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Statistical Optimization of Fermentation Conditions by Plackett–Burman Methodology for a New Extracellular Cholesterol Oxidase-Producing *Bacillus cytotoxicus* Strain

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CHOLESTEROL oxidase (CHO) is an oxidoreductase flavoenzyme with significant biotechnological applications. Recently, microbial CHO has received a great attention for their wide applications in medicinal field. The objective of the present work details the screening of extracellular cholesterol oxidase (CHO) producing microorganisms. Cholesterol oxidase producing bacteria were isolated from various local soil samples and some dairy products. Twelve isolates were tested for cholesterol oxidase activity using the well diffusion agar method to assess their ability to decompose cholesterol. One isolate was finally selected based on the highest productivity of cholesterol oxidase and high degree of cholesterol decomposition. The selected isolate was characterized using morphological, physical and biochemical techniques. The isolated bacterium was identified as *Bacillus cytotoxicus* strain and confirmed molecularly using 16S rRNA gene sequencing and deposited in GenBank with accession number KY367576. Classical (one-variable-at-a-time) and statistical method (Plackett–Burman design) were used to optimize growth medium to enhance the production of CHO from *Bacillus cytotoxicus*. The statistical optimized medium exhibited 2.63-fold increase in enzyme activity (2.31U/mL) and 2.57-fold increase in cholesterol decomposition (88.9%) in comparison with the un-optimized basal medium. The results revealed that *Bacillus cytotoxicus* has great potential for cholesterol degradation and produced significant levels of extracellular CHO in an optimized medium.

Keywords: *Bacillus cytotoxicus*, Cholesterol oxidase, Cholesterol decomposition, Optimization, Plackett-Burman design.

Introduction

Cholesterol oxidase (3β-hydroxysterol oxidase, EC 1.1.3.6) is a flavin adenine dinucleotide catalyzes the oxidation of cholesterol (cholest-5-en-3β-ol) to cholestenone (cholest-4-en-3-one), with simultaneous release of hydrogen peroxide (Devi & Kanwar, 2017; Yamada et al., 2019).

Bacterial cholesterol oxidases (CHO) have received a great attention due to its importance in clinical bioconversions of blood serum and food cholesterol (Doukyu, 2009) and because of its evidenced applications in various electrochemical biosensors (Vidal et al., 2004; Basu et al., 2007; Arya et al., 2008). In addition, the enzyme also finds applications in agriculture for insecticides (Purcell et al., 1993; Santos et al. 2002) and in bio-catalysis for the production of pharmaceutical steroids (Lee & Liu, 1992). More recently, CHO is also implicated in the bacterial pathogenesis and in the diagnosis of atherosclerosis, cardiovascular disease, cholesterol metabolism in cancer and other lipid disorders (Horiuchi et al., 1988; Khan et al., 2009; Molaei et al., 2014; Oggioni et al., 2015). The conversion of cholesterol by bacterial cholesterol oxidase can supply a solution for the treatment of various diseases related to cholesterol and its oxidized derivatives. These various applications processes explain the demand of isolation and selection of new CHO-producing bacterial

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strains and enhancing their productivity (Yazdi et al., 2001a). Many bacteria have been detected to produce extracellular CHO, the most common genera are *Arthrobacter*, *Brevibacterium*, *Rhodococcus*, *Streptomyces, Pseudomonas*, *Nocardia*, *Corynebacterium*, *Castellaniella* and *Shizophyllum* (Lee et al., 1989; MacLachlan et al., 2000; Yazdi et al., 2008; El-Naggar et al., 2018; Elsayed & Abdelwahed, 2020).

The optimization of the growth culture conditions is an important aspect to enhance and improve the microbial growth and productivity of cholesterol oxidase (Kim et al., 2002; Abd-Alla et al., 2018; Sahu et al., 2019). Optimization of the fermentation medium composition is generally achieved either by implementing one-factor-at-a-time (OFAT) or statistical optimization approaches (Chauhan et al., 2009; Kuddus, 2015; Nafady et al., 2015; Then et al., 2016; Latha et al., 2017; Singh et al., 2017; Hamid et al., 2018; Kepli et al., 2019; Djinni et al., 2022; Naik et al., 2022). On the other hand, optimization of growth conditions to elevate the biomass production of *Spirulina platensis* was achieved by El-Sheekh et al. (2021). The maximum glycerol productivity was achieved by optimizing the culture conditions of *Wickerhamomyces anomalus* AUMC 11687 using response surface methodology (Ali & Zohri, 2021). The best productivity of bioactive compounds by endophytic *Chaetosphaeronema* sp. KY321184 has been optimized using the experimental design methods (Osman et al., 2018). Furthermore, statistical medium optimization approaches allow investigating the interaction between different factors and reduce the number of experimental trials to be performed (Then et al., 2016; Hamid et al., 2018; Guo et al., 2019).

The objective of this current work was isolation, identification of cholesterol-decomposing bacteria and statistical optimization of fermentation conditions. *Bacillus cytotoxicu*s strain was isolated from soil sample and identified at molecular level by sequencing 16S rRNA gene region. In addition, a Plackett-Burman screening design (PBD) was adopted to optimize different parameters of growth conditions and estimate the optimum levels of the significant factors that influence the production of cholesterol decomposing enzyme by *Bacillus cytotoxicu*s.

Materials and Methods

Isolation of cholesterol decomposing bacteria

Different local garden soil samples were collected randomly from different locations and depths of Alexandria, Egypt. Also, various dairy products; raw milk and cheese samples were collected from a farm at Abies area of Alexandria, Egypt. Spread plate technique (Page et al., 1982) on basal cholesterol medium (Kumar et al., 2004) with some modifications was applied to estimate the ability of bacteria to decompose cholesterol. The composition of the basal cholesterol medium was as follows (g/L): NH_4Cl, 0.5; NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgSO_4$, 0.2; yeast extract, 0.1; agar, 18 and distilled water up to 1000mL. The pH was adjusted at 7.0 before sterilization. The medium without carbon source (cholesterol) was sterilized separately (Imshenetskii et al., 1968). Cholesterol was emulsified by dissolving 1g of cholesterol in 35mL of boiling acetone, and then added drop wise to 200mL of distilled water heated to 90-95°C. After that it was filtered, condensed under vacuum to remove the acetone. The resulting cholesterol emulsion was sterilized at 0.5atm. for 30 min and then it was added to the sterilized medium as 1g/L (Zanin, 1968). Suspensions of samples were prepared by shaking 10g of each sample with 90mL of sterile saline solution for about 30min. Serial dilutions of the range of 10^2 to 10^6 were prepared; 0.1mL of each dilution was spread on the surface of basal cholesterol agar medium plates using a sterile glass spatula under aseptic conditions. Duplicate sets of plates were used for each dilution. The plates were incubated at 37°C for 24h. The CHO producing colonies were picked up, purified and maintained in the basal media for further studies.

