration. Enzyme activity per unit of time

was measured at each substrate level. The data obtained was plotted using both the **Michaelis-Menten,1913** and **Lineweaver and Burk,1934** to calculate the values of Km and Vmax, as described **by Michaelis and Menten, 1913.** By assaying enzyme activity across a range of substrate concentrations and graphing the data, the maximum velocity (Vmax) and Michaelis constant (Km) kinetics values for the phospholipase enzyme could be determined. The Michaelis-Menten and Lineweaver-Burk plots allowed visualization and derivation of these important enzymatic parameters **[18].**

Michaelis and Menten Equation **[19].**

V= Vmax x **(S) /** Km + (S) The equation is nonlinear.

Where: V= Initial velocity of the reaction, Vmax = Maximum initial velocity, Km = Michaelis constant, (S) = Substrate concentration,

Lineweaver Burk plot; **[20].**
A plot is generated from 1/ V = (Km / Vmax)(1/ (S)) + 1/ Vmax
The equation of the straight line y = mx +b
The slope of the line = Km / Vmax the x and the y intercept is 1/ Vmax

2.10. Characterization of phospholipase C enzyme from *P. fluorescens* MICAYA2023 using BOX Behnken design of response surface methodology

The Box-Behnken design is a type of response surface methodology that allows efficient estimation of quadratic models for the response variable phospholipase C activity. It requires fewer experimental runs compared to a full factorial design with the same number of factors and levels. In this study, four independent variables (pH, calcium chloride concentration, egg yolk emulsion concentration, and temperature) were selected based on prior knowledge of their potential effects on phospholipase C activity. Each variable was studied at three levels: low, medium, and high, coded as -1, 0, and +1 respectively. The specific levels for each variable were: pH: 5 (-1), 6.5 (0), 8 (+1), Calcium chloride concentration: 0.1 g/l (-1), 0.55 g/l (0), 1 g/l (+1) , Egg yolk emulsion concentration: 0.1% v/v (-1), 1.05% v/v (0), 2% v/v (+1), Temperature: 37°C (-1), 48.5°C (0), 60°C (+1). This resulted in a total of 29 unique experimental runs, allowing estimation of linear, interaction, and quadratic effects of the variables on phospholipase C activity. The design is rotatable (points evenly distributed at all radial distances from the center) and allows unbiased estimation of model coefficients. For each experimental run, phospholipase C activity was measured as described in the assay procedure. The design matrix and corresponding phospholipase C activity data were then used to fit a second-order polynomial model relating the response to the independent variables. In summary, the Box-Behnken design allowed systematic and efficient optimization of the key variables influencing phospholipase C production, with a minimum number of experimental runs. The fitted model equation was then used to identify the optimal levels of each factor for maximum enzyme activity.

2.11. Toxicological activity of phospholipase C against Human Skin Fibroblast cells (HSF)

The cytotoxicity effect of phospholipase C was tested on the human fibroblast (HSF) cell line. This experiment was conducted at Nawah Scientific Inc. (Mokatam, Cairo, Egypt). The HSF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified 5% CO2 incubator. For the cytotoxicity assay, the cells were seeded in 96-well plates at a density of 1 x 104 cells per well and allowed to adhere for 24 hours. The purified phospholipase enzyme was filter sterilized using a 0.22 µm syringe filter and diluted to appropriate concentrations (0, 5, 10, 25, 50, 100, and 200 µg/ml) in serum-free DMEM. The culture medium was removed from each well and replaced with 100 µl of the enzyme dilutions in triplicate wells. Wells with only DMEM served as negative controls. After 24 hours of incubation, cell viability was determined by the MTT assay. Briefly, the treatment medium was removed, and 100 µl of MTT solution (0.5 mg/ml in PBS) was added to each well. After 4 hours, the supernatant was discarded, the formazan crystals were dissolved in 100 µl DMSO, and the absorbance at 570 nm was measured using a microplate reader. The cell viability (%) was calculated using the formula: (Absorbance of treated cells/Absorbance of control cells) X 100. Dose-response curves were constructed to determine the IC50 value, defined as the concentration of phospholipase that reduces cell viability to 50% of the untreated control. All assays were performed in triplicate.

2.12. Application of produced phospholipase C in oil degumming hydrolysis

Based on the optimized phospholipase C production conditions, a 200ml culture was grown and centrifuged at 15,000 rpm for 20 minutes under cooling to obtain the crude supernatant containing the phospholipase C enzyme. For oil degumming experiments. The crude oils (150 g) used in this study were taken in different conical flasks. The oil was heated in a water bath up to 80°C and then 0.1 ml of citric acid solution (30 g/100 ml) was added. After homogenization for 3 min at 10.000 rpm the mixture was incubated at 80°C for 15 min under mechanical stirring (500 rpm) and centrifuged at 10.000 rpm for 15 min. The oil supernatant so formed was allowed to cool to 40°C. Afterward 2 ml of deionized water and different volumes

(0.2,0.4,0.6,0.8,1) ml of PLC were added and the mixture was mixed under at a high rate (10.000 rpm for 3 min)to provide a large surface area for emulsification.The temperature was maintained at 40°C and the oil was stirred at 500 rpm **[21].**

2.13. Phospholipase assay

To determine phospholipase activity, the concentration of released phosphorus was measured by comparison to a standard phosphorus curve. One unit of phospholipase activity was defined as the quantity of enzyme required to liberate one micromole of phosphorus per minute at 37°C. Using this standard curve method, the amount of liberated phosphorus was quantified and used to calculate the corresponding phospholipase enzyme activity in international units **[22].**

2.14. Chemicals

Egg yolk used as substrates for the phospholipase assays were purchased from Sigma-Aldrich. Soybean meal, utilized as a nutrient source, and olive oil, used for enzymatic degumming experiments were obtained from local markets in Cairo, Egypt. These reagents and materials served as substrates, nutrients and samples for evaluating phospholipase activity and application.