Screening of extracellular CHO-producing bacterial isolates

Qualitative detection using well diffusion agar method

The ability of all bacterial isolates to decompose cholesterol was qualitatively detected using the well diffusion agar method (Espuny et al., 1980). A set of pour plates each containing 15mL of basal cholesterol medium were prepared. After solidification, wells of 1cm diameter were punched into the agar medium and filled with 0.2mL (20 x 10^6 CFU/mL) of previously prepared bacterial suspension (24h age). All plates were left for 30min in a refrigerator for diffusion and

then incubated at 37°C for 24-48h. Cholesterol decomposition was qualitatively estimated by the appearance of zone of translucency on the cholesterol agar medium around the wells. Diameters of the clear zones were measured as millimeter (mm) in triplicates.

Quantitative assaying in liquid medium

This experiment was performed to detect and select the most potent bacterial isolates that have the high percent of cholesterol decomposition in liquid medium. Sterile test tubes each contains 5mL of basal cholesterol liquid medium were prepared. Each tube was inoculated with 1% (24h age) bacterial suspension and incubated at 37°C for 24 and 48h. Quantitative determination method of cholesterol was measured calorimetrically according to Liebrmann-Burchard reaction (Tietz & Logan, 1987). The main idea of this method is the precipitation of the protein present in the sample and then dehydrate the sample to form cholesterol 3, 5 diene in the presence of conc. H_2SO_4. The residual cholesterol was estimated according to the equation of Aunpad et al. (2002) as follows:

Residual cholesterol (mg/dl)= Absorption of standard/Absorption of standard × Conc. of standard.

The amount of decomposed cholesterol (mg/dl)= Amount of cholesterol in control – Amount of residual cholesterol of sample.

The % of cholesterol decomposition= Amount of decomposed cholesterol/Amount of cholesterol in control × 100.

Cholesterol oxidase assay and protein estimation

The bacterial cultures were centrifuged at 4.000rpm for 10 min. The cell free culture supernatant was used as the crude enzyme source and was assessed for extracellular activity of CHO. The activity of the extracellular enzyme was estimated by a modified method of Lashkarian et al. (2010). The assay mixture was consisted of 1mM 4-aminoantipyrine, 5mM phenol, 5U/mL of horseradish peroxidase and sodium phosphate buffer (20mM, pH 7.0). Fifty µL of 6g/L cholesterol dissolved in dimethyl formamide containing 5% (v/v) Triton X-100 was added to 1mL of reaction mixture, then pre incubated for 3min. at 30°C. The reaction was initiated by adding 20µL of enzyme sample and was continued for 5min at 30°C. The assay mixture was boiled in a water bath for 2min. and then placed in an ice bath for 2min. The inactivated enzyme was considered as the blank. Absorbance of the reaction was measured at 500 nm. One unit (U) of cholesterol oxidase was defined as the amount of enzyme required to convert 1.0µmol of cholesterol to 4-cholesten-3-one / minute under the assay conditions.

Protein concentration was detected in the cell-free supernatant using bovine serum albumin as a standard at 750nm (Lowry et al., 1951).

Morphological, physical, biochemical, and molecular identification of the isolated strain

Primary identification was carried out by studying the morphological, physical and biochemical characteristics of the selected isolate by standard methods using VITEK® 2 Compact device at the Unit of Identification of Microorganisms of Mabaret El-Asafra Lab, Alexandria, Egypt. The 16S rDNA of the isolated strain was analyzed for molecular identification at Macrogene Lab, Korea. PCR amplification of the 16S rRNA gene of the strain was conducted using universal 16S ribosomal DNA primers (F: 5′-AGAGAGTTTGATCTGGCTCAG -3′ and R: 5′-TACGGTACCTTGTTACGACTT -3′). Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information (NCBI) web server (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997). The nucleotide sequence of 16s rRNA was deposited in NCBI database with accession number KY367576. The phylogenetic tree was constructed by the neighbor-joining program using MEGA software, version 4.1 (Kumar et al., 2008).

Optimization of some fermentation medium parameters affecting the growth and decomposition of cholesterol

Some factors affecting growth of the selected strain with respect to cholesterol decomposition were determined (OFAT) in their liquid medium. The study was carried out in 250mL Erlenmeyer flasks containing 50mL of basal cholesterol liquid medium and inoculated with 1mL (20 x 10^6 CFU/mL) of previously prepared bacterial suspension (24h age), then incubated at 37°C for 24h. Different parameters were detected including incubation periods at regular time intervals 12, 24, 36, 48, 60 and 72h at 37°C; different

incubation temperatures 25, 30, 37, 40 and 42°C; different pH values using phosphate buffer (pH 6 up to 8) to adjust the pH; static and shaking conditions at 120rpm and different inoculum size (0.5, 1, 5, 10, 30 and 50% v/v) of bacterial suspension (24h age) of the tested isolate. All the experiments were performed in triplicate.

Screening of significant factors affecting the production of cholesterol oxidase by Plackett–Burman design (PBD)

The PBD is a fractional design which was used for screening significant factors with respect to their main effect on cholesterol oxidase production (Plackett & Burman, 1946). In this experiment, eleven independent variables were screened in twelve combinations. Statistical Cohen's (d) values and the effect size (r) values were calculated for each variable. Each factor is represented at two levels, high and low, denoted by (+1) and (+1), respectively. The main assigned variables; glucose, sucrose, maltose, yeast extract, beef extract, $NaNO_3$ and different metal ions ($MgSO_4$, K_2HPO_4, Na_2HPO_4, $CaCl_2$ and $FeSO_4$) were investigated. The carbon sources were supplemented at concentration (1% w/v), organic nitrogen sources (0.5% w/v), inorganic nitrogen sources supplemented at a final concentration equimolecular of 3 g of $NaNO_3$ and metal ions (0.5% w/v). These designs gave an output of twelve experimental runs to detect the influence of each selected factor on cholesterol oxidase in triplicate. PBD is based on the first-order polynomial equation:

$$Y = \beta_0 + \sum \beta i \ Xi$$

where, Y is the activity of cholesterol oxidase, β_0 is the coefficient of the model, βi is the linear coefficient and X_i is the level of the independent variables.

Results and Discussion

Screening of bacterial isolates for cholesterol decomposition

Cholesterol degrading bacteria were enumerated and isolated from different sources; garden soil samples and dairy products (raw milk and cheese) using basal cholesterol medium (cholesterol as a sole carbon source) and dilution method. Twelve bacterial isolates were qualitatively screened for cholesterol decomposition on basal cholesterol agar medium using the well diffusion agar technique for 24-48h at 37°C. The appearance of clear zones around the well indicates the ability of bacteria to decompose cholesterol. The average diameters of clear zones were detected as shown in Table 1, out of 12 cholesterol decomposing isolates, only 4 isolates were selected for further screening for their high CHO productivity. The largest diameters of clear zones (25.3, 23.3, 21.3 and 20.5 mm) were recorded for bacterial isolates S-3, S-2, S-6 and S-1, respectively.

Screening of the recorded isolates (S-1, S-2, S-3 and S-6) to select the most potent bacterial isolates that have the high percent of cholesterol decomposition added in their liquid medium at 37 °C for 24 and 48 h. The residual cholesterol was quantitatively measured using a colourimetric method of Liebrman-Burchard reaction (Tietz & Logan, 1987). The highest percent of cholesterol decomposition (50.5%) was detected after 48h by isolate S-2 followed by S-3 (43.7%) as shown in Table 1. Therefore, the highly active cholesterol-decomposing isolate S-2 was considered for further studies.

Morphological, physical and biochemical properties of the potent isolate (S-2)

On the basis of morphological, physical and biochemical characteristics of the selected isolate, the results (Table 2) revealed that the isolated strain (S-2) is a rod-shaped, Gram-positive, spore former, and motile bacterium. The isolate showed positive results on catalase, sugar fermentation test and β-Glucosidase. For Antibiotic resistance, the isolate is resistant to Kanamycin and susceptible to Oleandomycine. D (+) glucose, D-ribose, D-Trehalose, Maltotriose, Glycogen and N-Acetyl-D-Glucosamine are utilized for growth but the others are not utilized. The results indicated that the selected isolate is belonging to the *Bacillus* genus.

Molecular identification of the isolated strain

The phylogenetic tree based on the 16S rDNA sequence was illustrated using the neighbor- joining method (Fig. 1). The results showed homologies with other relevant species sequences belonging to the *Bacillus* genus with higher similarity of 94% to *Bacillus cytotoxicus* NVH 39 1-98. The 16S rDNA sequence has been submitted to NCBI GenBank database under accession number KY367576.

TABLE 1. Screening of bacterial isolates for cholesterol decomposition (a) using well diffusion agar method and (b) in liquid medium

Isolate source	Isolate No.	Well diffusion agar method (a) Zone diameter (mm)	Liquid medium (b) Cholesterol decomposition (%)	
			24h	48h
Soil	S-1*	20.5	16.7	24.5
	S-2*	23.3	34.6	50.5
	S-3*	25.3	20.9	43.7
	S-4	12.0	--	--
	S-5	0.00	--	--
	S-6*	21.3	15.3	28.3
Raw milk	M-1	0.00	--	--
	M-2	0.00	--	--
	M-3	13.5	--	--
Cheese	C-1	14.0	--	--
	C-2	0.00	--	--
	C-3	0.00	--	--

S-soil sample, M-milk sample and C-cheese sample

* Isolates were tested for cholesterol decomposition in liquid medium

TABLE 2. Morphological, physical and biochemical properties of *Bacillus cytotoxicus*

Test	Result	Test	Result
Colony morphology	Circular, smooth and creamy colonies	**Antibiotic resistance**	
		Kanamycin resistance	Resistant
Cell shape	Rod-shaped	Oleandomycine resistance	Susceptible
Physical properties			
Gram staining	Gram +ve	**Utilization of carbon sources (1 % w/v)**	
Spore formation	+	D(+) Glucose	+
Motility	+	D(+) Galactose	-
Biochemical characterization		D(+) Mannose	-
		D-Melezitose	-
Oxidase	-	D-Ribose	+
Catalase	+	D-Tagatose	-
Sugar fermentation	+	D-Trehalose	+
β-Xylosidase	-	Palatinose	-
β-Galactosidase	-	L-Rhamnose	-
α-Galactosidase	-	Maltotriose	+
α-Glucosidase	-	D-Mannitol	-
β-Glucosidase	+	Myo-Inositol	-
β-Mannosidase	-	Inulin	-
α- Mannosidase	-	Glycogene	+
Putrescine assimilation	-	N-Acetyl-D-Glucosamine	+
Growth at 6.5 NaCl broth	+	Cyclodextrine	-
Esculin Hydrolyse	+	Methyl-D-Xyloside	-
Tetrazoliun red	-		

+ Positive, - Negative

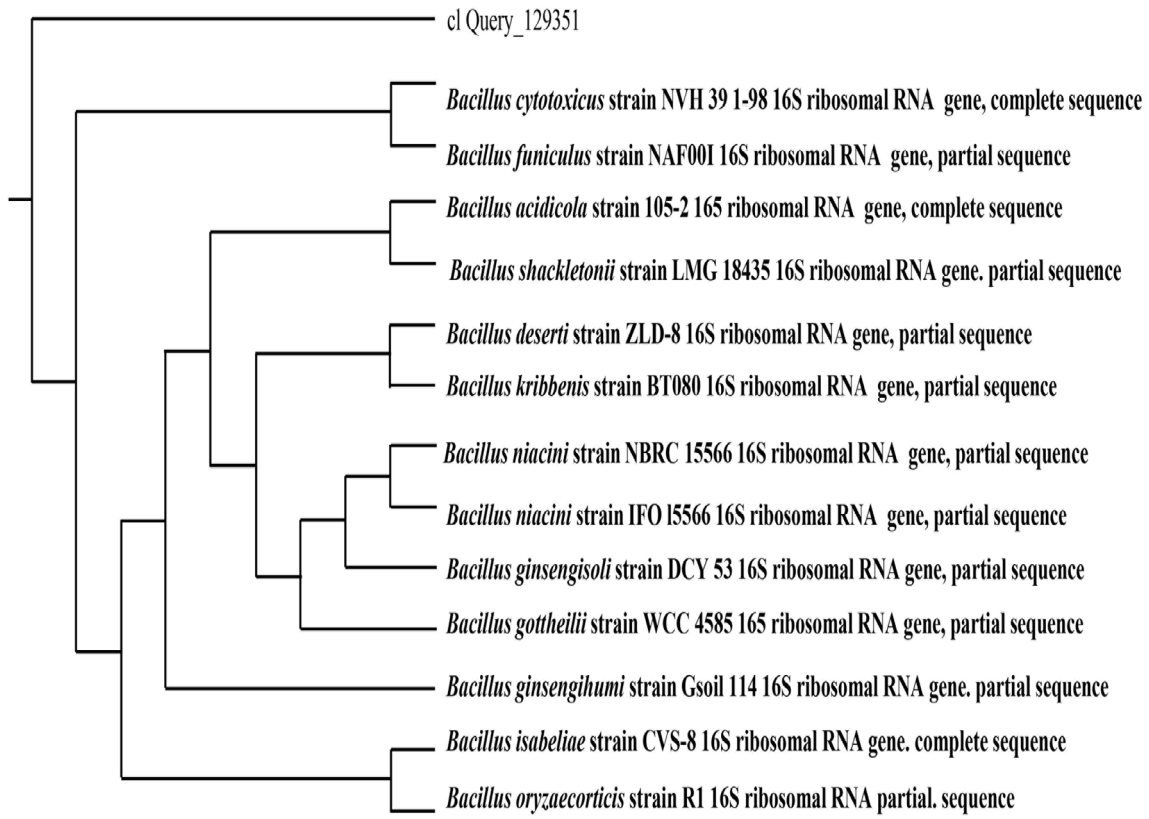

Fig. 1. Phylogenetic tree of *Bacillus cytotoxicus* and their closest relatives based on 16S rRNA gene

Optimization of fermentation medium parameters using one factor-at-a-time (OFAT) method for the production of cholesterol oxidase

OFAT method was used for different parameters to optimize fermentation medium components and other culture conditions to improve the productivity of CHO by *Bacillus cytotoxicus*. The measured CHO activity and cholesterol decomposition obtained from the initial un-optimized experiment were 0.88U/ml and 34.6%, respectively. All the results were demonstrated in Fig. 2. The effect of different incubation periods on CHO production (Fig. 2A) indicated that the highest enzyme activity of 1.20 U/ml was obtained after 36h fermentation time. This result was in agreement with Kuppusamy & Kumar (2016) reported that 32h was the optimum incubation period of *Bacillus cereus*. In contrast, one day incubation time was the optimum for *Enterococcus hirae* (Yehia et al., 2015). *Pediococcus acidilactici* showed the highest levansucrase activity after 2 days of fermentation Youssef et al. (2014).