2.15. Statistical analysis

Analysis of variance (ANOVA) followed by post hoc multiple comparisons using Tukey's HSD test was performed for statistical analysis.For the shake flask experiments, comparison of the means from the two replicate trials was done using SPSS 26.0 software.A significance level of $p<0.05$ was used to determine statistically significant differences between means **[23].**

3. Results

3.1. Isolation and screening of phospholipase C-producing microorganisms

Results in **Tables (3 to 6) and Fig (1)** show the number and percentage distribution of bacterial, fungal, and yeast isolates collected from different sources. A total of 60 isolates were collected from food and soil. Food included (butter, cooked cheese, ketchup, mayonnaise, rice, tomato, soybean, and wheat). The percentage distributions of isolates were 36.67, 8.33 and 20% for bacteria, yeast and fungi respectively. However 35% of isolates did not producing the enzyme. These isolates were tested for phospholipase C according to the hydrolysis zone

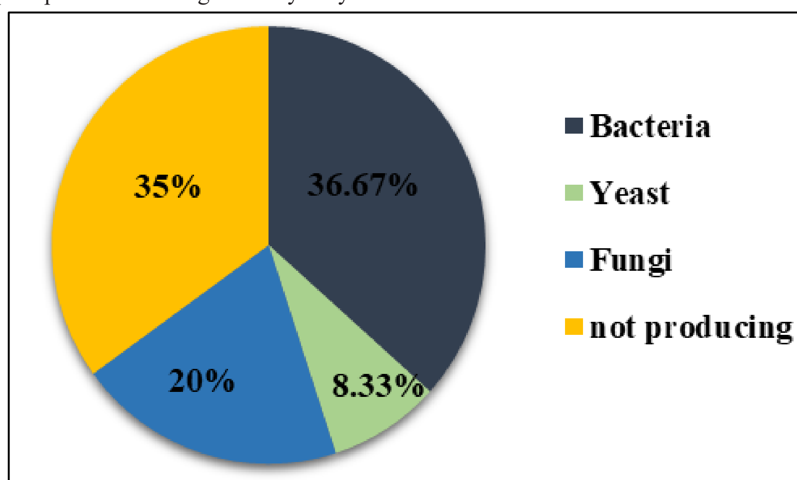

Fig (1): Percentage of Phospholipase C producing and not producing isolates.

Table (3): Number of high, moderate, and low Phospholipase C producer fungi according to the hydrolysis zone at 28° C for 3 days.

		Hydrolysis zone (mm)		
Sources of isolates	**Total number of isolates**	**High (More than 7.33)**	**Moderate (4.67 – 6.67)**	**Low (2.33 – 2.33)**
Cooked Cheese	1	-	1	-
Rice	1	1	-	-
Soil	4	2	-	2
Soybean	3	1	2	-
Tomato	1	-	1	-
Wheat	2	1	1	-
Total	12			

Table (4): Number of high, moderate, and low Phospholipase C producer bacteria according to the hydrolysis zone at 28° C and for 24 h.

		Hydrolysis zone (mm)		
Sources of isolates	**Total number of isolates**	**High (More than 6.67)**	**Moderate (4.33 – 5.67)**	**Low (1.33 – 4.33)**
Butter	1	-	-	1
Mayonnaise	1	-	-	1
Ketchup	1	-	-	1
Molasses	1	-	-	1
Cooked Cheese	1	-	1	-
Rice	3	-	1	2
Soybean	3	-	3	-
Wheat	4	1	1	2
Soil	**7**	2	2	3
Total	**22**			

Table (5): Number of high, moderate, and low Phospholipase C producer yeast isolates according to the hydrolysis zone at 28° C for 24h.

		Hydrolysis zone (mm)		
Sources of isolates	**Total number of isolates**	**High more than 7.67**	**Moderate (3.67 – 5.33)**	**Low (1.67 – 5.32)**
Soil	2	-	1	1
Soybean	1	-	1	-
Wheat	2	2	-	-
Total	**5**			

Table (6): Number of not-produced Phospholipase C isolates according to the hydrolysis zone.

Source	Total number of isolates	Hydrolysis zone (mm)
Cooked Cheese	1	0.0
Mayonnaise	1	0.0
Molasses	1	0.0
Rice	6	0.0
Soil	7	0.0
Soybean	3	0.0
Wheat	2	0.0
Total	**21**	

3.2. Screening of high-producing phospholipase C

Five isolates were selected for high producing phospholipase C. They were two bacterial isolates (*Pseudomonas* sp. (PLC32) & *Serratia* sp. (PLC31)), two fungal isolates (*Aspergillus* sp. (PLC17) & *penicillium* sp. (PLC5)) and *Saccharomyces* sp. (PLC14).

Table (7): High Phospholipase C producer's bacteria, fungi and yeast according to hydrolysis zone at 28° C and for 24/ 48/ 72 hours.

Microorganisms	Isolates	Source	Hydrolysis zone (mm)	Phospholipase activity U/ml
Bacteria	***Pseudomonas* sp. PLC 32**	**Soil**	**24.76**	**231.00**
	***Serratia* sp. PLC 31**	**Soil**	**11.33**	**92.66**
fungi	***Aspergillus* sp. PLC 17**	**Rice**	**9.33**	**79.94**
	***Penicillium* sp. PLC 5**	**Soybean**	**12.33**	**94.00**
yeast	***Saccharomyces* sp. PLC14**	**Wheat**	**7.67**	**65.70**

Fig (2): Phospholipase activity (PLC32) isolate on tryptone soya agar (TSA) medium supplemented with 2% egg yolk (as a substrate) incubated at 28°C for 24h by well diffusion method.

3.3. Identification of the selective phospholipase C (PLC 32) phenotypic identification genotypic identification

The *Pseudomonas* isolate phospholipase C (PLC 32) was identified by 16S rRNA gene sequencing and phylogenetic analysis using the neighbor-joining tree-building algorithm. As shown in **Fig. (3),** the 16S rRNA sequence of isolate phospholipase C (PLC 32) was highly like *Pseudomonas fluorescence* ATCC13525 in the NCBI database. Therefore, the isolate was identified as *P. fluorescens* and designated as strain MICAYA (accession No. OQ231499). The phylogenetic tree in **Fig (3),** illustrates the relationship of isolate MICAYA to its closest phylogenetic neighbors within other Pseudomonas species. Though the 16S rRNA gene tree contained many diverse sequence groups, the phylogenetic branches were derived from many reference sequences. Results showed 97% sequence similarity between *P. fluorescens* MICAYA and 10 other *Pseudomonas* strains. In summary, molecular identification by 16S rRNA gene sequencing and phylogenetic analysis established that isolate phospholipase C (PLC 32) belonged to *P. fluorescens* species based on its placement with the closest reference *Pseudomonas* strains

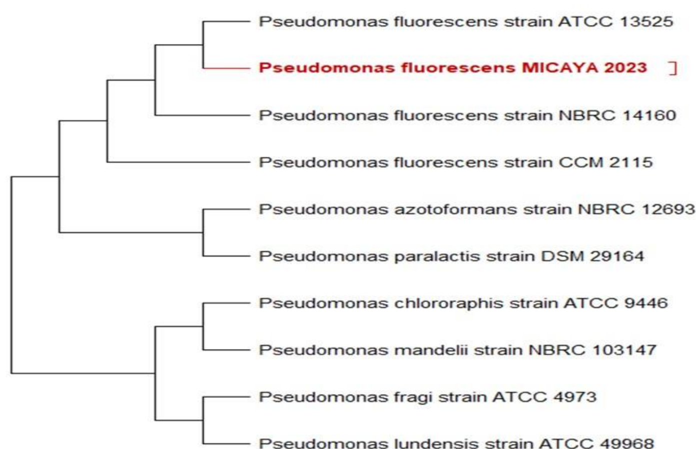

Fig (3): Phylogenetic neighbor-joining tree based on the 16S rRNA gene sequence of the most potent phospholipase C-producing *P. fluorescens* strain (marked in red color) was figured out. The DNA sequence of the isolate was deposited on the GenBank under accession number; OQ231499 (*P. fluorescens*). The phylogenetic tree was carried out using the MEGA X program, using the neighbor-joining algorithm.