The temperature profile of the CHO production from *Bacillus cytotoxicus* was illustrated in Fig. 2B. The maximal cholesterol decomposition (57.1%) was recorded at 25 °C. It is closer to that were achieved by Kuppusamy & Kumar (2016), Lashkarian et al. (2010) and Youssef et al. (2014) at room temperature for *Bacillus cereus* and at 30°C for *Bacillus subtilis SFF34* and *Pediococcus acidilactici*, respectively. In contrast, Yazdi et al. (2001b) detected that 34 °C incubation temperature was the optimum for maximum CHO production by *Streptomyces fradiae*.

The effect of initial pH on cholesterol decomposition was illustrated graphically in Fig. 2C. The optimal pH value required for attaining maximum decomposition of cholesterol (65.3%), growth (105mg/50mL) and protein content (1.22mg/mL) was pH 7.5 by *B. cytotoxicus*. This matched with Kuppusamy & Kumar (2016) recorded that pH 7.5 was the optimum for maximum CHO activity obtained from *Bacillus cereus*. Moreover, the maximum enzyme productivity was detected at a pH rang of 6.5-8.0 (Doukyu, 2009). It was previously reported that the optimal pH value for cholesterol decomposition is 7 for *Rhodococcus* sp. (Lee et al., 1997) and *Streptomyces rimosus* (Srivastava

et al., 2018). The pH may indirectly affect the dissociation degree of the medium components (Youssef et al., 2014).

The results in Fig. 2D estimated that the highest percentage of cholesterol decomposition under static condition was 65.3% versus 24.2% for shaking conditions indicating that static condition was preferable than shaking condition (120rpm) by *B. cytotoxicus*. This is in agreement with *Enterococcus hirae* that showed maximum cholesterol decomposition under static conditions (Yehia et al., 2015). Otherwise, shaking speeds of 120 and 150 rpm are optimal for *Pseudonocardia compacta* S-39 and *Streptomyces fradiae* (Ahmed, 1994; Yazdi et al., 2001b), respectively.

The highest percentage of cholesterol decomposition of 71% was achieved at inoculum size 5% with maximum protein content (1.58mgmL) as shown in Fig. 2E.

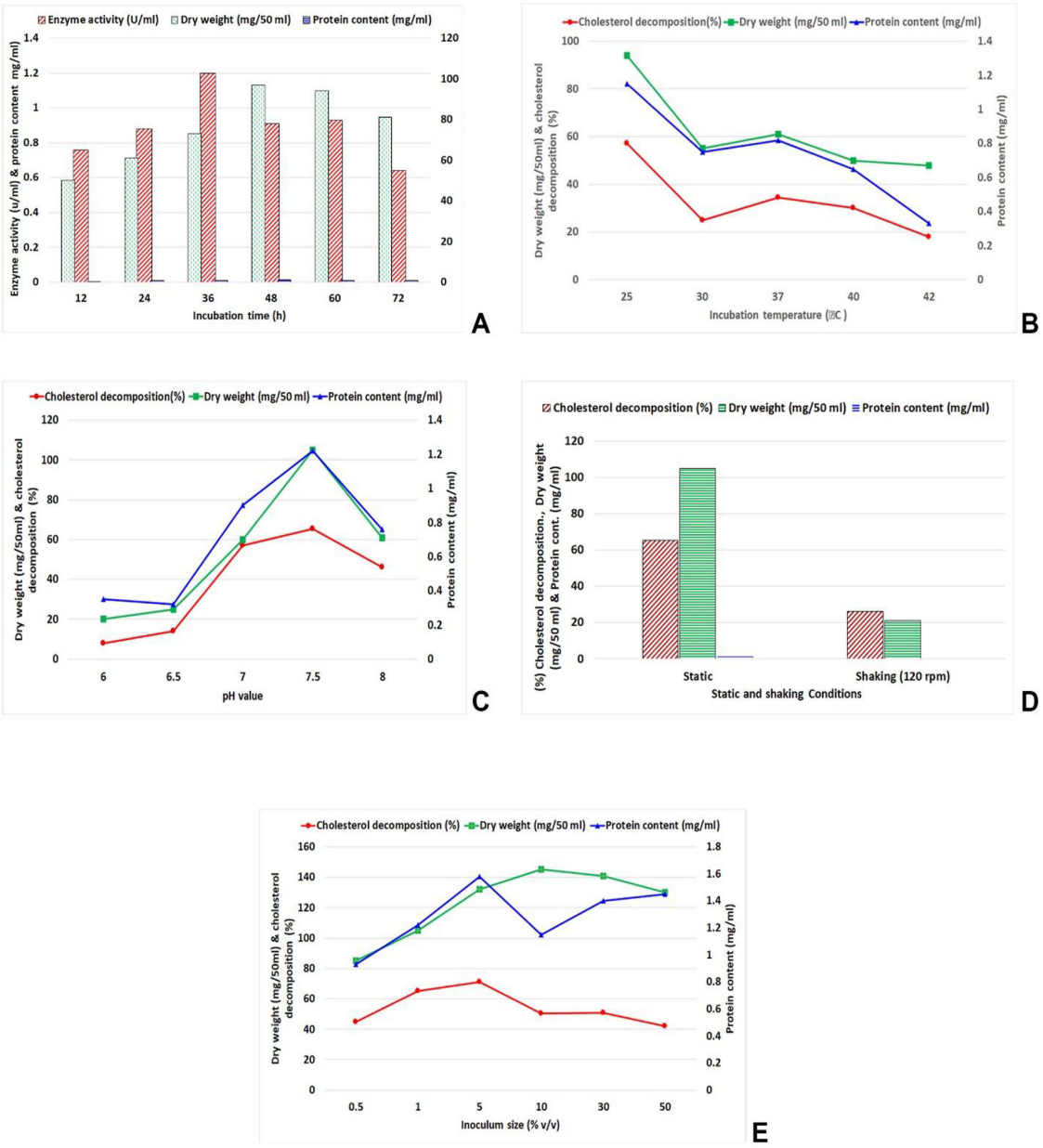

Fig. 2 Effect of different parameters (A) Incubation time, (B) Temperature, (C) pH value, (D) Static and shaking conditions and (E) Inoculum size on the biomass, protein content and cholesterol decomposition by *Bacillus cytotoxicus*

Placket-Burman experimental design

The Placket-Burman experimental design was used to assess the importance of different medium components on cholesterol oxidase production by *B. cytotoxicus*. The PBD is a quick and uncomplicated technique for screening a huge number of variables in one experiment to evaluate the significant variables affecting the cultural requirements to improve the production of cholesterol oxidase (Lolekha & Jantaveesirirat, 1992; Varma & Nene, 2003). Eleven independent variables, carbon sources (glucose, sucrose, maltose); metals ($MgSO_4$, $FeSO_4$, $CaCl_2$, K_2HPO_4, Na_2HPO_4); nitrogen sources (yeast extract, beef extract, $NaNO_3$) were screened by 12 runs to identify the key variables that enhance cholesterol oxidase production. Each variable being examined at two levels (-1) for low and (+1) for high level. As shown in Table 3, trials 6 and 9 recorded the highest cholesterol decomposition (88.9 and 86.6%), respectively. The wide variation of cholesterol decomposition (15 to 88.9%) reflects the significance of medium optimization to achieve higher enzyme productivity. Furthermore, the experimental runs investigate the interrelationship between different independent variables affecting the cell growth and enzyme production for solving problems run across the fermentation process (Varma & Nene, 2003; Guo et al., 2019).

Table 4, Fig. 3 illustrated the estimated effect of the tested variables on the production of cholesterol oxidase. Variables with large positive or negative influences indicate that variables have large impact on the cholesterol decomposition. From the obtained results, we can conclude that four of the eleven variables named $NaNO_3$, metals (Na_2HPO_4, K_2HPO_4 and $FeSO_4$) have a positive influence on the enzyme productivity, where the other seven variables including all carbon sources (glucose, maltose and sucrose) negatively affect the cholesterol oxidase production. As shown in Fig. 3, the inorganic nitrogen source ($NaNO_3$) is better stimulant for CHO production rather than organic nitrogen sources, which were also in accordance with the results of Sabry (1994) reported that ammonium nitrate, ammonium sulphate and sodium nitrate are the most favorable nitrogen sources used for cholesterol assimilation by *Pseudonocardia compacta* S-39. Furthermore, Tembhurkar et al. (2012) indicated that the inorganic nitrogen source is the best source of nitrogen for lipase production by *Pseudomonas* spp. According to El-Naggar et al. (2016), the initial pH and $(NH_4)_2SO_4$ were the most significant positive independent variables affecting cholesterol oxidase production by *Streptomyces cavourensis* NEAE-42 strain. While in contrast, *Rhodococcus equi* 2C and *Rhodococcus equi* no. 23 showed maximum production of enzyme with yeast extract at 0.3 %w/v and 0.4– 0.5 %w/v (Yazdi et al., 2001a; Lee et al., 1997), respectively). On the other hand, Elsayed & Abdelwahed (2020) reported that glucose, malt extract, and $CaCO_3$ have a positive influence on enzyme production by *Streptomyces rochei* NAM-19 Strain.

TABLE 3. Placket-Burman design with cholesterol decomposition response as influenced by the high and low levels of the eleven independent variables

Run	Carbon source			Nitrogen source		Metal ions						Cholesterol decomposition (%)
	Glucose	Sucrose	Maltose	Yeast extract	Beef extract	$NaNO_3$	$MgSO_4$	K_2HPO_4	Na_2HPO_4	$CaCl_2$	$FeSO_4$	
1	1	1	1	1	1	1	1	1	1	1	1	56.5
2	-1	1	-1	1	1	1	-1	-1	-1	1	-1	48.5
3	-1	-1	1	-1	1	1	1	-1	-1	-1	1	76.3
4	1	-1	-1	1	-1	1	1	1	-1	-1	-1	56.5
5	-1	1	-1	-1	1	-1	1	1	1	-1	-1	62.0
6	-1	-1	1	-1	-1	1	-1	1	1	1	-1	88.9
7	-1	-1	-1	1	-1	-1	1	-1	1	1	1	35.2
8	1	-1	-1	-1	1	-1	-1	1	-1	1	1	25.0
9	1	1	-1	-1	-1	1	-1	-1	1	-1	1	86.6
10	1	1	1	-1	-1	-1	1	-1	-1	1	-1	15.0
11	-1	1	1	1	-1	-1	-1	1	-1	-1	1	30.0
12	1	1	1	1	1	1	1	1	1	1	1	29.3

1: High level of variables and -1: Low level of variables

TABLE 4. Statistical analysis of Plackett–Burman design showing regression coefficient and estimated effect for each variable on cholesterol oxidase production by *Bacillus cytotoxicus*

Variable	Coefficient Cohen's *(d)*	Effect Size (*r*)
Glucose	- 0.48	- 0.23
Sucrose	- 0.08	- 0.04
Maltose	- 0.12	- 0.06
Yeast extract	- 0.67	- 0.32
Beef extract	- 0.09	- 0.05
$NaNO_3$	2.18	0.74
$MgSO_4$	- 0.04	- 0.02
K_2HPO_4	0.18	0.09
Na_2HPO_4	0.75	0.35
$CaCl_2$	- 0.48	- 0.23
$FeSO_4$	0.06	0.03

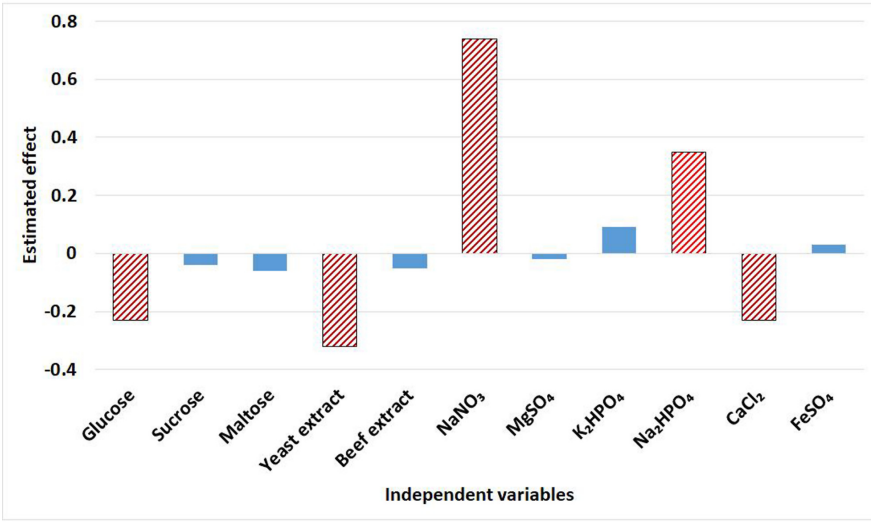

Fig. 3. Main effect of independent variables on cholesterol decomposition by *Bacillus cytotoxicus* using Plackett-Burman design [The striped red colors represent the most significant independent variables affecting cholesterol decomposition]