3.4. Effect of incubation period on Production of phospholipase C by P. Fluorescens MICAYA2023

To determine optimal growth and phospholipase C production by the selected *P. fluorescens* MICAYA strain, the effect of incubation period was studied by culturing at 28°C for 72h. As shown in **Fig (4)**, phospholipase C activity started after 16h and reached a maximum of 53.03 U/ml by 20h incubation. The high R2 value indicated that incubation time accounted for 99% of the variation in phospholipase C production, with a specific growth rate of 0.329 µ/h, doubling time of 0.47 days, and multiplication rate of 2.1h during logarithmic growth.

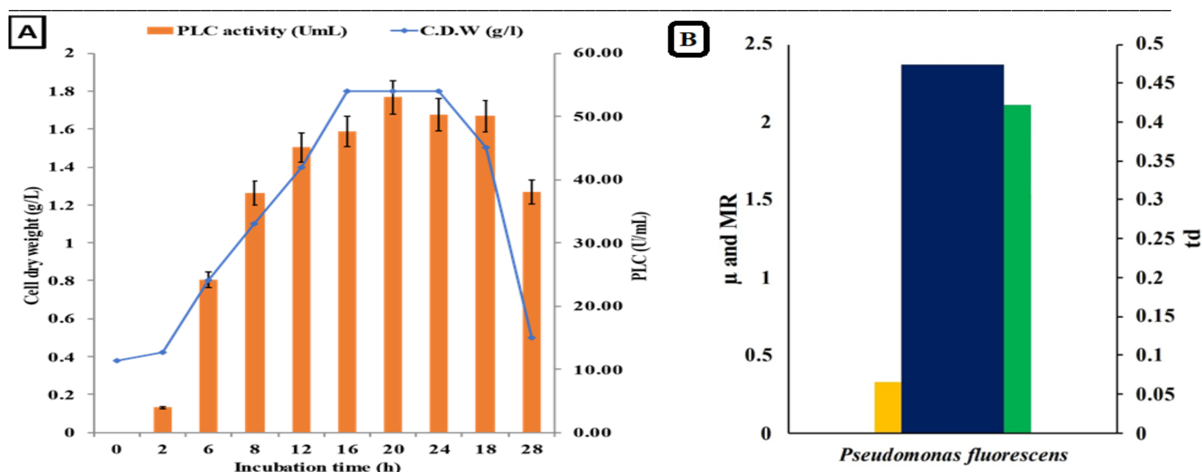

Fig 4 : Effect of incubation time on phospholipase C production by *P. fluorescens* MICAYA2023 isolate on LB medium supplemented with 2% egg yolk and incubated at 28° C for 24h. A: growth curve of *P. fluorescens* MICAYA2023 against phospholipase C production (U/ml) and cell dry weight (g/l). B: plot graph indicating the logarithmic phase of *P. fluorescens* MICAYA2023 growth against phospholipase C activity. C: Growth kinetics indicating specific growth rate (µ/h), td and MR.

3.5. Plackett- Burman design for screening variables affecting phospholipase C production.

A Plackett-Burman experimental design with 12 runs was implemented to screen 10 factors for their significance in phospholipase C production at high and low levels. The factors studied were egg yolk (A), soybean meal (B), tryptone (C), yeast extract (D), NH4Cl (E), MnSO4 (F), NaCl (G), agitation (H), inoculum size (J) and pH (K). As depicted in **Fig (5)**, the main effects plot and ANOVA analysis showed the level mean differences for each factor's effect on phospholipase C production. Results indicated soybean meal, egg yolk, and NaCl had positive effects on phospholipase C production at the high levels of 40 g/l, 5 ml/l, and 2 g/l respectively. The PBD screening was able to identify these three media components that enhanced phospholipase C production when present at higher concentrations. The main effects plot and ANOVA quantified their significant positive impacts. **Table (3)** Screens of the most significant parameters affecting phospholipase production using Plackett- Burman, Main effects plot of PBD indicating that soybean meal, Egg yolk and NaCl are the main significant factors affecting phospholipase production by *Pseudomonas fluorescence.* MICAYA2023.

Table 8: Screening of the most significant parameters affecting phospholipase C production using Plackett-Burman, Main effects plot of PBD indicating that soybean meal, Egg yolk and NaCl are the main significant factors affecting phospholipase C production by *Pseudomonas fluorescence.* MICAYA2023.

Std	Run	Factor 1 A: Egg Yolk g/l	Factor 2 B: Soybean meal ml/l	Factor 3 C: Tryptone g/l	Factor 4 D: Yeast Extract g/l	Factor 5 E: NH4Cl g/l	Factor 6 F: MnCL2 g/l	Factor 7 G: NaCl g/l	Factor 8 H: Agitation rpm	Factor 9 J: Inoculum size %	Factor 10 K: pH	Factor 11 L: Dummy	Response 1 Phospholipae Activity U/ml
10	1	20	40	35	35	0.2	0.2	0.2	200	3	8	1	350
5	2	20	20	35	15	2	2	0.2	200	7	8	-1	150
3	3	40	20	35	35	0.2	2	2	200	3	6	-1	380
8	4	40	40	15	15	0.2	2	0.2	200	7	6	1	250
11	5	40	20	35	35	2	0.2	0.2	100	7	6	1	85
9	6	40	40	35	15	0.2	0.2	2	100	7	8	-1	380
7	7	40	20	15	15	2	0.2	2	200	3	8	1	200
2	8	20	40	35	15	2	2	2	100	3	6	1	355
1	9	40	40	15	35	2	2	0.2	100	3	8	-1	350
6	10	20	20	15	35	0.2	2	2	100	7	8	1	280
4	11	20	40	15	35	2	0.2	2	200	7	6	-1	390
12	12	20	20	15	15	0.2	0.2	0.2	100	3	6	-1	75