A verification experiment was carried out to evaluate the efficiency of the Plackett–Burman design and verify the results. Comparison was performed to estimate the value of cholesterol decomposition using the basal medium and statistically optimized medium in static condition. Table 5 indicated that optimization of the fermentation medium compositions by *B. cytotoxicus* using the statistically BPD detected 2.63-fold increase in enzyme activity (2.31U/mL) and 2.57-fold increase in cholesterol decomposition (88.9%) in versus to the initial un-optimized basal medium. The experiment was performed in duplicate, and the average CHO activity was treated as response. This verification revealed a high degree of accuracy of PBD. Optimization of the medium composition of *Streptomyces rochei* NAM-19 increased maximal CHO production by about 2.55 times in comparison with initial un-optimized medium (25.5U/mL) Elsayed and Abdelwahed (2020). Furthermore, 3.6-fold increase in CHO production (5.41U/mL) by *Streptomyces rimosus* in comparison with the un-optimized medium (1.5U/mL) (Srivastava et al., 2018). CHO production increased with a fold of increase of 6.06 times compared to the production before applying the PBD (4.51U/mL) by *Streptomyces anulatus* strain NEAE-94 in shake flasks El-Naggar & El-Shweihy (2020).

TABLE 5. Comparative study of cholesterol decomposition and CHO production using statistically optimized medium in versus to the un-optimized basal medium by *Bacillus cytotoxicus*

Medium	Cholesterol decomposition (%)	Dry weight (mg/50 mL)	Protein content (mg/mL)	Enzyme activity (U/mL)	Specific activity (U/mg protein)
Un-optimized basal medium	34.6	61.0	0.89	0.88	0.99
Statistical optimized medium	88.9	115	1.57	2.31	1.47

Conclusion

Herein, a new bacterial strain was isolated from local soil showing promising potential for cholesterol oxidase production. The isolated strain was identified morphologically, biochemically and molecularly confirmed as *Bacillus cytotoxicus*. The genomic sequence was deposited in the NCBI database under the accession number KY367576. The Plackett-Burman optimization design increased cholesterol decomposition by 2.57-fold with CHO activity of 2.31 U/ml in comparison with the un-optimized medium composition (0.88 U/ml). Further investigations of the structural, biochemical properties of the enzyme and cloning of CHO genes are warranted for future experimental studies and applications.

Conflict of interest: Authors declare that there is no conflict of interest.

Authors' contributions: Samy El-Assar conceived the presented idea. Samy El-Assar and Ghada Youssef developed the theory and performed the computations. Waleed El-Magraby and Ghada Youssef performed the material preparation and carried out the experiments. Samy El-Assar and Ghada Youssef were involved in planning and supervised the work. All authors discussed the results and approved the final version of the manuscript.

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Ethics approval: This article does not contain any studies with animals performed by any of the authors**.**

References

Abd-Alla, M., Bashandy, S., Nafady, N., Hassan, A. (2018) Enhancement of exopolysaccharide production by *Stenotrophomonas maltophilia* and *Brevibacillus parabrevis* isolated from root nodules of *Cicer arietinum* L. and *Vigna unguiculata* L.(Walp.) plants. *Rendiconti Lincei. Scienze Fisiche e Naturali*, **29**(1), 117-129.

Ahmed, A. (1994) Decomposition of cholesterol by some organisms isolated from certain Egyptian soils. Egypt: N. p., Web.

Ali, M., Zohri, A.N. (2021) Optimization of glycerol production by a new osmotolerant *Wickerhamomyces anomalus* AUMC 11687 yeast strain using response surface methodology. *Egyptian Journal of Botany*, **61**(1), 53-60.

Altschul, S., Madden, T., Schäffer, A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**(17), 3389-340.

Arya, S., Datta, M., Malhotra, B. (2008) Recent advances in cholesterol biosensor. *Biosensors and Bioelectronics*, **23**(7), 1083-1100.

Aunpad, R., Muench, S., Baker, P., Sedelnikova, S., Panbangred, W., Doukyu, N., Aono, R., Rice, D. (2002) Crystallization and preliminary X-ray crystallographic studies on the class II cholesterol oxidase from *Burkholderia cepacia* containing bound flavin. *Acta Crystallographica* Section D: *Biological Crystallography,* **58**(12), 2182-218.

Basu, A., Chattopadhyay, P., Roychoudhuri, U., Chakraborty, R. (2007) Development of cholesterol biosensor based on immobilized cholesterol esterase and cholesterol oxidase on oxygen electrode for the

determination of total cholesterol in food samples. *Bioelectrochemistry*, **70**(2), 375-379.

Chauhan, A., Survase, S., Kishenkumar, J., Annapure, U. (2009) Medium optimization by orthogonal array and response surface methodology for cholesterol oxidase production by *Streptomyces lavendulae* NCIM 2499. *The Journal of general and applied microbiology*, **55**(3), 171-180.

Devi, S., Kanwar, S. (2017) Cholesterol oxidase: source, properties and applications. *Insights Enzyme Res*, **1**(1), 1-5.

Djinni, I., Djoudi, W., Harfi, N., Stambouli, I., Khamtache, S., Makhlouf, D., et al. (2022) Enhanced Anti-E. coli ST131 metabolites production by a novel *Streptomyces* sp. CMB51 strain isolated from a coal mininig soil using statistical optimization. *Geomicrobiology Journal,* **39**(1), 39-53.

Doukyu, N. (2009) Characteristics and biotechnological applications of microbial cholesterol oxidases. *Applied Microbiology and Biotechnology*, **83**(5), 825-83.

El-Naggar, N.E.-A., El-Shweihy, N. (2020) Identification of cholesterol-assimilating actinomycetes strain and application of statistical modeling approaches for improvement of cholesterol oxidase production by Streptomyces anulatus strain NEAE-94. *BMC Microbiology* **20**(1), 1-18.

El-Naggar, N.E.-A., El-Shweihy, N., El-Ewasy, S. (2016) Identification and statistical optimization of fermentation conditions for a newly isolated extracellular cholesterol oxidase-producing *Streptomyces cavourensis* strain NEAE-42. *BMC Microbiology*, **16**(1), 1-20.

El-Naggar, N.E.-A., Soliman, H., El-Shweihy, N. (2018) Extracellular cholesterol oxidase production by *Streptomyces aegyptia*, *in vitro* anticancer activities against rhabdomyosarcoma, breast cancer cell-lines and *in vivo* apoptosis. *Scientific Reports*, **8**(1), 1-1.

El-Sheekh, M., Morsi, H., Hassan, L. (2021) Growth Enhancement of *Spirulina platensis* through optimization of media and nitrogen sources. *Egyptian Journal of Botany*, **61**(1), 61-6.

Elsayed, E., Abdelwahed, A.N. (2020) Medium optimization by response surface methodology for improved cholesterol oxidase production by a newly isolated *Streptomyces rochei* NAM-19 Strain. *BioMed Research International*, **31**, 1870807.