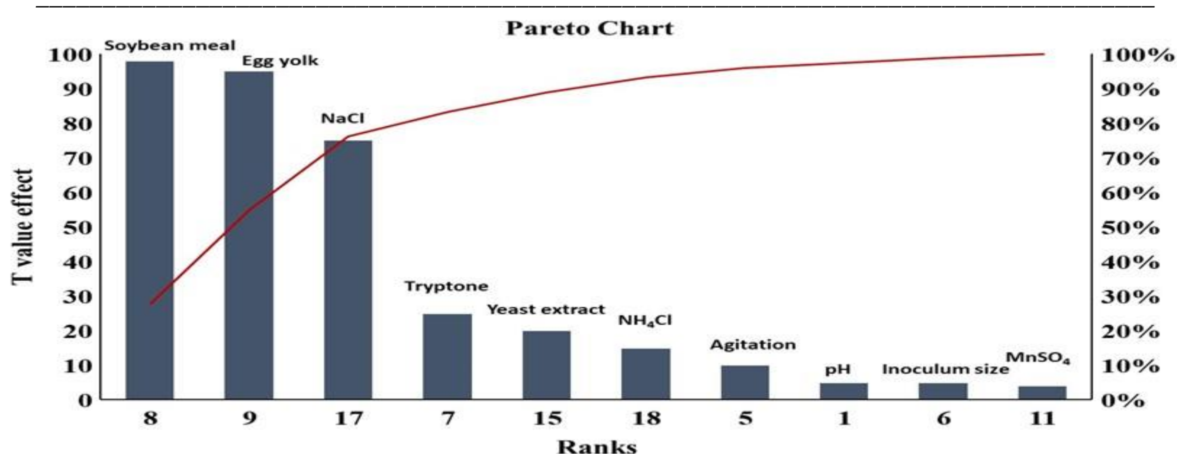

Fig (5): Pareto chart of PBD indicating the main significant factors affecting phospholipase Cproduction by *P. fluorescens* MICAYA2023.

3.6. Central Composite Design (CCD) for phospholipase production optimization

Based on the Plackett-Burman design results, the ranges for the independent variables were selected for optimization by central composite design. **Table (9),** and **Fig (6)** show the phospholipase C production for each CCD run along with predicted responses. **Table (9)** shows the results of using PBD model to predict phospholipase C production by *P. fluorescens* based on varying concentrations of four input variables: soybean meal, yeast extract, NaCl, and MnCl2. There are 35 total experimental trials shown, with the actual measured phospholipase C activity value and the predicted value from the model. Overall, the model provides fairly accurate predictions, with most of the predicted values within ±50 U/ml of the actual experimental results. The predicted and actual values align very closely in many cases, indicating good model performance. However, there are a few larger differences, like trial 32 where the model under-predicts by about 51 U/ml. While no clear trends are apparent in terms of which input variables lead to less accurate predictions, the model could likely be improved, especially to minimize the larger outliers. In general, the close alignment between most of the predicted and actual phospholipase C activities demonstrates that the model can reasonably predict phospholipase C production based on changes in medium composition. Further refinement could potentially improve model accuracy even more. The RSM software suggested levels above and below the coded values to improve the signal-to-noise ratio. Analysis of variance (ANOVA) of the CCD data gave the following regression equation in terms of actual factors: -147.245 + 7.54167 Soybean meal + 3.54167 Yeast extract + 26.3889 MnSO4 + 67.1296 NaCl with correlation coefficient of R2 0.82. The regression models were visualized as 3D response surfaces and 2D contour plots in **Fig (7), and (8),** showing the relative effects of soybean meal, egg yolk and NaCl based on the circular red contour lines. A fairly strong curvature of the 3D surfaces was observed, from which the optima were determined. An optimized medium composition was experimentally validated, giving 350 U/ml phospholipase C activity close to the 351.4 U/ml predicted by the model, indicating successful validation**. Fig (7)** shows the 3D surface plots of the interactions between the three media components (soybean meal, egg yolk, NaCl) on phospholipase C activity. **Fig (8)** illustrates the corresponding 2D contour plots of these interactions and their effect on phospholipase C production.

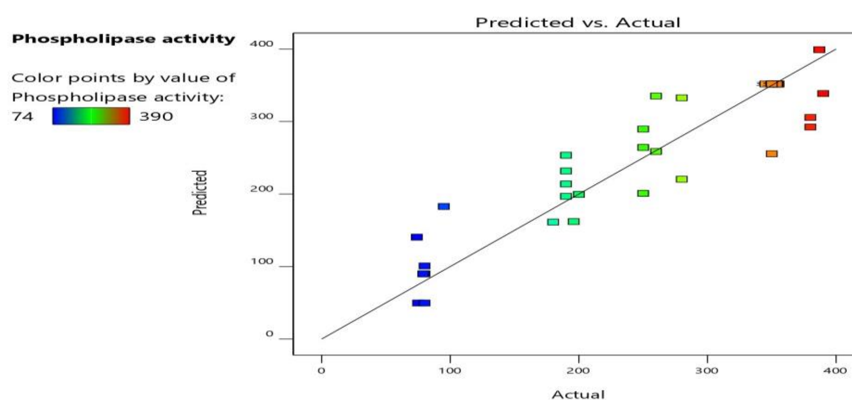

Fig 6 : Predicted against Actual activity of phospholipase C production by *P. fluorescens* MICAYA2023. Using CCD of the response surface methodology.

Table (9): Predicted against Actual activity of phospholipase C production by *P. fluorescens* MICAYA2023

	Soybean meal (g/l)	Yeast Extract(g/l)	NaCl (g/l)	MnCl2 (g/l)	Actual phospholipase C (PLC Activity) U/ml	Predicted phospholipase C (PLC Activity) U/ml
1	30	50	1.1	1.1	250	200.97
2	30	30	1.1	-0.7	380	305.64
3	30	10	1.1	1.1	250	264.30
4	30	30	2.9	1.1	380	292.47
5	30	30	-0.7	1.1	280	332.80
6	30	30	1.1	2.9	250	289.64
7	30	30	1.1	1.1	350	351.78
8	30	30	1.1	1.1	355	351.78
9	30	30	1.1	1.1	354	351.78
10	10	30	1.1	1.1	75	49.69
11	10	30	1.1	1.1	80	49.69
12	30	30	1.1	1.1	350	351.78
13	30	30	1.1	1.1	345	351.78
14	30	30	1.1	1.1	355	351.78
15	50	30	1.1	1.1	80	89.94
16	50	30	1.1	1.1	79	89.94
17	30	30	1.1	1.1	352	351.78
18	30	30	1.1	1.1	350	351.78
19	30	30	1.1	1.1	355	351.78
20	20	20	0.2	2	350	255.54
21	20	40	2	2	260	258.70
22	20	20	2	2	95	182.87
23	20	40	0.2	0.2	190	196.87
24	20	20	2	0.2	180	161.37
25	20	40	2	0.2	260	335.20
26	20	40	0.2	2	190	213.87
27	20	20	0.2	0.2	74	140.54
28	40	20	2	2	280	220.49
29	40	20	0.2	2	387	399.16
30	40	40	2	2	80	100.83
31	40	40	2	0.2	190	231.83
32	40	20	0.2	0.2	390	338.66
33	40	40	0.2	0.2	200	199.49
34	40	20	2	0.2	190	253.49
35	40	40	0.2	2	196	161.99

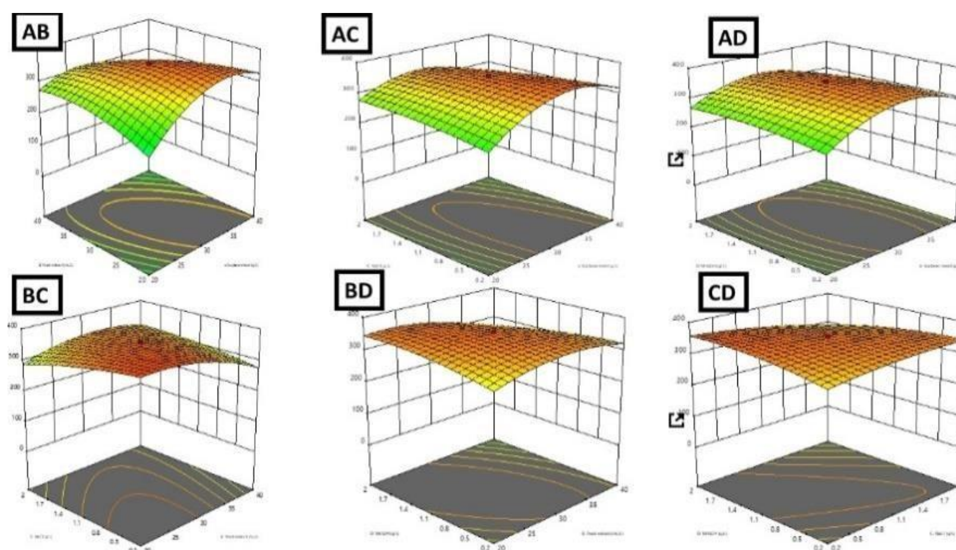

Fig (7): 3D surface plots of the CCD model for medium components showed the relative effects of soybean meal, egg yolk and NaCl on phospholipase C production by *P. fluorescens.* MICAYA2023

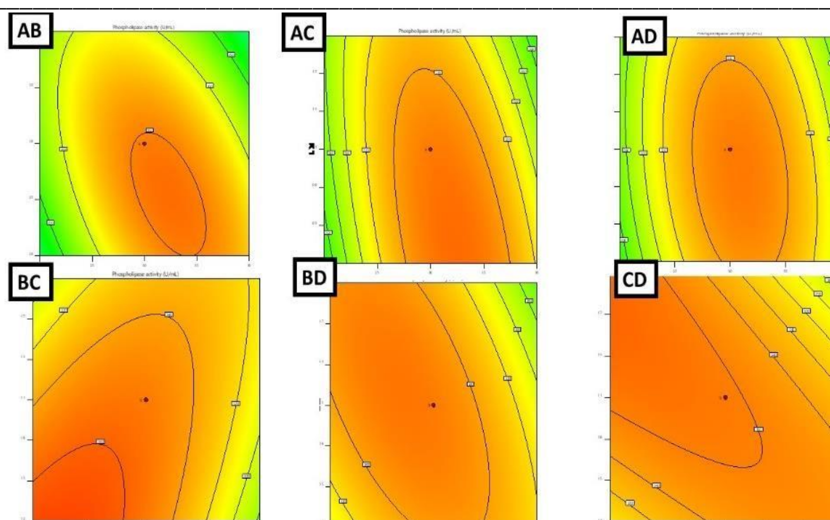

Fig (8): 2D contour lines of the CCD model for medium components showed the relative effects of soybean meal, egg yolk and NaCl on phospholipase C production by *P. fluorescens.* MICAYA2023.

3.7. Effect of egg yolk substrate concentration on enzyme velocity

As shown in **Fig (9)**, the velocity of the enzymatic reaction (first-order) increased with rising substrate concentration until saturation of the active sites occurred. Phospholipase C activity gradually enhanced with increasing egg yolk substrate up to 3 ml, giving a maximum value of 139.25 U/ml and 108.2% relative activity compared to the 2.5 ml control. At higher substrate levels, competitive binding reduced the reaction rate as sites became occupied. The kinetic parameters calculated from the Michaelis-Menten and Lineweaver-Burk plots were a Km of 0.4 mg/ml and a Vmax of 287.54 U/ml. The kinetic analysis demonstrated that phospholipase C velocity increased with substrate concentration until all sites were saturated, reaching optimal activity at 3 ml egg yolk before declining at higher levels. The Km and Vmax values signify the enzyme affinity and maximum rate under the assay conditions.

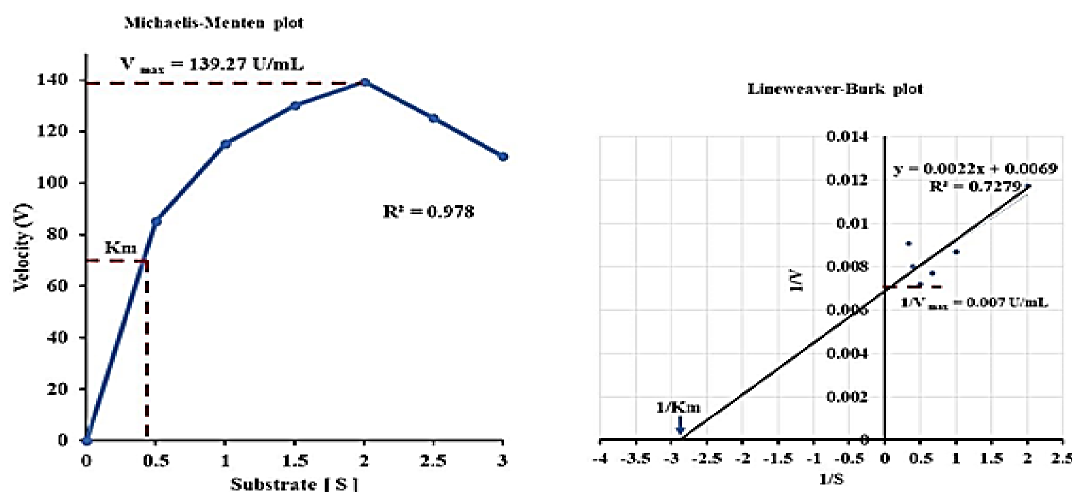

Fig (9): Phospholipase C Kinetic plots according to the specific substrate (egg yolk) concentrations for the partially purified Phospholipase C produced by *P. fluorescens*. MICAYA2023 Left: Michaelis-Menten plot, right: Lineweaver-Burk plot