Espuny, T., Simon, P., Congregado, F. (1980) Method to assess antagonism of soil microorganisms towards fungal spore germination. *Soil Biology & Biochemistry*, **12**(2), 197-198.

Guo, F., Li, X., Zhao, J., Li, G., Gao, P., Han, X. (2019) Optimizing culture conditions by statistical approach to enhance production of pectinase from *Bacillus* sp. Y1. *BioMed research International*, **1**, 1-10.

Hamid, K., Elsayed, E., El Enshasy, H., Esawy, M., Abd Malek, R. (2018) Bioprocess optimization for levan production by *Bacillus subtilis* B58. *Journal of Scientific and Industrial Research (JSIR)*, **77**(7), 386-393.

Horiuchi, N., Nakagawa, K., Sasaki, Y., Minato, K., Fujiwara, Y., Nezu, K., Ohe, Y., Saijo, N. (1988) *In vitro* antitumor activity of mitomycin C derivative (RM-49) and new anticancer antibiotics (FK973) against lung cancer cell lines determined by tetrazolium dye (MTT) assay. *Cancer Chemotherapy and Pharmacology*, **22**(3), 246-250.

Imshenetskii, A., Efimochkina, E., Zanin, V., Nikitin, L. (1968) Decomposition of cholesterol by Mycobacteria. *Mikrobiologia*, **37**, 31.

Kepli, A., Dailin, D., Malek, R., Elsayed, E., Leng, O., El-Enshasy, H. (2019) Medium optimization using response surface methodology for high cell mass production of *Lactobacillus acidophilus*. *Journal of Scientific and Industrial Research (JSIR)*, **78**(9), 608-614.

Khan, R., Solanki, P., Kaushik, A., Singh, S., Ahmad, S., Malhotra, B. (2009) Cholesterol biosensor based on electrochemically prepared polyaniline conducting polymer film in presence of a nonionic surfactant. *Journal of Polymer Research*, **16**(4), 363-373.

Kim, K., Rhee, C., Park, H. (2002) Degradation of cholesterol by *Bacillus subtilis* SFF34 isolated from Korean traditional fermented flatfish. *Letters in Applied Microbiology*, **35**(6), 468-47.

Kuddus, M. (2015) Statistical optimization of cold-active chitinase production by mutagenized cells of multi-enzyme producing *Bacillus cereus* GA6. *Rendiconti Lincei*, **26**(3), 271-280.

Kumar, C., Joo, H-S., Koo, Y-M., Paik, S., Chang, C-S. (2004) Thermostable alkaline protease from a novel marine haloalkalophilic *Bacillus clausii* isolate. *World Journal of Microbiology and Biotechnology*, **20**(4), 351-357.

Kumar, S., Nei, M., Dudley, J., Tamura, K. (2008) MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics*, **9**(4), 299-306.

Kuppusamy, A., Kumar, K. (2016) Optimization of cholesterol oxidase production and 16S rRNA partial sequence of *Bacillus cereus* strain KAVK4 isolated from butter. *Journal of Applied Pharmaceutical Science*, **6**(7), 61-66.

Lashkarian, H., Raheb, J., Shahzamani, K., Shahbani, H., Shamsara, M. (2010) Extracellular cholesterol oxidase from *Rhodococcus* sp.: Isolation and molecular characterization. *Iranian Biomedical Journal*, **14**(1-2), 49.

Latha, S., Sivaranjani, G., Dhanasekaran, D. (2017) Response surface methodology: A non-conventional statistical tool to maximize the throughput of *Streptomyces* species biomass and their bioactive metabolites. *Critical Reviews in Microbiology*, **43**(5), 567-58.

Lee, C.Y., Liu, W.H. (1992) Production of androsta-1,4-diene-3,17-dione from cholesterol using immobilized growing cells of *Mycobacterium* sp. NRRL B-3683 adsorbed on solid carriers. *Applied Microbiology and Biotechnology*, **36**(5), 598-603.

Lee, M-T., Chen, W-C., Chou, C-C. (1997) Nutritional factors that affect the production of cholesterol oxidase by *Rhodococcus equi* no. 23. *Biotechnology and Applied Biochemistry*, **26**(3), 159-162.

Lee, S-y., Rhee, H-i., Tae, W-C., Shin, J-C., Park, B-k. (1989) Purification and characterization of cholesterol oxidase from *Pseudomonas* sp. and taxonomic study of the strain. *Applied Microbiology and Biotechnology*, **31**(5) 542-546.

Lolekha, P., Jantaveesirirat, Y. (1992) *Streptomyces*: a superior source for cholesterol oxidase used in serum cholesterol assay. *Journal of Clinical Laboratory Analysis*, **6**(6), 405-409.

Lowry, O., Rosebrough, N., Farr, A., Randall, R. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.

MacLachlan, J., Wotherspoon, A., Ansell, R., Brooks, C. (2000) Cholesterol oxidase: sources, physical properties and analytical applications. *The Journal of Steroid Biochemistry and Molecular Biology*, **72**(5), 169-195.

Molaei, R., Sabzi, R., Farhadi, K., Kheiri, F., Forough, M. (2014) Amperometric biosensor for cholesterol based on novel nanocomposite array gold nanoparticles/acetone-extracted propolis/multiwall carbon nanotubes/gold. *Micro & Nano Letters*, **9**(2), 100-104.

Nafady, N., Bagy, M., Abd-Alla, M., Morsy, F., Mahmoud, G.A.-E. (2015) Improvement of medium components for high riboflavin production by *Aspergillus terreus* using response surface methodology. *Rendiconti Lincei*, **26**(3), 335-344.

Naik, B., Goyal, S. K., Tripathi, A. D., Kumar, V. (2022) Optimization of pullulanase production by Aspergillus flavus under solid-state fermentation. *Bioresource Technology Reports*, 100963.

Oggioni, C., Cena, H., Wells, J., Lara, J., Celis-Morales, C., Siervo, M. (2015) Association between worldwide dietary and lifestyle patterns with total cholesterol concentrations and DALYs for infectious and cardiovascular diseases: An ecological analysis. *Journal of Epidemiology and Global Health*, **5**(4), 315-325.

Osman, M., El-Beih, A., Khatab, O-K., Moghannem, S.A., Abdullah, N. (2018) Optimization of bioactive compounds production by endophytic *Chaetosphaeronema* sp.(KY321184) using experimental design method. *Egyptian Journal of Botany*, **58**(3), 343-343.

Page, A., Miller, R., Keeney, D. (1982) Methods of soil analysis part 2: Chemical and microbiological properties. Agronomy Monograph no. 9. American Society of Agronomy and Soil Science Society America Madison, Wisconsin, USA

Plackett, R., Burman, J. (1946) The design of optimum multifactorial experiments. *Biometrika*, **33**(4), 305-325.

Purcell, J., Greenplate, J., Jennings, M., Ryerse, J., Pershing, J., Sims, S., Prinsen, M., Corbin,

D., Tran, M., Sammons, R. (1993) Cholesterol oxidase: a potent insecticidal protein active against boll weevil larvae. *Biochemical and Biophysical Research Communications*, **196**(3), 1406-1413.