3.8. Characterization of Phospholipase C enzyme from *P. fluorescens* MICAYA2023 BOX Behnken design of response surface methodology

Table (5) shows 29 experimental trials testing different combinations of pH, calcium concentration, egg yolk concentration, and temperature on phospholipase activity produced *by P. fluorescens.* A BOX statistical model was used to predict the phospholipase activity based on the input variable conditions. Overall, the model provided accurate predictions, with most values within 10 U/ml of the actual experimental results. The closest alignment between actual and predicted was seen in run 22 where both were 95.84 U/ml. The largest difference was in run 28 where the model underpredicted by about 28 U/ml. Based on the variable ranges tested, pH and egg yolk concentration had the strongest effects on enzyme activity, with optimal conditions seeming to be pH 6.5, 0.55 g/l calcium carbonate, 1.05% egg yolk, and 48.5°C. The good agreement between most

of the actual and predicted phospholipase activities indicates the BOX model can reliably predict enzyme production across different medium conditions. A few larger discrepancies suggest some refinement may further improve accuracy. Overall, the model and experiments successfully define key factors and optimal levels that enhance phospholipase yield. The RSM software tested only the high, medium and low levels for each factor. Analysis of variance (ANOVA) of the BOX data gave the following regression equation in terms of actual factors:

-955.507 + 273.997 pH + -53.6992 Ca2+ + -1.88629 egg yolk Conc. + 7.44145 Temperature + 4.92963 pH Ca2+ + -1.46316 pH egg yolk Conc. + -0.65029 pH Temperature + -3.46784 Ca2+ egg yolk Conc. + 0.232367 Ca2+ Temperature + 0.150572 egg yolk Conc. Temperature + -18.5685 pH2 + 12.2695 Ca2 + +3.65605 egg yolk Conc.2 + -0.041109 Temperature2

The models were visualized as 3D surfaces and 2D contours in **Fig (10) and (11)**, showing the relative effects on phospholipase C activity based on the circular red contours. A strong curvature of the 3D plots was seen, from which optima were determined. Experimental validation of the BBD model gave 95.27 U/ml activity close to the 95.87 U/ml predicted, indicating successful validation. Fig. 11 and 12 show the 3D and 2D plots for the interactions between pH, Ca2+, egg yolk and temperature and their effects on phospholipase C. The key points from the ANOVA model analysis showed that, the Model F-value of 6.78 indicates the model is statistically significant. P-values less than 0.05 indicate significant model terms. In this case, A^2 is significant. In summary, the ANOVA results show a significant model.

The suggested model equation is:

$$95.89 + 3.36333\,A + -0.239167\,B + 1.5925\,C + -5.60167\,D + 3.3275\,AB + -2.085\,AC + -11.2175\,AD + -1.4825\,BC + 1.2025\,BD + 1.645\,CD + -41.7792\,A^2 + 2.48458\,B^2 + 3.29958\,C^2 + 5.43667\,D^2$$

Table 10: BOX model for phospholipase C characterization parameters showed the relative effects of pH, egg yolk, Ca++ and temperature on phospholipase C produced by *P. fluorescens.* MICAYA2023

Run	Factor 1 A: pH	Factor2 B: CaCO3	Factor 3 C: egg yolk	Factor 4 D: Temperature	phospholipase activity U/ml
1	6.5	1	0.1	48.5	97.52
2	8	1	1.05	48.5	67.91
3	8	0.1	1.05	48.5	67.19
4	6.5	0.55	1.05	48.5	95.27
5	5	0.55	0.1	48.5	45.74
6	6.5	0.1	0.1	48.5	92.66
7	8	0.55	2	48.5	69.26
8	6.5	0.55	0.1	60	93.13
9	5	1	1.05	48.5	45.54
10	5	0.55	1.05	60	43.29
11	6.5	1	1.05	37	92.55
12	6.5	0.55	1.05	48.5	92.39
13	6.5	0.55	2	60	98.13
14	6.5	1	2	48.5	97.18
15	6.5	0.55	1.05	48.5	98.7
16	8	0.55	1.05	37	65.95
17	6.5	0.1	1.05	37	92.35
18	6.5	1	1.05	60	100.28
19	6.5	0.55	2	37	97.28
20	5	0.55	2	48.5	55.13
21	8	0.55	0.1	48.5	68.21
22	6.5	0.55	1.05	48.5	95.84
23	5	0.55	1.05	37	57.35
24	6.5	0.55	1.05	48.5	97.25
25	5	0.1	1.05	48.5	58.13
26	6.5	0.1	2	48.5	98.25
27	6.5	0.1	1.05	60	95.27
28	8	0.55	1.05	60	7.02
29	6.5	0.55	0.1	37	98.86

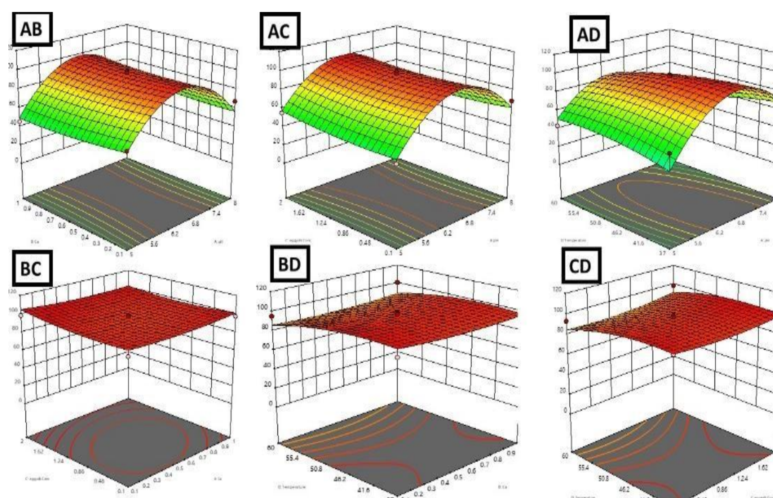

Fig 10 : 3D surface plots of the BOX model for phospholipase C characterization parameters showed the relative effects of pH, egg yolk, Ca++ and temperature on phospholipase C produced by *P. fluorescens.*

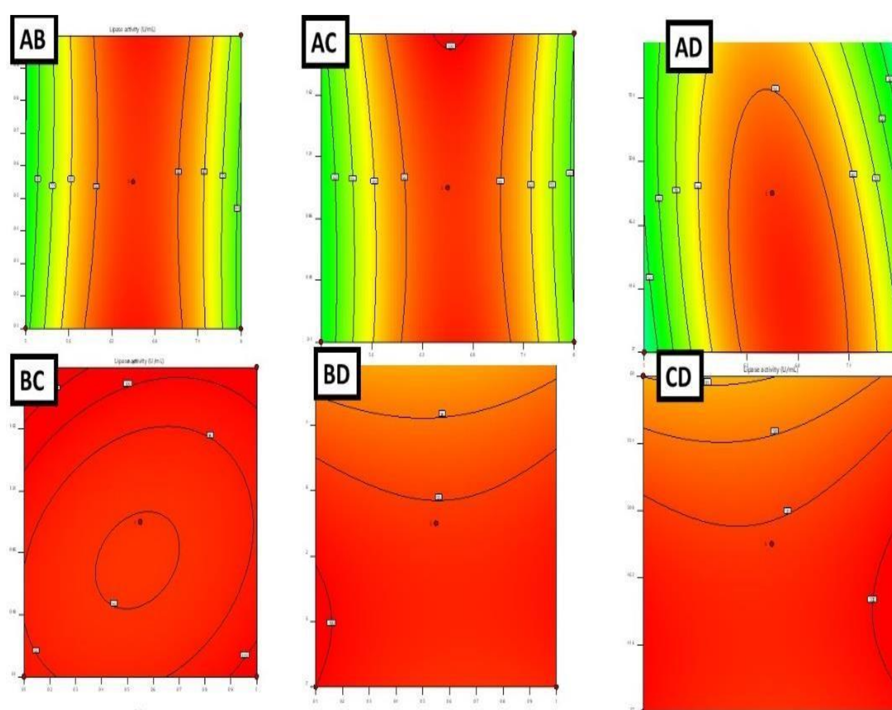

Fig 11: 3D surface plots of the BOX model for phospholipase C characterization parameters showed the relative effects of pH, egg yolk, Ca++ and temperature on phospholipase C produced by *P. fluorescens.*

3.9. Application of using phospholipase C from *P. fluorescens* in degumming different plant oils

Table 11 shows the results of using varying amounts of phospholipase C enzyme solution to degum different plant seed oils. The oils tested include Sesame, Chamomile, Watercress, Jojoba, Olive, Nigella sativa, and Castor. The amount of phospholipids remaining after enzyme treatment was measured by the Olsen method. For most oils, adding 0.2-1 ml of the phospholipase solution significantly reduced phospholipids compared to the untreated control. The optimal amount varied by oil type, with 0.4 ml working best for Sesame and 0.8 ml for olive and Nigella sativa oils. The enzyme degumming completely eliminated phospholipids in castor oil at 0.4 ml addition. Overall, the phospholipase C solution effectively degummed all the oils tested when added at suitable concentrations between 0.2-0.8 ml. The optimal amount likely depends on the initial phospholipid composition of each oil. This demonstrates the enzyme's utility for environmentally friendly degumming of diverse plant seed oils.

Table 11 : Varying amounts from phospholipase C solution were used in oil degumming process and measuring end product by Olsen method.

	Phospholipase C (ml)					
`Oil	Control	0.2	0.4	0.6	0.8	1
Sesame	0.9	3.75	8.25	5.1	3.6	3.6
Chamomile	1.2	2.55	5.55	4.8	6.15	5.25
Watercress	0.9	1.8	3.6	3.75	4.5	4.65
Jojoba	0.9	2.4	4.35	4.5	3.9	4.2
Olive	1.05	3.75	3.15	3.9	7.2	4.35
Nigella sativa	0.9	4.2	4.5	5.1	6.75	3.45
Castor	0.75	2.25	1.95	3.75	4.05	3.6

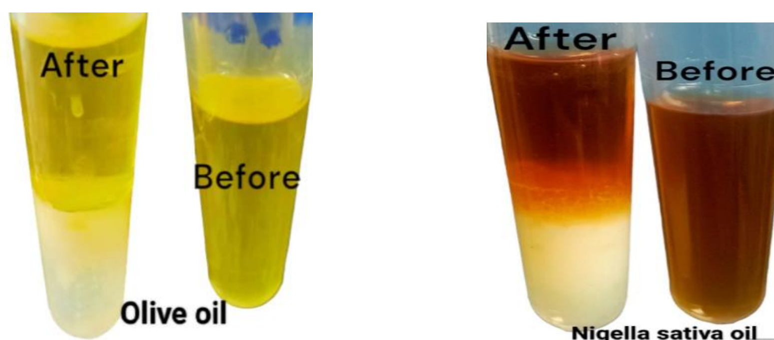

Fig 12: Phospholipase C solution effect on Nigella sativa and olive with 0.8 ml Phospholipase C solution in oil degumming process.

3.10. Cytotoxicity of phospholipase C produced from *P. fluorescens*

The cytotoxicity of *P. fluorescens* phospholipase was evaluated on human skin fibroblast (HSF) cells using the MTT assay at varying concentration from 20-200 µl/ml. As shown in **Fig (13)**, HSF cells treated with 10 µl/ml and 100 µl/ml phospholipase showed 98.2% and 96.3% viability respectively, compared to 100% viability in the untreated control. GraphPad Prism software calculated the LC50 as greater than 100% extract concentration. Microscopic imaging revealed no increased cytotoxicity of *P. fluorescens* phospholipase compared to control cells. In summary, the high HSF viability of 96-98% across a wide range of phospholipase concentrations, along with an LC50 over 100%, indicates low cytotoxicity. Microscopic examination also showed no negative impacts on HSF morphology. This demonstrates the phospholipase from *P. fluorescens* exhibits negligible cytotoxic action on human skin cells at the tested levels.