Sabry, A. (1994) Decomposition of cholesterol by some organisms isolated from certain Egyption soils, *M.Sc. Thesis*. Bot. and Microbiol. Cairo: Dept. Al-Azhar University; 1994.

Sahu, S., Shera, S., Banik, R. (2019) Optimization of process parameters for cholesterol oxidase production by *Streptomyces olivaceus* MTCC 6820.

Santos, R., Monnerat, R., Sá MdFGd, Cordeiro, C., Gomes, A., Gander, E. (2002) Cholesterol oxidase interference on the emergence and viability of cotton boll weevil larvae. *Pesquisa Agropecuária Brasileira*, **37**, 1525-1530.

Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti, M., Tripathi, C. (2017) Strategies for fermentation medium optimization: an in-depth review. *Frontiers in Microbiology,* **7**, 2087.

Srivastava, A., Singh, V., Tripathi, C. (2018) Scale up and optimization of cholesterol oxidase production from *Streptomyces rimosus* MTCC 10792 in a 3-L bioreactor. *Environmental Sustainability*, **1**(1), 99-107.

Tembhurkar, V.R., M.B., Kulkarni, Peshwe, S.A. (2012) Optimization of lipase production by Pseudomonas spp. in submerged batch process in shake flask culture. *Science Research Reporter,* **2**(1), 46-50.

Then, C., Wai, O., Elsayed, E., Mustapha, W., Othman, N., Aziz, R., Wadaan, M., El Enshsay, H. (2016) Comparison between classical and statistical medium optimization approaches for high cell mass production of *Azotobacter vinelandii*. *Journal of Scientific and Industrial Research,* **75**, 231-238.

Tietz, N., Logan, M. (1987) Reference ranges. "*Tietz NW Fundamentals of Clinical Chemistry*", 3rd ed Philadelphia: WB Saunders Company, pp. 944-975.

Varma, R., Nene, S. (2003) Biosynthesis of cholesterol oxidase by Streptomyces lavendulae NCIM 2421. *Enzyme and Microbial Technology*, **33**(2-3), 286-291.

Vidal, J-C., Espuelas, J., Garcia-Ruiz, E., Castillo, J-R. (2004) Amperometric cholesterol biosensors based on the electropolymerization of pyrrole and the electrocatalytic effect of Prussian-Blue layers helped with self-assembled monolayers. *Talanta,* **64**(3), 655-664.

Yamada, K., Koroleva, A., Laughlin, M., Oksanen, N., Akhgari, A., Safronova, V., et al. (2019) Characterization and overproduction of cell-associated cholesterol oxidase ChoD from *Streptomyces lavendulae* YAKB-15. *Scientific Reports*, **9**(1), 1-8.

Yazdi, M., Malekzadeh, F., Zarrini, G., Faramarzi, M., Kamranpour, N., Khaleghparast, S. (2001a) Production of cholesterol oxidase by a newly isolated *Rhodococcus* sp. *World Journal of Microbiology and Biotechnology*, **17**(7),731-737.

Yazdi, M., Zahraei, M., Aghaepour, K., Kamranpour, N. (2001b) Purification and partial characterization of a cholesterol oxidase from *Streptomyces fradiae*. *Enzyme and Microbial Technology*, **28**(4-5), 410-414.

Yazdi, M., Yazdi, Z., Zarrini, G., Ghasemian, A., Sepehrizadeh, Z., Olyaee, N. (2008) Purification and characterization of extra-cellular cholesterol oxidase from *Rhodococcus* sp. PTCC 1633. *Biotechnology*, **7**(4), 751-756.

Yehia, H., Hassanein, W., Ibraheim, S. (2015) Purification and characterisation of the extracellular cholesterol oxidase enzyme from *Enterococcus hirae*. *BMC Microbiology*, **15**(1), 1-12.

Youssef, Gh., Youssef, A., Talha, S., El-Aassar, S. (2014) Increased fructosyltranseferase (levansucrase) production by optimizing culture condition from *Pediococcus acidilactici* strain in shaking batch cultures. *Life Science Journal*, **11**(7), 33-47.

Zanin, V. (1968) Decomposition of cholesterol by actinomycetes. *Mikrobiologiia,* **37**(6), 1024-1029.

التعظيم الإحصائى للظروف البيئية التخمرية لتحفيز إنتاج إنزيم الكوليسترول أوكسيديز بإستخدام طريقة بلاكيت – برمان من سلالة *Bacillus cytotoxicus* الجديدة

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في الأونة الأخيرة، إكتسب إنزيم الكوليسترول أوكسيديز الميكروبي اهتمامًا كبيرًا بسبب استخدامه على نطاق واسع في التطبيقات الطبية المختلفة. يهدف هذا العمل إلى دراسة وتحسين إنتاج إنزيم الكوليسترول أوكسيديز الميكروبى. حيث تم عزل إثنتى عشرة عزلة بكترية مختلفة من عينات التربة المحلية المختلفة بالإسكندرية، مصر ومن بعض منتجات الألبان. تم فحص وتقييم قدرة هذة العزلات البكترية على انتاج إنزيم الكوليسترول اوكسيديز باستخدام طريقة الانتشارفى الأجار لتقييم قدرتها على تحلل الكوليسترول. ولقد أوصت النتائج بإختيار عزلة واحدة بناءا على أعلى إنتاجية لإنزيم الكوليسترول اوكسيديز وأعلى كفاءة لتحلل الكوليسترول. حيث تم التعرف على هذة العزلة على المستوى المورفولوجي (العياني والمجهرى) والبيوكيميائى وتأكيدها جزيئيا باستخدام التسلسل الجيني 16S rRNA وتم تعريفها على أنها سلالة *Bacillus cytotoxicus* وسجلت فى بنك الجينات برقم KY367576. ومن ثم تم تحسين وتعظيم الظروف البيئية المختلفة باستخدام الطريقة الكلاسيكية (متغير واحد في كل تجربة) والطريقة الإحصائية (تصميم بلاكيت - برمان) لتحسين وسط النمو ولتعزيز انتاجية إنزيم الكوليسترول اوكسيديز من العزلة المختارة. وأوضحت النتائج أن الطريقة الإحصائية حددت المستويات المثلى للعوامل المهمة التي تؤثر على انتاجية الإنزيم ورفعت كفاءة نشاط الإنزيم بحوالى 2.63 ضعف (2.31 وحدة / مل) كما زادت نسبة تحلل الكوليسترول بحوالى 2.57 ضعف (88.9%) مقارنة بالطريقة العادية الكلاسيكية. ومن ثم أكدت النتائج أن سلالة *Bacillus cytotoxicus* الجديدة لديها كفاءة عالية لتحلل الكوليسترول وإنتاج كميات كبيرة من إنزيم الكوليسترول أوكسيديز بإستخدام الطريقة الإحصائية (تصميم بلاكيت - برمان) حيث إن إنزيم الكوليسترول أوكسيديز يدخل فى العديد من تطبيقات البيوتكنولوجيا الطبية والدوائية.