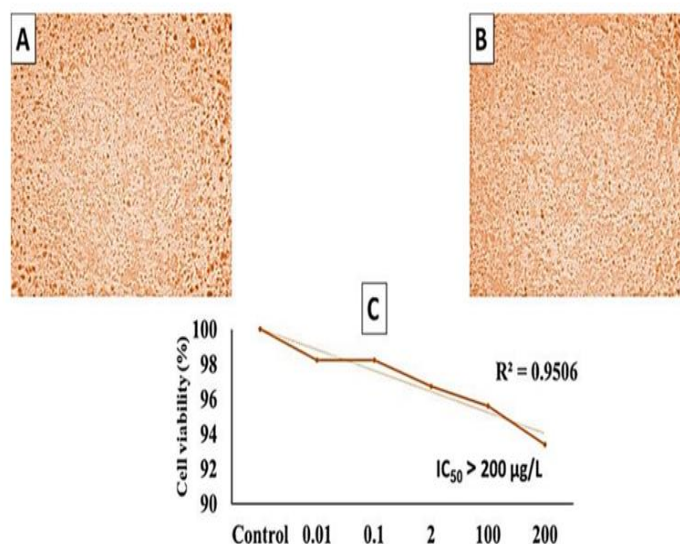

Fig 13: Toxicological activity of PLC against Human Skin Fibroblast cells (HSF) indicating normal aggregation of cell number alterations . A, control treatment. B, HSF treated with 200 µg/l of PLC produced by *P. fluorecens.* C, Cell viability test using different concentrations of produced PLC.

Discussion

The current study isolated and identified a potent phospholipase C producing bacteria from Egyptian food and soil sources. Molecular identification characterized the isolate as *Pseudomonas fluorescence* based on 16S rRNA gene sequencing and phylogenetic analysis. Statistical experimental design techniques including Plackett-Burman and central composite design were then implemented to optimize the culture medium composition and significantly enhance phospholipase C production .

The Plackett-Burman design identified soybean meal, yeast extract, and NaCl as having significant positive effects on phospholipase C yields, consistent with prior reports on microbial phospholipase C optimization **[24].** Central composite design optimization suggested an ideal combination of 30 g/l soybean meal, 30 g/l yeast extract, 1.1 g/l NaCl, and 1.1 g/l MnCl2 to achieve 350 U/ml phospholipase C activity, very close to the 351 U/ml predicted by the model. These results demonstrate the power of statistical design of experiments to systematically optimize medium formulations for improved microbial enzyme yields, as shown in earlier studies **[25].** The optimized medium can provide a cost-effective, sustainable substrte for large-scale phospholipase C production using *P. fluorescens* fermentation **[26].**

Enzyme kinetic analysis revealed a typical Michaelis-Menten curve for phospholipase C velocity with increasing egg yolk substrate concentration, reaching saturation at 3 ml. The calculated Km of 0.4 mg/ml indicates moderately high enzyme-substrate affinity, while the Vmax of 287 U/ml signifies the maximum catalytic rate under the assay conditions. These kinetic parameters provide insights into the catalytic efficiency and preferred substrate concentration range of the phospholipase C enzyme, consistent with previous kinetic analyses **[27].**Response surface methodology using Box-Behnken design effectively identified the optimal pH, temperature, calcium concentration, and egg yolk levels for peak phospholipase C activity as 6.5, 48.5°C, 0.55 g/l, and 1.05% respectively. Phospholipase C have shown wide variability in their pH and temperature optima depending on microbial source, as noted across earlier characterization studies **[28, 29].** The alkaline pH optimum aligns with previous reports of bacterial phospholipase C preferring mild basic conditions **[30]**, in contrast to acidic optima for fungal phospholipase C **[31].** The mesophilic temperature optimum is also consistent with many bacterial phospholipases **[32].** The strong influence of egg yolk and calcium highlights their essential cofactor roles in modulating phospholipase C catalytic activity, as described previously **[33].**

Significantly, the partially purified phospholipase C enzyme was able to effectively degum crude plant seed oils when added at suitable concentrations between 0.2-0.8 ml/l. All the oils tested showed substantial reductions in phospholipids compared to untreated controls, including up to 100% removal in castor oil. The optimized concentration ranged from 0.4-0.6 ml/L for the different oil types, likely depending on their initial phospholipid composition. Enzymatic degumming provides an attractive biodegradable alternative to replace the toxic chemical degumming agents currently used, as noted in earlier studies **[34, 35].** The microbial phospholipase allows mild, eco-friendly oil processing.

Cytotoxicity evaluation revealed high viability of human fibroblasts treated with up to 100% PLC, with an LC50 greater than 100% enzyme concentration. The low toxicity profile makes the PLC potentially suitable for food processing or biomedical applications, though more detailed cytotoxicity testing is needed to confirm its biosafety, as highlighted in toxicity analyses of other microbial PLC.

In conclusion, this study successfully optimized production conditions for a microbial phospholipase C from *Pseudomonas fluorescens* using statistical experimental design. Detailed enzymatic characterization found optimal activity at alkaline pH and moderate temperature. Most significantly, the phospholipase C demonstrated excellent bio-degumming capability for diverse crude plant oils. The high viability of human cells also indicates biocompatibility of the enzyme. With further protein engineering and immobilization, this microbial phospholipase C can serve as a versatile green processing aid for the food, pharmaceutical, and biofuel sectors.

Conclusion

In conclusion, this study isolated a potent phospholipase C producing *Pseudomonas fluorescence* strain from Egyptian sources. Statistical optimization significantly increased phospholipase C production levels by 3.5-fold compared to unoptimized conditions. The optimized medium contained soybean meal, yeast extract, NaCl and egg yolk. Kinetic analysis revealed increasing phospholipase C velocity with egg yolk substrate until saturation at 3 ml. Characterization found optimal phospholipase C activity at pH 6.5, 48.5°C, 0.55 g/l CaCO3 and 1.05% egg yolk. Importantly, the phospholipase C effectively degummed plant seed oils including sesame, chamomile and castor at 0.2-0.8 ml/l concentrations, demonstrating its potential as a biodegradable alternative to replace chemical degumming agents.

Author Contribution Statement

F.R, S.N, S.A conceived and designed the research. A.M and S.A. conducted experiments and collected data. SA, F.R and S.N analyzed and interpreted microbiological data. All authors wrote the draft manuscript. SA, F.R and S.N reviewed and edited the manuscript. All authors read and approved the manuscript.

Conflicts of interest

The authors have no competing interests to declare relevant to this article's content.

Ethical Statement
This article was approved by the Ethics Committee of the Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Also, the research does not contain any studies with human participants or animals performed by any of the authors.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request. Raw sequencing files and associated metadata have been deposited at NCBI's Sequence Read Archive *Pseudomonas fluorescens* MICAYA is deposited in Genbank https://www.ncbi.nlm.nih.gov/nuccore/OQ231499

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